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Mapping of genetic loci that change pheromone discrimination in *Drosophila* males

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Summary

Reproduction in individual animals of sexual species depends largely upon their ability to detect and distinguish specific signal(s) among those produced by various potential sexual partners. In *Drosophila melanogaster* males, there is a natural polymorphism for discrimination of female and male principal pheromones that segregates with chromosome 3. We have mapped two loci on chromosome 3 that change sex-pheromone discrimination in males. We successively exploited meiotic recombination, deficiencies and enhancer-trap strains; excision of the transposon in two selected enhancer-trap strains clearly reverted the discrimination phenotype. These results indicate that pheromonal discrimination is a character that can be genetically manipulated, and provide further insights into the evolution of the specific mate recognition system.

1. Introduction

An individual's ability to choose an adequate mate depends largely upon its ability to detect and distinguish specific signal(s) among those produced by various potential sexual partners. Each species possesses its own distinct specific mate recognition system (SMRS) that controls the exchange of sensory information sent and received by both sexual partners during interplay (Patterson, 1985). To understand the role of genes in courtship and mating behaviour, it is essential to determine how evolution has shaped the components of the SMRS. Sexual selection could have acted on these components and sexual isolation could have occurred between two populations if a new SMRS arose (Greenspan & Ferveur, 2000).

For *Drosophila melanogaster* and closely related species, cuticular hydrocarbons (HCs) represent an important part of the SMRS and are crucial for recognition of the specific mate (Cobb & Ferveur, 1996). In *D. melanogaster*, the main HCs are sexually dimorphic in both their occurrence and their effects: females produce 7,11-dienes whereas males produce 7-monoenes including 7-tricosene (7-T) and 7-pentacosene (7-P) (Antony & Jallon, 1982). 7-T strongly inhibits courtship of other conspecific males whereas 7,11-dienes stimulate male courtship only slightly (Ferveur & Sureau, 1996; Savarit *et al.*, 1999). However, 7-T stimulates the courtship of males from the sibling species *Drosophila simulans* (a monomorphic species with 7-monoenes in both sexes), whereas 7,11-dienes strongly inhibit these males (Jallon, 1984; Coyne *et al.*, 1994; Savarit *et al.*, 1999).

Within *D. melanogaster*, males exhibit a natural dimorphism for the production and perception of 7-P, or for the molecules associated with 7-P (Jallon, 1984; Ferveur & Sureau, 1996; Sureau & Ferveur, 1999). Both characters are co-adapted and are controlled by different chromosomes. The variation in 7-P amounts segregates mostly with chromosome 2, whereas the ability to court 7-P-rich males largely depends upon chromosome 3. As neither morph (high or low male courtship of 7-P-rich males) differs in their courtship towards control female objects (Sureau & Ferveur, 1999), chromosome 3 might carry the loci involved in the discrimination between male and female pheromones.

In *D. melanogaster*, a case of strong asymmetrical isolation has been found between a strain from Zimbabwe and strains from a nearby region (Wu *et al.*, 1995). The behavioural difference between these

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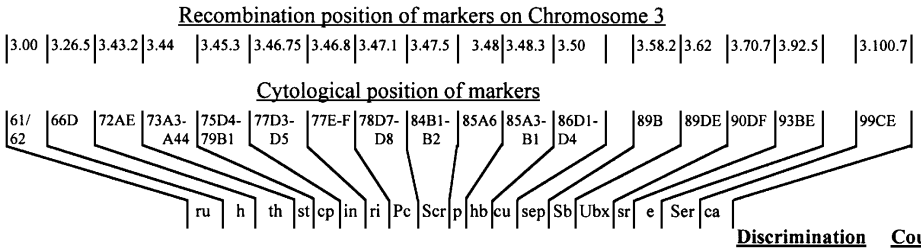

		Discrimination Index (DI)	Courtship Indices CI_{MT} (s.e.)	CI_F (s.e.)
	Strain: Tai (centromere)			
	F0 Tai/TM3	8·6	5 (1)	43 (4)
(A)	**Strain: 70900**			
#				
1	F0 70900/TM3 ru h th st ... cu sep Sb Ubx sr e Ser ca	1·4	28 (2)	39 (4)
2	F1 70900/Tai	6·5	6 (1)	39 (2)
	RECOMBINANTS			
3	F2 70900/ ru h	4·6	7 (1)	32 (2)
4	F2 70900/ ru h th	3·1	8 (1)	25 (2)
5	F2 70900/ th st	2·1	9 (2)	19 (2)
6	F2 70900/ ru h th st	2·9	9 (1)	26 (2)
7	F2 70900/ ru h th st	1·6	14 (2)	22 (2)
8	F2 70900/ cu sep Sb Ubx sr e Ser ca	1·2	15 (1)	18 (2)
9	F2 70900/ cu sep Sb Ubx sr e Ser ca	0·8	23 (2)	19 (3)
	synthesis: Factor 1 / Factor 2			
(B)	**Strain: 70760**			
#				
1	F0 70760/TM3 ru h th st ri Scr p | cu sr e ca	1·0	28 (3)	29 (3)
2	F1 70760/Tai	8·5	4 (1)	34 (3)
	RECOMBINANTS			
3	F2 70760/ th	6·7	4 (1)	27 (2)
4	F2 70760/ th st	2·5	11 (2)	28 (2)
5	F2 70760/ ru h th st	1·5	17 (2)	26 (2)
6	F2 70760/ ca	2·4	18 (2)	43 (4)
7	F2 70760/ cu sr e ca	1·3	20 (2)	25 (3)
	synthesis: Factor 1 / Factor 2			
(C)	**Strain: 72255**			
#				
1	F0 72255/TM3 th st cp in ri ... p hb |	0·9	39 (3)	37 (4)
2	F1 72255/Tai	7·6	5 (1)	38 (4)
	RECOMBINANTS			
3	F2 72255/ th	5·5	6 (1)	33 (4)
4	F2 72255/ th st	3·3	9 (1)	30 (3)
5	F2 72255/ th st cp	2·3	15 (2)	34 (3)
6	F2 72255/ th st cp in ri	1·2	30 (3)	37 (3)
	synthesis: Factor 1			
(D)	**Strain: 61700**			
#				
1	F0 61700/TM2 th st cp in ri Pc | sr	2·8	20 (4)	57 (2)
2	F1 61700/Tai	9·7	3 (0)	29 (3)
	RECOMBINANTS			
3	F2 61700/ th st cp	3·3	16 (2)	53 (4)
4	F2 61700/ th st cp in	1·8	20 (3)	36 (3)
	synthesis: Factor 1			
	FINAL SUMMATION: Factor 1 (73A3- 77F) / Factor 2			

