

Role of X Chromosomal Song Genes in the Evolution of Species-Specific Courtship Songs in *Drosophila virilis* Group Species

Seliina Päällysaho,^{1,2} Jouni Aspi,¹ Jaana O. Liimatainen,¹ and Anneli Hoikkala¹

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In the *Drosophila virilis* group the males of the *virilis* phylad species produce courtship song consisting of pulse trains with no pauses between successive sound pulses, whereas the males of the *montana* phylad species produce songs with clear pauses between the sound pulses. We obtained song data for F₁ hybrids between *D. virilis* (representing the *virilis* phylad) or *D. flavomontana* (representing the *montana* phylad) females and the males of several species of the *D. virilis* group to study the interaction of X chromosomal and autosomal song genes affecting species differences in song. In crosses with *D. virilis* females, X chromosomal (or maternal) factors masked variation in pulse length despite variation in heterospecific autosomal song genes. To the contrary, in crosses with *D. flavomontana* females, X chromosomal genes largely determined the pause length and interacted with autosomal genes to determine the pulse length. In the *montana* phylad species, pulse length showed dominance toward shorter pulses and pause length toward longer pauses. The first-mentioned trait also indicated the epistatic effects of X chromosomal and autosomal components.

KEY WORDS: Courtship song; dominance; *Drosophila virilis* group; epistasis; evolution.

INTRODUCTION

Recently, the genetic basis of factors maintaining pre-mating isolation mechanisms, especially sexual isolation, has attracted much attention (see Ritchie and Phillips, 1998). This kind of data is needed to determine whether speciation proceeds gradually or whether it involves fixation of genes with a large effect (Coyne *et al.*, 1994). Research on interspecific hybrids, however, is often complicated or hindered by defects in hybrid viability and fertility as well as by restricted recombination, and material for this kind of study is not easy to find.

In a review on sex linkage among genes controlling sexually selected traits, Reinhold (1998) states that these are influenced significantly more often by X chromosomal genes than traits not under sexual selection. As Hollocher and Wu (1996) and True *et al.* (1996) have shown, however, sex linkage may simply be caused by differential expression owing to the hemizygoty, rather than by accumulation of X-linked genes. Furthermore, as Cabot *et al.* (1994) have suggested, weak multilocus interactions are a very common cause of maintaining isolation between closely related species, and studying the effects of X chromosomal genes without studying their epistatic interaction with autosomal genes may be misleading.

Many behavioral genes in *D. melanogaster* are known to be located on the X chromosome (reviewed by Yamamoto *et al.*, 1997). In addition, earlier studies (Hoikkala and Lumme, 1987) have shown that, at least in three species of the *D. virilis* group (*D. littoralis*,

¹ Department of Biology, University of Oulu, P. O. Box 3000, 90014 Oulu, Finland.

² To whom all correspondence should be addressed at Department of Biology, University of Oulu, P. O. Box 3000, 90014 Oulu, Finland. Tel: +358-8-553-1786. Fax: +358-8-553-1061. e-mail: spaallys@paju.oulu.fi

D. flavomontana, *D. lacicola*), species-specificity of the courtship songs is largely caused by X chromosomal gene(s). Hoikkala and Lumme (1987) have suggested that an X chromosomal major change allowing variation in the interpulse interval has occurred during the separation of the two major *D. virilis* group phylads (*virilis* and *montana* phylad; Spicer, 1991; 1992), with the long interpulse interval also allowing variation in pulse length and cycle number in species of the latter phylad. Here we have studied how the X chromosomal song genes of *D. virilis* and *D. flavomontana*, representing two different phylads, interact with homo-/heterospecific autosomal genes, and whether the song traits show dominance, epistasis, or both in interspecific F_1 males.

MATERIALS AND METHODS

Flies and Crosses

For this study we chose four species of the *D. virilis* group, differing in their song characters. *D. virilis*, probably the most ancient species of the group, belongs to the *virilis* phylad (Spicer, 1991; 1992) and has dense pulse trains without pauses between pulses (Fig. 1) as do the rest of the species of this phylad (Hoikkala and Lumme, 1987). The other three species belong to the *montana* phylad: *D. littoralis* has very long pauses between sound pulses and belongs to the *littoralis* subphylad; *D. montana* has short pauses and *D. flavomontana* has intermediate pauses, and they belong to the *montana* subphylad (Fig. 1). The strains used in the present study had been maintained in the laboratory for at least 5 years before the present studies. *D. virilis* strain A originates from Moscow, Russia; *D. littoralis* strain li1 and *D. montana* strain mo1 are both from Kemi, Finland; and *D. flavomontana*, strain 0981.0 is from Idaho, (obtained from *Drosophila* Species Stock Center, Tucson, Arizona).

