Do Quantitative Trait Loci (QTL) for a Courtship Song Difference Between *Drosophila simulans* and *D. sechellia* Coincide With Candidate Genes and Intraspecific QTL?

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ABSTRACT

The genetic architecture of traits influencing sexual isolation can give insight into the evolution of reproductive isolation and hence speciation. Here we report a quantitative trait loci (QTL) analysis of the difference in mean interpulse interval (IPI), an important component of the male courtship song, between *Drosophila simulans* and *D. sechellia*. Using a backcross analysis, we find six QTL that explain a total of 40.7% of the phenotypic variance. Three candidate genes are located in the intervals bounded by two of the QTL and there are no significant QTL on the X chromosome. The values of mean IPI for hybrid individuals imply the presence of dominant alleles or epistasis. Because unisexual hybrid sterility prevents an F_2 analysis, we cannot distinguish dominant from additive genetic effects at the scale of QTL. A comparison with a study of QTL for intraspecific variation in *D. melanogaster* shows that, for these strains, the QTL we have identified for interspecific variation cannot be those that contribute to intraspecific variation. We find that the QTL have bidirectional effects, which indicates that the genetic architecture is compatible with divergence due to genetic drift, although other possibilities are discussed.

THE divergence of mating behaviors influencing sexual isolation plays a fundamental role in speciation. Understanding the genetic architecture of courtship is therefore essential for studying models of speciation (Shaw and Parsons 2002). The genetics underlying courtship behavior are poorly understood (Ritchie and Phillips 1998), yet the evolution of premating isolation may be a primary cause of speciation in many taxa (Butlin and Ritchie 1994; Panhuis *et al.* 2001).

The relative importance of different genetic architectures in speciation has been debated but remains unresolved because of a lack of empirical evidence (Barton and Charlesworth 1984; Carson and Templeton 1984; Barton and Turelli 1989; Orr and Coyne 1992). Two extreme types of genetic architecture have been described. Type I architecture is characterized by many genes of small effect contributing to differences between species (Templeton 1981). In type II architecture, major gene effects underlie relevant traits. Both architectures depend on the magnitude and direction of allelic effects as well as interactions among loci. Both conventional crosses and advances in quantitative trait loci (QTL) analysis allow us to address the relative importance of the two types for adaptive traits. Evidence for both type I and type II architectures has been found. For example, major gene effects have been reported for pheromonal communication, sexual isolation, and genitalic morphology differences in Drosophila (Coyne *et al.* 1994; True *et al.* 1997; Doi *et al.* 2001; Takahashi *et al.* 2001). In contrast, acoustical communication has been suggested to have a polygenic mode of inheritance (Ritchie and Phillips 1998; Shaw and Parsons 2002; but see Henry *et al.* 2002).

In addition to the number, type, and genomic placement of genes affecting behavioral traits, QTL analysis allows assessment of the potential contribution of candidate genes to the trait. A candidate gene is a gene that is identified through mutational analyses as having an effect on the trait. QTL for quantitative traits, such as Drosophila bristle number, are often concordant with candidate loci (reviewed in Mackay 1996). However, mutational effects may not reflect the same kind of changes that contribute to important natural variation, although polymorphism at a bristle locus has been shown to affect naturally occurring variation (Lai *et al.* 1994). In general, mutationally defined candidate genes have been implicated in explaining intraspecific variation, but for increasing phylogenetic distance, the candidate gene approach is less successful (reviewed in Haag and True 2001).

An additional question that can be addressed through QTL analysis is whether the locations of genes affecting a trait difference between species are the same as those for polymorphisms within a species. If the locations are the same, then the same genetic loci may be responsible for trait variability both within and between species. Alternatively, intraspecific variants may be deleterious mutations that have not yet been removed by natural

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selection. In that case, intraspecific polymorphisms would not necessarily be responsible for generating interspecific trait differences (Nuzhdin and Reiwitch 2000). In studies to date, QTL have been found that are shared within and between species (Nuzhdin and Reiwitch 2000; Kopp *et al.* 2003).

Courtship song in Drosophila provides opportunities to study the genetic architecture of species differences, to determine the direction of allelic effects, to examine the possible contribution of candidate genes, and to compare intra- and interspecific genetic architecture. Male Drosophila produce courtship song by wing vibration. *Drosophila melanogaster* males have two songs: pulse song and hum (or "sine") song. Pulse song consists of a series of low-frequency, short pulses that affect male and female mating behavior. The most important parameter of pulse song for species recognition in the *D. melanogaster* group is the interpulse interval (IPI), that is, the amount of time between each pulse (Ewing and Bennet-Clark 1968; Ritchie *et al.* 1999). Mean IPI is species specific (Kawanishi and Watanabe 1980) and has been demonstrated to affect female mating propensity in a species-specific manner (von Schilcher 1976b,c).

