

Variation in Male Courtship Song Traits in *Drosophila virilis*: The Effects of Selection and Drift on Song Divergence at the Intraspecific Level

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Abstract Genetic and phenotypic divergence of *Drosophila virilis* laboratory strains originating from different parts of the species range were studied with the aid of microsatellite markers and by analysing male courtship songs. The strains from America, Europe, continental Asia and Japan showed moderate geographic clustering both at the genetic level and in several traits of the male song. The genetic distances and the song divergence of the strains did not show significant association, which suggests that the songs have not diverged solely as a side-effect of genetic divergence. Comparison of the songs of the laboratory strains to those of freshly collected strains showed that pulse characters of the song are quite sensitive to culture conditions. While laboratory rearing of the flies had no effect on the number of pulses in a pulse train or the pulse train length, the tendency of the sound pulses to become longer during laboratory maintenance could explain the lack of geographic variation in pulse length and inter pulse interval. Sensitivity of songs to culturing conditions should be taken in account in studies on song divergence.

Keywords *Drosophila virilis* · Population structure · Geographic variation · Microsatellite · Courtship song

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Introduction

Evolution of species-specific mating rituals and male ornaments has long been a target of intense theoretical and empirical research. Coevolution of female preferences and male secondary sexual traits may cause rapid divergence between geographically isolated populations if females exercise selection on male traits and if both the male trait and the female preferences for it possess genetic variation (Lande 1981). Also, random genetic drift (Lande 1976) and adaptation to variable environmental conditions (Hoffmann and Merilä 1999) may give rise to population differentiation in mating signals, but their effects have been less studied than those of sexual selection. Stabilising selection, on the other hand, should lead to evolutionary stability of traits involved in the mate recognition system throughout the distribution of a species (Lambert and Henderson 1986).

Population comparisons provide a powerful way to study the first steps of divergence in species-specific phenotypic characters because genetic differentiation between populations is expected to be smaller than that between the species. Intraspecific genetic and phenotypic variation has been studied e.g. in *Drosophila melanogaster*, in which geographically isolated populations exhibit large variation at the molecular level (Begun and Aquadro 1993; Veuille et al. 1998; Andolfatto 2001; Kauer et al. 2002) and in several morphological traits (Capy et al. 1993; Long and Singh 1995; Prout and Baker 1993), as well as in traits important in the mate recognition system (Wu et al. 1995; Ferveur et al. 1996; Hollocher et al. 1997a, b; Capy et al. 2000). Since the path breaking work of Dobzhansky and his colleagues (e.g. Dobzhansky and Levene 1948), geographic variation also in species with extensive inversion polymorphisms (e.g. like *D. pseudoobscura*) has been under intensive study.

Most studies describing the songs of *Drosophila* species are based on the songs of a few laboratory strains, which do not give much information on geographical variation in songs (review on studies in Hoikkala 2005). Male courtship song traits have been shown to express genetic variation in several *Drosophila* species (e.g. Hoikkala 1985; Ritchie et al. 1994), but geographic variation in these traits has so far been demonstrated only in *D. melanogaster* (Colegrave et al. 2000), *D. mojavensis* (Etges et al. 2006) and *D. montana* (Mirol et al. 2007; Routtu et al. 2007). However, many studies dealing with geographic variation in songs (e.g. Ritchie et al. 1994; Colegrave et al. 2000; Mirol et al. 2007) have been performed using fly strains maintained in laboratory for a shorter or longer period before song recording, which may have affected the results. For example, Hoikkala (1985) found the songs of old *D. littoralis* laboratory strains from Eurasia to differ from each other more than the songs of the fresh isofemale strains. Also, while the songs of the old laboratory strains of *D. montana* studied by Mirol et al. (2007) showed significant geographic variation in only the carrier frequency of the song, the songs of freshly collected strains from Finland, Canada and USA showed variation in several song traits (Routtu et al. 2007).

The male courtship song of *D. virilis* represents the conservative song type shared by other species of the virilis subgroup of the *D. virilis* group (Hoikkala and Lumme 1987). *Drosophila virilis* females are able to recognize species-specific characters of the male song (Isoherranen et al. 1999), but contrary to the females of some other species of the group (e.g. Aspi and Hoikkala 1995) they do not require hearing the song before mating (Hoikkala 1988). Saarikettu et al. (2005) found that five *D. virilis* strains originating from different parts of the species distribution exhibit high inter-strain variation in mating rituals, including the male courtship song. Behavioural differences between the strains did not, however, give rise to sexual selection/isolation, as neither the males nor the females favoured the flies of their own strain over others (the males of one strain were incapable of producing song, but they had only a slight disadvantage in mating).

We studied genetic and phenotypic divergence of *D. virilis* laboratory strains originating from different parts of the species range with the aid of 48 microsatellite markers and by analysing the male courtship songs of the strains. We assume that if the interstrain and geographic variation in male song traits has evolved solely as a side effect of the genetic divergence of the strains, the songs should reflect the geographic origin and the genetic relatedness of the strains. Higher interstrain and geographic variation in song traits compared to neutral genetic variation could be due to sexual selection for different optimums, drift and/or adaptation to varying environmental conditions. We also

compared the songs of the laboratory strains to those of F_3 progenies of females collected from the wild in Japan and China 2002–2003, to find out how the songs are liable to change during laboratory maintenance, and whether such changes could increase/decrease geographic variation in songs.