To determine how the X chromosomal song gene(s) interacts with autosomal song genes of different species, we made reciprocal crosses between these four species using *D. virilis* (representing species with no pause between sound pulses) or *D. flavomontana* (representing species with a clear pause between pulses) always as one parent. All crosses, where *D. virilis* or *D. flavomontana* was the maternal species, were successful. When using *D. montana* as a maternal species, F_1 progeny were obtained; however, hybrid males did not court. If *D. virilis* or *D. flavomontana* was the paternal species, crosses were unsuccessful, except for the reciprocal crosses between these two species. The cross between

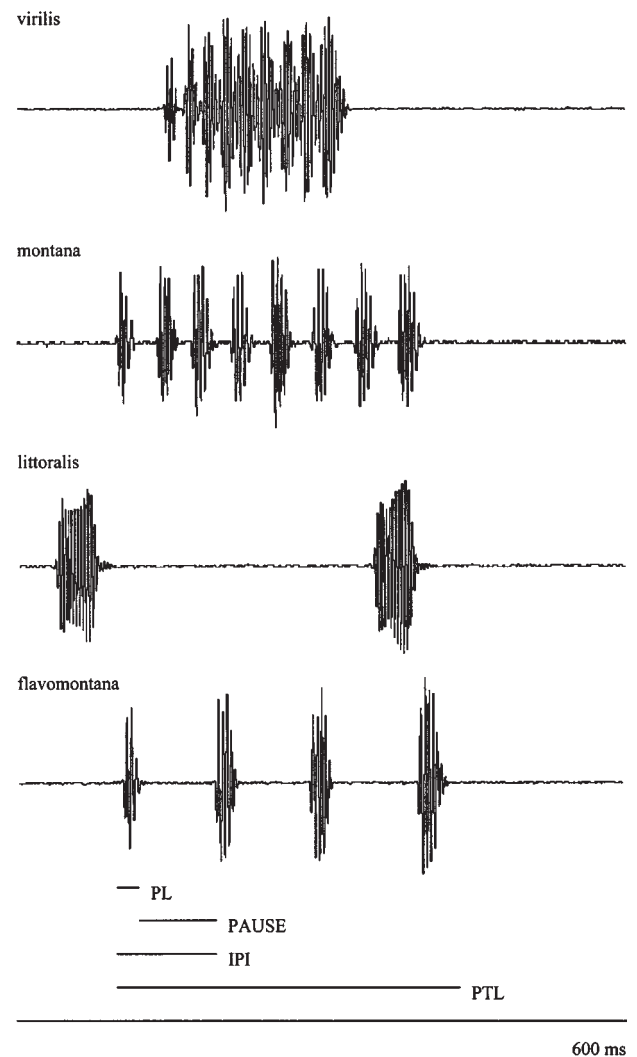


Fig. 1. Songs of *Drosophila virilis*, *D. montana*, *D. littoralis*, and *D. flavomontana* males. PL, pulse length; PAUSE, distance from end of pulse to beginning of next pulse; IPI, interpulse interval; PTL, pulse train length.

D. flavomontana females and *D. montana* males produced fertile progeny and was continued into the F_2 generation.

The strains were maintained in culture bottles containing malt medium in continuous light and at 19°C. Males and females were collected in separate vials no later than 2 days after their emergence. The flies were used in song recordings and in crosses at the age of about 2 weeks. Interspecific F_1 hybrids were obtained by making several mass-mating lines (10 males and 10 females per bottle) between the flies of different species. In each cross, the hybrid progeny was collected from at least three different mass-mating lines.

Song Recording and Analyses

Male courtship songs were recorded when the male courted a female, which could be of the same species or a hybrid involving the species in question. The recording chamber was made of a Petri dish (diameter, 5 cm; height, 0.7 cm), which was covered with nylon net. The floor of the chamber was covered with a moistened filter paper. Songs were recorded with a Sony TC-FX33 cassette recorder and a JVC-condenser microphone, when both the male and female were upside down on the roof of the chamber close to the diaphragm of the microphone.

