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Quantitative trait loci affecting a courtship signal in *Drosophila melanogaster*

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Courtship plays a major role in the sexual isolation of species, yet the genetics underlying courtship behaviour are poorly understood. Here we analyse quantitative trait loci (QTL) for a major component of courtship song in recombinant inbred lines derived from two laboratory strains of *Drosophila melanogaster*. The total variance among lines exceeds that between parental strains, and is broadly similar to that seen among geographic strains of the Cosmopolitan form of this species. Previous studies of the quantitative genetics of fly song have implied a polygenic additive inheritance with numerous genes spread throughout the genome. We find evidence for only three significant QTLs explaining 54% of the genetic variance in total. Thus there is evidence for a few large effect genes contributing to the genetic variance among lines. Interestingly, almost all of the candidate song genes previously described for *D. melanogaster* do not coincide with our QTLs.
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Introduction

Courtship plays a major role in the sexual isolation of species, yet the genetics underlying courtship behaviour are poorly understood (Ritchie and Phillips, 1998). Analysis of the genetics of courtship behaviour will contribute to our understanding of speciation by identifying the types of genetic change involved in reproductive isolation. For this, one should identify the crucial components of courtship behaviour and design appropriate genetic approaches. A first step in understanding species differences is elucidating the nature of genetic variation within a species.

Courtship in *Drosophila* has been described in detail, including analysis of differences among species (eg, Spieth and Ringo, 1983; Welbergen *et al*, 1992). Courtship song is an important species-specific behaviour that improves the mating success of *D. melanogaster* males (Ewing, 1964; Bennet-Clark and Ewing, 1967; Ritchie *et al*, 1999) and probably contributes to sexual isolation among species of the *melanogaster* group (Kyriacou and Hall, 1986; Tomaru *et al*, 2000). The influence of courtship song on species-specific mating behaviour has also been demonstrated to have this effect in other species groups (Grossfield, 1968; Tomaru *et al*, 1995; Tomaru *et al*, 1998; Doi *et al*, 2001).

Males produce courtship song by wing vibration. *Drosophila melanogaster* males have two songs: pulse song and hum (or 'sine') song. Of the two, pulse song affects male and female mating behaviour in a more conspicuous manner (Schilcher, 1976b, c). Pulse song consists of a series of low frequency, short pulses. The most important parameter of pulse song for species recognition is the interpulse interval (IPI), the amount of time between each pulse (Ewing and Bennet-Clark, 1968; Ritchie *et al*, 1999). Mean IPI is highly species-specific; for example, *D. melanogaster* mean IPI is approximately 33 msec while that of *D. simulans*, a sibling species, is around 50 msec, and the ranges of variation for each species do not overlap (Kawanishi and Watanabe, 1980).

Within *D. melanogaster*, variability in mean IPI among natural populations of the Cosmopolitan race is low (Kyriacou and Hall, 1986; Ritchie *et al*, 1994). Conventional crosses imply that population or laboratory strain differences in mean IPI have a polygenic additive genetic architecture (Cowling, 1980; Ritchie *et al*, 1994). Artificial selection for mean IPI results in an asymmetric response, increasing but not decreasing IPI for *D. melanogaster* (Ritchie and Kyriacou, 1996; Pugh, 1997). Heritabililty for mean IPI is high (26%) but evolvability (the coefficient of additive genetic variance (Houle, 1992) is low (2.1%; Ritchie and Kyriacou, 1996) because of low phenotypic variation. These results suggest that inheritance is consistent with a polygenic trait involving both the sex chromosome and autosomes. In contrast, comparing the Zimbabwe and Cosmopolitan races of *D. melanogaster*, which have songs that are more widely divergent in mean IPI than the selection lines, IPI differences are largely attributable to genes on chromosome 3 with significant interactions involving other chromosomes (Colegrave *et al*, 1999). As yet, the genetics of courtship song are poorly understood, predominantly because studies to date have not included sufficient numbers of lines or markers for good statistical and mapping analyses.