In a recent study (Gleason *et al.* 2002), we examined the genetic architecture of *D. melanogaster* mean IPI with QTL mapping using recombinant inbred lines derived from two laboratory strains. Three QTL accounted for 54% of the genetic variation in the trait. One of the QTL was located on the left arm of the second chromosome whereas the other two were on the left arm of the third chromosome. In another QTL study of mean IPI difference between *D. pseudoobscura* and *D. persimilis*, three QTL explained 95.8% of the genetic variation (Williams *et al.* 2001). All three QTL in this latter case were associated with nonrecombining portions of the X and second chromosomes, so it is possible that many genes contribute to each QTL.

Twelve mutationally defined candidate genes are known to affect courtship song in *D. melanogaster*. Certain alleles of *cacophony* (*cac*), *fruitless* (*fru*), *paralytic* (*para*), *maleless* (*mle*), and *slowpoke* (*slo*) affect mean IPI (von Schilcher 1976a, 1977; Wheeler *et al.* 1988; Villella *et al.* 1997; Peixoto and Hall 1998). The *period* (*per*) locus affects a species-specific cycle in mean IPI, but not the mean itself (Kyriacou and Hall 1980), whereas *Cysteine string protein* (*Cys*), *temperature-induced-paralytic-E* (*tipE*), and *slo* affect pulse amplitude (Peixoto and Hall 1998). Song is completely eliminated with some mutants: *doublesex* (*dsx*) alleles eliminate sine song (Villella and Hall 1996), whereas some *fru* alleles eliminate pulse song (Wheeler *et al.* 1988; Villella *et al.* 1997). Intrapulse frequency can be affected by alleles of *slo, Cys*, and *tipE* (Peixoto and Hall 1998). The shape of intrapulse cycles is affected by alleles of *cac*, *per*, *slo*, *Cys*, *croaker* (*cro*), *transformer* (*tra*), and *no-on-or-off-transientA* (*nonA*; von Schilcher 1976a, 1977; Kulkarni *et al.* 1988; Wheeler *et al.* 1988; Bernstein *et al.* 1992; Yokokura *et al.* 1995; Stanewsky *et al.* 1996). Most of these genes were originally isolated because they affect other phenotypes, for example, circadian rhythm (*per*; Kyriacou and Hall 1980), sex determination pathways (*fru*, *dsx*, *tra*; Kulkarni *et al.* 1988; Wheeler *et al.* 1988; Bernstein *et al.* 1992; Villella and Hall 1996; Villella *et al.* 1997), and locomotion (*para*, *slo*; Peixoto and Hall 1998).

The genes identified through mutational analyses serve as possible candidate genes for QTL studies of natural variation in the same traits. In the *D. melanogaster* study (Gleason *et al.* 2002), only *tipE* fell within a QTL. This does not necessarily mean that *tipE* is the gene affecting the trait at this QTL because many genes may underlie each QTL. The absence of candidate genes from most QTL regions does suggest that the candidate gene approach has not been successful in identifying the genes underlying natural variation in courtship song within these strains of *D. melanogaster*.

In this study, we examine the genetic architecture of the difference in mean IPI between *D. simulans* and *D. sechellia*. These closely related species differ significantly in their courtship song and form fertile hybrid females. Thus, through QTL backcross analyses we can assess the genetic architecture of an important behavioral trait in species discrimination (Ritchie *et al.* 1999), determine if candidate genes colocalize with QTL for the trait, identify directional effects to infer the possible role of drift *vs.* selection, and determine whether or not QTL responsible for intraspecific variability and interspecific differences fall in locations similar to those found in the *D. melanogaster* study.

MATERIALS AND METHODS

Strains and crosses: One strain each of two species was used in this study. The *D. sechellia* strain was kindly provided by Jean David. This strain, although probably already inbred, was inbred for a further 18 generations of brother-sister mating to produce the line used in the subsequent crosses. The inbred *D. simulans* line was kindly provided by Jerry Coyne and had five morphological markers, one per chromosome arm (Table 1). Previous studies had shown that this strain sings with the same mean IPI as wild-type strains (Pugh and Ritchie 1996).

