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Localization of genes affecting species differences in male courtship song between *Drosophila virilis* and *D. littoralis*

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Summary

The males of six species of the *Drosophila virilis* group (including *D. virilis*) keep their wings extended while producing a train of sound pulses, where the pulses follow each other without any pause. The males of the remaining five species of the group produce only one sound pulse during each wing extension/vibration, which results in species-specific songs with long pauses (in *D. littoralis* about 300 ms) between successive sound pulses. Genetic analyses of the differences between the songs of *D. virilis* and *D. littoralis* showed that species-specific song traits are affected by genes on the X chromosome, and for the length of pause, also by genes on chromosomes 3 and 4. The X chromosomal genes having a major impact on pulse and pause length were tightly linked with *white*, *apricot* and *notched* marker genes located at the proximal third of the chromosome. A large inversion in *D. littoralis*, marked by *notched*, prevents more precise localization of these genes by classical crossing methods.

1. Introduction

The founders of neo-Darwinism argued that most adaptive changes in populations are polygenic. They also suggested that the genetic basis of phenotypic differences among populations and individuals within a species is similar to that among the species themselves, the latter representing a summation of the former (Charlesworth *et al.*, 1982). There is, however, a growing amount of evidence suggesting that speciation may involve novel genetic processes such as mobilization of transposable elements among hybrids, genomic incompatibility, and rapid and coordinated divergence of regulatory regions of genes (Rose & Doolittle, 1983). Identifying the genes involved in species isolation allows one to find out how many genes need to change to isolate a new species, and where the genes are located in the genome. Even though there are still only a few examples of 'speciation genes' (e.g. Orr, 1992; Coyne *et al.*, 1994; Khadem & Krimbas, 1997), it seems likely that the increasing application of quantitative trait loci (QTL) marker methodologies to evolutionary studies will provide further examples of major genes for traits previously thought to be polygenic (Mitchell-Olds, 1995; Liu *et al.*, 1996).

Reproductive isolation between species involves both prezygotic (prevention of interspecific matings) and postzygotic (sterility or inviability of hybrids) isolation mechanisms. In *Drosophila*, prezygotic isolation is significantly stronger than postzygotic isolation between sympatric species (Coyne & Orr, 1997; Noor, 1997). The most effective mechanism preventing interspecific matings is sexual isolation relying on species differences in visual, olfactory and acoustic signals emitted by the males and females during courtship and on courtship interactions between the two sexes. According to Ritchie & Phillips (1998), the likelihood of major genes (and hence the possibility of rapid speciation) varies with the mode of signalling – pheromonal systems showing many more examples of major gene effects than acoustic systems. Peixoto & Hall (1998) have suggested that the constraints associated with the pleiotropy of genes affecting male courtship song in *D. melanogaster* might explain why there is so little evidence for genes with major effects on song in crosses between closely related species. They suggest that the lovesong differences between

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most such species are based on the cumulative effect of very mild and subtle changes in several genes involving interspecific variation for example at the *Dmca1A* (*cacophony*), *slowpoke* and *maleless* loci (i.e. loci with observed song mutations in *D. melanogaster*).

In *D. virilis* group species, the male courtship songs play an important role both in species recognition (Liimatainen & Hoikkala, 1998) and in sexual selection within the species in the wild (Aspi & Hoikkala, 1995). Thus the genes affecting male song can, for a good reason, be called 'speciation genes'. The males of six species of this group (including *D. virilis*) keep their wings extended while producing a train of successive sound pulses with no pauses between pulses. The males of the remaining five species produce only one sound pulse during each wing extension/vibration, which results in species-specific songs with long pauses (in *D. littoralis* about 300 ms) between successive sound pulses (Hoikkala *et al.*, 1982). Hoikkala & Lumme (1987) have earlier found that the differences between the songs of *D. virilis* and *D. littoralis* are largely due to sex-linked genes. In the present paper we have studied the relative contributions of the X chromosome and autosomes to differences in male song traits between the two species mentioned above. We have also studied whether different traits of the male song are affected by the same genes or gene clusters, and whether the X chromosomal song genes can be localized on a certain chromosome segment and possibly classified as 'a major gene'.