Recent improvements in molecular and statistical tools

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have shown that the traits considered polygenic by more traditional studies might in fact be influenced by few quantitative trait loci (QTL) (reviewed in Barton and Turelli, 1989; Orr and Coyne, 1992; Mitchell-Olds, 1995), including *Drosophila* song (Williams *et al*, 2001) so here we ask whether there is evidence for significant QTLs affecting variation in mean IPI within *D. melanogaster*. Our first step is to map mean IPI QTL in recombinant inbred (RI) lines of *D. melanogaster*. Further progress may be achieved by comparing QTL positions with the position of candidate genes for *Drosophila* courtship song.

Potential candidate genes include any that have been identified as affecting *D. melanogaster* courtship song when mutated (though few of these influence mean IPI). The *period* locus affects the length of the cycle of mean IPI, without directly influencing overall mean IPI (Kyriacou and Hall, 1980). In contrast, *cacophony* affects mean IPI, but it also influences the structure of pulses (Schilcher, 1976a, 1977; Wheeler *et al*, 1988). Alleles of *no-on-transient-A* (Kulkarni *et al*, 1988; Wheeler *et al*, 1988; Stanewsky *et al*, 1996) and *croaker* (Yokokura *et al*, 1995) also influence song through increasing the number of cycles within each pulse. Alleles of *doublesex* eliminate sine song, but do not affect pulse song (Villella and Hall, 1996). Some alleles of *fruitless* eliminate pulse song while other alleles affect the length of mean IPI (Wheeler *et al*, 1988; Villella *et al*, 1997). Recent analysis of previously identified locomotor mutants has implicated *paralytic*, *no-action-potential* and *slowpoke* as genes affecting the length of mean IPI (Peixoto and Hall, 1998). In addition, *slowpoke*, as well as *Cysteine-string-protein*, affects the number of cycles per pulse. These two genes, along with *temperature-induced-paralysis-E*, affect both amplitude and intrapulse frequency (Peixoto and Hall, 1998).

Here we examine RI lines to detect QTLs affecting song in *D. melanogaster.* Recombinant inbred lines have a great advantage over crosses between divergent lines in that environmental effects are minimised through repeated measures on lines. Such measurements are often not possible in individual progeny. In addition, RI lines can be made with natural variants, whereas selection lines can only mimic natural variation so that there is no guarantee that the same genes would be involved. Furthermore, deleterious effects fixed by artificial selection are avoided when using RI lines.

The RI lines used here are derived from two established laboratory strains. The use of RI lines derived from parental strains that are not significantly different for the trait in question, has been quite successful in *D. melanogaster*. The same RI lines used here have been used in QTL studies of life history and morphological traits, which typically show limited interspecific variation, including longevity (Nuzhdin *et al*, 1997; Vieira *et al*, 2000), bristle number (Gurganus *et al*, 1998), reproductive success (Fry *et al*, 1998; Wayne *et al*, 2001), sex combs (Nuzhdin and Reiwitch, 2000) and ovariole number (Wayne *et al*, 2001). We ask if the RI lines vary significantly in mean IPI and analyse the genetic basis of this variation.

Materials and methods

Drosophila strains

The construction of the RI lines is described in Nuzhdin *et al* (1997). Briefly, hybrids of two unrelated, homozygous strains, Oregon R and 2b, were backcrossed to 2b. The backcross progeny were randomly mated for four generations and then inbred by full-sib mating for 25 generations. This generated ninety-eight lines, of which 92 were studied here (insufficient recordings were obtained from four of the lines and two lines have smaller than normal wings and cannot sing). All fly culturing was at 25° using standard techniques.