Male songs were analyzed using the SIGNAL Sound Analysis System (© Engineering Design). For each male we analyzed three pulse trains of song and calculated the means of different pulse song traits over these trains. The songs were analyzed (Fig. 1) by measuring the length of the pulse train (PTL) and counting the number of pulses per train (PN) in oscillograms made for the songs (for *D. littoralis* only the first two pulses of the train are shown). We also measured the length of the fourth (or third in short songs) sound pulse of the pulse train (pulse length, PL), and we counted the number of cycles in this pulse (CN). The interpulse interval (IPI) was measured as a distance from the beginning of the analyzed pulse to the beginning of the next pulse, and PAUSE was measured as a distance from the end of the pulse to the beginning of the next one. The four last-mentioned song traits were measured from the enlarged oscillograms showing only the respective sound pulses. The carrier frequency of the song was measured from Fourier spectra of the pulse train. For statistical analysis PTL, PL, IPI, and PAUSE were ln-transformed to achieve normal distribution. We added 1 to all values of PAUSE before transformation, because *D. virilis* always had 0 for PAUSE.

RESULTS

Songs of the Parental Species and F₁ Hybrids

The songs of *D. virilis*, *D. montana*, *D. littoralis*, and *D. flavomontana* are shown in Fig. 1. Each song character differed significantly between species (Kruskal–Wallis test; $\chi^2 = 26.66\text{--}37.21$; $df = 3$ and $P < 0.001$ in each case). The song characters showing the clearest species differences were the PAUSE, PL, and IPI, with the IPI being the sum of the two first-mentioned traits (Table I). In multiple comparison (Dunn's test) these three characters grouped the species

Table I. Means and Standard Deviations of Different Song Traits in Milliseconds in *Drosophila virilis*, *D. montana*, *D. littoralis*, and *D. flavomontana* and All Obtained Interspecific Hybrid Males^a

Species/cross	N	PAUSE	PL	IPI
<i>virilis</i>	10	0	19.7 ± 1.25	19.7 ± 1.25
<i>montana</i>	10	15.0 ± 2.21	20.7 ± 2.71	35.7 ± 2.11
<i>littoralis</i>	10	245 ± 52.40	41.2 ± 4.96	286 ± 51.50
<i>flavomontana</i>	10	85.7 ± 9.94	27.1 ± 2.38	113 ± 8.93
vir × mon	5	10.2 ± 0.84	20.0 ± 2.74	30.2 ± 3.27
vir × lit	10	20.0 ± 2.18	19.4 ± 2.90	39.4 ± 2.32
vir × fla	10	12.0 ± 2.00	17.5 ± 2.07	29.5 ± 2.22
fla × vir	10	19.7 ± 6.57	22.9 ± 2.18	42.6 ± 5.21
fla × mon	8	39.0 ± 8.88	16.6 ± 0.92	55.6 ± 9.02
fla × lit	5	128 ± 19.10	20.8 ± 0.84	148 ± 19.10
F ₂ (fla × mo) ^b	11	43.7 ± 15.7	22.2 ± 2.89	65.9 ± 16.6

^a N is the number of males, PAUSE is the distance from the end of the pulse to the beginning of the next one, PL is the pulse length, and IPI is the interpulse interval. In the interspecific crosses, the female is always mentioned first.

^b A single male with *D. montana* X chromosome not included.

unambiguously, the only exception being PL between *D. montana* and *D. virilis*. In addition, discriminant analysis with PAUSE and PL classified the species with 100% confidence. Accordingly, these two characters have been used in the analysis of hybrid songs below.

The only species pair that we succeeded to cross reciprocally was *D. virilis* and *D. flavomontana*. The hybrids having the X chromosome of *D. virilis* (Fig. 2a) had shorter PAUSEs (t -test: $t = 4.166$; $df = 18$; $P = 0.001$) and PLs ($t = 5.323$; $df = 18$; $P = 0.001$) than the hybrids having the X chromosome of *D. flavomontana* (Fig. 2b), which refers to sex linkage.

Next we studied the possible interactions between the X chromosomal and autosomal song genes. Table I and Fig. 3 (note that Fig. 3 is in ln-logarithmic scale) show the data on the songs of the hybrid males having the X chromosome and one set of autosomes from the maternal species (*D. virilis* or *D. flavomontana*) and the second set of autosomes from the paternal species (*D. virilis*, *D. montana*, *D. flavomontana*, or *D. littoralis*). The two sets of hybrid data with the X chromosome of either *D. virilis* or *D. flavomontana* enabled us to check whether the maternal genotype of the hybrid males functions in a different way with the autosomes of different species.

