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QTL analysis of variation in male courtship song characters in *Drosophila virilis*

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We have used a quantitative trait locus (QTL) mapping approach to study the genetic basis of differences between two *Drosophila virilis* strains representing extreme phenotypes in two song characters, the number of pulses in a pulse train (PN) and the length of a pulse train (PTL). Variation in these characters among 520 F_2 males was studied by single-marker analysis and composite interval mapping (CIM) using a recombination linkage map constructed for 26 microsatellite markers. In single-marker analysis, two adjacent microsatellite markers on the third chromosome, msat19 and vir84 explained 13.8 and 12.4% of the variation in PN and 9.9 and 6.5% of the variation in PTL, respectively. CIM analysis revealed significant QTLs affecting PN, located on the X and the second, third and fourth chromosome of *D. virilis*, while variation in PTL was attributable to QTLs located only on the third chromosome.
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Introduction

Genetic factors affecting phenotypic traits are typically divided into genes with a major effect (major genes) and genes with a minor effect (polygenes). According to Fisher's (1918) infinitesimal model, variation in phenotypic characters within and between species is mainly caused by polygenes. Data supporting this neo-Darwinian view are available, for example, in Drosophila (Orr and Coyne, 1992; Mackay and Fry, 1996; True *et al*, 1997; Nuzhdin *et al*, 1999; Weber *et al*, 1999; Nuzhdin and Reiwitch, 2000; Vieira *et al*, 2000; Zeng *et al*, 2000). There is, however, also an increasing amount of evidence for variation in quantitative traits caused by a few major and several minor genes even within these species (Orr and Coyne, 1992). The sensory bristle number in *Drosophila melanogaster* is a good example of this kind of genetic architecture (reviewed by Mackay, 2001). Also, the rover/sitter larval foraging behaviour in *D. melanogaster* is influenced by one major gene, *foraging*, modified by several polygenes (de Belle *et al*, 1989; de Belle and Sokolowski, 1989), and the cuticular hydrocarbon profile in *D. melanogaster* is mainly controlled by a single gene, *desaturase2* (Takahashi *et al*, 2001).

Most studies on quantitative measurements of male courtship song in Drosophila species have concentrated on interspecific differences in the interpulse interval (IPI), which may play an important role in species recognition in some species (Tomaru and Oguma, 1994). In the *D. virilis* group of species, IPI does not seem to have important role in species recognition or in female choice (Hoikkala and Lumme, 1987). In five species of the *virilis* subgroup (*D. a. americana*, *D. a. texana*, *D. novamexicana*, *D. lummei* and *D. virilis*) IPI is relatively constant, whereas the most variable parameters are the number of pulses in a pulse train (PN) and the length of a pulse trains (PTL; Hoikkala and Lumme, 1987). Song simulation studies have shown that in *D. virilis*, females are able to recognise some species–specific characters in male song, and that there is variation among females in their preferences (Isoherranen *et al*, 1999). Unidirectional dominance in both PN and PTL suggests that these song characters have been affected in past history by directional selection favouring short and dense pulse trains (Huttunen and Aspi, 2003).

In our previous paper (Huttunen and Aspi, 2003), we used two biometrical approaches to study the genetic basis of courtship song variation in PN and PTL between two *D. virilis* strains showing large differences in these song characters. The joint scaling test revealed significant additive and dominance components, and also significant additive interaction between maternal and progeny genotypes. In addition, planned comparisons (contrast analyses of variance) between different generations revealed significant Y chromosomal and transient maternal factors and their epistatic interactions contributing to differences in the means of song characters. Furthermore, the Castle–Wright estimators for the effective number of loci contributing to mean differences between the strains suggested a low number of genes affecting variation both in PN and PTL (about 7 and 1, respectively). In this paper, we have used a quantitative trait locus (QTL) mapping approach with the aid of a recombination linkage map constructed for 26 microsatellite markers to identify the number and the chromosomal location of QTLs affecting variation in these courtship song characters in *D. virilis*.