2. Materials and methods

(i) *Flies*

All the fly strains used in the present study were isofemale strains, which had been kept in the laboratory for several years. *D. virilis* strain A (from Moscow, Russia) and *D. littoralis* strain 1016 (from Kuopio, Finland) were wild-type strains. Mutations in *D. virilis* marker strains have been listed by Alexander (1976). *D. virilis* strain MM ('Majors Marked') had the following recessive mutant genes on its major chromosomes: *white* (*w*) on the X chromosome, *broken* (*b*) on chromosome 2, *gapped* (*gp*) on chromosome 3, *cardinal* (*cd*) on chromosome 4 and *peach* (*pe*) on chromosome 5. *D. virilis* YPE strain had the same autosomal markers, but instead of *white*, it had *yellow* (*y*) marker on the X chromosome.

Homology of some mutations (*cn*, *v*, *ap*) present in *D. littoralis* marker strains with mutations described for *D. virilis* has been ascertained by complementation tests. *D. littoralis* marker strain ARPE (*ar*; *cn ev*; *pe*) had markers on all major autosomes: *arrow* (*ar*) on chromosome 2, *cinnabar* (*cn*; homologous with *cn* of *D. virilis*) and *extra vein* (*ev*) on fused chromosome 3–4 and *peach* (*pe*) on chromosome 5. *D. littoralis* strain VAP (*v Cv nd ap*) had four markers – *vermilion* (*v*), *Crossveinless* (*Cv*), *notched* (*nd*) and *apricot* (*ap*) – all on the X chromosome.

The strains were maintained in culture bottles containing Lakovaara's malt medium in continuous light, at 19 °C. Males and females were collected in separate vials no later than 2 days after their emergence and used in song recordings and/or crosses at the age of about 2 weeks. Interspecific F1 and backcross hybrids were obtained by making several mass-mating lines (about 10 males and 10 females of different species/hybrid class per bottle) between the flies of different species/hybrid types.

(ii) *Song recording and analysis*

Male courtship songs were recorded as the male courted a female in a single pair courtship. The recording chamber was made of a Petri dish (diameter 5 cm, height 0·7 cm) covered with a 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.

Male courtship songs were analysed with the SIGNAL Sound Analysis System (Engineering Design). For each male we analysed one pulse train of the song, measuring the length of the pulse train (PTL) and counting the number of pulses per train (PN) in oscillograms made for the songs (Fig. 1). We also counted the number of cycles (CN) in the fourth sound pulse of the pulse train, and measured the length of this pulse (PL) and the distance from the end of this pulse to the beginning of the next one (PAUSE). Carrier frequency of the song was measured from Fourier spectra.

(iii) *Statistical analysis*

Statistical analyses on songs were made on log (ln) transformed data. Differences between species (three strains per species) were tested with hierarchial ANOVA. Differences between the songs of reciprocal F1 hybrids (where obtained), as well as between the songs of the males of backcross progenies, were tested with a two-tailed *t*-test.

Genetic mapping of QTLs (i.e. genes affecting the studied trait) tests whether the genetic markers are transmitted together with a specific value of the trait. In single-marker analysis the null hypothesis is that a given marker is unlinked to the putative QTL, i.e. the recombination fraction between the marker and the gene(s) affecting the studied trait is 0·5. This hypothesis can be tested by testing for a non-zero slope to the regression line of trait value on marker indicator

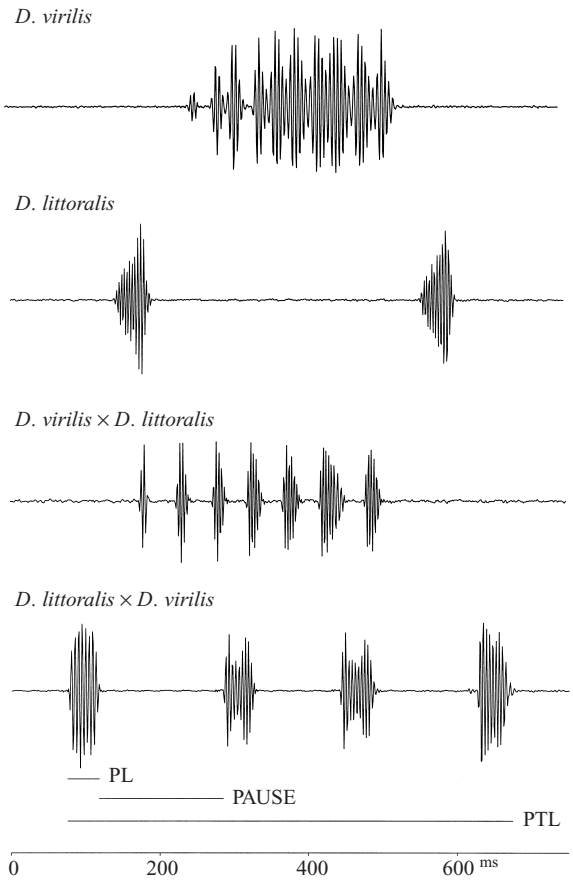

Fig. 1. Oscillograms of the male courtship songs of *D. virilis*, *D. littoralis* and the reciprocal F1 hybrids having the X chromosome of *D. virilis* (*D. virilis* × *D. littoralis*) or *D. littoralis* (*D. littoralis* × *D. virilis*).