Song analysis

Five females and one male of the line to be tested were placed in a vial (75×23.5 mm) for 5 days, after which they were removed. Progeny males were collected on the day of eclosion and isolated in another vial (95 mm × 16.5 mm) until recording. All flies were recorded at 4–6 days post-eclosion using a custom built 'insectavox' microphone (Gorczyca and Hall, 1987) and Marantz CP430 cassette tape recorder. The male to be recorded, along with a wingless Oregon-R female, was introduced by aspiration into a mating chamber and recordings were made for approximately 5 min from the first burst of pulse song. Temperature was recorded as the average of that at the beginning and the end of each recording. Recordings were made between 23.7° and 28.8° with a mean of 26.66° (and IPI values reported here were corrected to this temperature). Song was digitised using a Cambridge Electronic Design 1401 A/D converter (at 2 kHz after bandpass filtering at around 100 Hz to 1 kHz). Individual pulses of song were detected using an automatic procedure, with subsequent manual monitoring of data points and song pattern by the experimenter. All analyses used custom-written scripts in the 'Spike2' language (copyright C.E.D.). Histograms of the IPIs detected in each recording were examined and the IPI values of each male entered into the analysis was the mean IPI from the recording. These procedures have been shown to be accurate (Ritchie and Kyriacou, 1994).

Recordings were carried out in two non-overlapping temporal 'blocks' consisting of 84 lines and 29 lines, separated by about 4 months. To allow comparisons across blocks, 23 lines were recorded in both blocks. The number of individuals recorded per line varied between five and 23 with a mean of 7.4 for a total of 682 individuals.

Molecular markers and map

Each line had been previously scored for the position of *roo* transposable elements as described in Nuzhdin *et al* (1997). There were a total of 93 markers polymorphic between the parental lines with an average spacing of 2.9 cM (Gurganus *et al*, 1998). Seventeen markers were completely linked with neighbouring markers and were excluded from the analysis. Of the remaining 76 markers, 16 are on the X chromosome, 22 on the second chromosome, 37 on the third chromosome and one on the fourth chromosome. Heterozygous markers within an RI line were treated as missing data for that line. Construction of the genetic map is described in (Nuzhdin *et al*, 1997).

Statistical analyses to calculate the mean IPI of each line were conducted using GLM routines of Minitab v11. QTL Cartographer (Basten *et al*, 1994, 1997) was used for composite interval mapping of QTLs and permutations of the data set.

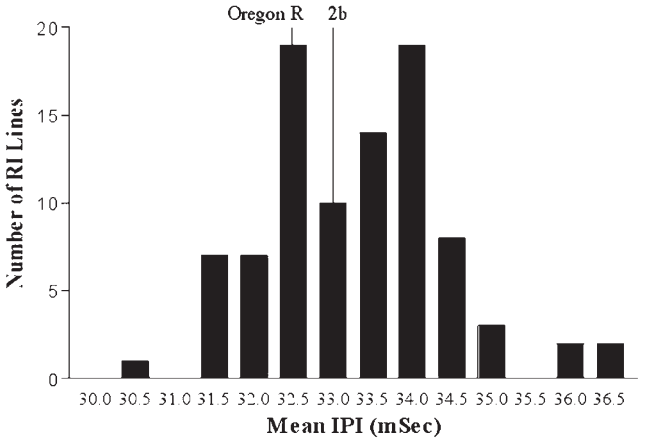

Figure 1 Variation in mean interpulse interval among the RI lines. The values of the parental strains, Oregon R and 2b, are indicated by lines.