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Materials and methods

Flies and crosses

We used three *D. virilis* strains: a multimarker strain YPE, a wild-type strain 1431 from England (collected 1986) and a wild-type strain B22 from Japan (collected 1986). *D. virilis* strains 1431 and B22 represent extreme song types of *D. virilis* (Huttunen S, Aspi J, Hoikkala A, Päällysaho S and Schlötterer C, unpubl. results) differing significantly in two song characters, the PN and the PTL.

The marker strain YPE had the following recessive visible mutant markers on each chromosome: *yellow* (*y*) on the X chromosome, *broken* (*b*) on chromosome 2, *gapped* (gpL_2) on chromosome 3, *cardinal* (*cd*) on chromosome 4 and *peach* (*pe*) on chromosome 5. Microsatellites were assigned into five linkage groups (corresponding to the major chromosomes of *D. virilis*) by crossing the females of the marker strain YPE with the males of the strain 1431 and by backcrossing the F_1 hybrid males with the females of the YPE marker strain. The position of the visible marker in relation to the microsatellites in each linkage group was localised by crossing the F_1 hybrid females with the males of the YPE strain.

Courtship songs

The data on courtship song of the strains 1431 (45 males) and B22 (50 males) as well as F_2 generation (890 males) are published in Huttunen and Aspi (2003). The courtship songs were recorded with a Sony TC-FX33 cassette recorder and a JVC-condenser microphone at 20 ± 1°C. Two song characters, the PN and the PTL were analysed using Signal Sound Analysis System (©Engineering Design). The means over three songs were used for both song characters for each individual to decrease within-male variation.

The means of the parental strains 1431 and B22 differ significantly both in PN (10.2 and 7.0, respectively, $F_{1,93} = 212.37$, $P < 0.0001$) and in PTL (213.6 and 159.5 ms, respectively, $F_{1,93} = 83.88$, $P < 0.0001$; Huttunen and Aspi, 2003). The differences in the other song characters, pulse length (PL), number of sound cycles (CN), IPI and carrier frequency (FRE) between these two strains were not significant (Huttunen and Aspi, 2003). F_2 males were obtained from four reciprocal crosses (220, 219, 203 and 248 males obtained from each cross, Huttunen and Aspi, 2003). The mean PN varied between 9.6 and 9.9 (overall mean 9.7, SD 1.24 units) and PTL 207.1 and 214.2 (overall mean 209.2, SD 27.86 ms) among the reciprocals, differences between the mean values of both PN and PTL being nonsignificant (Huttunen and Aspi, 2003). As the frequency distributions of both song characters were close to normal distribution both in parental strains and in F_2 progeny (Figure 1), untransformed data were used in the QTL analysis.

Microsatellite genotyping

In total, 34 microsatellite markers were used for genotyping: vir7, vir19, vir32, vir34, vir35, vir36, vir37, vir38, vir44, vir69, vir84, vir6cs, vir12cs, vir17cs, vir24cs, vir35cs, msat1, msat5, msat8, msat11, msat19, msat21, msat28, msat34, v10-47, v11-23, v11-48.1, v11-53 and v68-06.1 (Huttunen and Schlötterer, 2002), and v68-86.1, v68-4, v68-74, v71-38 and v93-93 (Schlötterer and Harr, 2000, at http://www.i122server.vu-wien.ac.at/). Details of primer sequences, optimal annealing temperatures and cytological positions of microsatellites (if known) are described in the corresponding papers.

Single fly DNA was extracted using the high salt extraction method (Miller *et al*, 1988). Microsatellite genotyping with γ^{32}P end-labelled primer was made in a 10 μl volume with 10 ng of single fly 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. The PCR products were separated on 7% denaturing polyacrylamide gel (32% formamide, 5.6 M urea) at 90 W and visualised by autoradiography after 12–24 h. The origin of alleles in F_2 progeny was determined using individuals of the strains 1431 and B22 as size standards.

