A Quantitative Genetic Analysis of Male Sexual Traits Distinguishing the Sibling Species *Drosophila simulans* and *D. sechellia*

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Manuscript received March 15, 1999
Accepted for publication July 21, 1999

ABSTRACT

A quantitative trait locus (QTL) genetic analysis of morphological and reproductive traits distinguishing the sibling species *Drosophila simulans* and *D. sechellia* was carried out in a backcross design, using 38 markers with an average spacing of 8.4 cM. The direction of QTL effects for the size of the posterior lobe was consistent across the identified QTL, indicating directional selection for this trait. Directional selection also appears to have acted on testis length, indicating that sexual selection may have influenced many reproductive traits, although other forms of directional selection cannot be ruled out. Sex comb tooth number exhibited high levels of variation both within and among isofemale lines and showed no evidence for directional selection and, therefore, may not have been involved in the early speciation process. A database search for genes associated with significant QTL revealed a set of candidate loci for posterior lobe shape and size, sex comb tooth number, testis length, tibia length, and hybrid male fertility. In particular, *decapentaplegic* (*dpp*), a gene known to influence the genital arch, was found to be associated with the largest LOD peak for posterior lobe shape and size.

THERE have been various published studies, using primarily model organisms, that examine interspecific differences for single morphological or hybrid incompatibility traits. Few have attempted the genetic dissection of several characters together. Here we describe one of the first studies to investigate a highly integrated set of sexually selected morphological traits and measures of hybrid sterility in two closely related sibling species of Drosophila. This design allows us to simultaneously investigate the genetic complexity underlying both prezygotic and postzygotic isolating mechanisms, and by examining the genetic architecture of the characters we can ask questions about evolutionary change during speciation.

Detailed examination of the genetic factors underlying quantitative traits, referred to as quantitative trait loci (QTL), has become feasible through the development of robust statistical methods for associating markers and phenotypes. In combination with the availability of high-density genome-wide molecular maps, methods such as interval mapping (Lander and Botstein 1989) and composite interval mapping (Zeng 1994) have greatly increased the resolution for the detection and localization of QTL compared with that of early linkage methods based on associations between single markers and traits.

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Although relatively few genetic studies of interspecific differences in traits exhibiting continuous variation have been carried out, the consensus view is that most have a polygenic basis (Coyne and Orr 1998). Liu *et al.* (1996) used composite interval mapping and a genetic map of 18 markers to elucidate the genetic basis of morphological shape variation of a male limited cuticular genital structure in *Drosophila simulans* and *D. mauritiana*. Eight of the 15 intervals showed evidence of QTL acting in a largely additive manner.

The study reported here uses two species from the *simulans* clade of the *melanogaster* complex of Drosophila (*D. melanogaster* is an outgroup to the clade consisting of *D. simulans*, *D. mauritiana*, and *D. sechellia*). The most reliable character distinguishing the species is the shape of the posterior lobe of the male genital arch (Ashburner 1989). Both *melanogaster* and *simulans* are cosmopolitan in distribution, while *mauritiana* is endemic to Mauritius and *sechellia* is endemic to the Seychelles archipelago (Lachaise *et al.* 1988). Of the four species, *sechellia* is the only specialist, showing a range of adaptations related to its exploitation of *Morinda citrifolia* as a host plant (Louis and David 1986).

The species of the *simulans* clade are homosequential and differ from *melanogaster* by a large paracentric inversion on the right arm of chromosome 3 and several short rearrangements (Lemeunier and Ashburner 1976). In terms of nucleotide sequence the three species are very similar, having an ambiguous phylogenetic branching pattern (Coyne and Kreitman 1986; Cariou *et al.* 1990). Hey and Kliman (1993) estimate that the ances-

tor of the *simulans* clade diverged from *melanogaster* ~2.5–3.4 mya, with *mauritiana* and *sechellia* diverging from *simulans* ~0.6–0.9 mya (see also Bodmer and Ashburner 1984; Harr *et al.* 1998). The clade is amenable to genetic analysis as all three interspecific crosses (in at least one direction) give fertile female hybrids (Lachaise *et al.* 1986). Among those traits that have been genetically analyzed are the male external genitalia (Coyne 1983; Coyne and Kreitman 1986; Liu *et al.* 1996; True *et al.* 1997), the male sex comb (Coyne 1985; True *et al.* 1997), testis length (Joly *et al.* 1997), and female ovariole number (Coyne *et al.* 1991). The genetic basis of hybrid incompatibility has also been examined by numerous authors in recent years (Zeng and Singh 1993a; Hollocher and Wu 1996; True *et al.* 1996; reviewed by Wu and Palopoli 1994).

Traits under sexual selection, such as male ornamentation, are thought to be important in the divergence of species (Wu *et al.* 1996; Coyne and Orr 1998). Recently, however, Civetta and Singh (1998a) suggested a major role for all sexual traits, not just those traditionally believed to be sexually selected, in establishing barriers to reproduction. They found higher between-species divergence for sexual over nonsexual traits among species of the *melanogaster* complex. A similar pattern of high between-species variation was also found for male reproductive tract proteins (Civetta and Singh 1995). These findings are supported by the observation of high rates of evolution in genes involved in male reproductive functions (Civetta and Singh 1998b; Nurminsky *et al.* 1998).

The traits chosen for analysis here reflect the significance of reproductive characters in speciation: shape and size of the posterior lobe of the male genital arch, the male sex comb, and size of the testis and cysts (sperm bundles). To investigate postzygotic isolating mechanisms, we also took several measures of hybrid sterility.

Posterior lobe of the genital arch: Eberhard (1985) has suggested that divergent male genital structures, such as the posterior lobe, may evolve rapidly via sexual selection mediated by female choice. However, the lobe may also have a functional role in determining asymmetrical mating propensities among the *melanogaster* complex of species. Robertson (1988) has suggested that partial mechanical isolation is caused by the shape of the posterior process. He showed that during mating the arch is inserted under the base of the ninth abdominal segment of the female (see Figure 3 of Robertson 1988) and that the size of these insertion points is positively correlated across species with arch size (see Figure 2 of Robertson 1988). This may help explain why *sechellia* males, having a narrow process, readily mate with *simulans* females, which have a large insertion point, while the reciprocal cross is difficult. Such asymmetrical reproductive isolation is also thought to be influenced by the cuticular hydrocarbon profile (Coyne *et al.* 1994).

Sex comb tooth number: Both Cook (1977) and Coyne (1985) found that males lacking the foreleg tarsal segment holding the sex comb have great difficulty grasping female genitalia, substantially reducing male mating ability. Further support for their role as precision grasping organs is provided by the presence of small cuticular projections at the base of each tooth (see plate B of Coyne 1985), which may provide mechanosensory information to the male, and by a row of bristles near the tip of the female ovipositor (see plate C of Coyne 1985) that may anchor the male sex comb bristles during copulation.

Sperm and testis size: Significant variation in sperm length, evaluated by measuring sperm bundle, or cyst length has been found among members of the *melanogaster* complex (Joly 1987). A positive correlation between sperm length and female seminal receptacle length has been reported in species of the *nannoptera* group of drosophilids, indicating that sperm size may be subject to sexual selection (Pitnick and Markow 1994). It is unlikely that sperm could develop correctly unless the testes are sufficiently large, and Joly and Bressac (1994) found a strong correlation between testis and sperm lengths in numerous Drosophila species, with sperm length frequently being less than that of the testis. Nevertheless, Joly *et al.* (1997) report that the two traits may be influenced by different sets of genes.

Hybrid male sterility: Coyne and Orr (1989) used data on 119 pairs of closely related Drosophila species to show that recently diverged taxa tend to produce inviable or sterile male hybrids, while female hybrids are more often viable and fertile. This pattern conforms to Haldane's rule, which states that in the hybrid generation, when only one sex is sterile or inviable, that sex is the heterogametic one (Haldane 1922). Haldane's rule is a general pattern with few exceptions, the causes of which have been debated for the last 20 years (Laurie 1997). By studying the genetic basis of postzygotic isolation in a species pair obeying Haldane's rule, we hope to help elucidate the evolutionary mechanisms responsible for this widespread phenomenon.

Our goal is thus to use a correlated set of traits distinguishing *D. sechellia* and *D. simulans* to examine the genetic changes occurring early in speciation via the formation of both pre- and postzygotic barriers to reproduction. The use of an array of characters will enable us to detect phenotypic correlations among traits and allow us to assess pleiotropy, the degree to which different traits are influenced by overlapping sets of genetic factors. We can ask whether traits differ in their level of genetic complexity, possibly indicating different tempos or modes of evolution during speciation, and with subsequent study we can compare the genetic architecture of reproductive and nonreproductive traits. The work is facilitated by using a dense genetic map and high-resolution composite interval mapping. This study is the first in a series examining the nature of genetic

variation between species in the *melanogaster* complex and its relationship to intraspecific genetic variation.

MATERIALS AND METHODS

Drosophila stocks: Two isofemale lines were used in the backcross mapping experiment, sim 132 and sec S-9. Both have been maintained in the laboratory for several years. To look for within-line homozygosity, we genotyped several individuals of many lines at molecular marker loci. To achieve maximal QTL resolving power, we then examined pairs of homozygous lines for differences at numerous marker loci between lines and for high between-line trait variation.

Crosses for QTL interval mapping analysis: Females from the *simulans* isofemale line sim 132 were crossed to males of the *sechellia* isofemale line sec S-9, and the resultant fertile F_1 females were backcrossed to males of each parental line. All crosses were carried out at 25 ± 1° on standard maize yeast agar medium.

The following crosses were set up with 10 female and 10 male parents in each bottle: five bottles each of *simulans* × *simulans*, *sechellia* × *sechellia*, and *simulans* females × *sechellia* males, from each of which 5 male progeny were collected; and eight bottles each of F_1 females × *simulans* males and F_1 females × *sechellia* males, from each of which 25 male progeny were collected. This gives 25 males from both parental strains and the F_1, and 200 from each backcross.

