ABSTRACT

MOEHRING, AMANDA JEAN. The Quantitative Genetic Basis of Mating Behavior and Speciation in *Drosophila*. (Under the direction of Trudy F. C. Mackay).

The widely-accepted Biological Species Concept defines species as populations that are reproductively isolated, i.e. are unable to mate with one another or produce viable and fertile progeny when given the opportunity. Speciation is characterized by the evolution of prezygotic (ethological barriers to interspecific mating) and postzygotic (reduced viability and fertility of interspecific hybrids) reproductive isolating mechanisms. Although recent progress has been made towards understanding the genetic basis of postzygotic isolation, little is known of the genetic architecture of sexual isolation – arguably the most important form of reproductive isolation in animals. In addition, it has not been determined if reproductive isolation occurs due to selection acting on variation within a species or arises from novel mutations. In order to understand how new species arise, the genetic basis of variation in mating behavior within a species, as well as the genetic basis for prezygotic reproductive isolation between species, must be known.

The mating behavior of *Drosophila* consists of a series of actions that exchange auditory, visual and chemosensory signals between males and females. Although mating behavior has been studied extensively in *Drosophila*, most known genes affecting mating behavior have been located through the mutation of single genes. The wide range of variation in courtship behavior in natural populations is believed to arise from the joint segregation of multiple quantitative trait loci (QTL) with varying effects that can be influenced by the environment. Here, we identified QTL that affect courtship occurrence, courtship latency, copulation occurrence and copulation latency that

segregate between a *D. melanogaster* strain selected for reduced male mating propensity (2b) and a standard wild-type strain (Oregon-R). Mating behavior was assessed in a population of 98 recombinant inbred lines derived from these two strains and QTL affecting mating behavior were mapped using composite interval mapping. There were four QTL affecting male mating behavior at cytological locations 1A;3E, 57C;57F, 72A;85F and 96F;99A. We used deficiency complementation mapping to map the autosomal QTL with much higher resolution to five QTL at 56F5;56F8, 56F9;57A2, 70E1;71F4, 78C5;79A1, and 96F1;97B1. Quantitative complementation tests performed for 45 positional candidate genes within these intervals revealed seven genes which failed to complement the QTL: *eagle, 18 wheeler, Enhancer of split, Polycomb, spermatocyte arrest*, *l(2)05510* and *l(2)k02206*. None of these genes have been previously implicated in mating behavior, demonstrating that quantitative analysis of subtle variants can reveal novel pleiotropic effects of key developmental loci on behavior.

In a separate experiment, we mapped QTL contributing to pre-zygotic reproductive isolation between *Drosophila simulans* and *D. mauritiana*. We mapped at least seven QTL affecting discrimination of *D. mauritiana* females against *D. simulans* males, three QTL affecting *D. simulans* male traits against which *D. mauritiana* females discriminate, and six QTL affecting *D. mauritiana* male traits against which *D. simulans* females discriminate. QTL affecting sexual isolation are largely different in males and females and between the two species, and are not preferentially located on the *X* chromosome. Relatively few QTL with moderate to large effects associated with pre-zygotic isolation facilitates future positional cloning of the underlying genes. In contrast

to results for postzygotic isolation, no epistasis was detected between QTL for prezygotic isolation.

Several of the intraspecific *D. melanogaster* mating behavior QTL overlap those found to affect reproductive isolation between *D. simulans* and *D. mauritiana*. Future testing of these positional candidate genes for their effect on reproductive isolation could provide evidence that speciation arises in response to selection acting on naturally-occurring variation in a population.

THE QUANTITATIVE GENETIC BASIS OF MATING BEHAVIOR AND SPECIATION IN *DROSOPHILA*

by
AMANDA JEAN MOEHRING

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GENETICS

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APPROVED BY:

_____
Chair of Advisory Committee
Trudy F. C. Mackay

_____
Co-chair of Advisory Committee
Gregory C. Gibson

_____ _____
Coby Schal Robert R. H. Anholt

DEDICATION

This work is dedicated with my whole heart to my husband, Thomas. I am in awe of your limitless love, understanding and support. I can not thank you enough for the hundreds of small sacrifices you have made and all of the times in which you went the extra mile so that I would not have to, allowing me to follow this dream. You are my rock, my sense of calm, and my voice of reason. You are also my co-conspirator, my companion in adventure, and my partner in crime. I am thankful for the moment that I met you, for each subsequent day that we have spent together, and for the future that awaits us. You are, and always will be, my best friend.

BIOGRAPHY

I was born in a suburb of Los Angeles, California, in 1976. My parents fled the largest city in the U.S. for the peaceful tranquility of one of the smallest: Bandon, Oregon. For a youth, this tranquility translated into boredom, whose only savior was a vivid sense of imagination. A tree house would become a boat lost at sea, a military fortress, or a castle in the clouds. My brothers and I would play in the forest for hours, our imaginations taking the place of television and video games. Reading, too, allowed me to develop my imagination as I traveled outside of the small town to places far away. While I rarely have the opportunity to play in the forest today, my imagination continues to be stimulated through reading, traveling, painting, and the everyday learning that an academic environment provides.

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In the outstanding academic environments that I have experienced, there are many people who deserve recognition for assisting me my development as a scientist, and in this research in particular.

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My family has also been a great source of support and inspiration. My husband, Thomas Winkeler, who has given me more than I thought a person could give. My parents, Carl and Chris, for raising me into the person I have become. Your lifetime

examples of love, honesty and hard work demonstrate who you are more eloquently than any words. My brothers, Carl and Jon, for driving me to be more, and my sister-in-law Rachelle, who is truly a sister to me, for always believing I am capable of accomplishing anything. I strive to be worthy of the respect you have shown me and to grow into the person you all think I am capable of becoming.

I would also like to thank Habitat for Humanity and the people of Cantel, Guatemala, for shaping me as a human being. My experiences with you taught me to be grateful for the opportunities I have, for the life that is available to me, and how minor the troubles of my life are compared to those who truly have to struggle. Your perseverance in the face of repeated hardship is an example I have never forgotten.

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CHAPTER 1

Literature Review:

The Genetics of *Drosophila* Mating Behavior

"Studies of courtship and mating in the fruit fly offer a window on the ways genes influence the execution of complex behaviors"
R. J. Greenspan (1995)

SUMMARY

The mating behavior of *Drosophila* consists of a series of actions that exchange auditory, visual and chemosensory signals between males and females. Although mating behavior has been studied extensively in *Drosophila*, most known genes affecting mating behavior have been located through the mutation of single genes. The genes affecting genetic variation for mating behavior in natural populations are not known. The wide range of variation in courtship behavior in natural populations is believed to arise from the joint segregation of multiple quantitative trait loci (QTL) with varying effects that can be influenced by the environment. Locating the QTL responsible for behavior has been limited due to the complexity of behavior and the multiple factors involved in its production. Due to its intricate nature, large sample sizes and numbers of molecular markers are necessary to unravel the genetic basis of a behavior, which is why QTL have not previously been identified for courtship behavior to the level of an individual gene. This review will discuss the single genes that are known to affect mating and courtship behavior in *Drosophila* and the methods by which QTL can be identified.

INTRODUCTION

The range of genetic variation within a species both underlies and limits the degree to which a population can respond to selection. Populations evolve when selection acts upon genetic variation, causing a shift in the allele frequency of polymorphic genes affecting a particular trait. If two subpopulations diverge in the frequency of a gene that can affect mating behavior to the degree that they no longer are able to reproduce with one another, they will be devoid of gene flow and can then evolve along separate paths. This reproductive isolation is therefore the basis of the formation of new species. Hence, in order to understand the genetic basis of speciation, we must first identify the genes producing variation in mating and courtship behavior, for it is divergence in these traits that is thought to be responsible for the initiation of reproductive isolation, and subsequently speciation.

Although behavioral traits have been studied for decades in an attempt to identify the genetic basis of the variation seen within and among species, these studies were limited until recently due to the difficulty of separating the individual behavioral components, the necessity for a large panel of molecular markers, and the need for an accurate statistical mapping technique and publicly available computational software to implement the statistical analysis. With the recent availability of sequence data providing a multitude of molecular markers and improved analytical techniques, we are beginning to understand the genetic basis of naturally occurring variation in behavior, at least in model genetic organisms. *Drosophila* is ideally suited for the study of complex behavioral traits such as courtship behavior due to its wide repertoire of stereotypical

behaviors and the genetic tools available for understanding them.

The courtship behavior of *Drosophila* involves the exchange of information and a dynamic mutual stimulation involving several sensory modalities. There are multiple factors that comprise the whole of mating behavior. In *Drosophila*, this behavior follows a series of steps (**Figure 1.1**) (Dethier, 1971). First, the male becomes aware of the female and aligns himself with her. Then he taps her abdomen, the major site of female pheromone production (Coyne and Oyama, 1995), sensing the pheromones through his prothoracic legs (Dethier, 1971; Pikielny and Wang, 1996; Wang and Pikielny, 1997). This transfer of pheromones is partially responsible for gender and species recognition. *Drosophila* do not mate in flight, and as the female moves around, the male will follow her and begin his wing vibration display, producing a courtship "song" (Wheeler *et al.*, 1991). The antennae permit the specialized perception and processing of this acoustical information in the female while acting in the male to process some of the pheromonal cues (Clyne *et al.*, 1997). Several seconds to minutes after courtship initiation, the male will then extend his proboscis and lick the female's genitalia, creating another transfer of pheromones (Dethier, 1971). Finally, the male attempts to copulate, curling his abdomen by contracting the Muscle of Lawrence (MOL). If he is unsuccessful because the female rejects him through avoidance (running away) or by extruding her ovipositor (if she has previously mated), the male will either return to the following or singing stage and repeat the cycle or begin anew with a different female (Hall, 1994). Receptive females raise their wings, allowing the male to grip them and open the vaginal plate (Yamamoto and Nakano, 1998). When the male successfully copulates, he transfers sperm, seminal fluid,

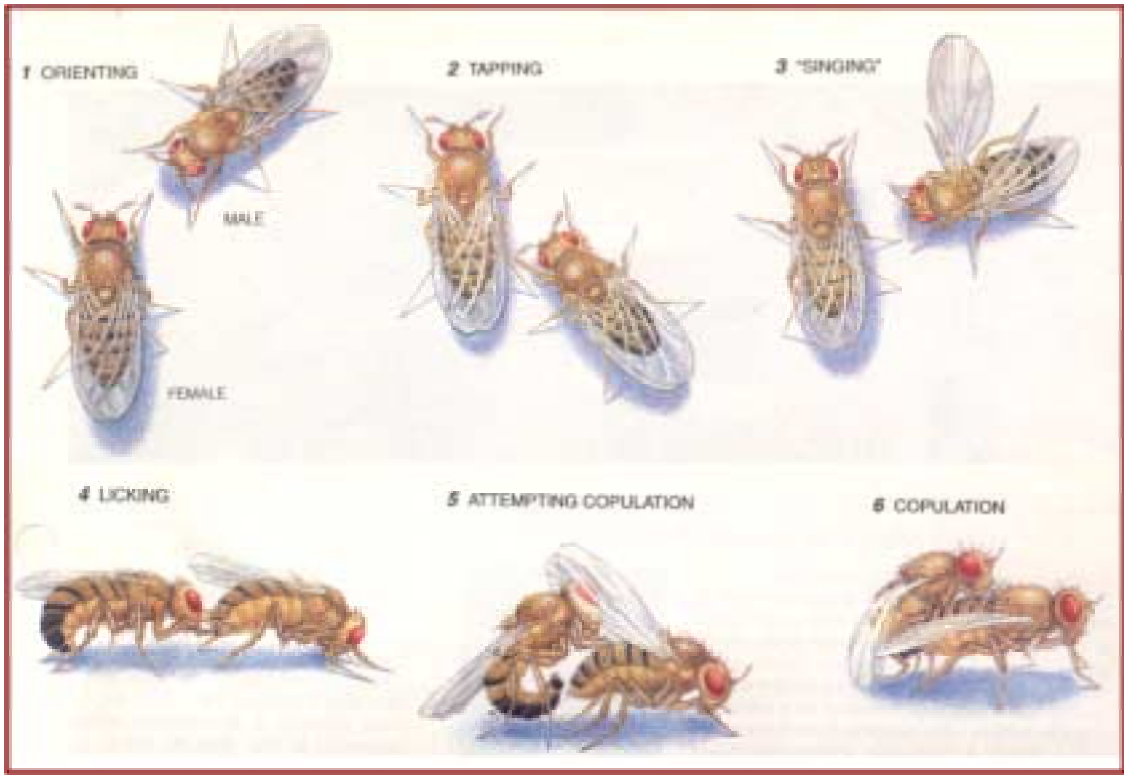

Figure 1.1: The various stages of mating behavior. Figure taken from R. J. Greenspan (1995).

and even "anti-aphrodisiac" pheromones that make the female less appealing to other males (Jallon, 1984).

Mating ability is a critical component of reproductive fitness in males (Partridge *et al.*, 1985), and can undergo significant inbreeding depression (Sharp, 1984). However, there is a considerable amount of segregating variation for the individual components of mating behavior, as well as for mating preference (Manning, 1961, 1963; Parsons, 1964); this variation most likely persisting through genotype by sex interactions (Casares *et al.*, 1993; Nuzhdin *et al.*, 1997; Vieira *et al.*, 2000). Estimates of heritability (h^2) for male mating speed range from 0.3-0.6 (Manning, 1961, 1963; Parsons, 1964; Collins and Hewitt, 1984; Casares *et al.*, 1993), suggesting a strong genetic component for mating behavior in *Drosophila*.

MUTANTS

By examining flies that have a mutation in a single gene, individual components of development, morphology and behavior can be assessed. To locate single genes involved in *Drosophila* mating behavior, point mutations can be created through treatment with *X*-rays or ethyl methane sulfonate (EMS), through *P*-element-mediated germ-line transformation (Bate and Martinez, 1993), or through the use of RNA interference (RNAi) (Fire *et al.*, 1998). Treatment with the ionizing radiation of *X*-rays causes the release of electrons, and subsequently the formation of positively charged free radicals that are highly chemically reactive. Ionizing radiation is mutagenic due to this increased reactivity of atoms present in DNA molecules, usually resulting in gross

chromosomal rearrangements. EMS is an alkylating agent that induces chemical mutagenesis through the transfer of an ethyl group to a DNA base, transforming its chemical structure and resulting in point mutations due to altered base pairing.

P-elements are transposable elements whose transposition can be controlled by simple crosses to strains containing a stable source of transposase (Engles, 1984). *P*-elements typically insert in 5' regulatory regions of genes, causing hypomorphic mutations, but can also disrupt the coding sequence and produce null mutations (Spradling *et al.*, 1999). Several *P*-element insertions are known to disrupt genes that are involved in courtship behavior. *P*-elements have also been constructed that enable temporally restrictive gene expression by engineering the wild-type gene with a heat shock promoter. If such a *P*-element construct is inserted into a stock homozygous for a null allele, the only functional copy of that gene is the one present on the *P*-element. 'Rescue' of the mutant phenotype by applying a mild heat shock constitutes proof of identity of the mutant gene. The *P*-element can be a vessel for the addition of gene function as well when used in a binary system where the *P*-element is joined with an upstream activating sequence (*UAS*) promoter. When the *UAS* line is crossed to a line containing the *GAL4* driver, the *UAS* transgene will be activated in all of the tissues that express *GAL4*. The *GAL4* driver can be universal or tissue-specific, allowing for the expression of the gene in the whole organism or in a localized area, and can be fused to a heat shock promoter, causing activation only when the flies are exposed to a dose of heat.

While eliminating gene function can provide clues as to the nature of that gene's purpose in an organism, null mutations often have pleiotropic effects unrelated to their

original function, producing extraneous phenotypes (Sokolowski, 2001). Null mutations also do not tell us how natural (functioning) allelic variants of that gene, which have the most evolutionary relevance, affect the organism (Sokolowski, 2001).

Sensory mutants

While studying *Drosophila* almost a century ago, Thomas Hunt Morgan was rewarded with the first of many mutants: the visual mutant *white*. A few years later, Sturtevant showed that this mutation (and the mutations *yellow* and *curved*) reduced the vigor of male courtship (Sturtevant, 1915). To date, many single-gene mutations have been identified that affect behavior by disrupting the fly's ability to sense its surroundings (**Table 1.1**). Most of these "mating behavior" genes were located through the mutation of single genes associated with abnormal development. Given that courtship behavior is complex and utilizes most or all of the sensory modalities, it is not surprising that mutations which disrupt vision, olfaction, and hearing produce behavioral defects. A mutation in the development of the eye, causing a breakdown in the physical ability of the fly to sense its surroundings, does not truly isolate the founding genetic factors of courtship behavior, but rather shows that physical components are necessary in order to carry out these behaviors. While it is difficult to separate those genes that are not truly involved with the production of the underlying behavior itself from those that are, this review will mainly focus on mutations that have been shown to have a distinct behavioral effect beyond the cause-and-effect scenario seen with the disruption of a sensory modality.

Table 1.1. An alphabetical list of genes shown to have an affect on courtship or copulatory behavior. C.L. = cytological location. Molecular functions from The Flybase Consortium. All descriptions refer to the affect on male behavior, unless otherwise noted. References: (1) Ackerman and Siegel, 1986; (2) Arthur *et al.*, 1998; (3) Baba, 1989; (4) Baker *et al.*, 2001; (5) Balakireva *et al.*, 1998; ; (6) Bernstein *et al.*, 1992; (7) Bien-Willner and Doane, 1997; (8) Billeter *et al.*, 2002; (9) Bubis *et al.*, 1998; (10) Buchner, 1991; (11) Burnet and Wilson, 1980; (12) Castrillon *et al.*, 1993; (13) Clark *et al.*, 1995; (14) Cook, 1980; (15) Cowan *et al.*, 1984; (16) Crossley, 1988; (17) Crossley and Zuill, 1970; (18) Dauwalder *et al.*, 2002; (19) Dockendorff *et al.*, 2002; (20) Emmons and Lipton, 2003; (21) Ewing, 1988; (22) Ferveur and Jallon, 1993; (23) Ferveur *et al.*, 1997; (24) Finley *et al.*, 1997; (25) Finley *et al.*, 1998; (26) Fleischmann *et al.*, 1995; (27) Friedman *et al.*, 1995; (28) Gailey *et al.*, 1982; (29) Gailey *et al.*, 1984; (30) Gailey *et al.*, 1986; (31) Gailey and Siegel, 1989; (32) Gailey *et al.*, 1991; (33) Gaines *et al.*, 2000; (34) Grosjean *et al.*, 1991; (35) Hall, 1984; (36) Heifetz *et al.*, 2000; (37) Jackson *et al.*, 1983; (38) Jallon, 1984; (39) Joiner and Griffith, 1997; (40) Kerr *et al.*, 1997; (41) Krejci *et al.*, 1994; (42) Kulkarni *et al.*, 1988; (43) Kuniyoshi *et al.*, 2002; (44) Kyriacou, 1990; (45) Kyriacou and Hall, 1980; (46) Kyriacou and Hall, 1984; (47) Kyriacou and Hall, 1985; (48) Kyriacou and Hall, 1986; (49) Kyriacou and Hall, 1988; (50) Kyriacou *et al.*, 1978; (51) Lilly and Carlson, 1989; (52) Lung and Wolfner, 2001; (53) Markow, 1987; (54) McRobert and Tompkins, 1985; (55) Nakano *et al.*, 2001; (56) Nakayama *et al.*, 1997; (57) Neckameyer, 1998; (58) Nitasaka, 1995; (59) Nitasaka and Yamakazi, 1994; (60) O'Dell, 1993; (61) O'Dell *et al.*, 1989; (62) O'Dell and Kaiser, 1995; (63) Orgad *et al.*, 1997; (64) Orgad *et al.*, 2000; (65) Orgad and Segal, 1995; (66) Peixoto and Hall, 1998; (67) Rendahl, 1992; (68) Rendahl and Hall, 1996; (69) Rendel, 1951; (70) Ringo *et al.*, 1991; (71) Ringo *et al.*, 1993; (72) Romanova *et al.*, 2000; (73) Ryner *et al.*, 1996; (74) Sandrelli *et al.*, 2001; (75) Saudan *et al.*, 2002; (76) Siegel and Hall, 1975; (77) Sturtevant, 1915; (78) Suzuki *et al.*, 1997; (79) Takahashi *et al.*, 2001 (80) Taylor *et al.*, 1994; (81) Tempel *et al.*, 1984; (82) Toivonen *et al.*, 2001; (83) Tompkins *et al.*, 1980; (84) Tompkins *et al.*, 1982; (85) Tompkins *et al.*, 1983; (86) Tompkins and McRobert, 1995; (87) Villella and Hall, 1996; (88) von Schilcher, 1976; (89) Waterbury *et al.*, 1999; (90) Wheeler *et al.*, 1989; (91) Wilson *et al.*, 1976; (92) Wolfner *et al.*, 1997; (93) Wood and Butterworth, 1972; (94) Yamamoto *et al.*, 1997; (95) Yokokura *et al.*, 1995; (96) Zhang and Odenwald, 1995.

Table 1.1

Name	Abbr.	C. L.	Molecular Function	Behavior	Description
a la Voila et a la vapeur	*Voila*	86E1-2	unknown	Courtship behavior	Male-male courtship[5, 34]
Accessory gland-specific peptide 26Aa	*Acp 26Aa*	26A1	hormone	Postmating behavior	Acts in the female to stimulate the release of oocytes by ovaries[36]
Accessory gland-specific peptide 26Ab	*Acp 26Ab*	26A1	hormone	Postmating behavior	Acts to reduce female receptivity; resists displacement of sperm by subsequent sperm[13]
Accessory gland-specific peptide 32CD	*Acp32CD*	32D1	hormone	Female receptivity	Reduced female receptivity[92]
Accessory gland-specific peptide 33A	*Acp33A*	33A	hormone	Female receptivity	Reduced female receptivity[92]
Accessory gland-specific peptide 36DE	*Acp36DE*	36E2	hormone	Postmating behavior	Resists displacement of sperm by subsequent sperm in the female reproductive tract.[13, 52]
Accessory gland-specific peptide 53Ea	*Acp 53Ea*	53C14	hormone	Postmating behavior	Resists displacement of sperm by subsequent sperm in the female reproductive tract.[13]
Accessory gland-specific peptide 70A	*Acp70A*	70A4	hormone	Female receptivity	Reduces female receptivity;[56, 26] stimulates oviposition[56, 26]
Accessory gland-specific peptide 98AB	*Acp98AB*	98B1	hormone	Female receptivity	Reduces female receptivity[92]
amnesiac	*amn*	18F4-19A2	neuropeptide hormone	Conditioning; memory	Reduced female song memory;[46] males not conditioned by courtship of fertilized females[1, 76]
apterous	*ap*	41F8	zinc ion binding; specific RNA polymerase II activity	Courtship behavior; Female receptivity	Reduced courtship behavior;[17, 71] reduced female receptivity[70, 71]
bifold (aka *optimotor blind*)	*bi*	4C3-4	transcription factor; RNA polymerase II transcription factor; transcription regulator	Courtship behavior	Reduced ability to track females[14, 84]
Btk family kinase at 29A (aka *fickleP*)	*Btk29A*	29A1	protein tyrosine kinase; receptor signaling protein tyrosine kinase	Copulation	Reduced copulation duration[3]

Table 1.1 (continued)

Calcium calmodulin kinase II	*CaMKII*	102D1-2	protein serine/threonine kinase; calmodulin binding; calcium/calmodulin-dependent protein kinase	Courtship behavior	Males not conditioned by courtship of fertilized females[39]
cabbage	*cab*	11A2-3	unknown	Courtship behavior	Reduced courtship behavior[28]
cacophony	*cac*	10F8-11	voltage-gated calcium channel	Courtship behavior; song	Abnormal song pulse[6, 88]; courtship defective[6]
celibate	*cel*	12E	unknown	Copulation	Males court but do not copulate[8]
chaste	*chaste*	54A	unknown	Female receptivity	Reduced female receptivity[94]
coitus interruptus	*coi*	7D	unknown	Courtship behavior; Copulation	Reduced courtship behavior;[8] reduced copulation duration[8]
courtless	*crl*	14F1	ubiquitin-conjugating enzyme	Courtship behavior	Reduced courtship behavior[63, 64]
croaker	*cro*	45E	unknown	Courtship behavior; Song	Reduced mating success;[95] aberrant courtship song[95]
cuckold	*cuc*	27F6	unknown	Courtship behavior	Males can not court or mate[12]
curved	*c*	52D3-7	unknown	Courtship behavior	Reduced courtship behavior[66]
desaturase 2	*desat2*	87B10	stearol-CoA desaturase activity	Pheromones	Removes isolation between two populations[79]
dissatisfaction	*dsf*	26A1-2	ligand-dependant nuclear receptor; transcription factor; steroid hormone receptor	Courtship behavior; Female receptivity	Reduced female receptivity;[24, 25] defective male abdominal curling;[25] male-male courtship[24, 25]
don giovanni	*dg*	5C2	unknown	Conditioning	Males not conditioned by courtship of fertilized females[31]
Dopa decarboxylase	*Ddc*	37C1	aromatic-L-amino acid decarboxylase	Conditioning	Males not conditioned by courtship of fertilized females[81]

