

Complex Inheritance of Male Courtship Song Characters in *Drosophila virilis*

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We analyzed the inheritance of two male courtship song characters, number of pulses in a pulse train, and length of a pulse train in *Drosophila virilis*. Biometric analyses of song differences among 16 crosses over three generations (parental and reciprocal F₁, F₂, and backcrosses) were performed using two different approaches. The joint scaling test revealed significant additive and dominance components, and also significant additive interaction between maternal and progeny genotypes. The direction of dominance was toward shorter and denser pulses in *D. virilis*. In addition, planned comparisons (contrast analyses of variance) between different generations revealed significant Y chromosomal and transient maternal factors and their interactions, with all other factors contributing to differences in the means of song characters. These results suggest that the genetic basis of courtship song characters in *D. virilis* is primarily of autosomal polygenic nature, with small but significant Y chromosomal and nonchromosomal maternal effects.

KEY WORDS: Courtship song; dominance; *Drosophila virilis*; transient maternal effect; Y chromosome.

INTRODUCTION

Courtship is a complex set of behaviors in *Drosophila* recruiting a wide range of genes for its realization. Results from different experimental approaches sketch the outlines of a set of pleiotropic genes acting on a distributed system in the brain and nervous system to produce the species-specific sequence of responses and actions (Greenspan and Ferveur, 2000). An important component of the courtship is the species-specific male courtship song produced by the male wing vibration (Aspi and Hoikkala, 1995; Hoikkala, 1988; Hoikkala *et al.*, 1998; Kyriacou and Hall, 1982; Ritchie *et al.*, 1999). However, the importance of these acoustic signals during the courtship seems to vary even between closely related species. Females of two *D. virilis* group

species, *D. virilis* and *D. montana*, have different requirements for the courting male. The stimulatory effect of wing vibration is not necessary for *D. virilis* females to accept the courting male (Hoikkala 1988, Isoherranen *et al.*, 1999a); whereas, for *D. montana* females, the song is a prerequisite to copulation (Liimatainen *et al.*, 1992). However, song simulation experiments show that *D. virilis* females are able to recognize species-specific characteristics in male song. The proportion of females responding to species-specific song is about twice that of females responding to songs with modified sound pulses and interpulse intervals (Isoherranen *et al.*, 1999b).

The inheritance pattern of interspecific differences in several song characters has been investigated in hybrids between *D. virilis* and *D. littoralis*. By conventional segregation analysis, X chromosomal genes near the proximal end have been found to account for most of the differences in song characters (pause and pulse length) even though there are also autosomal factors controlling them (Hoikkala *et al.*, 2000; Päällysaho *et al.*, 2001). *D. virilis* females have also been crossed with males of

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several species of the *D. virilis* group to study the interaction of X chromosomal and autosomal song genes affecting species differences in song. These crosses suggest that the X chromosomal (or maternal) factors of *D. virilis* prevent variation in the length of a sound pulse despite variation in heterospecific autosomal song genes (Päällysaho *et al.*, 2003). On the other hand, the genes contributing to the species differences in the number of pulses in a pulse train (PN) between *D. virilis* and *D. lummei* are distributed on all autosomes but not on the X chromosome (Hoikkala and Lumme, 1984).

A worldwide study of ~40 *D. virilis* laboratory strains has revealed significant interstrain and geographic variation in male song characters (A. Hoikkala, unpublished). The PN and length of a pulse train (PTL) show the largest coefficient of variation (15.5% and 16.1%, respectively; A. Hoikkala, unpublished). However, there is no prior information on the genetic basis of intraspecific variation in these song characters.