(Doerge *et al*., 1997). Single-marker analysis is better than conventional interval mapping for analysing the data on interspecific hybrids. Due to differences between *D. virilis* and *D. littoralis* in their gene arrangement (see Hsu, 1952), the distances between homologous loci can not be measured.

Regression analysis assumes that the trait values are distributed normally within each marker class. This requirement was fulfilled after logarithmic transformation of the trait values. After calculating the significance of regression slopes, we made Bonferroni corrections for each trait within each marker class for multiple comparisons. *t*-values and their significance as well as the proportion of variation explained (*R* squares) are given for each significant marker – song trait regression in Table 2.

3. Results

(i) *Variation in male song traits*

Songs of *D. virilis* and *D. littoralis* males differed from each other in several song traits (Fig. 1, Table 1). *D. littoralis* males produced one sound pulse and *D. virilis* males a train of sound pulses during each wing extension/vibration bout, sound pulses (PL) and pauses between pulses (PAUSES) being much longer in *D. littoralis* song than in *D. virilis* song. Hierarchical ANOVA showed that the species differed significantly in both of these traits ($F = 2099$, $df_1 = 1$, $df_2 = 59$, $P < 0{\cdot}0001$ and $F = 76519$, $df_1 = 1$, $df_2 = 59$, $P < 0{\cdot}0001$, respectively), even though variation between conspecific strains was significant ($F = 14{\cdot}21$, $df_1 = 2$, $df_2 = 59$, $P < 0{\cdot}0001$ and $F = 27{\cdot}14$, $df_1 = 2$, $df_2 = 59$, $P < 0{\cdot}0001$, respectively). The songs of the two species differed from each other significantly also in the number of cycles in a pulse (CN; $F = 1198$, $df_1 = 1$, $df_2 = 59$, $P < 0{\cdot}0001$) and in the length of the pulse train (PTL; $F = 536{\cdot}7$, $df_1 = 1$, $df_2 = 59$, $P < 0{\cdot}0001$). In the first-mentioned trait variation between strains was also significant ($F = 13{\cdot}91$, $df_1 = 2$, $df_2 = 59$, $P < 0{\cdot}0001$). In the carrier frequency of song (FRQ), variation between conspecific strains was large and significant ($F = 30{\cdot}17$, $df_1 = 2$, $df_2 = 59$, $P < 0{\cdot}0001$), variation between species remaining non-significant. In pulse number (PN) variation both between conspecific strains and between species was non-significant.

(ii) *Species differences in pulse and pause length*

We have presented in Fig. 2 bivariate scattergrams and 90% equal-frequency ellipses of pulse and pause lengths (PL and PAUSE) for *D. virilis* and *D. littoralis* males of the strains used in the present study, and for F1 and backcross hybrids between these strains. PL and PAUSE were chosen for the analysis because they describe species differences well, and because these traits can be measured independently (Fig. 1; Table 1). Together PL and PAUSE make up the interpulse interval (IPI), which is a commonly used trait in song analysis (e.g. Ewing, 1969).

Our data for most hybrid classes consist of hybrids from several crosses; only F1 hybrid males having the X chromosome of *D. littoralis* were obtained from a single cross, *lit*1016 × *vir*MM (see Table 1). The songs of the two hybrid males obtained from this cross resembled the song of *littoralis* × *virilis* F1 hybrids recorded in our earlier study (Hoikkala & Lumme, 1987), which confirms that the songs are typical for the males of this hybrid class.

The songs of the reciprocal F1 hybrid males differed from each other in both PL ($t = -2{\cdot}995$, $df = 50$, $P < 0{\cdot}01$) and PAUSE ($t = -8{\cdot}259$, $df = 50$, $P < 0{\cdot}001$), which suggests that these traits are affected by X chromosomal or maternal factors. For a sex-limited male trait the difference between reciprocal F1 hybrids should be $1/(2n-1)$ if all chromosomes had an equal contribution on the studied trait (Ritchie & Phillips, 1998), n being the chromosome number of the species.