Table 1.1 (continued)

doublesex	*dsx*	84E5-6	DNA binding activity; transcription factor; RNA polymerase II transcription factor; specific RNA poly. II transcription factor; mRNA binding activity; zing ion binding	Courtship behavior; pheromones; Song; Female receptivity	Defective female pheromone production;[38] defective male courtship and song;[2] male-male courtship;[2] increased female receptivity.[87]
Ductus ejaculatorius peptide 99B	*Dup99B*	99B	Unknown	Female receptivity	Reduces female receptivity;[75] stimulates oviposition[75]
dunce	*dnc*	1A-3C10	cAMP-specific phosphodiesterase; 3',5'-cyclic-nucleotide phosphodiesterase	Female receptivity; Memory	Reduced female song memory;[44, 47] increased female mating[35, 44. 47]
ebony	*e*	93D1	beta-alanyl-dopamine synthase	Courtship behavior; Song	Reduced courtship behavior;[17] reduced mating success;[69] abnormal song[50]
ether a go-go	*eag*	13A2-5	voltage-gated potassium channel	Courtship behavior; Conditioning	Males not conditioned by courtship of fertilized females;[15] reduced courtship behavior[15]
flamenco	*flam*	20A1-2	unknown	Courtship behavior	Reduced courtship and altered sequence of behaviors[72]
FmrI	*Fmr1*	85F10-11	mRNA binding activity; RNA binding activity	Courtship behavior	Reduced courtship behavior[19]
freeze	*fez*	22D	unknown	Courtship behavior	Reduced courtship behavior[58, 59]
fruitless	*fru*	91A8	Zinc finger family transcription factor; RNA polymerase II transcription factor	Courtship behavior; Song	Abnormal song production;[73, 90] defective male abdominal curling;[32] reduced courtship;[4] male-male courtship[4]
he's not interested	*hni*	89E7-90A7	unknown	Courtship behavior	Reduced courtship behavior[9, 27]
icebox	*ibx*	7E10-8A5	unknown	Female receptivity	Reduced female receptivity[40]
inactive	*iav*	7A5-C1	ion channel	Courtship behavior; Female receptivity; Conditioning	Reduced courtship behavior;[61] Reduced female receptivity;[60] males not conditioned by courtship of fertilized females[60]

Table 1.1 (continued)

Intersex	*ix*	47F5	protein binding	Courtship behavior	Reduced courtship behavior [54]
lingerer	*lig*	44A4	unknown	Courtship behavior; Copulation	Reduced courtship behavior;[43] Reduced frequency of copulation[43]
nerd	*nerd*	3rd Chr.	unknown	Copulation; Pheromones	Reduced frequency of copulation;[22] reduced pheromone production[22]
no on or off transient A (aka *dissonance*)	*nonA*	14B18-C1	RNA binding; pre-mRNA splicing factor; poly-pyrimidine tract binding	Courtship behavior; Song	Reduced mating success;[67, 68] aberrant courtship song[6, 41, 42, 74, 90]
okina	*okina*	2nd Chr.	unknown	Copulation	Reduced copulation duration[94]
pale	*ple*	65C3	tyrosine 3-monooxygenase	Courtship behavior	Abnormal courtship behavior[10, 57]
paralytic	*para*	14D1-E1	voltage-gated sodium channel	Courtship behavior; Song; Female receptivity; Conditioning	Reduced mating success;[30, 51, 53, 83] aberrant courtship song;[66] Reduced female receptivity;[30, 53, 84] males not conditioned by courtship of fertilized females[85]
period	*per*	3B2-3	transcription co-repressor; transcription cofactor	Courtship behavior; Song	Defective courtship song,[16, 21, 45, 48, 49] reduced courtship success[37, 44]
platonic	*plt*	unknown	unknown	Copulation	Reduced frequency of copulation[94]
quick-to-court	*qtc*	25C3-4	unknown	Courtship behavior	Quick initiation of courtship in males;[33] male-male courtship[33]
Rutabaga	*rut*	12F4	adenylate cyclase; calcium/calmodulin-responsive adenylate cyclase	Courtship behavior; Conditioning; Female memory	Reduced courtship behavior;[29] Females have reduced song memory;[46] males not conditioned by courtship of fertilized females[29, 46]
Sex lethal	*Sxl*	6F5	RNA binding activity; pre-mRNA splicing factor; translation repressor; nucleic acid binding; mRNA 5' UTR binding	Courtship behavior; Pheromones	Reduced courtship behavior;[86] Females synthesize inhibitory pheromones[86]

Table 1.1 (continued)

Shaker	*Sh*	16F4-5	voltage-gated potassium channel	Conditioning	Males not conditioned by courtship of fertilized females[15]
slowpoke	*slo*	96A14-17	calcium-activated potassium channel	Song	Aberrant courtship song[66]
spinster	*spin*	52E5-8	membrane protein	Female receptivity	Reduced female receptivity[55, 78]
stuck	*sk*	3rd Chr.	unknown	Copulation	Inability to terminate copulation[20]
takeout	*to*	96C7	unknown	Courtship behavior	Reduced courtship behavior[18]
tan	*t*	8A1-B8	beta-alanyl-dopamine hydrolase	Courtship behavior	Reduced courtship behavior[14, 84]
tapered	*ta*	46C3-11	unknown	Courtship behavior	Reduced courtship behavior[7, 93]
technical knockout	*tko*	3A3	structural constituent of ribosome	Courtship behavior	Reduced courtship success[20, 82]
timeless	*tim*	23F6	interacts with Per	Song	Defective courtship song[49]
transformer	*tra*	73A10	pre-mRNA splicing factor	Courtship behavior; Song;	Reduced courtship behavior;[23, 80, 89] abnormal song;[6] male-male courtship[23, 25, 62, 89]
transformer 2	*tra2*	51B6	RNA binding activity	Female receptivity	Reduced female receptivity[59]
turnip	*tur*	18A5-D1	unknown	Conditioning	Males impaired in conditioning after courting fertilized females[28, 29]
Ubiquitin-conjugating-enzyme-47D	*Ubc47D*	47D	ubiquitin conjugating enzyme	Courtship behavior	Defective male courtship behavior[65]
white	*w*	3C1	ATP-binding cassette (ABC) transporter; eye pigment precursor transporter; transmembrane receptor	Courtship behavior	Reduced courtship behavior;[77, 96] male-male courtship[96]
yellow	*y*	1A5	unknown	Mating success	Reduced male mating success[11, 91]

Courtship anomalies

There are several mutations in genes for which males rarely court females. These include *courtless* (*col*) (Yamamoto and Nakano, 1998), *cuckold* (*cuc*) (Coyne, 1989), *he's not interested* (*hni*), (Yamamoto *et al.*, 1997), *pale* (*ple*) (Buchner, 1991; Neckameyer, 1998), and *tapered* (*ta*) (Bien-Willner and Doane, 1997). While courtship activity is not completely blocked in any of these mutants, some show a delayed age-of-onset of courtship, and all show a reduced frequency of courtship. Several of the mutants also court males as well as females. For example, males mutant at the *ta* locus do not court females for about a week after eclosion and show a low level of courtship thereafter without copulation. *Voila1/+* mutants exhibit strong bisexual behavior while *Voila1 UAS-tra* mutants show a complete block of courtship behavior that is correlated with the manipulation of a restricted part of the nervous system, namely the mushroom bodies (Balakireva *et al.*, 1998). In contrast, while *quick-to-court* males have a high level of male-male courtship, they also have an abnormally quick onset of courtship when placed with virgin females (Gaines *et al.*, 2000).

The average copulation duration for *D. melanogaster* is about 18 minutes, but averages range from species to species in *Drosophila* from 30 seconds (*D. mulleri*) to over two hours (*D. acanthoptera*) (Markow, 1996). Multiple mutations have been identified that alter the duration of copulation. Copulations involving the *coitus interruptus* (*coi*) male are an average of 40% shorter than those of a wild-type male (Hall *et al.*, 1980). On the other hand, males mutant for *stuck* (*sk*) cannot withdraw even though they try to dismount. Copulation can last anywhere from minutes to days, the

longest cases ending in death (Hall *et al.*, 1980).

Typically, virgin females over three days old are very receptive to courting males (Markow, 1996). However, virgin females mutant at the *spinster* (*spin*) (Suzuki *et al.*, 1997) or *chaste* (*cht*) (Yamamoto *et al.*, 1997) loci persistently reject male advances. The mutation *dissatisfaction* (*dsf*) (Finley *et al.*, 1997) not only reduces a female's receptivity, but also prevents her from laying eggs after successful copulation due to a lack of motor innervation in the uterus.

Song mutants

One of the most successful examples of mutant behavioral studies involves the rhythmicity genes *period* (created through EMS mutagenesis) and *timeless* (created through *P*-element mutagenesis) (Konopka and Benzer, 1971; Kyriacou and Hall, 1980; Sehgal *et al.*, 1994). While these genes were originally discovered to affect the circadian rhythm of *Drosophila* (Konopka and Benzer, 1971), it was later shown that they affect the oscillation frequency of the courtship song as well (Kyriacou and Hall, 1980).

The courtship song consists of two species-specific components: the pulse song, which is comprised of tone pulse trains, and the sine song, which is a modified sine wave of around 160 Hz manifested as occasional bursts of humming (Yamamoto *et al.*, 1997). In *Drosophila*, there is a succession of temporally regulated events that regulate the sine wave oscillation frequency, involving delays between peak levels of *period* (*per*) and *timeless* (*tim*) mRNA and protein, subsequent post-translational modification, PER-TIM dimerization, and final transport of PER-TIM into the nucleus where it acts to modulate

the circadian and wing beat rhythms (Rosato *et al.*, 1997). A handful of auxiliary genes have also been identified that encode proteins that assist in the formation and transport of the PER-TIM dimer (Rosato, *et al.*, 1997; Yamamoto *et al.*, 1997). Mutation of the *per* or *tim* genes, or one of their associated factors, can lengthen or shorten the sine curve of the courtship song, or abolish the song's rhythm altogether (**Figure 1.2**).

The sex-determination pathway

The primary determinant of sex in *Drosophila* is the ratio of X chromosomes to sets of autosomes (X:A ratio). Information about this ratio is facilitated through maternal and genetic components and then passed through a cascade involving *Sex-lethal* (*Sxl*), *transformer* (*tra*), *transformer-2* (*tra-2*), *doublesex* (*dsx*), and *fruitless* (*fru*) (**Figure 1.3**) (Cline, 1993; Barbash and Cline, 1995; MacDougall *et al.*, 1995; Finley *et al.*, 1997; Schütt and Nöthiger, 2000). The execution of sexual behavior depends upon the integrity of *Sxl*, *tra*, *tra-2*, and *fru*, but not on *dsx* (Schütt and Nöthiger, 2000). It was known that the sex-determination pathway could affect courtship behavior, so when *dsx* mutants were shown to have normal courtship behavior and normal formation of the Muscle of Lawrence, features that are disrupted when the preceding parts of the sex-determining pathway were interrupted, the *fru* branch of the sex-determining pathway was discovered (Taylor *et al.*, 1994). A disruption of *fru* or one of its predecessors can cause abnormal courtship behavior by altering the "sexual orientation" of regions of the fly responsible for generating the behavior. *fru* males court other *fru* and wild-type males, form long "courtship chains" with other *fru* males, stimulate normal males to court them, and do

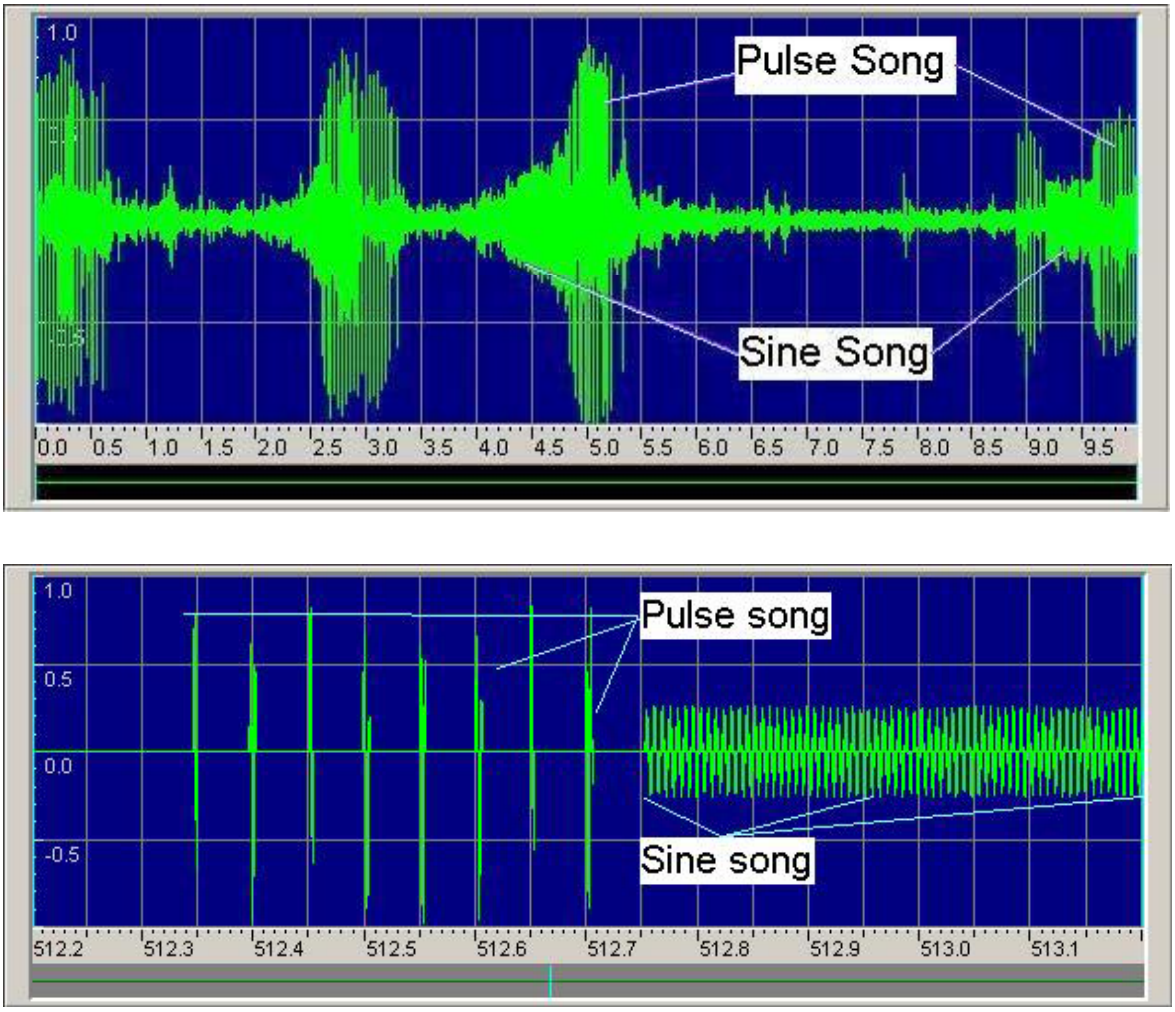

Figure 1.2: The sine wave of the *D. melanogaster* courtship song. In the normal song the interval between each beat (top) increases gradually over about 27 seconds and then decreases equally gradually. The top figure shows naturally-occurring courtship song while in the bottom figure the pulse and sine components have been artificially separated to allow for visualization of the separate components. Units on the X axis are in seconds. Figures from the web page of Becky Talyn <http://www.denison.edu/~talynb/Drosophila.html>

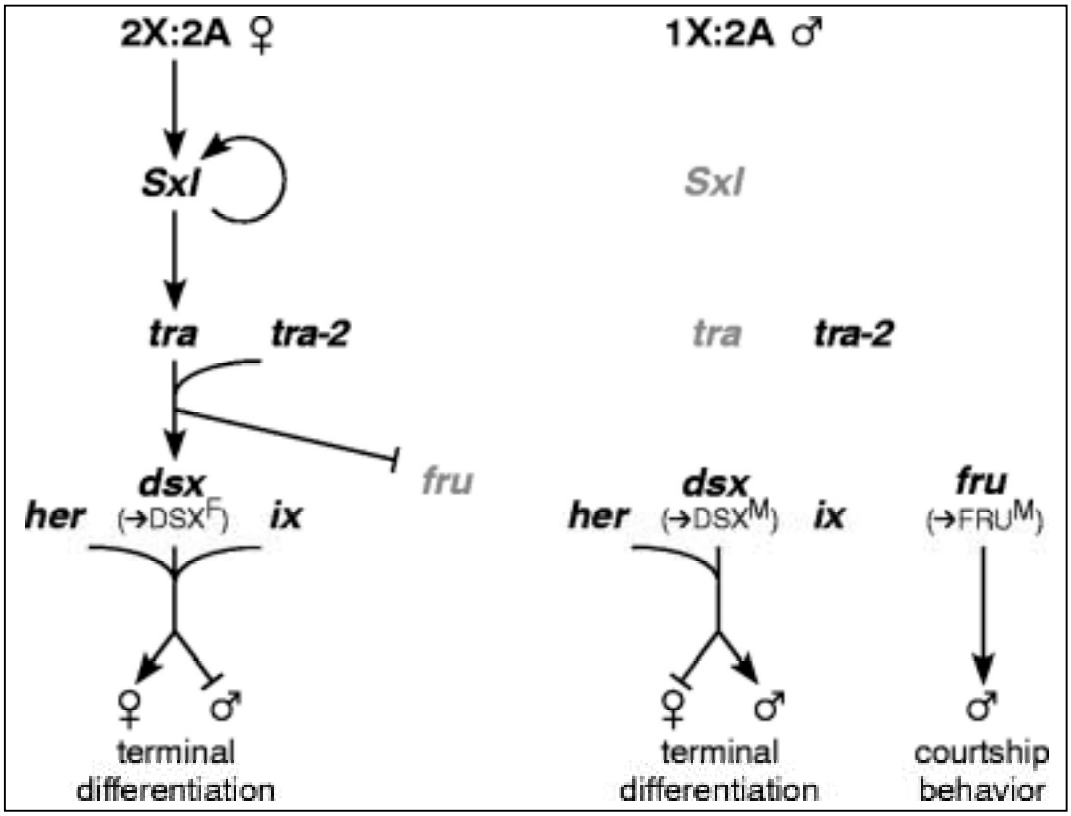

Figure 1.3. Genetic network regulating sexual differentiation. The genes forming the primary signal encode transcription factors; *Sxl*, *tra*, and *tra-2* encode proteins involved in RNA processing and act at the post-transcription level. Figure 1 from Garrett-Engele *et al.* (2002).

not attempt copulation with females (Kyriacou and Hall, 1994; MacDougall *et al.*, 1995). Males with severe mutations in *fru* no longer follow other flies (male or female), play a courtship song, or attempt copulation (Roush, 1996).

To identify the regions of the brain that are responsible for the male courtship behavior, a *UAS-transformer* construct has been utilized (Ferveur *et al.*, 1995). The female-spliced form of the *transformer* gene switches cells in an otherwise male fly to a female pattern of development. Lines in which *GAL4 P*-element constructs have been mobilized to different genomic locations were crossed to those carrying the *UAS-tra* construct. The *transformer* gene was activated in all of the tissues that express *GAL4*, creating tissue-specific feminization. Behavioral assays revealed that some of the transformed male flies courted males as well as females due to feminization of regions of the antennal lobe or mushroom bodies (Balakireva *et al.*, 1998), and that some of the transformed males were courted by other males due to the feminization of the pheromone profile (Ferveur *et al.*, 1997). Feminization of the antennal lobe and mushroom bodies disrupted the male's ability to sense male-specific (courtship inhibitory) pheromones, suggesting that these regions are involved in processing pheromonal signals. It has since been determined that pheromonal cues are first received in the antennal lobe of the brain, then sent to the mushroom bodies, where the olfactory information is processed (Balakireva *et al.*, 1998; Hall *et al.*, 1980). A mutation in the mushroom bodies could therefore remove the essential ability to identify the sex and mating status of another fly, drastically altering the courtship behavior.

Drosophila pheromones

Pheromones are chemical signals produced by an organism that modify the behavior or physiology of another organism of the same species (Karlson and Lüscher, 1959). Although it was long suspected that a chemical form of communication existed, it was not until the late 1950's that these molecules were isolated when Karlson and Butenandt extracted pheromones from the gland of the female silk moth (*Bombyx mori*) that attracted conspecific males from a long distance (Karlson and Butenandt, 1959). Since that time, pheromones have been discovered in many species, principally in insects. Although the major focus of research has been in moths, there has also been a concerted effort to characterize pheromones in *Drosophila* due to the large amount of supplemental information available on other molecular systems in the organism.

Like other insects, *Drosophila* have an outer layer of lipids and cuticular hydrocarbons to prevent desiccation. In *Drosophila*, this outer layer also acts as female and male contact pheromones (Cobb and Jallon, 1990; Coyne, 1996a). *Drosophila* males receive species-specific pheromone cues that allow them to distinguish the female's species, reproductive maturity, and most importantly, if they are a female and not a male (Jallon, 1984). *Drosophila* pheromonal hydrocarbons are long-chain (20-30 carbons) relatively non-volatile compounds (Cobb and Ferveur, 1996). *Drosophila* express 12 to 17 different hydrocarbons (Cobb and Jallon, 1990), of which 1-3 predominate and act as sex pheromones during courtship behavior (Ferveur, 1997). For example, females of the *D. melanogaster* species sub-group primarily express *cis* 7-pentacosene (7-P), *cis* 7-tricosene (7-T), *cis*,*cis* 7,11-heptacosadiene (7,11-HD) or *cis*,*cis* 5,9-heptacosadiene (5,9-

HD) with minor presence of one, two or all three of the other pheromone compounds. Males of this sub-group show major expression of 6-tricosene, 7-T or 7-P. These compounds differ only in the total number of carbons and the position of the double bond. The female's pheromones stimulate a conspecific male, and can be inhibitory when the male is heterospecific if the two species' females differ in their pheromone profiles. However, males will still court and mate females who have had all of their cuticular hydrocarbons eliminated (Savarit *et al.*, 1999), suggesting that although pheromones can act to inhibit courtship and copulation, they alone do not elicit a behavioral response. Male pheromones, on the other hand, can act to inhibit the courtship behavior of other males, reducing the frequency of homosexual courtship.

Drosophila pheromones are hydrocarbons, not proteins, so there is an indirect genetic control of pheromone production. Mutations that change the phenotypic sex of the fly (*Sxl*, *tra*, *dsx*) similarly alter the pheromone profile. A mutation in the *fru*, *nerd*, or *seven monoene-quantity* genes of *D. melanogaster* decrease the amount of 7-T produced by the male, while a mutation at the *seven pentacosene* locus will alter the 7-T:7-P male hydrocarbon ratio (Cobb and Ferveur, 1996). A naturally-occurring polymorphism at the *desaturase 2* locus affects a pheromonal polymorphism that reduces mating between two divergent populations of *D. melanogaster* (Takahashi *et al.*, 2001).

The male can also identify if the female has mated another male recently through the presence of anti-aphrodisiac pheromones deposited by another male (Jallon, 1984). The male then "remembers" not to copulate with a previously-mated female, decreasing his chance of encountering sperm competition (Price, 1997; Tully *et al.*, 1994). In fact, a

male will not court even a virgin female after exposure to a mated female, either due to a residual effect of the anti-aphrodisiac pheromones or due to learning (Kyriacou and Hall, 1994). The memory mutants *dunce* (Dunwalder and Davis, 1995), *turnip* (Tully, 1987), *rutabaga* (Levin *et al.*, 1992), *amnesiac* (Feany and Quinn, 1995), *cabbage* (Davis and Dunwalder, 1991) and *Don Giovanni* (Gailey and Hall, 1989) are all deficient in the experience-dependent modification of courtship, mostly through the disruption of CaM kinase which is necessary for learning and memory (Griffith *et al.*, 1993).

Commonalities of genes coding for mating behavior

Genes that affect mating behavior are scattered throughout the genome (**Table 1.1**), but this is not an even distribution. While the second and third chromosomes are much larger than the *X*, the *X* chromosome contains the same number of mating behavior genes as chromosome *2* and almost twice the number of genes found on chromosome *3*. This could mean that genes affecting mating behavior are located preferentially on the *X*, or more likely, that studies on mating behavior have focused primarily on the *X*, and therefore more genes have been identified on that chromosome than the autosomes. The genes also code for a wide array of products, ranging from membrane proteins and hormones to transcription factors. Although many of the gene products are unknown, it is still clear that a large proportion of mating behavior genes code for transcription factors. This supports the notion that genes that control mating behavior in *Drosophila* often have pleiotropic effects (Sokolowski, 2001), which could easily result if their gene products affect the transcription of genes other than those coding for behavior. It also explains

how a single mutation can affect a complex trait such as behavior: a single mutation preventing the formation of a transcription factor can affect the transcription of many downstream genes.

