Experimental Introgression To Evaluate the Impact of Sex Specific Traits on *Drosophila melanogaster* Incipient Speciation

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**ABSTRACT** Sex specific traits are involved in speciation but it is difficult to determine whether their variation initiates or reinforces sexual isolation. In some insects, speciation depends on the rapid change of expression in desaturase genes coding for sex pheromones. Two closely related desaturase genes are involved in *Drosophila melanogaster* pheromonal communication: *desat1* affects both the production and the reception of sex pheromones while *desat2* is involved in their production in flies of Zimbabwe populations. There is a strong asymmetric sexual isolation between Zimbabwe populations and all other “Cosmopolitan” populations: Zimbabwe females rarely copulate with Cosmopolitan males whereas Zimbabwe males readily copulate with all females. All populations express *desat1* but only Zimbabwe strains show high *desat2* expression. To evaluate the impact of sex pheromones, female receptivity and desat expression on the incipient speciation process between Zimbabwe and Cosmopolitan populations, we introgressed the Zimbabwe genome into a Cosmopolitan genome labeled with the *white* mutation, using a multi-generation procedure. The association between these sex-specific traits was determined during the procedure. The production of pheromones was largely dissociated between the sexes. The copulation frequency (but not latency) was highly correlated with the female—but not with the male—principal pheromones. We finally obtained two stable white lines showing Zimbabwe-like sex pheromones, copulation discrimination and desat expression. Our study indicates that the variation of sex pheromones and mating discrimination depend of distinct—yet overlapping—sets of genes in each sex suggesting that their cumulated effects participate to reinforce the speciation process.

**KEYWORDS**
cuticular hydrocarbon tricosene heptacosadiene

The quality of sensory cues exchanged by sex partners is crucial with regard to sexual isolation and selection (Darwin 1871; Andersson 1994; Coyne and Orr 2004). Chemical signals emitted by conspecifics (pheromones) are used by many insects to assess the sex, species, population and reproductive status of their potential partner (Howard and Blomquist 2005). The intraspecific variation of pheromones (and of other sensory signals) can enhance the divergence between partly sexually isolated populations, this ultimately leading to distinct species (Wyatt 2014). The mechanisms initiating and/or reinforcing speciation can either occur before copulation [altered mate discrimination; morphological alteration of genital parts (Eberhard 1993; Arnqvist 1998)] or after copulation [gametic or genomic incompatibility (Mayr 1963; Hurst and Pomiankowski 1991; Presgraves et al. 2003)]. However, the chronological involvement of these alterations and their relative contribution to sexual isolation remain difficult to determine together with the potential impact of novel sensory signals (Wyatt 2014).

Pheromone natural variants have been discovered in several insect orders. In the European corn borer moth (*Ostrinia nubilalis*), some
populations diverge both for the ratio between two pheromonal iso- 
mers (E) and (Z) of the 11-tetradecenyl acetate, and for the male be-
avioral response to the blends with different (E)/(Z) ratio (Klun and 
Maini 1979). Also, in some moths, the production of a variant (or 
新型) pheromone was caused by the reactivation of a silent desaturase 
gene (Roelofs et al. 2002) or by a sex- and species-specific variation in 
the expression of this gene (Lassance et al. 2013). Since the apparition 
of the “novel” female pheromone was not directly linked to the gene(s) 
coding for the male behavioral preference for this pheromone (Löststedt 
et al. 1989), male preference may have pre-existent prior to the appari-
tion of this novel female pheromone (Butlin and Ritchie 1989; Roelofs 
et al. 2002). Indeed, desaturases are fast evolving genes related to spe-
ciation events in moths and in Drosophila species (Fang et al. 2009; 
Shirangi et al. 2009; Xue et al. 2012).

In the cosmopolitan species Drosophila melanogaster, most—but not 
all—populations show random mating (Henderson and Lambert 
1982; Begun and Aquadro 1993; Koroil et al. 2000; Haerty et al. 2002; 
Yukilevich and True 2008). Among the few known exceptions to this 
parasitic rule, a strong case of asymmetrical sexual isolation was 
reported in Zimbabwe populations (Z) where Z females rarely mate 
with cosmopolitan (M) males whereas Z males readily mate with all 
females (Wu et al. 1995). A link between this incipient speciation case 
and the variation of expression in a desaturase gene (desat2) was pro-
posed, based on the presence of a functional desat2 gene in Z, but not 
in M strains (Takahashi et al. 2001; Fang et al. 2002). However, the 
expression of desat2 is not totally abolished in M strains but seems to be 
“only” strongly repressed (Michalak et al. 2007). While the desat2 gene 
is largely involved in the production of the variant female cuticular 
hydrocarbon (CH) 5,9-heptacosadiene (5,9HD Coyne et al. 1999; 
Dallerac et al. 2000), its effect in the increased production of 
5-tricosene (5T) in Z males remains unknown (Grillet et al. 2012). 
While both Z females and males show high levels of C5-desaturated 
CHs, in several west-African strains only females—but not males— 
produce high levels of C5-desaturated CHs (5,9HD in Tai strain; 
Pechine et al. 1988; Sureau and Ferveur 1999). The desat1 gene, flank-
ing desat2, is expressed in all D. melanogaster strains (Z and M) and 
codes for the production of C7-desaturated CHs in males (7-tricosene = 
7T) and in females (7,11-heptacosadiene = 7,11HD; Jallon 1984; 
Wicker-Thomas et al. 1997; Marcillac et al. 2005a). Surprisingly, desat1 
is also involved in the discrimination of sex pheromones and in the 
emission of other yet unidentified mating cues (Marcillac et al. 2005b; 
Bousquet et al. 2012; Bousquet et al. 2016).