Construction of a microsatellite marker map

Microsatellite markers were used to construct a recombination linkage map for *D. virilis*. The order and the genetic distances (in centiMorgans) between the microsatellite loci were determined using the MapMaker/Exp 3.0 program (Lander *et al*, 1987) with the Kosambi mapping function. Grouping was done with a minimum LOD score of 3.0 and maximum interval of 50 centimorgans (cM) as threshold values. The relative order of microsatellite markers within each linkage group was determined using 520 out of 890 F_2 males used in the biometrical study (Huttunen and Aspi, 2003). The microsatellite marker map was visualised using MapChart v.2.1 program (Voorrips, 2002). Localisation of microsatellites into linkage groups was also made separately for each reciprocal F_2 cross (Huttunen and Aspi, 2003) to detect possible differences between the reciprocals.

In addition to the F_2 data, the backcross individuals (see Flies and crosses) were used for assigning microsatellite markers into five linkage groups and localising the position of the visible marker in each chromosome in relation to the microsatellite markers. Owing to the lack of recombination in Drosophila males, the visible and microsatellite markers on the same chromosome always segregate together and therefore the linkage group of each microsatellite marker can be determined. In total, three females and three males for each phenotype class were used for scoring the alleles of each microsatellite locus (see Microsatellite genotyping). For determining the position of the visible marker in each linkage group, two males and two females from each phenotype class in backcross progeny (see Flies and crosses) were genotyped for the microsatellite loci (see Microsatellite genotyping).

QTL mapping

Single-marker analysis was used for detecting linkage between microsatellite markers and variation in song characters using linear regression in SPSS v. 10 (©SPSS Inc. 1989–2000; eg Liu, 1998). The coefficient of determination (R^2) was used as a measure for a marker explaining a fraction of the variation in PN and PTL among 520 F_2 males. The composite interval mapping method (CIM; Zeng, 1994) was applied to the F_2 data using QTL Cartographer v.1.12 software (Basten *et al*,