FUTURE PROSPECTS

Most studies which use mutants to identify genes affecting mating behavior did not create the mutants specifically for that purpose. Usually known mutants that are readily available from a stock center are assayed, rather than a specific broad saturation screen for mutants affecting behavior. In fact, the identification of behavioral mutants often occurs by accident while the researcher is investigating another trait. Since these studies were not focused on the complex behavior, the genetic background of the mutant flies is not controlled for, nor are there co-isogenic controls used as a comparison. Epistatic interactions present in some mutant stocks and not others could therefore be common, which can confound results. This necessity for a co-isogenic background is confirmed in studies that have screened a large number of individuals for each line containing a single *P*-element insertion (Lyman *et al.*, 1996; Anholt *et al.*, 2001). Under these conditions, mutations with subtle effects on quantitative traits, which are more likely to mimic natural variants, can be discriminated. In spite of these complications, many mutants affecting mating behavior have still been identified. If this technique were used to its full potential, with a large panel of mutants in an isogenic background and co-isogenic control lines, the number of genes known to affect mating behavior would undoubtedly be expanded.

Novel genes affecting mating behavior could potentially be identified through the use of microarrays, which have already been used to identify putative candidate genes affecting olfaction (Anholt *et al.*, 2003). By removing or altering expression of a single gene in an isogenic background and comparing whole-genome expression levels to a co-isogenic control, one can reveal the epistatic effects that gene has on other genes involved in that behavior pathway. The genes that are uncovered by this technique could then hypothetically be tested individually for their test on behavior via RNA interference (RNAi).

An RNAi construct can be used to eliminate the function of a gene suspected to affect behavior in *Drosophila* (Fire *et al.*, 1998; Kennerdell and Carthew, 1998). The RNAi construct is composed of a cDNA and a genomic construct that are ligated together into a vector that can be injected into *Drosophila* eggs. The presence of double-stranded RNA interferes with the normal RNA produced by the gene of interest, eliminating its ability to function. Original RNAi constructs involved the injection of double-stranded RNA, whose effect is largely embryonic due to its eventual degradation. Genomic cDNA fusions of RNAi constructs containing introns allow for the formation of hairpin dsRNA, enabling for a continuous supply of RNAi (Kalidas and Smith, 2002). This provides the means to study adult phenotypes. The RNAi construct could potentially be paired with a heat shock promoter, which could allow for the study of genes that cause lethality when eliminated during development, but produce a milder adult phenotype if gene function is removed after development is complete. This technique has been used to successfully target the *Drosophila* olfaction gene *lush* (Kalidas and Smith, 2002), demonstrating

RNAi's substantial potential in the discovery or characterization of behavior genes.

QUANTITATIVE GENETICS OF BEHAVIOR

While the disruption of single genes has been used to identify a plethora of genes that can affect mating behavior, it is only through naturally-occurring allelic variation that a population can respond to selection. *Drosophila* typically show a wide intra- and inter-specific variation in most aspects of courtship, such as copulation latency (time to mating) and duration (total copulation time) (Markow, 1996). This broad range in phenotypes suggests that quantitative trait loci (QTL), multiple loci whose effects can be influenced by the environment, are responsible for the extensive variation seen in mating behavior. However, the variation and complexity of mating behavior complicates the process of separating out the individual genetic components that add together to form the whole of courtship behavior.

Studies that seek to identify QTL responsible for mating behavior have tended to focus on loci contributing to reproductive isolation in pairs of species that exhibit normal mating when males of one species are paired with females of the other, but an absence of mating when the reciprocal cross occurs. In this way, recombinants can be created and tested for mating or no mating to identify loci contributing to mate preference, a relatively simple behavioral trait to measure. Due to the inadequate number of molecular markers available for genotyping at the time, chromosome substitution lines had previously been employed to localize candidate regions to the *Y* chromosome (Zouros, 1981), *X* chromosome (Coyne, 1989; Coyne, 1993; Coyne, 1996c), and each of the major

autosomes (Coyne *et al.*, 1999; Coyne, 1989; Coyne, 1992; Coyne, 1993; Coyne, 1996c; Zouros, 1981). Other studies on pheromone perception have isolated genes responsible for this trait to the *X* (Coyne, 1996a), second (Coyne, 1996a), and third chromosomes (Coyne and Charlesworth, 1997; Coyne *et al.*, 1994; Coyne, 1996a; Coyne, 1996b). Combined, these studies have found candidate regions in almost the entire *Drosophila* genome, the chromosome of the largest effect depending on the species being examined and the laboratory in which the study was performed. To date, the smallest "candidate region" for mating behavior QTL using this method has been the larger portion of a chromosome arm (Ting *et al.*, 2001). Given the large number of candidate genes that fall within a region of that size, the localization of QTL for mating behavior through the more precise mapping techniques currently available is necessary.

It has long been hypothesized that the loci contributing to quantitative traits vary in the size of their effect (Robertson, 1967). Large sample sizes are required to detect QTL with small effects, and to map QTL to the level of individual genes (McClearn, 1997). While QTL analysis has led to the identification of new sets of loci, ones with important biological roles (Mackay, 2001), molecular characterization of these loci remains difficult (Glazier *et al.*, 2002), hindering the identification of genes that might affect behavior (Liu, 1997).

In order to locate QTL, one must have two lines with divergent behavioral phenotypes and a large number of molecular markers spanning the genome (Zeng, 1993). Crossing the two lines to create backcross, F_2 or recombinant inbred progeny produces a segregating mapping population. Recombinant inbred lines are especially useful for traits

that have a low heritability, such as mating behavior, since a single genotype can be tested multiple times. Each individual (or multiple individuals per line, in the case of recombinant inbred lines) is assayed for behavior. For a behavior that is easily affected by the environment, a large sample size is necessary in order to surmount the high level of standing variation and subsequently localize QTL.

Alleles that are polymorphic between the two parental lines, but fixed within each line, can be used as molecular markers to genotype all individuals of the mapping population. To have a high level of confidence in the estimated QTL position, one would want markers to be evenly-distributed and at a high density (Jayakar, 1970). When using visible markers to locate QTL for a trait, the effects of the markers themselves on that trait must either be readily assessed or generally ignored; this is difficult to accomplish for behavioral traits. Several studies have shown effects of the markers themselves on the mating behavior of *Drosophila*, such as the eye-color marker *white* (Hall, 1994). This is no longer a problem, however, with the advent of multiple molecular markers, which are presumably neutral.

QTL are mapped by linkage to molecular markers if the difference in behavior in the mapping population is associated with the marker genotype. A comparison between genotype and phenotype can be done using several different statistical techniques. The simplest approach is single marker analysis, which examines differences in trait means between marker genotypes one marker at a time. This technique tends to underestimate the QTL effect and confounds effect and position (Falconer and Mackay, 1996). Interval mapping examines whether a QTL lies between two adjacent markers. While this is an

improvement in single marker analysis in that it can estimate the position and effect of QTLs, it does not account for the effects of QTL outside of the interval (Falconer and Mackay, 1996). Composite interval mapping (CIM) allows for the identification of QTL in the same manner as interval mapping by testing whether an interval between two markers contains a QTL affecting the trait, but simultaneously controls for the effect of QTL located outside of the interval by using a likelihood estimate (Zeng, 1994; Xu and Atchley, 1996). The power of CIM in detecting QTL has been repeatedly demonstrated by follow-up studies to fine-map QTL (e.g. Pasykova *et al.*, 2000; Fanara *et al.*, 2002; De Luca *et al.*, 2003). The most advanced technique to date is multiple interval mapping (MIM) (Kao *et al.*, 1999) which is similar to CIM but can include both main effects and epistatic interactions. MIM is limited in practicality, however, since a very large sample size is required for its application and no studies have confirmed its accuracy through higher resolution mapping to date. For these reasons, CIM is the QTL mapping technique of choice. CIM does not in itself allow for the identification of any specific candidate genes since the mapping populations tend to be small and the intervals between markers tend to be large. The identification of candidate genes for mating behavior is further hindered due to the notion that almost any gene could potentially have a direct or indirect effect on behavior. Fine-mapping techniques are necessary to further reduce the potential number of candidate genes.

In most organisms, high-resolution recombination mapping is necessary in order to further reduce the size of a candidate region, which has been done to successfully identify a gene (*fw2.2*) involved in tomato fruit size (Frary *et al.*, 2000). This technique

can be time consuming and difficult since a large number of recombinants and a large number of molecular markers are needed in order to narrow down candidate regions to a manageable size. For example, 100 flies are needed to get just one recombinant in a 1 cM interval, and 1000 flies are needed to get a single recombinant in a 0.1 cM interval, and a trait with a high level of variation, such as behavior, will require multiple recombinants in order to definitively map a region. Markers are also needed for each interval tested – the smaller the refinement, the greater the number of markers. The use of recombinant isogenic lines makes the task of mapping more manageable since behavioral assays are only performed for informative recombinants and all flies of a single line are of the same genotype and therefore a particular genotype can be tested multiple times.

Quantitative genetic mapping with CIM has not been used to test for mating behavior directly, but two studies that focused on aspects that could potentially affect mating success have been completed. In a study by Nuzhdin *et al.* (2000), recombinant inbred lines were used to map loci affecting variation in sex comb tooth number in *Drosophila* to two regions on the *X* chromosome (3E-6E and 12E-19A). Gleason *et al.* (2002) mapped loci for interpulse interval in the *Drosophila* courtship song using these same recombinant inbred lines and identified one QTL on the second chromosome (22F-29F) and two on the third (61A-65D and 67D-69D). It is immediately apparent that the QTL for these two traits do not overlap, suggesting that each component of mating behavior might be controlled by different loci, although it is not certain that the traits that were measured have any effect on mating success in the two parental lines used to create the recombinants.

In *Drosophila*, there are unique tools beyond recombination mapping available for QTL mapping. One such technique is the use of deficiency lines (Pasyukova *et al.*, 2000; Fanera *et al.*, 2002; De Luca *et al*., 2003). In these lines, a small region of the genome is hemizygous, or possessing only one allele with the other being completely absent. These lines are usually homozygous lethal, and are therefore maintained over a balancer (*Bal*) chromosome which suppresses recombination and contains a dominant visible marker so one can determine whether an F_1 fly received the wild-type chromosome or the deficiency (*Df*) chromosome. One can select multiple deficiency lines with short overlapping segments within the candidate regions to determine which portions of the region contributed to the variation in behavior. One can cross these deficiency lines to the parental lines (P_1, P_2) to create four genetic classes of offspring: Df/P_1, Df/P_2, Bal/P_1, Bal/P_2. By using a 2-way ANOVA, one can detect if quantitative complementation occurs when $(Df/P_1 - Df/P_2) = (Bal/P_1 - Bal/P_2)$ and there are no QTL present in a region, or if quantitative failure to complement occurs when $(Df/P_1 - Df/P_2) \neq (Bal/P_1 - Bal/P_2)$ and there is a QTL within the interval. Lines that exhibit the alternate behavioral phenotype have a candidate gene within the region of the deficiency. Comparing across the overlapping regions of the deficiencies, one can narrow the region further (if one deficiency shows the alternate phenotype while a partially overlapping deficiency does not, one can also rule out the overlap region as a candidate region). Although this method does not usually reduce candidate areas to the gene level, it does reduce the potential number of candidate genes. Genes for which a mutant stock is available can then be tested in the same manner as the deficiencies to further refine the list of candidate genes.

One downside to deficiency mapping techniques is that epistatic interactions, which undoubtedly occur among behavior QTL, can not be easily identified or isolated. New methods of mapping QTLs that can recognize and isolate epistatic interactions would greatly facilitate the search for behavioral QTL. An additional difficulty occurs when attempting to identify putative candidate genes since there are many genes for which there is no mutant stock available, making it impossible to test every gene within a candidate region.

CONCLUSIONS

Understanding the genetic basis for behavior in *Drosophila* can open the door for behavioral genetics research in a multitude of other species including humans. The recently completed *Drosophila* genome project will allow further insight into the underlying genetic components of behavior. The genome project identified ~13,600 genes, of which only a portion have been molecularly or phenotypically characterized (Adams *et al*., 2000). Even though less than one-third of mutated genes have obvious phenotypes, there are a multitude of single genes that have been identified as influencing mating and courtship behavior, providing the genetic framework by which the formation of sexual orientation, neural processing of external stimuli, and manifestation of response are built. With the genome sequence in hand, transposon-induced mutations can now easily be physically mapped by identifying the short segment of genomic sequence flanking either side of the insertion site, expanding our current knowledge of the location of genes affecting behavior and their distribution in the genome. Sequence

polymorphisms between strains of *Drosophila* can also be directly identified for recombination mapping of induced mutations and QTL (Kornberg and Krasnow, 2000).

The formation of large panels of mutants in isogenic backgrounds will provide a valuable tool with which to identify genes that have a subtle effect on behavior by reducing variation due to epistatic interactions with genes elsewhere in the genome. Genome-wide screens specifically designed for the identification of mating behavior genes will allow for a less biased and more focused identification of the genetic components of behavior. Studying allelic variants of genes affecting mating behavior will provide a more accurate picture of how these genes affect behavior in naturally-occurring populations. The use of binary *P*-element constructs or RNAi can allow for tissue-specific or adult-specific expression, or ihnhibition of expression, of putative candidate genes, providing a mechanism by which these genes can not only be tested, but also allow for the precise characterization of where in the fly these genes are having their effect.

The ability to study the quantitative genetic basis of behavior is relatively new due to the only recent advent of analytical software and large numbers of molecular markers. These advances have caused a heightened interest in studying naturally-occurring variation, since it is this variation which affects subtle changes within populations as well as evolutionary changes between populations. Identification of QTLs for mating behavior will enable further characterization of the components responsible for the variation seen within and among species in the courtship ritual, enhancing the picture of how genes and environment interact to produce the behavior that is expressed. By characterizing the

genetic basis of variation in these traits, we can begin to understand the source of reproductive isolation and speciation.

In Chapter 3, I map QTL affecting courtship occurrence, courtship latency, copulation occurrence and copulation latency which segregate between a *D. melanogaster* strain selected for reduced male mating propensity and a standard wild-type strain. Mating behavior was assessed in a population of recombinant inbred (RI) lines derived from these two strains and QTL affecting mating behavior were mapped by linkage to polymorphic markers using CIM. Subsequent deficiency complementation mapping and quantitative complementation tests at candidate loci revealed seven putative candidate genes that had not previously been implicated as affecting mating behavior: *eagle*, *18 wheeler*, *Enhancer of split*, *Polycomb*, *spermatocyte arrest*, *l(2)05510* and *l(2)k02206*.

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CHAPTER 2

Literature Review:

The Genetic Basis of Speciation in *Drosophila*

SUMMARY

The widely-accepted Biological Species Concept defines species as populations that are reproductively isolated, i.e. are unable to mate with one another or produce viable and fertile progeny when given the opportunity (Dobzhansky, 1935). Reproductive isolation can either arise in allopatry, where populations are separated by a geographical barrier, or in sympatry, where reproductive isolation arises between populations living in the same geographical location. The inability to have progeny can arise from two separate, but not mutually exclusive, paths: prezygotic isolation, where mating or fertilization does not occur, and postzygotic isolation, where mating can occur but the resulting progeny are either inviable or infertile. The concept of reproductive isolation as the definitive basis for speciation has founded numerous theories as to how this isolation arises, and created controversy as to which theory is the most accurate due to the inadequate amount of empirical evidence for the genetic basis of speciation in nature, making it impossible to irrefutably support one particular theory over another. Here I discuss the different theories of speciation and summarize what is known about the genetic basis of speciation.

INTRODUCTION

Species arise when there is no longer any gene flow between two subpopulations, allowing them to evolve along separate paths. In order for speciation to occur, there must be naturally occurring variation for a reproductive trait as well as a period of selection that allows for new mutations affecting reproductive isolation to arise and become distributed throughout the population. While the first requirement is clearly met, the process by which the second requirement is fulfilled, as well as the duration of time necessary for isolation to occur, is the source of much debate.

The first survey of the genetics of species difference was by Haldane in 1938. This was soon followed by one of the first mathematical models for the evolution of reproductive isolation with Wright's analysis of underdominant chromosome rearrangements (reciprocal translocations) arriving at fixation through random drift (Wright, 1941). Genetic drift can potentially produce morphological innovations and adaptive radiation in certain groups, such as the Hawaiian *Drosophila* (Templeton, 1989). Studies of discrete inbred populations have also been used to determine if genetic drift can lead to reproductive isolation. However, these studies have been inconclusive since they have shown that sampling drift can both contribute to and detract from reproductive isolation (Rice and Hostert, 1993).

Reproductive isolation could arise as a chance byproduct of changes in the genetic background that arose through genetic drift, as Wright proposed, or it could appear in response to selection (Muller, 1939). There are two major types of selection to consider, natural and sexual, each of which induce a different systematic response. Natural

selection occurs when individuals that are best suited to a particular environment produce the most offspring; if these offspring also contain genetic combinations that allow them to be well suited to that particular environment, they subsequently will produce the most offspring, and so forth, leading to adaptation (Darwin, 1859). Natural selection could involve a single bout of adaptation to a new optimum (Orr and Coyne, 1992), causing the development of a new species if two subpopulations arrive at different optima. It has been argued that natural selection may not be the driving force behind reproductive isolation since there is a low correlation between adaptive phenotypic traits and premating and postmating isolation (Treganza, 2002). Recombination can also work against natural selection by breaking apart favorable combinations of genes unless nonrandom mating through sexual selection ensures that alleles at different loci remain together. Reproductive isolation can occur through natural selection, however, and has been documented in two populations of *Rhagoletis pomonella*: the haw fly and apple maggot fly. *Rhagoletis* mate and oviposit exclusively on their host plant (Feder *et al.*, 1994). While hawthorn (*Crataegus* spp.) is the haw flies' native host, apple flies have adapted to the introduced, domesticated apple tree (*Malus pumila*). These sympatric populations rarely have the opportunity to mate with one another due to these different mating locales and due to the shorter duration of pupal diapause in apple flies during winter (Filchak *et al.*, 2000; Feder *et al.*, 2003), resulting in their current state of incipient speciation.

Sexual selection occurs when there is differential mating success among individuals within a population, and may underlie a distinct mode of speciation (Coyne,

1992b). This can be through mate choice (preference for one individual over another) or direct competition between members of the same sex (for food resources, through male-male competition, or through sperm competition). Sexual selection can also occur when a change in mate choice has a corresponding secondary sexual trait, as demonstrated in taxa ranging from fish (Basolo, 1990) and frogs (Ryan and Rand, 1993) to spiders (McClintock and Uetz, 1996). This can lead to speciation when the divergence in traits (and subsequently mate choice) can be amplified by female preferences along a cline, leading to prezygotic reproductive isolation (Lande, 1982; Panhuis *et al.*, 2001). Unlike natural selection, sexual selection might involve repeated bouts of co-evolution between male and female traits, yielding a more polygenic basis of speciation (Orr and Coyne, 1992) although the sexually selected trait is not always what is used for species recognition (Boake *et al.*, 1997).

One form of sexual selection is the sensory exploitation model, where females respond to certain signals because of pre-existing properties of their sensory system (Endler and McLellan, 1988; Ryan 1990; Basolo 1990), and so male traits tend to evolve after female preference (Ryan and Rand, 1993). Female sensory biases tend to be generalized, where a variety of male traits could satisfy their requirements; a different male trait could evolve in two populations in response to the same sensory bias. For example, a wing vibration or a leg hum could both satisfy a female's auditory requirement. Each of these modalities will satisfy the female requirement, and therefore not in themselves cause reproductive isolation. But if these divergent traits become associated with another aspect of mating behavior, that trait could lead to isolation.

Aside from whether selection or drift drives speciation is the consideration of what types of populations are able to undergo speciation events. Is a smaller or larger population more likely to segregate into new species? A small population size is associated with increased genetic drift and the subsequent breaking up of coadapted gene complexes, allowing a population to evolve onto new peaks in the adaptive landscape that might be inaccessible to a larger population (Wright, 1931; Mayr, 1963). Larger populations, on the other hand, have a greater level of genetic diversity, reducing the chance of inbreeding depression and inefficient selection, making it more likely for speciation to be able to occur (Fisher, 1930). It seems that the likelihood of speciation depends on both genetic drift, which is negatively correlated with effective population size, and on genetic variation, which is positively correlated with effective population size (Hartl and Clark, 1988), but no general effect of effective population size was found to influence the likelihood of allopatric speciation in a review of 25 laboratory experiments examining allopatric speciation (Florin and Ödeen, 2002). In addition to population size, one must also consider the number and size of bottlenecks (Rundle *et al.*, 1998), selection intensity (Schluter, 1996), and the number of generations which subpopulations are kept isolated from one another (Hostert, 1997; Mooers *et al.*, 1999). Low population density can relax sexual selection since individuals cannot be as choosy when encounters with mates are rare. The mode of speciation therefore might depend on mate choice as well. Speciation may therefore be associated with long periods of allopatry in populations where there is little chance for mate choice, while bottlenecks or

strong selection may be necessary in order for speciation to occur in populations that have mate choice.

GEOGRAPHICAL CONDITIONS FOR SPECIATION

When discussing mechanisms and modes of speciation, it is important to note that there are hundreds of theories of speciation. For example, in the last 25 years, there have been about 100 different mathematical models for the evolution of prezygotic isolation by selection (Kirkpatrick and Ravigné, 2002). In a simplified context, reproductive isolation can arise in allopatry, whereby the populations are completely separated due to a geographical barrier, or in sympatry, where there is no geographical barrier and reproductive isolation results due to the inability to mate or produce viable offspring. This geographical context of mating, either allopatric or sympatric, can itself be viewed as a type of assortative mating (Kirkpatrick and Ravigné, 2002). These theories often go beyond the simple scenario of allopatry or sympatry, and can be divided into three major categories: reinforcement, divergence with gene flow, and bottleneck speciation (for excellent reviews, see Rice and Hostert, 1993; Turelli *et al.*, 2001).

Allopatric Speciation

Allopatric speciation is the most widely-accepted model of speciation (Mayr 1942, 1963; Lynch, 1989; Coyne, 1992b; Rice and Hostert, 1993). Allopatric speciation occurs when new species arise from subpopulations that remain separated by a geographical barrier over a long period of time. Reproductive isolation in allopatry can

potentially arise as a by-product of genetic changes. When a mutation or translocation that arises in one group is absent in the other, allelic incompatibilities could result in sterile or inviable offspring when hybridization occurs (Dobzhansky, 1936; Mayr, 1954; Carson, 1975; Templeton, 1980). The concept of reproductive isolation arising due to the formation of cytological incompatibilities (also called Dobzhansky-Muller incompatibilities) in allopatry is supported since long periods of allopatry are associated with postmating isolation (hybrid inviability or sterility) in the grasshopper *Chorthippus parallelus* (Treganza, 2002) as well as there being a relatively large amount of postzygotic isolation in allopatric *Drosophila* species pairs when compared to sympatric species pairs (Coyne and Orr, 1989, 1997).

Alternatively, reproductive isolation can arise in response to selection. Through natural selection, alleles that are favorable in one environment might be disadvantageous in another. Sexual selection, on the other hand, can cause individuals in each population to mate selectively with their own population (Kaneshiro, 1989). Simply put, given enough time, speciation in allopatry seems inevitable since there is no longer any selection towards maintaining reproductive compatibility. Reduced hybrid fitness under the divergent selection paradigms of pH tolerance (de Oliviera and Cordeiro, 1980) and ethylenediaminetetraacetate (EDTA) tolerance (Robertson, 19661, 1966b) demonstrates that environment-dependent postzygotic isolation can occur in allopatric laboratory scenarios.

Sympatric Speciation

Whether species can arise in sympatry, without the hallmark geographical barrier preventing gene flow in allopatric species formation, has been highly controversial. It has been theorized that isolation can arise in sympatric populations if divergent selection is strong in relation to gene flow; therefore gene flow does not have to be entirely absent for speciation to occur. There are three major factors that could cause sympatric speciation: competition for mates (Higashi *et al.*, 1999; Turner and Burrows, 1995), competition for resources (Kondrashov and Kondrashov, 1999; Dieckman and Doebeli, 1999; Drossel and McKane, 2000), and habitat-specific adaptation (Kawecki, 1996, 1997). Models of mate competition leading to sympatric speciation seem unlikely since the improbable assumption that female preferences are not subject to direct selection must be made in order for sexual selection to render a group to split into reproductively isolated groups rather than shift the entire population towards one optimum or the other (Turelli *et al.*, 2001). However, resource competition is strongly favored as a model due to its ability to cause disruptive natural selection. When a continuum of resources are distributed across a range smaller than that able to be used by the average individual, disruptive selection can arise (Dieckman and Doebeli, 1999). In habitat-specific adaptation models, populations adapt to specific habitats within an environment, as seen in *Rhagoletis*, discussed previously. When there is preferential within-habitat mating, linkage disequilibrium can develop between alleles that are beneficial for surviving in a habitat and behavioral alleles for choosing that habitat (Kawecki, 1996, 1997).

