A Mutation in the Promoter of Desaturase 2 Is Correlated With Sexual Isolation Between Drosophila Behavioral Races

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ABSTRACT

Examples of genes governing behavioral isolation are scarce. Here we report that a regulatory mutation in desaturase 2, known to determine a pheromonal polymorphism in *Drosophila melanogaster* females, may be such an example. This mutation is strongly correlated with the mating pattern between the Zimbabwe and cosmopolitan races of this species. These two behavioral races appear to be at the incipient stage of speciation. The desaturase 2 mutation may be one of the many loci underlying the behavioral differences between the two races.

SOME progress has been made toward the molecular elucidation of "speciation genes" in the last decade, mostly in systems of gamete recognition (Swanson and Vacquier 1998) or spermatogenesis (Ting *et al.* 1998, 2000; see Wu 2001 for a review). However, no study appears to have successfully identified genes governing behavioral isolation between natural populations or species. A possible "entry point" may be genes controlling the production of cuticular hydrocarbons (CHs), which are contact pheromones in Drosophila. It has been shown that interspecific differences in the CH profile of female Drosophila play a large role in male mate choice (Coyne *et al.* 1994). Unfortunately, CH differences between species have a complex genetic basis (Coyne and Charlesworth 1997) and cloning the underlying genes may not be straightforward.

It may be easier to unravel the genetic basis of CH differences between conspecific behavioral races, which are at the incipient stage of sexual isolation. Females of many *Drosophila melanogaster* lines from southern/central Africa (Zimbabwe- or Z-type flies) do not mate with males from other continents (M type, for common *m*elanogaster) in double-choice experiments (Wu *et al.* 1995; Hollocher *et al.* 1997a). Although the Zimbabwe and worldwide populations are still at an incipient stage of speciation, the number of genes controlling the mating behavioral differences exceeds 15 (Hollocher *et al.* 1997b; Ting *et al.* 2001).

African and non-African *D. melanogaster* females are also known to have different CH profiles (Ferveur *et al.* 1996). In Africa and the Caribbean, most females carry the contact pheromone 5,9 heptacosadiene (5,9 HD). In the rest of the world, the major cuticular CH on females is 7,11 heptacosadiene (7,11 HD). We and others have previously determined the genetic basis of this pheromonal difference in females (Dallerac *et al.* 2000; Takahashi *et al.* 2001). In most African and Caribbean flies, an active desaturase gene (*desat2*) is responsible for the production of 5,9 HD. In non-African flies, a 16-bp deletion in the promoter region of *desat2* has led to its inactivation and, as a result, female flies produce predominantly 7,11 HD. We shall refer to the active form of *desat2* as wild type (+) and the deletion mutation as the "D" type. The association between the +/D genotype and the 5,9 HD/7,11 HD phenotype is complete among >40 lines measured (Takahashi *et al.* 2001).

The main question addressed here is whether the *desat2* gene is one of the many loci underlying the mating behavioral differences between the Z- and M-type flies. Since one of the many female mating loci in the Z/M system of *D. melanogaster* has been crudely mapped to the *desat2* region (Ting *et al.* 2001), a positive answer is plausible. In addition, wing vibration, a key element of male courtship, is most efficiently induced by 7,11 HD. The intensity of wing vibration by Canton-S males was a function of the quantity of 7,11 HD on females (Antony and Jallon 1982; Antony *et al.* 1985; Ferveur and Sureau 1996) and 7,11 HD-rich females induce mating by M-type males more readily than 5,9 HD-rich females (Ferveur *et al.* 1996). Two lines of observations have been held against the hypothesis of an association between the Z/M behavioral differences and the CH profile on females. First, the Caribbean lines have the 5,9 HD (Ferveur *et al.* 1996) but are behaviorally of the M type. Second, CH rub-off experiments do not have a detectable effect on female mating behavior (Coyne *et al.* 1999). Nevertheless, it is prudent not to prematurely

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reject the hypothesis on the basis of these observations. In association studies of complex trait mapping, comparisons should not be made across divergent populations due to linkage disequilibrium between the candidate locus and background variation (Lander and Schork 1994). Thus, the Caribbean flies could have a component of the Z-type behavior that is associated with the *desat2* allele but the overall behavior is masked by their M-type genetic background. The rub-off experiments essentially attempted to convert typical M-type females into Caribbean-like females.

To answer the question about the association (or lack of) between the *desat2* gene and the Z/M behavior more definitively, we have incorporated the above considerations in a different experimental design. First, we used a double-choice design to include all behavioral components (male choice, female choice, and interactions). Second, we compared lines from the same populations. Although the 7,11 HD type (or the D *desat2* allele) is nearly fixed outside of Africa (except in the Caribbean), the + and D alleles segregate at appreciable frequencies in several African populations. In this study, we compared 22 lines from all three populations known to be commonly polymorphic for the *desat2* alleles for their mating pattern.