Morphological traits: Bottles were cleared and male flies were harvested 7 hr later, remaining virgin because young females are completely unreceptive to male courtship (Manning 1967). The male flies were then aged for 4 days in groups of between 5 and 10 individuals. For each male the following traits were examined: foreleg sex comb tooth number, shape and size of the posterior lobe of the genital arch, testis length, and cyst length. Foreleg tibia length was ascertained to provide a measure of body size, as leg segment length is significantly correlated with body mass in male *melanogaster* (Catchpole 1994). Flies were also scored for quantity of sperm, sperm motility, testicular atrophy, and the presence or absence of cysts.

Each male was etherized and dissected in Ringer solution. The seminal vesicles, which hold mature sperm (Miller 1950; Lindsley and Tokuyasu 1980), were removed and squashed in a droplet of Ringer solution, and the sperm were then examined under a phase-contrast microscope. The dissected testes were transferred to a droplet of Ringer solution on a fresh slide. One testis was left intact for subsequent length measurement; the other was dissected to liberate cysts following the method of Joly (1987). These slides were left to dry, fixed in 95% ethanol for 5 min, allowed to dry, stained in 1:1 2% aceto-orcein solution:distilled water, and mounted in DPX (Merck, St. Louis). The genital arch and both forelegs were dissected from each male and mounted in Hoyer's medium, with a coverslip serving to flatten the arch. These slides were incubated at 68° overnight.

Phenotypic data acquisition: Sperm quantity was categorized as none, reduced, or full amount for each fly, and a fly was scored as "motile" if even a single sperm was seen to move. Withered, degenerate testes corresponding to "type 1 testes" of Zeng and Singh (1993b, see Figure 2) were scored as atrophied, and the presence or absence of cysts in the single dissected testis was noted.

A video camera attached to a compound microscope was used to capture all testis, cyst, tibia, and genital arch images. Subsequent measurements were carried out using the image analysis software SigmaScan Pro 4.0 (Jandel Scientific, Inc., San Rafael, CA). Each testis was measured twice along the median line from the apical end to the junction with the seminal vesicle. For each fly all mature cysts were measured. These can be recognized by a slightly distended waste-bag at one end and a brush of emerging sperm heads at the other. Due to the difficulty of visualizing individual sperm and to avoid measuring broken sperm, we chose to measure cysts rather than individual sperm.

Where possible, both left and right tibia and both sides of the genital arch were measured. All arches were oriented with artificial horizontal baselines, coinciding with the relatively flat region between the posterior lobe and the lateral plate. The thus-enclosed outline was then digitized, providing 400–700 Cartesian coordinate pairs per arch. Outlines from the left side of the fly were reflected in the vertical axis to produce outlines of the same handedness, and each was placed in a standard configuration by translating the origin of the coordinate system to the centroid of the outline.

An elliptic Fourier series was used to represent arch shape in the absence of reliable landmarks (Ferson *et al.* 1985; Liu *et al.* 1996). Elliptic Fourier functions use a parametric representation of the *x*- and *y*-projections of the outline, treating each independently as a function of contour length. This circumvents most of the constraints of conventional nonparametric Fourier descriptors, such as the requirement for equally spaced data points, allowing more complex shapes to be analyzed (reviewed by Lestrel 1997). Fourier coefficients were calculated using the public domain program Elliptic Fourier Analysis (Rohlf and Ferson 1992), which implements the algorithms developed by Kuhl and Giardina (1982).

The coefficients were normalized to remove any influence of outline starting position and rotation, leading to representations based only on internal shape properties of the outlines (Kuhl and Giardina 1982). A size-adjusted data set was constructed by dividing each coordinate by the square root of the arch area prior to performing the elliptic Fourier analysis (see Liu *et al.* 1996).

This procedure allows recreation of the original outline with arbitrary accuracy, depending on the number of harmonics. Here 25 harmonics were used, giving high precision in outline reconstruction (see Figure 2 of Liu *et al.* 1996). To encapsulate the between-arch variation in fewer variables, the 100 coefficients for each arch (4 coefficients for each of 25 harmonics) were used in a principal components analysis. This was performed on covariance matrices using pooled data from both parental lines, the F_1, and both backcrosses, with one randomly selected arch used per fly.

The principal components analyses, as well as all other statistics presented, unless otherwise stated, were performed using modules of the Statistica 6.0 package (StatSoft, Inc., Tulsa, OK).

Within-species morphological variation: To examine variation of the morphological traits within the two species, individuals of a further nine isofemale lines of *simulans* and *sechellia* were dissected as described in the previous sections. The fertility traits were not scored in these extra lines.

Molecular markers: Genetic databases of Drosophila genome sequences were searched for various microsatellite repeat sequences, and primers were designed for 107 loci. Each was tested for a length difference between the parental inbred lines, and 37 out of 71 polymorphic markers were selected. To fill the interval between 65D1-D3 and 73A1-B7 on chromosome 3, we used a single base pair difference between the parental lines at the *DROLAMB2A* locus (Colson and Goldstein 1999). This difference was used to create a pair of forward primers differing only by the 3′-terminal base. Conditions were found such that in combination with a common reverse primer, each amplified the allele of only one line. Testing each DNA extract with both primer pairs allowed homo- and heterozygotes to be distinguished in both back-

TABLE 1

Molecular markers

Marker[a]	Cytological location	Genetic location[b]	Gel condition[c]
AF047180	1B1-B14	1-0.0	I
DMU56661	4F1-F2	1-9.0	I
G00630	8B5-B8	1-17.7	I
DROSEV	10A1-A2	1-24.4	I
DMTENA	11A6-A9	1-28.4	I
DMU18774	13D	1-33.9	II
DMTROPONI	16F3-F6	1-41.4	I
DROPASSOV	19E3	1-50.7	II
DROEXPAND	21C4	2-0.0	I
DROYANETSB	22D1-D2	2-4.1	I
AC005732	24D1-D2	2-9.5	II
DROGPDHA	26A1	2-21.6	I
AC005555	29A5-B4	2-31.4	I
G410	33E9-E10	2-44.5	I
DRODORSAL	36C	2-54.3	I
AC004759	38E1-E9	2-64.0	I
DROGPAD	47A	2-72.8	I
DMMP20	49F9-F13	2-80.8	I
AC004248	52D2-D15	2-89.3	I
DS00361	54B1-B2	2-93.1	I
DSO8687a	57C5-D1	2-102.4	I
AC004365	58A4-B1	2-107.6	I
DS08011	59A1-B2	2-111.9	I
DMRHOb	62A1-A3	3-0	I
AC004658	63D2-E1	3-6.5	I
DRODSRC	64B	3-16.5	I
DMU14395	65D1-D3	3-28.3	I
DROLAMB2A[d]	67C	3-37.9	II
DM22F11T	73A1-B7	3-55.2	II
DMCATHPO	75D4	3-60.4	I
AE001573	84	3-69.4	I
DRONANOS	91E-F	3-77.8	I
DMEHAB	90B1-B2	3-84.4	I
AC006414	89A1-A5	3-94.7	I
DMTRXIII	88B3	3-100.3	I
DROPROSA	86E3	3-107.1	I
DMTF125	95C6-C8	3-119.7	I
DMU43090	99D6-D9	3-132.8	I

[a] For accession numbers and primer sequences see Colson *et al.* (1999).

[b] Genetic map positions inferred from the progeny of *simulans-sechellia* F_1 hybrid females.

[c] I, 4.25% acrylamide gel; II, 2% agarose gel.

[d] sim 132-positive forward primer, CTG GGA ATC TAT CAA TTA; sec S-9-positive forward primer, CTG GGA ATC TAT CAA TTG; common reverse primer, AAT TTG CAG TTG ATA GGC AGC.

crosses. As the possibility of false negatives is high with such a marker, each DNA extract was tested twice with each primer. This gives a total of 38 markers (Table 1).

Genotyping: PCR reactions were carried out in 11 μl 2.5 mm $MgCl_2$ prealiquoted reaction mixture (Advanced Biotechnologies, Ltd., Surrey, U.K.), with 0.5 μl primers (7 pmol/μl), and 1 μl template DNA from a single fly; the temperature program was as follows: 94° for 4 min; 40 cycles of 94° for 1 min, 55° for 1 min, 72° for 1 min; 72° for 8 min (the annealing temperature was reduced to 54° for the sec S-9-positive version of *DROLAMB2A* and to 51° for the sim 132-positive version). Fragments were run on either 2% agarose or 4.25% acrylamide using an ABI Prism 377 DNA sequencer (Perkin-Elmer, Norwalk, CT), depending on allele size differences. For those markers typed on acrylamide, fluorescently labeled forward primers were used in the PCR.

Genetic marker map: For each locus from each backcross the segregation ratio was tested for deviation from the expected 1:1 using a chi-square test. Where two adjacent markers both showed significant deviation, most likely due to viability differences, a corrected recombination fraction was calculated (see Bailey 1961, p. 53). For the *simulans* backcross, seven intervals on the X chromosome, eight intervals on chromosome 2, and six intervals on chromosome 3 showed deviation; for the *sechellia* backcross, no intervals on the X chromosome, two intervals on chromosome 2, and four intervals on chromosome 3 showed such deviation.

The Kosambi (1944) mapping function, which assumes moderate interference, was used to convert the recombination fractions to map distance. The map distances (in centimorgans) for each interval were averaged over both backcrosses; that is, they were inferred from the progeny of 400 F_1 hybrid females and are shown in Table 1. The average spacing of the 38 markers is 8.4 cM, with a maximum interval of 17.4 cM. This hybrid map shows slightly reduced recombination distances compared with the *simulans-mauritiana* genetic map (see Figure 3 of Liu *et al.* 1996).