Materials and methods

Genetic variation among *D. virilis* strains

Genetic variation in 48 microsatellite loci (Orsini et al. 2004) was studied among 30 *D. virilis* strains originating from different parts of the species distribution area. The details for six of the loci (v68-74, v71-38, v68-4, v71-6, v68-86-1 and v93-93) are given in Schlötterer and Harr (2000). The remaining 42 loci are described in Huttunen and Schlötterer (2002).

DNA from one individual of each strain was extracted by the method described in Miller et al. (1988). Radioactive genotyping of microsatellites followed standard protocols described in Schlötterer (1998). PCR was performed using $\gamma^{32}\text{P}$ -ATP labelled primer in a 10 μl volume with 50–100 ng of DNA, 1 μM of each primer, 200 μM of dNTP, 1.5 mM of MgCl_2 and 1 U of *Taq* DNA Polymerase. The PCR profile was 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 30 s at 45–59°C (depending on the locus), and 30 s at 72°C, and finally one cycle of 30 min at 72°C. PCR products were separated in 7% denaturing polyacrylamide gels (32% formamide, 5.6 M urea) at 90 W and visualised by autoradiography after 12–24 h. Allele sizes were determined by running a ‘PCR slippage ladder’ and a known size standard adjacent to the samples (Schlötterer and Zangerl 1999).

Basic measures of microsatellite variability, the expected heterozygosity and variance in the repeat number (calculated by averaging over 200 randomly discarded data sets) were calculated using Microsatellite Analyzer (MSA; Dieringer and Schlötterer 2003). On the basis of multilocus microsatellite data we constructed a neighbor-joining tree using the proportion of shared alleles (Bowcock et al. 1994) as a distance measure. The tree was constructed using the Neighbor program in Phylip v. 3.6 (Felsenstein 1993) and visualised using TreeView (Page 1996).

Population structure of *D. virilis*, as well as the patterns of possible admixture between genetically divergent groups, was investigated using the BAPS 3.2 program (Bayesian Analysis of Population Structure; Corander and Marttinen 2005). The mixture model of this program clustered the strains into four groups, America, Europe, continental Asia (Asia from hereon) and Japan, on the basis of their multilocus genotypes (see Corander et al. 2004,

2006). We then used the admixture analysis (see Corander and Marttinen 2006) to estimate individual strains' coefficients of admixture (q) with regard to the previously detected groups (proportion of alleles showing ancestry with each of these groups). In this analysis, we used 100 iterations to estimate the admixture coefficients for the individuals representing different strains, 200 simulated reference individuals from each group and 20 iterations to estimate the admixture coefficients for the reference individuals, following the advice in the software manual (Corander and Marttinen 2005). The Bayesian clustering in BAPS assumes that the marker loci are unlinked and that the source populations contributing to the observed sample are in Hardy–Weinberg equilibrium (e.g. Corander et al. 2003). Because these assumptions are not necessarily appropriate in our laboratory populations, the results of this analysis should be interpreted with caution.

Finally, we estimated the proportion of total variance within and between the four geographic groups based on hierarchical variance of allele frequencies (Excoffier et al. 1992) using AMOVA (Arlequin 2.000; Schneider et al. 2000). F_{ST} values (Weir and Cockerham 1984) were estimated for each locus separately and for pair-wise group comparisons with MSA. The significances of these values were determined by permuting genotypes among groups.

Recording and analysing the male courtship songs

The male courtship songs were recorded for the above-mentioned 30 strains originating from different parts of the species distribution area, for five additional strains from China and for F_3 progenies of females collected from China (four progenies) and Japan (13 progenies). All flies were maintained in the culture room for at least one generation before recording their song (continuous light, 70% humidity and 19°C temperature). The flies were sexed at the age of two days (or less) and the males and the females were stored separately in vials containing malt medium (Lakovaara 1969) until they were sexually mature (14 ± 2 days). The songs of five males per strain were recorded in a single-pair courtship at $20 \pm 1^\circ\text{C}$ between 9 and 12 am, and analysed for six song characters. Details of the recording methods are described e.g. in Saarikettu et al. (2005). Data for the songs of 12 of the 35 laboratory strains have been published earlier (Huttunen et al. 2002; Saarikettu et al. 2005; see Appendix 1).

The male courtship song of *D. virilis* consists of dense pulse trains with short pauses between successive sound pulses normally only at the beginning of the pulse train (see Fig. 1). Due to the irregularity of the first sound pulses of the pulse trains and the fact they usually were of lower intensity and included less sound cycles than the rest of the

pulses (see e.g. Campesan et al. 2001), they were not included in pulse structure analysis. The songs of different fly strains were analysed with the SIGNAL Sound Analysis System (©Engineering Design) by measuring the lengths of the pulse trains (PTL) and by counting the number of pulses in each train (PN) from the oscillograms. The traits for single sound pulses (i.e. CN = number of cycles in a pulse, PL = length of a pulse and IPI = length of an inter pulse interval) were measured for the fourth sound pulse of each pulse train. Due to the dense structure of pulse trains the IPIs of most strains were of the same length as PLs (see Fig. 1 and Table 2). The carrier frequency (FRE) was measured from the Fourier spectra of the analysed pulse trains. For each strain the means of different song traits were calculated over the means of five males (the mean for each male was first calculated over the traits measured for three pulse trains).