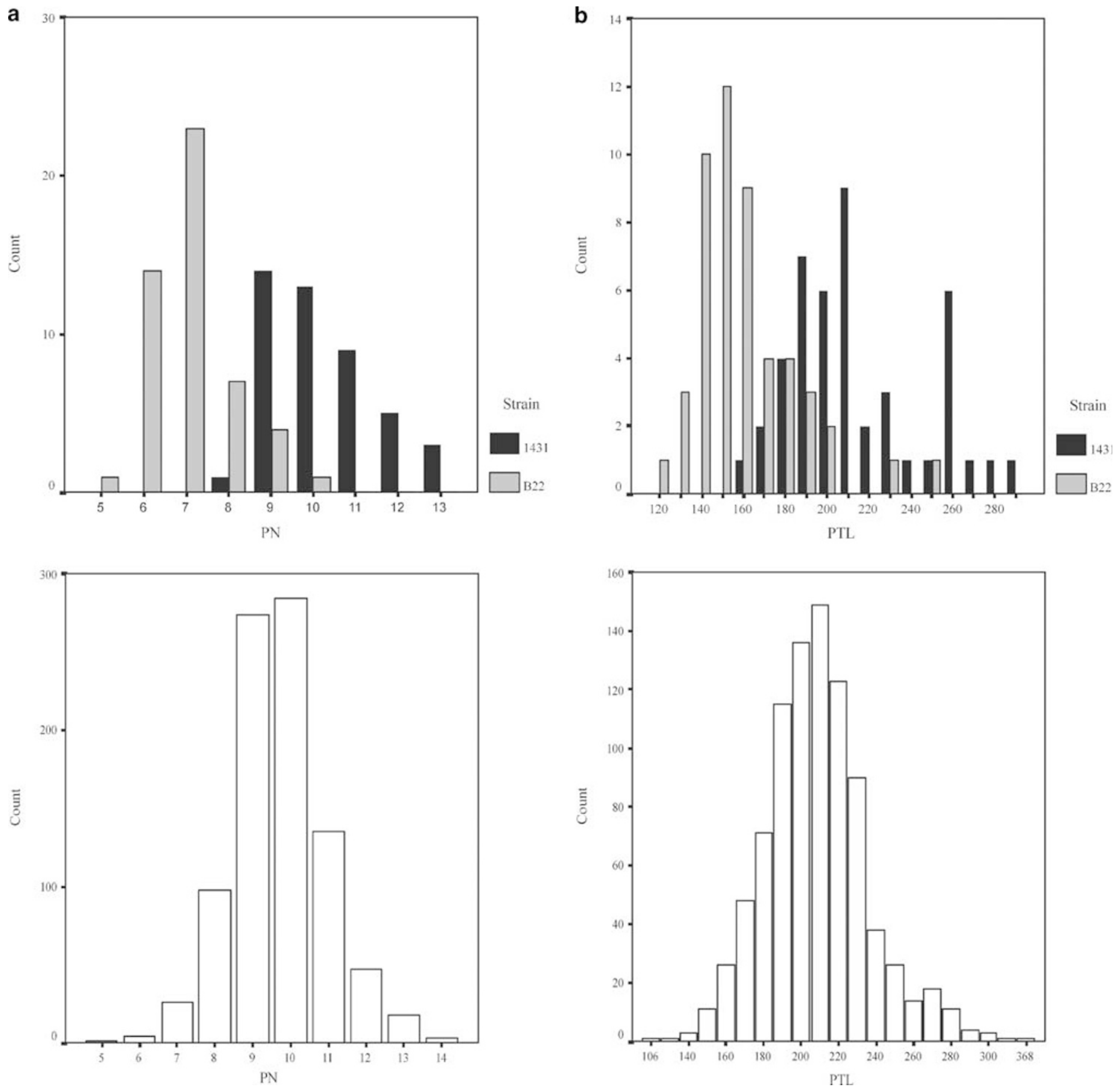

Figure 1 Frequency distributions of male courtship song characters (**a**) the number of pulses in a pulse train, PN and (**b**) the length of a pulse train, PTL in strain B22 and 1431 and F_2 generation males. Data from Huttunen and Aspi (2003).

1997). A backward–forward stepwise regression method in the SRmapqtl program of the QTL Cartographer package was used to obtain the number of markers to control for the genetic background in CIM. Six microsatellite loci (vir36, vir34, msat19, msat21, msat34 and v11-48.1) were used as genetic background markers in CIM in PN and one marker (vir36) in PTL. The significance of a QTL was tested by estimating a likelihood ratio (LR) score, defined as $-2\ln(L_0/L_1)$, which is the ratio of likelihood that a QTL is present in a test interval relative to the null hypothesis of no QTL in the interval. The LR scores were converted to LOD scores (likelihood of odds; Lander and Botstein, 1989), which were plotted against the marker position on each linkage group. The experiment-wise significance level ($P = 0.05$) for each analysis was obtained by randomly permuting the trait data 1000 times.

Results

Recombination linkage map for *D. virilis*

Initially, 34 microsatellite loci were genotyped for 520 F_2 individuals to construct a recombination linkage map for *D. virilis*. Nine of the markers (vir19, vir35, vir37, vir44, vir24cs, msat5, msat34, v68-86.1 and v93-93) did not group with any of the markers in the five linkage groups, and thus were not used in the map construction.

Recombination mapping of the remaining 26 microsatellites using F_2 data resulted in two markers being located on X chromosome, six on chromosome 2, seven on chromosome 3, eight on chromosome 4 and three on chromosome 5 (Figure 2).

The linkage group for the X chromosomal markers is not shown since the two microsatellite loci, msat34 and v68-06.1, were not linked with each other (distance >50 cM). However, these two markers showed 1:1 segregation pattern in both F_2 and backcross flies, and were also linked to the X chromosomal visible marker *yellow* (*y*) in the backcross data. The localisation of microsatellites was also tested in each of the four reciprocal crosses separately (see Materials and methods).

In the fourth linkage group, there were discrepancies between cytological and linkage map positions for some microsatellite markers (v68-74, v71-38 and v68-4, Figure 2), which have been cytologically mapped to sections 41D, 42E and 43A, respectively, on the fourth chromosome of *D. virilis*. However, the likelihood ratio for the cytological order of the markers in the fourth linkage group was over 10-fold lower compared to the likelihood ratio for the most likely order of the microsatellites given in Figure 2.