QTL mapping: All analyses were performed using the QTL Cartographer suite of programs (Basten *et al.* 1994, 1997). For each trait the backcrosses were analyzed separately, and two forms of composite interval mapping were employed. Composite interval mapping, model I, uses all markers outside the interval under test to control for background genetic variation, while semicomposite interval mapping, model II, uses all unlinked markers (described fully in Zeng 1994).

Model II has the higher statistical power for detecting QTL-marker linkage, with the power of model I reduced due to fitting closely linked markers (Zeng 1993). However, only model I provides a test in which the interval is unaffected by QTL located outside the test interval and its two adjacent intervals, effectively eliminating most of the variation due to segregating QTL, giving unbiased estimates of QTL position and effect (Zeng 1994). Using both models we achieve a high probability of QTL detection as well as good estimates of position and effect for the stronger QTL.

Following convention the likelihood ratio test statistics were converted to LOD scores (Lander and Botstein 1989). To achieve a genome-wide type I error rate of 5%, the experimentwise critical values were calculated using permutation tests (Churchill and Doerge 1994), in which the phenotypes are shuffled relative to the genotypes, and the analyses are redone. For each trait per backcross 1000 permutations were carried out for each test.

Maximum-likelihood interval mapping assumes that the data analyzed are normally distributed, which was not the case for the categorical fertility data. However, we have assumed that the traits are likely to be threshold traits influenced by an underlying normal distribution. In this case interval mapping is appropriate, though it may lead to reduced power of QTL detection (see Xu and Atchley 1996).

Comparison of QTL locations in different species pairs: To quantitatively evaluate the similarity of the locations of identified QTL for posterior lobe area in the two species pairs, *simulans-sechellia* (data from our study) and *simulans-mauritiana* (data from True *et al.* 1997), we performed two resampling tests. First, we took the four highest QTL peaks from the *simulans-mauritiana* experiment and summed the LOD scores for these locations on a *simulans-sechellia* LOD profile. The *simulans-mauritiana* map locations were transposed to the *simulans-sechellia* map using the ratio of the total length of each

TABLE 2

Variation among lines in mean trait values

Line	Provided by	Testis length (mm)	Cyst length (mm)[a]	Tibia length (mm)	Sex comb tooth number	Posterior lobe area ($\times 10^{-3}$ mm^2)	adjPC1[b]
D. simulans							
simiso 1	A. G. Clark	1.22	1.06	0.461	9.60	12.17	−1.09
simiso 4	A. G. Clark	1.39	1.05	0.484	10.89	14.31	−0.85
simiso 21	A. G. Clark	1.18	1.09	0.465	9.40	11.48	−0.91
sim 130	DSCU	1.29	1.04	0.473	10.15	12.78	−1.06
sim 131	DSCU	1.29	1.10	0.461	10.45	12.48	−0.66
sim 132[c]	DSCU	1.20	0.99	0.425	10.38	12.80	−1.22
sim 134	DSCU	1.33	1.07	0.471	10.20	12.91	−0.87
sim 135	DSCU	1.23	1.09	0.449	10.65	9.78	−1.01
sim 145	DSCU	1.19	1.12	0.475	11.85	11.59	−0.96
SI-14	E. Nevo	1.23	1.07	0.474	10.10	12.82	−1.03
Species mean		1.26	1.07	0.464	10.37	12.31	−0.97
Among line SD		0.07	0.04	0.017	0.69	1.19	0.15
D. sechellia							
sec 1	NDSRC	1.72	1.40	0.492	12.58	5.33	0.43
sec 7	NDSRC	1.76	1.67	0.467	10.78	5.64	0.61
sec 8	NDSRC	1.73	1.65	0.455	11.22	4.65	1.13
sec 12	NDSRC	1.95	1.49	0.483	11.56	5.35	1.33
sec 15	NDSRC	1.92	1.11	0.489	12.80	7.03	1.03
sec 19	NDSRC	1.71	1.60	0.496	11.83	6.54	1.23
sec 3588	NDSRC	1.65	1.62	0.486	10.40	4.91	0.89
sec S-9[c]	J. Roote	1.89	1.52	0.494	12.44	6.40	1.28
sec S-32	J. Roote	1.98	1.66	0.471	11.10	5.47	0.74
JDsec	J. David	1.56	1.42	0.481	12.14	5.70	0.77
Species mean		1.79	1.51	0.481	11.69	5.70	0.94
Among line SD		0.14	0.17	0.013	0.81	0.75	0.30

For each line, 10 individuals were examined, except for sec 1 and JDsec, where 6 and 7 flies were used, respectively, and sim 132 and sec S-9 where 25 flies were used; in some cases loss of trait information occurred due to damage during dissection. DSCU, Drosophila Stock Centre, University of Umeå, Umeå, Sweden; NDSRC, The National Drosophila Species Resource Center, Bowling Green State University, Bowling Green, OH.

[a] Two cysts were measured for each individual.

[b] From a principal components analysis using pooled data from these 20 lines.

[c] These two lines were used in the QTL mapping experiment.

chromosome in each cross. The summed LOD was compared with that obtained by placing four points at random on the *simulans-sechellia* profile in 10,000 simulations.

Similarly, to focus only on location, we measured the absolute distance between the four highest *simulans-mauritiana* QTL and the nearest significant peak on the *simulans-sechellia* profile.

RESULTS

Variation within and between species: The trait means for each of the 20 lines examined, including those for the 2 lines used for creating the backcross individuals, are shown in Table 2. For all traits, aside from tibia length, variation between species is considerable (*t*-test; $P < 0.001$) compared with intraspecific variation. No significant difference was found between *simulans* and *sechellia* tibia length means, although the *sechellia* lines tended toward slightly longer tibia.

The lines chosen for creating the backcross flies, sim 132 and sec S-9, are generally representative of the species. However, as tibia length variation within each species is large relative to that between species, interval mapping of this trait will identify QTL involved in both inter- and intraspecific variation, some of which may be deleterious alleles affecting a number of traits.

Morphological measurements: For the bilateral traits, arch area, tibia length, and sex comb tooth number, an average was taken of the left- and right-side measurements wherever possible and was used in all subsequent analyses. Left-right correlations in the *simulans* and *sechellia* backcross populations, respectively, were 0.86 and 0.94 for arch area, 0.83 and 0.88 for tibia length, and 0.11 and 0.37 for comb tooth number. In each case the correlation is greater in the *sechellia* backcross, and the difference is significant for area and tooth number. This implies that introduction of *simulans* genes into a *sechellia* background perturbs bilateral symmetry less than the reciprocal exchange.

The low correlation between left and right combs, coupled with the high within-line variation for this trait, suggest a large environmental component contributing to the variance of this character.

TABLE 3

Trait differences between parental lines

Trait	*sechellia* mean minus *simulans* mean	Line difference in SD units
Testis length[a]	0.69	5.0
Cyst length[a]	0.53	4.5
Tibia length[a]	0.06	5.7
Sex comb tooth number	2.06	3.1
Posterior lobe area[b]	−6.40	10.4
PC1	2.70	12.1
adjPC1	2.33	7.5

[a] Values in millimeters.
[b] Values in units $\times 10^{-3}$ mm^2.

For both backcrosses, left and right correlations of the first principal component (PC1) and the first principal component obtained from the size-adjusted data (adj PC1) were $r > 0.60$. To reduce the complexity of the subsequent QTL analysis, one randomly selected arch was chosen from each fly to enter into a principal components analysis. Each fly is thus represented by one value for each PC axis.

All clearly identifiable mature cysts (varying between 1 and 15 with mean ≈ 3.5) were measured for each fly and an average was taken.

Differences between parental strains: Table 3 shows the differences between parental lines for each of the morphological traits included in the QTL analysis. For all seven the difference between *simulans* and *sechellia* was highly significant in *t*-tests ($P < 0.00001$). When size, measured by tibia length, was treated as a covariate, highly significant differences were still found for all traits except sex comb tooth number ($P = 0.09$), suggesting that some of the within-line variation in tooth number is due to body size.

The variances of *simulans* and *sechellia* were homogeneous for tibia length and comb teeth (*F*-test; $P > 0.05$). For posterior lobe area the variance within *simulans* is greater than that within *sechellia*, while for both testis and cyst lengths the reverse is true. Applying a log transform to the data removes the dependency of the variance on the mean. *F*-tests using this transformed data showed that the variances of *simulans* and *sechellia* are not significantly different for comb tooth number, testis length, or cyst length, while for both tibia length and arch area the variance of *simulans* is greater than the variance of *sechellia*. The difference in variance for tibia length is eliminated by removing a single, particularly low datum from the *simulans* observations. For posterior lobe area, as measurement error is low, the difference in variance may imply that lines with larger arches have greater inherent variability, possibly indicating increased developmental instability when a large morphological structure is produced.

To highlight the line differences, the sums of squares from the two lines were pooled and used to compute the "environmental standard deviation" (True *et al.* 1997). The line differences are given as the number of these standard deviations (Table 3). All of the traits differ by at least three SD, with the largest differences being in the genital arch characters.

Descriptive results for the backcrosses: As expected, the backcross classes display higher levels of variation than the parentals and hybrids for all traits (Figure 1). No trait shows overdominance because the F_1 mean is always between those of the parental lines.

For cyst length (Figure 1E), both backcross means are lower than expected based on additivity, because cyst length altered with fly fertility in the backcrosses. No such pattern could be observed in the F_1 because all males possessed normal cysts, but only 2 out of 25 had very few, immotile sperm. In contrast, backcross flies frequently either lacked cysts or showed very small and malformed cysts. We found that mean cyst length increased in both backcrosses with sperm quantity and was also higher for those males having motile sperm. Thus, to some extent cyst length may be used as an indicator of male fertility. As testis length is not depressed in the backcrosses to the same degree as cyst length, these two characters may be under separate genetic control.