The variation of the female heptacosadiene ratio (7,11HD/5,9HD) has 
apparently no, or very little effect, on male copulation (Ferveur et al. 
1996; Coyne et al. 1999). Moreover, the experimental variation of the 
main tricosene ratio (7T/5T) only partly affects mating preference in Z 
females (Grillet et al. 2012). This suggests that the asymmetrical sexual 
interval between Z and M populations involves female perception of 
other male—non acoustic—sensory cues (Colegrafe et al. 2000; Grillet 
et al. 2012; Grillet et al. 2018). The hypothesis of sexual isolation based 
on multiple sensory signals and/or systems is somewhat supported by 
the finding that the divergence of mating preference between Z and M 
populations depends on a highly polygenic control (Hollocher et al. 
1997; Ting et al. 2001; Kauer and Schlötterer 2004). Also, among several 
genes showing mating-dependent variation of expression, desat2 is 
up-regulated in Z flies, but down-regulated in M flies, after mating 
(Michalak et al. 2007). Post-copulatory isolation mechanisms inducing 
partial gametic incompatibilities were also detected between Z and M 
populations (Alipaz et al. 2001).

Since the relationship between (i) the variation of desat2 expression, 
(ii) the production of cuticular sex pheromones in males and (iii) the 
asymmetrical sexual isolation of Z populations remains unclear, we 
explored the genetic relationship between these traits in both sexes. 
To mimic a natural process, which may have taken many more genera-
tions, we carried out a multi-generations "backcross-selection" pro-
cEDURE in the laboratory to progressively introgress a Z genome into 
a M line carrying the white mutation (w). We measured, at different 
time points of this procedure, the relationship between these sex spe-
cific traits. This allowed us to evaluate their potential influence on the 
incipient speciation process observed between D. melanogaster popu-
lations. Our final goal consisted to obtain stable ZW lines associating 
Z-like sex pheromones, copulatory discrimination and desat expression 
with the w mutation. Such lines could be used to test the effect of 
transgenes associated with the w+ marker.

MATERIAL & METHODS

Flies and stocks

Drosophila melanogaster strains were raised in 15 ml glass vials con-
taining 4 ml of yeast/cornmeal/agar medium and kept in a breeding 
room at 24.5 ± 0.5°C with 65 ± 5% humidity on a 12:12h light/dark 
cycle (subjective day from 8:00 AM to 8:00 PM). M-type flies were transferred every two/three days (and Z-type flies every five to seven days) to 
avoid larval competition and to regularly provide abundant progeny for 
testing and breeding. All behavioral experiments were performed under 
similar conditions.

We used two M-type strains: Canton-S (Cs), an old-established strain 
widely used in fly laboratories, and the Dij/w strain which was established 
in (2011) after ten backcrosses between the Dijon 2000 (Di2) strain and 
the w118 strain which carries the white118 mutation [w; carried by the 
X chromosome and providing white eyes; (Green 1996) in a Cs genetic 
background].

We also used the Zimbabwe 30 strain [Z30; also noted Z6 in Grillet 
et al. 2012], a Z-type line which was collected in 1990 in the Wildlife 
Reserve of Sengwa in Zimbabwe (kindly provided by Profs Jerry Coyne, 
Chicago Univ. and Aya Takahashi, Tokyo Metropolitan Univ.). Both 
Z30 male and female flies produce relatively high level of variant desa-
turated cuticular hydrocarbon isomers.

All tested flies were screened under light CO2 anesthesia 0-4h after 
adult emergence and kept in fresh food vials for four or five days before 
testing. Males used in behavioral tests were held individually while 
females used in all the tests (behavior, CH analysis, mating) were kept in fresh food vials for four or five days before testing.

Genetic Procedure: backcrosses, selection and 
line establishment

We designed a genetic procedure including three series of backcrosses 
(BC1, BC2, BC3) alternating with three “Analysis & Selection” sessions 
(A&S #1-3; Figure 1). The procedure was initiated with a cross between 
Di/w females (M-type; w) and Z30 males (Z-type; throughout our ms, 
all crosses and pairs are indicated as “females x males”; we used this 
mating scheme since the reciprocal cross would have produced rare 
mating and fewer progeny). Our aim consisted to create and select lines 
associating the w mutation in an increasing proportion of Z genome 
(ZW lines). Each BC series (consisting of a 7-generations procedure; 
Fig. S1) was followed by a “A&S” session—lasting 3 to 4 generations— 
during which male and female pheromones and mating ability were 
measured in BC lines (successively producing ZW-BC1, ZW-BC2 and 
ZW-BC3 flies). More precisely, during each BC series, the male pro-
genomic resulting of the previous generation was backcrossed either to ZW 
siblings (with w eyes) or to Z30 males (this mating scheme was chosen 
given that meiotic recombination only occurs in Drosophilas females).
The initial “Di/w × Z30” cross produced F1 flies with 100% red eye females (w⁺/w heterozygous) and 100% white eyes males (w hemizygous). F1 x F1 crosses yielded about 50% F2 white eyes females (w⁺/w homozygous) and 50% F2 red eyes males, which were backcrossed to Z30 males. A similar mating scheme was used for the two following generations: F3 females (w⁺/w) were mated with F3 males to produce F4 w females backcrossed to Z30 males. This yielded F5 w⁺/w females and w males, which were statistically produced 50% F6 w flies. Small groups of F6 w sisters and brothers were intra-line mated to induce several inbred ZW-BC1 lines (only producing w flies) whose individuals were tested during the subsequent “A&S session #1”. Then, only ZW-BC1 lines showing the best Z-like characters were kept for the next BC session. A similar procedure was used to produce and select ZW-BC2 and then ZW-BC3 lines. To reduce the remaining genetic variability, pairs of sisters and brothers were intra-line mated to induce several inbred ZW-BC1 lines—among the two lines showing the best Z-like characters (during the “A&S session #3”—were mated to induce 20 isoparental sublines (ZW-Isop).