Fig. 1. For legend see opposite.

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Table 1. *Variation in male courtship and discrimination in strains carrying various deficiencies (Df) in the chromosomal region 73A–78B*

Deficiency	Location	Discrimination index (DI)[a]	Courtship to Tai males (CI_{mT})[b]	Courtship to control females (CI_f)[b]
Df 54643	73A3–74E5	1·6	29 (±3)	45 (±2)
Df 55340	75B11–75C4	1·7	22 (±3)	37 (±3)
Df 55330	76A6–76A7	2·0	25 (±4)	50 (±3)
Df 54682	76B2–76D4	3·7	4 (±1)	15 (±3)
Df 54740	77A1–77D1	1·1	39 (±3)	44 (±4)
Df 54750	77B4–78B1	1·1	27 (±3)	29 (±4)

[a] DI (CI_f/CI_{mT}) is the discrimination index.
[b] The mean courtship index (CIs; ±standard error) of heterozygous Df/TM3 males was measured towards Tai males (CI_{mT}) and control shi females (CI_f). $11 < n < 16$ for each strain.

strains has been explored with stable chromosome substitution lines and has revealed that chromosome 3 carries at least seven loci that influence mating success (Ting *et al*., 2001).

Here, we have used four genetic approaches to map on chromosome 3 the loci that change pheromone discrimination in males. (1) Meiotic recombination of the chromosome 3 between variant strains. (2) Use of deficiencies covering the chromosomal region segregating with the largest part of the variation. (3) Screening of enhancer-trap strains with a single transposon inserted in that region. (4) Excision of the transposon in two selected strains. We found that the transposon in these two enhancer-trap lines drastically altered the amplitude of pheromone discrimination.

2. Material and methods

(i) Drosophila *stocks and crosses*

All strains were raised in glass tubes on standard cornmeal and yeast medium and maintained at 24–25° at 50% relative humidity under a 12-hour day, 12-hour night photoperiod.

The initial step in localising regions on the third chromosome responsible for male courtship was carried out with four strains (provided by the University of Umea, Sweden) carrying several recessive markers on chromosome 3: #61700, *th* [1] *st* [1] *cp* [1] *in* [1] *ri* [1] *Pc* [2] *sr* [61j]/TM2, *ri* [1] *Ubx* [130] *e* [s] *ca* [1]; #70760, *ru* [1] *h* [1] *th* [1] *st* [1] *ri* [1] *Scr* [XF9] *p* [p] *cu* [1] *sr* [1] *e* [s] *ca* [1]/TM3; #70900, *ru* [1] *h* [1] *th* [1] *st* [1] *cu* [1] *sr* [1] *e* [s] *ca* [1]/TM3, *y* [+] *ri* [1] *p* [p] *sep* [1] *Ubx* [bx-34e] *e* [s] *Ser* [1]; and #72255, *th* [1] *st* [1] *cp* [1] *in* [1] *ri* [1] *p* [p] *hb* [13]/TM3 (for all markers and balancers, see Lindsley & Zimm, 1992). Except in the 70900 strain, all chromosomes 3 were homozygous lethal and were held opposite a balancer (TM2 or TM3). Chromosomal mapping was performed by meiotic recombination (Fig. 1). This was achieved by first exchanging chromosome 3 of the multiply marked chromosome (M) held opposite a balancer (B) with the Tai strain to yield F1 females with the Tai/M genotype. Recombination occurred in these females, which were then backcrossed with M/B males of the parental multiply marked strain to give recombinant F2 males carrying a recombinant Tai-M chromosome opposite an M chromosome. Each F2 recombinant male of interest was then backcrossed with M/B females and resulting Tai-M/B male and female progeny was mated within each line to produce stable recombinant lines. This procedure allows the production of F3 males carrying the same Tai-M recombinant chromosome 3, and the isogenization of