All fly culturing was at 25° and a 12 hr light:12 hr dark cycle using standard techniques. Female *D. simulans* flies were crossed to male *D. sechellia* flies and the female progeny were backcrossed to *D. simulans* males. For each cross, one female was paired with a single male in a vial (95 × 16.5 mm) for 7 days. Multiple crosses were performed to produce 554 males whose songs were subsequently recorded. In total, 58 crosses and 99 backcrosses were used. Using the five morphological markers, we attempted to record songs for all of the 32 backcross phenotypes so that we did not study only the most frequent intraspecific chromosomal combinations.

Courtship song recordings and analysis: Males were collected on the day of eclosion, genotyped for the five morphological markers, and isolated in another vial (95 × 16.5 mm) until recording. Males were recorded 8–10 days posteclosion using a custom-built "insectavox" microphone (Gorczyca and Hall 1987) and a Marantz CP430 cassette tape recorder. The male to be recorded, along with a wingless *D. simulans* or *D.*

sechellia female (the female courted does not influence song parameters, M. G. Ritchie, unpublished observation), was introduced by aspiration into a mating chamber and recordings were made for ~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 24.0° and 28.1° with a mean of 26.4°. Song was digitized using a Cambridge Electronic Design 1401 A/D converter (at 2 kHz after bandpass filtering at ~100 Hz–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 analysis used custom-written scripts in the "Spike2" language (Cambridge Electronic Design). Histograms of the IPIs detected in each recording were examined and the mean IPI value of each male entered into the analysis. These procedures have been shown to be accurate for determining mean IPI (Ritchie and Kyriacou 1994).

The number of IPIs obtained for each male recorded ranged from 4 to 770. By randomly resampling a selection of songs, we determined that at least 30 IPI values were necessary to accurately estimate mean IPI for an individual. Thus, subsequent analyses were performed only on individuals for which we had at least 30 IPI values. Mean IPI is strongly influenced by temperature (Shorey 1962). All mean IPI values were corrected to common temperature of 25° using the formula $-1.6(25\text{-}T) + I$, where T is the mean temperature of the recording and I is the mean IPI of the recording. The coefficient of 1.6 was empirically derived from other studies (Ritchie *et al.* 1994; Ritchie and Kyriacou 1996). After temperature correction, four outliers were removed and the data were log transformed to remove a right skew. Variance components reflect the transformed data (*i.e.*, they have not been back transformed). Both untransformed and backtransformed effects are presented (see below). The final sample size for the quantitative trait was 429 individuals.

Marker scoring: After recording, males were frozen at −20°. DNA was isolated from frozen individual males ($N = 433$) using the method of Gloor and Engels (1992). Forty molecular markers were scored for each individual (Table 1). These markers were all PCR amplified and had different-sized fragments for *D. sechellia* and *D. simulans* on 2% agarose, 4% Metaphor agarose (Cambrex), or acrylamide gels. Size differences were caused by natural variation in sequence length (indels or microsatellites) or by differences in restriction enzyme sites (Table 1). Hybrids were easily distinguished from homozygotes.

Genetic mapping and QTL analysis: Together with the morphological markers (see above), 45 markers were scored on 433 individuals. These markers were mapped using MAPMAKER (Lander *et al.* 1987). The map obtained was subsequently used in QTL analyses using QTL Cartographer version 1.16c (Basten *et al.* 1997) to map QTL.

Calculation of effects in milliseconds: Because our data were transformed by natural logs, the resulting effect of each QTL is dimensionless. The effect, as calculated, is the difference between the mean of the logs for the group (MLG) and the mean of the logs for the traits of all backcross individuals (MLT). This difference is equivalent to the log of ratios of the geometric mean for the group (GMG) and the grand geometric mean (GMT), that is, MLG − MLT = log(GMG/GMT). Therefore, if exp(effect) is multiplied by GMG, the result is GMT. Subtracting GMT from GMG yields the effect in milliseconds.