Results

Statistical analysis of mean IPI

The mean IPIs of the parental strains were not significantly different from each other: 32.48 ± 0.45 (SE) msec for Oregon R males and 33.08 ± 0.45 msec for 2b males. In contrast, the range of mean IPI for the RI lines, 30.31–36.63 msec (Figure 1), was much greater than that of the parents. Mean IPI for each RI strain was calculated from a general linear model with recording block as a blocking factor, recording temperature as a covariate and the number of IPIs per recording as a weighting factor (Table 1). This analysis showed significant variation among lines ($P < 0.001$). Previous studies have shown that IPI can be prone to environmental and block effects (Ritchie and Kyriacou, 1996), so a separate analysis was carried out on the 23 lines recorded in both blocks. When analysed using a general linear model with recording block treated as a factor nested within lines (within block recordings could not be exactly simultaneous), plus the covariate and weights described before, line effects remained significant using recording blocks as the error mean square ($F_{22,23} = 3.76$, $P = 0.001$, Table 2). Although the analysis detected environmental effects between blocks, the differences between lines were significant when tested against these block effects. Therefore, we are confident that the mean IPIs calculated here reflect the genetic effects (furthermore, if blocks are treated as a fixed crossed effect, there was no significant interaction term between blocks and strains, $F_{22,\ 227} = 0.97$, NS).

Mapping of QTLs for mean IPI

The second chromosome was divided into two sections in the genetic map (Figure 2) because no linkage disequilibrium was found between the markers at 50F and 57C (Nuzhdin *et al*, 1997). To map QTLs affecting mean IPI, we used composite interval mapping, as implemented in QTL Cartographer (Basten *et al*, 1994, 1997). Composite interval mapping (Jansen and Stam, 1994; Zeng, 1994) combines interval mapping (Lander and Botstein, 1989) with multiple regression. Each interval flanked by adjacent markers is tested for the presence of a QTL affecting the trait while statistically accounting for the effects of additional segregating QTLs outside the interval. The significance level of $P = 0.05$ was calculated from 1000 permutations of the trait data among marker classes (Churchill and Doerge, 1994) and corresponds to a LOD score of 3.59.

Parameters potentially affecting the detection of QTLs using composite interval mapping include the number of background markers used and the size of the window around the tested interval within which linked markers are excluded from multiple regression. Using a wide range of window sizes did not influence the result. Forward/backwards stepwise regression resulted in 11 significant markers that could be used in composite interval mapping. Over a wide range of number of markers, which were subsequently used in composite interval mapping, the same three QTLs were significant after per-

Table 1 Results of generalized linear model for all strains

Source of variation	*Degrees of freedom*	*Adjusted mean squares*	*F*	*P*
Temperature	1	96153.7	284.22	<0.001
Block	1	5734.6	16.94	<0.001
Strain	91	1305.8	3.86	<0.001
Error	588	338.3		

Table 2 Results of generalized linear models for strains in both blocks (blocks nested within strains)

Source of variation	*Degrees of freedom*	*Adjusted mean squares*	*F*	*P*
Temperature	1	63902.8	163.12	<0.001
Strain				
(against error)	22	2058.3	5.26	<0.001
(against Block)			3.76	<0.001
Block (within strains)	23	667.2	1.70	0.027
Error	227	391.8		

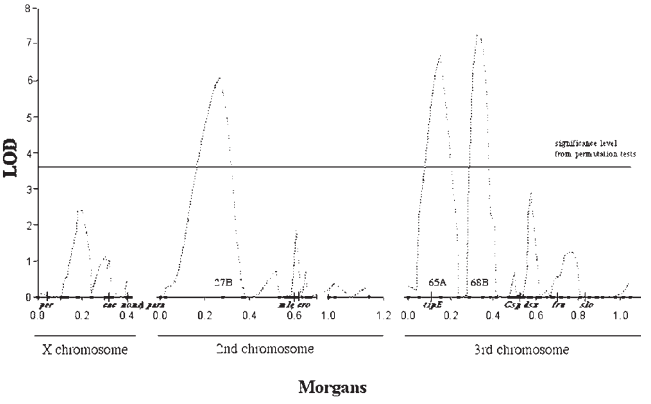

Figure 2 Plot of likelihood of odds (LOD) statistics from composite interval mapping against map distance (in Morgans) for all three chromosomes of *Drosophila melanogaster*. Marker positions are indicated on the absissa by a dot. The dashed lines are the LOD scores. The solid line shows the significance level ($P = 0.05$) for the data set as determined by 1000 permutations of the data set. Marker positions for estimating effects of QTLs are shown (Table 3). In addition, the locations of potential candidate genes for song are indicated.