Fig. 1. Mapping genetic segments of chromosome 3 that change male courtship behaviour and discrimination of sexual partners. Mean courtship indices (CIs; ±standard error) of subject males from different lines were measured towards Tai males (CI_{mT}) and control shi females (CI_f). All markers borne by chromosome 3, their genetic location, cytology and abbreviation (Lindsley & Zimm, 1992) are shown on top of the figure. The behaviour corresponding to each multiply marked chromosome was analysed in association with a balancer chromosome (TM3 or TM2) and with a Tai chromosome (#1 and #2, respectively, for each series). The recessive markers carried by the four multiply marked chromosome 3 strains (dark lines) were only visible in the progeny of F2 recombinant males and their absence was used to follow the segregation of a high discrimination index ($DI = CI_f/CI_{mT}$) with the Tai chromosome (grey lines). This mapping procedure included the uncertainty of crossing over between two consecutive markers (blank space between dark and grey lines). Factor 1 and Factor 2 (shown in bold below each series of recombinant lines) represent the chromosomal segments carrying factor(s) that could change the DI. The final summation represents the overlap between the four experiments and indicates that Factor 1 is probably found in the cytological region 73A3–77F.

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Table 2. *Mean courtship indices and discrimination index of males carrying a transposable element inserted on the chromosome 3, between 75E and 78C*

P-element strain[a]	Transposon location	Discrimination index (DI)[b]	Courtship to Tai males (CI_{mT})[c,d]	Courtship to control females (CI_f)[c,d]
Cs/TM2[e]		3·1	22 (±4)	69 (±4)
Cs		2·4	26 (±4)	63 (±5)
10189	75E1–75E2	1·8	35 (±5)	64 (±9)
12105	75E3–75E5	2·9	20 (±5)	57 (±3)
10019	75F	1·6	38 (±6)*	62 (±6)
12108	76B6–76B7	1·7	38 (±8)	64 (±6)
10190	76B9–76B10	1·2	34 (±5)	40 (±5)***
10627	76D	2·9	19 (±9)	56 (±7)*
10196	76D3–76D4	1·4	45 (±5)**	62 (±5)
12106	76D3–76D4	1·2	57 (±5)***	68 (±3)
12107	77A1–77A2	1·8	24 (±8)	42 (±2)*
10076	77B	3·6	17 (±7)	61 (±8)
10266	77B6–77B7	1·4	50 (±6)**	69 (±4)
10021	77C	3·8	13 (±4)*	50 (±9)*
10197	78A1–78A2	10	4 (±2)***	40 (±5)***
10198	78A1–78A2	2·3	22 (±7)	51 (±6)*
12109	78A1–78A2	2·1	30 (±11)	64 (±5)
10022	78A	1·5	47 (±7)**	69 (±6)
10199	78A5–78A6	1·6	37 (±6)	59 (±9)
10267	78C	2·1	29 (±9)	60 (±7)

[a] Strains are organized following the cytological position of the transposon that was found in Flybase (Bloomington, IN).
[b] DI (CI_f/CI_{mT}) is the discrimination index.
[c] The mean courtship index (CI; ±standard error) was measured towards Tai males (CI_{mT}) and control females (CI_f) ($15 < n < 46$).
[d] Significant differences compared with the Cs/TM2 control were detected with a Mann–Whitney *U* test (*, $p < 0{\cdot}05$; **, $p < 0{\cdot}01$; ***, $p < 0{\cdot}001$). Discrimination was also tested and only strains 10190 and 12106 yielded no significant difference between CI_{mT} and CI_f (Mann–Whitney *U* test; $0{\cdot}2 > p > 0{\cdot}4$). The courtship of 10190/TM2 and 12106/TM2 was also tested with Cs males (CI_{mCs} were 6±3 and 42±7, respectively; compare with the CI_{mCs} of Cs/TM2 males (6±4)).
[e] Because strains were homozygous lethal, subject males, except the Canton-S (Cs) control strain, carried the TM2 balancer opposite the chromosome carrying the transposon.

their genetic background by eliminating most of the genome arising from the Tai strain.

Six strains (also supplied by the University of Umea) carrying a homozygous lethal deficiency spanning the region 73A3 to 77F: #54643, Df(3L)81k19/TM6B; #55340, Df(3L)W4, *ru* [1] *h* [1] *e* [1] *ca* [1]/TM6B, *e* [1] *Tb* [1] *ca* [1]; #55330, Df(3L)VW3/TM3; #54682, Df(3L)kto2/TM6B; #54740, Df(3L)*rdg*C-co2, *th* [1] *st* [1] *in* [1] *ri* [1] *p* [p]/TM6C, *Sb* [1] *cu* [1] *e* [s]; and #54750, Df(3L)*ri*-79c/TM3. Each deficiency strain was outcrossed with a laboratory strain carrying the TM3 balancer, and all progeny carried the Df(3L)/TM3 genotype (Table 1).