The PAUSE in male song increased in crosses using *D. flavomontana* as a maternal species by about 7.5 ms (original scale) when compared with the reciprocal cross with *D. virilis* as a mother. The two-way

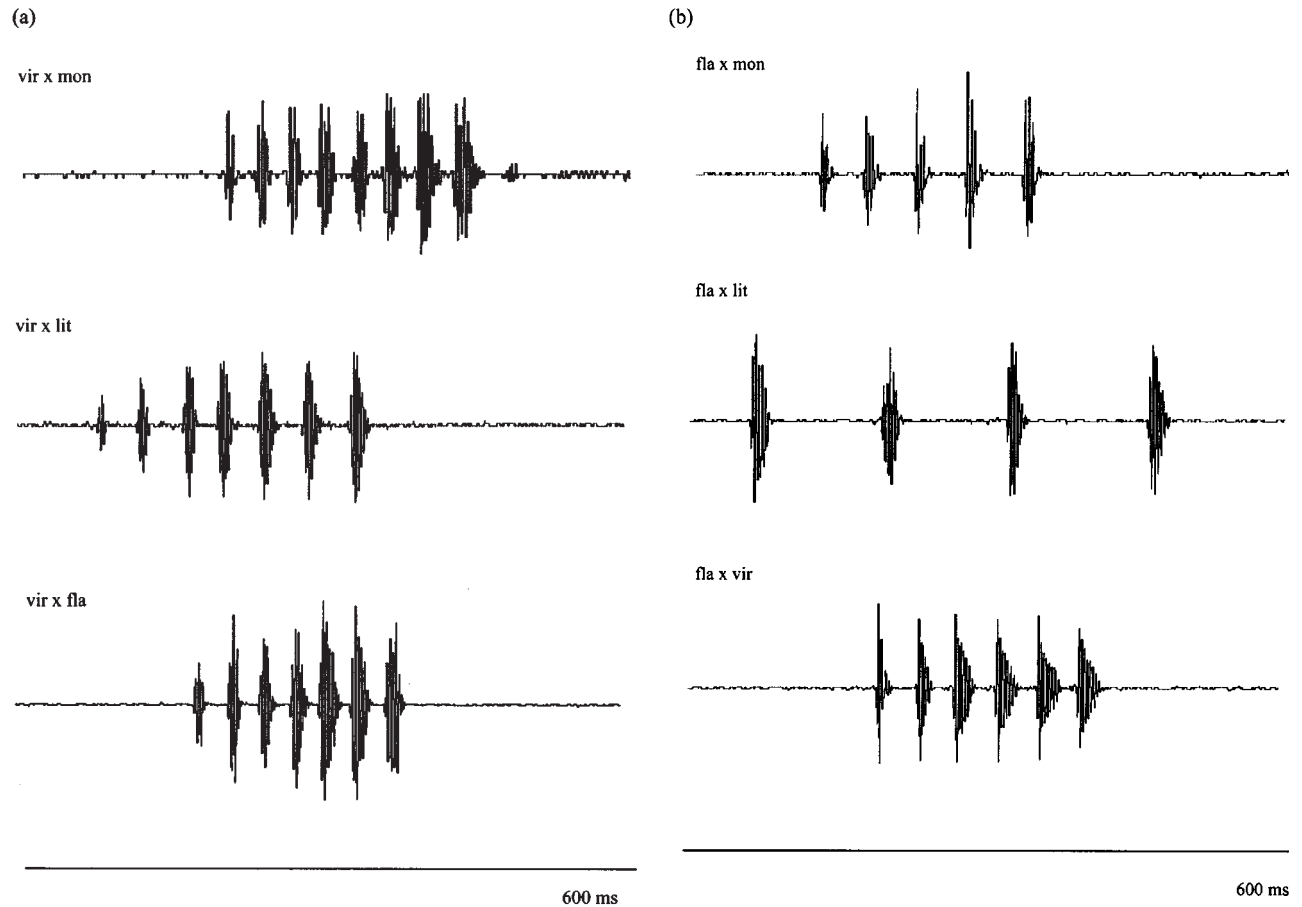


Fig. 2. Songs of F₁ hybrids (a) between *Drosophila virilis* females and *D. montana*, *D. littoralis*, and *D. flavomontana* males, and (b) between *D. flavomontana* females and *D. montana*, *D. littoralis*, and *D. virilis* males.

analysis of variance (ANOVA) revealed that the contribution of both maternal (X + cytoplasmic factors + one set of autosomes) and paternal (Y + one set of autosomes) chromosomes was significant (Table II). A significant interaction also occurred between maternal and paternal genotypes. In ANOVA, the highest percentage of variation was among maternal factors (60.9%), followed by variation among paternal factors (32.9%), and the interaction component between maternal and paternal factors (7.1%). The contribution of autosomal and Y chromosomal effects and their interaction with maternal factors was much smaller than the effects of X chromosomal and other maternal factors. Thus, long pauses seem to be caused mainly by the X chromosomal song gene(s) of *D. flavomontana*.