**Mating behavior, fertility and fecundity**

Behavioral tests took place 1–4 hours after lights on and were completed over several days for each genotype pair. Each male was individually anesthetized (without anesthesia) under a watch glass used as an observation chamber (1.6 cm³) followed by a virgin female, 10 min later. Each test, performed under white light, lasted 120 min. For each pair, we noted the eventual copulation (time lapse between the test start and copulation onset) and the overall frequency of copulating pairs for each treatment (for the sake of clarity, we do not show the copulation duration). In some experiments, when copulation occurred within the two-hour observation period, single mated females were individually placed into a food vial. Also, each non-mating pair (during the 2-hour test observation) was placed into a food vial and the male was discarded (with a mouth aspirator) after 24 hr. We only kept vials in which the mated female (in case of mating during the 2-hour period) or the two flies of the pair were still alive after the end of the mating test. In each vial, we determined both the fertility and fecundity based on the presence and number of adult progeny, respectively. The total number of viable adults yielded by each fertile pair was counted once, two weeks after the day of the mating test, and according to the mating status (mating or not during the 2-hours test).

**Cuticular hydrocarbons**

5-Day old flies were frozen for 5 min at -20° and individually extracted for 10 min at room temperature using 30 µl of hexane containing 3.33 ng/µl of n-C26 (n-hexacosane) and 3.33 ng/µl of n-C30 (n-triacontane) as internal standards. Male cuticular hydrocarbons (CHs) were quantified by gas chromatography using a Varian CP3380 gas chromatograph fitted with a flame ionization detector and an apolar CP Sil 5CB column (25 m by 0.25 mm; internal diameter: 0.1 µm film thickness; Agilent). In females, given that 5,9HD coeluted with 27-Br, we used a pooling CP-Wax 58 FFAP column [25 m by 0.25 mm; 0.2 µm film thickness; Agilent]. In both cases, a split—splitless injector (60 ml/min split-flow; valve opening 30 sec after injection) with helium as the carrier gas (50 cm/sec at 120°C) was used. The temperature program began at 120°C, ramping at 10°C/min to 140°C, then ramping at 2°C/min to 280°C and holding for 10 min. The chemical identity of the peaks was determined using a gas chromatography–mass spectrometry system equipped with a CP Sil 5CB or a polar CP-Wax 58 FFAP column as previously described (Everaerts et al. 2010). The amount (ng/insect) of each component was calculated based on the readings obtained from the internal standards. The overall sums of all the CHs (ΣCHs) and of the principal CH groups were noted: desaturated CHs (ΣDesat), linear saturated CHs (ΣLin) and branched CHs (ΣBranch). We also show the ratio between ΣDesat and ΣLin (D/L ratio). Ten to 20 flies were analyzed per sex. CH nomenclature is provided in Table S1.

Although the complete cuticular hydrocarbon profile was analyzed in all flies, for the sake of clarity, in most cases, we only show the predominant CHs (and their ratio) diverging between M and Z strains. This simplified CH analysis allowed us to screen in real time during successive generations, the CH profiles of many ZW-BC lines simultaneously. However, the complete CH profiles of parental strains (Z30, Di/w, Cs) and of the two stable ZW-Isop lines (1W14, 3W1) are shown (Table S1).

**Gene expression**

To measure the relative amount of the five desat1 transcripts (RA, RC, RE, RB and RD) and of the desat2 transcript, we first extracted RNAs from 30 whole 5-day-old flies using the Trizol method (GIBCO BRL) and RNase-free DNase treatment to avoid contamination by genomic DNA (Bogart and Andrews 2006). Total RNA (2µg) was reverse transcribed with the iScript cDNA Synthesis Kit (Biorad). Quantitative 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 20µl volume, by 40 cycles (95°C for 30 sec, TM°C for 30 sec and 72°C for 30 sec), preceded by a 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 (Houot et al. 2010). Each reaction was triplicated and the mean was calculated using three independent biological replicates. All results were normalized to the Actine5C mRNA level.

**Statistics**

All statistical analyses were performed using XLSTAT 2012 (Addinsoft 2012). Frequencies were compared using a Wilks G² likelihood ratio test completed with a computation of significance by cell (Fisher’s test). Comparison of fecundity, copulation latency and duration was carried out with a Kruskall-Wallis test with Conover-Iman multiple pairwise comparisons ($P = 0.05$, with a Bonferroni correction). CH and/or behavioral data were analyzed using Principal Components Analysis (PCA; type Pearson’s correlation matrix) with standardized values. For qPCR analysis, significant differences in transcript levels ratio between control and sample strain were detected with the Relative Expression Software Tool (REST, REST-MCS beta software version 2; Pfaffl 2001) where 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 Randomization Test©; Pfaffl et al. 2002).

**Data availability**

The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article and its tables and figures. All raw data are available in the annexed “Data_Set.xlsx” file. All supplementary information is included in the 9 Supplemental figure files. Supplemental material available at FigShare: https://doi.org/10.25387/g3.8230493.

**RESULTS**

**F0 and F1 flies phenotypes**

Our genetic procedure consisted to progressively introgress the genome of a *D. melanogaster* wild-type strain collected in Zimbabwe (Z30 = Z) into the genome of a wild-type strain from Dijon (Di2) representative of M strains and carrying the white mutation (w; Di/w; Figures 1 & S1; see Material and methods). We first measured the (i) cuticular pheromones and (ii) mating phenotypes in males and females of parental strains (F0 = Z30, Di/w, and Canton-S = Cs) and in the F1 progeny resulting of their reciprocal crosses (Z30 x Di/w; Z30 x Cs; Di/w x Z30; Cs x Z30; all crosses are shown as “females x males”).

For the sake of clarity, our pheromone analysis was mostly based on the measure of the 7T/5T ratio (T-ratio) in males and of the 7,11HD/5,9HD ratio (HD-ratio) in females. Both T- and HD-ratio showed much lower values in Z30 male and female flies (1.24 ± 0.02 and 0.20 ± 0.01, respectively) compared to M-type flies (for Cs: 14.85 ± 0.34 and 31.31 ± 0.63; for Di/w: 9.62 ± 0.18 and 8.47 ± 0.09, respectively). The absolute amounts (in ng) of these compounds, as well as the complete hydrocarbon profile of parental strains, are provided in supplemental information (Table S1).