Eighteen strains containing a single transposon inserted in the cytological region 75E–78C (Table 2, based on data from Flybase) were obtained from the Bloomington Stock Center. These enhancer-trap strains were produced in three different screens: (1) #10019, #10021, #10022, #10076, #10266, #10267, #10627 (donated by HHMIP Stock Center, 1/8/1990); (2) #12105, #12106, #12107, #12108, #12109 (donated by Berkeley *Drosophila* Genome Project on 30/9/1994); and (3) #10189, #10190, #10196, #10197, #10198, P#10199 (donated by Berkeley *Drosophila* Genome Project on 4/10/1997). All strains were homozygous lethal for the chromosome carrying the transposon and all were re-balanced with TM2 after a cross with a laboratory stock Canton-S (Cs)/TM2. The TM2 balancer chromosome was chosen because (1) it does not alter male courtship behaviour (Ferveur & Jallon, 1993), and (2) it was not present in any original P-element stock, allowing us to distinguish TM2 clearly from other balancers in the progeny. The effect of TM2 was also measured on Cs/TM2 males.

To generate excision lines of strains 10190 and 12106, we used the scheme described by Cooley *et al.* (1988) to mobilize the transposon. Males of the supplied P-element strain were mass mated with females carrying a Δ2-3 transposase (Robertson *et al.*, 1988) on the third chromosome associated with the marker *Drop* (held opposite the balancer TM6). To identify flies without the transposon, mosaic *Drop* males from the F1 progeny (which did not carry a balancer) were individually crossed with *w*; +/TM3 females. The F2 male progeny were then scored for the loss of *w*+ (the presence of white-eyed progeny after transposase activity represents at least a partial excision of the P-element). Finally, these *w*− males

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were individually crossed with virgins from the Cs/TM2 laboratory stock to yield stable lines with the excised region held over the TM2 balancer chromosome. An average of two excision lines was obtained from each fertile F1 mosaic *Drop* male. Some 30 excision lines were maintained after transposon remobilization from each of the two enhancer-trap strains. The occurrence and fertility of homozygous progeny in each line was also recorded.

(ii) *Behavioural tests*

Flies were isolated 0–4 h after eclosion under CO_2 anaesthesia. Subject male flies (i.e. those whose sexual responses to object flies were measured) were held individually in fresh glass food vials for 4–5 days before testing. Male object flies from two wild-type strains, the 7-T-rich Cs and the 7-P-rich Tai, were similarly isolated and held in groups of ten for the same period. The female object flies were from the shibire strain (shi^{ts7}).

Tests were performed simultaneously over several days for subject flies of each strain with male and female objects and always took place 1–4 h after lights on. Object flies were decapitated 10–60 min before observation under CO_2 anaesthesia. Decapitated flies are rarely observed to copulate and produce fewer visual and acoustic signals than intact flies, and so this procedure allows behavioural observations to be standardised and thus enhances the influence of chemical signals on male courtship (Ferveur *et al.*, 1995). Subject males were individually aspirated (without anaesthesia) under a watch glass used as an observation chamber (1·6 cm^3). After 10 min, a decapitated object fly was also introduced and stereotypical male courtship behaviours (tapping, wing vibration, licking and attempted copulation) were noted for 10 min. The courtship index (CI), calculated for each male, is the sum of the duration of all these courtship behaviours. Courtship indices in the presence of Tai male objects (CI_{mT}) and shi female objects (CI_f) were measured for each strain. The ratio of CI_f to CI_{mT} for a given strain was termed the 'discrimination index' (DI). The DI is a useful way to circumvent the variation of general activity (generally measured with locomotor activity) indirectly affecting courtship because CI_f and CI_{mT} are theoretically altered in similar proportions. However, a previous study based on crosses between Cs and Tai strains revealed that the variation of CI_f, but not that of CI_{mT}, was correlated with the variation of locomotor activity (Sureau & Ferveur, 1999). For the sake of clarity, the values of locomotor activity are not shown here.

(iii) *Statistical analysis*

Courtship indices (CI_{mT} and CI_f) were compared using the non-parametric Mann–Whitney *U* test (two-tailed), because the frequencies of the samples to be compared were not usually normally distributed. Correlations between CI_{mT} and CI_f were tested using the non-parametric Spearman test (two-tailed).

3. Results

In a previous study, some of us found that the variation of male courtship intensity (or courtship index) toward 7-P-rich Tai males (CI_{mT}), but not the response toward control females (CI_f), mostly segregated with chromosome 3 (Sureau & Ferveur, 1999). Here, we have mapped some of the loci that change the male discrimination index ($DI = CI_f/CI_{mT}$) using a four-step approach.

(i) *Mapping the segment(s) controlling DI variation on chromosome 3*

Previous studies showed an important difference in DI between Cs males and stable Cs-autosome substitution lines ($DI = 1{\cdot}5$; for details, see Sureau & Ferveur, 1999) and males with Tai chromosomes ($10 < DI < 37$). Most of the variation segregated with chromosome 3. Therefore, we hybridized the Tai strain with four different Cs-like strains (70900, 70760, 72255 and 61700; Fig. 1) carrying a multiply marked chromosome 3, in order to map the genetic factor(s) controlling DI. In all cases, heterozygous males that carried a single multiply marked chromosome 3 with a balancer chromosome (TM3 or TM2; see Material and methods) showed low or moderately low DIs (0·9–2·8) that mainly resulted from their relatively high CI_{mT} (20–39). The effect of a single chromosome 3 from the Tai strain was largely dominant with respect to the DI phenotype. Heterozygous Tai/TM3 and the four Tai/multiply-marked males had a CI_f that was considerably higher than their CI_{mT}, and their DI was 6·5. The dominant effect of Tai chromosome 3 was therefore favourable for studying the segregation of high DI: we measured the courtship of males carrying a recombinant Tai/multiply-marked chromosome associated with the homologous multiply marked chromosome 3. The behavioural response corresponding to each recombinant chromosome 3 was the mean response of a population of males (and not the response of a single individual) arising from a line established from a single recombinant fly.