For PL, the two-way ANOVA did not reveal any constant maternal or paternal effects, and only the inter-

action was significant, suggesting that the X chromosomes of *D. virilis* and *D. flavomontana* do not interact similarly with the different sets of autosomes (Table II). The crosses with *D. flavomontana* as a maternal species, when analyzed separately, differed significantly from each other (one-way ANOVA: $F = 55.086$; $df = 1, 31$; and $P < 0.001$), while the crosses with *D. virilis* as a maternal species showed no differences (one-way ANOVA: $F = 2.336$; $df = 1, 31$; and $P < 0.93$) (Fig. 3b). This suggests that autosomal genes are important in causing species differences in PL in the *D. montana* phylad and that they interact with the *D. flavomontana* X chromosome, while the X chromosomal (or maternal) factors of *D. virilis* mask variation in PL despite variation in autosomal components.

Possible dominance or epistasis in song characters was tested by comparing the means of the F₁ males with

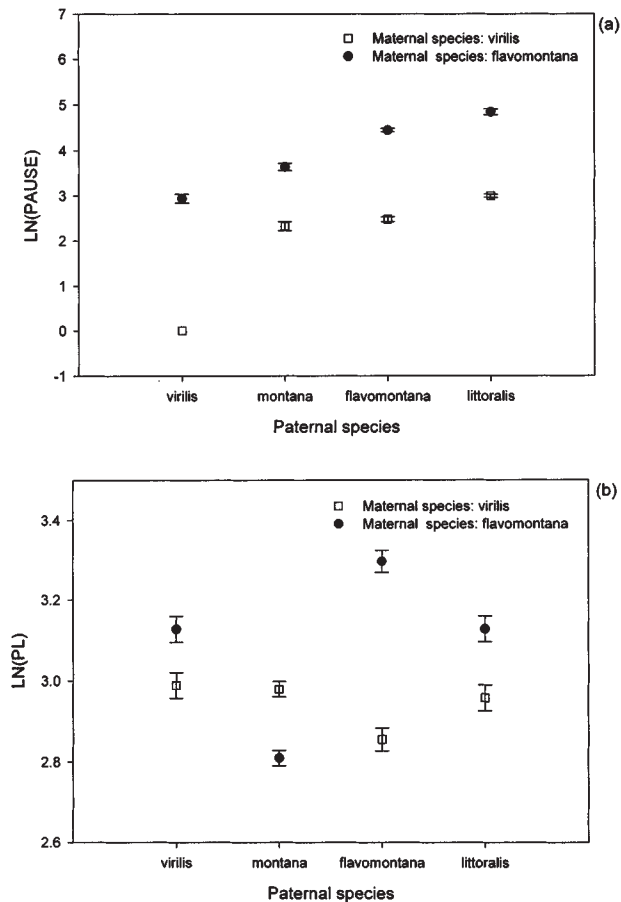


Fig. 3. The (a) PAUSE (mean \pm SE) and (b) PL (mean \pm SE) in crosses with *Drosophila virilis* or *D. flavomontana* as a maternal and *D. virilis*, *D. montana*, *D. flavomontana*, or *D. littoralis* as a paternal species (note the logarithmic scale). PAUSE, distance from end of pulse to beginning of next pulse; PL, pulse length.

the average of the parental species (dominance effects can be detected only from ln-transformed data in Fig. 4, and not from the Table I because of scale effect; see, e.g., Lynch and Walsh, 1998). PAUSE (Figs. 4a and 4b) showed significant dominance toward longer pauses (i.e., PAUSEs in the songs of F₁ males exceeded the average of the parental species) in all crosses irrespective of the maternal species (*t*-test; $P < 0.01$ in each case), except in the cross between *D. flavomontana* and *D. littoralis* ($t = 4.8405$; $df = 4$; $P = 0.1431$). However, dominance was quite small because the means of the F₁ males were close to the averages of the parental species.

