Genetic analysis of *Drosophila virilis* sex pheromone: genetic mapping of the locus producing Z-(11)-pentacosene

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Summary

Z-(11)-pentacosene, *Drosophila virilis* sex pheromone, is predominant among the female cuticular hydrocarbons and can elicit male courtship behaviours. To evaluate the genetic basis of its production, interspecific crosses between *D. novamexicana* and genetically marked *D. virilis* were made and hydrocarbon profiles of their backcross progeny were analysed. The production of Z-(11)-pentacosene was autosomally controlled and was recessive. Of the six *D. virilis* chromosomes only the second and the third chromosomes showed significant contributions to sex pheromone production, and acted additively. Analysis of recombinant females indicated that the locus on the second chromosome mapped to the proximity of position 2-218.

1. Introduction

Female cuticular hydrocarbons in *Drosophila* play an important role in stimulating males and can elicit male courtship behaviours, that is, some can act as a sex pheromone (Antony & Jallon, 1982; Jallon, 1984; Antony *et al.* 1985; Oguma *et al.* 1992; Nemoto *et al.* 1994). Sex pheromones, in most cases, can stimulate only conspecific males, acting as species-specific mate recognition signals. The signal differences between close relatives could cause mate discrimination and contribute to sexual isolation (Lofstedt & Van Der Pers, 1985; Cobb & Jallon, 1990). Sex pheromones have now been identified in many insects, but the evolutionary mechanisms involved in their diversity and fixation processes among species are unclear (review by Roitberg & Isman, 1992).

One reason for the lack of knowledge on how sex pheromones have diverged and been fixed is the lack of data on their genetic control. There have been relatively few reports on genetic analysis of pheromonal signals because of the difficulty of genetic work in many taxa. However, in *Drosophila*, genetic techniques can be used, and many marker mutants and cross experiments are available. Sex pheromones have already been identified in four species – *D. melanogaster*, *D. simulans* (Jallon, 1984), *D. pallidosa* (Nemoto *et al.* 1994) and *D. virilis* (Oguma *et al.* 1992) – and some work on the genetic basis of their control has been done.

In *D. simulans*, intrastrain hydrocarbon polymorphism is very marked, and two loci that are involved in controlling the hydrocarbon variations have been identified. One is *Ngbo*, mapped to position 65·3 on the second chromosome (Ferveur, 1991), the other is *kété*, mapped to position 18·5 on the X chromosome (Ferveur & Jallon, 1993). Scott & Richmond (1988) showed that in *D. melanogaster* males production of the predominant hydrocarbon was controlled by all three major chromosomes. Thus there are at least three factors responsible for hydrocarbon production and variation in this male. Coyne *et al.* (1994), however, showed that the hydrocarbon difference between the very close relatives *D. simulans* and *D. sechellia* mapped to only the third chromosome. They concluded that isolating mechanisms caused by hydrocarbon differences might have a fairly simple genetic basis. Therefore, although many genes might contribute to pheromone production, a small number of genes may determine the species-specificity.

(Z)-11-pentacosene (11-P; 25 carbons with a single double-bond in position 11) is the major sex pheromone component in *D. virilis* females (Oguma *et al.* 1992). It comprises over one-third of the total, being the predominant female cuticular hydrocarbon, and can elicit courtship behaviours in *D. virilis* males. *D. virilis* is a widely distributed cosmopolitan species and belongs to the *virilis* species group (consisting of 11

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species; Throckmorton, 1982). This group has been well investigated as regards many aspects of evolutionary history, phylogeny, population dynamics and ecology using cytogenetic, population and biochemical approaches (Alexander, 1976). Many studies have evaluated the phylogenetic relationships among the group (reviewed by Spicer, 1992). Their cuticular hydrocarbons, especially in *D. virilis*, have also been investigated (Jackson & Bartelt, 1986; Bartelt *et al.* 1986). This group is very suitable for examining the evolution of sex pheromones. We examined the genetic control of 11-P production in *D. virilis* by chromosomal and recombinant analyses using *D. virilis* and *D. novamexicana*.

2. Materials and methods

(i) Drosophila *stocks*

Two *D. virilis* strains (National *Drosophila* Species Resources Center nos. 15010-1051.83, 15010-1051.54) and the *D. novamexicana* wild-type strain (National *Drosophila* Species Resources Center no. 15010-1031.4) were used in the crosses. Strain 1051.83, used in the chromosomal analysis, had all its autosomes except for the sixth chromosome (which is a tiny dot chromosome only about one map unit long) marked with recessive markers. The genetic markers and their map positions are as follows: *broken* (*b*, 2-188.0), *gapped* (*gp*, 3-118.5), *cardinal* (*cd*, 4-32.2), *peach* (*pe*, 5-203.0) (Alexander, 1976; Gubenko & Evgen'ev, 1984). The other strain (1051.54), used in genetic mapping on the second chromosome, has four markers: *Confluent* (*C*, 2-45.0), *broken*, *brick* (*bk*, 2-248.0), *detached* (*dt*, 2-255.0) (Alexander, 1976; Gubenko & Evgen'ev, 1984).