MATERIALS AND METHODS

Fly stocks: The Zimbabwe isofemale lines were collected from the Sengwa Wildlife Reserve around 1990 (the ZS lines) and near Harare (the ZH lines). The Luangua (LA) lines were from southern Zambia and the Okavanga (OK) lines were from Botswana. These are described in Hollocher *et al.* (1997a). We include all of the lines used in Hollocher *et al.* (1997a) with the exception of ZH16, ZH33, ZH34, ZH42, and LA69. (These lines were excluded because they are polymorphic for the *desat2* allele. The polymorphism complicates the interpretation of the behavioral measurements as correlants of the *desat2* genotype.) One isofemale line from Zimbabwe, Z30, and one from France, FrV3-1 (Fr), were chosen as the standard Z and M lines, respectively.

DNA genotyping: The genotype at the *desat2* locus was determined by the presence or absence of a 16-bp deletion ~150 bp upstream from the *desat2* coding sequence (Takahashi *et al.* 2001). The determination was done by PCR followed by restriction fragment length polymorphism analysis.

Measurement of mating behavior: We use a double-choice mating design, which measures multiple components of mate choice. Briefly, 55–65 females of the tested line and the same number of M-type females (the Fr line; Hollocher *et al.* 1997a,b) were released into a cage containing 55–65 males of a strong Z line and the same number of males from the M line. All flies were fed colored food prior to the experiment and the copulating pairs were aspirated out of the cage for identification by food coloring in the gut.

We use the discrimination index (DI; Wu *et al.* 1995; Hollocher *et al.* 1997a) to present the relative mating preference or success in each experiment. $DI = -\ln(n_{UM} \times n_{MZ}/n_{UZ} \times n_{MM})$, where n_{ij} is the number of copulating pairs between type i females and type j males; U, Z, and M designate the tested (U, for unknown), pure Z lines, and pure M lines, respectively. DI = 0 indicates no difference in mating preference and a positive value means the tested females are more Z-like in terms of their mating pattern.

The design is nearly identical with that of Hollocher *et al.* (1997a) except that the males used here are Z males rather than M males. In Hollocher *et al.* (1997a), we used males of the tested line against M males. The new method is more sensitive in detecting the mating pattern of females with intermediate Z behavior. When a line is of intermediate Z femaleness, its Z maleness is usually intermediate as well (Hollocher *et al.* 1997a). Such females often mate much more randomly when offered M males and intermediate Z males than when offered M males and strong Z males. Therefore, for the intermediate Z behavior lines, the DI values measured by the modified method are higher than those measured by the method of Hollocher *et al.* (1997a). For example, $DI = -\ln[5 \times 15/40 \times 45] = 3.18$ for LA2 in Table 1. In contrast, the DI value is 1.99 for the same line by the method of Hollocher *et al.* (1997a). For the strong Z lines, the DI values measured by these two methods are similar because males from the tested lines are most often strong Z males. We remeasured all of the lines with a DI value of <2.5 from the three African populations in Hollocher *et al.*'s study (1997a).

RESULTS AND DISCUSSION

In total, 22 lines from the three African populations that are polymorphic for the *desat2* alleles were used in this association study. Each of these 22 lines is monomorphic for either the + or the D *desat2* allele. Previous studies have shown that the allelic status (+ or D) at the *desat2* locus accurately predicts the CH phenotype of females from >40 different lines (Takahashi *et al.* 2001; see also Coyne *et al.* 1999; Dallerac *et al.* 2000). Monomorphic populations are of course uninformative. In Table 1, we rank these 22 lines according to the DI value in the double-choice mating design. Measurements are listed separately for each population. Among the ZH lines, the Wilcoxon rank test yields $P < 0.05$, suggesting a strong correlation between the mating pattern and the *desat2* allele carried by the females. The correlation is complete in both the LA and OK populations although the number of lines in each population (6 and 4, respectively) is too small to yield a significant result.

We may also pool the observations from the three polymorphic populations for a statistical test. This is a conservative practice as pooling observations across populations can only weaken the correlation. In other words, an imperfect correlation within each population will always be preserved in the pooled data but a perfect correlation may be destroyed by pooling. Thus, pooling across populations should be done only if the populations are not too divergent in their mean DIs. The ZH, OK, and LA populations broadly overlap in this measurement and are geographically the most adjacent among all collections. In the pooled data, the top 13 lines with high DI values all carry the wild-type *desat2* allele (+) and the bottom 7 lines all carry the inactive D allele. Both the Mann-Whitney and Wilcoxon rank tests give $P < 0.001$.