The studies on hereditary differences in behavioral traits are often designed to test the autosomal additive/dominance model, even though the behavioral characters may have a very complex genetic basis. Nonautosomal effects could be caused by sex chromosomes, which may counteract or superimpose each other (de Belle and Sokolowski, 1987), and epistatic interactions within the sets of functionally related loci may be common and may hide the potential for strong phenotypic effects (Moreno, 1994). In addition to genes transmitted in subsequent generations, nonchromosomal cellular and cytoplasmic factors are also transmitted from maternal parent to progeny (de Belle and Sokolowski, 1987). Wahlsten (1979) distinguishes between two types of maternally inherited cytoplasmic components showing different patterns of heredity. Permanent cytoplasmic factors include any nonchromosomal component of the egg, that persists throughout the lifetime of the organism and is transmitted over generations (e.g., mitochondrial DNA). Transient maternal factors are nonchromosomal components of the egg that are not transmitted to subsequent generations and include maternal gene products and nutrients. Examples of inheritance of both types of maternal factors have been described in *Drosophila* (e.g., Bauer and Sokolowski, 1988; Gonzalez, 1990).

In this paper we have investigated the inheritance of two male courtship song characters, the PN and PTL between two strains of *D. virilis*. The relative contributions of autosomal and maternal additive, dominance, and epistatic effects to differences in two courtship song characters were examined using the joint scaling test (Kearsey and Pooni, 1996; Lynch and Walsh, 1998). In addition,

we examined the influence of Y chromosome and cytoplasmic factors by contrast analysis of variance. We also estimated the effective or minimum number of loci (n_E) contributing to variance in *D. virilis* male courtship song characters. The Quantitative Trait Loci (QTL) analysis with molecular markers will be discussed in a separate paper.

MATERIALS AND METHODS

Strains and Crosses

We used two *D. virilis* strains, 1431 and B22, to investigate the genetic basis of male courtship characters. These strains originate from different parts of the species distribution range (strain 1431 from England, and strain B22 from Japan) and in the preliminary analysis showed very divergent songs with respect to PN and PTL (A. Hoikkala, unpublished).

We raised parental, F_1 , F_2 , and backcross generations, including all reciprocals (Table I) in culture bottles with a malt medium at 19°C in continuous light. The flies were sexed under carbon dioxide anesthesia at age 1 to 2 days and used in courtship song recordings and crossing experiments when reproductively mature (7–10 days old).

Song Recording and Analysis

We recorded the male courtship song of each individual male when the male was courting a laboratory-reared female in a mating chamber (diameter, 5.5 cm; height, 1.3 cm), covered with nylon net and the floor moistened with a filter paper. We made the song recordings with a Sony TC-FX33 cassette recorder and a JVC-condenser microphone at $20 \pm 1^\circ\text{C}$, and analyzed the oscillograms of the songs using the Signal Sound Analysis System (©Engineering Design). We counted the PN and measured the PTL for each song. To decrease the within male variation, the values of both characters for each male were calculated as a mean over three songs.

The differences in the other song characters, pulse length (PL), number of sound cycles (CN), interpulse interval (IPI), and carrier frequency (FRE) between these two strains were not significant (data not presented; *t*-test, $P > 0.05$ in each case).

Biometric Methods

We estimated the effects of different genetic factors by using two basically different biometric methods. The effects of additive, dominance, epistatic, and ma-

Table I. Generation Means (\pm Standard Deviation) of Male Courtship Song Characters in Different Crosses of *Drosophila virilis*

Crosses	N^a	PN ^b	PTL ^c (ms)
Parental strains			
1 1431	45	10.2 \pm 1.2	213.6 \pm 32.3
2 B22	50	7.0 \pm 0.9	159.5 \pm 25.1
Reciprocal F ₁ hybrids			
3 1431 \times B22	45	9.2 \pm 0.7	178.4 \pm 18.1
4 B22 \times 1431	84	8.8 \pm 0.9	171.9 \pm 20.7
Reciprocal backcrosses			
5 1431 (1431 \times B22)	105	9.9 \pm 1.0	216.3 \pm 22.9
6 1431 (B22 \times 1431)	89	10.2 \pm 1.3	210.8 \pm 29.5
7 B22 (1431 \times B22)	109	8.4 \pm 1.1	191.4 \pm 23.1
8 B22 (B22 \times 1431)	104	8.2 \pm 1.0	191.4 \pm 23.7
9 (1431 \times B22) 1431	107	10.6 \pm 1.1	232.2 \pm 27.1
10 (B22 \times 1431) 1431	110	10.5 \pm 1.2	236.0 \pm 27.7
11 (1431 \times B22) B22	100	8.5 \pm 1.0	198.4 \pm 23.8
12 (B22 \times 1431) B22	104	8.3 \pm 1.0	197.9 \pm 24.8
Reciprocal F ₂ hybrids			
13 (1431 \times B22) (1431 \times B22)	220	9.9 \pm 1.3	214.0 \pm 29.2
14 (1431 \times B22) (B22 \times 1431)	219	9.6 \pm 1.2	207.1 \pm 26.6
15 (B22 \times 1431) (1431 \times B22)	203	9.7 \pm 1.2	214.2 \pm 26.3
16 (B22 \times 1431) (B22 \times 1431)	248	9.5 \pm 1.2	201.5 \pm 27.3