The experiment performed with strain 70900 indicates that one or more factors changing DI are segregating on the left arm of the chromosome 3 between the *th* marker and the centromere, and some others are located on the right arm (Fig. 1A). The differences in DI between F2 strains #3 and #4, and between #3 and #5 suggest that at least one factor is located on the [*th–st*] segment (i.e. the borders of this

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segment include both *th* and *st* markers). The difference in DI observed between the recombinant strains #6 and #7, which carry the same set of markers, also indicates the presence of another factor changing DI on the segment]*st–cu*[(i.e. the borders of this segment are between and do not include the *st* and *cu* markers), and the low DIs produced by strains #8 and #9 suggest that one or more factors changing DI are located on the right arm.

The recombination of Tai chromosome 3 with the multiply marked chromosome 3 of the three other strains (70760, 72255 and 61700) allowed a more precise mapping of the main factor segregating with the variation of male DI. These three multiply marked chromosomes were chosen because they carry a series of markers on the segment of the left arm that seems to segregate with a substantial variation of DI (see above). The recombinant F2 lines arising from the cross between Tai and 70760 strains suggest the existence of a main factor (or group of factors) around the *th* marker (on]*h–th*[and/or]*th–ri*[; compare DIs of #3 and #4). A second series of factors largely changing DI could be on]*e–ca*] (the segment excluding *e* and including *ca* markers; compare DIs of #2 and #6; Fig. 1B) and [*cu–e*] (compare DIs of #6 and #7).

The recombinant lines obtained with the 72255 strain indicate that one factor controlling a large part of the DI variation segregates with the]*cp–ri*] segment (compare DIs of #5 with #6; Fig. 1C). Another factor could be located nearby, because a substantial variation of DI segregated with the [*st–cp*] segment (compare DIs of #3 and #4–5). The recombinant F2 lines obtained with the 61700 strain pinpoint the substantial role played by a factor segregating with a small chromosomal segment including the *in* marker ([*in*]; compare DIs of #3 and #4; Fig. 1D).

Overall, these data indicate that a large part of the DI variation segregates with two chromosomal segments that overlap at]*st–ri*[and on most of the right arm, especially the distal part including the]*e–ca*] segment. These results are somewhat supported by the findings of Ting *et al.* (2001), who found that male mating success segregates with two distincts regions of chromosome 3, IIIc and IIIe, which correspond to the [*st–th*] and [*sr–ca*] segments, respectively.

We decided to focus our mapping on the]*st–ri*[segment because it was relatively accurately delimited (between cytological positions 73A3-4 and 77E-F) and it segregated with a substantial part of the DI variation in the four experiments.

(ii) *Deficiency study on the*]*73A–77F*[*chromosomal segment*

The previous experiment indicates that a single copy of the]*st–ri*[segment from the Tai chromosome 3 greatly increases the DI value. However, it was not clear whether this effect was caused only by the dominant effect of a copy of Tai chromosome and/or by the absence of the second Cs-like chromosome 3. For this reason, we have checked the DI phenotype of males carrying only a single copy of the chromosomal region]*st–ri*[originating from a Cs (or Cs-like) chromosome 3. We used six strains carrying deficiencies (Df) uncovering various parts of this region spanning the cytological positions 73A–78B and measured the CI_f and CI_{mT} of heterozygous Df/TM3 males (Table 1). Males from five of the six strains showed a low DI (1·1–2·0); Df54740/TM3 and Df54750/TM3 males had the lowest values, whereas Df54682/TM3 males showed a relatively high DI (3·7), a result of the ratio between two very low courtship indices (15/4). These data suggest that (i) the loss of one copy of Cs-like chromosome 3 does not switch the DI value from Cs to Tai phenotype and (ii) the deletion of various portions of the chromosomal segment]*st–ri*[can change male courtship discrimination and intensity. For the next experiment, we focused on the cytological region including the breakpoints of Df54682, Df54740 and Df54750, because these contiguous deletions intriguingly induced very different courtship and discrimination phenotypes: Df54682 showed the highest DI, whereas the other two strains had the lowest DI.

(iii) *Effect on DI of a single P-element inserted between 75E1–2 and 78C*

We tested 18 strains containing a single P-element inserted on chromosome 3, between 75E1–2 and 78C (Table 2). All enhancer-trap strains were outcrossed with Cs/TM2 and their original chromosome 3 balancers replaced with TM2. Therefore, with the exception of their chromosome 3, all 'P-element/TM2' strains shared half of their genes. As courtship variation could also be caused by several factors borne by chromosome 3 (other than the transposon), we checked for DI differences between the 18 strains according to their origin (produced by three screens; see Material and methods). No significant effect on courtship intensity was found relative to the origin of chromosome 3 (data not shown).

The courtship of heterozygous 'P-element/TM2' males was measured. CI_f ranged between 40 and 69, and CI_{mT} between 13 and 57. Except for the 10197 strain, which had an high DI (10, or 40/4), DIs ranged between 1·2 and 3·8. These moderately low DIs can be explained by the relatively high CI_{mT} shown by heterozygous males of these strains. These values are very close to those obtained in the previous experiment for males carrying one balancer and one incomplete Cs-like chromosome 3.