Geographical variation in the songs was studied with a multivariate MANCOVA test using the geographic origin of the strain (i.e., the four groups: America, Europe, Asia and Japan) as a factor and the age of the strain as a covariate. Variation in male song traits was partitioned to differences within and among the four geographic groups with nested ANOVA. We also performed a Mantel (1967) test with 10,000 permutations (Genepop; Raymond and Rousset 1995) to examine the relationship between genetic and song distances between the strains. Song divergence between each pair of strains was estimated using an Euclidean distance measure based on the four song characters showing geographic variation (CN, FRE, PN and PTL).

The effect of laboratory maintenance on male song traits was studied by comparing the songs of the 35 strains maintained in the laboratory for 10–58 years with those of the F_3 progenies of females collected in Japan and China 2002–2003, using a discriminant analysis.

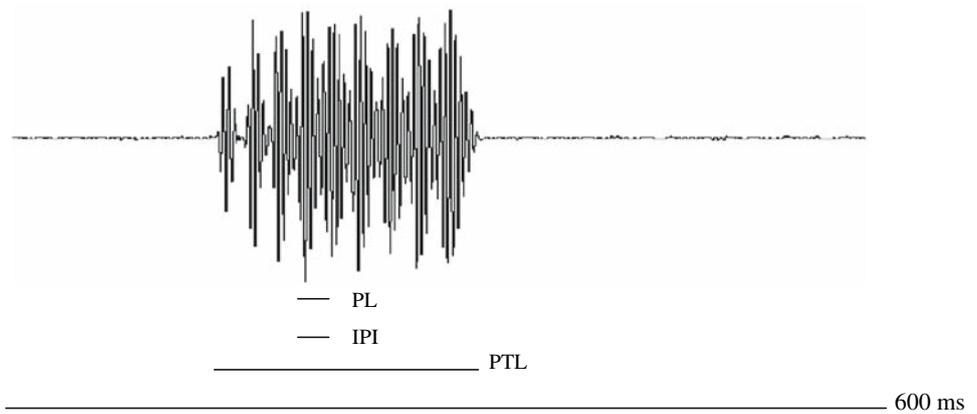
Results

Patterns of genetic variation among the strains

Genetic variation in 48 microsatellite loci was studied in 30 laboratory strains of *D. virilis*. All loci, except *tra*, were polymorphic among the studied strains, the mean number of alleles per locus ranging from 3 to 16 (average 9). The average heterozygosity of polymorphic loci was 0.69 and the mean variance in repeat number was 13.32.

Geographic structuring of *D. virilis* was examined by constructing a Neighbor-joining tree of individual strains of this species and using seven *D. lummei* strains (Orsini et al. 2004) as an outgroup. This tree showed moderate geographic clustering among the *D. virilis* strains

Fig. 1 Oscillogram of the male courtship song of *D. virilis*. PL = the length of a pulse, IPI = the length of an interpulse interval and PTL = the length of a pulse train. In the songs of most strains PLs and IPIs were of equal length



originating from America, Europe, Asia and Japan (Fig. 2). The Bayesian mixture analysis suggested the same grouping and the admixture analysis confirmed the levels of admixture between the genomes of the strains of these groups to be very low. Only three strains showed significant admixture to a different group: one Asian strain (1051.47) had admixture coefficients of 0.31 to the American group (i.e. 0.31 of its microsatellite alleles showed common ancestry with the American strains) and 0.07 to the European group and two Japanese strains showed admixture to the American group (strain B22, admixture coefficient of 0.24) or to the European group (strain W158, admixture coefficient of 0.18).

F_{ST} -statistics calculated from the microsatellite data showed significant differentiation between American, European, Asian and Japanese strains, varying from 0.058

to 0.140 (Table 1). The lowest differentiation was detected between American and Japanese strains and the highest was between European and Asian strains. The global F_{ST} value over all polymorphic loci was 0.097 and highly significant ($P < 0.001$). Significant differences between the strains from the four localities were detected at 22 out of 47 polymorphic loci ($P < 0.05$). Variance components were highly significant both between and within the geographic groups (AMOVA; 1.169 and 9.034, respectively, $P < 0.001$; 1,000 permutations). Microsatellite variation between the groups explained 11.5% of the total variation, whereas most of the variation (88.5%) was within the geographic groups (see Table 3).

Variation in male song traits among the *D. virilis* strains

The 35 laboratory strains of *D. virilis* (30 strains with microsatellite information plus 5 additional strains from China) showed high variation in male song traits, especially in the pulse train characters, PN and PTL (see Appendix 1). The mean number of pulses per train (PN) varied from 6.2 to 12.6 and mean pulse train length (PTL) varied from 142 ms to 270 ms. Among the pulse characters, the mean number of sound cycles per pulse (CN) varied from 4.9 to 6.4, mean carrier frequency (FRE) varied from 242 Hz (A13) to 311 Hz (strain 59) and mean pulse length (PL) and interpulse interval (IPI) varied from 18.4 to 21.6 ms. Variation among the songs of the