A plot of the first two size-adjusted principal components represents posterior lobe shape only (Figure 2) and shows that the five genotypic classes separate well. The adjPC1 axis, which accounts for 61.5% of the shape variation, mainly distinguishes *simulans* and the *simulans* backcross; adjPC2, accounting for just 15.2% of the variation, appears to separate *sechellia* and the *sechellia* backcross. A similar pattern was observed for the plot of PC1 against PC2.

Figure 2 indicates a partial dominance of the *sechellia* genome for posterior lobe shape as the hybrids cluster with the *sechellia* and *sechellia* backcross populations. More formally, one can examine dominance by assessing the difference between observed F_1 and backcross population means for a trait and their expected values under additivity based on parental line means. For both PC1 and adjPC1, the F_1 and backcross means are significantly larger (more *sechellia*-like) than expected, confirming the pattern of Figure 2. In contrast, for posterior lobe area, the F_1 and backcross means are significantly more like *simulans*. This implies that a distinction between size and shape of the posterior lobe may be possible in our study.

Sex comb tooth number showed no deviation from additive gene action, and tibia length exhibited dominance of *sechellia* alleles, while for both testis and cyst lengths there was no significant difference between the observed F_1 means and their expected values. However, for testis length the *simulans* backcross showed dominance of *sechellia* alleles, and the *sechellia* backcross

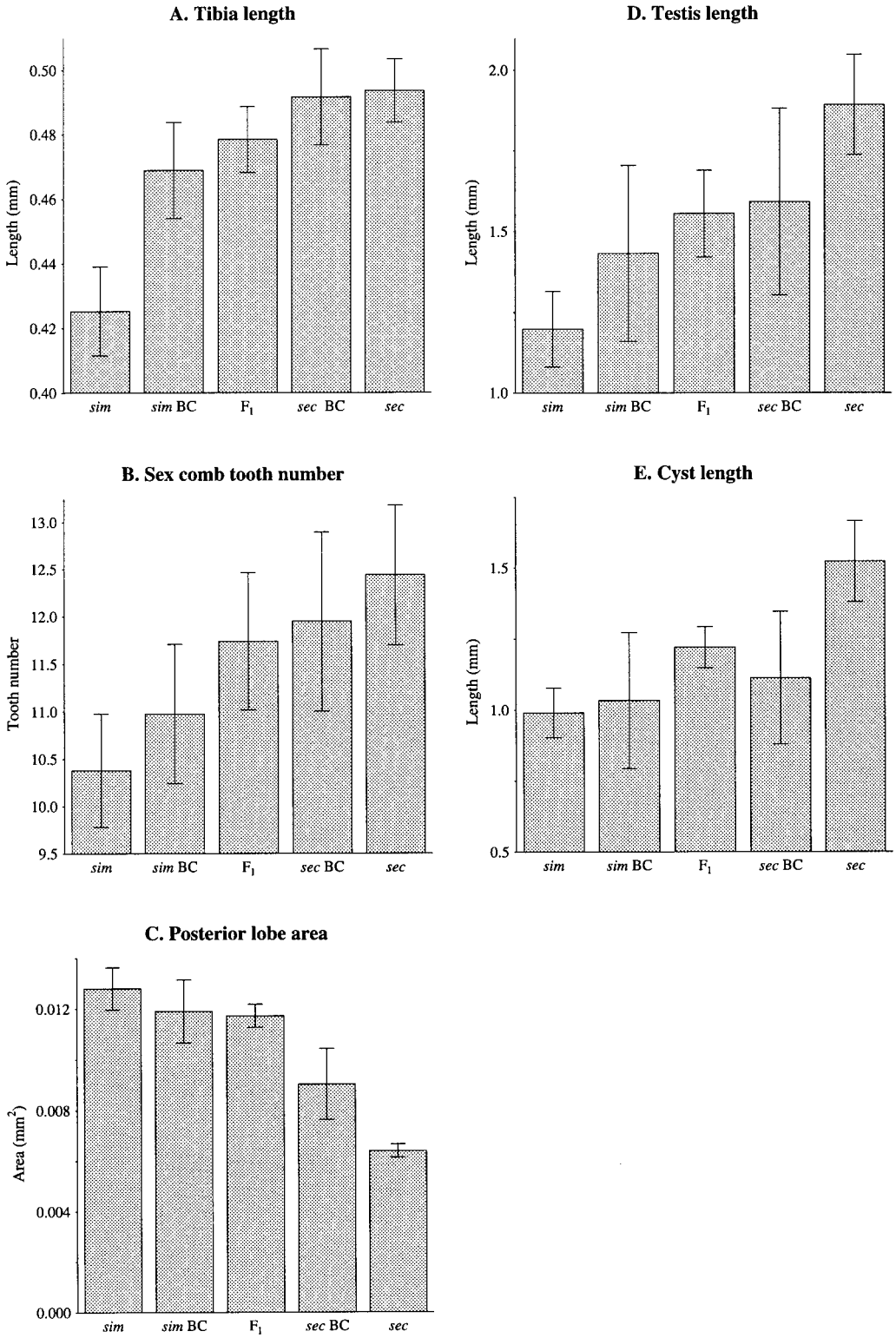

Figure 1.—Graphical representation of the morphological data arranged by genotypic class. Each bar shows the mean ± SD of the measurements, from 25 flies for the *simulans*, *sechellia*, and F_1 populations and from 200 flies for each backcross population. Due to absence of cysts in some flies, $n = 151$ and $n = 160$ for the *simulans* and *sechellia* backcrosses, respectively, in E.

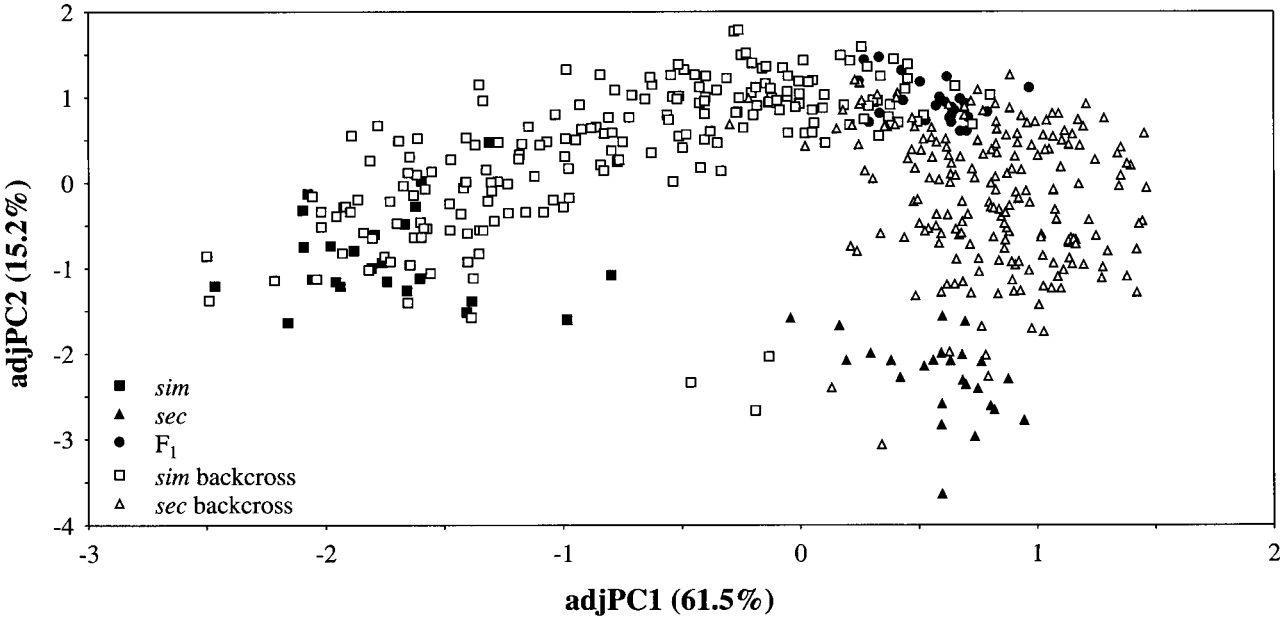

Figure 2.—Plot of the first two principal components for the elliptic Fourier coefficients derived from the size-adjusted data set of posterior lobe outlines. Each point represents a single arch chosen at random from each fly. A total of 25 individuals from the *simulans*, *sechellia*, and F_1 and 200 individuals from each backcross are presented. The percentage of shape variation explained by each principal component is shown in parentheses.

showed dominance of *simulans* alleles. Both backcross cyst length means were lower than expected but, as noted, the means are reduced due to low-fertility flies having abnormally short cysts.

Meaning of the principal component axes: By plotting the outlines of arches having equal values of adjPC1, we attempted to determine by eye the effect of changing adjPC2 on arch shape and vice versa. The size-adjusted data set was used to prevent confounding size and shape variation. It was not possible to distinguish any specific changes associated with only one principal component axis. All the features of the posterior lobe that show differences between the parental shapes, such as height, width, angle of rotation, and specific head shape, appear to change in a correlated fashion along both axes.

Fertility measurements: Both parental lines were fully fertile, while all F_1 males lacked motile sperm. A summary of the fertility measures for the backcrosses is presented in Table 4. Infertility was caused by a hierarchical set of traits: flies scored as having atrophied testes had neither cysts nor sperm, while flies having no cysts had no sperm. Therefore, an absence of sperm may be due to malformed testes, any of various problems occurring during spermatogenesis, or a failure to initialize sperm from sperm bundles.