All flies were kept on standard cornmeal and yeast medium under a 14L (0700–2100 hours):10D cycle at 25 ± 1 °C.

(ii) *Crosses*

All crosses were performed as mass mating with 40 flies of each sex in a vial. To examine the chromosomal contribution to 11-P production, first *D. novamexicana* females were crossed to *D. virilis b;gp;cd;pe* males, and then F_1 males were backcrossed to *b;gp;cd;pe* females. The backcross progeny were collected at 24 h intervals and female flies carrying only one homozygous *D. virilis* chromosome, whose phenotypes were [*b*], [*gp*], [*cd*], [*pe*] and [+], were selected. For all genotypes, five females were maintained in each food vial until GC analysis.

For the mapping on the second chromosome, *D. novamexicana* females were crossed to *D. virilis C b bk dt* males. F_1 females were then backcrossed to *C b bk dt* males and their progeny, classified into eight genotypes (see Table 2), were separately maintained until hydrocarbon analysis. Since all chromosomes except the second could not be controlled, their chromosome constitutions were mixed with *novamexicana/virilis* and *virilis/virilis*.

(iii) *Hydrocarbon analysis*

In both experiments all females used for hydrocarbon analysis were 9–13 days old. Cuticular hydrocarbons were removed from an individual female by washing for 5 min in 50 μl hexane in a microtube and agitation for 1 min. The fly was removed immediately after washing and the solvent fully evaporated from the microtube. These microtubes were stored at −40 °C until GC analysis. Before GC analysis, 20 μl hexane containing 50 ppm docosane (C22), an internal standard, was added to each microtube. After agitation for 1 min, 1 μl of the solution was injected into a GC380 gas chromatograph (GL Science) equipped with a FFAP column (0·25 mm ID × 25 m, D.F. = 0·25 μm, Quadrex), with a Flame Ionization Detector for peak detection. Temperature was programmed from 160 °C to 250 °C at 2·5 °C/min. We compared the peak area of 11-P with that of the internal standard C22 and measured the 11-P/C22 ratio. Differences in the 11-P/C22 ratio between genotypes were tested with an analysis of variance (ANOVA) followed by the Tukey multiple comparison and *t*-tests (Zar, 1984).

3. Results

(i) *Chromosomal contributions to 11-P production*

Chromosomal contributions to 11-P production were examined by measuring the 11-P/C22 ratios (Table 1). The mean ratio ± S.E. in *D. virilis b;gp;cd;pe* females was 0·92 ± 0·044, indicating that one *D. virilis* female contains about 5·0 μg 11-P. This value was consistent with that of *D. virilis* wild strain (TK) reported by Oguma *et al.* (1992). The mean 11-P/C22 ratio ± S.E. of *D. novamexicana* females was 0·02 ± 0·001, less than one-tenth that of *D. virilis*. F_1 hybrid females also showed a low 11-P/C22 ratio (0·09 ± 0·009), suggesting that the production of 11-P was recessive. Preliminary analysis in F_1 males indicated that the factor controlling 11-P production was autosomal, because both F_1 males from the reciprocal crosses had a small amount of 11-P (data not shown).

Table 1 also shows the mean 11-P/C22 ratio for each genotype. An ANOVA was performed between five genotypes of backcross progeny ($F_{4,145} = 204{\cdot}15$, $P < 0{\cdot}001$), and the Tukey multiple comparison test revealed that the second and the third chromosome have significant effects on 11-P production. No significant effect was found for the fourth or the fifth chromosome. The 95% confidence limits for the mean ratio in *b;gp;cd;pe* females were 0·837–1·009, and those for the sum of the mean ratio in *b* females and in *gp* females were 0·813–0·959. This indicates that the

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Table 1. *The mean ratios of 11-P/C22 for* D. virilis, D. novamexicana, F_1 *female and backcross females carrying only one homozygous* D. virilis *chromosome*

Female	*n*	11-P/C22 ratio (mean ± SE)*	Range
D. virilis (1051.83)	24	0·92 ± 0·044	0·837–1·009
D. novamexicana (1031.4)	21	0·02 ± 0·001	0·021–0·025
F_1	20	0·09 ± 0·009	0·077–0·111
b (2)†	30	0·56 ± 0·023[a]	0·511–0·601
gp (3)	30	0·33 ± 0·017[b]	0·291–0·359
cd (4)	30	0·13 ± 0·007[c]	0·119–0·145
pe (5)	30	0·09 ± 0·009[c]	0·073–0·109
+	30	0·09 ± 0·007[c]	0·078–0·104

* The values with the same superscript letters are not significantly different (5% level, Tukey multiple comparison test).
† Numbers in parentheses are those of the homozygous *D. virilis* chromosomes.