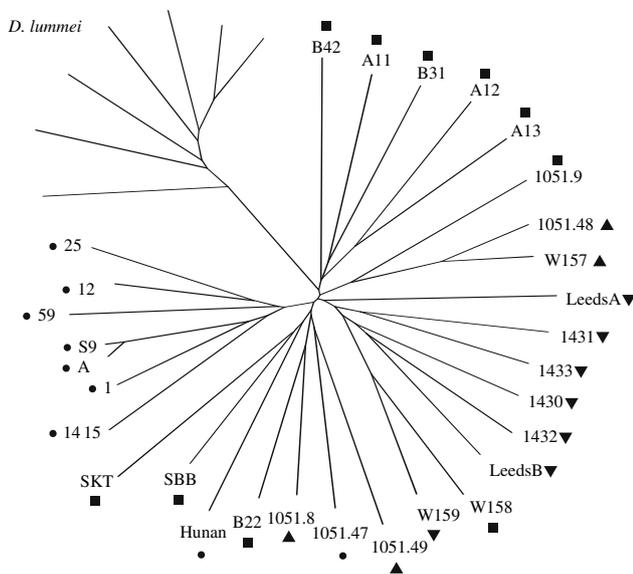


Fig. 2 Neighbor-joining tree of *D. virilis* strains from America (▲), Asia (●), Europe (▼) and Japan (■) using *D. lummei* (7 strains, Orsini et al. 2004) as an outgroup species. Analysis was based on 48 microsatellite loci, with distances measured as 1—the proportion of shared alleles) (Bowcock et al. 1994)

Table 1 Pairwise population differentiation (F_{ST} values obtained by 1,000 permutations; Weir and Cockerham 1984) among *D. virilis* strains grouped according to their geographic origin

	America	Asia	Europe
Asia	0.101 ^{NS}		
Europe	0.118 ^{**}	0.140 ^{***}	
Japan	0.058 ^{NS}	0.087 ^{***}	0.069 ^{**}

*** $P < 0.001$; ** $P < 0.01$; NS = not significant

laboratory strains was significant after sequential Bonferroni correction in PN ($F_{34} = 10.17$, $P < 0.001$), PTL ($F_{34} = 8.94$, $P < 0.001$), CN ($F_{34} = 3.70$, $P < 0.001$) and FRE ($F_{34} = 7.49$, $P < 0.001$), but not in PL ($F_{34} = 1.38$, $P = 0.10$) and IPI ($F_{34} = 1.29$, $P = 0.15$).

Divergence of *D. virilis* strains at genetic vs. phenotypic (song) levels

Geographical variation in the songs of the 30 laboratory strains for which genetic data were available was studied with a multivariate MANCOVA—test using the geographic origin (America, Europe, Asia and Japan) of the strain as a factor and the age of a strain (10–58 years, see Appendix 1) as a covariate. The song characters CN, FRE, PN and PTL showed significant variation between the geographic groups (MANCOVA; F -transformed Wilks lambda; $F_{18, 255} = 5.69$, $P < 0.001$), and the age of the strain had a significant negative effect on FRE and CN (F -transformed Wilks lambda; $F_{6, 90} = 4.106$, $P = 0.01$). The means of the strains from America, Europe, Asia and Japan showed significant variation in CN ($F_3 = 5.63$, $P < 0.001$), FRE ($F_3 = 17.08$, $P < 0.001$), PN ($F_3 = 9.06$, $P < 0.001$) and PTL ($F_3 = 8.23$, $P < 0.001$) even when the effect of the age was controlled. The American and Japanese strains showed lower values in all the above-mentioned song traits than the strains from Asia and Europe (Table 2). In IPI the proportion of variation between groups was of the same level as in PTL (see Table 3), but it remained nonsignificant, since it was mainly caused by three strains with longer IPIs than PLs (1051.8 from America, A12 from Japan and 1430 from Europe).

Variation in male song traits of *D. virilis* laboratory strains was partitioned into differences within and among the four geographic groups with nested ANOVA to compare the magnitudes of the two variance components with those measured for genetic variance with AMOVA. PL showed no between-group variation, whereas most of the other song characters showed two- to nearly four-fold higher variance between the groups compared to genetic

variance in microsatellite markers (Table 3). Variance among the four geographic groups was highest in the two negatively correlated song traits, FRE and CN.

The divergence in male song traits of the *D. virilis* strains was compared to the genetic distances of the respective strains to investigate if song evolution could have occurred as a side-effect of genetic divergence. The genetic distances (the proportion of shared alleles) and the song distances between the strains did not show significant association (Mantel test; $r = 0.105$, $P = 0.714$).

Changes in male song traits due to laboratory maintenance

Conditions in culture bottles are quite different from those in market places, breweries and timber yards, where *D. virilis* is found in the wild. To study the effects of laboratory rearing on male song traits, we compared the songs of the 35 strains maintained in the laboratory for 10–58 years (see Appendix 1) with those of the 17 F_3 progenies of females collected in Japan and China 2002–2003. Figure 3 shows a scattergram of a discriminant analysis of the overall song variation for the mean values of each song trait of all 52 *D. virilis* strains (laboratory strains and the F_3 progenies). The first and second discriminant axes (DA) accounted for 72.4% and 20% of the variance, respectively (Table 4). Pulse length (PL) and inter pulse interval (IPI) were highly associated with the 1st DA, whereas the number of cycles (CN) showed highest correlation with the 2nd DA. The old laboratory strains showed neither inter-strain nor geographic divergence in IPI and PL, but the songs of the F_3 progenies from Japan and China had clearly shorter IPIs and PLs than the laboratory strains. The lack of significant interstrain/geographic divergence in PL and IPI in laboratory strains may, indeed, have been caused by the tendency of sound pulses to get longer during laboratory maintenance. The second discriminant factor showed that the Japanese (both recently established and old strains) and American strains have in general lower CNs and FREs than the strains from Asia and Europe (see also Table 2).