RESULTS

Marker mapping: The markers were chosen to have an average spacing of ~6 cM on the basis of the *D. melanogaster* map. The average spacing realized between markers was 19.06 cM, because of segregation distortion and our selection for recombinants. Most markers mapped in the same linear order (Table 1) as in *D. melanogaster* with the exception of a marker pair on the end of the left arm of each chromosome (*sc* and *Pgd* on the X chromosome, *ex* and *nt* on the second chromosome, and *ve* and *Cdc37* on the third chromosome). In a companion study of cuticular hydrocarbon QTL in the females derived from these crosses, the map order for the X and third chromosomes is the same as for *D. melanogaster*, although the order for the second chromosome is reversed as it is here (J. M. Gleason, J.-M. Jallon, J. Rouault and M. G. Ritchie, unpublished results). The reversals are most likely artifacts of being at the end of chromosomes, and as they are not near QTL, the order does not affect the results. On the right arm of the third chromosome, there is an inversion in *D. simulans* and *D. sechellia* relative to *D. melanogaster* and five markers (*Mtn*, *pros, gl*, *nos*, and *e*) show this inversion.

The length of the genetic map found in this study is quite long. Studies of backcross hybrids between *D. simulans* and *D. mauritiana* have resulted in maps longer than those of the original species (*e.g.*, Liu *et al.* 1996; True *et al.* 1997). Our long map length may be derived in part from selection for recombinant individuals and also from epistatic inviability interactions among loci. Genetic incompatibilities leading to inviability have been found in interactions between the X chromosome of *D. sechellia* and the autosomes of *D. simulans* (Joly *et al.* 1997; our personal observation). In addition, there was a reduction in viability when the third chromosome was recombinant (Joly *et al.* 1997). Such interactions will affect observed map lengths in interspecific studies.

Mean IPI: The strain of *D. simulans* had a mean IPI of 56.15 ± 1.699 msec and *D. sechellia* had a value of 67.06 ± 1.789 msec. F_1 hybrid males had a mean IPI value of 50.53 ± 1.090 msec. The final data set, of 429 individual, backcrossed males, had a mean IPI value of 50.78 ± 6.331 msec. Evidently, the trait displays dominance for low values of IPI or there are hybrid incompatibility influences on the trait. Using a backcross design, we cannot distinguish dominant from additive genetic effects, but because of the sterility of F_1 males, backcross analysis is the only crossing scheme possible with these species.

QTL analysis: The marker on the fourth chromosome (*ey*) was not significantly associated with the trait, and results for this chromosome, which comprises only ~1% of the genome, are not shown. Composite interval mapping (CIM) was performed for the rest of the genome. CIM (Jansen and Stam 1994; Zeng 1994) combines interval mapping (Lander *et al.* 1987) 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 QTL outside the interval. The significance