Table 2. *The mean ratios of 11-P/C22 in recombinant females*

Genotype	*n*	11-P/C22 ratio (mean ± SE)	*t* value
C + + +	28	0·35 ± 0·037	$t_{59} = 6·418$*
+ *b bk dt*	32	0·74 ± 0·048	
C b + +	40	0·54 ± 0·046	$t_{68} = 0·286$
+ + *bk dt*	29	0·56 ± 0·075	
C b bk +	3	0·88 ± 0·363	NA
+ + + *dt*	5	0·62 ± 0·111	
C b bk dt	11	0·66 ± 0·078	$t_{19} = 3·421$*
+ + + +	9	0·31 ± 0·057	

* $P < 0·001$; NA, not available.

second and the third chromosomes additively contribute to 11-P production.

(ii) *Genetic mapping of the second chromosome factor*

Table 2 shows each complementary pair and their 11-P/C22 ratios. If one phenotype showed a significantly higher value for the 11-P/C22 ratio than the other of the pair, we could expect that the females with the higher ratio were homozygous for the factor on the second chromosome, because this factor showed a larger contribution to 11-P production than that on the third, and the number of females homozygous or heterozygous for the third chromosome factor were not different. Since [*b bk dt*] and [*C b bk dt*] females had significantly higher ratios than their complementary pairs, they were homozygous for the factor on the second chromosome. Although statistical analysis for [*C b bk*] and [*dt*] females could not be done because of the small sample size, we suspected homozygosity for the second chromosome factor in the former female genotype. The [*C b*] and [*bk dt*] females were not significantly different, however, suggesting that the locus controlling 11-P production is located between *b* and *bk*.

Fig. 1 shows the frequency distributions based on the 11-P/C22 ratios of the *b* and *gp* females in chromosomal analysis (Table 1), where only the second or the third chromosome is homozygous respectively, and the ratios for each recombinant female (Table 2). On the basis of *b* and *gp* female distribution patterns, the empirical cut-off point was established at 0·4: females showing a ratio of less than 0·4 were heterozygous, and those with a ratio of more than 0·4 were homozygous for the second chromosomal factor. With this classification criterion, most [*b bk dt*] females are distributed on the homozygous side and most [*C*] females on the heterozygous side, showing that the factor is located at a more proximal position than *b*. This result agreed with those in Table 2. In [*C b*] and [*bk dt*] females the ratios showed scattered distributions. Of 40 [*bk dt*] females 23 were homozygous ($P = 0·58$; probability that the genotypes have the factor); of 29 [*C b*] females 16 were homozygous ($P = 0·55$). Thus the factor was mapped to position 2-218, midway between *b* (2-188.0) and *bk* (2-248.0).

4. Discussion

The present results strongly suggest that the production of *D. virilis* female sex pheromone, (Z)-11-pentacosene, is not controlled by a complicated genetic mechanism but rather can be explained by a simple one. Only the second and the third chromosomes of *D. virilis* contributed to the production of the hydrocarbon; the other four made no contribution. Moreover, the factor on the second chromosome was mapped to a single locus or a tightly linked group of loci at map position 2-218. These gene(s) on the second chromosome and factor(s) on the third chromosome are recessive and acted additively. They may produce the 11-P independently and change the females' cuticular hydrocarbon profile.