Table 2 The means \pm standard deviations within geographic groups in six male song characters

Geographic group	<i>N</i>	PN	PTL	PL	IPI	CN	FRE
America	4	8.9 \pm 0.8	191.3 \pm 14.5	20.2 \pm 1.3	20.8 \pm 0.6	5.3 \pm 0.1	262 \pm 10.1
Europe	7	10.6 \pm 1.6	228.1 \pm 33.9	19.9 \pm 0.8	20.1 \pm 0.8	5.9 \pm 0.4	295 \pm 8.4
Asia	9	9.8 \pm 1.0	207.8 \pm 23.4	19.6 \pm 0.5	19.7 \pm 0.5	5.7 \pm 0.4	292 \pm 13.5
Japan	10	8.7 \pm 1.1	191.5 \pm 26.0	20.2 \pm 0.8	20.3 \pm 0.7	5.4 \pm 0.3	271 \pm 14.1

N = the number of strains originating from each geographic area. PN = number of pulses in a train, PTL = length of the pulse train, PL = length of a pulse, IPI = length of an inter pulse interval, CN = number of cycles in a pulse and FRE = carrier frequency of the song

Table 3 The proportion of variance between and within the geographic groups of *D. virilis* (America, Europe, Asia and Japan) in six male courtship song traits from nested ANOVA and in variance components derived from 48 microsatellite loci using AMOVA (Excoffier et al. 1992)

	PN	PTL	PL	IPI	CN	FRE	Microsatellites
Between groups	30.6	23.9	0	25.0	40.3	41.0	11.5
Within groups	69.4	76.1	100.0	75.0	59.7	59.0	88.5

PN = number of pulses in a train, PTL = length of the pulse train, PL = length of a pulse, IPI = length of an inter pulse interval, CN = number of cycles in a pulse and FRE = carrier frequency of the song

Discussion

Evolution of male secondary sexual characters playing a role in sexual selection within the species and/or in species recognition may have a major impact on speciation. The present study shows that variation in male song traits in *D. virilis* has not arisen purely as a side-effect of genetic divergence of populations, even though the females of this species show only weak selection on male song (Saarikettu et al. 2005). Another important finding is that the pulse characters of the song are quite sensitive to culture conditions, which has to be taken into account in studies on song divergence.

Throckmorton (1982) suggested that *D. virilis* originated in Asia, where the most primitive species of the *virilis-repleta* section have been found. Vieira and Charlesworth (1999) found evidence for this in their study of sequence variation in six X-linked genes of 21 *D. virilis* strains from different continents; all the genetic variants found outside Asia were also present in Asia, but not vice versa. P.M. Mirol et al. (submitted) analysed mitochondrial DNA

sequence data (*COI* and *COII* genes) of 35 *D. virilis* strains and found no geographic structure in haplotype distribution. The species showed very low nucleotide diversity with haplotypes distributed in a star-like network, consistent with a recent world-wide exponential expansion possibly associated with either domestication or post-glacial colonisation. However, although our phylogenetic analysis based on microsatellites did not reveal the ancestry of the strains, it did show moderate geographic clustering. The fact that *D. virilis* strains showed population structure in microsatellite but not in mtDNA analysis is not surprising, since rates of mutation of microsatellites are high compared to rates of point mutations (Hancock 1998) and so microsatellite variation usually has a more recent origin than mitochondrial DNA variation.

The relationship between genetic and quantitative phenotypic variation is frequently analysed by comparing F_{ST} values for genetic variation with an analogous measure, Q_{ST} (Spitze 1993), for phenotypic traits. This method enables one to infer the effects of selection on phenotypic characters assuming that allelic effects at each locus are additive (reviewed by Merilä and Crnokrak 2001; McKay and Latta 2002). Our *D. virilis* data are not applicable for this kind of analysis because the data consist mainly of old laboratory strains and the number of groups to be compared is quite small (four). Instead, we used a multivariate analysis to test the divergence of the strains at the genetic and phenotypic (song) level, and Mantel tests to study the relationship between genetic and song distances. The main finding in these comparisons was that geographic variation in most male song traits exceeds the amount of genetic variation between the strains. Furthermore, the genetic distances and the song distances of *D. virilis* strains did not show significant association, i.e. the songs had not diverged solely as a side-effect of the genetic divergence of the strains. This kind of trend has been found in several animal species (see e.g. Butlin and Tregenza 1998; Tregenza et al. 2000), with phenotypic traits being sensitive especially to sexual selection. For example, the songs of laboratory strains of *D. montana*, in which the females exercise strong selection on male song (e.g. Aspi and Hoikkala 1995), show high interstrain and geographic variation in several traits (Routtu et al. 2007).