Our mating experiments always involved pairs of flies (female x male; Figure 2, Table S2). Since the w mutation carried by Di/w and ZW flies causes a visual defect inducing delayed mating latency, our behavioral observations lasted 2 hr. In parallel, and to control for a potential effect induced on mate discrimination by the w mutation, we also tested a second M-type strain with red eyes: the wild-type Canton-S strain (Cs). In the parental crosses, Z30 females showed, as expected, a contrasted mating frequency, which was very low with both M-type males (4% with Cs; 0% with Di/w) and high with Z30 males (74%). Differently, both M-type females frequently mated with all males (60–98%), except in Cs x Di/w pairs (38%). F1 females more frequently mated with Z30 males (69–85%) and with Cs males (59–83%). With Z30 and Cs females, most F1 males showed very high mating frequencies (73–100%) except for w F1 “Di/w x Z30” males with Z30 females (38%). Based on the fact that the F1 females mated with both types of males, this suggests that the female preference is co-dominant.

Both the fertility (ability to leave progeny) and fecundity (number of adult progeny left) were measured in F0 pairs according to their copulation status (mating vs. not mating during the 2-hour test). While fertility and fecundity were not affected by copulation status ($r$ range: -0.046 to 0.045; $P = NS$), both parameters were highly correlated in pairs mating within 2 hr or in the following 24h ($r = 0.689$ and 0.828, respectively; $P < 0.05$; Fig. S2). Z30 females showed a much higher fertility with Z30 males (74%) than with Cs and Di/w males (39 and 12%, respectively; $G^2$ Wilks $(10df) = 234.84$ whereas M-type females always showed a very high fertility regardless of the male strain (88–98%) (Table S3). Moreover, Z30 x Z30 pairs copulating during the 2 hr period showed a much lower fecundity (median value = 20 adults) compared to all other pairs involving M-type females (50–90 adults; $KW_{(10df)}=70.03; P < 0.0001$; Fig. S3).

**Selection of ZW-BC1 lines**

As mentioned above, the progeny of the “Di/w x Z30” cross was mated following the genetic procedure designed to establish ZW-BC1 lines.
with white eyed flies (w; see supra; Fig. S1 and Material and methods section).

We obtained 21 ZW-BC1 lines in which we measured during the “A&S session #1”: (1) male and female cuticular pheromones and (2) ZW male copulation with Z30 and Cs females. In particular, the T- and HD-ratio were analyzed during 3 successive generations (F1, F2, F3; see “BC1” in Figure 3). Given time limitation, we could only analyze one or two fly pool(s) per generation (pool size = 3-11 for males; 2-6 for females).

The T-ratio showed a wide variation in F1 (1.4-19.0), F2 (2.3-18.5) and F3 males (3.4-20.1). Similarly, the HD-ratio varied in F1 (0.1-47.9), F2 (0.1-4.9) and F3 females (0.2-21.1). Despite the presence of some extreme data points, the variability of both T- and HD-ratios tended to decrease between generations (Figs. S4 and S5A, respectively). A significant positive correlation between both T- and HD-ratio was found in F1 (r = -0.60; P = 0.03), but not in F2 and F3 flies (r = 0.48; P = 0.10, and 0.29; P = 0.21, respectively; Fig. S5B). This effect may partly explained by the negative correlation between absolute amounts of 7,11HD and...
5.9HD, which were higher in F1 (r = -0.77; P = 0.002) than in F2 and F3 flies (r = -0.46; P = 0.12, and -0.50; P = 0.026, respectively). Similarly, but to a lesser extent, the amounts of C5- and C7-desaturated CHs showed higher level of correlation in F1 flies than in F2 and F3 flies (Fig. S6). The values for T- and HD-ratios are shown in Table S4.

While no F2 male copulated with Z30 females (within two hours), their mating frequency was highly variable with Cs females (between 9.8% for #1 line to 70.2% for #2 line; Table S5). However, given that these mating data were not enough discriminant between lines, we selected the three best ZW-BC1 lines only based on their CH phenotypes. Therefore, the #1, #3, #12 lines showing the lowest and most stable T- and HD-ratio values (especially in F3 flies) were used to initiate the BC session #2 (BC2).

In summary, this first phenotyping round (ZW-BC1), involving 4 backcrosses between M-type females and Z30 male hybrids (Z/M), yielded ZW males which did not yet acquire enough Z-like characteristics to be accepted by Z females and ZW females not accepting M-type males. We therefore selected 3 ZW-BC1 lines based on their T-ratio phenotypes close to Z-type (i.e., Low T-ratio).

Selection of ZW-BC2 lines
ZW-BC1 flies of #1, #3 and #12 lines reciprocally mated to Z30 parents yielded 6 ZW-BC2 main lines (#1, #3 and #12, resulting of Z30 x ZW-BC1 crosses; #B1, #B3 and #B12, from ZW-BC1 x Z30 crosses). Each of these six main lines was subdivided into five sublines (labeled A to E; 1A, 1B, 1C, 1D, 1E; ... B12D, B12E) representing a total of 30 ZW-BC2 sublines.

During the “A86” session #2, both the CH profile and mating performance were analyzed in F1, F2 and/or F3 flies. After screening both CH (on pools) and mating phenotypes (see below) in F1 flies, we only kept the 15 sublines derived from the #1, #3 and #B3 ZW-BC2 lines [1A-1E; 3A-3E; B3A-B3E; Figures 3 & 4; the 15 sublines deriving from the three other main lines, #B1 (B1A-B1E); #12 (12A-12E); #B12 (B12A-B12E), were further discarded]. In most of the 15 sublines, the HD-ratio in F1 and F3 females was relatively low and stable (often < 1.5; range = 0.2-3.0), whereas the T-ratio showed a broader variability between and within sublines (for F1: 1.2-10.3; F2: 2.8-15.6; F3: 3.1-12.3; “BC2” in Figure 3). The relatively high correlation value between T- and HD-ratios in F1 flies (r = 0.60; P = 0.03; Fig. S5B) strongly decreased in F2 flies (r = 0.29; P = 0.21). Such decrease maybe due to the low correlation between 7.11HD and 5.9HD and/or null correlation between 7T and 5T in F2 flies (Fig. S6). The values for T- and HD-ratios are shown in Table S6.