The linkage grouping of the microsatellite markers was also confirmed using the backcross data. There were no large differences in the assignment and the order of the markers within each linkage group between the F_2 and the backcross data, despite the smaller sample size used in latter case. However, the average distances between the markers varied between the two data sets (results not shown).

Single-marker analysis

Single-marker analysis was used for studying linkage between microsatellite markers and variation in courtship song characters among F_2 males (Table 1). In total, 13 microsatellite markers were linked with genes affecting variation in PN (R^2 ranged from 1.7–12.4%) and eight markers with genes affecting variation in PTL (R^2 ranged from 1.6–9.9%). Altogether, the markers explained 75.8 and 39.6% of the variation in PN and PTL, respectively. Two adjacent microsatellite markers on the third chromosome, msat19 and vir84, explained more variation than any other markers, for both characters. The R^2 coefficients for markers msat19 and vir84 were 13.8 and 12.4% with PN, and 9.9 and 6.5% with PTL, respectively. Finding the same markers showing association with variation in PN and PTL is not unexpected, since there is a significant phenotypic correlation between the song characters in the segregating F_2 generation ($r = 0.86$, $P = 0.01$). In *D. virilis*, PTL is mainly determined by PN but when PN is very high, the IPI will be slightly shorter, decreasing PTL ($r = -0.22$ between PN and IPI; Hoikkala and Lumme, 1987).

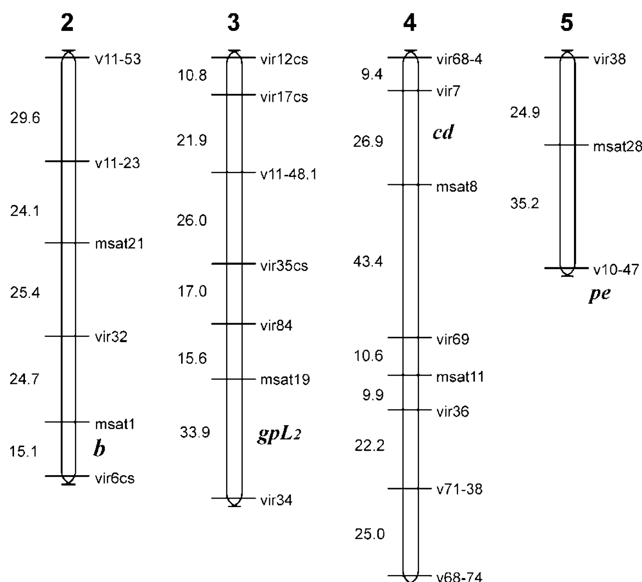

Figure 2 Recombination linkage map of microsatellite markers in *D. virilis*. The distance between markers is shown in centiMorgans. The position of the visible marker in relation to the microsatellites in each linkage group is shown in bold.

QTL mapping results

The CIM showed the presence of eight significant QTLs affecting PN locating on the X (one), second (one), third (five) and fourth chromosome (one) of *D. virilis* (Figure 3a). Four adjacent QTLs on the third chromosome were observed to affect pulse train length (PTL, Figure 3b). The QTLs on the third chromosome may not be independent, that is they may represent only a single genomic region since in most cases the LOD score curve between identified QTL peaks did not fall below significance threshold in either PN or PTL (Figure 3a and b). Furthermore, the number of QTLs detected is a conservative estimate, as the linkage map does not completely cover the genome of *D. virilis*. The window size in CIM can have an effect on the results obtained from CIM. The mapping results were, however, the same irrespective of the window size used (10 or 20 cM).