TABLE 1

Markers used and their resulting map order

Order[a]	Gene[b]	Gene abbreviation[c]	Location[d]	Size relation[e]	Source[f]
		X chromosome			
1	*Phosphogluconate dehydrogenase*	*Pgd*[6]*	2D4	*Rsa*I cuts *sim*	Intron
2	*scute*	*sc*	1A8	*sec* > *sim*	1
3	*period*	*per*	3B2–3	*sim* > *sec*	Repeat
4	*defective chorion*	*dec1*	7C1	*Dde*I cuts *sim*	X96929, X96931
5	*DS01391 (microsatellite)*	*DS01391*	9A1–2	*sec* > *sim*	2
6	*no-on-or-off-transient A*	*nonA*[4]*	14B18–C1	*sec* ≫ *sim*	Intron
7	*forked*	*f*[2]	15F7–9	Morphological	
8	*Zwischenferment (G6PD)*	*Zw*	18D13	*sec* > *sim*	Intron
		Second chromosome			
1	*expanded*	*ex*	21C5–6	*sec* > *sim*	3
2	*net*	*nt*	21A5	Morphological	
3	*anterior open*	*yan*	22D1	*sec* > *sim*	2
4	*odd skipped*	*odd*	24A1	*sim* > *sec*	4
5	*Mst26Aa*	*Mst*	26A1	*sec* > *sim*	X70899, X72630; intron
6	*neither inactivation nor afterpotential C*	*ninaC*	27F3	*Hsp*92II cuts *sim*	5
7	*big brain*	*bib*	30F5	*sec* > *sim*	Repeat
8	*spalt*	*sal*	32F1–2	*sim* > *sec*	Intron
9	*Suppressor of Hairless*	*Su(H)*	35B8	*sim* > *sec*	4
10	*caudal*	*cad*	38E6	*sec* > *sim*	Repeat
11	*Phosphoglucose isomerase*	*Pgi*[1]*	44F6	*Taq*I cuts *sim*	Intron
12	*G protein oα47A*	*Dgα*	47A7–9	*sim* > *sec*	1
13	*slit*	*sli*[3]*	52C9–D1	*sec* > *sim*	2
14	*grainy head*	*grh*	54E1–F1	*sim* > *sec*	4
15	*plum*	*pm*	59E2–3	Morphological	
16	*twist*	*twi*	59C2	*Taq*I cuts *sim*	Repeat
		Third chromosome			
1	*Cdc37*	*Cdc37*	62B4	*Alw*26I cuts *sim*	*6*
2	*veinlet*	*ve*	62A1–2	*sim* > *sec*	1
3	*temperature-induced-paralytic-E*	*tipE*[7]*	64A10	*sim* > *sec*	Intron
4	*Laminin B2*	*LamB2*	67C2	*Mfe*I cuts *sec*	*7*
5	*Superoxide dismutase*	*Sod*	68A7	*Hae*III cuts *sim*	Intron
6	*Esterase 6*	*Est6*	69A1	*Bsr*GI cuts *sec*	Intron
7	*Accessory gland peptide 70A*	*Acp70A*	70A4	*sim* > *sec*	X99414, X99417; intron
8	*scarlet*	*st*	73A3	Morphological	
9	*transformer*	*tra*	73A10	*sec* > *sim*	Intron
10	*Catalase*	*Cat*	75E1	*sim* > *sec*	1
11	*Glucose dehydrogenase*	*Gld*	84D3	*Hae*III cuts *sim*	Noncoding
12	*ebony*	*e*	93D1	Morphological	
13	*nanos*	*nos*[2]*	91F7	*sim* > *sec*	3
14	*glass*	*gl*	91A3	*Rsa*I cuts *sec*	*6*
15	*prospero*	*pros*	86E5–6	*Cfo*I cuts *sim*	*1*
16	*Metallothionein A*	*Mtn*	85E9	*Dra*I cuts *sec*	Intron
17	*slowpoke*	*slo*	96A14–17	*sec* > *sim*	Intron
18	*Myosin alkali light chain 1*	*Mlc1*	98A14–15	Cuts *sec*	L49010, L49009; intron
19	*janus*	*jan*	99D3	*Dde*I cuts *sec*	*5*
20	*similar*	*sima*[5]*	99D3–7	*Hsp*92II cuts *sec*	*8*
		Fourth chromosome			
1	*eyeless*	*ey*	102C2	*sim* ≫ *sec*	Intron

[a] The order is that on the mapped chromosome (Figure 1).

[b] Candidate genes are identified by underlining.

[c] Significant markers from forward/backward stepwise regression are designated by an asterisk and their rank order (1–7).

[d] Cytological locations were obtained from FlyBase (http://flybase.bio.indiana.edu/) and are for *D. melanogaster*.

[e] Size of PCR products of *D. simulans* (*sim*) relative to *D. sechellia* (*sec*) or restriction enzyme used to digest PCR products. Five markers are morphological, not molecular, and are designated as such.

[f] Sequences of primers can be found in these references: 1, Schug *et al.* (1997); 2, http://i122server.vu-wien.ac.at/; 3, Goldstein and Clark (1995); 4, Michalakis and Veuille (1996); 5, Liu *et al.* (1996); 6, Schug *et al.* (1998); 7, Colson and Goldstein (1999); 8, Colson *et al.* (1999). Of these, numbers in italic designate primers for microsatellite sequences for which we did not find PCR product length differences. Instead, a restriction enzyme was used to resolve the two species. The designations "intron," "repeat," and "noncoding" indicate markers new to this study that are PCR products crossing an intron, incorporating a repeat, or in a noncoding sequence, respectively. GenBank accession numbers are given for sequences available from both species. Primer sequences and PCR conditions are available by request from the authors.