Table 1. *Means and standard deviations of male song traits in* D. virilis *and* D. littoralis *strains and their crosses*

		PL		PAUSE		CN		FRQ		PN		PTL	
Strain/cross	*N*	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
*vir*A	10	19·1	1·37	0	—	5·10	0·57	262	8·78	10·2	1·32	237	30·6
*vir*MM	10	17·8	1·40	0	—	6·00	0·47	336	11·1	8·90	0·74	183	17
*vir*YPE	10	19·4	1·65	0	—	5·60	0·52	290	15·6	9·30	0·48	205	13·0
*lit*106	10	41·5	3·54	266	27·6	12·2	1·48	302	27·2	7·60	2·46	2083	946
*lit*ARPE	10	52·8	4·21	369	40·6	14·5	1·18	270	19·9	11·5	6·31	4871	3065
*lit*VAP	10	48·3	3·56	269	34·5	14·0	1·41	304	18·2	10·5	5·00	3478	1841
A × 1016	10	25·8	2·04	17·1	9·19	7·40	0·52	279	9·61	8·20	1·23	337	64·5
(A × 1016) × A	17	21·4	7·59	14·2	17·4	7·12	3·16	333	41·8	8·71	2·80	260	59·5
(A × 1016) × 1016	31	35·8	9·40	108	46·8	11·4	3·34	330	36	6·16	2·66	793	645
MM × 1016	10	23·0	2·87	20·9	4·93	7·40	0·97	327	20·1	7·50	1·72	320	73·9
1016 × MM	2	32·0	7·07	132	12·7	9·50	2·12	309	7·78	8·00	4·24	1019	314
(MM × 1016) × MM	39	25·1	7·52	12·4	14·1	7·72	2·49	320	35·7	8·64	1·86	335	161
YPE × 1016	10	20·8	3·19	25·2	7·16	6·30	1·06	301	12·5	9·80	1·40	465	71·7
(YPE × 1016) × YPE	17	22·2	6·75	19·7	19·9	6·53	2·12	298	36·5	9·41	3·06	352	120
A × ARPE	10	22·4	2·72	17·0	5·14	6·60	0·70	281	12·7	8·70	2·06	360	97·4
(A × ARPE) × ARPE	23	32·4	17·6	83·9	91·7	8·70	4·51	274	52·2	6·70	4·23	583	540
A × VAP	10	24·4	3·75	17·4	4·81	6·90	1·37	281	13·6	7·50	1·08	305	44·8
(A × VAP) × VAP	151	33·2	10·6	89·3	66·1	10·3	4·60	314	36·4	6·91	3·55	674	559

N refers to the number of males studied. Cross designations are female × male.
PL, pulse length; PAUSE, distance from the end of one pulse to the beginning of the next; CN, number of cycles in the fourth pulse; FRQ, carrier frequency of song; PN, number of pulses per train; PTL, length of pulse train.

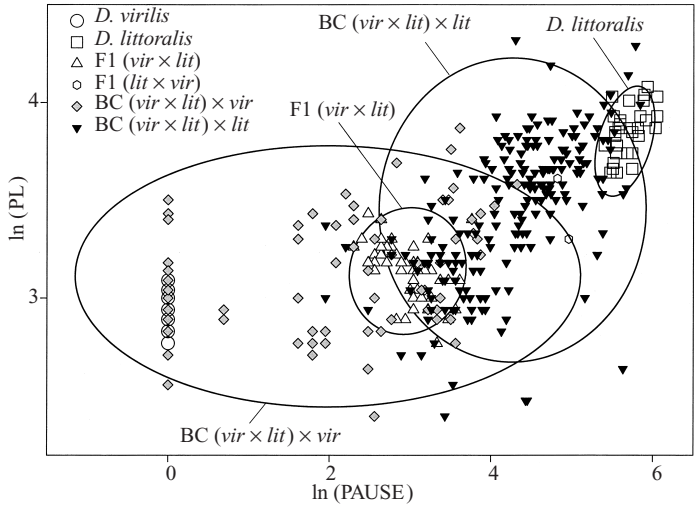

Fig. 2. Bivariate scattergrams and 90% equal-frequency ellipses of pulse and pause lengths (logarithms) of *D. virilis* and *D. littoralis* strains and interspecific F1 and backcross (BC) hybrids. Ellipses could not be drawn for *D. virilis*, because in this species the sound pulses follow each other without a pause, or for *D. littoralis* × *D. virilis* hybrids, because of the scarcity of the data points.