^a Number of individuals analyzed.^b Number of pulses in a pulse train.^c Pulse train length.

ternal factors have often been analyzed by the joint scaling test, in which the amount and significance of these factors are estimated simultaneously from several generation means using a maximum likelihood method. It is possible to include Y chromosomal, cytoplasmic, and maternal genotype effects, as well as their interactions, to the joint scaling models. However, the more parameters that are added, the more difficult and more prone to systematic errors the model becomes. Therefore, the effects of these factors are often tested using the contrast analysis of variance (de Belle and Sokolowski, 1987) in which planned comparisons are conducted between crosses differing only with regard to the considered factor. These comparisons may reveal smaller effects, which are not necessarily significant when all generation means are considered simultaneously.

We analyzed the means of different generations with a joint scaling test using the weighted least squares method (Mather and Jinks, 1982; Kearsey and Pooni, 1996; Lynch and Walsh, 1998). This technique was used to derive estimates of midparent (m), additive ($[a]$), dominance ($[d]$), additive \times additive ($[aa]$), additive \times dom-

inance ($[ad]$), dominance \times dominance ($[dd]$), maternal genotype ($[a]_m$, $[d]_m$, and cytoplasmic $[c]$), and genotype/maternal interaction ($[a.am]$, $[a.dm]$, $[d.am]$, and $[d.dm]$) effects for male song characters (Mather and Jinks, 1982; Kearsey and Pooni, 1996). The observed generation means were first tested for fit to the models by successively incorporating only m ; m and $[a]$; and m , $[a]$, and $[d]$. The expected means of the six generations were calculated using the parameter estimates, the goodness-of-fit of the observed generation means was tested with the χ^2 -statistic, and the significance of each parameter was tested using the χ^2 -test (Mather and Jinks, 1982; Kearsey and Pooni, 1996). The more complex model was applied only if the test revealed that this model is more adequate than the simpler one.

To further determine the mode of inheritance of male song characters, we also performed a contrast analysis of variance. We made *a priori* comparisons of crosses to permit detection of significant contributions of autosomes, the Y chromosome, permanent cytoplasmic factors, transient maternal factors, and interaction effects. Differences between crosses, which share three

of the four factors in common were compared to detect the effect of the fourth factor. We performed nine possible comparisons (see de Belle and Sokolowski, 1987) between different crosses (Table I) for male courtship song data:

1. B22 vs. 1431 (1 vs. 2, Table I) to investigate the difference between the parental strains;
2. F₁ crosses (3 vs. 4) to investigate the deviation from autosomal mode of inheritance;
3. Parental strains vs. F₁ crosses (1 + 2 vs. 3 + 4) to investigate dominance;
4. F₂ crosses (13 + 15 vs. 14 + 16) for Y chromosomal effects;
5. Backcross to females (5 + 8 vs. 6 + 7) to examine interactions between the Y chromosome and all other factors;
6. F₂ crosses (13 + 14 vs. 15 + 16) for significance of permanent cytoplasmic factors;
7. Backcrosses to males (9 + 12 vs. 10 + 11) to investigate interaction between permanent cytoplasmic and all other factors;
8. Backcrosses (6 + 12 vs. 7 + 9) for transient maternal factors; and
9. Backcrosses (6 + 7 vs. 9 + 12) to investigate interaction between transient maternal factors and all other factors.

Note that it is not possible to examine the effect of the X chromosome separately from other maternal factors in male data.