Table 3 Effects and contribution to genetic variance for QTLs for mean interpulse interval in *Drosophila melanogaster*

Marker	*Map position*	*Boundary markers* [a]	*Boundary map positions*	*Effect (msec)*[b]	*Vg of each QTL* [c]	*% total Vg* [c]
27B	2–22	22F, 29F	2–6, 2–34	0.826	0.078	19.4
65A	3–16	61A, 65D	3–1, 3–18	–0.925	0.100	24.9
68B	3–35	67D, 69D	3–32, 3–38	0.560	0.039	9.7

[a]Approximate bounds on the intervals were determined by the position of the first nonsignificant marker flanking the significant regions as determined by regression.
[b]Additive effects (a) were estimated from composite interval mapping.
[c]The genetic variance (Vg) was calculated from $0.5pqa^2$. The total Vg was 0.402.

mutation and no others were ever found. Figure 2 depicts the results using the Ri2 design (recombinant inbred lines, sib mated), the Kosambi map function, 11 background markers and a window size of 5 cM. This analysis provides support for the presence of three QTLs that affect mean IPI, one on the left arm of the 2nd chromosome and two on the left arm of the 3rd chromosome.

QTL effects

The approximate intervals for each QTL were determined by the position of the first non-significant markers flanking the significant regions (Table 3). For each of the most significant markers in each of the three QTL regions, as determined by linear regression, the proportion of the genetic variance contributed by each QTL was estimated as $0.5p(1–p)a^2$, where p is the marker frequency and a is the difference between genotypes for the marker. Effects are given as the deviation from strain 2b. The estimate assumes strict additivity and that the markers and QTLs are tightly linked so that the frequencies of the markers and the QTLs are the same. The genetic variance was calculated as the variance component among lines and is equal to 0.402, thus the effect of the first and second QTLs is approximately one standard deviation each and for the third QTL about 0.6 standard deviations. Together, the QTLs account for 54.0% of the genetic variance among the RI lines. The effect of the QTL on the second chromosome is 0.826 msec, which corresponds to 0.409 phenotypic standard deviations (σ_p). The adjacent QTLs of the third chromosome have opposite effect of –0.925 and 0.560 msec from left to right, which correspond to 0.458 and 0.277 σ_p, respectively.

Discussion

Previous studies of the quantitative genetics of IPI in *D. melanogaster* have suggested that IPI has a polygenic genetic architecture (Cowling, 1980; Ritchie *et al*, 1994; Ritchie and Kyriacou, 1996; Pugh, 1997). Here we have looked for the presence of QTLs affecting mean IPI in RI strains of *D. melanogaster* and found three located on the chromosome arms 2L and 3L. This is similar to the 3rd chromosome attributable difference between Zimbabwe and Cosmopolitan *D. melanogaster*, which have a larger difference in mean IPI than is present in these RI lines (Colegrave *et al*, 1999).

Because the three significant QTLs account for 54.0% of the genetic variance in mean IPI and the effects are a relatively large proportion of σ_p, the implication is that few genes of relatively large effect account for most of the genetic variance among these lines. We cannot be sure how many genes contribute to each QTL because the resolution of factors is determined by the number of recombination breakpoints. In addition, there certainly must be genes of smaller effect contributing to the remaining 46% of the genetic variance. However, these result imply that the three QTL detected clearly have a major influence on IPI variation among these lines.