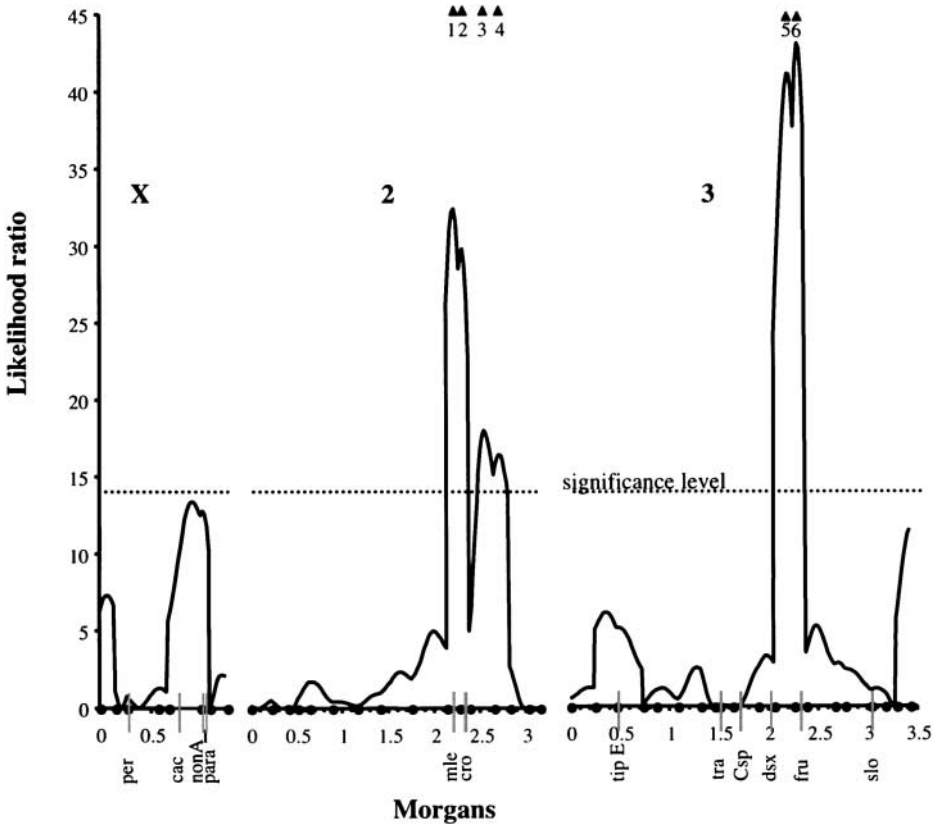

FIGURE 1.—Composite interval mapping of the difference in mean IPI between *D. simulans* and *D. sechellia* for the three major chromosomes. The positions of the markers are denoted by solid circles along the *x*-axis. The identity of each marker is given in Table 1. Solid triangles at the top of the graphs indicate the positions of each QTL and are numbered for reference to the text. The significance level at $P = 0.05$ was determined by 1000 permutations of the data set. The locations of candidate genes are indicated by shaded vertical lines along the *x*-axis. Abbreviations for candidate genes are given in Table 1.

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 likelihood ratio of 14.029.

Parameters potentially affecting the detection of QTL using CIM include both 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. We tested a range of window sizes from 2.5 to 20 cM and found that the window size did not influence the results. Forward/backward stepwise regression resulted in seven significant markers (Table 1) that could be used in CIM. Results varied slightly with the addition of markers. Figure 1 depicts the results using a backcross design, the Kosambi map function, seven background markers, and a window size of 5 cM. This analysis provides support for the presence of six QTL that affect mean IPI, four on the right arm of the second chromosome and two on the right arm of the third chromosome. The two peaks farthest right on the second chromosome (QTL 3 and QTL 4, Figure 1) were present in all analyses but were significant only when four or more markers were included in the CIM. QTL 1 and QTL 2 were present in all analyses but were much more significant (likelihood ratio of >50) in analyses using just one or two markers than in analyses with more markers. The third significant marker (Table 1) is located between QTL 3 and QTL 4 and thus has a large effect on the size of these two QTL on the second chromosome.

The two QTL on the third chromosome (QTL 5 and QTL 6) were present in all analyses and the addition of markers increased the likelihood score from ~30, with one marker, to >40, with seven markers. The addition of each marker increased the score. There were no other significant QTL except in the analysis using just one marker. In this case, there were two more QTL, one adjacent to QTL 5 and one adjacent to QTL 6. Because the second significant marker, *nanos* (Table 1), is positioned between QTL 5 and 6, adding this marker had a large effect on the resolution of QTL 5 and 6. For the X chromosome, the analysis with five markers produced a barely significant QTL at ~0.85 M. Adding in the sixth significant marker, located on the far left of the X chromosome, dropped this QTL below significance. Therefore, by using all of the significant markers in the analysis, we are giving a conservative estimate of the number of QTL.

Twelve song candidate genes have been identified in previous studies. Markers were made for five of these genes and the positions of the others have been inferred by their location relative to the markers used (Table 2). Three candidate genes fall within the QTL of this study: *mle*, *cro*, and *fru*. In a previous QTL study of the mean IPI of *D. melanogaster*, a different candidate gene, *tipE*, fell within a QTL (GLEASON *et al.* 2002). None of the six QTL from this study overlaps with the three QTL of the previous study.