TABLE 1

Association between Z femaleness and the *desat2* alleles

	Degree of Z femaleness DI (n_{UZ}, n_{UM}, n_{MZ}, n_{MM})[a]	*desat2* allele		
Lines from populations monomorphic at the *desat2* locus				
Zimbabwe, Sengwa ($n = 12$)	3.89 ± 2.20 (1.05–5.76)[b,c]	+		
Caribbean ($n = 4$)	0.37 ± 0.68 (−0.51–1.05)[c]	+		
Cosmopolitan ($n = 5$)[b]	0.89 ± 0.44 (0.12–1.19)[b,c]	D		
Lines from polymorphic populations in Africa[d]		ZH	OK	LA
ZH21	5.05 (38, 1, 10, 41)	+		
ZH12	4.55 (78, 3, 16, 58)[b]	+		
ZH18	4.49 (37, 1, 17, 41)	+		
OK87	4.47 (39, 0, 16, 36)[b]		+	
ZH32	4.25 (33, 0, 31, 66)[b]	+		
ZH23	3.29 (33, 4, 12, 39)	+		
LA2	3.18 (40, 5, 15, 45)			+
LA34	3.02 (20, 3, 14, 43)			+
ZH1	2.69 (42, 12, 9, 38)	+		
OK59	2.64 (96, 15, 26, 57)[b]		+	
LA79	2.61 (24, 6, 5, 17)[b]			+
ZH19	2.49 (38, 15, 8, 38)	+		
ZH29	2.38 (35, 10, 11, 34)	+		
ZH40	2.23 (35, 11, 14, 41)	D		
ZH20	1.93 (37, 14, 15, 39)	+		
ZH13	1.39 (34, 14, 20, 33)	D		
ZH27	1.32 (28, 10, 21, 28)	D		
OK91	1.08 (31, 17, 21, 34)		D	
LA47	0.16 (29, 30, 24, 29)			D
LA20	−0.06 (20, 27, 26, 33)			D
OK17	−0.29 (24, 32, 26, 26)		D	
LA66	−0.43 (23, 33, 29, 27)			D

[a] Z femaleness is the DI value defined by $-\ln(n_{UM} \times n_{MZ}/n_{UZ} \times n_{MM})$, where n_{ij} is the number of copulating pairs between type i females and type j males; U, Z, and M designate the tested lines (U, unknown), pure Z lines, and pure M lines, respectively. The four n_{ij} numbers are given in the parentheses.

[b] Z femaleness as recorded in Hollocher *et al.* (1997a); see MATERIALS AND METHODS.

[c] Numbers in parentheses show the Z femaleness range among tested lines.

[d] Zimbabwe, Harare (ZH); Zambia, Luangua (LA); Botswana, Okavanga (OK).

Higher DI values indicate an excess in mating of the following two combinations, (tested females × Z males) and (M females × M males), over the other two, (tested females × M males) and (M females × Z males). The DI value does not differentiate female choice, male choice, or interactions between them. We thus refer to a high DI value as high Z femaleness without making a distinction between her attractiveness, her mate choice, and the interactions. The interpretation in favor of any mechanism usually entails supplementary observations. In this case, it is more straightforward to interpret the results as male choice (or female attractiveness), in light of the behavioral observations (Ferveur *et al.* 1996) as well as in light of the female-limited expression of *desat2* (Dallerac *et al.* 2000). The exact mechanism may be more complex, involving male choice, female response, and their synergism.

In a separate experiment that allowed the Z- and M-type genomes to mix for 60 generations in five replicates, the resultant lines varied in the degree of Z femaleness, which indeed is strongly correlated with the presence of the wild-type *desat2* allele (J. Alipaz, S. Fang and C.-I Wu, unpublished data). The *desat2* allele carried by the female determines her CH phenotype, which affects the relative courtship intensity of Z and M males toward her. It should also be noted that the *desat2* locus is only one of the many loci (>7; Ting *et al.* 2001) that determine Z femaleness. Populations may differentiate all, or a subset, of these loci (Hollocher *et al.* 1997a).

The above conclusion necessitates a reevaluation of the genetic make-up of the Caribbean flies, which carry the + *desat2* allele but exhibit M-like behavior (Table 1). Since a measurable degree of Z femaleness requires the presence of the Z-type allele at several loci (Hollocher *et al.* 1997b; Ting *et al.* 2001), the effect of a single locus can be observed only when the alleles at other Z-behavior loci are equivalent in the comparison. By this criterion, lines from the same population are

appropriate. On the other hand, the Caribbean lines are likely to be of the M genotype at most of the loci. This study suggests that association studies can be adequate for gene mapping, even for very complex traits like mating bias, but the comparison has to be made strictly within populations.

Are there genetic variants other than the +/D difference that may also account for the pattern of Table 1? Because *D. melanogaster* generally exhibits low levels of linkage disequilibrium and because the sequences in the *desat2* region indeed show extensive recombination (Takahashi *et al.* 2001), the observation of Table 1 is not likely to be associated with polymorphism outside of the *desat2* region. It should also be noted that the +/D variant in the promoter of *desat2* is the only variant that shows a complete concordance with the CH phenotype in the entire 13-kb region where the CH difference was mapped (Takahashi *et al.* 2001). In light of previous phenotypic studies (Coyne *et al.* 1994; Ferveur *et al.* 1996; Dallerac *et al.* 2000; Takahashi *et al.* 2001), the most parsimonious explanation for the correlation between the mating pattern and the *desat2*-region genotype is the inactivation of the *desat2* gene by the 16-bp deletion in its promoter. That the nonfunctionalization of a gene may play a role in sexual isolation during incipient speciation merely reveals a small corner of the fascinating unknowns in the molecular genetics of species differentiation.

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