QTL effects

The effects of significant QTLs detected in CIM analysis for both PN and PTL are shown in Table 2. Altogether, eight significant QTLs (on X, second, third and fourth chromosome) affecting PN and four significant QTL (on the third chromosome) affecting PTL were detected in CIM. The highest effect on variation in PN was shown in QTLs located on third and X chromosome, explaining 26.6 and 26% of the variance in the F_2, respectively. QTL within the interval msat19-vir34 (on the third chromosome) explained 17.2% of the variance in PTL in F_2 males. Overall, single QTL effects were positive in sign, additive effects being larger than dominance effects. Dominance was detected in three adjacent QTLs on the third chromosome located between markers vir35cs and vir34, in both song characters. The X chromosomal QTL showed also dominance for PN. As mentioned earlier, the effects of the QTLs are not necessarily independent since the linkage map is sparse (Figure 2).

Discussion

The recombination linkage map of 26 microsatellite markers used in this study is the first molecular marker map constructed for *D. virilis*. Undoubtedly, more markers are required and their cytological position in each chromosome should be determined to obtain a denser and more precise molecular map, especially for

Table 1 Percentage of variance in the number of pulses (PN) and in the length of a pulse train (PTL) among F_2 males explained (as R^2 and coefficient $\pm$SE) by the microsatellite loci in single-marker analysis

Marker	*Location*	*PN*			*PTL*		
		R^2 *(%)*	P-*value*	*Coefficient* $\pm$*SE (units)*	R^2 *(%)*	P-*value*	*Coefficient*$\pm$*SE (ms)*
v11-53	2	1.8	0.003	0.27$\pm$0.09	—	—	—
msat21	2	3.5	0.000	0.38$\pm$0.09	—	—	—
vir32	2	1.7	0.003	0.27$\pm$0.09	—	—	—
msat1	2	2.3	0.001	0.29$\pm$0.09	1.6	0.004	5.42$\pm$1.89
vir17cs	3	6.4	0.000	0.50$\pm$0.09	3.7	0.000	8.39$\pm$1.92
vir12cs	3	5.3	0.000	0.45$\pm$0.08	3.1	0.000	7.50$\pm$1.87
v11-48.1	3	9.4	0.000	0.64$\pm$0.09	5.6	0.000	10.89$\pm$2.00
msat19	3	13.8	0.000	0.74$\pm$0.08	9.9	0.000	14.05$\pm$1.89
vir84	3	12.4	0.000	0.77$\pm$0.09	6.5	0.000	15.00$\pm$2.12
vir35cs	3	6.7	0.000	0.50$\pm$0.09	4.1	0.000	8.74$\pm$1.98
vir36	4	1.8	0.002	0.26$\pm$0.09	—	—	—
vir34	4	8.8	0.000	0.61$\pm$0.09	5.1	0.000	10.22$\pm$1.99
vir41	NA	1.9	0.002	0.21$\pm$0.07	—	—	—

Only the markers with a significant association ($P<0.05$ after Bonferroni correction) with variation in at least one of the song characters are shown. The chromosomal location of each marker is based on recombination mapping (NA; unknown location).