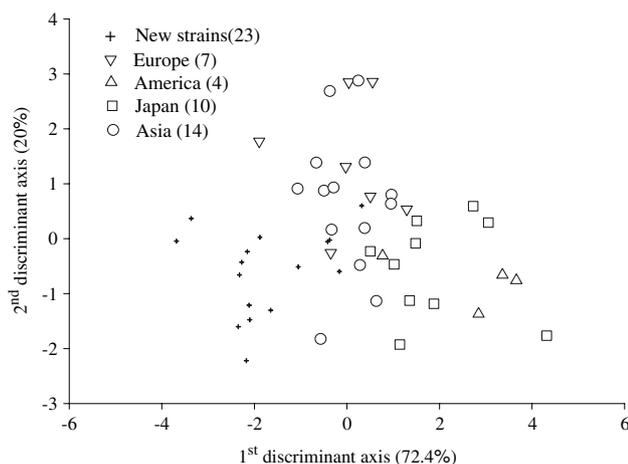


Fig. 3 Discriminant analysis on male courtship song characters of *D. virilis*. The number of strains in each group is shown in parenthesis. New strains refer to the progenies of females recently collected in Japan and China; others are old laboratory strains from different geographic areas

Table 4 Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions for each song character for the 1st and 2nd discriminant axis (DA)

	PN	PTL	PL	IPI	CN	FRE
DA1	−0.436***	−0.237*	0.625***	0.747***	0.042	−0.502***
DA2	0.241*	0.354*	0.507***	0.523***	0.956***	0.524***

PN = number of pulses in a train, PTL = length of the pulse train, PL = length of a pulse, IPI = length of an inter pulse interval, CN = number of cycles in a pulse and FRE = carrier frequency of the song. * $P < 0.05$ and *** $P < 0.0001$

During laboratory maintenance, the quality of male song traits could have been affected by drift, sexual selection, inbreeding and changed environmental conditions. In crowded culture bottles the females have a variety of mating partners to choose from, which in principle could lead to increased sexual selection. The songs of the males of laboratory strains could have longer sound pulses due to changes in muscle physiology taking place during adaptation to laboratory conditions and/or due to inbreeding depression (see Aspi 2000). The fact that changes in song traits during laboratory maintenance had occurred in all *D. virilis* laboratory strains in the same direction, i.e. towards longer PLs and IPIs, suggests that the changes have not been caused by random genetic drift. It is also worth noting that laboratory maintenance had a significant effect only on the pulse characters of the song, and not on the pulse train characters PN and PTL.

Studies of interspecific hybrids between *D. virilis* species with unique courtship songs have shown that X chromosomal genes have an important role in the evolution of species-specific song (e.g. in allowing the IPIs to become longer than PLs in most species of the montana phylad; Hoikkala and Lumme 1987; Päällysaho et al. 2003). At the intraspecific level, song variation in *D. virilis* is determined mainly by autosomal genes (Huttunen and Aspi 2003; Huttunen et al. 2004). In a recent biometric analysis of song differences between two *D. virilis* strains diverging in PN and PTL (strains 1431 and B22) Huttunen and Aspi (2003) suggested significant additive and dominance components and a significant additive interaction between maternal and progeny genotypes. The direction of dominance in PN was towards a lower number of pulses and in PTL it was towards shorter pulse trains. This direction is different from that observed at the species level in the virilis phylad species (Hoikkala and Lumme 1987), among which *D. virilis* has the shortest pulse trains. In the present study the lowest PNs and the shortest PTLs were found in American and Japanese strains, which might represent derived populations, if *D. virilis* originated in continental Asia as Throckmorton (1982) suggested.

In our study PL and IPI of the *D. virilis* song (which had the same mean value in most study strains) appeared not to vary significantly among the geographic groups. Lack of intra- and interspecific variation in the length of the inter-pulse interval (IPI) has been observed also in other *Drosophila* species, e.g. in *D. ananassae* and *D. pallidosa*, and the phenomenon was suggested to be due to selection on other species-specific song parameters requiring constant IPI (Yamada et al. 2002). The fact that the pulse characters of the song may change during laboratory rearing should be taken into account in song studies. While the songs of the laboratory strains give important information on factors affecting male song evolution and also provide good material for genetic crosses, their use in studies on the genetic and geographic variation in male songs may lead to false interpretations (see Introduction). These studies should always be done within a natural population framework.

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Appendix 1

Geographical origin and collection year (if known) of *D. virilis* strains and the means \pm standard deviations of their song traits. Number of males analysed for each strain is five. Abbreviations: PN = number of pulses in a pulse train, PTL = length of a pulse train, PL = length of a pulse, IPI = length of an inter pulse interval, CN = number of cycles in a pulse and FRE = carrier frequency of the song.