In *D. virilis* group species this difference would be about 11% of the difference between parental species, as these species have five major chromosomes (or chromosome arms) of about equal size, plus one dot chromosome (Throckmorton, 1982). In the cross between *vir*MM and *lit*1016 the difference between reciprocal F1 hybrids was 38·5% of the parental difference in PL and 32% in PAUSE, which is high even if the X chromosomal genes were doubled in expression due to dosage compensation (Baker *et al.*, 1994).

In the joint data (three strains per species plus their hybrids), the songs of the backcross hybrids (*virilis* × *littoralis*) × *virilis* and (*virilis* × *littoralis*) ×

Table 2. *Percentage of variation in different song traits explained* (R *square*) *by marker genes in the songs of the males from backcrosses towards* D. virilis *and towards* D. littoralis. *Only markers with significant regression slopes on song traits are included*

Cross	Marker strain	Marker gene	Chromosome	Song trait	Variance explained (%)	t^a
(*vir* × *lit*) × *vir*	MM	*w* (*vir*)	X	PL	20·5	−3·088*
				PTL	30·6	−4·035***
(*vir* × *lit*) × *vir*	YPE	*cd* (*vir*)	4	PAUSE	30·5	−2·568*
(*vir* × *lit*) × *lit*	ARPE	*cn* (*lit*)	3–4	PAUSE	22·1	2·443*
(*vir* × *lit*) × *lit*	VAP	*v* (*lit*)	X	PL	10·2	4·112***
				PAUSE	6·1	3·100**
				CN	13·9	4·902***
				FRQ	6·1	3·106**
		cv (*lit*)	X	PL	16·0	5·335***
				PAUSE	18·0	5·721***
				CN	20·5	6·206***
				PN	6·0	−3·072*
		nd (*lit*)	X	PL	39·0	9·758***
				PAUSE	40·4	10·06***
				CN	36·6	9·267***
				PN	19·7	−6·039**
				PTL	5·2	2·873*
		ap (*lit*)	X	PL	34·9	8·942***
				PAUSE	34·9	8·937**
				CN	33·7	8·704**
				PN	21·7	−6·433**

Significance of *t*-tests after Bonferroni corrections: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ and *** $P < 0{\cdot}001$.
Abbreviations as in Table 1.
[a] Negative *t*-values indicate that the species with a marker gene has a lower value in the studied trait.

littoralis differed from each other both in PL ($t = -7{\cdot}188$, df = 276, $P < 0{\cdot}001$) and in PAUSE ($t = -13{\cdot}07$, $df = 87{\cdot}1$, $P < 0{\cdot}001$). This shows that in addition to X chromosomal genes, autosomal genes also have a major impact on the studied traits. The songs of some backcross (*virilis* × *littoralis*) × *virilis* males resembled the song of *D. virilis*; in these songs the sound pulses followed each other without a pause (Fig. 2). The rest of the males of this hybrid class were distributed around the songs of *virilis* × *littoralis* F1 hybrid males. For (*virilis* × *littoralis*) × *virilis* backcross hybrids Spearman's correlation coefficient between PL and PAUSE was 0·376 ($n = 73$, $P < 0{\cdot}01$). These hybrids have received recombined X chromosome and autosomes from their hybrid mother and one homologue of each autosome pair from *D. virilis*.

The pulse and the pause lengths of the backcross hybrids (*virilis* × *littoralis*) × *littoralis* covered the whole range of variation between *virilis* × *littoralis* F1 hybrids and *D. littoralis*. The songs of only a few of these males reached the pause length of *D. littoralis*, which suggests that the autosomal genes of *D. littoralis* affecting this trait are at least partly recessive and must be in a homozygous condition to have a full effect on male song. In this male group, Spearman's correlation coefficient between PL and PAUSE was as high as 0·660 ($n = 205$, $P < 0{\cdot}01$).

The fact that most backcross hybrid males had long PLs and PAUSEs in spite of all these males having the cytoplasm of *D. virilis* suggests that maternal factors do not play a role in determining song characteristics.

(iii) *Localization of genes affecting male song traits*

The locations of loci affecting male song within the X chromosome and on the autosomes was studied with the aid of visible marker genes. Strain *vir*A and *lit*1016 were wild-type strains. These strains were crossed with each other to produce F1 and backcross hybrids both towards *D. virilis* and *D. littoralis*. These strains were also used in crosses with marker strains. In crosses with *D. virilis* marker strains we produced backcross hybrids only towards *D. virilis*, and in crosses with *D. littoralis* marker strains, only towards *D. littoralis*.