Pulse length showed dominance toward shorter pulses irrespective of the maternal species in each of the five crosses, where the paternal species was from the *D. montana* phylad (Figs. 4c and 4d). The means

Table II. Two-Way Analysis of Variance for the Maternal Factors (*Drosophila virilis*, *D. flavomontana*), Paternal Factors (*D. virilis*, *D. montana*, *D. flavomontana*, *D. littoralis*) and Their Interaction Affecting PAUSE and PL in the Interspecific F₁ Hybrids^a

	PAUSE		PL	
	$F_{(1,60)}$	P	$F_{(1,60)}$	P
Maternal factors	35.021	0.010	0.931	0.406
Paternal factors	10.797	0.041	0.352	0.793
Interaction	61.717	0.001	24.17	0.001

^a PAUSE, Distance from the end of the pulse to the beginning of the next one; PL, pulse length.

of the F₁ males differed from the average of the parental species in all crosses (*t*-test; $P < 0.001$ in each case), except in the cross between *D. virilis* and *D. montana* ($t = -0.287$; $df = 4$; $P = 0.789$), where the PLs of the parental species did not differ significantly from each other. The fact that the PLs of F₁'s were much shorter than those of either of the parental species indicates that dominance could not be a sole explanation and that there must also be epistatic effects of X chromosome and autosomal components. The crosses with *D. virilis* as a paternal species showed no significant dominance ($t = -0.526$; $df = 4$; $P = 0.626$).

F₂ Hybrids Between *D. flavomontana* and *D. montana*

To determine how much the song traits vary in hybrids having the X chromosome of one species and the autosomes of the two parent species in various combinations of homo- and heterozygosity, we produced an F₂ generation from the cross *D. flavomontana* \times *D. montana* and obtained 38 males. This species pair was chosen for further analysis because it had been reported to give fertile progeny of both sexes (Throckmorton, 1982), and because the salivary gland chromosomes of interspecific F₁ females showed conjugation at the proximal end of the X chromosome (Päällysaho, 2002), where major song gene(s) has earlier been localized in *D. littoralis* (Päällysaho *et al.*, 2001). Restriction fragment length polymorphism (RFLP) analysis with three markers (*fused*, *nonA*, and *paralytic*; see Päällysaho *et al.*, 2001) distributed along the proximal half of the X chromosome, however, showed no sign of recombination among the F₂ males (Päällysaho, 2002). Even though the data could not be used for localizing any X chromosomal song genes in *D. flavomontana*, the