In order to isolate genes that change the DI, we selected strains 10190 and 12106, whose males showed

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Table 3. *Courtship and discrimination in excision lines arising from the* 10190 *strain. Mean* (± *standard error*) *courtship indices* (*CIs*) *and discrimination index* (*DI*) *of heterozygous* '*excision/TM2*' *males are shown with viability of homozygous males. For abbreviations and statistics, see Tables 1 and 2*

Strain	Discrimination index (DI)	Courtship to Tai males (CI_{mT})[a]	Courtship to control females (CI_f)[a]	Viability[b]
10190 (parental)	1·2	34 (±5)	40 (±4)	L
31·1	43·0	1 (±1)**	43 (±3)	L
26·1	22·5	2 (±2)***	45 (±10)	V
4·1	18·0	2 (±2)***	36 (±7)	V
17·4	13·4	5 (±3)**	67 (±7)*	L
9·4	10·5	6 (±4)**	63 (±9)*	L
30·2	9·4	5 (±2)**	47 (±4)	L
3·2	8·2	6 (±3)***	49 (±9)	L
28·2	7·1	11 (±5)**	65 (±8)*	L
17·1	6·8	6 (±6)**	41 (±8)	L
22·1	6·3	7 (±7)**	44 (±8)	L
28·1	5·9	10 (±7)**	59 (±9)	V
9·1	5·9	11 (±4)*	60 (±9)	L
17·2	4·8	9 (±5)**	43 (±8)	V
9·2	4·3	9 (±6)*	39 (±9)	L
1·3	4·3	11 (±5)*	47 (±10)	V
7·1	3·7	15 (±8)*	55 (±8)	L
3·1	3·2	9 (±5)***	29 (±7)	L
4·3	2·4	16 (±7)*	39 (±7)	L
17·3	3·8	15 (±5)	57 (±7)	L
32·1	3·5	14 (±4)	49 (±9)	V
30·1	3·4	15 (±6)	51 (±9)	L
36·1	3·4	19 (±5)	64 (±9)*	L
32·2	3·3	18 (±9)	60 (±9)	V
31·2	3·2	19 (±7)	61 (±2)*	L
32·3	3·1	14 (±8)	44 (±8)	L
5·1	2·9	15 (±8)	44 (±11)	L
35·1	2·8	22 (±9)	61 (±9)	L
40·1	2·5	23 (±9)	58 (±10)	V
2·2	2·5	28 (±8)	71 (±7)**	L
37·1	2·4	30 (±9)	71 (±4)**	L

[a] Asterisks indicate that CI was significantly different with 10190/TM2 control towards the same sex-object (*, $p < 0{\cdot}05$; **, $p < 0{\cdot}01$; ***, $p < 0{\cdot}001$; Mann–Whitney *U* test). Excision lines were grouped according to their CI_{mT} difference with parental 10190 strain, and then ranked in order of decreasing DI, within each group. For courtship tests: $12 < n < 20$.
[b] L, lethal; V, viable.

no discrimination (Table 3). Our choice was also influenced by the fact that 10190 males showed the lowest CI_f (40), whereas 12106 males showed the highest CI_{mT} (57).

(iv) *Reversion of the DI phenotype after remobilization of the P-element*

Remobilization of the P-element in strains 10190 and 12106 clearly indicates that, in both strains, the transposon was inserted in, or nearby, a gene that changes pheromonal discrimination in male flies. In the case of the 10190 strain, we obtained 30 stable excision lines whose DIs ranged between 2·4 and 43 (Table 3). Males of all excision lines significantly discriminated male and female sex-objects (Mann–Whitney *U* test; $p < 0{\cdot}05$) and their DI was always higher than that of the parental 10190 strain. CI_{mT} was always lower than for the 10190 strain and the decrease was significant for 18 of the 30 lines. By contrast, seven of the 30 strains showed a significant increase of CI_f compared with parental males, and only four lines had a $CI_f < 40$. If we only consider the 18 lines with a CI_{mT} significantly different from the 10190 strain, no correlation was found between their CI_{mT} and CI_f values. However, CI_{mT} and CI_f showed a high level of correlation in the 12 other excision lines (Spearman test; $\rho = 0{\cdot}9$; $p < 0{\cdot}01$).

In the 12106 strain, we also observed the reversion of DI after transposon remobilization (Table 4). The great majority of excision males (31 of 33) showed a rescue of discrimination between Tai males and control females (Mann–Whitney *U* test; $p < 0{\cdot}05$) and yielded DIs (1·9–31) that were higher than parental 12106 males (1·2). Only two lines (#18·2 and #20·1)

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Table 4. *Courtship and discrimination in excision lines arising from the 12106 strain. Mean* (± *standard error*) *courtship indices* (*CIs*) *and discrimination index* (*DI*) *of heterozygous* '*excision/TM2*' *males are shown with viability of homozygous males. For abbreviations and statistics, see Tables 1, 2 and 3*