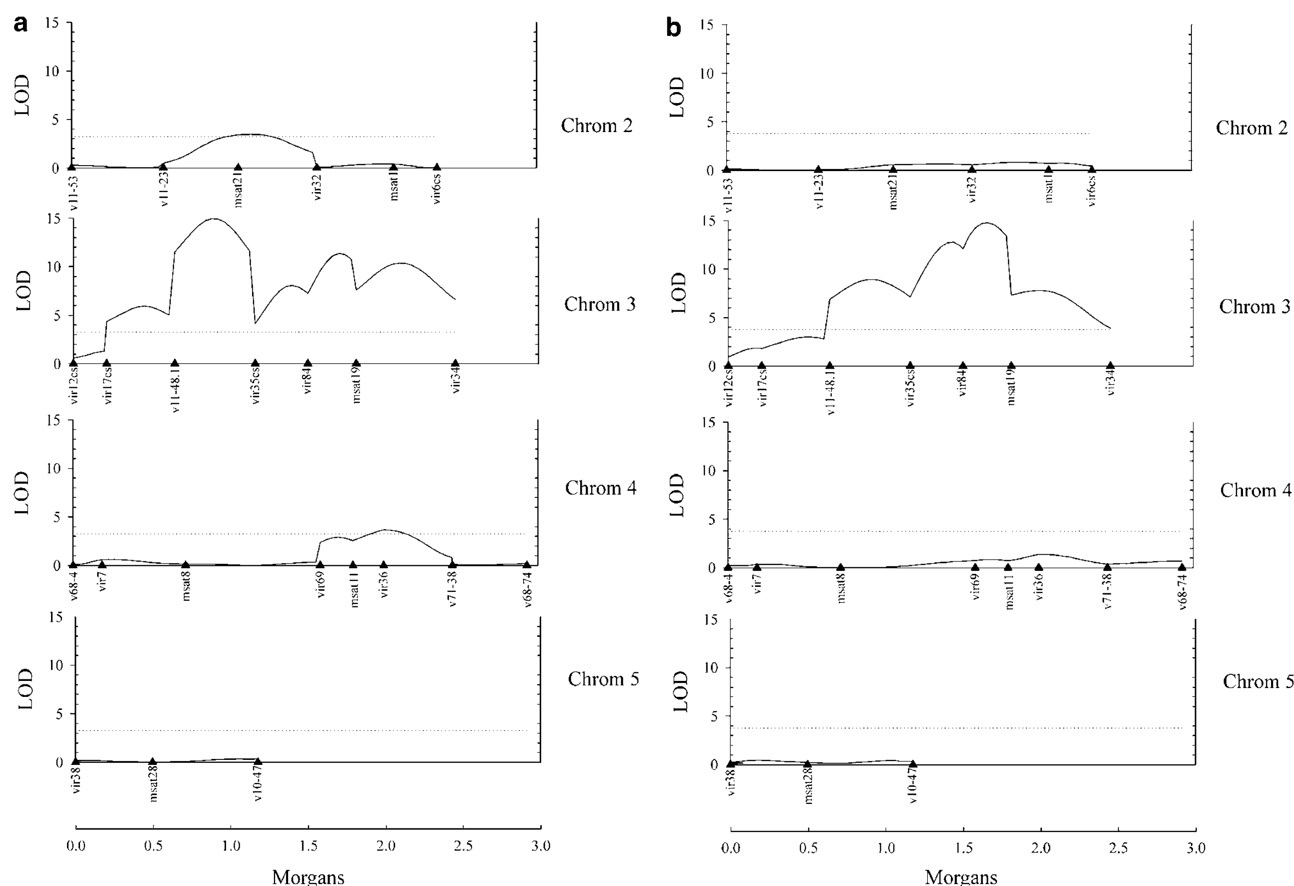

Figure 3 Plot of the LOD statistics against the marker map position (in Morgans) for (**a**) the number of pulses, PN and (**b**) the length of a pulse train, PTL using composite interval mapping. The horizontal lines (LOD score 3.25 for PN and 3.24 for PTL) show the 5% significance threshold level.

the chromosomal regions having QTLs affecting the song characters.

Our previous biometrical study showed directional dominance towards a lower number of pulses and a shorter pulse train length, strain B22 having more dominant (negative) alleles than strain 1431 (Huttunen and Aspi, 2003). In the present study, low dominance effects of the single QTLs were in a different direction. Difference between the two studies can be explained by the fact that the QTL study gives additive and dominance effects of individual QTLs, whereas dominance in the biometrical study represents the overall

Table 2 Additive and dominance effects of significant QTLs (shown between markers) on variation in the number of pulses (PN, in units) and the length of a pulse train (PTL, in milliseconds) obtained from composite interval mapping

Character	*Boundary markers (chromosome)*	*Additivity*	*Dominance*	*%Var*
PN	v68-06.2 – msat34 (X)	−0.020	2.413	26.0
	msat21 – vir32 (2)	0.345	—	4.1
	vir17cs – v11-48.1 (3)	0.593	—	13.5
	v11-48.1 – vir35cs (3)	0.777	—	18.4
	vir35cs – vir84 (3)	0.662	0.315	16.7
	vir84 – msat19 (3)	0.75	0.272	19.6
	msat19 – vir34 (3)	0.755	0.350	26.6
	vir36 – v71-38 (4)	0.272	—	1.8
PTL	v11-48.1 – vir35cs (3)	12.81	—	11.4
	vir35cs – vir84 (3)	17.34	7.89	12.7
	vir84 – msat19 (3)	17.81	8.39	14.9
	msat19 – vir34 (3)	14.66	7.92	17.2