All the QTL on the second chromosome had a negative additive effect whereas those on the third chromo-

TABLE 2

Song candidate genes

Gene	Abbreviation[a]	Location[b]	This study[c]	*D. melanogaster* song QTL[c]
period	*per*	Marker	No	No
cacophony	*cac*	Between *DS01391* and *nonA*	No	No
no-on-or-off-transientA	*nonA*	Marker	No	No
paralytic	*para*	Between *nonA* and f^2	No	No
maleless	*mle*	Between *cad* and *Pgi*	Yes	No
croaker	*cro*	Between *Pgi* and *Dg*α	Yes	No
temperature-induced-paralytic-E	*tipE*	Marker	No	Yes
transformer	*tra*	Marker	No	No
Cysteine string protein	*Cys*	Between *Cat* and *Gld*	No	No
doublesex	*dsx*	Between *Gld* and *e*	No	No
fruitless	*fru*	Between *nos* and *gl*	Yes	No
slowpoke	*slo*	Marker	No	No

[a] Abbreviations are also used to identify position in Figure 1.
[b] Some of the candidate genes were used as markers and are indicated as such. The flanking markers for the others are listed.
[c] The presence of each candidate gene within a QTL is noted for this study and also for a QTL analysis of *D. melanogaster* mean IPI (Gleason *et al.* 2002).

some had a positive additive effect, with respect to *D. simulans* (Table 3). In total, the QTL explain 40.66% of the phenotypic variance and range individually from 3.44 to 9.38%. The magnitude of the QTL ranged from 22.1 to 36.8% of the phenotypic difference between the parents. If a threshold of >25% of the phenotypic variance explained is used to designate a major QTL (Bradshaw *et al.* 1995, 1998), then the trait here is not influenced by major QTL.

DISCUSSION

QTL studies of interspecific differences in adaptive quantitative traits have found QTL with both large (Laurie *et al.* 1997; MacDonald and Goldstein 1999) and minor effects (*e.g.*, Fishman *et al.* 2002). Many interspecific trait differences have been shown to be polygenic (*e.g.*, Kim and Rieseberg 1999; Zeng *et al.* 2000) and it has been hypothesized that the time since species divergence is positively correlated with the number of QTL found (Kim and Rieseberg 1999). In the present study, none of the QTL for mean IPI identified has a major effect and the majority of the phenotypic variation is not explained, indicating that the study lacked sufficient resolution to detect additional small-effect QTL that also influence this trait difference. This is in contrast to the study of *D. melanogaster* in which major QTL were found for the same trait (Gleason *et al.* 2002). Overall, the difference in QTL effect between the studies follows the Kim and Rieseberg (1999) hypothesis, because the divergence between species (*D. simulans* and *D. sechellia*) is greater than that between *D. melanogaster* strains. The parental lines for the intraspecific study did not differ significantly for mean IPI, and the QTL reflect transgressive segregation (Gleason *et al.* 2002). Differences in the time scales of within *vs.* between species comparisons will confound conclusions concerning the nature of genotypic differences within

TABLE 3

QTL locations and effects

QTL	Chromosome	Position (cM)	Effect[a]	%Vp[b]	Effect[c]	% PD[d]
1	2	221.77	−0.0683	6.85	−3.35	30.7
2	2	231.25	−0.0672	6.14	−3.30	30.2
3	2	254.27	−0.0685	5.73	−3.36	30.8
4	2	270.75	−0.0486	3.44	−2.41	22.1
5	3	219.76	0.0762	9.38	4.02	36.9
6	3	229.96	0.0750	9.12	3.95	36.3

[a] Effects are given in natural-log-transformed units.
[b] Percentage of phenotypic variance explained.
[c] Effect transformed back into milliseconds (see materials and methods for details of calculation).
[d] Percentage of parental difference.

species and those contributing to speciation (Wu and Hollocher 1998). Our data are compatible with the suggestion that the large-effect QTL of the within-species study reflect recent mutations at deleterious genes that have not yet been fixed, whereas between-species differences arise over a much longer timescale and are therefore more likely to reflect numerous QTL of more minor effect (Orr 1998a).

The greater absolute trait values for between-species divergence than for within-species variation could mean that genes of similar absolute effect on the trait would appear as major-effect genes in one study and as minor-effect genes in another. In this study, effects range from 2.41 to 4.02 msec, whereas for the *D. melanogaster* study (Gleason *et al.* 2002) effects ranged from 0.56 to 0.826 msec. The nature of the genes involved is thus not conclusively different within and between species, because our minor-effect genes explain a greater absolute value of the trait than do the major-effect genes found within species.