All backcross flies that have a full, parental amount of sperm also show motile sperm, with the proportion of such individuals being greater for the *simulans* backcross (36% fully fertile) than for the *sechellia* backcross (11.5% fully fertile). Overall, males of the *sechellia* backcross appear far more likely to be infertile (as indicated by sperm quality), implying that the introduction of *simulans* genes into a *sechellia* genetic background has more deleterious consequences for the individual than the reverse.

Taking the mean value of morphological traits from those backcross flies with and without atrophied testes suggests atrophy may be linked to a more general lack of fitness. Within both backcrosses, comb tooth number, lobe area, and testis and tibia lengths are reduced in individuals exhibiting atrophy (data not shown).

TABLE 4

Summary of fertility measurements

Fertility measure	*simulans* backcross	*sechellia* backcross
Atrophied testes	11.0	3.0
Cysts absent[a]	13.5 (24.5)	17.0 (20.0)
Sperm absent[b]	29.0 (53.5)	59.0 (79.0)
Reduced quantity of sperm		
Motile	1.5	7.5
Immotile	9.0	2.0
Full quantity of sperm		
Motile	36.0	11.5
Immotile	0.0	0.0

The values represent the percentage of individuals exhibiting the character state in each backcross ($n = 200$ for each).

[a] The values in parentheses include individuals lacking cysts due to testicular atrophy.

[b] The values in parentheses include individuals lacking sperm due to both testicular atrophy and absence of cysts.

Phenotypic correlations: Pearson correlation coefficients among all morphological traits in the two backcross populations were calculated (data not shown). PC1 and adjPC1 are highly correlated ($r = 0.95$ and $r = 0.85$ for the *simulans* and *sechellia* backcrosses, respectively), while their correlations with posterior lobe area are slight ($-0.18 < r < -0.09$), again suggesting that we may be able to separate posterior lobe size and shape variation. Posterior lobe area is correlated with tibia length only in the *sechellia* backcross and, given that in both parental lines tibia length and lobe area are significantly correlated ($r = 0.81$ and $r = 0.44$, in *simulans* and *sechellia*, respectively), it is unlikely that general body size variation is an important element contributing to posterior lobe size variation.

A notable significant positive correlation in both backcross populations is that between comb tooth number and lobe area. In the parental lines and hybrids, although they are not significant, the correlations between these traits are negative ($r = -0.02$, $r = -0.27$, and $r = -0.23$ in *simulans*, *sechellia*, and the F_1, respectively). The change in sign in the backcrosses suggests that the traits may be genetically correlated.

QTL mapping: The LOD profiles for selected morphological traits are shown in Figures 3–6. Plots are presented for each backcross and for each of the two models used. For all plots, significant peaks or significant regions (within which the profile does not dip below critical value) are indicated (see legend to Figure 3). The two mapping models are generally consistent, with model II having higher LOD scores and identifying intervals missed by model I in many cases. A further model, model VI, was also tested for several of the traits. This model fits background markers found to be significant by stepwise regression and is recommended by the authors of QTL Cartographer (Basten *et al.* 1994, 1997). Using this method did not alter the QTL detected, and the results are not reported in detail.

Table 5 summarizes information on the positions and effects of QTL for the morphological traits. QTL supported by model I are significant for model II also, and the results are presented for model I because these are unbiased. For those wide regions of above-threshold LOD score in model II, information for the highest peak, or that supported by the more stringent model I, is given. It may be that all intervals in these regions harbor QTL, but because the test statistic for adjacent intervals is interdependent (Zeng 1994), two adjacent, significant intervals do not show evidence for more than one QTL.

Table 5 also gives a support interval for the QTL. Either these are two-LOD drop-offs, which have been shown by simulation to provide a good estimate of the 95% confidence interval for QTL position (van Ooijen 1992), or they are derived by bootstrapping. Bootstrap replicates were created and analyzed with QTL Cartographer (Basten *et al.* 1994, 1997), and the 95% confidence interval for position was found for several QTL simultaneously. Two hundred replicates were used because this is sufficient to provide an unbiased estimate of the confidence interval (Visscher *et al.* 1996). The sizes of the support intervals for lobe area given by bootstrapping or the LOD drop-off method are comparable.

Figure 3 presents the mapping results for posterior lobe area. Comparison of the LOD profiles for lobe area and adjPC1 (not presented) and the results from Table 5 show that lobe area and shape (adjPC1) are largely nonindependent, both revealing similar QTL peaks. Notable differences exist on the X chromosome, however, which appears to have a greater effect on size. On chromosome 3R, at genetic position 3-77.4, model II for the *sechellia* backcross shows a significant peak for area that is not present for shape. Also on 3R a factor affecting only area is present at 3-110.1, while a factor influencing only shape is present at 3-126.7. Such inconsistencies imply that while the genetic coupling between posterior lobe shape and size, either due to pleiotropy or tight linkage of loci, may be significant, it is not absolute.

As expected from Figure 2, the LOD profile for adjPC2 (not shown) has peaks identified primarily by the *sechellia* backcross. Slight differences between the adjPC2 and adjPC1 profiles are apparent, and the adjPC2 profile has much in common with that for lobe area. The adjPC2 axis reveals mainly the same QTL as the two other lobe traits, aside from a significant peak at 2-57.3.

In general all the lobe QTL act in the same direction (Table 5), including those from the *sechellia* backcross for adjPC2. Substitution of a *sechellia* allele for a *simulans* allele usually causes the posterior lobe to decrease in area and move toward the *sechellia*-specific lobe shape.

The hypothesis of directional selection acting on the arch was tested using a sign test proposed by Orr (1998). The test finds the probability that n_1 factors of the same sign would be found by chance out of a total of n_2 factors, conditioned on the observed phenotypic difference and the distribution of QTL effects.

For the observed difference in posterior lobe area, with 11 QTL (Table 5), and assuming an exponential distribution of QTL effects (scale parameter, $\alpha = 1.61$; shape parameter, $\beta = 1$), the probability of finding 10 QTL of the same sign by chance is $P = 0.039$, supporting our view that directional selection has acted upon the arch. This result was robust to modest changes in mean QTL effect and QTL effect detection threshold. A similar result was found by Orr (1998), using the posterior lobe area data of True *et al.* (1997).

We repeated the test for adjPC1, with $\alpha = 4.21$ and $\beta = 1$, and found the probability that seven out of nine QTL of the same sign would occur by chance was $P = 0.408$, so there is no significant evidence of directional selection acting upon posterior lobe shape.

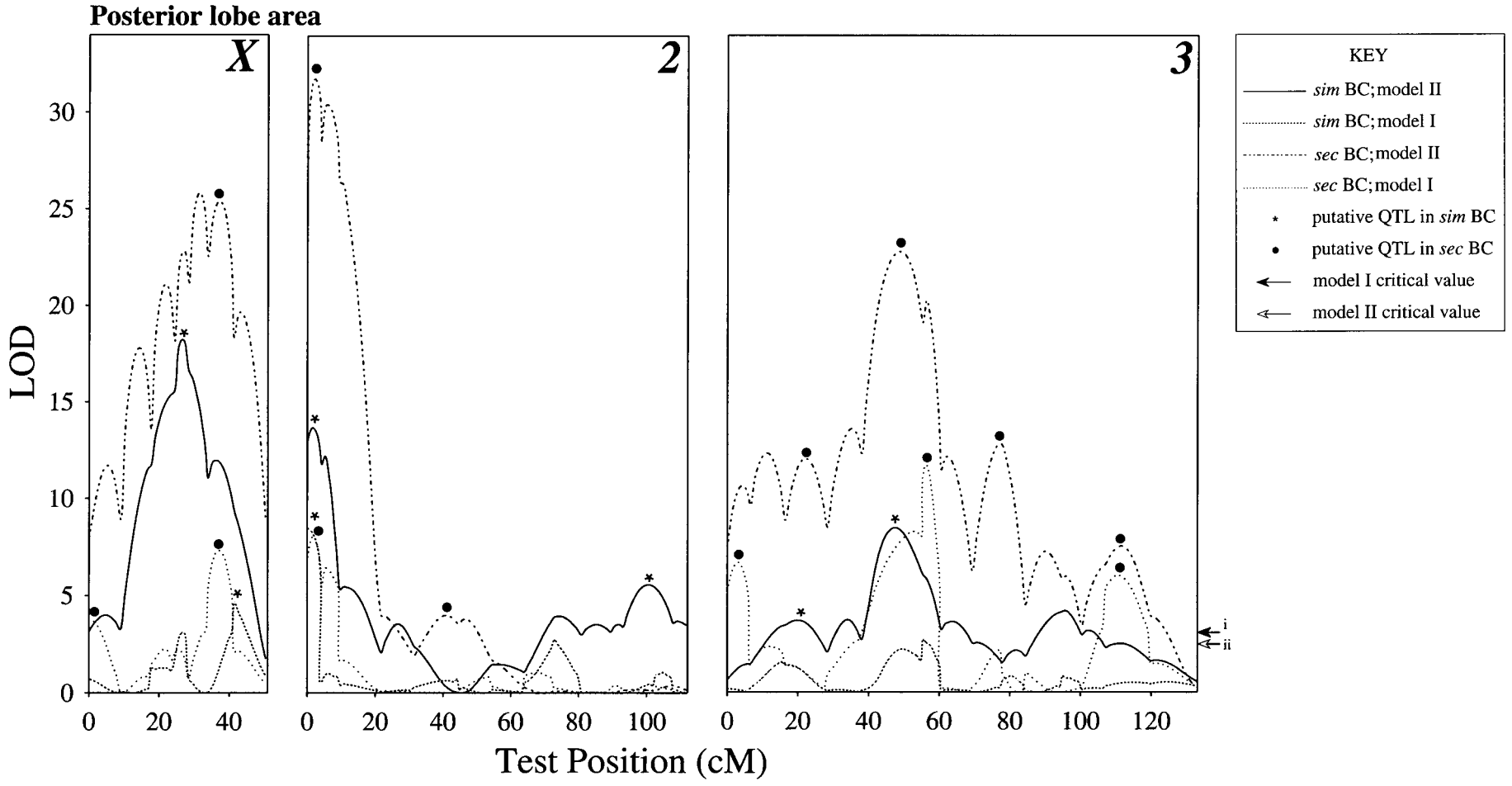

Figure 3.—LOD profile for posterior lobe area. The four curves represent the two backcrosses and the two mapping models used with each. See key for details of plot style for Figures 3–6. X, 2, and 3 refer to the three major chromosomes. Significant peaks, or regions above threshold, are marked, and the higher of the two experiment-wise critical values calculated for the backcrosses by permutation is indicated with arrows at the right axis. (i) Model I critical value = 3.12; (ii) model II critical value = 2.73.