The mating performance of ZW-BC2 flies with M-type and Z30 flies substantially varied between lines (Figure 4; Table S7). At F1 generation, ZW x Cs pairs showed higher mating frequency in #1 (average = 29.7% — 36.4%, #3 (25.4%; 9.1–56.6%) and #B3 (20.0%; 8.3–33.3%). At F3 generation, most Z30 x ZW pairs showed an increased mating frequency (#1 = 29.7% — 25.0–33.3%; #3= 42.5% — 33.3–56.3%; #B3 = 25.6% — 12.5–43.8%).

At F2 generation, a highly significant negative correlation was detected between the T-ratio of ZW males and their mating frequency with Z30 females (r = -0.71; P = 0.004; Fig. S7A). Moreover, the copulation frequency in these pairs was negatively correlated with their copulation latency (Fig. S7B). However, no other significant correlation was found between any other CH-ratio and mating parameter in Z30 x ZW pairs (F2 and F3), or in ZW x Cs pairs (F1 and F2; Fig. S7). Based on these data, and as indicated above, we kept the 15 sublines derived from the #1, #3 and #B3 main ZW-BC2 lines to initiate the backcross series #3 (ZW-BC3; Figure 1).

In summary, after the second round of backcrossing of these 3 ZW-BC1 lines, some ZW-BC2 females did not mate with M-type males indicating that they took Z-like qualities, while ZW-BC2 males started to mate with Z females. Therefore, we selected 15 ZW-BC2 sublines showing the best Z-like phenotypes (mating and CHs: 1A-1E; 3A-3E; B3A-B3E).

Selection of ZW-BC3 lines
Backcrossing ZW-BC2 females of the 15 selected sublines to Z30 males yielded 65 ZW-BC3 lines. More precisely, single females yielded by the last “BC3” generation were individually mated with 2-3 sibling males to initiate BC3-ZW isofemale sublines (labeled with I-VII roman numerals for each BC2 “mother” line). Each subline was first characterized by its T-ratio, given that this parameter seems to reflect best the proportion of “Z30” genome in ZW recombinant lines. The T-ratio was first measured (at F1 and F2) in two fly pools and subsequently—but only in lines showing a low T-ratio—characterized in individual males (at F3 and F4; “ZW-BC3” in Figure 3). When the four F1-F4 generations were considered, the T-ratio remained constantly low in only two sublines (#1BIII and #3BIII; Table S8) while others produced at least some individuals (or pools) with higher T-ratio (≥5.0). The HD-ratio remained low in several ZW-BC3 sublines including #1BIII and #3BIII, at least until F4 (≤5.0; Figure 3; Table S9).

No significant “T-ratio/HD-ratio” correlation was found in F2 or F3 flies, despite a slight increase between these generations (r = 0.04; P = 0.89, r = 0.25; P = 0.43, respectively; Fig. S5B). This variation may be due to the increased correlation between the absolute amounts of 7.11HD and (i) 5.9HD or (ii) 5T (both negative) or (iii) 7T (positive) in F3 flies compared to F2 flies (Fig. S6).

We measured the mating performance of F1 and F2 flies showing the best Z30-like CH-ratio (#1BIII, #1CIV, #1DIII, #1DIV, #1EIII and #3BIII “BC3” in Figure 4). At the F1 generation, #1BIII and #3BIII females did not copulate with Cs males (differently from the four other ZW-BC3 females), while #1BIII and #3BIII males substantially mated with Z30 females. At F2, #1BIII and #3BIII females showed a 2-like sexual discrimination: no copulation (within 2 hr) with Cs males and a high mating frequency with Z30 males (62 and 77%, respectively; Figure 4; Table S10). The mating frequency within, and between, the two ZW-BC3 lines was relatively low. While no correlation was detected in F3 ZW-BC3 x Cs pairs between any mating parameter and CH-ratio (Fig. S7A), a significant correlation was found between their copulation frequency and latency (Fig. S7B).

In summary, after this third round of backcrossing, we selected to ZW-BC3 sub-lines (#1BIII and #3BIII) based both on their Z-like CH profiles and mating discrimination.

Selection of isoparental lines (ZW-Isop)
To reduce as much as possible the potential intra-line genetic variability, pairs of ZW-BC3 flies were mated to initiate 20 inbred lines (one female x one male, called “isoparental” or Isop) derived from each #1BIII and #3BIII line (ZW-Isop: 1W1-1W20 and 3W1-3W20, respectively). In each 1W and 3W line, the “F1 to F4” stability of the T-ratio was assessed using individual males (“ZW-Isop” in Figure 3; Table S11). Based on these data, only four 1W isoparental sublines (#1, 2, 6 and 14) and four 3W isoparental sublines (#1, 13, 15 and 16) were kept for subsequent testing.
In these 8 ZW-IsoP sublines, F6 and/or F7 females showed a low HD-ratio ($r < 0.3$; Table S12) whereas the T-ratio diverged between lines. Based on these data, we only retained the “1W14” and “3W1” lines, which showed a stable T-ratio until F9. A great similarity for the complete CH profiles was found between 1W14, 3W1 flies and parental Z30 flies, in both sexes (Table S1). Our most recent analysis carried out with F75 flies show a very stable profile (data not shown).

Simultaneous testing of the 8 ZW-IsoP lines (F6–F7) revealed a weak correlation between T- and HD-ratio ($r = 0.12$, $P = 0.79$) caused by the opposite correlation between 1W and 3W lines (respectively, $r = -0.65$; $P = 0.35$ and $r = 0.54$; $P = 0.46$) (Fig. S5B). The correlation between the absolute amounts of single CHs was relatively low and not significant between 5T and either 7,11HD ($r = 0.33$; $P = 0.42$) or 5,9HD ($r = 0.35$; $P = 0.40$; Fig. S6). However, a very high correlation was found within the 3W1 line (but not within the 1W14 line) between 7- and 5T ($r = 0.64$; $P = 0.01$) and between 7,11- and 5,9HD ($r = 0.71$; $P = 0.002$).