Reinforcement

Whether populations that are allopatric are reproductively isolated can only be tested when those individuals are again placed in contact, either artificially in a laboratory, through the removal of the geographical barrier, or through migration. When these species do come into contact in a natural environment, the (controversial) process of reinforcement may occur. Reinforcement occurs when natural selection directly increases the reproductive isolation between two incipient allopatric species when they become sympatric (Dobzhansky, 1937; Noor, 1995). If hybrids have reduced fitness, there will be selection for positive assortative mating. This is believed to arise when cytological incompatibilities exist since there is a benefit to avoiding maladaptive heterospecific mating and the formation of less fit hybrids. If selection results in complete reproductive isolation, the divergence between populations that began in allopatry will be complete in spite of the potential for gene flow.

It is more common for similar species living in sympatry to be separated by a chromosomal inversion than those living in allopatry (Noor *et al.*, 2001b), suggesting that chromosomal inversions might be a prerequisite for species to be able to endure in sympatry. Assuming a linear relationship between genetic distance and divergence time (Nei, 1987), one can deduce that taxa that become secondarily sympatric speciate in less than a tenth of the time required for allopatric taxa: speciation should take ~200,000 years for taxa that become sympatric and 2.7 million years among taxa that remain allopatric (Coyne and Orr, 1997). There appears to be heightened prezygotic sexual isolation in sympatric species pairs in nature, assumed to arise due to natural selection reinforcing

mate discrimination among some sympatric species (Coyne and Orr, 1989). Increased sexual isolation among sympatric versus allopatric taxa provides minimal evidence of reinforcement (Coyne and Orr, 1989; Butlin, 1995; Noor, 1995), leaving only a tentative inference as to the plausibility of reinforcement models. However, it is possible that increased prezygotic isolation in allopatry is a prerequisite for species to be able to persist in sympatry, rather than it being a cause of sympatry (Templeton, 1981).

One method for experimentally evaluating the feasibility of reinforcement is to pair two genetically marked strains and eliminate all hybrids that result. One study found complete prezygotic isolation as a correlated response to disruptive selection on bristle number in two separately housed populations of *Drosophila* (Thoday and Gibson, 1962), but these results have not been repeated despite numerous attempts (reviewed in Thoday and Gibson, 1970, and Scharloo, 1971). While studies implementing the removal of hybrids have shown that most *Drosophila* laboratory strains have the prerequisite additive genetic variation required for homotypic mating to arise (Rice and Hostert, 1993), the removal of hybrids prevents gene flow (akin to postzygotic isolation) and therefore does not truly test whether reinforcement can bring about reproductive isolation. Studies using lines adapted to different toxic media then placed in a sympatric environment containing both media did not show any incipient reproductive isolation (Robertson, 1996a; Wallace, 1982; Ehrman *et al.*, 1991).

Divergence with Gene Flow

Divergence with gene flow could occur when there is an incomplete or absent

barrier between populations. This can result from a single large population in a uniform environment that experiences divergent selection due to niche-specific adaptations or from a population that has been divided into subpopulations due to partial geographic barriers that restrict gene flow; the two populations evolve according to their specific environment in spite of gene flow. Directional selection on an adaptive trait with a correlated change in a reproductive trait within each subpopulation could result in reproductive isolation. Studies examining reproductive isolation as a pleiotropic response to divergent selection have had both positive and negative results, showing that divergent selection can lead to assortative mating, but not in all cases (Rice and Hostert, 1993).

Bottleneck Speciation

In bottleneck speciation, a major reduction in population size (for example, through colonization) creates the platform for a 'genetic revolution', or major reorganization of the genome, to occur. The new population is genetically distinct, resulting in reproductive isolation from the original population. Studies testing this theory have shown that bottlenecks followed by exponential growth can lead to low levels of reproductive isolation (Powell, 1978; Dodd and Powell, 1985; Meffert and Bryant, 1991), but these levels do not even approach those required for speciation to occur, suggesting that bottlenecks can facilitate, but not induce, speciation (Rice and Hostert, 1993).

MECHANISMS OF REPRODUCTIVE ISOLATION

Populations can remain reproductively isolated through factors prior to fertilization (prezygotic) preventing successful copulation from occurring, or through factors acting after fertilization (postzygotic) resulting in infertile or inviable offspring. Premating and postmating isolation are believed to arise independently due to the presence of only one form of isolation or the other in certain populations (Coyne and Orr, 1989, 1997; Treganza, 2002), and since genes known to affect postzygotic isolation have not been shown to affect prezygotic isolation, suggesting a different genetic basis for these two traits.

Prezygotic Isolation

Prezygotic isolation, or isolation due to factors prior to fertilization, can arise through several different paths. Prezygotic isolation is often asymmetric between two species, with strong isolation only present in one direction of hybridization (Watanabe and Kawanishi, 1979; Kaneshiro, 1980). In one instance, the males of a population might not be attracted to females of another population, and therefore copulation is not even attempted. In another scenario, it is the females who are disinterested, and any advances from the males are firmly rejected. This discrepancy in male traits versus female preference can lead to male-female co-evolution, whereby genes for male characteristics and female preference for those characteristics evolve simultaneously. In terms of selection, individual males that do not display the correct trait with which to attract mates, or females which to not prefer the traits which represent the best mate, would be at a

selective disadvantage since their offspring would either be nonexistent or of a lower overall fitness than the average.

In conflict with male-female co-evolution is antagonistic co-evolution (Rice, 1998). Rather than the genes of males and females evolving along a mutually beneficial path, there is an arms race between male sex peptides that serve to increase female ovulation and reduce her receptivity to subsequent males (Chen, 1984), usually at the expense of female longevity and fitness due to the toxic nature of these sex peptides (Fowler and Partridge, 1989; Chapman *et al.*, 1995). Females that have not evolved a response to the male's peptides would experience reduced fitness, not only in the harmful side-effects to the female's health, but also to the reduced number of mates that she would subsequently be receptive to resulting in a smaller number of total progeny.

A review of 178 species pairs of *Drosophila* found that prezygotic isolation was a stronger barrier to reproduction than postzygotic isolation when species live in sympatry, suggesting that prezygotic isolation is the first step in speciation (Coyne and Orr, 1989, 1997, 1998). Although prezygotic and postzygotic isolation both increase with divergence time between taxa (Coyne and Orr, 1989, 1997), prezygotic isolation evolves faster than postzygotic isolation (Coyne and Orr, 1989, 1997). Allopatry is not necessary for prezygotic isolation to arise (Feder *et al.*, 1994; Feder *et al.*, 2003; Filchak *et al.*, 2000; Treganza, 2002); prezygotic isolation is actually predominantly present in sympatric taxa, either due to direct selection for mate discrimination or through indirect selection triggered by postzygotic isolation (Coyne and Orr, 1989, 1997).

By comparing populations living at different altitudes, it has been suggested that

adaptation to an environment does not drive premating isolation, therefore natural selection is not believed to be the driving force behind prezygotic isolation (Treganza 2002), although this theory is repudiated by the incipient speciation seen in sympatric populations of *Rhagoletis* discussed previously. It is challenging to make a case for sexual selection as well due to the need to demonstrate that prezygotic isolation is the direct result of changes in a sexually selected trait and not the result of other evolutionary forces, such as genetic drift or evolutionary history.

Postzygotic Isolation

Postzygotic isolation is believed to develop when genes or chromosomes evolve along discrete paths while species are separated in allopatry. Later, when the two populations merge, hybrid offspring will either be infertile or inviable due to genetic incompatibilities. On rare occasions, hybrid offspring are better adapted to an environmental niche and are no longer genetically compatible with either parental species, causing hybrid speciation (Turelli *et al.*, 2001), sometimes seen in polyploid plants (Rieseberg *et al.*, 2003).

An allele which produces a normal phenotype in its own genetic background can interact epistatically with the genetic background of another species, causing hybrid inviability or sterility. These factors for sterility or inviability can perpetuate within inversions that differentiate taxa by creating linkage groups that persist when these taxa hybridize since recombination between inverted regions is suppressed (**Figure 2.1**). When recombination is also reduced or prevented between loci conferring hybrid sterility and those underlying mate discrimination, it is more likely for there to be additional

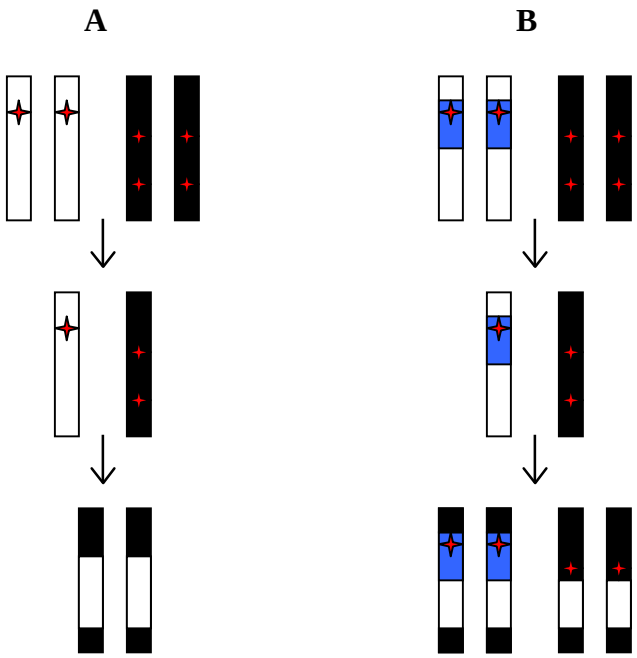

Figure 2.1: Model for the persistence of hybrid sterility when an inversion is present. Two species are designated by diploid chromosomes of a single color (black or white) with or without an inversion (blue). Genes conferring hybrid sterility (red star) are removed through recombination in scenario A, while they persist in the presence of an inversion in scenario B. Figure adapted from Noor *et al.* (2001b).

reinforcement of barriers to gene flow (Trickett and Butlin, 1994; Servedio, 2000; Felsenstein, 1981). These inversions act to reduce gene flow through the prevention of recombination rather than through a reduction in fitness (Johnson and Wu, 1993; Noor *et al.*, 2001b), leading to the continuation of separate taxa in spite of ongoing gene flow (Noor *et al.*, 2001b).

Alternatively, duplicate genes located at different cytological locations can provide a mechanism for hybrid dysfunction. If one copy is rendered functionless in one speciesand the other species loses function from the second copy, the offspring of hybrids can completely lack a functional copy of the gene (Lynch and Force, 2000) (**Figure 2.2A**). If there are separate regulatory regions for different tissues, an extension of this concept can be applied to regulatory regions of genes as well. If one copy loses the ability to be expressed in a particular tissue, but is compensated by the other copy, the organism does not lose any fitness. However, if its gametes are paired with a species that has lost the function in different tissues, then future generations could lack the proper expression of the gene product (Lynch and Force, 2000) (**Figure 2.2B**).

If hybrid sterility appears in a single sex, it is almost always the heterogametic sex (Haldane, 1922), a phenomenon referred to as 'Haldane's Rule'. In males and females, hybrid sterility seems to arise due to changes in the same genes; this also appears true for hybrid inviability (Coyne and Orr, 1989). Genes for hybrid sterility appear to evolve more rapidly than those for hybrid inviability (Wu, 1992), and research using chromosomal introgression lines (explained below) has found that hybrid male sterility is highly polygenic (**Table 2.1**) (True *et al.*, 1996; Hollocher and Wu, 1996; Tao *et al.*,

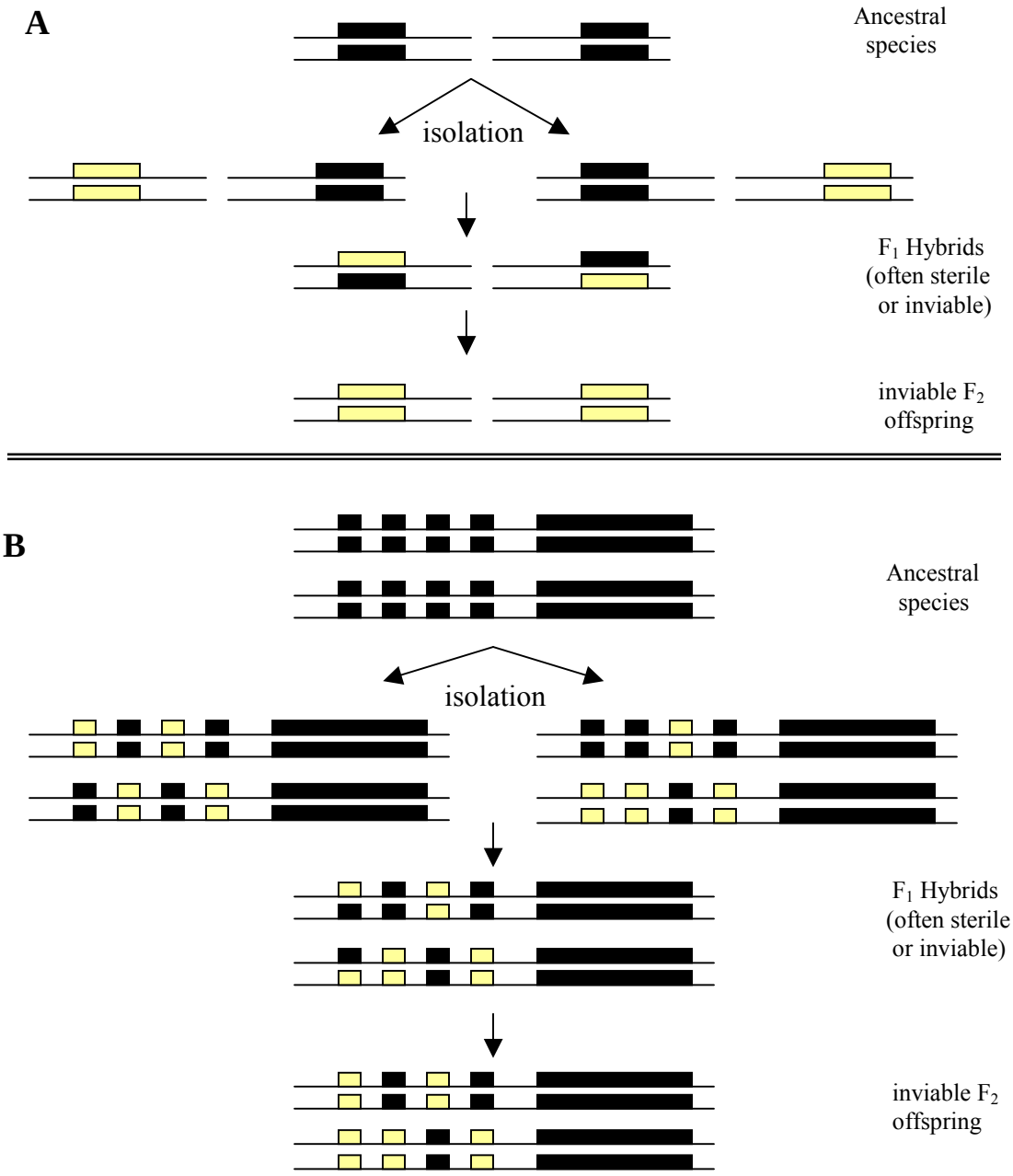

Figure 2.2: Duplicate genes as the mechanism for hybrid dysfunction. Black boxes are functional genes or regulatory regions, open boxes are nonfunctional. (A) Two genes on different chromosomes with duplicate function. (B) Two genes with multiple regulatory regions. The four small boxes on the left denote independently mutable regulatory regions, and the long boxes to the right are coding regions. For both (A) and (B), 1/16 of all F_2 hybrid offspring will be completely inviable, and 5/16 will be inviable if two copies of the gene or regulatory region are required for proper function. Figure adapted from Lynch and Force (2000).

Table 2.1: Summary of existing genetic analyses of species pairs that are reproductively isolated. The current mode of isolation, trait studied, experimental design (E. D.) and number of loci affecting speciation are listed. E. D.'s are: chromosome substitution (C); recombination mapping (R); introgression (I); deficiency complementation mapping (D); complementation mapping of single genes (G) and quantitative trait locus mapping (Q). Table 2.assisted by Coyne and Orr (1998), Orr, (2001) and Coyne (personal communication). References: (1) Barbash *et al.*, 2003 (2) Cabot *et al.*, 1994 (3) Carvajal *et al.*, 1996 (4) Civetta and Cantor, *in press* (5) Coyne, 1989 (6) Coyne, 1992a (7) Coyne, 1993 (8) Coyne *et al.*, 1994 (9) Coyne, 1996a (10) Coyne 1996b (11) Coyne, 1996c (12) Coyne and Charlesworth, 1986 (13) Coyne and Charlesworth, 1997 (14) Coyne and Kreitman, 1986 (15) Davis and Wu, 1996 (16) Doi *et al.*, 2001 (17) Heikkinen and Lumme, 1991 (18) Hoikkala and Lumme, 1984 (19) Hoikkala *et al.*, 2000 (20) Hollocher and Wu, 1996 (21) Hollocher *et al.*, 1997 (22) Khadem and Krimbas, 1992 (23) Lamnissou *et al.*, 1996 (24) Macdonald and Goldstein, 1999 (25) Mitrofanov and Sidorova, 1981 (26) Naviera and Fontdevila 1986 (27) Naviera and Fontdevila 1991 (28) Noor, 1997 (29) Noor and Coyne, 1996. (30) Noor *et al.*, 2001a (31) Noor *et al.*, 2001b (32) Orr, 1987 (33) Orr, 1989a (34) Orr, 1989b (35) Orr, 1992 (36) Orr and Irving, 2001 (37) Palopoli and Wu, 1994 (38) Pantazidis *et al.*, 1993 (39) Patterson and Stone, 1952 (40) Presgraves, 2003 (41) Presgraves *et al.*, 2003 (42) Sawamura *et al.*, 1993c (43) Shäfer, 1978 (44) Takahashi *et al.*, 2001 (45) Tao *et al.*, 2003a (46) Tao *et al.*, 2003b (47) Templeton, 1977 (48) Ting *et al.*, 1998 (49) Ting *et al.*, 2001 (50) Tomaru and Oguma, 1994 (51) True *et al.* 1996 (52) True *et al.*, 1997 (53) Val, 1977 (54) Williams *et al.*, 2001 (55) Wu *et al.*, 1995 (56) Yamada *et al.*, 2002 (57) Zeng *et al.*, 2000 (58) Zouros, 1981.

Table 2.1

Species Pair	Isolation	Trait	E. D.	# of Loci
D. melanogaster (two 'races')	allopatric	male prezygotic isolation[21, 49, 55]	C, I	≥ 5
		female prezygotic isolation[21, 49, 55]	C, I	≥ 4
		female pheromone production[44]	R	1
D. melanogaster *D. mauritiana*	allopatric	hybrid inviability[1, 42]	G	1
		hybrid sterility[1]	G	1
D. melanogaster *D. simulans*	sympatric	female pheromone production[9]	D	≥ 5
		hybrid inviability[1, 40, 41, 42]	D, G	≥ 1-191
		hybrid sterility[1, 35]	C, D, G	≥ 1-20
D. melanogaster *D. sechellia*	allopatric	hybrid inviability[42]	C	≥ 1
D. simulans *D. sechellia*	allopatric	male prezygotic isolation[4]	Q	≥ 1
		male copulation duration[4]	Q	≥ 1
		male genital morphology[24]	Q	≥ 7-11
		male sex comb tooth number[24]	Q	≥ 4
		male pheromone production[4, 8, 11]	Q, C	≥ 1-5
		female prezygotic isolation[6]	C	≥ 2
		hybrid inviability[20]	I	≥ 2
		hybrid male sterility[2, 14, 20]	C, I	≥ 6
D. simulans *D. mauritiana*	allopatric	male prezygotic isolation[5, 6, 10]	C	≥ 2
		male copulation duration[7]	C	≥ 3
		male sex comb tooth number[52]	Q	≥ 2
		male genital morphology[52, 57]	Q	≥ 9
		female prezygotic isolation[5, 6, 10]	C	≥ 3
		hybrid male sterility[12, 15, 20, 37, 45, 46, 48, 51]	I, R	≥ 15-120
		hybrid female sterility[20, 51]	I	≥ 4
		hybrid inviability[20, 51]	I	≥ 5
D. mauritiana *D. sechellia*	allopatric	female pheromone production[13]	R	≥ 6
D. mojavensis *D. arizonae*	sympatric	male prezygotic isolation[58]	C	≥ 2
		female prezygotic isolation[58]	C	≥ 2
		hybrid male sterility[38]	C	≥ 3

Table 2.1 (continued)

D. heteroneura *D. silvestris*	sympatric	male head shape[47, 53]	C	≥ 9-10
D. pseudoobscura *(two 'races')*	allopatric	hybrid male sterility[33, 34, 36]	C, I	≥ 5
D. pseodoobscura *D. persimilis*	sympatric	male prezygotic isolation[28, 30]	C, R	≥ 3
		male courtship song[54]	Q	≥ 2-3
		female prezygotic isolation[31]	I	≥ 2
		pheromone production[29]	CS	≥ 2
		hybrid male sterility[32, 34]	C	≥ 9
		hybrid female sterility[32, 34]	C	≥ 3
D. subobscura *D. madeirensis*	sympatric	hybrid male sterility[22]	R	≥ 6
D. buzatti *D. koepferae*	sympatric	hybrid male inviability[3]	I	≥ 4
		hybrid male sterility[26, 27]	I	≥ 7
D. virilis *D. littoralis*	sympatric	hybrid female viability[25]	C	≥ 5
		male song production[19]	C	≥ 3
D. virilis *D. lummei*	sympatric	male courtship song[18]	C	≥ 4
		hybrid male sterility[17]	C	≥ 6
D. virilis *D. texana*	sympatric	hybrid male sterility[23]	C	≥ 3
D. texana *D. montana*	sympatric	hybrid female inviability[39]	C	≥ 2
D. hydei *D. neohydei*	sympatric	hybrid male sterility[43]	C	≥ 5
		hybrid female sterility[43]	C	≥ 2
D. auraria *D. biauraria*	sympatric	male courtship song[50]	C	≥ 2
D. ananassae *D. pallidosa*	sympatric	female prezygotic isolation[16]	C, I	≥ 2
		male song production[56]	C	≥ 2

2003a, 2003b). Genes for inviability act as partial recessives in hybrids, which males succumb to more rapidly since the alleles are fully expressed on their single *X* chromosome (Coyne and Orr, 1997). A study using chromosomal introgressions found that homozygous autosomal introgressions were more likely to cause male sterility than female sterility or inviability, suggesting that genes for sterility evolve more rapidly in males (True *et al.*, 1996). This implies that there would subsequently be more sterility genes in males than females. Since hybrid males also succumb first to sterility and inviability mutations, the production of postzygotic isolation arises first in hybrid males, then later in females with a long time lag in between (Coyne and Orr, 1989), explaining Haldane's rule.

METHODS OF GENETIC ANALYSIS

It is difficult to address the genetic basis of reproductive isolation since the very nature of this isolation inhibits the formation of hybrids. This difficulty is compounded further since genes for reproductive isolation could be any type of gene, or even an external factor, such as the incompatibility-causing symbiont *Wolbachia* (Hurst and Schilthuizen, 1998). There are several approaches, however, one can use to address the question of the genetic basis of reproductive isolation. These studies, summarized in **Table 2.1**, utilize related species pairs that are only partially reproductively isolated, and can therefore be made to mate under no-choice laboratory conditions, creating hybrids that can be used for genetic analysis.

While genes affecting prezygotic isolation have yet to be discovered, great advances have been made in research that strives to identify factors for postzygotic

isolation. Several mutations have been identified that rescue hybrid inviability in *Drosophila*. *Zygotic hybrid rescue* (*Zhr*) (Sawamura *et al.*, 1993c) and *Nucleoprotein 98* (*Nup98*) (Presgraves *et al.*, 2003) in *D. melanogaster* and *maternal hybrid rescue* (*mhr*) in *D. simulans* (Sawamura *et al.*, 1993a) rescue embryonic hybrid inviability, while larval hybrid inviability is rescued by *Hybrid male rescue* (*Hmr*) in *D. melanogaster* (Hutter and Ashburner, 1987; Hutter *et al.*, 1990; Barbash *et al.*, 2003), and *Lethal hybrid rescue* (*Lhr*) in *D. simulans* (Watanabe, 1979). That embryonic and larval inviability are rescued by mutations at different loci suggests a different developmental basis for these two forms of postzygotic isolation (Sawamura *et al.*, 1993b). The putative candidate gene *Odysseus*-site homeobox gene (*OdsH*) (Ting *et al.*, 1998), discussed in more detail below, has been shown to confer sterility to hybrids between *D. simulans* and *D. mauritiana*.