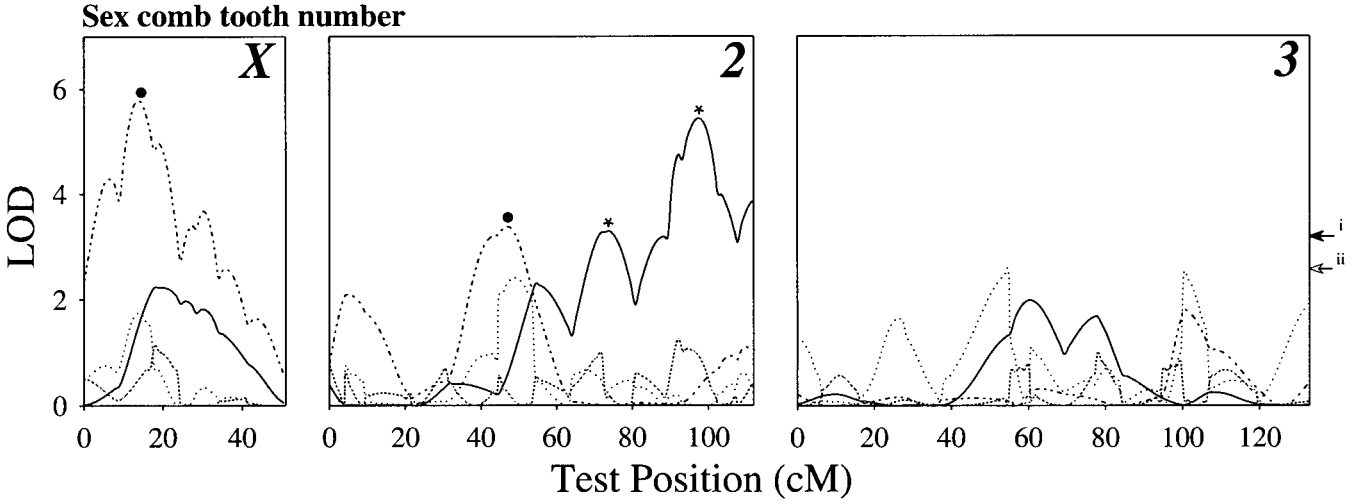

Figure 4.—LOD profile for sex comb tooth number. See key and legend accompanying Figure 3 for details of plot. (i) Model I critical value = 3.21; (ii) model II critical value = 2.67.

To look at the type of effect of the QTL found with model I for posterior lobe area, we used the bootstrap replicates to find 95% confidence intervals for the effect of those QTL. For five of the six QTL the confidence intervals of the estimated effect in the two backcrosses overlapped, suggesting that the QTL act in an additive fashion. The one that did not, at 3-3.0, is significant only in the *sechellia* backcross. The failure to find evidence for nonadditive QTL action is in contrast to the result presented earlier when considering purely the phenotypic information. From Figure 1C it can be seen that the F_1 and *simulans* backcross means cluster, indicating dominance of the *simulans* genome.

The plots in Figure 3 are similar to those found by Liu *et al.* (1996) and True *et al.* (1997), using a lower density map for backcrosses between *simulans* and *mauritiana*. These authors also found little evidence for dominance of the QTL and observed that all QTL for the posterior lobe act in the same direction. This indicates that the genital arch may be generally subject to directional selection during speciation in the *simulans* clade.

The two resampling tests for coincidence of the QTL found by True *et al.* (1997) and those presented here for posterior lobe area were not significant.

The LOD profile for sex comb tooth number (Figure 4) is generally quite low, likely due to the large environmental component to the variance of this trait. As noted, some of the variation in tooth number is explained by general body size variation, so the analysis was repeated after factoring out tibia length (not shown). It was seen

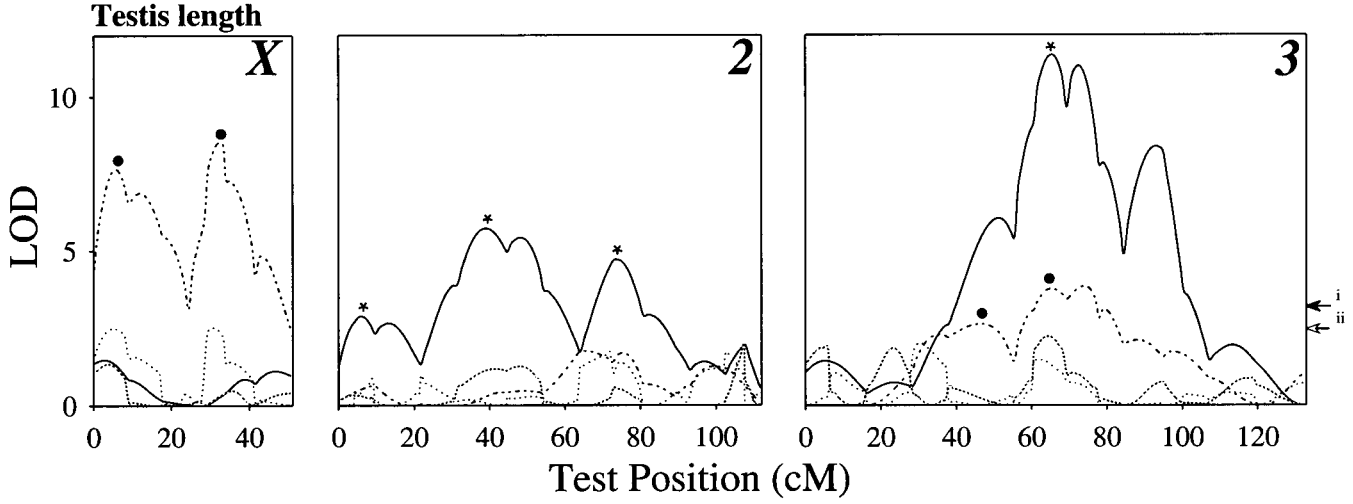

Figure 5.—LOD profile for testis length. See key and legend accompanying Figure 3 for details of plot. (i) Model I critical value = 3.33; (ii) model II critical value = 2.59.

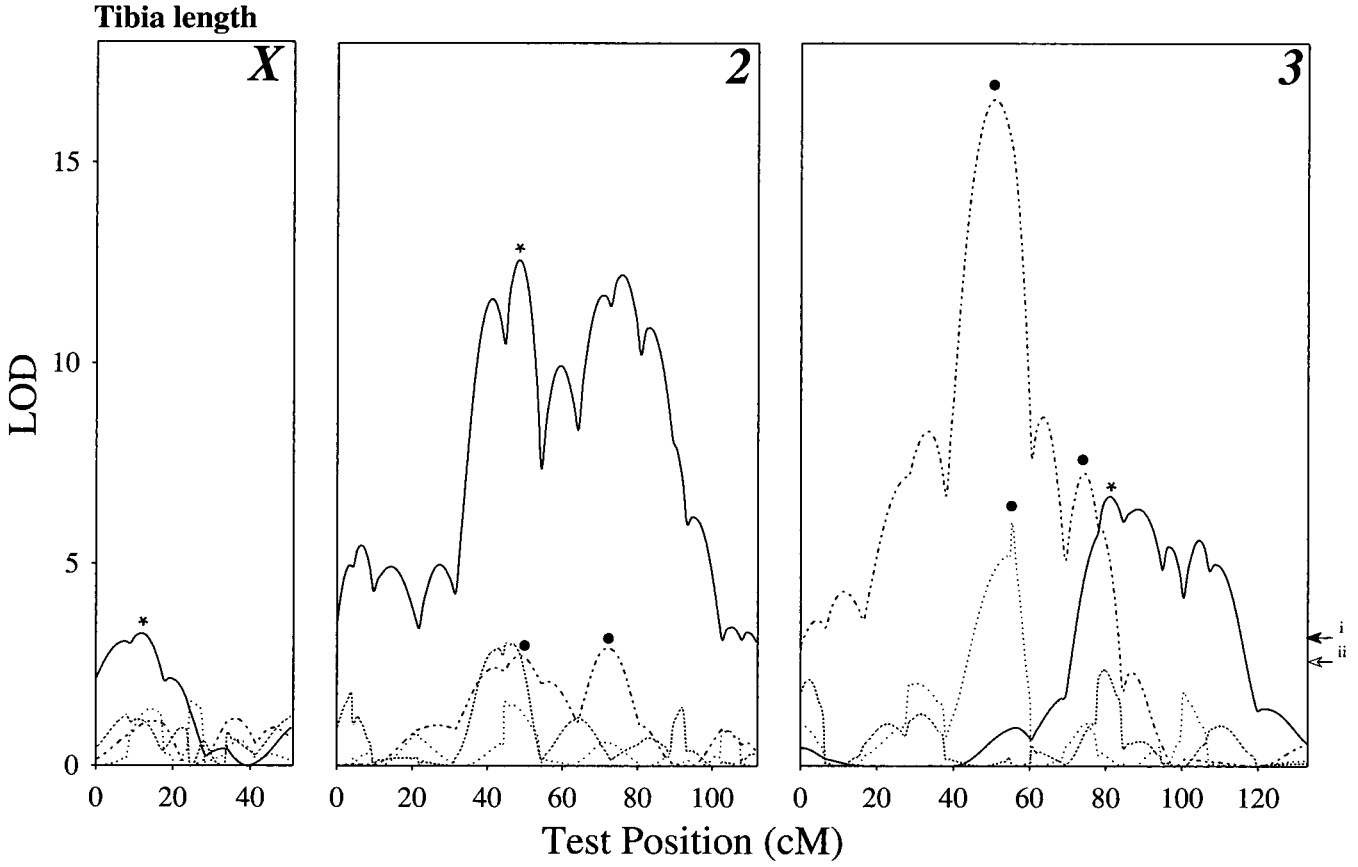

Figure 6.—LOD profile for tibia length. See key and legend accompanying Figure 3 for details of plot. (i) Model I critical value = 3.18; (ii) model II critical value = 2.69.

that the formerly significant peaks at 2-73.8 and 2-97.1 were removed, and a peak at 3-54.9 (possibly equivalent to a QTL peak found by True *et al.* 1997) achieved significance. It is interesting that the regions losing significance in the reanalysis are above the critical value in the tibia LOD profile (Figure 6). This seems to indicate that tibia length and comb tooth number are genetically as well as environmentally correlated and that the peaks at 2-73.8 and 2-97.1 have pleiotropic effects on both traits.