In backcross between *vir*MM (*w*; *b*; *gp*; *cd*; *pe*) and *lit*1016, *cd* and *pe* were not useful as markers, because *w* is epistatic to both these markers and the number of individuals expressing *cd* and/or *pe*, but not *w*, was very low. As Table 2 shows, only the X chromosomal marker gene, *w*, was linked with genes affecting male song. It explained about 20% of variation in PL and about 30% of variation in PTL in the songs of the backcross hybrids. The difference between males

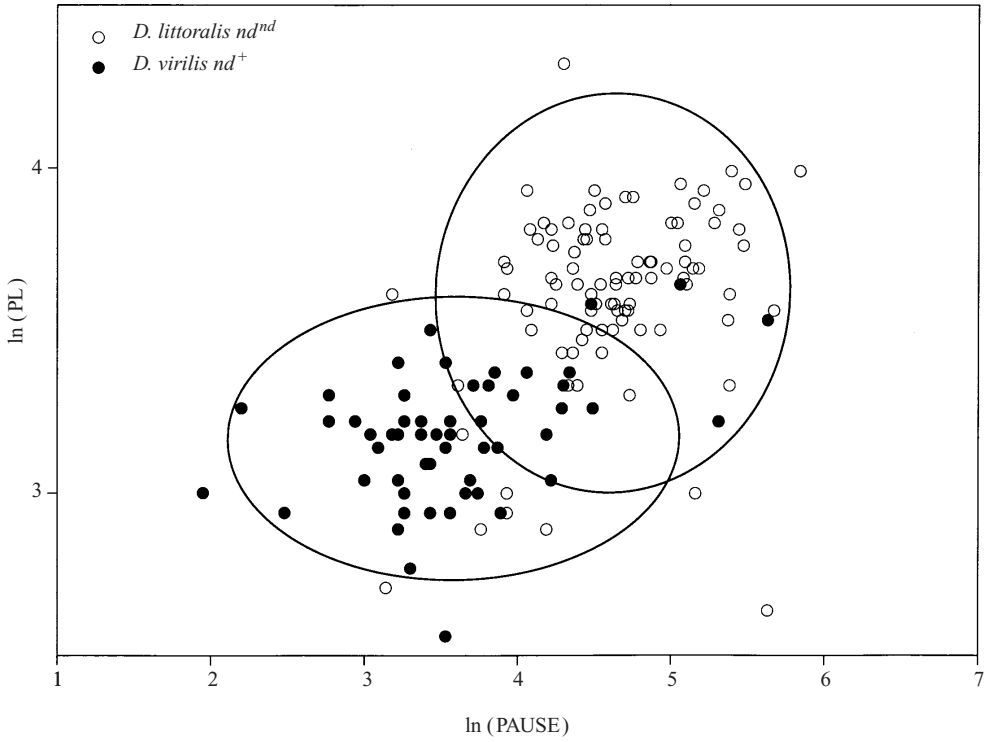

Fig. 3. Bivariate scattergrams and 90% equal-frequency ellipses of pulse and pause lengths of (*D. virilis* × *D. littoralis*) × *D. littoralis* backcross hybrids expressing/not expressing an X chromosomal marker gene, *nd*, of *D. littoralis*.

expressing/not expressing *w* was 6·73 ms in PL and 164 ms in PTL. *white* is located at the proximal end of the X chromosome of *D. virilis* (site 13C; Lozovskaya *et al.*, 1993).

The strain *vir*YPE (*y*; *b*; *gp*; *cd*; *pe*) had *yellow* (*y*) marker located at the distal end of the X chromosome (site 1D; Lozovskaya *et al.*, 1993). The fact that this marker was not linked with male song genes, even though *w* was, shows that the song genes are not randomly distributed on the X chromosome, and that they are closer to the *white* than the *yellow* locus. Among the autosomal markers, only *cardinal* (*cd*) was linked with genes affecting male song, explaining about 30% of variation in PAUSE. The difference between *cd*/+ and *cd*/*cd* males in this song trait was 18·1 ms. *cd* is located close to site 41E on the fourth chromosome of *D. virilis* (Gubenko & Evgen'ev, 1984).