Variation between inbred laboratory lines for any trait is a result of the fixation of different alleles that were segregating in the population from which they were derived, and to fixation of new mutations that occurred during laboratory culturing. Quantitative genetic studies can only estimate genetic effects of the study population. However, the variation among these lines reflects the extent of naturally occurring variation within the Cosmopolitan form of *D. melanogaster*. The range of mean IPI among the RI lines is slightly larger than that found for European populations of *D. melanogaster* (32.9–36.0 msec; Ritchie *et al*, 1994) indicating that mean IPI studied here is phenotypically analogous to natural variation in *D. melanogaster*. Furthermore, the success many authors have had in QTL studies with these same lines for a variety of fitness traits (see Introduction) illustrates the potential power of using RI lines derived from strains that do not differ in the trait being examined.

The range of mean IPI found in these RI lines exceeds that of the original parental strains, another example of transgressive segregation, as has previously been seen for mean lifespan and bristle number in these lines (Gurganus *et al*, 1998; Nuzhdin *et al*, 1997). The increased variance in mean IPI among the RI lines compared with the parental strains is not symmetrical, with more lines with longer IPI (Figure 1). This reflects the finding of Ritchie and Kyriacou (1996) in which there was a greater response to selection for longer mean IPI than for shorter mean IPI. Interestingly, of the mutations found thus far that affect mean IPI, all make IPI longer (Wheeler *et al*, 1989; Villella *et al*, 1997; Peixoto and Hall, 1998). This is further evidence for the idea that direct or indirect selection on IPI has been favouring shorter mean IPI. Female preferences favouring males with shorter IPI (and hence more song per unit time) might be the source of such selection (Ritchie and Kyriacou, 1996).

The QTL on 2L overlaps with one for abdominal bristles (Gurganus *et al*, 1998). The first QTL on 3L overlaps with QTLs for ovariole number (Wayne *et al*, 2001) and sternoplural bristles (Gurganus *et al*, 1998). The last QTL overlaps with QTLs for male life span (Nuzhdin *et al*, 1997) and abdominal bristles (Gurganus *et al*, 1998). In general, the different placement of our QTLs versus ones for male fitness traits implies that we have not measured a general feature of male fitness but a unique character. As sensory bristles are neurogenic traits, and mean IPI is

a behaviour trait with underlying neurogenic causes, any relationship with those QTLs, while intriguing, remains to be demonstrated.

Only one of the candidate genes described as affecting song (see Introduction) falls within the region of one of the three QTLs. Alleles of temperature-induced paralytic E (*tipE*) affect song amplitude and intrapulse frequency (Peixoto and Hall, 1998) and this gene maps to 64B2 which is within the boundaries of the first QTL on the 3rd chromosome (Figure 2, Table 3). This gene has not been shown to affect mean IPI, the trait studied here. In many studies of bristle number, most QTL regions are associated with regions containing known candidate genes for the trait (eg, Long *et al*, 1995; Nuzhdin *et al*, 1999). These sites repeatedly are picked up in QTL studies, suggesting they are polymorphic in many populations (Mackay, 1996). In contrast, our results suggest that mutational analyses have not identified the genes affecting natural variation in mean IPI in these RI lines.

More studies have examined the genetics of IPI differences between species than within species. Studies with few markers imply that mean IPI is affected by genes on all chromosomes (Tomaru and Oguma, 1994). More recently, studies with a greater number of markers, have implicated few, localised regions contributing to song differences. In a study of *D. virilis* and *D. littoralis* (Hoikkala *et al*, 2000), chromosomal regions on the X, chromosome 3 and chromosome 4 (homologous to the X, 2L and 3L in *D. melanogaster*, Powell, 1997) were identified in courtship song differences. For the song difference between *D. pseudoobscura* and *D. persimilis* (Williams *et al*, 2001), three significant QTLs were found for IPI, but all three are associated with inversions between the species. It is not possible to resolve the number of genes involved to a high level, but the implications is that only three chromosome arms are involved: the ones homologous to the *D. melanogaster* X, 3L and 3R. Clearly, more high resolution studies of both within and between species difference are required to assess whether there are general patterns to the number or location of genes affecting species specific mating signals, but our results suggest that these important genes contributing to species differences can be localised and potentially characterised on the molecular level.

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