Number of Effective Factors Contributing to Male Song Characters

We estimated the effective or minimum number of loci (n_E) contributing to variance in two male courtship song characters. Several statistical methods can be used to estimate the effective number of loci (e.g. Lande, 1981; Zeng *et al.*, 1990; Lynch and Walsh, 1998). The most widely used method, originally suggested by Wright, relates the difference in the means of two inbred lines to the segregational variance and is known as a Castle–Wright estimator (e.g., Wright, 1968). Segregational variance in a character describes the excess variance that appears in the F₂ and backcross generations as a consequence of the segregation of parental strain genes. We used the iterative process of Hayman (1960) to calculate the maximum likelihood estimates of the parental and segregational variances, as described in Lynch and Walsh (1998, pp. 226–231). Goodness-of-fit was tested by the χ^2 -test, which com-

pares observed values with the maximum likelihood estimates of those variances in the additive model (Lynch and Walsh, 1998). We used the estimated segregational variances to calculate the Castle–Wright estimators (Lynch and Walsh, 1998) for the number of effecting factors contributing to differences in means of courtship song characters in PN and PTL. We also calculated the approximate variance and standard error of n_E by using the formula (Eq. 9.25) derived in Lynch and Walsh (1998).

RESULTS

We recorded and analyzed the courtship songs of 1942 individual males from 16 different crosses over three generations (Table I) between two *D. virilis* strains. The phenotypic means of parental strains 1431 and B22 differed significantly in both PN and PTL (Fig. 1; see Table III, comparison 1), males of strain 1431 having longer PN and PTL than males of strain B22 (Table I).

The generation means analysis by the joint scaling test suggested that a simple additive-dominance model was not an adequate representation of the data in either song character. The analysis also revealed a significant and quite large additive \times additive interaction ($[a.am]$) between maternal and progeny genotypes (Table II). Dominance in PN was toward lower number of pulses and in PTL toward shorter pulse trains. In both cases the potence ratio ($[d]/[a]$) was negative (-0.51 for PN, and -1.95 for PTL), indicating that the

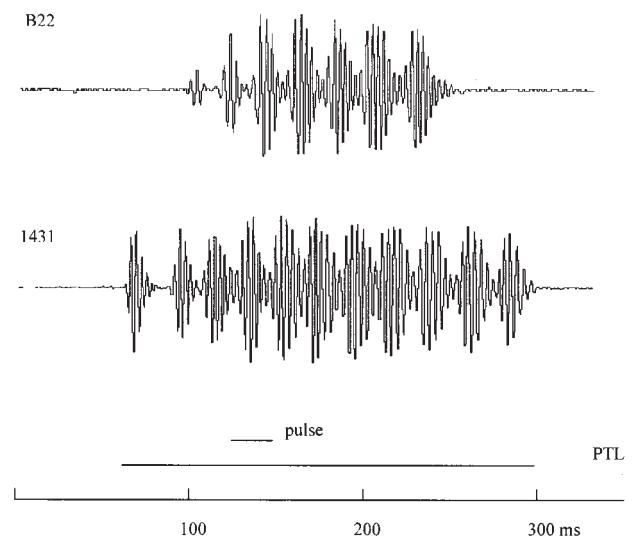


Fig. 1. Oscillograms of male courtship songs in strains B22 and 1431 of *Drosophila virilis*. PTL is the pulse train length.

Table II. Joint Scaling Test Estimates for Mean [*m*], Additive [*a*], Dominance [*d*], and Additive × Additive Maternal [*a.am*] Interaction Components (±standard error) of the Generation Means for Male Courtship Song Characters PN and PTL in *Drosophila virilis*

Character	<i>m</i> ±SE	[<i>a</i>] ±SE	[<i>d</i>] ±SE	[<i>a.am</i>] ±SE	R ^{2,a}
PN ^b	10.062 ± 0.221	1.878 ± 0.149	-0.949 ± 0.347	-1.116 ± 0.253	0.940
PTL ^c	239.980 ± 8.674	29.427 ± 6.048	-57.510 ± 13.954	-31.064 ± 9.888	0.781

^a R² is the proportion of the variation explained.