Appendix 1

Strain number	Strain	Origin	Collection year	PN	PTL	PL	IPI	CN	FRE
<i>Strains with microsatellite data</i>									
1	15010–1051.8 ^{a, b}	Truckee, California, USA	–	9.1 ± 0.8	188 ± 26	18.4 ± 1.3	21.0 ± 0.7	5.1 ± 0.2	261 ± 20
2	15010–1051.9 ^{a, b}	Sendai, Japan	–	9.1 ± 0.3	212 ± 9	19.6 ± 2.0	19.6 ± 2.0	5.2 ± 0.5	261 ± 19
3	15010–1051.47 ^{a, b}	Hangchow, China	1948	8.8 ± 0.6	192 ± 18	20.2 ± 2.3	20.2 ± 2.3	5.9 ± 0.9	285 ± 9
4	15010–1051.48 ^{a, b}	Texmelucan, Puebla, Mexico	1947	8.6 ± 0.4	194 ± 9	21.4 ± 1.1	21.4 ± 1.1	5.3 ± 0.2	259 ± 8
5	15010–1051.49 ^{a, b}	Chaco, Argentina	1950	9.9 ± 0.3	209 ± 4	20.0 ± 1.0	20.0 ± 1.0	5.4 ± 0.4	276 ± 11
6	A ^c	Batumi, Georgia	–	9.0 ± 0.7	187 ± 24	19.6 ± 0.9	19.6 ± 0.9	5.6 ± 0.4	289 ± 11
7	A11 ^c	Matsuyama, Ehime, Japan	1973	9.9 ± 0.8	205 ± 24	20.4 ± 1.1	20.4 ± 1.1	5.4 ± 0.4	266 ± 14
8	A12 ^c	Matsuyama, Ehime, Japan	1973	8.3 ± 0.8	168 ± 17	18.8 ± 1.6	19.2 ± 0.8	5.0 ± 0.5	264 ± 24
9	A13 ^c	Matsuyama, Ehime, Japan	1973	7.5 ± 1.0	168 ± 16	21.6 ± 1.1	21.6 ± 1.1	5.1 ± 0.4	242 ± 16
10	B22 ^c	Matsuyama, Ehime, Japan	1986	6.2 ± 0.2	142 ± 13	20.4 ± 1.5	20.4 ± 1.5	5.9 ± 0.7	294 ± 10
11	B31 ^c	Matsuyama, Ehime, Japan	1986	8.9 ± 0.7	189 ± 15	19.4 ± 1.1	19.4 ± 1.1	5.5 ± 0.5	285 ± 17
12	B42 ^c	Matsuyama, Ehime, Japan	1986	9.1 ± 0.8	190 ± 11	20.2 ± 1.8	20.2 ± 1.8	5.1 ± 0.5	271 ± 12
13	SBB ^c	Sapporo, Hokkaido, Japan	1986	10.1 ± 0.5	228 ± 13	20.4 ± 1.8	20.4 ± 1.8	5.4 ± 0.4	273 ± 14
14	SKT ^c	Sakata, Yamagata, Japan	1987	9.1 ± 0.8	199 ± 21	20.6 ± 1.8	20.6 ± 1.8	5.7 ± 0.3	274 ± 16
15	S9 ^b	Batumi, Georgia	1970	10.0 ± 0.5	222 ± 17	20.0 ± 1.2	20.0 ± 1.2	5.9 ± 0.2	299 ± 13
16	Human ^d	Hunan, China	–	9.0 ± 0.4	180 ± 10	19.4 ± 0.9	19.4 ± 0.9	5.9 ± 0.2	304 ± 12
17	LeedsA ^b	Leeds, England	1995	11.2 ± 1.3	240 ± 32	20.4 ± 1.5	20.8 ± 1.6	6.3 ± 0.4	305 ± 9
18	LeedsB ^b	Leeds, England	1995	8.0 ± 0.8	175 ± 23	20.0 ± 1.6	20.0 ± 1.6	5.8 ± 0.7	289 ± 15
19	W157 ^b	Mexico	–	8.0 ± 1.1	174 ± 27	20.8 ± 1.3	20.8 ± 1.3	5.2 ± 0.2	252 ± 10
20	W158 ^b	Mishima, Japan	–	8.9 ± 1.8	214 ± 32	20.8 ± 2.4	21.0 ± 2.1	5.5 ± 0.7	278 ± 2
21	W159 ^b	Holland	–	10.7 ± 1.6	245 ± 31	21.2 ± 0.8	21.2 ± 0.8	6.4 ± 0.3	289 ± 3
22	1 ^b	Erevan, Caucasus, Armenia	–	9.5 ± 0.5	195 ± 8	19.5 ± 1.1	19.5 ± 1.0	5.4 ± 0.4	280 ± 9
23	12 ^b	Tashkent, Middle Asia	–	10.9 ± 0.5	234 ± 12	19.4 ± 1.1	19.4 ± 1.1	5.5 ± 0.5	287 ± 26
24	25 ^b	Mzheta, Caucasus	–	8.9 ± 0.5	184 ± 14	19.4 ± 1.1	19.4 ± 1.1	5.3 ± 0.4	270 ± 15
25	59 ^b	Seishel Islands	1986	10.2 ± 1.6	219 ± 32	20.2 ± 1.3	20.2 ± 1.3	6.3 ± 0.4	311 ± 14
26	1415 ^b	Jalta, Russia	1973	10.3 ± 0.7	222 ± 18	19.2 ± 2.8	19.2 ± 2.8	5.7 ± 0.7	303 ± 11
27	1430 ^b	England (53°N, 1°E)	1981	12.6 ± 0.6	270 ± 20	19.4 ± 0.6	20.4 ± 1.5	5.4 ± 0.6	285 ± 11
28	1431 ^b	England (53°N, 1°E)	1981	12.0 ± 0.7	253 ± 16	19.4 ± 0.9	19.4 ± 0.9	6.0 ± 0.6	297 ± 12
29	1432 ^b	England (53°N, 1°E)	1981	9.3 ± 0.8	193 ± 18	18.8 ± 0.5	18.8 ± 0.5	5.5 ± 0.2	293 ± 7
30	1433 ^b	England (53°N, 1°E)	1982	10.4 ± 1.7	221 ± 28	19.8 ± 1.8	19.8 ± 1.8	5.8 ± 0.4	307 ± 17
<i>Strains without microsatellite data</i>									
1	TOY3F2	Toyama, Japan	2003	10.3 ± 1.3	209 ± 31	17.4 ± 1.3	17.4 ± 1.3	5.1 ± 0.4	290 ± 23
2	TOY3F3	Toyama, Japan	2003	9.7 ± 0.6	198 ± 14	16.8 ± 1.1	16.8 ± 1.1	5.0 ± 0.4	302 ± 20
3	TOY3F6	Toyama, Japan	2003	12.2 ± 0.8	244 ± 19	17.2 ± 1.3	17.2 ± 1.3	5.4 ± 0.4	306 ± 12
4	TOY3F7	Toyama, Japan	2003	10.7 ± 0.6	208 ± 14	17.8 ± 1.1	17.9 ± 1.1	5.4 ± 0.5	305 ± 18