Chromosome Substitution

The first studies that attempted to identify speciation genes used lines of one species that had been bred to contain a single chromosome of a sister species (**Figure 2.3**). This process requires one of the species to have a recessive visible marker on each of the chromosomes or chromosome arms. In this way, individuals with a normal genetic background containing a single chromosome or chromosome arm of another species can be tested for mating preference similar to that other species, identifying whether that chromosome contains genes influencing reproductive isolation. Chromosome substitution lines have been employed to localize candidate regions for prezygotic isolation to the *Y* chromosome (Zouros, 1981), *X* chromosome (Coyne, 1989; Coyne,

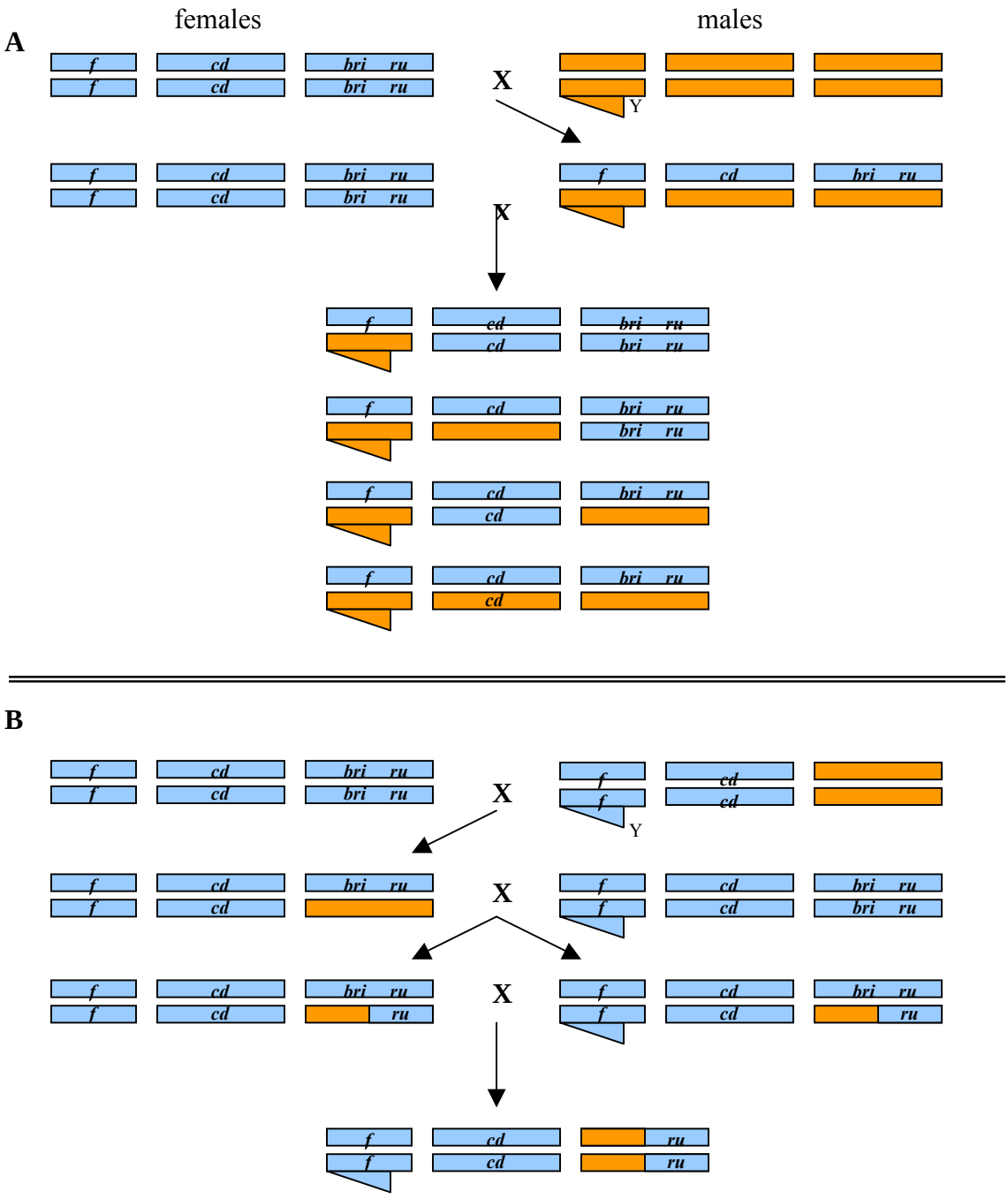

Figure 2.3: The creation of chromosomal introgression lines. The three major chromosomes of the *Drosophila melanogaster* sister species are shown; different strains are represented by different colors. One visible marker is shown for the *X* (*f*) and *2*nd chromosomes (*cd*), and two visible markers are shown on chromosome *3* (*bri* and *ru*), which allow one to track the chromosomal regions in subsequent generations. (A) simple introgression looking at whole chromosomes. (B) Introgression of segments of chromosome *3*.

1993; Coyne, 1996c), and each of the major autosomes (Coyne *et al.*, 1999; Coyne, 1989; Coyne, 1992; Coyne, 1993; Coyne, 1996c; Zouros, 1981). Combined, these studies have found candidate regions in almost the entire *Drosophila* genome, the chromosome of the largest effect depending on the species being examined and the laboratory in which the study was performed. To date, the smallest "candidate region" of genes for reproductive isolation using this method has been the larger portion of a chromosome arm (Ting *et al*., 2001), which contains thousands of candidate genes that could potentially affect reproductive isolation. While this is the simplest technique, and the only one available prior to the advent of more advanced molecular techniques for mapping, its scope is limited, providing little detailed information as to the genetic basis of speciation.

Chromosome Introgression

The process of chromosome substitution can be taken a step further. Rather than substituting a chromosome or chromosome arm, smaller portions of the genome of one species can be introgressed into the background of another. A study by True *et al.* (1996) on the genetic basis of hybrid sterility and inviability among crosses of *Drosophila mauritiana* and *D. simulans* is the most elegant example of this technique. *D. mauritiana* individuals that were homozygous for a *P-lacZ* vector inserted in different locations and containing the wild-type allele of *white* were repeatedly backcrossed to *D. simulans* males containing the *X*-linked *white* mutation (**Figure 2.4**). The *white* gene is used each generation as a visible eye color marker to select for heterozygous females. The 103 homozygous lines that resulted had genomes that were almost entirely composed of *D.*

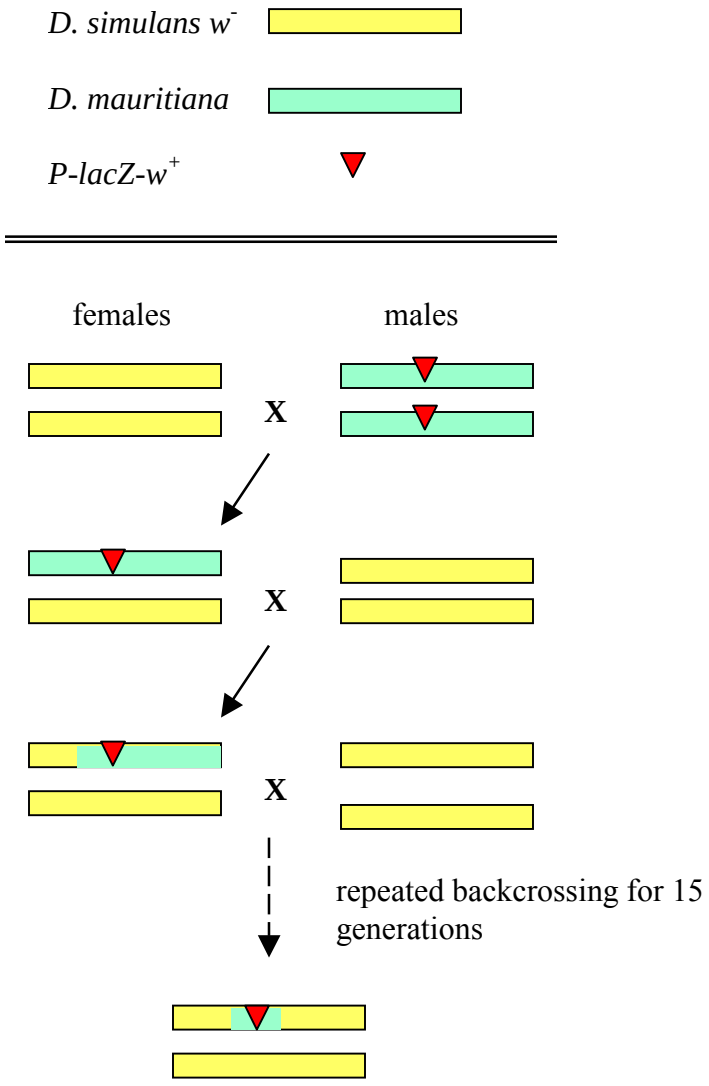

Figure 2.4: The introgression of a *P*-element marked segment of *D. mauritiana* into a *D. simulans* background by repeated backcrossing. Figure adapted from True *et al.* (1996).

simulans, with only a small introgressed region (about 9.4 cM) of *D. mauritiana* genome surrounding the *P-lacZ-w*$^{+}$ insert. Each insert that was assayed for viability and fertility was located in a distinct position in one of the four chromosomes, the cytological location being determined through *in situ* hybridization to the polytene chromosomes. Although a precise number of loci can not be determined from this study since exact breakpoints of the introgressed segments are not known and since the introgressions did not cover the entire genome, it is clear that there is a large number of genes (≥15) that affect sterility and fertility, the largest effect being when the introgressed segment lies on the *X* chromosome. These genes do not act as complete dominants or complete recessives, but display an intermediate degree of dominance. Recent experiments (Tao *et al*., 2003a, 2003b) have confirmed and refined these results.

Recombination Mapping

Although hybrid inviability can prevent the formation of hybrids, it is more common to see hybrid sterility maintaining species separation (Wu, 1992; Wu and Davis, 1993), yet far fewer of these genes have been characterized. In fact, only one putative 'sterility gene' has been identified: *Odysseus*-site homeobox gene (*OdsH*) (Ting *et al.*, 1998), which causes hybrid sterility in crosses between *D. simulans* and *D. mauritiana*. The *OdsH* gene was identified through high-resolution recombination mapping (**Figure 2.5**). Recombination mapping requires molecular markers evenly spaced throughout the region of interest in combination with a sample size large enough to contain individuals that have experienced recombination between those markers. The smaller the desired

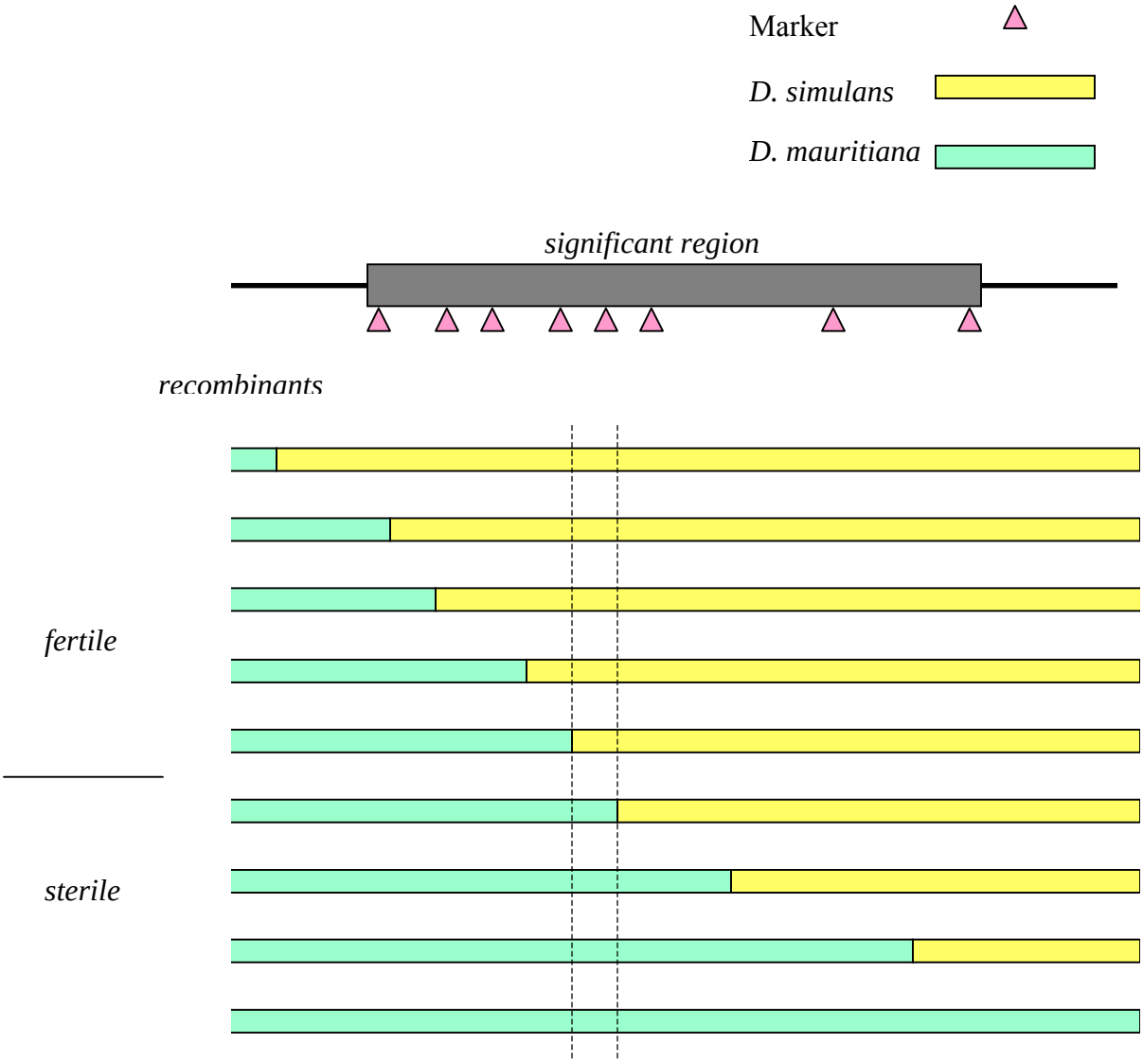

Figure 2.5: Identifying candidate regions or genes through recombination mapping. The delineation between *D. simulans* and *D. mauritiana* genotypes is approximate since the precise location of recombination between two markers is unknown. The dashed vertical line indicates a significant region. Figure adapted from Ting *et al.* (1998).

refinement, the greater the number of markers and recombinants needed. The small cytological region of 16D had been identified previously through chromosome introgression and recombination mapping as affecting male sterility (Perez *et al*., 1993; Perez and Wu, 1995). Using eight molecular markers and 84 individuals that had recombination within this region, there was a clear delineation of 8.4 kb separating sterile and fertile recombinants. While a single gene falls within this region, it should be noted that the final step, the creation of transformants, has not been done to confirm the causality of this gene for sterility. Evaluation of sequence data revealed that *OdsH* has homologs in species as divergent as *Caenhorabditis elegans* and *Mus musculus*, yet it is the most highly divergent between *D. simulans* and *D. mauritiana*, suggesting that it has undergone a dramatic increase in evolutionary rate in these two species. Due to the considerable prevalence of substitutions that cause an amino acid change over those that do not, the increase in rate is most likely caused by directional selection.

Deficiency Mapping

A completely different approach to identifying candidate regions for reproductive isolation is through the use of deficiency complementation mapping, a technique only available in *Drosophila*. Rather than relying on visible or molecular markers, one can utilize *Drosophila melanogaster* stocks that are hemizygous for a small portion of their genome, possessing only one allele with the other being completely absent, in order to elucidate whether that region affects the trait of interest. The non-deficient chromosome of the deficiency stock contains a balancer (*Bal*) chromosome to suppress recombination,

with a dominant visible marker so one can determine whether an F_1 fly received the wild-type chromosome or the deficient (*Df*) chromosome. These deficiency lines can be crossed to the parental line to create offspring that have small exposed regions of the parental genome in the candidate areas under study, in a heterozygous background.

This technique has been used successfully by Coyne (1996a) to identify four regions on the left arm of chromosome *3* that contribute to cuticular hydrocarbon (pheromone) differences between females of *D. melanogaster* and *D. simulans* (**Table 2.2**). It has also been used by Presgraves (2003) to uncover 20 autosomal regions conferring hybrid lethality in crosses between *D. melanogaster* and *D. simulans* and by Presgraves *et al.* (2003) in combination with candidate gene complementation mapping to further refine one of these regions to the level of genetic locus, *Nucleoprotein 98* (*Nup 98*). The finest example of deficiency complementation mapping was employed by Barbash *et al.* (2003) to identify a gene that confers lethality to *D. melanogaster*/*D. mauritiana* and *D. melanogaster*/*D. simulans* hybrids. The breakpoints of the flanking deficiencies that showed failure to complement were cloned, and four candidate genes within the refined region were used to transform *D. melanogaster*/*D. mauritiana* and *D. melanogaster*/*D. simulans* hybrids. The gene that suppressed rescue of the lethal phenotype in the Hmr^1 loss-of-function background is called *Hybrid male rescue* (*Hmr*).

One problem with this technique is that the deficiency stocks contain different genetic backgrounds which can interact epistatically with the line one is interested in, requiring additional confirmation of positive results, either through the use of alternate stocks containing a deficiency in the region or through positive confirmation by testing of

Table 2.2: Quantitative genetic studies of speciation for which there is a molecular marker map. Chromosome positions are given relative to *D. melanogaster*. U = unknown. No cytological information is known for *mhr* or *Lhr*, and they are therefore not included in this table. References: (1) Barbash *et al.*, 2003 (2) Civetta and Cantor, *in press* (3) Coyne, 1996a (4) Macdonald and Goldstein, 1999 (5) Orr, 1992 (6) Presgraves, 2003 (7) Presgraves *et al.*, 2003 (8) Ting *et al.*, 1998 (9) True *et al.*, 1997 (10) Williams *et al.*, 2001 (11) Zeng *et al.*, 2000.

Table 2.2

Species pair	Trait	Cytological Region	Effect
D. melanogaster *D. mauritiana*	hybrid inviability[1]	9D3 (*Hmr*)	U
D. melanogaster *D. simulans*	female pheromone production[3]	62B8; 62D1	U
		63C6; 63D3	U
		65F3; 66B10	U
		71F3; 72D12	U
	hybrid male sterility[5]	101E-F; 102B2-5	U
	hybrid inviability[1, 6, 7]	9D3 (*Hmr*)	U
		21C2-3; 21C8-D1	U
		27C2-9; 28A	U
		31F; 32A	U
		32F1-3	U
		36E4-F1; 36F7-9	U
		37D5; 37F5	U
		38A7-B1; 39C2-3	U
		41A	U
		41E2-F1; 42A2-B1	U
		44D3-8; 44F10	U
		47E3; 48A3-4	U
		49D-E; 50C23-D2	U
		51A5; 51B6	U
		57D8-9; 57F5-6	U
		64E1-13; 65C	U
		72D10-11; 73A3-4	U
		86E2-4; 86F6-7	U
		88F7-89A2; 89A11-13	U
		89E1-F4; 91B1-2	U
		95A5-7; 95C10-11	U
		95B1-5 (*Nup98*)	U
	hybrid semi-inviability[6]	47A1; 47D1-2	U
		60C5-6; 60D1	U
		79E5-F1; 79F2-6	U
D. simulans *D. mauritiana*	genital morphology[11]	1A8; 3C2	8.4
		7C; 10A	8.3
		21A-B; 22D-23C	5.1
		59C; 60F	5.9
		64B-C; 65E	8.0
		68E; 70D	10.2
		82C; 84B	12.4

Table 2.2 (continued)

Species pair	Trait	Cytological Region	Effect
D. simulans *D. mauritiana*	genital morphology[11]	97A-B; 100E	7.5
	male sex comb tooth number[9]	73A; 87F	53.6
		97A; 99E	-21.5
	hybrid male sterility[8]	16D1-3 (*OdsH*)	U
D. simulans *D. sechellia*	courtship latency[2]	84A; 86B	U
	copulation latency[2]	84A; 86B	U
	copulation duration[2]	84A; 86B	U
	male pheromones[2]	70A; 72C	U
	male sex comb tooth number[4]	4F1-8B7	U
		25A9-B4; 36C	U
		38E1-9; 49F9-13	U
		54B1-2; 58A4-B1	U
D. pseudoobscura *D. persimilis*	male courtship song (interpulse interval)[10]	*X* chromosome	274.5
		U ; 98C	1208.5
	male courtship song (interpulse frequency)[10]	*X* chromosome	2.0
		U ; 93E	1.4

candidate genes.

Quantitative Trait Locus (QTL) Mapping

QTL mapping is essentially a method for recombination mapping applied to the whole genome rather than a targeted genomic region. This method requires a large number of molecular markers evenly spaced throughout the genome and an advanced statistical model to assess genotype-phenotype associations (e.g. Zeng, 1993). The more divergent the trait in the two populations and the greater number of markers, the better one will be able to identify QTL. While recombination mapping tends to be most useful when focusing on genes of large effect in a small region of the genome, with significantlylarge sample sizes, QTL mapping has the power to detect genes of small effect, allows for genome-wide localization of multiple loci simultaneously, and enables the detection of epistatic interactions among loci. Since prezygotic isolation is believed to be controlled by many genes (Coyne and Orr, 1998) that might interact epistatically, QTL mapping can provide an efficient method by which to localize the factors involved in reproductive isolation.

Genes associated with reproductive isolation can be mapped using pairs of related species that do not normally mate, but will do so in the laboratory when given only non-specifics with which to mate. Previously, quantitative genetic studies on prezygotic isolation were limited to those involving chromosome substitution lines due to the inadequate number of molecular markers available for genotyping. There is a limited number of quantitative studies of either prezygotic or postzygotic reproductive isolation

that have made significant progress towards identifying speciation genes (**Table 2.2**). However, the recent completion of the *Drosophila melanogaster* genome sequence (Adams *et al.*, 2000) provides a multitude of single nucleotide polymorphisms (SNPs) and insert/deletions (InDels) that can be used as molecular markers in *D. melanogaster* and its sibling species, making high-resolution quantitative genetic mapping possible.

There are several interesting features of the studies implementing higher-resolution QTL mapping. A single trait that acts as an isolating mechanism between species, for example genital morphology, can have multiple QTL contributing to that trait. If each trait that affects reproductive isolation were to be examined, it is possible that hundreds of genes could directly or peripherally affect speciation. When the same trait is examined in two different species pairs, for example male sex comb tooth number, the regions controlling variation do not overlap, implying that the genetic basis of reproductive isolation could be different for different species pairs.

By looking at two species that have diverged, we know that some of the observed differences are those that are preventing gene flow. However, we cannot determine whether the differences are causal for speciation or if they arose after speciation occurred. It is also difficult in laboratory experiments to distinguish between divergence as a result of pleiotropic effects due to adaptation in differing environments and divergence as a result of the random accumulation of mutations (Rice and Hostert, 1993). By taking into account the known differences between species and evaluating whether the QTL effects are all in the same direction, it can be determined if selection played a role in shaping species differences since one would not expect QTL effects to be in the same direction if

random genetic drift was responsible (Orr, 1998), and in fact there appears to be strong selection for reproductive traits (Orr, 1998).

The effect of each QTL can be measured relative to the phenotypic difference between species that the QTL accounts for, or it can be compared to the standing variation within a species. Since it is the variation within a species that selection 'sees', this is what is relevant in terms of selection (Orr, 2001). However, even if one focuses on the large amount of standing variation within a species, species differences might be built from new (or rare) mutations rather than from polymorphisms segregating at the higher frequencies able to be observed in most experiments. This is supported by the finding that a large amount of standing variation is due to large insertions (such as transposable elements) which are presumably deleterious, and therefore not likely to cause the subpopulation within which they arise to become a new species. (Orr, 2001; see Mackay and Langley, 1990; Long *et al.*, 2000 for the contribution of molecular variation to phenotypic variation).

Some caveats regarding the use of QTL mapping to identify loci affecting species divergence should be noted. In addition to the limitations set by marker number and sample size, most studies use inbred lines, which contain less genetic variation than wild-type lines. Since allopatric speciation begins with variation between two geographical isolates, the level of standing variation within a subpopulation is a critical component of speciation, and therefore inbred lines do not provide a true representation of the differences between species. Laboratory conditions are also limited, and do not necessarily reflect conditions for reproductive isolation in nature. The evolution of

reproductive isolation might be inhibited by adverse experimental conditions, whereby the derived populations could be less fit and therefore have a preference for the unaffected control population. Finally, in the event that QTL mapping, or any other type of experiment, uncovers genes that contribute to reproductive isolation, it is difficult to tell which factors are causal of, and which arose in response to, isolation.