^b Number of pulses in a pulse train.

^c Pulse train length.

parental strain B22 had, on average, more dominant alleles than strain 1431 and that B22 thus was more potent in the cross. The analysis did not reveal any direct additive or dominant maternal effects in the song characters. Both models appeared to be adequate representations of the data, with the model explaining about 94% of variation in generation means in PN and about 78% in PTL. Not all generation means were within two standard errors from the expected means (Fig. 2), however, suggesting there also may be other factors contributing to the variation between generation means.

The overall pattern given by the contrasts analysis of variance was similar to the results of the joint scaling test. The reciprocal F₁ hybrid males differed significantly from each other with respect to PN (Table III,

comparison 2), revealing that the autosomal mode of inheritance is not an adequate representation of the data. On the other hand, the difference in PTL between the reciprocal F₁'s was nonsignificant (*P* = 0.08). However, the negative result does not necessarily prove that PTL is affected only by autosomal factors, because maternal and Y chromosomal effects may counteract each other. Significant dominance was detected in both song characters by comparing the means of the parental and F₁ generation (Table III, comparison 3), as also observed in the joint scaling test.

Comparison of 890 F₂ males showed direct influence of the Y chromosome in both PN and PTL (Table III, comparison 4), even though the effect of the Y chromosome explained only 1.3% of the variation between

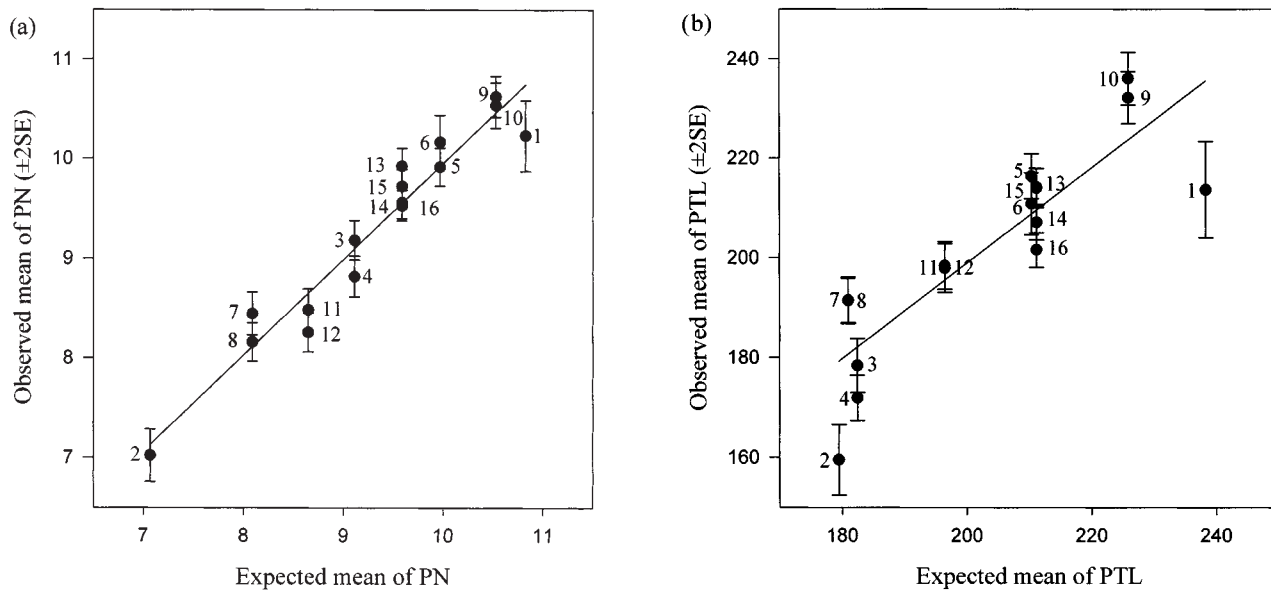


Fig. 2. Comparison of observed and expected means for (a) number of pulses in a pulse train (PN) and (b) pulse train length (PTL) for different crosses (parental, F₁, F₂, and backcrosses; cross number as in Table I) using the model obtained from the joint scaling test.