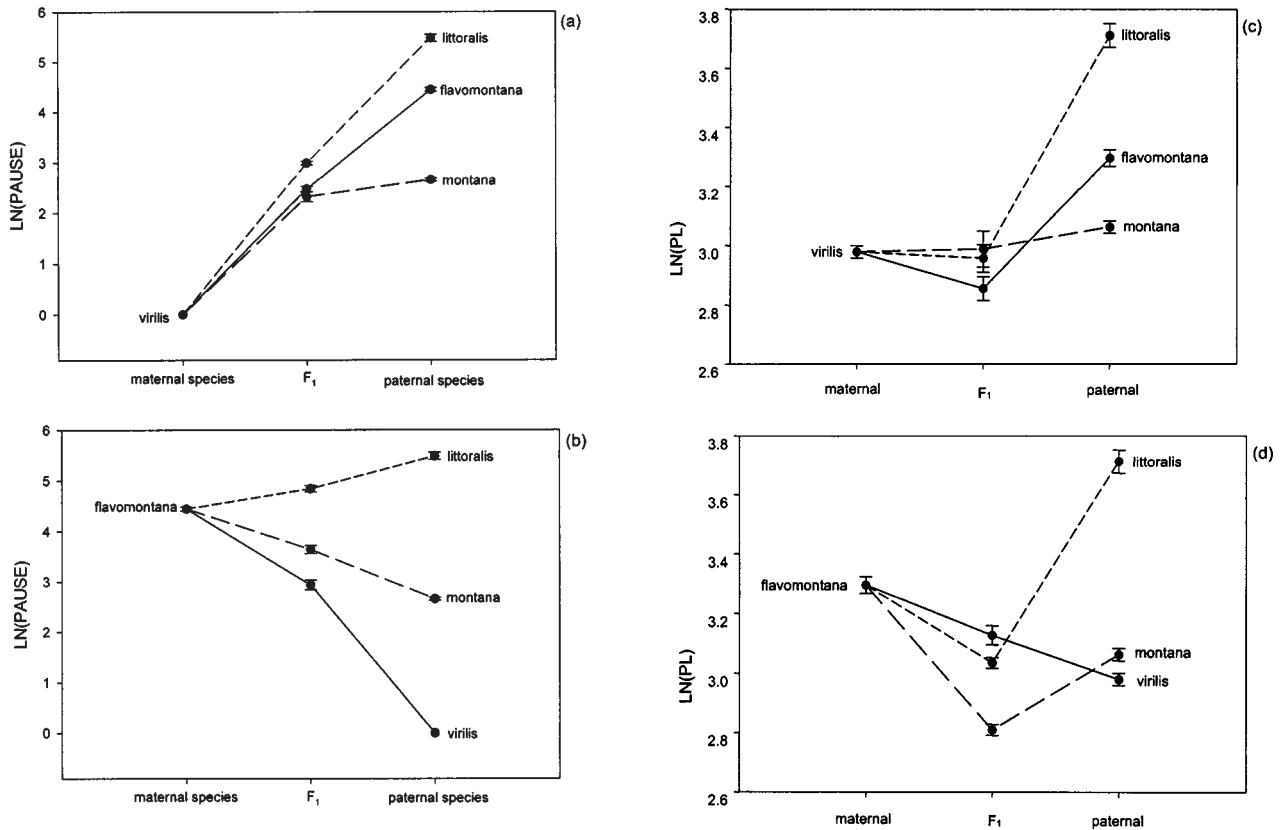


Fig. 4. PAUSE (mean \pm SE) of the male courtship song in parental species and F₁ hybrids with (a) *Drosophila virilis* or (b) *D. flavomontana* as a maternal species, and PL (mean \pm SE) of the male courtship song in parental species and F₁ hybrids with (c) *D. virilis* or (d) *D. flavomontana* as a maternal species (note the logarithmic scale). PAUSE, distance from end of pulse to beginning of next pulse; PL, pulse length.

markers enabled us to identify the origin of the X chromosome (or at least the proximal part of it) in F₂ males.

According to their markers, 13 F₂ males resembled *D. flavomontana* and 25 F₂ males resembled *D. montana* (Päälylsaho, 2002). Only one of 25 males with a *D. montana* X chromosome began to court and sing to the female (Fig. 5), even though each male was given a trial on three separate days, at least 2 hours at a time. Analysis of F₂ males consists of only hybrids having the *D. flavomontana* X chromosome ($n = 11$; Table I). Interspecific F₁ hybrids between *D. flavomontana* and *D. montana* had intermediate PAUSEs and very short PLs compared with those of the parental species (Fig. 5). In F₂ generation the PAUSEs of the males were intermediate to those of the parental species, with the mean PAUSE being similar to that of the F₁ generation. On the other hand, F₂ males had significantly longer sound pulses than did F₁ males ($F = 14.15$; $df = 1, 18$; $P = 0.001$; Fig. 5), which suggests that in addition to X chromosomal genes, PL is also affected by the autosomal genes of the parental species

whose autonomous or interactive effects become visible in homozygous conditions. In F₂ males, the ranges of PAUSE and PL were much wider than the ranges of respective traits of the parental species, suggesting that there is a substantial amount of segregational variance in these characters. The song of the only singing F₂ male with a *D. montana* X chromosome very much resembled the song of *D. montana* (Fig. 5).

DISCUSSION

The species-specific characters of courtship songs of *Drosophila* males are typically controlled by multiple genes with minor effects (e.g., Pugh and Ritchie, 1996; Noor and Aquadro, 1998; Hoikkala *et al.*, 2000; Williams *et al.*, 2001). Hoikkala and Lumme (1987) have suggested that during the separation of the two *D. virilis* group phylads, however, there has occurred a major X chromosomal change, allowing variation in the IPI of the male courtship song in *montana* phylad species. In the present study, we dissected the IPI into its compo-