While there have been numerous studies on the genetic basis of reproductive isolation, only a few causal genes have been located for postzygotic isolation, and a gene affecting prezygotic isolation has yet to be identified. While theories abound as to the cause of speciation, it has become clear that it is not new theory that will answer the question of how speciation occurs, but rather new data. In Chapter 4 we perform genome scans for QTL contributing to prezygotic reproductive isolation between *Drosophila simulans* and *D. mauritiana*. We address the number and cytological location of loci contributing to reproductive isolation, whether the loci are the same in males and females and whether the loci are the same in the two species. We mapped at least seven QTL affecting discrimination of *D. mauritiana* females against *D. simulans* males, four QTL affecting *D. simulans* male traits against which *D. mauritiana* females discriminate, and five QTL affecting *D. mauritiana* male traits against which *D. simulans* females discriminate. QTL affecting sexual isolation are different in males and females and between the two species, and are not preferentially located on the *X* chromosome. While QTL mapping has not previously been used to locate putative candidate genes for reproductive isolation, it is highly feasible for high-resolution recombination mapping to be used here to further refine these QTL maps to the level of genetic locus. Relatively few

QTL with moderate to large effects associated with prezygotic isolation facilitates future positional cloning of the underlying genes.

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CHAPTER 3

The Quantitative Genetic Basis of Male Mating Behavior in *Drosophila melanogaster*

Amanda J. Moehring* and Trudy F. C. Mackay*

**** Department of Genetics, North Carolina State University, Raleigh, NC 27695, USA***

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ABSTRACT

Although mating behavior has been studied extensively in *Drosophila*, locating genes directly responsible for variation in behavior has been limited due to the multiple factors involved in behavior production. Here, we have mapped quantitative trait loci (QTL) affecting courtship occurrence, courtship latency, copulation occurrence and copulation latency that segregate between a *D. melanogaster* strain selected for reduced male mating propensity (2b) and a standard wild-type strain (Oregon-R). Mating behavior was assessed in a population of 98 recombinant inbred lines derived from these two strains and QTL affecting mating behavior were mapped using composite interval mapping. We found four QTL affecting male mating behavior at cytological locations 1A;3E, 57C;57F, 72A;85F and 96F;99A. We used deficiency complementation mapping to map the autosomal QTL with much higher resolution to five QTL at 56F5;56F8, 56F9;57A2, 70E1;71F4, 78C5;79A1, and 96F1;97B1. Quantitative complementation tests performed for 45 positional candidate genes within these intervals revealed seven genes which failed to complement the QTL: *eagle*, *18 wheeler, Enhancer of split*, *Polycomb*, *spermatocyte arrest*, *l(2)05510* and *l(2)k02206*. None of these genes have been previously implicated in mating behavior, demonstrating that quantitative analysis of subtle variants can reveal novel pleiotropic effects of key developmental loci on behavior.

INTRODUCTION

Quantitative traits demonstrate a continuous range of phenotypes in natural populations, resulting from the combined effects of multiple genes whose expression is influenced by the environment. A major challenge of modern geneticists is to identify the quantitative trait loci (QTL) and environmental factors causing variation in quantitative traits. Most aspects of morphology, physiology and behavior are quantitative traits, as are many human diseases and disorders, including schizophrenia and susceptibility to addictive behaviors. These behaviors can be further understood through the quantitative genetic dissection of behavioral traits in a model organism, such as *Drosophila melanogaster*.

Drosophila mating behavior provides an excellent model system for determining the genetic architecture of behavioral traits. The courtship behavior of *Drosophila* is composed of sequential actions that exchange auditory, visual and chemosensory signals between males and females, allowing for the individual components of the behavior to be separated (Hall, 1994; Greenspan, 1995). First the male aligns himself with the female. Then he taps the female with his foreleg, performs a "courtship song" by vibrating one wing, extends his proboscis to lick the female's genitalia, attempts to copulate, and (if the female accepts his advances) copulates. These actions vary within and among species in the duration of courtship (Greenspan and Ferveur, 2000) and copulation (Markow, 1996).

P-element insertional and chemical mutagenesis have been used to identify genes involved in *Drosophila* courtship behaviors (Hall *et al.*, 1980; Yamamoto and Nakano, 1998). Mutations in *courtless* (*col*) (Yamamoto and Nakano, 1998), *cuckold* (*cuc*)

(Coyne, 1989), *he's not interested* (*hni*), (Yamamoto *et al.*, 1997), *pale* (*ple*) (Buchner, 1991; Neckameyer, 1998), and *tapered* (*ta*) (Bien-Willner and Doane, 1997) exhibit a decrease in male courtship intensity, while mutations in *spinster* (*spin*) (Suzuki *et al.*, 1997), *chaste* (*cht*) (Yamamoto *et al.*, 1997) and *dissatisfaction* (*dsf*) (Finley *et al.*, 1997) display decreased female receptivity. Mutations in *period* (*per*) (Kyriacou and Hall, 1980) and *cacophony* (*cac*) (von Schilcher, 1976) result in altered rhythmicity in courtship song, and disruptions in components of the sex-determination pathway genes *Sex-lethal* (*Sxl*), *transformer* (*tra*), *transformer-2* (*tra-2*) or *fruitless* (*fru*) (Cline, 1993; Barbash and Cline, 1995; MacDougall *et al.*, 1995; Finley *et al.*, 1997; Schütt and Nöthiger, 2000) result in altered sexual orientation.

In *Drosophila*, male mating ability is a critical component of reproductive fitness (Partridge *et al.*, 1985), and exhibits significant inbreeding depression (Sharp, 1984). However, there is a considerable amount of segregating variation for the individual components of mating behavior, as well as for mating preference (Manning, 1961, 1963; Parsons, 1964), with estimates of heritability (h^2) for male mating speed ranging from 0.3-0.6 (Manning, 1961, 1963; Parsons, 1964; Collins and Hewitt, 1984; Casares *et al.*, 1993). This variation is plausibly maintained by genotype by sex interactions (Casares *et al.*, 1993; Nuzhdin *et al.*, 1997; Vieira *et al.*, 2000).

We do not know what loci contribute to naturally occurring variation in behavior, their effects, and their interactions. Possibly, alleles at loci identified through analysis of mutations contribute to variation in mating behavior in natural populations. However, mutations generated in an isogenic background have not yet been screened for their

quantitative effects on mating behavior. Such screens for subtle allelic effects of hypomorphic mutations are likely to reveal novel loci affecting behavior (Anholt *et al.*, 1996; Lyman *et al.*, 1996; Norga *et al.*, 2003) since genes affecting behavioral traits are highly pleiotropic, with null mutations leading to homozygous lethality (Sokolowski, 2001). Alternatively, we can directly address the question of what genes affect variation in mating behavior by mapping QTL by linkage to polymorphic molecular markers in populations that have been selected for reduced courtship or copulation latency.

Here, we have mapped four QTL affecting male mating behavior in a population of recombinant inbred lines derived from the wild-type strain Oregon-R (Ore), and a strain selected for low male mating activity, 2b (Kaidanov, 1990). We used deficiency complementation mapping (Pasyukova *et al.*, 2000; Fanara *et al.*, 2002; De Luca *et al*., 2003) to fine-map the autosomal QTL regions with higher resolution, and quantitative complementation tests (Long *et al.*, 1996; Mackay and Fry, 1996; ; Fanara *et al.*, 2002; De Luca *et al*., 2003) to mutations at positional candidate genes to identify seven novel candidate genes contributing to the difference in mating behavior between Ore and 2b.

MATERIALS AND METHODS

Genome scan for QTL affecting mating behavior

***Drosophila* stocks:** An inbred *Drosophila melanogaster* line, 2b, was selected over 550 generations for reduced male mating activity (Kaidanov, 1990). While the average copulation latency of Oregon R (Ore), a standard wild-type and unrelated stock (Lindsley and Zimm, 1992), is around 44 minutes, the 2b line rarely mates within 2½ hours (the

actual copulation latency is not known since only one mating out of 40 was ever observed for 2b within the 2 ½ hour observation period). Ore and 2b were crossed and the F_1 progeny were backcrossed to 2b and allowed to randomly mate for four generations. 200 individual pairs were selected in the fifth generation and their offspring were inbred by full sib mating for 25 generations to create 98 recombinant inbred (RI) lines, each with a unique combination of Ore and 2b genomes (Nuzhdin *et al.*, 1997). The genetic composition of these RI lines has been determined using 80 *roo* transposable elements with polymorphic insertion sites between the parental lines and an average spacing of 3.2 cM, and a visible marker *sparkling* (*spa*) that is fixed within Ore on Chromosome *4* but is absent in 2b (Nuzhdin *et al.*, 1997; Vieira *et al.*, 2000) (**Table 3.1**, **Appendix 1**). Chromosome *2* is divided into two linkage groups because the recombination between markers 50F and 57C was >0.5 cM (Nuzhdin *et al.*, 1997).

Mating behavior assays: All flies were maintained at 25°C, under 12 hour light-dark cycles. Virgin flies were collected under brief CO_2 exposure, separated by sex, and aged 5-7 days to ensure reproductive maturity. Behavioral assays were performed for each line by aspirating (i.e. not anesthetizing) three females and one male of an RI line into 8-dram food vials containing about 3ml of standard cornmeal-agar-molasses medium. All experiments were performed at 25°C, 75% humidity, within 3½ -5½ hours of "lights on" to eliminate the known temperature, humidity and circadian influences. We recorded courtship latency (time from the entry of the male into the vial to the initiation of courtship), copulation latency (time from male vial entry to copulation), and courtship

Table 3.1. Cytological locations of *roo* transposable elements. The visible mutation *sparkling* (*spa*) marked Chromosome *4*.

***X* Chromosome**		**Chromosome *2***			**Chromosome *3***				
1B	11C	21E	38A	50D	61A	71E	85A	92A	99A
3E	11D	22F	38E	50F	63A	72A	85F	93A	99B
4F	12E	27B	43A	57C	65A	73D	87B	93B	99E
5D	14C	29F	43E	57F	65D	76A	87E	94D	100A
6E	15A	30AB	46A	60E	67D	76B	87F	96A	
7D	16D	30D	46C		68B	77A	88E	96F	
7E	17C	33E	48D		68C	77E	89B	97D	
9A	19A	34EF	49D		69D	78D	91A	97E	
10D		35B	50B		70C	82D	91D	98A	

duration (copulation latency - courtship latency) for a period of 60 minutes. Since only 40 test vials could be observed within each session, one vial for each of 40 lines was observed, the 40 lines being chosen at random each day, thus randomizing the environmental variation within RI lines. A total of 20 independent measurements were performed per RI line.

Quantitative genetic analysis: The latency and duration data were analyzed by single classification, random effects analysis of variance (ANOVA) among the RI lines using the GLM and VARCOMP procedures in SAS (Version 8.2; SAS Institute, 1988). Examination of residuals from these tests indicated that the assumptions of the ANOVA were satisfied without transformation. The categorical trait of occurrence was analyzed with a G test as well as with ANOVA. Broad sense heritability (H^2) for the traits in this population of RI lines was computed as $\sigma^2_L/(\sigma^2_L + \sigma^2_E)$, where σ^2_L is the among-line, and σ^2_E the within-line variance component. Pairwise genetic correlations (r_G) between traits were calculated as $r_G = cov_{12}/(\sigma_1\sigma_2)$, where cov_{12} is the product moment covariance between line means for traits 1 and 2 and σ_1 and σ_2 are the square roots of the among-line variance components for each trait separately.

QTL affecting mating behavior: For each of these measurements, the mean value per line was calculated in seconds. Since the observation period lasted 3600 seconds (one hour), flies that did not mate within the time period were given a score of 3601. Line means were not transformed prior to analysis since they approximated a normal

distribution. Genome scans for QTL were performed for courtship latency, courtship occurrence, copulation latency and copulation occurrence using composite interval mapping (CIM) (Zeng, 1993) as implemented by QTL Cartographer (version 1.13 <ftp://esssjp.stat.ncsu.edu/pub/qtlcart/>) software (Basten *et al.*, 1994; Basten *et al.*, 1999). CIM computes the likelihood ratio (LR) test statistic, $-2\ln(L_0/L_1)$ where (L_0/L_1) is the ratio of the null hypothesis (there is no QTL within the test interval) to the alternative hypothesis (there is a QTL within the test interval), taking into account the segregation of unlinked QTL by multiple regression. Marker co-factors were chosen for each trait by forward-backward step-wise regression. LR test statistics were computed every 0.1 cM using a 'window size' (i.e., the distance from the test interval within which marker co-factors are included) of 10 cM. Empirical significance thresholds were determined by randomly permutating the data 1000 times and calculating the maximum LR across each marker interval for each permutation (Doerge *et al.*, 1997). If the maximum LR statistic for the permutated data exceeded the original LR statistic less than 50 times, the marker interval was declared to be significant at $P \leq 0.05$ (Churchill and Doerge, 1994; Doerge and Churchill, 1996).

Test for epistasis among the random effects of markers: Pairwise epistatic interactions were tested by running ANOVA models including each marker closest to the significant QTL and one pair-wise interaction between markers (Dilda and Mackay, 2002). A Bonferroni correction was applied to account for multiple tests.

Quantitative complementation tests to deficiencies and mutations

Deficiency complementation mapping: We used deficiency complementation mapping (Pasyukova *et al.*, 2000) to fine-map the three autosomal QTL affecting mating behavior. **Table 3.2** provides a list of all deficiencies tested and their cytological locations. Deficiency complementation can not be used to map traits expressed in males on the *X* chromosome; fine-scale recombination will be necessary to map this QTL. Female flies containing deficiencies (*Df/Bal*) uncovering autosomal QTL regions were crossed to Ore or 2b males. Virgin males of the four resulting genotypes (Ore/*Df*, 2b/*Df*, Ore/*Bal*, and 2b/*Bal*) were collected, where *Df* denotes the deficiency and *Bal* the balancer chromosome. Some stocks also contained a *white* mutation, which could modify behavior due to decreased visual acuity. For these stocks, deficiency males were first crossed to *Samarkand Cy/Pm* or *Samarkand H/Sb* females (depending on whether the deficiency was present on the second or third chromosome). Virgin females (*Df/Cy* or *Df/Sb*) were collected and crossed to Ore and 2b, and w^+ males from this cross were collected and assessed for mating behavior.

Mating behavior assays: Behavioral assays were performed as described above with the following exceptions: Three *Samarkand* females were combined with one test male; 20 assays were performed per deficiency per genotype (Ore/*Df*, 2b/*Df*, Ore/*Bal*, and 2b/*Bal*); and one male of each genotype were observed per deficiency in each observation period, the deficiencies to be tested per day chosen at random (40 vials maximum = 10 deficiency stocks per observation period). For example, one observation was made for each of the

Table 3.2: Stocks used for deficiency complementation mapping of QTL affecting mating behavior.

Chromosome *2*			**Chromosome *3***		
QTL	**Genotype**	**Cytogenetic location**	**QTL**	**Genotype**	**Cytogenetic location**
57C-57F	*Df(2R)CX1*	49C1-4; 50C23-D2	**72A-85F**	*Df(3L)vin7*	68C8-11; 69B4-5
	Df(2R)L48	50F6-9; 51B3		*Df(3L)iro-2*	69B1-5; 69D1-6
	Df(2R)trix	51A1-2; 51B6		*In(3LR)C190*	69F3-4; 70C3-4
	Df(2R)03072	51A5; 51C1		*Df(3L)fz-CAL5*	70C2-6; 70E1
	Df(2R)Jp1	51D3-8; 52F5-9		*Df(3L)Brd6*	70E; 71F
	Df(2R)vg89e88	52B3-C1; 53E2-F2		*Df(3L)brm11*	71F1-4; 72D1-10
	Df(2R)Jp6	52E3-5; 52F		*Df(3L)st-F13*	72C1-D1; 73A3-4
	Df(2R)Jp8	52F5-9; 52F10-53A1		*Df(3L)81k19*	73A3; 74F
	Df(2R)Pcl7B	54E8-F1; 55B9-C1		*Df(3L)W10*	75A6-7; 75C1-2
	Df(2R)PC4	55A; 55F		*Df(3L)VW3*	76A3; 76B2
	Df(2R)P34	55E2-4; 56C1-11		*Df(3L)kto2*	76B1-2; 76D5
	Df(2R)017	56F5; 56F15		*Df(3L)XS-533*	76B4; 77B
	Df(2R)min	56F8-17; 56F8-17		*Df(3L)rdgC-co2*	77A1; 77D1
	Df(2R)AA21	56F9-17; 57D11-12		*Df(3L)ri-79c*	77B-C; 77F-78A
	Df(2R)D4	57A1-3; 57B13		*Df(3L)ME107*	77F3; 78C8-9
	Df(2R)exu1	57A2; 57B1		*Df(3L)Pc-2q*	78C5-6; 78E3-79A1
	Df(2R)Pu-D17	57B4; 58B		*Df(3L)Delta1AK*	79F; 80A
	Df(2R)X58-7	58B1-2; 58E4-10		*Df(3R)ME15*	81F3-6; 82F5-7
	Df(2R)X58-12	58D1-2; 59A		*Df(3R)3-4*	82F3-4; 82F10-11
	Df(2R)59AB	59A1-3; 59B1-2		*Df(3R)Tp110*	83C1-2; 84B1-2
	Df(2R)59AD	59A1-3; 59D1-4		*Df(3R)roe*	84A6-B1; 84D4-D9
	Df(2R)twi	59C3-4; 59D1-2		*Df(3R)dsx2*	84C1-3; 84E1
	Df(2R)bw-S46	59D8-11; 60A7		*Df(3R)p712*	84D4-6; 85B6
	Df(2R)or-BR6	59D5-10; 60B3-8		*Df(3R)p-XT103*	85A2; 85C1-2
	Df(2R)Chi[g230]	60A3-7; 60B4-7		*Df(3R)by10*	85D8-12; 85E7-F1
	Df(2R)Px1	60B8-10; 60D1-2		*Df(3R)by62*	85D11-14; 85F16
	Df(2R)Px2	60C5-6; 60D9-10		*Df(3R)M-Kx1*	86C1; 87B1-5
	Df(2R)M60E	60E2-3; 60E11-12		*Df(3R)T-32*	86E2-4; 87C6-7
	Df(2R)ES1	60E6-8; 60F1-2		Df(3R)ry615	87B11-13; 87E8-11
	Df(2R)Kr10	60F1; 60F5	**96F-99A**	*Df(3R)96B*	96A21; 96C2
	Df(2R)Kr14	60F2; 60F5		*Df(3R)Espl3*	96F1; 97B1
				Df(3R)Tl-P	97A; 98A1-2
				Df(3R)D605	97E3; 98A5
				Df(3R)3450	98E3; 99A6-8
				Df(3R)Dr-rv1	99A1-2; 99B6-11
				Df(3R)01215	99A6; 99C1
				Df(3R)L127	99B5-6; 99E4-F1
				Df(3R)B81	99C8; 100F5
				Df(3R)awd-KRB	100C;100D

four genotypes used to evaluate *Df(2R)CX1* (Ore/*Df(2R)CX1*, 2b/*Df(2R)CX1*, Ore/*Bal*, and 2b/*Bal*) for each observation period in which *Df(2R)CX1* was randomly chosen.

Statistical analysis: The test for quantitative failure to complement is whether the difference between the mating behavior of the Ore/*Df* and 2b/*Df* flies is the same as the difference in mating behavior between Ore/*Bal* and 2b/*Bal*. In other words, quantitative complementation occurs when (Ore/*Df* - 2b/*Df*) = (Ore/*Bal* - 2b/*Bal*), and quantitative failure to complement occurs when (Ore/*Df* - 2b/*Df*) > (Ore/*Bal* - 2b/*Bal*). Quantitative failure to complement results in a significant line by genotype interaction term in a two way ANOVA cross-classified design:

$$y = \mu + L + G + L \times G + E$$

where μ is the overall mean, L is the main effect of the parental line (Ore or 2b), G is the main effect of genotype (*Df* or *Bal*), L×G is the interaction term, and E is the error variance within L×G. If the variance of the differences (L×G) is significant (ANOVA, $P \leq 0.05$), and the difference between Ore/*Df* – 2b/*Df* is greater than the difference between Ore/*Bal* – 2b/*Bal*, i.e., consistent with an allelic interaction, then we conclude that the deficient region failed to complement Ore and 2b QTL (Pasyukova *et al.*, 2000). Error variances for the categorical traits of courtship and copulation occurrence are not normally distributed and could violate the ANOVA assumption of normality, yet ANOVA has been shown to be robust in spite of departures from normality (Robertson and Lerner, 1948; Lush *et al.*, 1949; Dempster and Lerner, 1950). Deficiency stocks that were found to be significant were re-tested and the two replicates (40 observations) were

analyzed jointly for significance. QTL locations were inferred using proximal and distal breakpoints of non-significant deficiencies overlapping significant deficiencies. Failure of deficiencies to complement QTLs confirms the presence of QTL in the candidate region.

Complementation tests to candidate genes: Quantitative complementation tests to candidate genes in the QTL regions defined by deficiency mapping were used to further identify putative candidate genes corresponding to the QTL (**Table 3.3**). All 34 candidate genes within regions defined by deficiency complementation mapping that had healthy stocks available were assayed and analyzed using the procedure described above for deficiency complementation mapping. An additional 11 candidate genes generated by insertion of the *P{GT1}* element (Lukacsovich *et al.*, 2001) in an isogenic derivative of Canton S as part of the Berkeley Gene Disruption Project (<www.fruitfly.org>; Norga *et al.*, 2003) were tested using a slightly altered paradigm. For the BG lines, single males were paired with one female and whether or not copulation occurred in 40 minutes was recorded.

Table 3.3: The genes that were tested for failure to complement and their cytological locations.

Significant QTL	Gene tested	Cytological Location
56F5; 57B4	*BG00756*	56D15-E1
	l(2)k08002	56F6-9
	18 wheeler	56F8
	humpy	56F9; 58A1
	mus209	56F10-11
	BG01288	56F11
	l(2)s1866	56F11-12
	BG02518	56F16
	l(2)s4831	57A3-4
	l(2)k09920	57A3-6
	l(2)k16204	57A5-6
	l(2)05510	57A5-6
	BG02102	57A5-6; 57A6
	bancal	57A6-7
	l(2)k02206	57A8-9
	BG01609	57A9
	l(2)k06409	57B1-3
	BG02471	57B1; 57B2-3
	inscuteable	57B3
78C5; 79A1	*spermatocyte arrest*	78A2; 78C9
	l(3)04063	78A3; 78D2
	l(3)neo29	78C
	l(3)ry3	78C; 78D
	BG01493	78C3-4; 78C7
	Polycomb	78C6-7
	Aef1	78C8
	l(3)78Da	78C8-9
	SR3-7	78D; 79A
	Hr 78	78D1
	l(3)00534	78D1-2
	M6	78D4
	eagle	78E5-6
	CG7145	78F1
	BG01362	78F4-79A1
	BG01919	79A7
96F; 97A	*taxi*	96A20; 96F9
	l(3)rQ197	96F1-2
	Enhancer of split	96F8
	BG02029	96F8
	groucho	96F8-9
	Prickly	96F11-14
	spindle-D	97A1-2
	goulash	97A1; 98A2
	Aldolase	97A6
	BG01280	97B6; 97B9

RESULTS

Mating behavior phenotypes and genetic variation in mating behavior in RI lines: The average time to the initiation of courtship behavior (1440 ± 39.3 seconds = 24.0 minutes) and copulation (2645 ± 25.8 seconds = 44.1 minutes), as well as the occurrence of courtship (0.781 ± 0.012) and copulation (0.495 ± 0.011) (**Table 3.4**) are similar for the population of RI lines and the Ore parental strain (1371 ± 238.1; 2637 ± 171.1; 0.950 ± 0.050; 0.524 ± 0.078, respectively). A comparison to the 2b parental strain is not possible due to the lack of courtship or copulation seen for this strain within the observation period. There was significant variation between the 98 RI lines for courtship latency ($P \leq 0.0001$) (**Table 3.4**, **Figure 3.1**), courtship occurrence ($P \leq 0.0001$) (**Table 3.4**, **3.5**, **Figure 3.2**), copulation latency ($P \leq 0.0001$) (**Table 3.4**, **Figure 3.3**), copulation occurrence ($P \leq 0.0001$) (**Table 3.4**, **3.5**; **Figure 3.4**), and courtship duration ($P \leq 0.0001$) (**Table 3.4**, **Figure 3.5**) (see **Appendix 2**). Heritabilities ranged from 0.188 to 0.282 (**Table 3.4**). There was a positive genetic correlation between courtship and copulation latency (r_G=0.70) (**Table 3.6**, **Figure 3.6a**), as well as between courtship and copulation occurrence (r_G=0.72) (**Table 3.6**, **Figure 3.6b**). However, there is a negative genetic correlation between courtship latency and courtship occurrence (r_G=-0.89) (**Table 3.6**, **Figure 3.6c**) as well as between copulation latency and copulation occurrence (r_G=-0.94) (**Table 3.6**, **Figure 3.6d**), demonstrating that the later courtship or copulation is initiated, the less likely it is to be successful. Additionally, there is a stronger correlation, albeit negative, between latency and occurrence (for both courtship and copulation) than between courtship and copulation occurrence and courtship and copulation latency,

Table 3.4: ANOVA for each mating behavior trait. Mean values (± standard error) are given in seconds for courtship and copulation latency and as a proportion for copulation and courtship occurrence. d.f., degrees of freedom; MS, mean square; σ^2, variance; H^2, broad sense heritability.