Table III. Summary of Planned Comparisons in Contrast Analysis of Variances of Courtship Song Characters PN and PTL^a

Comparison	Component	PN			PTL		
		F	df	P	F	df	P
1	Parental strains	212.37	1,93	<0.000	83.88	1,93	<0.000
2	Autosomal inheritance	5.39	1,127	0.022	3.11	1,127	0.08
3	Dominance	4.503	1,222	0.035	7.45	1,222	0.007
4	Y chromosome	11.64	1,888	0.001	29.04	1,888	<0.000
5	Y chromosomal interactions	36.15	1,616	<0.000	38.39	1,616	<0.000
6	Permanent cytoplasmic	2.34	1,888	0.127	3.23	1,888	0.073
7	Permanent cytoplasmic interactions	0.46	1,419	0.500	0.84	1,419	0.359
8	Transient maternal	6.67	1,407	0.010	6.68	1,407	0.010
9	Transient maternal interactions	2.52	1,407	0.113	26.79	1,407	<0.000

^a Comparisons 1–9 are described in Materials and Methods. PN, number of pulses in a pulse train; PTL, pulse train length.

the males in PN and 3.2% in PTL. The Y chromosome of parental strain B22 had an increasing effect on both characters by lengthening the PTL by 9.92 ms, on average and increasing the PN by 0.28 pulses. The comparison of 407 backcross males was significant for both song characters, indicating that 5.5% of the variation in PN and 5.9% of the variation in PTL are due to interaction with the Y chromosome and all other factors (Table III, comparison 5).

Comparisons of the means of different F₂ (Table III, comparison 6) and backcross males (Table III, comparison 7) suggest that permanent cytoplasmic factors and their interactions with other factors do not contribute to song differences either in PN or PTL. The contribution of transient maternal factors to the differences in song characters was assessed by comparing the means of reciprocal backcrosses. The comparisons (Table III, comparisons 8 and 9) were significant in both cases, explaining 1.6% of the variation between different backcrosses in both song characters. Interaction of maternal components and all other factors contributing to differences in song characters explained 6.2% of the variation in PTL.

The Castle–Wright estimates suggest that there is a moderate number of segregating loci contributing to the variation in PN ($n_E = 6.97 \pm 0.42$), whereas it is under unity for PTL ($n_E = 0.15 \pm 0.01$).

DISCUSSION

Inheritance of the courtship song characters in *D. virilis* appears to be rather complex. The model with additive, dominance, and additive interaction between progeny and maternal genotypes is an adequate de-

scription of means of different crosses in both song characters. Dominance is toward shorter pulse and pulse train length, that is, parental strain B22 having on average more dominant alleles than strain 1431. Unidirectional dominance in PN and PTL strongly suggests that these characters have been affected in the past by directional selection favoring short and dense pulse trains. This argument is based on selection theory, where fitness traits are presumed to attain a high degree of directional dominance (see Lynch and Walsh, 1998). However, the direction of dominance in PN and PTL in the intraspecific level in the *D. virilis* subgroup species is opposite. Crosses between the species in the *virilis* subgroup (*D. virilis*, *D. a. americana*, *D. a. texana*, *D. novamexicana*, and *D. lummei*) have shown directional dominance toward longer pulse trains and a higher pulse number (Hoikkala and Lumme, 1987). This suggests that dominance relationships are opposite in intraspecific and interspecific crosses or, alternatively, that different loci are contributing to the differences in these song characters between the species rather than within the species in the *D. virilis* group.