F6 and F7 ZW-IsoP females showed a high mating discrimination: they rarely mated with Cs males (0–10%) and much more frequently with Z30 males (44–90%; “IsoP” in Figure 4). F7 ZW-IsoP males moderately mated with Z30 females (11–36%) or with F7 ZW-IsoP sibling females (6–28%; Table S13).

We performed a further cross-examination of CH profile with mating performance in ZW-IsoP lines using Principal Component Analysis (PCA; Fig. S9A). This revealed that while (i) both T- and HD-ratios were not correlated together ($r = 0.026$), (ii) the copulation frequency of Z30 x ZW-IsoP pair was correlated with HD-ratio ($r = 0.524$), (iii) the
copulation frequency of both ZW-IsoP x ZW-IsoP and ZW-IsoP x Cs pairs was correlated with the T-ratio \( r = 0.420 \) and 0.414, respectively, but (iv) the copulation frequency of ZW-IsoP x Z30 was not correlated with any CH ratio \( r = -0.041 \) and -0.075).

We also determined the fertility and fecundity of F6- and F7 ZW-IsoP flies used in mating tests. ZW-IsoP females showed higher fertility with Z30 males (65–92%) than with Cs males (16–32%; Table S14) whereas ZW-IsoP male fertility was high with both Z30 or ZW-IsoP females (71–85% and 78–100%, respectively). This suggests that mating events occurring after the two-hours observation period were more frequent in ZW-IsoP x ZW-IsoP and ZW-IsoP x Z30 pairs than in ZW-IsoP x Cs pairs.

The fecundity, measured with regard to the genotypes and mating status of pairs (Fig. S8), only revealed slight difference: ZW-IsoP x Z30 and Z30 x ZW-IsoP fertile pairs left around 20 and 30 adult progeny per cross, respectively with no difference between IsoP lines. A slight effect of mating status was detected in ZW-IsoP x Z30 pairs: copulating pairs showed a slightly higher fecundity than non-copulating pairs \( U = 3588, P = 0.037 \). ZW-IsoP x ZW-IsoP fertile pairs generally produced a low progeny number (<20), but no effect was detected between lines.

**desat gene expression in IsoP lines**

The transcriptional profiles of the desat1 and desat2 genes were compared between the three F0 lines and the 1W14 and 3W1 lines (sampled at F8 and F9 generations). More precisely, we measured the level of the five desat1 transcripts (RA, RC, RE, RB and RD) and the unique desat2 transcript, in mature flies of both sexes. Males and females of the Z-type lines (Z30, 1W14 and 3W1) showed significantly higher levels of the desat2 transcript compared to both M-type parental strains (Cs; Di/w as shown in the baseline of Figure 5). The desat1 transcripts showed much less interstrain difference. In both sexes, the three Z-type lines showed a similar—although reduced—variation compared to M-type strains. The only notable exception was detected for the RE transcript, which decreased (i) in Z-type females compared to M-type females and (ii) in Cs males compared to the four other males.

**DISCUSSION**

The experimental introgression of a Z genome into a white-labeled M genome allowed us to follow, during many generations, the evolution of the relationship between several sex specific traits diverging between *D. melanogaster* populations. In particular, we found that cuticular pheromones and female sexual receptivity—two sex specific traits potentially involved in incipient speciation—depend of distinct sets of genes. After more than 20 generations of introgression and 10 generations of experimental selection, we obtained two white-eyes lines (1W14 and 3W1) showing the principal Z-type characters: female sexual discrimination, male copulation with Z30 female, male and female desaturated CHs, fecundity and desat1-desat2 gene expression.

Based on our data, the chronological scenario of the events involved in this specific case of incipient speciation remains hypothetical. Together with previous reports, our data indicate that Z- and M-type strains diverge for several aspects involved in both pre- and postzygotic isolation. Postzygotic isolation is revealed by the lower fertility and fecundity found in Z30 and ZW-IsoP pairs. This is supported by other studies (Alipaz et al. 2001). The prezygotic isolation is reflected by the positive correlation between mating frequency and HD-ratio. More specifically, high 7,11HD level was positively correlated with mating frequency, while increased (thus delayed) copulation latency was correlated with high levels of 5,9HD (Figure 6). However, while increased levels of the two HD isomers induced reciprocal effect on both copulation parameters, the HD-ratio variation was only correlated with the frequency of copulation but not with its latency (Fig. S9B). This apparent conundrum suggests that the behavioral effect induced by both HD isomers does not only depend on their absolute amounts per se but also on their relative contribution to the pheromonal bouquet, this determining their ratio. Other studies have shown that the ratio between two compounds with potential pheromonal effect, or their relative contribution to a more complex bouquet, can strongly influence insect ability to discriminate between closely related individuals, colonies, sub-species or species (Klun and Maini 1979; Collins and Cardé 1985; Adams and Holt 1987; Oguma et al. 1992; Ferveur and Sureau 1996; Ayasse et al. 1999; Marcillac et al. 2005c; Lin et al. 2010; Van Zweden and D’ettorre 2010). Given that the correlation between the absolute amounts of the two HD isomers tended to decrease during the introgression procedure (except in ZW-BC3-F3; Fig. S6), this suggests that the production of these compounds does not only depend of desat2, which may nevertheless exerts a major influence on the HD-ratio (Coyne et al. 1999).
Differently, no clear relationship was detected between the mating ability and either the absolute amounts of two principal tricosene isomers (7T, 5T; Figure 6) or their ratio (T-ratio; Fig. S9B). This may be due to the fact that our global statistical analysis including all ZW-BC sessions masked more subtle effects. For instance, during the introgression procedure the sign of the correlation between the absolute amounts of 7T and 5T changed: it was negative in F1 ZW-BC1 (r = -0.40) and became positive in IsoP lines (r = 0.25; Fig. S6). Despite the absence of a global significant correlation, our genetic procedure largely based on the selection of "low T-ratio" in ZW males, allowed us to isolate two stable IsoP lines combining the w mutation with all main Z-like sex specific phenotypes. This suggests that some of the genes (with Z-type alleles) involved in the low T-ratio and in mate discrimination are closely linked. This suggests that these hypothetic "low T-ratio" coding genes could also affect non-pheromonal Z male traits (sensory signals, behavioral postures) together with traits underlying female receptivity to Z male traits.