Trait	Mean	Source	d.f.	MS	σ^2	F	P	H^2
courtship latency	1439.7 ± 39.3 sec.	line	97	7149874	501073	5.61	<0.0001	0.282
		error	1051	1275294	1275294			
courtship occurrence	0.781 ± 0.012	line	97	0.512	0.032	3.71	<0.0001	0.188
		error	1051	0.138	0.138			
copulation latency	2645.2 ± 25.8 sec.	line	97	7553962	328716	7.71	<0.0001	0.251
		error	1862	979637	979637			
copulation occurrence	0.495 ± 0.011	line	97	1.289	0.055	6.58	<0.0001	0.218
		error	1862	0.196	0.196			
courtship duration	1978.9 ± 46.4 sec.	line	97	5099508	385006	3.22	<0.0001	0.198
		error	804	1558336	1558336			

Table 3.5: G test results for courtship and copulation occurrence.

Trait	χ^2	***P***
courtship occurrence	334.5	<0.0001
copulation occurrence	559.4	<0.0001

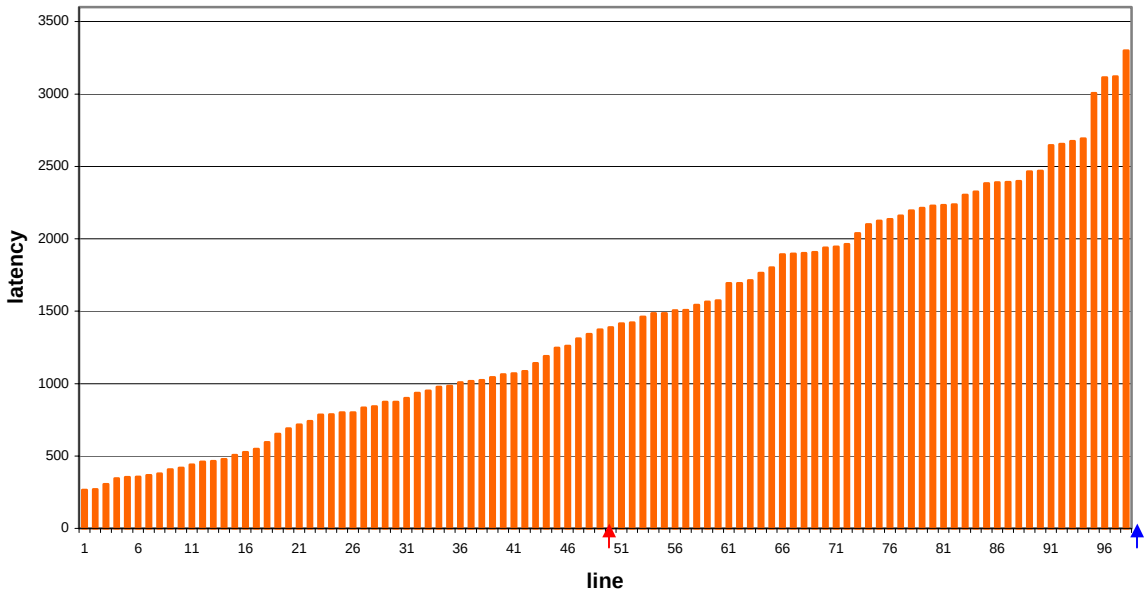


Figure 3.1: Continuous distribution of courtship latency means among 98 RI lines, sorted by shortest to longest courtship latency. The arrows represent the mean of the Ore (red) and 2b (blue) parental lines.

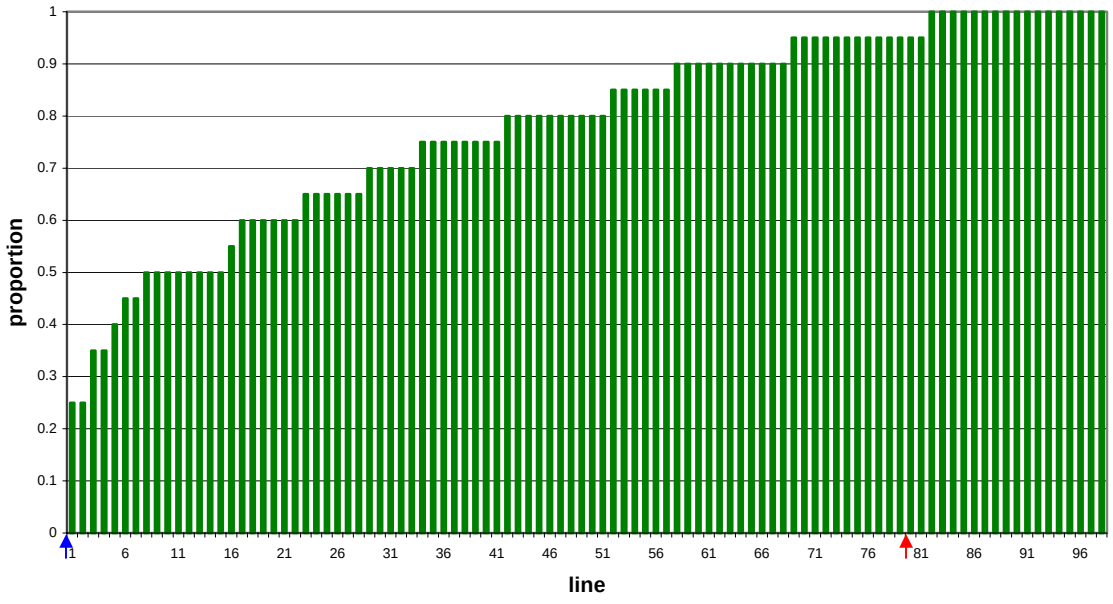


Figure 3.2: Continuous distribution of courtship occurrence means among 98 RI lines, sorted by shortest to longest courtship occurrence. The arrows represent the mean of the Ore (red) and 2b (blue) parental lines.

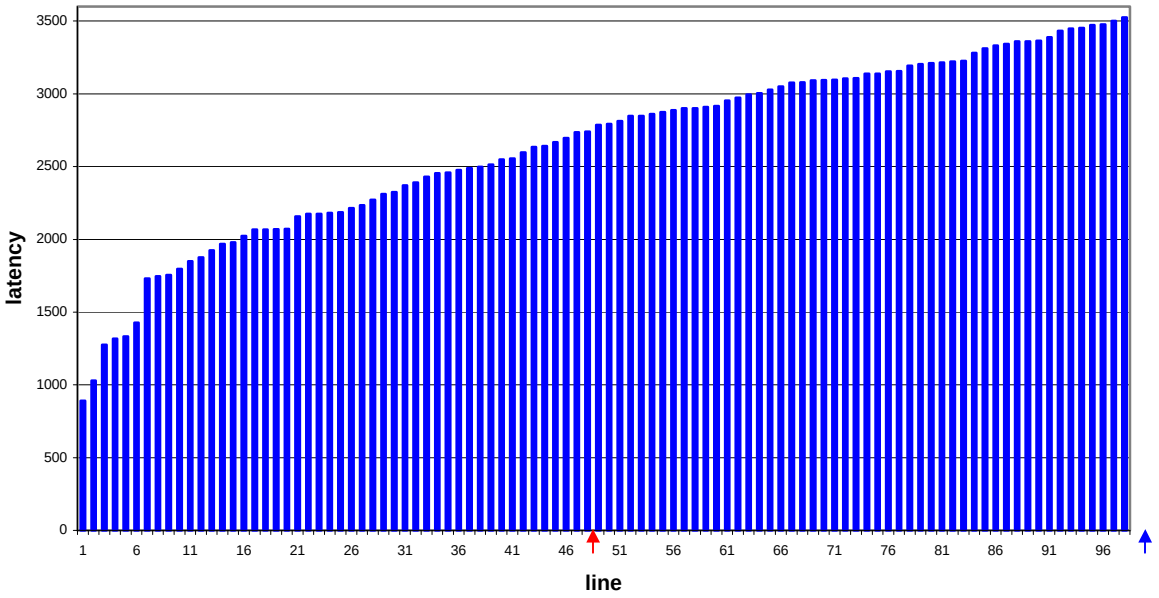


Figure 3.3: Continuous distribution of copulation latency means among 98 RI lines, sorted by shortest to longest copulation latency. The arrows represent the mean of the Ore (red) and 2b (blue) parental lines.

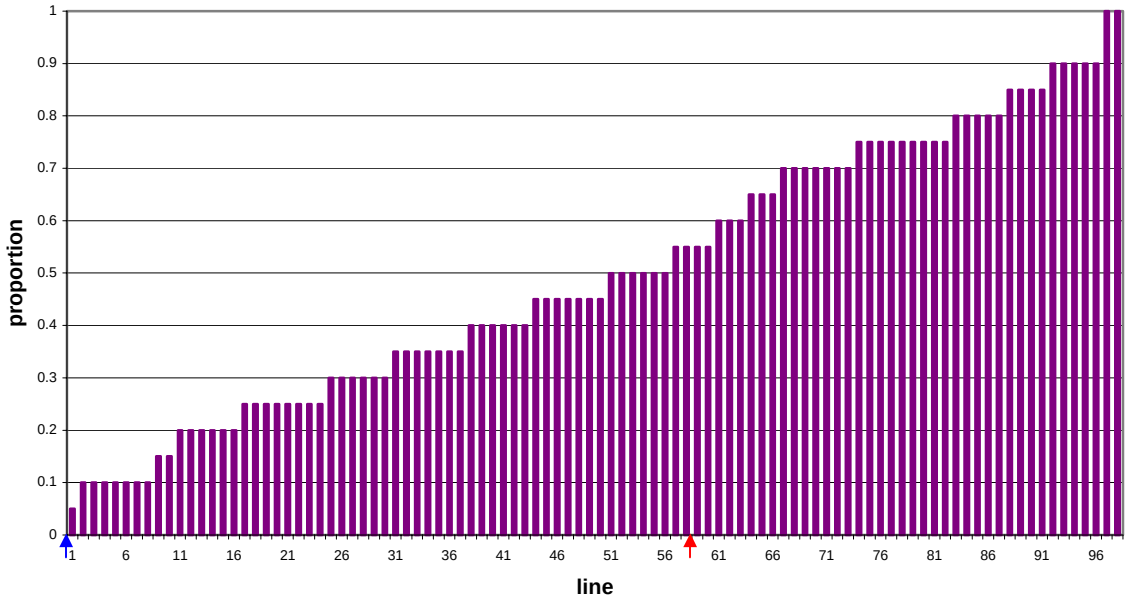


Figure 3.4: Continuous distribution of copulation occurrence means among 98 RI lines, sorted by shortest to longest copulation occurrence. The arrows represent the mean of the Ore (red) and 2b (blue) parental lines.

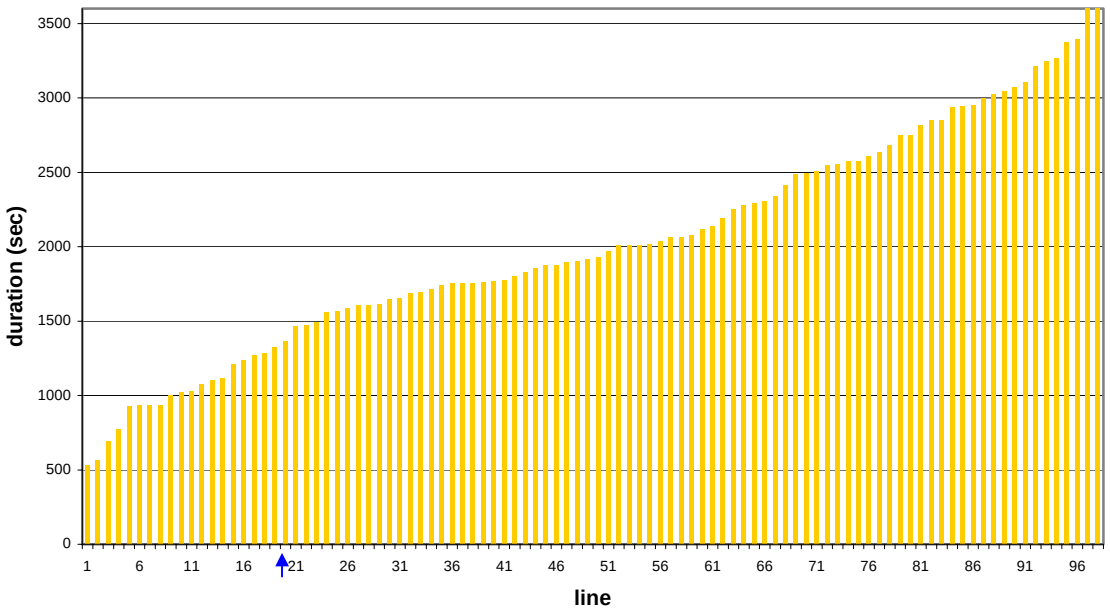


Figure 3.5: Continuous distribution of courtship duration means among 98 RI lines, sorted by shortest to longest courtship duration. The blue arrow represents the mean of the Ore parental line.

demonstrating that the simpler measure of occurrence can be used as a correlate for latency.

Genome scan for QTL affecting mating behavior: There is significant variation among RI lines for all of the traits measured, making it possible to implement QTL mapping to identify candidate loci. We used composite interval mapping (CIM) to localize QTL affecting *Drosophila* mating behavior (Zeng, 1994; Xu and Atchley, 1996). Four QTL affecting courtship occurrence and latency, as well as copulation occurrence and latency, were significant based on permutation-derived significance thresholds: 1A;3E, 57C;57F, 72A;85F, and 96F;99A. A fifth QTL at 61A;65A affecting copulation latency had a LR ratio (16.41) that was only slightly less than the threshold (16.75). These regions range from 2-21 cM and encompass 87-1,467 candidate genes. While two of the regions contribute to all four mating behavior traits (**Table 3.7, Figure 3.7**), the remaining two QTL contribute to only copulation latency or courtship latency. We did not detect any QTL for courtship duration despite the significant V_G for this trait. The effects of this trait must be too small to detect given the limited number of measurements taken for courtship duration, which required both courtship latency and copulation latency to occur, in many of the lines. It is possible that there are additional QTL for mating behavior, but their effects are too small to be detected given the sample size used. Even if this is true, it is still quite clear that of the genes that have the greatest effect there are some genetic factors that overlap in their contribution to mating behavior as a whole, while others are specific to individual components of mating behavior.

Table 3.6: Genetic correlations among mating behavior traits.

	courtship latency	**courtship occurrence**	**copulation latency**
courtship occurrence	-1.06		
copulation latency	0.82	-0.75	
copulation occurrence	-0.87	0.85	-1.09

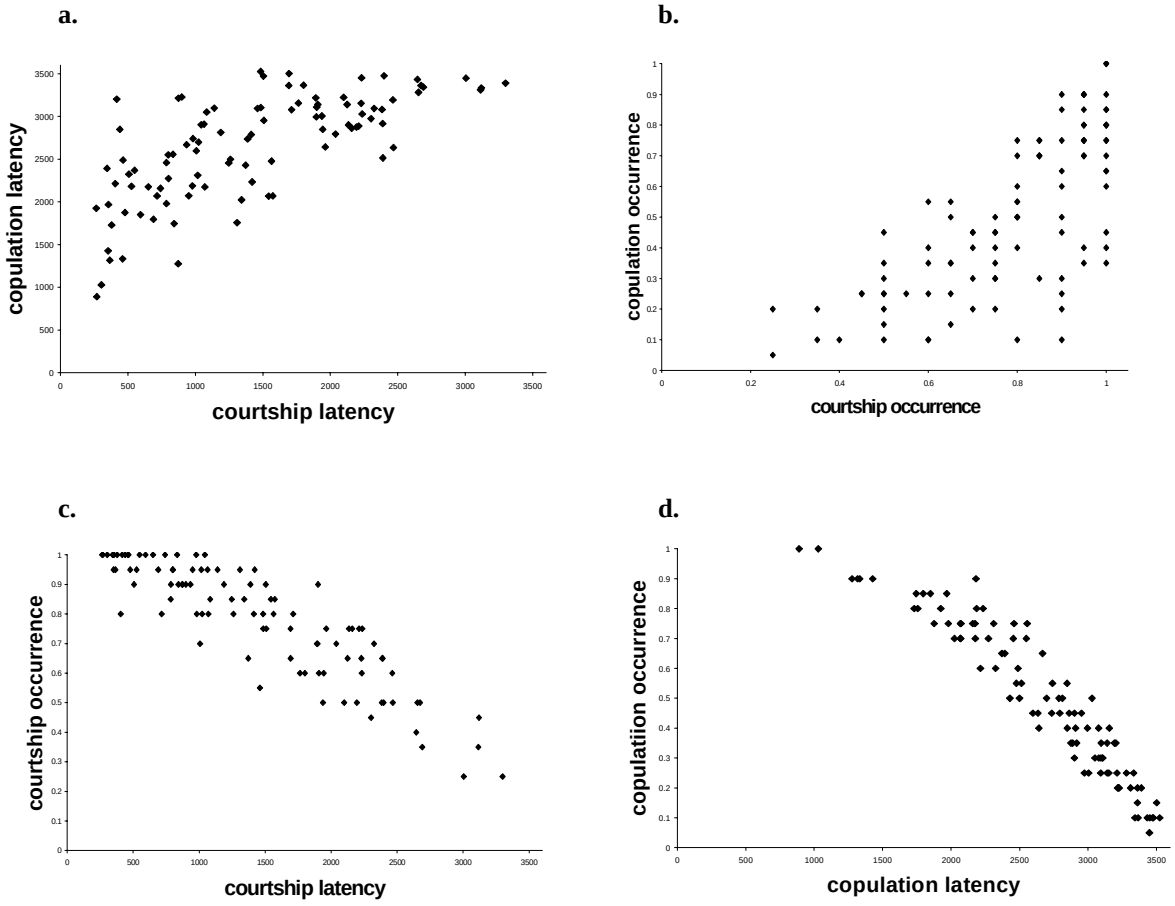


Figure 3.6: Correlations among line means for (a) courtship and copulation latency, (b) courtship and copulation occurrence, (c) courtship occurrence and latency and (d) copulation occurrence and latency.

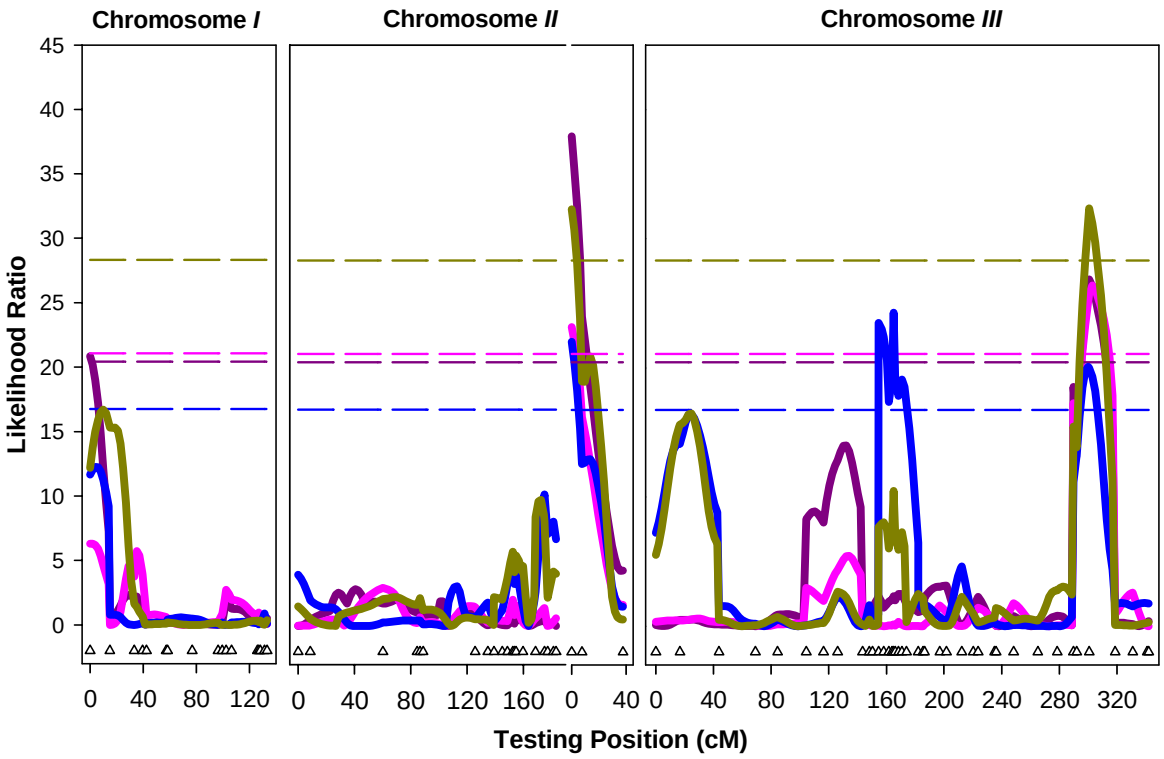


Figure 3.7: Likelihood ratio (LR) scores and significance thresholds plotted against chromosome location from multiple-trait composite interval mapping for courtship latency (purple), courtship occurrence (pink), copulation latency (dark blue) and copulation occurrence (gold). Horizontal lines represent the significance thresholds for each trait and the triangles on the x-axis represent the locations of cytogenetic markers.

Table 3.7: Summary of QTL mapping results. *Peak Likelihood Ratio (LR). †Size of the QTL region given by the 95% Confidence Limits (CL) (=2LOD support interval), in kilobase (kb) pairs (Sorsa, 1988). ‡Size of the QTL region given by the 95% CL, in CentiMorgans (cM) (Lindsley and Zimm, 1992). §Average number of genes in QTL region defined by the 95% CL, based on a total eukaryotic genome size of 120 megabase pairs and 13,600 genes and predicted genes; 1 gene on average = 8.8 kb (Adams *et al.*, 2000).

QTL	Marker*	95% CL	Trait	LR*	kb†	cM‡	Number Loci§
1	1B	1A-3E	Courtship latency	20.84	3,689	5	419
2	57C	57C-57F	Courtship latency Courtship occurrence Copulation latency Copulation occurrence	37.93 23.16 22.02 32.28	768	2	87
3	63A	61A-65A	Copulation latency	16.41	5,336	21	606
4	76A	72A-85F	Copulation latency	23.50	12,911	6	1,467
5	98A	96F-99A 97E-99A	Courtship latency Courtship occurrence Copulation latency Copulation occurrence	26.87 26.38 20.10 32.37	2,885	10	328

Epistasis between mating behavior QTL: We tested for epistatic interactions between the marker closest to each significant QTL (**Table 3.7**) and all of the other markers. After Bonferroni correction for multiple tests, none of the epistatic interactions were significant.

Deficiency complementation mapping: The sizes of the QTL intervals range from 2-21 cM (with an average of 8.8 cM), and include 87 to 1,467 genes. We therefore utilized deficiency complementation mapping to reduce the size of each QTL interval (**Table 3.8**, **3.9**; **Figure 3.8**, **3.9**). The QTL from 57C;57F fractionated into two much smaller regions at 56F5;56F8 and at 56F9;57A2. The region from 72A;85F was reduced from ~13,000 kb to two smaller regions. One region at 70E1;71F1-4 was 25 kb containing 22 genes for which no mutant stocks were available, and another region from 78C5-6;78E3-79A1 was 675 kb and contained 94 genes. The region from 96F; 99A was reduced from ~2,900 kb to a single smaller region at 96F1;97B1, with 580 kb and 98 genes. While each refined region could represent the effect of a single gene, it is quite possible that there are multiple closely linked genes within a region contributing to mating behavior. It should be noted that there are no candidate genes within these refined regions that have been previously implicated as affecting mating behavior.