Our interspecific QTL are different from the intraspecific QTL of *D. melanogaster*. For *D. melanogaster*, one QTL was on the left arm of the second chromosome and two were on the left arm of the third chromosome. In the present study, all QTL are on the right arms of the second and third chromosomes (Figure 1, Table 3). Thus, the QTL for intraspecific variation and intraspecific differences for mean IPI are located on different chromosome arms. This result differs from others for morphological traits (Nuzhdin and Reiwitch 2000; Kopp *et al.* 2003) in which intraspecific and interspecific traits are sometimes affected by overlapping QTL. Because the study of *D. melanogaster* QTL is only for a single pair of strains, many other QTL might be implicated in other crosses of different populations, but our results do confirm that there are many loci with the potential to influence IPI.

Comparisons of these QTL locations to other quantitative genetics studies of song loci are difficult. Pugh and Ritchie (1996), in a low-resolution study of the difference in IPI between *D. simulans* and *D. mauritiana*, found contributions of all of the chromosome arms. For the *D. pseudoobscura* and *D. persimilis* comparison (Williams *et al.* 2001), QTL were found in the inverted regions of the X chromosome (equivalent to the *D. melanogaster* X and 3L; Powell 1997) and the second chromosome (equivalent to *D. melanogaster* 3R). There is potential overlap between our QTL 5 and 6 with the *D. pseudoobscura* and *D. persimilis* X chromosome QTL; however, lack of common markers between the two studies precludes exact comparisons. In addition, the large bias in interspecies gene flow between these two species in uninverted areas of the genome (Machado and Hey 2003) makes the comparison between species groups uninformative. Yamada *et al.* (2002) found a large effect of the second chromosome on song differences between *D. anannassae* and *D. pallidosa*. Although the *D. anannassae* second chromosome is equivalent to the third chromosome of *D. melanogaster* (Powell 1997), the resolution of those loci is not sufficient to determine whether the same genes might be involved in this study.

Three candidate loci for song fall within the interspecific QTL. Two of these candidate genes, *mle* and *fru*, have alleles that affect mean IPI in *D. melanogaster* (Villella *et al.* 1997; Peixoto and Hall 1998). The third gene, *croaker*, causes polycyclic pulses (Yokokura *et al.* 1995). Presence within a QTL interval does not confirm that the candidate gene is involved in the trait because many genes underlie the QTL regions, but finding that a candidate gene underlies a QTL indicates that these genes are worth examining further for interspecific differentiation. Four of the six QTL detected do not contain candidate genes.

In the study of QTL for *D. melanogaster* mean IPI (Gleason *et al.* 2002), a different candidate gene was implicated; the gene, *tipE*, influences song amplitude and intrapulse frequency (Peixoto and Hall 1998). The other two QTL did not coincide with candidate genes. Thus, at one level, the candidate gene approach has not been successful for identifying genes affecting natural variation in mean IPI within or between species. If candidate genes influence genetic variability in natural populations, we would expect them to be equally likely to contribute to within- and between-species differences, unless variability was transient due to selection, in which case they would be more likely to be detected in interspecific analyses. Other studies have clearly demonstrated that candidate genes can influence natural variation between species (*e.g.*, Sucena and Stern 2000; Haag and True 2001), including studies of Drosophila song (Kyriacou 2002). Interestingly, the *nonA* gene has been shown to influence interspecific differences in courtship song when introgressed from *D. melanogaster* to *D. virilis* (Campesan *et al.* 2001), but natural variation at this locus within and among *D. virilis* group species does not correlate with song variation (Huttenen *et al.* 2002). Most mutants at such genes might be rapidly eliminated by strong selection.

The genetic architecture of species differences may give insights into the speciation process. We have found that a type I genetic architecture (many small-effect genes) underlies the mean IPI species difference. Combined with the bidirectional allelic effects, our data are most compatible with a history of gradual divergence without strong selection, possibly by drift (Orr 1998b; Shaw and Parsons 2002). Directional selection might be expected, given that the trait is sexually dimorphic, is under selection from female mating preferences, and contributes to sexual isolation (Ritchie *et al.* 1998). However, if preferences were stabilizing or often changed direction (under an unstable Fisherian scenario, for example), drift might still predominate in the long term. In addition, as these species have a long history of allopatry (although *D. simulans* has recently

been introduced to the Seychelles), character displacement is unlikely to have generated consistent directional selection.

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