Planned comparisons of different crosses have shown a significant Y chromosomal effect, and a Y chromosome interaction effect with all other factors on the variation in phenotypic means in both song characters in *D. virilis*. Interestingly, the direction of the Y chromosomal effect is opposite to that of the influence of autosomal factors. Only a few studies so far have shown a relationship between the Y chromosome and behavior with sufficient statistical power to detect small genetic effects on behavior in *Drosophila*. Åslund *et al.* (1978) has reported that normal *D. melanogaster* XY males have a significantly higher mating capacity and activity (in

number of copulations) than do males differing by their Y chromosome number (i.e., XO and XYY). Even though the heterochromatic Y chromosome contains genes essential for male fertility (Carvalho *et al.*, 2001), it is not known whether the Y chromosome contains genes directly affecting mating behavior of normal XY males. Another study of *Anopheles* mosquitoes has shown direct Y chromosomal control of precopulative flight mating behavior (Fraccaro *et al.*, 1977). Stoltenberg and Hirsch (1997) have detected a small Y chromosomal effect depending on genetic or cytoplasmic background, or both on geotaxis in *D. melanogaster*.

A significant transient maternal effect was also found to affect both song characters in *D. virilis*. These nonchromosomal factors are mainly nutritional and maternal gene products that affect the phenotypic values of traits even when measured at the adult stage, causing offspring of the same mother to resemble each other. However, data for maternal effects on behavioral traits are sparse. Maternal effect has been detected in larval pupation behavior in *D. melanogaster* (Bauer and Sokolowski, 1988), and a significant effect of the interaction between cytoplasmic/maternal factors and the chromosomal genotype in determining the oviposition behavior in *D. melanogaster* has been found (Gonzales, 1990).

Even though the planned comparisons cannot reveal direct X chromosomal effects in male data, our joint scaling test suggested no direct maternal effects on PN and PTL in *D. virilis*. This is consistent with the observation that the genes contributing to the difference in PN between *D. virilis* and *D. lummei* (both belonging to *virilis* subgroup) are located on all autosomes, but not on X chromosomes (Hoikkala and Lumme, 1987).

The estimated number (minimum number) of effective factors was reasonably large in PN but under unity in PTL. Even though the characters are positively correlated in F_2 generation males ($r^2 = 0.862$; $P = 0.01$), these estimates suggest that many loci are contributing to PN song character while the genetic basis for PTL could be due to one or few major loci. Both estimates of the number of loci affecting the characters, however, are probably biased downward for at least two reasons. First, the method used assumes there is no dominance in the alleles affecting the studied characters (e.g., Wright, 1968; Lande, 1981; Zeng *et al.*, 1990). We observed significant dominance effect in both characters, which may have led to some bias. Second, the method assumes that one parental strain contains all the increasing alleles and the other all the decreasing alleles (Lande, 1981; Zeng *et al.*, 1990). Even though the strains were chosen to be as

dissimilar as possible with respect to male courtship song characters, the strains have not been artificially selected to the opposite directions for expressing the most different phenotypes in song characters. Thus, the strains used do not represent the extremes in phenotypic variation with respect to the pulse train observed within the species (A. Hoikkala, unpublished). Accordingly, the estimates of the effective factors for PTL and probably also for PN are biased downward.

Epistatic interactions, dominance, and the Y chromosomal and cytoplasmic effects combined with opposite effects of different factors also complicate the classical biometric analysis of factors contributing to phenotypic traits. The analyses conducted usually are based on a simple additive polygenic inheritance of phenotypic traits and series of assumptions, which are not necessarily valid. The possible role of the Y chromosome has often been considered insignificant because of its small genomic contribution. Although the effects of single factors may be small, their combined effect could still be considerable. The statistical power for detecting these small genetic effects depends on sample size, which is an important aspect in designing quantitative genetic studies. Possible failure to detect these effects could be due to lack of statistical power because detection of interactions and small genetic effects requires larger sample sizes than does detection of main effects. Our analyses with reasonably large sample sizes suggest that, in addition to polygenic autosomal factors, both transient maternal traits and the Y chromosome may make significant contributions to male courtship song characters. These observations may have interesting evolutionary consequences. For example, the effect of female condition on sexually selected traits has never been considered in the theoretical models of sexual selection (e.g., Andersson, 1994).

Biometric methods based on phenotypic means and variances of different generations (Wright, 1968; Lande, 1981) also have little power to distinguish whether minor or major genes contribute to differences in phenotypic traits. To detect major genes, we are now constructing a microsatellite marker map for *D. virilis*, which will be used for QTL mapping to further study the genetic basis of the courtship song in *D. virilis*.

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