It seems likely that testis and cyst lengths are under separate genetic control, because cyst length QTL are confined to the X chromosome (plot not shown), while factors influencing testis length are present on all three chromosomes tested (Table 5 and Figure 5). This is confirmed by the low correlations seen between the traits in the backcrosses ($r = 0.02$ and $r = 0.15$ in the *simulans* and *sechellia* backcrosses, respectively) and supports the conclusions of Joly *et al.* (1997). The paucity of detected QTL for cyst length is not due to any variation associated with heterogeneity in the number of cysts measured per fly, because when the analysis was repeated using just one cyst per fly, the plot was very similar (not shown).

Table 5 reveals that the testis length QTL effects are generally in the same direction, implying, as for the genital arch, that testis size has been under directional selection. As with the genital arch characteristics, this was tested using Orr's (1998) sign test, with $\alpha = 9.04$ and $\beta = 1$. The probability of all seven QTL acting in the same direction was $P = 0.040$, which remains essentially unchanged for similar values of mean QTL effect and detection threshold.

The only nonsexual morphological trait investigated was tibia length, an indicator of body size (Catchpole 1994). The X chromosome appears to have little effect, while both autosomes show many significant QTL peaks, implying that numerous scattered autosomal genes influence body size (Figure 6). The QTL effects are not all of the same sign (and Orr's 1998 sign test shows no significant directionality), so it is unlikely that there has been directional selection for increased body size in *sechellia*, consistent with the variance observed across *simulans* and *sechellia* lines. One QTL at 3-80.8 has effects in opposite directions in each backcross, suggesting it has different (epistatic) effects depending upon the genetic background.

Table 6 shows the positions and effects of the QTL found for the set of fertility traits (LOD plots not shown), aside from sperm motility, which did not reveal

TABLE 5

Positions and estimated effects of putative QTL for morphological traits

Trait	QTL position (cM)[a]	Support interval for position[a]	Model[b]	QTL effect[c] *sim* BC	QTL effect[c] *sec* BC
Posterior lobe area	X-0.0	0.0-4.0[d]	I	−3.5[e]	−6.1
	X-27.4	25.2-28.4	II	−18.5	−19.7
	X-36.9	33.9-39.9[d]	I	−7.0	−13.6
	2-0.0	0.0-4.0[d]	I	−25.5	−28.8
	2-40.4	31.7-44.5	II	−6.5[e]	−19.2
	2-100.1	93.4-107.6	II	+21.7	−3.6[e]
	3-3.0	0.0-6.0[d]	I	+3.7[e]	−22.1
	3-22.5	17.7-26.7	II	−15.7	−36.9
	3-56.2	55.2-57.2[d]	I	−14.8[e]	−30.7
	3-77.4	73.1-80.3	II	−9.5[e]	−36.3
	3-110.1	107.1-119.1[d]	I	−6.7[e]	−23.3
adjPC1	X-22.7	14.2-28.4	II	−1.6[e]	−6.0
	X-49.4	43.0-50.4	II	−9.3[e]	−7.1
	2-2.0	0.0-3.8	I	+1.6[e]	+24.5
	2-11.5	9.5-16.1	II	+36.5	+27.9
	2-47.5	44.5-54.3	II	+34.0	+15.6
	3-6.0	1.3-6.5	II	+38.4	−13.5
	3-45.9	40.5-51.5	I	+63.6	−6.3[e]
	3-102.3	100.3-107.1	II	+43.1	+5.5[e]
	3-126.7	121.3-132.7	I	+30.0[e]	+19.1
Sex comb tooth number	X-14.0	9.0-17.7	II	−13.5[e]	−34.4
	2-46.5	34.8-56.9	II	+15.9[e]	−58.1
	2-73.8	64.0-80.8	II	+39.3	−0.4[e]
	2-97.1	93.1-106.5	II	+53.2	−16.5[e]
Testis length	X-6.0	1.2-9.0	II	+12.0[e]	+37.0
	X-32.4	28.8-38.2	II	−5.3[e]	+35.0
	2-6.1	0.0-9.5	II	+35.4	−15.8[e]
	2-39.4	29.1-44.5	II	+51.8	−15.3[e]
	2-73.8	66.5-80.8	II	+44.7	−30.3[e]
	3-50.9	42.1-55.2	II	+58.5	+40.9
	3-65.4	60.7-69.4	II	+72.6	+46.1
Cyst length	X-4.0	0.0-9.0	II	−35.8	+23.8[e]
	X-28.4	20.7-33.9	II	−37.3	+31.9
	X-47.4	41.4-50.4	II	−22.3[e]	+34.1
Tibia length	X-12.0	9.0-17.7	II	−9.6	+5.8[e]
	2-48.5	44.6-52.2	II	+44.5	+19.1
	2-71.0	65.5-72.8	II	+40.6	+19.0
	3-55.2	47.4-56.5	I	+2.8[e]	−34.9
	3-80.8	74.4-84.4	II	+27.2	−33.3

[a] When the QTL is significant in only one backcross, the position and support interval are given for that QTL. When both backcrosses have significant QTL, the position and support interval are presented for the QTL with the highest LOD score.

[b] The estimated positions and effects for model II can be influenced by linked QTL, if present.

[c] Defined as that effect caused by the replacement of a *simulans* allele by a *sechellia* allele. Given as a percentage of the species difference for X-linked QTL and as a percentage of half the species difference for autosomal QTL.

[d] Support interval is a 95% confidence interval based on 200 bootstraps of the original data. All other intervals are two-LOD drop-offs from the QTL peak. See text for details.

[e] LOD score was not significant.

any significant peaks. The three categorical fertility traits were treated independently, and it can be seen that there are slight differences in the QTL detected, which may influence different hierarchical levels of fertility.

The fertility traits indicate that X-linked factors are largely responsible for conferring sterility in the backcross populations, and this pattern is consistent with fertility factors being generally recessive. Indeed, Hollocher and Wu (1996) compared homozygote autosomal regions with hemizygous X regions and found no evidence to support an X effect stronger than autosomal effect in the evolution of hybrid male sterility. It is worth

TABLE 6

Positions and estimated effects of putative QTL for fertility traits

Trait	QTL position (cM)[a]	Support interval for position[a]	Model[b]	QTL effect[c]	
				sim BC	*sec* BC
Testicular atrophy	X-15.0	10.3-17.7	II	−0.279	−0.022[d]
	X-27.4	24.4-33.4	II	−0.285	−0.018[d]
	2-49.5	44.5-58.6	II	+0.187	−0.007[d]
Absence of cysts	X-15.0	11.8-17.7	II	−0.453	−0.315
	X-31.4	28.6-33.6	I	−0.401	−0.103[d]
	X-43.4	41.4-48.6	II	−0.235	−0.306
	3-79.8	72.8-84.4	II	+0.063[d]	+0.229
Quantity of sperm	X-22.7	19.3-27.3	II	−1.165	−0.470
	X-33.4	30.5-37.6	I	−0.943	+0.255[d]
	3-95.7	94.7-100.3	II	−0.228[d]	+0.448

[a] When the QTL is significant in only one backcross, the position and support interval (two-LOD drop-off) are given for that QTL. When both backcrosses have significant QTL, the results are presented for the QTL with the highest LOD score.

[b] The estimated positions and effects for model II can be influenced by linked QTL, if present.

[c] Effects, reported as given by QTL Cartographer, represent deviation from the fertile parental phenotype, so QTL with negative effects confer sterility.

[d] LOD score was not significant.

noting that the results for cyst length also indicate primarily X-linked effects, in contrast to Joly *et al.* (1997) who found no X effect for this trait.

Candidate genes: A FlyBase search (FlyBase 1999), conducted using a keyword search and, for some regions, directly examining all associated genes, revealed numerous candidate genes for the most obvious QTL peaks (Table 7). Those regions for which we found no reliable candidates are not shown.

One particular gene of interest is *decapentaplegic* (*dpp*). This was the only reliable candidate in our search found to be associated with the largest LOD peak for both lobe area and adjPC1 and is a locus known to be involved in determining the conformation of the genital arch (Spencer *et al.* 1982). Liu *et al.* (1996) also found a QTL peak about this region, though it did not stand out as sharply as in the *simulans-sechellia* cross presented.