When using *D. littoralis* marker strains, we made backcross hybrids by crossing F1 hybrid females with *D. littoralis* males. From the cross with *lit*ARPE (*ar*; *cn ev*; *pe*) we obtained only 22 backcross males, and only the marker gene *cn* showed significant linkage with song genes. This marker explained more than 20% of variation in PAUSE, the difference between homo- and heterozygous males being 98·2 ms. The location of *cn* in *D. littoralis* is not known but it may be close to the centromeric region of the fused chromosome 3–4. In *D. virilis cn* is located near site 30C on chromosome 3 (Gubenko & Evgen'ev, 1984).

All X chromosomal marker genes *v Cv nd ap* of *lit*VAP strain showed significant linkage with song genes (Table 2). The linkage was strongest for markers *nd* and *ap*, which explained 30–40% of variation in PL, PAUSE and CN. Differences between males homo- and heterozygous for *nd* were in these traits 13·7 ms, 68·5 ms and 4·84 cycles (see Fig. 3), and between males homo- and heterozygous for *ap*, 12·7 ms, 59·4 ms and 4·55 cycles, respectively. Also *v* and *Cv* showed linkage with X chromosomal song genes, but this linkage was considerably weaker than that for *nd* and *ap* (Table 2). For example, differences between *v*/*v* and *v*/+ males in PL and PAUSE were 6·9 ms and 28·3 ms, and between *Cv*/*Cv* and *Cv*/+ males 8·4 ms and 49·9 ms, respectively. It is worth noting that the values given in Table 2 are not independent values, i.e. the effects of different marker genes can not be added.

The X chromosomal markers of *lit*VAP were mapped relative to each other: *v*–27·5–*Cv*–44·7–*nd*–46·8–*ap* (percentage recombination in homozygous Standard chromosomes, $n = 850$). They were also mapped in females heterozygous for an X chromosomal inversion polymorphic in *D. littoralis*: *v*–27·5–*Cv*–29·1–[inversion *nd*]–0·09–*ap* ($n = 1125$). In the linkage map of *D. virilis*, *vermilion* is located at the distal part of X chromosome (Alexander, 1976), but its cytological location is not known. *ap* is located near site 16C in *D. virilis* (Gubenko & Evgen'ev, 1984).

4. Discussion

Our data suggest that the traits by which the songs of *D. virilis* and *D. littoralis* differ from each other are strongly affected by major genes on the X chromosome and autosome(s). X chromosomal markers of *D. virilis* (*y* and *w*), and *D. littoralis* (*v*, *Cv*, *nd* and *ap*) made it possible roughly to localize the genes with a major impact on male song to the proximal third of the X chromosome. The fact that the linkage between marker genes and song genes was strongest for *nd* suggests that the searched gene/genes is inside the same inversion as *nd*. Autosomal genes with a significant impact on PAUSE are located on the third and/or fourth chromosome (chromosomes separate in *D. virilis* and fused in *D. littoralis*). Species differences in gene arrangement prevent exact localization of the song genes by classical crossing experiments.

Studies on the genetic variation within and between species are important for determining whether the latter could represent a summation of the former, as suggested by Charlesworth *et al.* (1982). Detailed studies on the genetic basis of interspecific differences in sexual characters known to be important in species recognition are, however, rare (Coyne *et al.*, 1994). One problem with interspecific crosses is that the species differences in gene arrangement lead to restrictions in intrachromosomal recombination. Evgen'ev (1971) has shown that while crossing-over is restricted in inverted areas in interspecific *D. virilis* group hybrids, it may be enhanced at the areas of chromosome conjugation. In our cross with *D. littoralis* X chromosomal multimarker strain, about 45% of 151 backcross progeny males expressed recombination between the markers in spite of extreme rearrangements of the chromosomes. This kind of recombination does not allow regular genetic mapping. Another problem with species hybrids is that all genotype classes may not be produced due to hybrid dysgenesis or to incompatibility of the genes (e.g. Lumme & Heikkinen, 1990).

In the present study we found both PL and PAUSE to be affected by X chromosomal genes. PAUSE was linked also with autosomal markers *cinnabar* of *D. littoralis* and *cardinal* of *D. virilis*, which both are located on chromosome 3–4. Even though PL and PAUSE were correlated among the backcross progeny males, the two traits seem to be inherited partly independently. This is expected as these traits vary independently also in the songs of the *D. virilis* group species (Hoikkala *et al.*, 1982). Among the species of *D. montana* subgroup, the lengthening of pulses has occurred only in *D. littoralis* and *D. ezoana*, PLs of several species (*D. lacicola*, *D. flavomontana* and *D. montana*) being short despite long pauses between sound pulses. Interpulse interval (i.e. PL + PAUSE) has been found to be an important factor in separating the songs of different species in other *Drosophila* groups also (Cowling & Burnet, 1981). It has also been found to help the females to recognize the songs of conspecific males (Greenacre *et al.*, 1993; Tomaru *et al.*, 1995).