Several D. melanogaster studies revealed the involvement of single genes in female sexual discrimination and receptivity (Suzuki et al. 1997; Carhan et al. 2005; Ditch et al. 2005; Juni and Yamamoto 2009; Sakai et al. 2009). This indicates that accurate female discrimination of male characters depends of many genes organized in networks (Greenspan 2001; Ferveur 2005; Houot et al. 2012). Therefore, any alteration in each of these genes could induce detrimental effect on female mating behavior. Most of these genes may determine the production or the reception of each of the multiple sensory signals reciprocally exchanged during the courtship interplay by the two partners (Markow 1987; Arienti and Jallon 1991; Welbergen et al. 1992; Lasbleiz et al. 2006; Krstic et al. 2009). Taken together, these observations indicate that Z30 female discrimination depends of multiple genes shaping her accurate ability to perceive and integrate, during different phases of courtship, the multiple male sensory signals. Based on this, we believe that the origin of assortative mating between Z- and M-type females is linked to their divergent perception and/or integration of male cues. Indeed, while Z females only respond to a precise and complete multisensory set of signal provided by homotypic males (Grillet et al. 2012; Grillet et al. 2018), M-type females only need part of these cues to be sexually receptive (Colegrave et al. 2000; Grillet 2009; Ma et al. 2010; Grillet et al. 2012; Grillet et al. 2018).

Our hypothesis that genes underlying the production of cuticular pheromones and mating responses at least partly differ, is also supported by the divergent dominance status of their Z- and M-type alleles: they both semi-dominantly control the production of C5- and C7-desaturated CHs (Figure 2; Table S1) while M-type alleles dominantly act over Z-type alleles with regard to female discrimination (Figure 2). Our data also reveal that the production of C5- and C7-desaturated CHs depends on a distinct genetic determination between the sexes: a stable Z-like HD-ratio was obtained after a relatively low number of generations in ZW-BC females while it took many more generations to stabilize a Z-like T-ratio in ZW-BC males. As a consequence, the variation between HD- and T-ratios was not correlated through the introgression process. This indicates that the production of C5- and C7-desaturated CHs at least partly depends of a sex specific control: the HD-ratio mostly—but not only (see above)—depends of the desat2 gene (Coyne et al. 1999), while the T-ratio may additionally depends on many other genes. Some of the "low T-ratio coding genes" diverging between Z and M populations, and potentially involved in sexual isolation, maybe influenced by mating activity (Hollocher et al. 1997; Ting et al. 2001; Kauer and Schlötterer 2004; Michalak et al. 2007). Indeed, two genes detected in the later study (CG12400; Cypp4p2) code for enzymes potentially involved in hydrocarbon biosynthesis (Jallon 1984; Qiu et al. 2012). The hypothesis of a sex specific control for the production of C5- and C7-desaturated CHs isomers is also supported by the dissociation of their production between the sexes in West-African strains (Tai strain females predominantly produce 5,9HD, while Tai males produce 7T and 7P; Pechine et al. 1985; Ferveur et al. 1996; Sureau and Ferveur 1999).
We still do not understand how Z strains have been able to survive in nature given all their potentially disadvantageous reproduction-related characters compared to M strains which have spread all over our planet. The Z-type populations may be very strictly adapted to local ecological conditions only found in Zimbabwe forests. Based on their genetic diversity and the simultaneous expression of both desat1 and desat2 genes, Zimbabwe populations have been proposed to represent ancestral D. melanogaster populations from which M-type populations have derived. Then, during their global expansion on earth (maybe in relation with human migration; David and Capy 1988), M populations may have progressively adapted their chemosensory perception system together with other non-chemosensory perception systems (Arguello et al. 2016).

In conclusion, the present study offers a “real-time measure” of the evolution of several sex-specific traits potentially involved in sexual isolation. Our data provide a statistic view on their relative involvement and also on their inter-sex relationship. We are now planning to introduce transgenes targeting chemosensory genes (including desat1) into the genome of our two stable ZW lines to further dissect the mechanisms underlying the incipient speciation process between D. melanogaster populations to better understand the recent evolution and worldwide adaptation of this species.

ACKNOWLEDGMENTS

We want to thank two anonymous referees for their extensive work to improve the ms. This work was partly funded supported by the Centre National de la Recherche Scientifique (INSB), the Burgundy Regional Council (PARI 2012), the Université de Bourgogne, the ANR (Gustaille) and the CONICYT (MEC 80140013).

LITERATURE CITED

Adams, T. S., and G. G. Holt, 1987 Effect of pheromone components when applied to different models on male sexual behavior in the housefly, Musca domestica. J. Insect Physiol. 33: 9–18. https://doi.org/10.1016/0022-1910(87)90099-0

Addinso, 2012 XLSTAT 2012, Data analysis and statistics with Microsoft Excel, pp. Addinso, Paris, France.

Alipaz, J. A., C. Wu, and T. L. Karr, 2001 Gametic incompatibilities between races of Drosophila melanogaster. Proc. Biol. Sci. 268: 789–795. https://doi.org/10.1098/rspb.2000.1420

Andersson, M., 1994 Sexual Selection, Princeton University Press, Princeton.

Arguello, J. R., M. Cardoso-Moreira, J. K. Grenier, S. Gottipati, A. G. Clark et al., 2016 Extensive local adaptation within the chemosensory system following Drosophila melanogaster’s global expansion. Nat. Commun. 7: ncomms11855. https://doi.org/10.1038/ncomms11855

Arienti, M., and J. M. Jallon, 1991 Intrasepecific Variability Of Drosophila Chemical Signals. J. Neurogenet. 7: 115–116.