Strain[a]	Discrimination index (DI)	Courtship to Tai males (CI_{mT})[b]	Courtship to control females (CI_f)[b]	Viability[c]
12106 (parental)	1·2	57 (±5)	68 (±4)	L
1·2	31·0	2 (±1)***	62 (±6)	L
16·1	10·8	5 (±3)***	54 (±9)	L
6·1	9·8	6 (±4)***	59 (±7)	S
9·1	9·3	8 (±4)***	74 (±5)	S
19·2	8·4	7 (±4)***	59 (±6)	S
4·3	6·6	9 (±6)***	59 (±6)	L
11·1	5·1	11 (±6)***	56 (±7)	S
17·3	4·9	11 (±5)***	54 (±3)	S
7·3	4·9	14 (±6)***	69 (±4)	S
13·2	4·8	11 (±4)***	53 (±8)	L
3·3	4·5	14 (±6)***	63 (±7)	S
7·2	4·4	16 (±6)**	70 (±5)	L
1·1	4·3	12 (±5)***	52 (±8)*	V
8·1	4·3	14 (±6)***	60 (±8)	S
24·1	3·8	18 (±8)**	68 (±6)	S
8·2	3·6	16 (±6)**	57 (±6)	S
3·2	3·5	15 (±8)**	53 (±9)	V
17·1	3·5	19 (±8)**	67 (±2)	S
13·1	3·2	18 (±5)***	58 (±10)	S
24·2	3·1	19 (±7)**	59 (±7)	S
23·1	2·7	15 (±6)***	40 (±7)**	S
5·2	2·7	27 (±7)*	74 (±6)	S
16·2	2·4	24 (±8)*	57 (±7)	S
15·2	2·4	26 (±7)**	63 (±9)	S
21·1	2·3	22 (±6)**	50 (±10)	S
5·1	2·3	27 (±7)**	61 (±8)	S
15·1	2·2	29 (±8)*	65 (±7)	L
3·1	2·2	24 (±9)**	52 (±7)*	V
10·1	2·2	26 (±8)**	56 (±9)	S
21·2	2·0	27 (±10)*	53 (±9)	S
18·1	1·9	35 (±8)*	66 (±2)	S
18·2	1·6	36 (±8)	58 (±8)	S
20·1	1·2	44 (±10)	54 (±8)	S

[a] The three groups shown here roughly correspond to three DI phenotypes.
[b] For courtship tests, $12 < n < 20$. Strains were ranked in order of decreasing DI.
[c] L, lethal; S, semi-lethal; V, viable.

retained a low DI. If the enhanced discrimination among most excision lines was largely due to a decreased CI_{mT}, it was not significantly changed in the two latter excision lines. By contrast, the remobilization induced a significant decrease of CI_f in only three strains. As for the 10190 excisions, CI_f and CI_{mT} were not correlated ($p > 0{\cdot}2$, $n = 21$) among 12106 excision lines showing high discrimination (Tai-like behaviour; DI = 2·7) but were correlated ($\rho = 0{\cdot}7$; $p < 0{\cdot}05$; Spearman test, $n = 10$) in the lines showing moderately low discrimination (Cs-like behaviour; $2{\cdot}7 \leqslant DI \leqslant 1{\cdot}6$).

Finally, rescue of adult viability was noted for homozygous males of several excision lines, in both enhancer-trap strains. However, no relationship was found between the rescue of this character in homozygous males and the level of courtship in heterozygous males.

4. Discussion

We found two genetic factors that changed courtship behaviour toward specific sex partners because the remobilization of the transposon inserted in the DNA of both corresponding enhancer-trap strains rescued a high index of discrimination (DI) in excision males. Both mutant strains had a similarly low DI, but the 12106 strain showed higher courtship indices toward females and Tai males than the 10190 strain. Although transposon remobilization decreased CI_{mT} in both strains, it produced opposite effects on CI_f, which rarely decreased (but rather increased) in 10190

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excision strains, whereas it rarely increased (and rather decreased) in 12106 excision strains. These results support the idea that male response toward male and female flies is controlled by distinct genetic factors (Sureau & Ferveur, 1999).

How can we interpret these results? Despite their different origin, both strains probably share a Cs-like chromosome 3 (inducing a low DI) because the probability that a Tai-like strain was used for transposon hopping is very low. The transposon inserted in the 10190 strain may directly affect DI because its remobilization drastically decreased CI_{mT} and increased CI_f in many excision lines. It is possible that the transposon (or a secondary event following its remobilization) affects another factor controlling general activity because 12 of 30 excision lines exhibited correlated change on CI_{mT} and on CI_f, as among 10 of 33 excision lines originating from the 12106 strain. Nevertheless, the high level of homosexual courtship observed toward 7-tricosene-rich Cs males (CI_{mCs}) by males of the 12106 strain suggests that the original transposon increases the sexual activity regardless of the pheromones of the sex-object, as has been reported for PGal4-*Voila*[1] (Balakireva *et al*., 1998).

The fact that few or no excision line(s) produced a behavioural phenotype similar to that of the parental strain indicates that the abnormal courtship phenotype of both enhancer-trap strains is sensitive to the size of the DNA fragment that remained inserted, as for *Voila* excision lines, in which the remobilization always produced lines that had lower CIs than the parental strain (Grosjean *et al*., 2001). The decrease of CI_{mT} in all excision males could reflect the partial or complete loss of the *miniwhite* transgene (the screen of excision was based on the loss of w^+ activity), whose presence was correlated with high level of male homosexual courtship (Zhang & Odenwald, 1995). However, here, CI_{mT} varied from very low to moderately high among the excision lines, indicating that the intensity of courtship towards Tai males is independent of *miniwhite*, as reported for CI_{mCs} in Voila excision lines (Grosjean *et al*., 2001).