Among the markers used, *white*, *cardinal*, *peach*, *cinnabar* and *apricot* are eye-colour mutations, *broken*, *gapped*, *arrow*, *extra vein*, *Crossveinless* and *notched* are wing mutations, and *yellow* affects the body colour (see Alexander, 1976). None of the wing mutations radically affected wing morphology and/or male song. Even though the songs of the strains used in the present study differed from each other in some song characters, this variation was small compared with species differences in PL, PAUSE, CN and PTL. The songs of all marker strains varied within the limits found in wild-caught flies or the wild-type strains of the two species (e.g. Hoikkala & Lumme, 1984; Hoikkala, 1985).

Rice (1984) has proposed that X-linked genes play a predominant role in coding for sexually dimorphic traits facilitating the evolution of sexual dimorphism, and Reinhold (1998) that about one-third of the phenotypic variation in sexually selected traits is caused by X chromosomal genes. Ewing (1969) and Charlesworth *et al.* (1987) have suggested that substitution of selectively favourable mutations proceeds more rapidly for sex-linked loci than for the autosomes, provided that mutations are partially or fully recessive. In the present study the autosomal genes of *D. littoralis* affecting pause length were found to be at least partly recessive to *D. virilis* genes. Dominance relationships of X chromosomal genes could not be studied, because these genes in *Drosophila* males are in the hemizygous condition. X-chromosomal inheritance of interspecific differences in male song traits has been detected by Ewing (1969) for *D. subobscura* and *D. persimilis* and by Hoikkala & Lumme (1987) for *D. virilis* group species. Also, Ford & Aquadro (1996) have suggested that the greater differentiation of the X-linked loci compared with autosomal loci in the two semispecies of the *D. athabasca* complex is due to X-linked selective sweeps, where the target of selection may have been X-linked mating song differences among the semispecies.

Hoikkala & Lumme (1987) have proposed that a major genetic change on the X chromosome occurred during the separation of the *D. virilis* and *D. montana* subgroups, allowing variation in interpulse interval. Our data give support to this idea. Both studies also refer to the existence of autosomal genes affecting some song traits on chromosomes 3 and 4 of *D. virilis* and *D. littoralis*. Even though *D. virilis* and *D. littoralis* are too divergent to assign a role to song genes in the initial stages of species divergence, studying the genetic basis of interspecific differences in

song gives valuable information on song evolution. In *D. montana* subgroup species with species-specific songs, genes lengthening PL and PAUSE could have formed a new coordinated genetic system and built up 'a new behavioural phenotype' (setting the wings in a resting position after the production of each sound pulse). Another alternative is that these genes have blocked the function of some genes controlling the production of sound pulses in continuous trains. Even though the songs consisting of dense pulse trains are very common among *Drosophila* species, the song of *D. littoralis* is not unique. Also *D. robusta* males (Hoikkala, unpublished results) and the males of *D. melanica* group species (Ewing, 1970) produce songs with long pauses between sound pulses. These species are relatives to the *D. virilis* group (Throckmorton, 1975).

In *D. melanogaster*, several mutant genes (e.g. *period*, *cacophony*, *dissonance*) have been found to affect the male courtship song (studies reviewed by Yamamoto *et al.*, 1997). Peixoto & Hall (1998) have raised the question of whether these genes could also have played a role in inducing species differences in male song. In the light of the results of the present study it seems unlikely that the song differences between *D. virilis* and *D. littoralis* could be due to cumulative effects of mild changes in several genes as suggested by these authors. However, among the *D. melanogaster* song genes at least *cacophony* (*Dmca1A* locus) and *dissonance* (*nonA* locus) are located on the same area on the X chromosome as our gene(s) affecting species differences in male song (Päällysaho, unpublished results). The next steps in our work will be to locate our 'song genes' more thoroughly with the aid of molecular markers and P1 clones on the area of inversion marked by the *nd* gene. We also plan to study the homology between *D. melanogaster* song genes and the genes inducing species differences in the songs of *D. virilis* group species.

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