Appendix 1 continued

Strain number	Strain	Origin	Collection year	PN	PTL	PL	IPI	CN	FRE
5	TOY3F9	Toyama, Japan	2003	10.5 ± 2.0	207 ± 40	17.6 ± 0.6	17.6 ± 0.6	5.3 ± 0.2	298 ± 9
6	TOY3F10	Toyama, Japan	2003	10.3 ± 1.2	209 ± 31	17.4 ± 1.3	17.4 ± 1.3	5.1 ± 0.4	290 ± 23
7	TOY3F11	Toyama, Japan	2003	10.3 ± 1.0	201 ± 23	17.4 ± 1.1	17.4 ± 1.1	5.1 ± 0.6	292 ± 30
8	TOY3F12	Toyama, Japan	2003	10.6 ± 0.8	219 ± 14	17.0 ± 1.0	17.0 ± 1.0	4.8 ± 0.3	278 ± 25
9	TOY3F15	Toyama, Japan	2003	9.5 ± 0.4	185 ± 13	16.8 ± 0.8	16.8 ± 0.8	5.1 ± 0.1	298 ± 14
10	TOY3F16	Toyama, Japan	2003	9.1 ± 1.2	180 ± 23	18.2 ± 1.1	18.2 ± 1.1	5.4 ± 0.4	298 ± 10
11	TOY3F17	Toyama, Japan	2003	11.6 ± 1.3	242 ± 27	19.8 ± 1.3	19.8 ± 1.3	5.4 ± 0.3	275 ± 19
12	TOY3F19	Toyama, Japan	2003	11.6 ± 0.7	219 ± 24	17.6 ± 0.9	17.6 ± 0.9	5.5 ± 0.4	316 ± 31
13	TOY3F20	Toyama, Japan	2003	9.8 ± 0.3	188 ± 6	17.6 ± 0.9	17.6 ± 0.9	5.4 ± 0.5	303 ± 24
14	V-EH-01	Lanzhou, Gansu Prov., China	2002	11.7 ± 1.3	249 ± 31	19.8 ± 1.5	19.8 ± 1.5	5.4 ± 0.4	273 ± 23
15	V-WW-03	Wuwei, Gansu Prov., China	2002	10.7 ± 1.0	222 ± 30	19.4 ± 0.9	19.4 ± 0.9	5.3 ± 0.3	278 ± 12
16	V-WW-05	Wuwei, Gansu Prov., China	2002	11.2 ± 1.7	233 ± 2	18.4 ± 1.5	18.4 ± 1.5	5.5 ± 0.3	290 ± 20
17	V-WW-08	Wuwei, Gansu Prov., China	2002	10.5 ± 1.1	226 ± 30	20.2 ± 1.1	20.2 ± 1.1	5.7 ± 0.5	279 ± 12
18	V-DNH	Dunhuang, Gansu Prov., China	–	10.3 ± 0.5	216 ± 22	19.6 ± 1.1	19.6 ± 1.1	5.9 ± 0.3	300 ± 18
19	V-HUNAN	Hunan Prov., China	–	9.9 ± 0.9	217 ± 18	20.6 ± 0.6	20.6 ± 0.6	6.4 ± 0.3	310 ± 12
20	V-NANJIIN	Nanjing, Jiangsu Prov., China	–	10.9 ± 1.3	218 ± 32	18.7 ± 1.3	18.8 ± 1.1	4.9 ± 0.6	268 ± 17
21	V-QUFU	Qufu, Shangdong Prov., China	–	7.9 ± 1.3	170 ± 29	19.8 ± 1.1	19.8 ± 1.1	5.9 ± 0.4	292 ± 19
22	V-ZZP-01	Zeziping, Hunan Prov., China	–	11.7 ± 1.3	253 ± 27	19.4 ± 2.0	19.4 ± 2.0	5.7 ± 0.4	290 ± 16

For strains with microsatellite data

^a Strains obtained from Bowling Green Drosophila Stock Center

^b Song data published in the present study

^c Song data published in Huttunen et al. (2002)

^d Song data published in Saarikettu et al. (2005)

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