We believe, for several reasons, that discrimination between different potential sexual partners by courter males is largely based upon their ability to perceive the pheromones of object flies. (1) Previous rub-off experiments performed to transfer HCs from donor flies onto object flies deprived of HCs made the donor as attractive as the receiver toward control males (Savarit *et al*., 1999). (2) Decapitation of object flies increases the preponderant influence of chemical signals over the other signals produced by intact flies (Ferveur & Sureau, 1996; Ferveur *et al*., 1997; this study). We do not know yet whether the difference in CI_{mT} corresponds to a difference of perception and response towards 7-P itself or towards other chemical(s) linked to 7-P. The next step of our work will aim to characterize the molecules involved. We suspect that such signal(s) do differ between genotypes because *Drosophila* males can distinguish between them. Olfactory receptors have been found in *Drosophila* (Clyne *et al*., 1999; Vosshall *et al*., 2000) but the nature of the stimulatory volatile pheromones binding to these receptors remains to be determined. As these substances should not differ between Tai males and control females (*ur*-pheromones; Savarit *et al*., 1999) and therefore not induce a discriminatory response in tester males, we strongly believe that contact pheromones are the crucial discriminatory cues that modulate male courtship intensity (Savarit *et al*., 1999).

Studies in several invertebrates have shown that the nervous system involved in chemical perception is highly specialized (Hansson *et al*., 1992; Hildebrand, 1995; Wu *et al*., 1996). In *D. melanogaster*, male pheromonal discrimination has been changed following feminization of precise CNS regions that are probably involved in recognition of male inhibitory cues (Ferveur *et al*., 1995). In this scenario, the effect on male pheromonal discrimination between the 10190 and 12106 strains would be caused by the abnormal detection and/or integration of gustatory signals by the nervous system.

We are currently characterizing the molecular structure of the genetic region flanking the site of the transposon insertion in both enhancer-trap strains in order to identify the altered genes. Both insertions are disrupting genes that are essential for adult viability and that are ubiquitously expressed in the organism (Spradling *et al*., 1999). Moreover, the 10190 transposon is inserted near the *Sha1* gene, which encodes a voltage-dependent potassium channel (76B9; Tanouye *et al*., 1981), and further from the *lush* gene, which controls fly olfaction (76B11; Kim *et al*., 1998). The closest known gene to the site of the 12106 insertion is the *Mi-2* gene, which codes for an ATP dependent DNA helicase (76D3; Saurin *et al*., 2001). The fact that another transposon studied here (#10196; produced in a different screen), which is inserted near the 12106 insertion, yielded very similar CI_{mT} and CI_f supports their behavioural role. Once the identity of the genes corresponding to both insertions has been revealed, we will examine their pattern of expression and focus on the regions of the nervous system that are involved in pheromonal perception. The study of homologous genes in the closely related species *D. simulans* will be very interesting because the response to sex pheromones of *D. simulans* males is exactly reciprocal to that observed in *D. melanogaster* males (Jallon, 1984; Coyne & Oyama, 1995; Savarit *et al*., 1999).

Finally, the maintenance of a natural polymorphism for discrimination of pheromones in *D. melanogaster*

males remains uncharacterized. We do not know if a low or a high DI provides a better adaptive value for a male fly. Recent studies have shown that a single genetic locus allows females to discriminate between male songs of closely related *Drosophila* species (Doi *et al*., 2001). Song discrimination can therefore cause premating isolation between these species that are not yet isolated by postmating mechanisms. This finding supports the idea that behavioural loci may evolve concurrently in the incipient stage of speciation before hybrid sterility occurs (Ting *et al*., 2001). With regard to female HCs polymorphism and to other genetic traits, the Tai strain belongs to the ancestral morph whereas the Cs strain belongs to the derived morph (Lachaise *et al*., 1988; Takahashi *et al*., 2001). Given that low production of 7-P has evolved with high CI_{mT} (Sureau & Ferveur, 1999), the ancestral morph had probably a high DI (as for Tai males). In the derived strains (like Cs), the DI has decreased, probably because of increased CI_{mT}. We do not know whether the homosexual response toward 7-P rich-males has increased in 7-T-rich males because the latter were not exposed to the former. In light of our results and those of Ting *et al*. (2001), who mapped at least seven genes on chromosome 3 that are involved in courtship behaviour, we propose that the increased CI_{mT} phenotype in Cs-like males corresponds to a 'recently acquired' behavioural response that is polygenically controlled by factors segregating on chromosome 3. The fact that the Cs strain has been kept in the lab for so many years (since 1935) has undoubtedly allowed it not only to acquire new mutations but also to exist without the rigours of selection on any of these traits, as would occur in the wild. However, it may be argued that Tai has also been domesticated for long enough (more than a decade) to fall into the same category, and this is apparently not the case.

Here, we have shown that DI is a phenotype amenable to genetic manipulation. To test whether the level of DI can be predicted by the molecular structure of the alleles coding for this phenotype, we are currently sequencing the DNA flanking the insertion point of 10190 and 12106 in order to see whether the rescue of a high DI correlates with all clean events of excision, in both enhancer-trap strains.

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