DISCUSSION

Using a high-resolution interval mapping approach, the analyses presented show that the closely related sibling species *D. simulans* and *D. sechellia* have strongly diverged in certain morphological reproductive traits, *i.e.*, major prezygotic isolation has built up. The genes affecting these characters are not confined to any particular chromosomes but rather are scattered across the genome. Many of the marker intervals show significant QTL for more than one trait, indicating close linkage of genes or pleiotropy. Thus, to some extent the traits may be genetically correlated.

Directional selection seems to have acted upon the genital arch and testis, as for each the QTL effects act in the same direction. However, the same pattern of QTL effects was not seen for the sex comb, where there was significant evidence for QTL acting in both directions. This shows that despite its correlation with lobe area, there is no evidence for directional selection having altered comb tooth number.

If, as suggested by Cook (1977) and Coyne (1985), the male sex comb does act as a grasping organ during mating, it may be that it does not require specificity to the female and that the exact number of teeth is not important—consistent with the large environmental variance seen for this trait. This suggests sex comb tooth number variation may be influenced primarily by genetic drift and may not have been involved in the very early stages of speciation. This seems also to be true of body size, where again the QTL effects are in different directions and no concerted difference in size exists between the species.

The many similarities between the results presented here and those involving crosses between *D. simulans* and the third member of the clade, *D. mauritiana* (Liu *et al.* 1996; True *et al.* 1997), suggest that similar genetic responses to selection occurred during the two speciation events. The two different crosses exhibit evidence for directional selection acting on the genital arch, and our study has suggested the same may be true for the testis. Together this provides support for the theory of Civetta and Singh (1998a) that reproductive traits are generally subject to directional selection during speciation, particularly within the *simulans* clade.

These results show that it is possible to test evolutionary hypotheses using QTL mapping, even when there is little or no information on the loci involved.

TABLE 7

Putative candidate genes

Trait	Chromosome	Interval posistion (cM) *sim-sec* hybrid[a]	Interval posistion (cM) mel[b]	Cytological location in *mel*[c]	Candidate genes[d]
Posterior lobe area	X	0.0-4.0	0.0-4.9	1B1-3E8	*ctt*, 1-0.3; *ge*, 1-0.1
	X	25.2-28.4	34.3-38.0	10B5-11A1	*ano*, 1-35.7; [*hep*, 11D1-2]; *twg*, 11A1-5
	X	33.9-39.9	51.0-56.3	13E1-15C2	*ber*, 1-52.4; *thvd*, 1-55.2
	2	0.0-4.0	0.1-4.9	21C4-22C2	[*dpp*, 22F1-2]
adjPC1	2	0.0-3.8	0.1-4.6	21C4-22C2	[*dpp*, 22F1-2]
	3	1.3-6.5	1.8-8.0	62C3-63D1	[*R*, 62B11]
	3	40.5-51.5	30.5-41.0	67C1-70C1	[*gem*, 66D10-F15]
	3	121.3-132.7	83.8-100.1	95E6-99D3	*Ser*, 97F1-2
Sex comb tooth number	X	9.0-17.7	11.0-25.7	4F1-8B7	[*scd*, 1-30.6]; *Sxl*, 6F5; *tbd*, 8A1-C1
	3	46.5-55.9[e]	36.2-44.7	68C5-73A8	[*klu*, 68A2]
Testis length	X	1.2-9.0	1.5-11.0	3B1-4F1	[*mit(l)15*, 3A9]
	2	29.1-44.5	28.6-47.0	28C6-33E10	*Nup154*, 32D1-2; *zk*, 32D1-2
	2	66.5-80.8	55.9-68.0	42F1-49F1	*Wnt2*, 45E
	3	60.7-69.4	45.8-48.1	76A1-84A4	*fbl*, 77B1-9; *ms(3)HO5A*, 3-45.9
Cyst length	X	20.7-33.9	29.1-51.0	8F1-13D1	*l(1)10Bh*, 10B10-17; *sbr*, 9F5-11
Tibia length	2	44.6-52.2	47.1-51.6	33F2-35D5	*CycE*, 35D4; *Idgf1*, *Idgf2*, *Idgf3*, 36A2-4
	2	65.5-72.8	55.3-60.0	41E1-45A1	*babo*, 45A1-2; [*chl*, 2-60.8]; *dpa*, 43C7
	3	47.4-56.5	37.1-44.8	68E1-73B3	*app*, 69A3-4; *CycA*, 68E1-2; *gnu*, 70E8-71E5; l(3)SG13, 3-25.9; l(3)SG14, 3-26.6; *l(3)SG26*, 3-42.9
Testicular atrophy	X	10.3-17.7	13.2-25.7	5B1-8B7	*ag*, 7A5-C1; *gs*, >7B3; *Sxl*, 6F5
	X	24.4-33.4	33.4-49.8	10A1-13A9	*hop*, 10B6
Cyst presence/absence	X	11.8-17.7	15.7-25.7	5D4-8B7	*mxc*, 8D8-9; *l(1)8*, 1-19.0/23.6
	X	28.6-33.6	38.5-50.3	11A6-13B7	[*Chc*, 13F2]
	X	41.4-48.6	57.6-62.6	16F3-18C1	[*ms(1)4*, 1-63]; *scu*, 16F3-7
Quantity of sperm	X	19.3-27.3	25.7-36.7	8D7-10E5	*l(1)10Bh*, 10B10-17; *ms(1)10A*, 10A2

[a] Represents the 95% confidence support interval about the putative QTL.
[b] Interval from hybrid converted to *melanogaster* genetic distance using information from Table 1 and the genetic location of the markers in *D. melanogaster*.
[c] Cytological position inferred from *melanogaster* genetic position using FlyBase (1999).
[d] All candidate genes were taken from FlyBase (1999), aided by Lindsley and Zimm (1992). Brackets indicate that the genes are very close to, though not within, the support interval. Where the cytological location of the gene is unavailable the genetic position is presented.
[e] Interval significant in reanalysis of comb tooth number data after factoring out tibia length.

This study makes use of a more dense molecular map than has previous work on this species complex, and it is apparent that many marker intervals have LOD peaks above threshold. We have chosen not to discuss in detail the number of genes influencing the traits, or give more than an indication of the type and magnitude of the effects, as the average intermarker distance is 8.4 cM. As such, even the smallest intervals may contain clusters of genes with related functions, and unless one can be certain the effects pertain to a single locus, distinctions between "major" and "minor" QTL are meaningless.

A preliminary search for genes in the major QTL regions revealed a set of candidates (FlyBase 1999). We do not claim that the candidates listed are necessarily the correct genes, but the regions examined for them collectively represent only a small fraction of the genome. In the case of posterior lobe area, for example, seven regions were examined, representing 12.6% of the genome. For the other traits this value ranges from 4.5 to 18.8%. Therefore, on the basis of this QTL study we have been able to eliminate large sections of the genome and have defined short regions that should be good starting points for future studies investigating the effect of single genes on the morphological traits studied here.

Perhaps the most compelling candidate for study is *dpp*, which is associated with the highest QTL peak for genital arch shape and size, and which is known from analyses of mutants to influence the conformation of the genital arch (Spencer *et al.* 1982).

A variety of methods are available to test the influence of candidate genes on particular traits: comparisons of the spatial and temporal patterns of expression of the transcript and gene product, complementation analysis, mutagenesis, and genetic transformation (for examples, see Mackay 1995; Stern 1998). With the possible ex-

ception of complementation analysis, these methods will probably be useful only for major factors.

Along with the morphological traits, fertility was also examined. For the purpose of discovering all factors with effects on fertility, a backcross design is inefficient because autosomal recessive factors will be missed. An introgression analysis of fertility factors between *simulans* and *mauritiana* revealed that autosomal introgressions are not completely dominant, because the majority of heterozygous introgressions are fertile (True *et al.* 1996). Backcross studies (for example, Coyne 1984) have further shown that fertility factors are not completely recessive because heterozygous autosomes have significant effects on male fertility. Nevertheless, the X chromosome in Drosophila is known to hold numerous fertility factors (True *et al.* 1996), and our study upheld this.

The high degree of prezygotic isolation between *simulans* and *sechellia*, coupled with the use by *sechellia* of a toxic resource (Louis and David 1986), suggests that the two species may have diverged in sympatry. The ancestor of *sechellia* is likely to have utilized Morinda as a competition-avoidance strategy, with sexual selection acting to increase the distinction between the partially ecologically isolated incipient species. Postzygotic isolation may then have evolved as a pleiotropic effect of the change in reproductive morphology of the two species and also may have evolved in *sechellia* due to its adaptation to Morinda.

Our study has shown that using an integrated set of characters provides a great deal of information on the genetic relationships of the traits and allows inference of their relevance to speciation. Further study of nonreproductive traits would be useful to substantiate the theory that they are less often subject to directional selection in comparison with reproductive traits. More high-resolution work using heterozygous and homozygous introgressions, together with quantitative measures of fertility, would help to determine whether postzygotic reproductive isolation has emerged as a consequence of diversifying selection on reproductive trait morphology.

Using a high-density molecular map has allowed us to identify regions of the genome important in determining species differences and justifies our proposal of various candidate loci for further investigation. Finer scale mapping is also possible if numerous markers are available in regions already supported by genome scans such as that presented. This should be facilitated by the sequence divergence between *D. simulans* and *D. sechellia*, allowing the development of very dense single nucleotide polymorphism maps that could be characterized by allele-specific amplification, as was used for the *DROLAMB2A* locus in our study. Fine mapping should be done in combination with designs, such as recombinant inbred lines that increase the recombinational distance, or with an introgression design, transferring short genomic regions between species.

We thank Isabelle Colson for help in constructing the microsatellite marker map and for helpful advice on the genotyping; the Drosophila workers in Table 2 for providing the fly stocks; and two anonymous referees for constructive criticism. This work was supported by a Biotechnology and Biological Sciences Research Council grant to D. B. Goldstein.

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Communicating editor: L. Partridge