Arnqvist, G., 1998 Comparative evidence for the evolution of genitalia by sexual selection. Nature 393: 784–786. https://doi.org/10.1038/31689

Ayasse, M., W. Engels, G. Lübke, T. Taghizadeh, and W. Francke, 1999 Mating expenditures reduced via female sex pheromone modulation in the primitive eusocial halitcine bee, Lasiosglumus (Eulaeus) malachurum (Hymenoptera: Halictidae). Behav. Ecol. Sociobiol. 45: 95–106. https://doi.org/10.1007/s002650050543

Begun, D. J., and C. F. Aquadro, 1993 African and North American populations of Drosophila melanogaster are very different at the DNA level. Nature 365: 548–550. https://doi.org/10.1038/365548a0

Bogart, K., and J. Andrews, 2006 Extraction of total RNA from Drosophila. CGB Technical Report 10.

Bousquet, F., I. Chauvel, J. Flaven-Pouchon, J.-P. Farine, and J.-F. Ferveur, 2016 Dietary rescue of altered metabolism gene reveals unexpected Drosophila mating cues. J. Lipid Res. 57: 443–450. https://doi.org/10.1194/jlr.M064683

Bousquet, F., T. Nojima, B. Houot, I. Chauvel, S. Chaudy et al., 2012 Expression of a desaturase gene, desat1, in neural and nonneural tissues separately affects perception and emission of sex pheromones in Drosophila. Proc. Natl. Acad. Sci. USA 109: 249–254. https://doi.org/10.1073/pnas.1109166108

Butlin, R. K., and M. G. Ritchie, 1989 Genetic coupling in mate recognition systems - what is the evidence. Biol. J. Linn. Soc. Lond. 37: 237–246. https://doi.org/10.1111/j.1095-8312.1989.tb01902.x

Carhan, A., F. Allen, J. D. Armstrong, S. F. Goodwin, and K. M. C. O’Dell, 2005 Female receptivity phenotype of icebox mutants caused by a mutation in the L1-type cell adhesion molecule neuroglian. Genes Brain Behav. 4: 449–465. https://doi.org/10.1111/j.1601-183X.2004.00117.x

Colegrave, N., H. Hollocher, K. Hinton, and M. G. Ritchie, 2000 The courtship song of African Drosophila melanogaster. J. Evol. Biol. 13: 143–150. https://doi.org/10.1046/j.1420-9101.2000.00148.x

Collins, R. D., and R. T. Cardé, 1985 Variation in and heritability of aspects of pheromone production in the pink bollworm moth, Pectinophora gossypiella (Lepidoptera: Gelechiidae). Ann. Entomol. Soc. Am. 78: 229–234. https://doi.org/10.1093/aesa/78.2.229

Coyne, J. A., and H. A. Orr, 2004 Speciation, Sinauer Associates Inc., Sunderland, MA.

Coyne, J. A., C. Wicker-Thomas, and J. M. Jallon, 1999 A gene responsible for a cuticular hydrocarbon polymorphism in Drosophila melanogaster. Genet. Res. 73: 189–203. https://doi.org/10.1017/S0016672398003723

Dallarac, R., C. Leubre, J. M. Jallon, D. C. Knippie, W. L. Roelofs et al., 2000 A Delta 9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 97: 9449–9454. https://doi.org/10.1073/pnas.150243997

Darwin, C., 1871 The Descent of Man, and Selection in Relation to Sex, John Murray, London, England.

David, J. R., and P. Capy, 1988 Genetic variation of Drosophila melanoget natural populations. Trends Genet. 4: 106–111. https://doi.org/10.1016/0168-9525(88)90098-4

Ditch, L. M., T. Shirangi, J. L. Pittman, K. L. Latham, K. D. Finley et al., 2005 Drosophila retained/dead ringer is necessary for neuronal patb-finding, female receptivity and repression of fruitless independent male courtship behaviors. Development 132: 155–164. https://doi.org/10.1242/dev.01568

Eberhard, W. G., 1993 Evaluating models of sexual selection - Genitalia as a test-case. Am. Nat. 142: 564–571. https://doi.org/10.1086/285556

Eversaets, C. J. P. Farine, M. Cobb, and J. F. Ferveur, 2010 Drosophila cuticular hydrocarbons revisited: mating status alters cuticular profiles. PLoS One 5: e9607. https://doi.org/10.1371/journal.pone.0009607

Fang, S., A. Takahashi, and C. I. Wu, 2002 A mutation in the promoter of desaturase 2 is correlated with sexual isolation between drosophila behavioral races. Genetics 162: 781–784.

Fang, S., C. T. Ting, C. R. Lee, K. H. Chu, C. C. Wang et al., 2009 Molecular evolution and functional diversification of fatty acid desaturases after recurrent gene duplication in Drosophila. Mol. Biol. Evol. 26: 1447–1456. https://doi.org/10.1093/molbev/msp057

Ferveur, J. F., 2005 Cuticular hydrocarbons: Their evolution and roles in Drosophila pheromonal communication. Behav. Genet. 35: 279–295. https://doi.org/10.1007/s10519-005-3220-5

Ferveur, J. F., M. Cobb, H. Boulkela, and J. M. Jallon, 1996 World-wide variation in Drosophila melanogaster sex pheromone: Behavioural effects, genetic bases and potential evolutionary consequences. Genetica 97: 73–80. https://doi.org/10.1007/BF00132583

Ferveur, J. F., and G. Sureau, 1996 Simultaneous influence on male courtship of stimulatory and inhibitory pheromones produced by live sex-mosaic Drosophila melanogaster. Proceedings of the Royal Society Biological Sciences Series B 263: 967–973. https://doi.org/10.1098/rspb.1996.0143

Green, M. M., 1996 The “Genesis of the white-eyed mutant” in Drosophila melanogaster: a reappraisal. Genetics 142: 329–331.

Greenspan, R. J., 2001 The flexible genome. Nat. Rev. Genet. 2: 383–387. https://doi.org/10.1038/35072018