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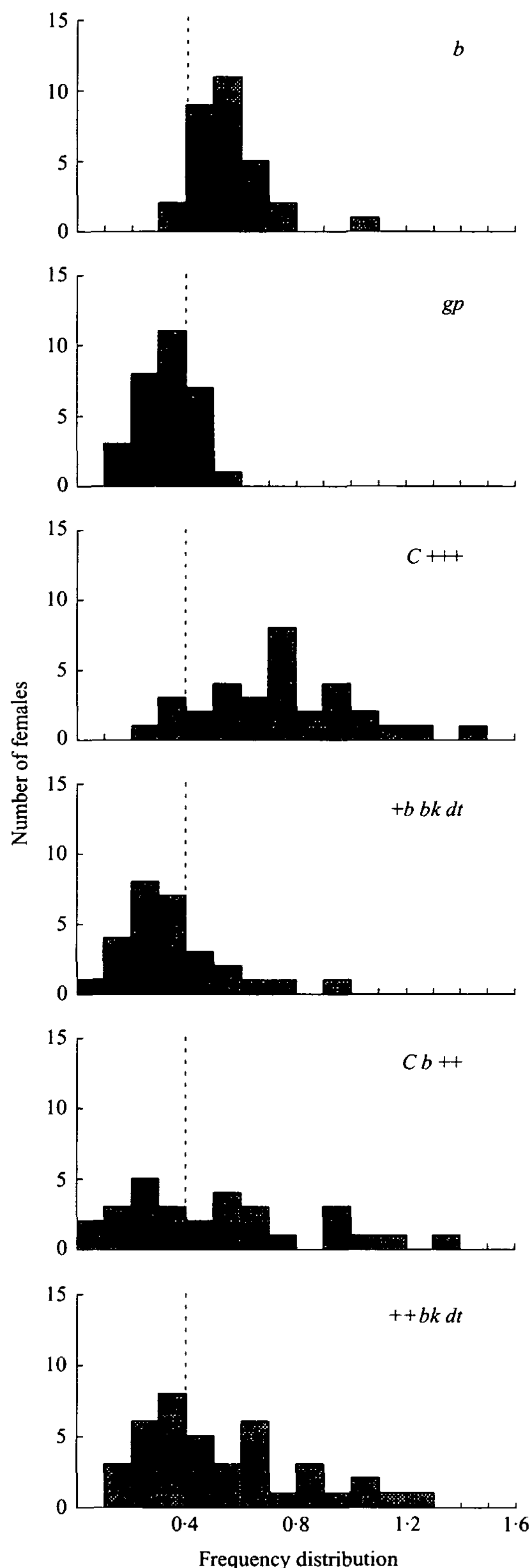

Fig. 1. Frequency distribution of the 11-P/C22 ratio for backcross and recombinant females. *b* and *gp* females are backcross females homozygous for the second or the third chromosome, respectively (Table 1), and the other four genotypes are recombinant females (Table 2). The dashed line indicates the empirical cut-off point (0·4) between the homozygous (right-hand side) and heterozygous (left-hand side) phenotypes for the second chromosome factor.

Precise data, suggesting that there was no epistatic interaction between the second and third chromosomes on sex pheromone production, are not shown in this study because we did not analyse female cuticular hydrocarbon profiles of all the genotypes genetically distinguished by four autosome combinations. However, we conclude that there is no interchromosomal epistasis because the range of the sum of the ratio in [*b*] females and that in [*gp*] females is nearly identical to the range of *D. virilis b;gp;cd;pe* females. The sum of all the ratios among genotypes should be much greater than the ratio in *b;gp;cd;pe* females if chromosomal epistasis acts on sex pheromone production.

In the study of hydrocarbon differences between two closely related species of *Drosophila*, *D. simulans* and *D. sechellia*, it was concluded that a single locus might control their hydrocarbon difference, and interchromosomal epistasis was not observed (Coyne *et al.* 1994). The pheromonal difference between the two species has a simple genetic basis and is not apparently derived from evolutionary changes spread throughout the genome. Similar results have been observed in *D. simulans*. Intraspecific hydrocarbon polymorphism is responsible for a single gene on the second chromosome, *Ngbo* (Ferveur, 1991). This gene controls the ratio between 7-tricosene (7-T; sex pheromone of most *D. simulans* strains) and 7-pentacosene (7-P; predominant hydrocarbon in a Cameroon strain).

Biosynthesis of hydrocarbons, including sex pheromones, must be controlled by many factors. *Ngbo*, for example, involved in determining the 7-T/7-P ratio, could act on either the elongation or decarboxylation step of their source hydrocarbon (Ferveur, 1991). *kété*, which also controls the amount of 7-T, might act on the early steps in fatty acid synthesis (Ferveur & Jallon, 1993). Jallon (1984) proposed a scheme for *Drosophila* cuticular hydrocarbon biosynthesis. These genes must be involved in some processes in the scheme. The gene on the third chromosome in Coyne's study (1994), *Ngbo*, and the gene(s) on the second chromosome in this study may be key factors in biosynthesis. These genes could cause a pheromonal difference between two species.

Contrary to the simple genetic basis of sex pheromone production, that of the dorsal abdominal stripe of *D. novamexicana* is controlled by many factors (Spicer, 1991). This character is not a species-specific mate recognition signal but a species-specific morphological character in the *virilis* species group. All five major chromosomes (four autosomes and the sex chromosome) showed significant effects on dorsal stripe formation. This means that a minimum of five loci are involved in the character difference between the two species, and this character seemed to be derived from many evolutionary changes over a long period. Since our study has shown that 11-P is controlled by only two chromosomes, the genetic

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basis of this species-specific character concerning reproductive isolation must be much simpler than that of other characters. This simple genetic basis probably indicates that the species-specific mate signal developed much more rapidly than other characters.

To construct a process of signal evolution in *virilis* species group, further analyses including the identification of sex pheromones in other members of this group and their genetic basis are required. Therefore, this study may be an example and a step in the evaluation of the genetic basis of the *virilis* group speciation process.

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