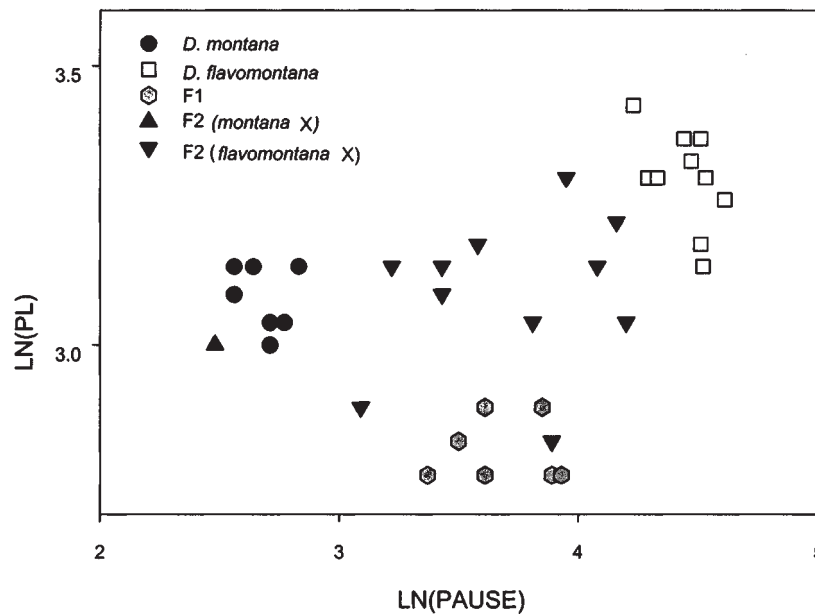


Fig. 5. Scattergram for PAUSE and PL for the songs of *Drosophila montana* and *D. flavomontana*, and their interspecific F₁ and F₂ hybrid males (note the logarithmic scale). PAUSE, distance from end of pulse to beginning of next pulse; PL, pulse length.

nents, PAUSE and PL, and studied how large an effect the X chromosomal genes had on these song traits alone and in interaction with autosomal genes. If the hybrid had X chromosomal (or maternal) factors of *D. virilis*, the genotype (species) of the father had only a minor effect on PL, while the X chromosomal factors of *D. flavomontana* caused long pauses and interacted with the autosomal genes in determining the PL. Our findings confirm that the X chromosomal gene(s) has (have) an important role in song evolution in *D. montana* phylad species.

We have previously shown that a gene or a gene cluster affecting song differences between *D. virilis* and *D. littoralis* is located at the proximal region of the X chromosome (Hoikkala *et al.*, 2000; Päällysaho *et al.*, 2001). Multiple chromosome rearrangements restricting recombination between homologous X chromosomes, however, prevent precise localization of the song gene(s) with the aid of these species or with the aid of *D. flavomontana* and *D. montana* hybrids (Päällysaho, 2002). The present finding that the X chromosomal genes of *D. virilis* (a species of *virilis* phylad) and *D. flavomontana* (a species of *montana* phylad) show different epistatic effects with autosomal genes refers to a central role of these genes in song evolution. Wide variation in the songs of the F₂ males having the X chromosome of *D. flavomontana* also suggest a substantial amount of segregational variance in both PAUSE and PL in the autosomal genes of *D. flavomontana* and *D. montana*.

Previously, Hoikkala and Lumme (1987) found unidirectional dominance in PTL and CN in the songs of the *virilis* phylad species and proposed that the song evolution in this phylad has gone toward longer and denser pulse trains. This argument was based on the selection theory, where high fitness traits are presumed to attain directional dominance and epistasis (see Lynch and Walsh, 1998). In the present study, we found strong epistasis and unidirectional dominance toward shorter pulses and longer pauses in *montana* phylad species, which suggests that song evolution in the *montana* phylad species has favored longer pauses and shorter pulses. Empirical evidence on dominance indicating the direction of evolution comes mainly from studies with pesticide resistance (Bourguet and Raymond, 1998) and the evolution of lepidopteran wing patterns (Wagner and Bürger, 1985).

It has been proposed that sexual selection exercised by the females could be a driving force in song evolution. *D. montana* females have been found to prefer males with short and dense (high-frequency) sound pulses (Aspi and Hoikkala, 1995; Ritchie *et al.*, 1998), and the song of this species shows directional dominance in the same direction (Suvanto *et al.*, 2000). Female preferences, however, may vary in different species, and sexual selection within the species may not be the only selection pressure affecting male songs. The song of *D. littoralis* with exceptionally long pauses

and sound pulses is a puzzle. Evolution of long sound pulses in this species, however, may be a side effect of genes causing the long pauses (Hoikkala *et al.*, 2000). Songs resembling the song of *D. littoralis* are found outside *D. virilis* group species at least in some species of *D. melanica* group (Ewing, 1970) and in *D. robusta* (Hoikkala, unpublished results).

Moreno (1994) has suggested that interlocus interactions within the sets of functionally related loci can hide a potential for strong phenotypic effects. Epistatic interactions are a central component of Wright's shifting balance hypothesis that postulates a creative role for small populations and founder events in character evolution and speciation (Wright, 1977). While the shifting balance theory and its role in speciation generally are still controversial (see, e.g., Wade and Goodnight, 1998), more data on epistatic interactions are needed on interspecific level (see Cabot *et al.*, 1994) in addition to intraspecific studies (e.g., Gonzalez, 1990; Hard *et al.*, 1992; Aspi, 1999).

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