The total variance explained by each QTL among F_2 individuals is shown as percentage (%Var).

dominance in the variation in mean PN and PTL. Above all, even though these characters are phenotypically correlated, both our previous biometrical and QTL study reveal that the genetic basis is not entirely influenced by the same QTLs. This is in agreement with the results of Hoikkala and Lumme (1987), who showed that the genetic correlation between these characters to be low ($r = 0.344$) in crosses between *D. virilis* group species.

Significant QTLs on the third chromosome had the highest effect on variation both in PN and PTL, and significant QTLs on the X chromosome, as well as on the other autosomes (second and fourth chromosome) affected variation in PN. Our QTL study confirms the genetic basis of song differences between *D. virilis* strains to be largely autosomal and probably affected by both major and minor genes, as observed in the same data using biometrical analysis (Huttunen and Aspi, 2003). Furthermore, several adjacent QTLs were observed in both song characters. Whether the adjacent QTLs detected in CIM analysis represent only one significant QTL region needs to be studied with the aid of a denser molecular map.

Most studies on male courtship song inheritance in *Drosophila* have concentrated on IPI, which has a polygenic additive inheritance with numerous genes spread throughout the genome (Kawanishi and Watanabe, 1980; Cowling and Burnet, 1981; Kyriacou and Hall, 1986; Pugh and Ritchie, 1996; Hoikkala *et al*, 2000). However, recently Gleason *et al* (2002) found evidence for only three significant QTLs explaining 54% of the genetic variance in mean IPI between recombinant inbred lines of *D. melanogaster*. Species differences in IPI between *D. pseudoobscura* and *D. persimilis* were also shown to be determined by a few QTLs (Williams *et al*, 2001). Our present results show somewhat similar results, especially for PTL, which is mainly determined by QTLs on a single autosome. A low number of QTLs affecting variation in other behavioural characters in Drosophila, for example in odor-guided behaviour (Fanara *et al*, 2002) and in mating discrimination (Civetta *et al*, 2002), has also been reported.

One of the major unresolved questions in quantitative genetics is the relationship between intraspecific variability and interspecific differences in phenotypic characters. The same QTLs may, then, account for the trait variability both within and between the species. Intraspecific variation in a quantitative character may serve as a source for selection to act on during speciation. In contrast, the vast majority of intraspecific variants could be deleterious mutations that have not yet been selected out. In this case, intraspecific variation would not ultimately generate interspecific trait differences (Nuzhdin and Reiwitch, 2000).

Our present results suggest that PN and PTL in *D. virilis* is controlled mainly by autosomal QTLs, even though there was a significant contribution of an X chromosomal QTL locating at the distal part of the chromosome. Hoikkala *et al* (2000) have studied the genetic basis of PN and PTL in crosses between *D. virilis* and *D. littoralis* using visible markers in single-marker analysis. No significant autosomal factors were found to contribute to the species differences in either song character, whereas significant X chromosomal genes at the proximal end of the chromosome (linked with *white*, *apricot* and *notched* visible markers) had a major impact on PN and PTL. Hoikkala and Lumme (1987) have shown that the species specificity of the courtship song in several species of the *D. virilis* group is largely caused by X chromosomal genes. Thus, these previous results compared with the present study strongly suggest that the loci contributing to variation in these song characters within *D. virilis* are not necessarily the same as the ones causing species differences in male song in the *D. virilis* group.

Chromosomal analysis has revealed polygenic autosomal inheritance in PN also between closely related species, *D. virilis* and *D. lummei* (Hoikkala and Lumme, 1984). We shall continue QTL mapping of genes affecting this song character in *D. virilis* and between *D. virilis* and *D. lummei* to find out whether the same loci are responsible for variation within and divergence between the species in this character.

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