Seven candidate genes are associated with variation in mating behavior: Of the mutations in 45 genes that were tested (**Table 3.3**), mutations in seven genes were found to fail to complement Ore and 2b QTL alleles for mating behavior (**Table 3.10, 3.11**;

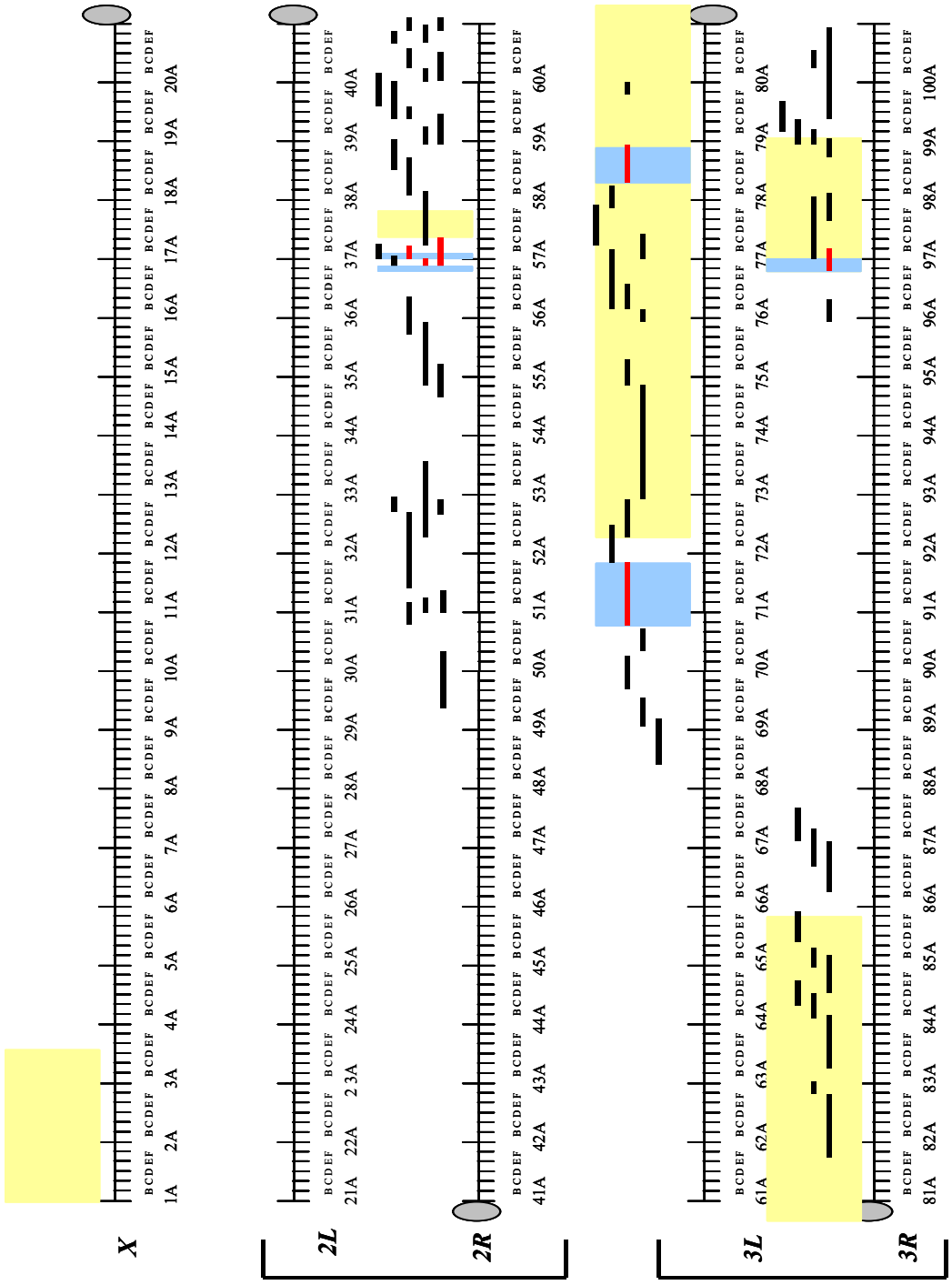


Figure 3.8: The significant regions from QTL mapping (yellow) and deficiency complementation mapping (blue) for the three major *Drosophila* chromosomes. Individual deficiencies that were tested are black lines, while those that showed failure to complement are in red. Centromeres are represented by a gray circle.

Table 3.8: Deficiency mapping results. L, line effect (Ore or 2b); G, genotype effect (*Df* or *Bal*); LxG, interaction term, denotes significance for the line tested. Significance underneath the following thresholds are indicated by asterisks: *=0.05, **=0.01, ***=0.001, ****=0.0001. Gray * for LxG indicates significance for the *Bal*, and therefore not considered failure to complement. †, lines that have had a replicate test due to significance in the initial assay. NS, not significant

Genotype	Cytological Location	courtship latency			courtship occurrence			copulation latency			copulation occurrence		
		L	G	LxG	L	G	LxG	L	G	LxG	L	G	LxG
QTL 57C;57F													
Df(2R)CX1	49C1-4; 50C23-D2	****	*	NS	**	NS	NS	***	**	NS	*	NS	NS
Df(2R)L48	50F6-9; 51B3	****	NS	NS	***	NS	NS	****	NS	NS	***	NS	NS
Df(2R)trix	51A1-2; 51B6	****	NS	NS	*	NS	NS	****	NS	NS	**	NS	NS
Df(2R)03072	51A5; 51C1	****	NS	NS	****	NS	NS	****	*	NS	****	NS	NS
Df(2R)Jp1	51D3-8; 52F5-9	****	**	NS	***	*	NS	****	NS	NS	**	NS	NS
Df(2R)vg89e88	52B3-C1; 53E2-F2	****	*	*	****	*	*	****	**	*	***	**	NS
Df(2R)Jp6	52E3-5; 52F	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Df(2R)Jp8	52F5-9; 52F10-53A1	NS	****	NS	NS	****	NS	NS	****	NS	NS	****	NS
Df(2R)Pcl7B	54E8-F1; 55B9-C1	***	NS	NS	*	*	NS	**	NS	NS	NS	NS	NS
Df(2R)PC4	55A; 55F	**	***	NS	NS	NS	***	****	NS	NS	***	NS	NS
Df(2R)P34	55E2-4; 56C1-11	****	NS	NS	NS	*	NS	****	NS	NS	NS	NS	NS
Df(2R)017 †	56F5; 56F15	****	***	*	****	***	**	****	*	*	****	*	*
Df(2R)min	56F8-17; 56F8-17	****	**	NS	**	NS	NS	****	****	*	***	*	NS

Table 3.8 (continued)

Genotype	Cytological Location	courtship latency			courtship occurrence			copulation latency			copulation occurrence		
		L	G	LxG	L	G	LxG	L	G	LxG	L	G	LxG
QTL 57C;57F													
Df(2R)AA21 †	56F9-17; 57D11-12	****	**	***	****	*	***	****	***	**	****	**	***
Df(2R)D4	57A1-3; 57B13	****	NS	NS	***	NS	NS	****	NS	NS	**	NS	NS
Df(2R)exu1 †	57A2; 57B1	****	NS	*	****	*	**	****	NS	NS	****	NS	*
Df(2R)Pu-D17	57B4; 58B	****	NS	NS	NS	NS	NS	**	*	NS	NS	NS	NS
Df(2R)X58-7	58B1-2; 58E4-10	****	NS	NS	**	NS	NS	NS	NS	NS	NS	NS	NS
Df(2R)X58-12	58D1-2; 59A	*	NS	NS	*	NS	NS	*	NS	NS	*	NS	NS
Df(2R)59AB	59A1-3; 59B1-2	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(2R)59AD	59A1-3; 59D1-4	**	**	NS	NS	NS	NS	**	***	NS	NS	NS	NS
Df(2R)twi	59C3-4; 59D1-2	****	NS	NS	****	*	NS	****	***	NS	****	***	NS
Df(2R)bw-S46	59D8-11; 60A7	****	***	NS	****	NS	NS	****	NS	NS	****	*	NS
Df(2R)or-BR6	59D5-10; 60B3-8	****	NS	NS	**	NS	NS	****	NS	NS	**	NS	NS
Df(2R)Chi[g230]	60A3-7; 60B4-7	****	**	NS	NS	**	NS	NS	NS	NS	NS	NS	NS
Df(2R)Px1	60B8-10; 60D1-2	NS	****	NS	NS	NS	NS	NS	****	NS	NS	NS	NS
Df(2R)Px2	60C5-6; 60D9-10	****	NS	NS	****	NS	NS	****	*	NS	****	*	NS
Df(2R)M60E	60E2-3; 60E11-12	****	NS	NS	****	**	NS	****	NS	NS	****	**	NS
Df(2R)ES1	60E6-8; 60F1-2	****	****	NS	**	****	*	***	****	*	*	****	NS

Table 3.8 (continued)

Genotype	Cytological Location	courtship latency			courtship occurrence			copulation latency			copulation occurrence		
		L	G	LxG	L	G	LxG	L	G	LxG	L	G	LxG
QTL 57C;57F													
Df(2R)Kr10	60F1; 60F5	****	**	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(2R)Kr14	60F2; 60F5	****	NS	NS	***	NS	NS	****	NS	NS	****	NS	NS
QTL 72A;85F													
Df(3L)vin7	68C8-11; 69B4-5	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(3L)iro-2 †	69B1-5; 69D1-6	****	**	NS	****	NS	NS	****	NS	NS	****	NS	NS
In(3LR)C190	69F3-4; 70C3-4	***	NS	**	NS	NS	*	****	NS	NS	**	NS	NS
Df(3L)fz-CAL5 †	70C2-6; 70E1	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(3L)Brd6 †	70E; 71F	****	****	**	****	****	****	****	****	*	****	****	****
Df(3L)brm11	71F1-4; 72D1-10	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(3L)st-F13	72C1-D1; 73A3-4	****	NS	NS	****	**	*	****	NS	*	****	*	NS
Df(3L)81k19	73A3; 74F	****	**	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(3L)W10	75A6-7; 75C1-2	****	NS	NS	****	NS	NS	****	*	NS	****	*	NS
Df(3L)VW3	76A3; 76B2	***	NS	*	NS	NS	NS	**	NS	*	**	NS	NS
Df(3L)kto2	76B1-2; 76D5	****	****	****	**	**	**	****	**	*	***	NS	NS
Df(3L)XS-533	76B4; 77B	****	**	*	***	**	*	****	NS	NS	***	NS	NS
Df(3L)rdgC-co2	77A1; 77D1	****	**	NS	****	*	NS	****	**	NS	****	*	NS

Table 3.8 (continued)

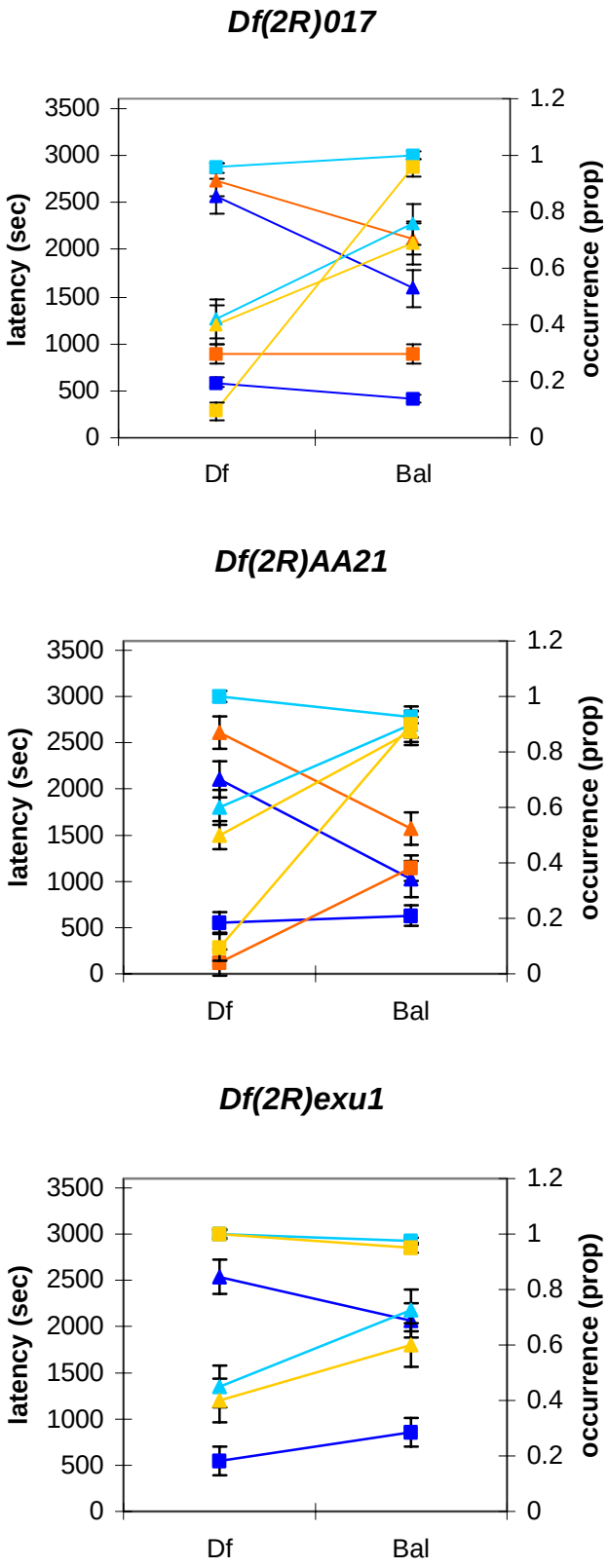
Genotype	Cytological Location	courtship latency			courtship occurrence			copulation latency			copulation occurrence		
		L	G	LxG	L	G	LxG	L	G	LxG	L	G	LxG
QTL 72A;85F													
Df(3L)ri-79c	77B-C; 77F-78A	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(3L)ME107	77F3; 78C8-9	****	NS	NS	***	NS	NS	****	NS	NS	****	NS	NS
Df(3L)Pc-2q †	78C5-6; 78E3-79A1	****	****	****	****	****	****	****	****	****	****	**	****
Df(3L)Delta1AK	79F; 80A	****	NS	NS	****	*	NS	****	*	NS	****	**	NS
Df(3R)ME15	81F3-6; 82F5-7	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(3R)3-4	82F3-4; 82F10-11	****	****	*	***	****	**	****	NS	NS	****	****	**
Df(3R)Tp110	83C1-2; 84B1-2	****	*	NS	****	*	*	****	****	*	****	NS	NS
Df(3R)roe	84A6-B1; 84D4-D9	****	***	NS	****	****	**	****	*	NS	****	*	NS
Df(3R)dsx2M	84C1-3; 84E1	****	NS	NS	****	NS	NS	****	*	NS	****	NS	NS
Df(3R)p712	84D4-6; 85B6	****	****	***	****	**	**	****	NS	NS	****	NS	**
Df(3R)p-XT103	85A2; 85C1-2	****	***	NS	****	*	NS	****	**	NS	****	*	NS
Df(3R)by10	85D8-12; 85E7-F1	****	**	NS	****	*	NS	****	***	NS	****	*	NS
Df(3R)by62	85D11-14; 85F16	***	NS	NS	**	NS	NS	****	*	NS	***	NS	NS
Df(3R)M-Kx1	86C1; 87B1-5	****	NS	NS	****	NS	NS	****	NS	*	****	NS	NS
Df(3R)T-32	86E2-4; 87C6-7	****	***	NS	****	*	NS	****	****	NS	****	***	NS
Df(3R)ry615	87B11-13; 87E8-11	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS

Table 3.8 (continued)

Genotype	Cytological Location	courtship latency			courtship occurrence			copulation latency			copulation occurrence		
		L	G	LxG	L	G	LxG	L	G	LxG	L	G	LxG
QTL 96F;99A													
Df(3R)96B	96A21; 96C2	**	NS	NS	NS	NS	NS	****	NS	NS	*	NS	NS
Df(3R)Espl3 †	96F1; 97B1	****	****	****	****	****	****	****	****	****	****	****	****
Df(3R)Tl-P	97A; 98A1-2	****	NS	NS	****	NS	NS	****	*	NS	****	NS	NS
Df(3R)D605	97E3; 98A5	****	*	NS	NS	NS	NS	****	NS	NS	**	NS	NS
Df(3R)3450	98E3; 99A6-8	****	NS	NS	**	NS	NS	**	NS	NS	*	NS	NS
Df(3R)Dr-rv1	99A1-2; 99B6-11	****	*	NS	****	*	NS	***	*	NS	**	*	NS
Df(3R)01215	99A6; 99C1	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Df(3R)L127	99B5-6; 99E4-F1	****	NS	NS	**	NS	NS	****	NS	NS	****	NS	NS
Df(3R)B81	99C8; 100F5	****	****	NS	****	****	*	****	****	NS	****	****	NS
Df(3R)awd-KRB	100C;100D	***	NS	NS	NS	NS	NS	**	**	NS	NS	NS	NS

Table 3.9: *P* values from ANOVA for deficiencies that showed failure to complement. NS, not significant.

Genotype	courtship latency	courtship occurrence	copulation latency	copulation occurrence
Df(2R)017	0.0144	0.0071	0.0491	0.0119
Df(2R)AA21	0.0009	0.0002	0.0020	0.0002
Df(2R)exu1	0.0146	0.0078	NS	0.0350
Df(3L)Brd6	0.0033	<0.0001	0.0133	<0.0001
Df(3L)Pc-2q	<0.0001	<0.0001	<0.0001	<0.0001
Df(3R)Espl3	<0.0001	<0.0001	<0.0001	<0.0001



△ 2b

□ Ore

courtship latency

courtship occurrence

copulation latency

copulation occurrence

Figure 3.9: Deficiency complementation tests and the standard error for the deficiencies and traits that showed failure to complement. Latency is measured in seconds; occurrence is measured as a proportion.

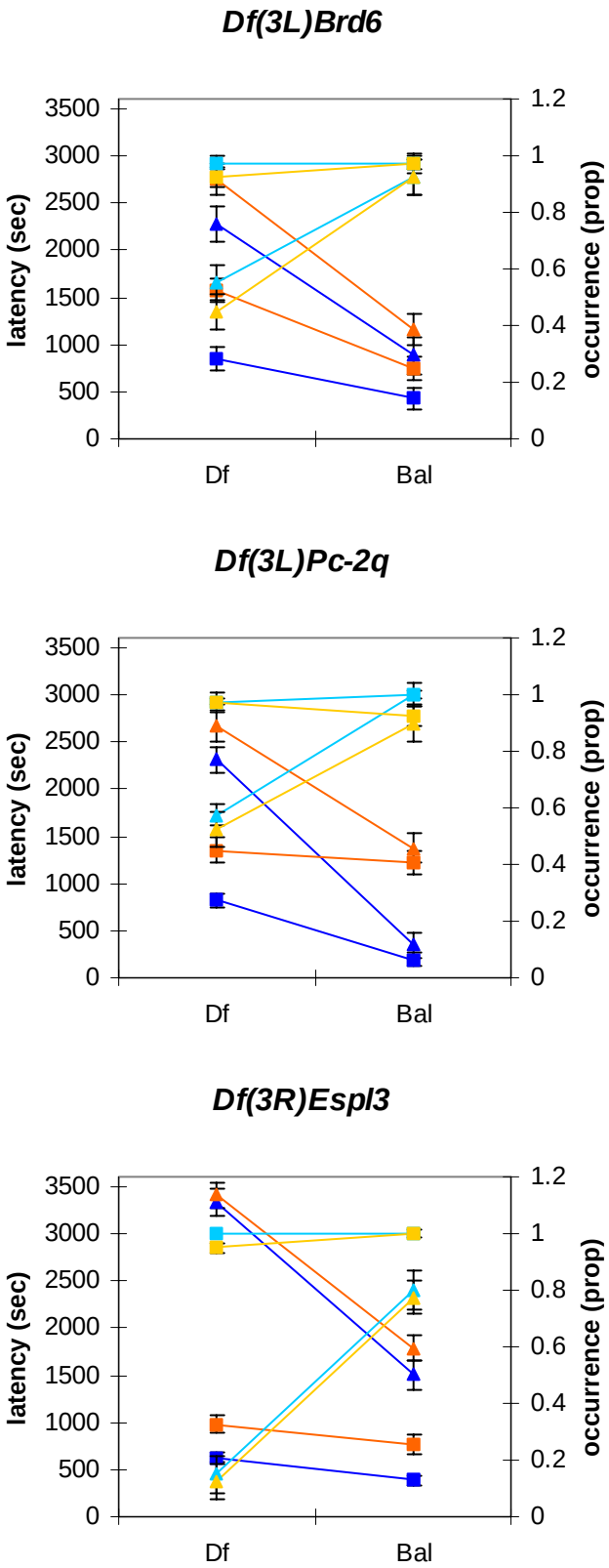


Figure 3.9
(continued)

Figure 3.11). None of these genes have previously been implicated for this trait. The gene products of *l(2)k02206* and *l(2)05510* have not been characterized. *18 wheeler* (*18w*), located cytologically at 56F8, encodes a transmembrane receptor localized to the plasma membrane. It is involved in morphogenesis during pattern formation and imaginal cell determination, and mutants often display morphological defects in their appendages. It is also involved in the development of the humoral immune response (Williams *et al*., 1997, Eldon *et al*., 1994).

spermatocyte arrest (*sa*), located at 78A2-C9, encodes a product involved in spermatid development (Fuller, 1998). It is required for male meiotic cell cycle progression and the initiation of postmeiotic differentiation (Lin *et al*., 1996). Mutants have multipolar spindles in male meiosis and irregular mitotic figures in the larval neuroblasts, which is the result of aberrant behavior of the mitotic spindle during embryonic cleavage (Wilson and Fuller, 1991). The meiotic arrest phenotype is similar to that seen for meiosis I maturation arrest infertility in human males, suggesting that the pathway control is conserved from flies to man (Lin *et al*., 1996).

Polycomb (*Pc*), located at 78C6-7, is named for the addition of sex comb teeth on the second and third legs of mutants (Duncan and Kaufman, 1975). It interacts with at least 59 other genes, including *Antennapedia* and *trithorax* (The Flybase Consortium). *Polycomb* expression is localized to the nucleus where it encodes a transcriptional repressor (Gould *et al.*, 1990; Roseman *et al.*, 2001).

eagle (*eg*), located at 78F3, is aptly named for the spread wing phenotype of mutants (Duncan and Kaufman, 1975). It encodes a nuclear transcription factor involved in fate determination of sister serotonin neurons in the central nervous system (Dittrich *et*

Table 3.10: The genes that were tested for failure to complement. Significance underneath the following thresholds are indicated by asterisks: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001. † , lines that have had a replicate test due to significance of the initial assay. NS, not significant; NA, not applicable.

Genotype	Cytological Location	courtship latency			courtship occurrence			copulation latency			copulation occurrence		
		L	G	LxG	L	G	LxG	L	G	LxG	L	G	LxG
QTL 56F5;57B4													
BG00756	56D15-E1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NS	NS	NS
l(2)k08002	56F6-9	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
18 wheeler †	56F8	****	NS	*	****	NS	NS	****	NS	NS	**	NS	NS
humpy	56F9; 58A1	****	NS	NS	****	NS	NS	****	NS	NS	***	NS	NS
mus209	56F10-11	****	NS	NS	****	NS	NS	****	NS	NS	**	NS	NS
BG01288	56F11	NA	NA	NA	NA	NA	NA	NA	NA	NA	**	NS	NS
l(2)s1866	56F11-12	****	NS	NS	**	NS	NS	****	*	NS	**	NS	NS
BG02518	56F16	NA	NA	NA	NA	NA	NA	NA	NA	NA	*	NS	NS
l(2)s4831	57A3-4	****	*	NS	****	NS	NS	****	NS	NS	****	NS	NS
l(2)k09920	57A3-6	**	NS	NS	****	NS	NS	****	NS	NS	**	NS	NS
l(2)k16204 †	57A5-6	****	NS	NS	****	NS	NS	****	NS	NS	***	NS	NS
l(2)05510 †	57A5-6	****	****	****	****	****	****	****	**	**	****	***	***
BG02102	57A5-6; 57A6	NA	NA	NA	NA	NA	NA	NA	NA	NA	****	NS	NS
bancal	57A6-7	****	NS	NS	****	NS	NS	****	NS	NS	***	NS	NS

Table 3.10 (continued)

Genotype	Cytological Location	courtship latency			courtship occurrence			copulation latency			copulation occurrence		
		L	G	LxG	L	G	LxG	L	G	LxG	L	G	LxG
QTL 56F5;57B4													
l(2)k02206 †	57A8-9	****	NS	NS	****	NS	*	****	NS	NS	****	NS	NS
BG01609	57A9	NA	NA	NA	NA	NA	NA	NA	NA	NA	****	NS	NS
l(2)k06409	57B1-3	****	NS	NS	****	NS	NS	****	NS	NS	NS	NS	NS
BG02471	57B1; 57B2-3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NS	NS	NS
inscuteable	57B3	****	NS	NS	****	NS	NS	****	NS	NS	**	NS	NS
QTL 78C5;79A1													
spermatocyte arrest †	78A2; 78C9	****	**	***	NS	NS	*	****	NS	*	NS	NS	NS
l(3)04063	78A3; 78D2	****	NS	NS	**	NS	NS	****	NS	NS	**	NS	NS
l(3)neo29	78C	****	NS	NS	****	NS	**	****	NS	NS	****	NS	NS
l(3)ry3	78C; 78D	****	NS	NS	**	NS	NS	****	NS	NS	*	NS	NS
BG01493	78C3-4; 78C7	NA	NA	NA	NA	NA	NA	NA	NA	NA	***	**	NS
Polycomb †	78C6-7	****	NS	***	****	NS	NS	***	****	****	**	**	**
Aef1	78C8	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
l(3)78Da	78C8-9	****	NS	NS	**	NS	NS	****	NS	NS	**	*	*
SR3-7	78D; 79A	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Hr 78	78D1	**	NS	NS	*	NS	NS	***	*	NS	**	NS	NS
l(3)00534	78D1-2	****	NS	NS	***	NS	NS	****	NS	NS	*	NS	NS

Table 3.10 (continued)

Genotype	Cytological Location	courtship latency			courtship occurrence			copulation latency			copulation occurrence		
		L	G	LxG	L	G	LxG	L	G	LxG	L	G	LxG
QTL 78C5;79A1													
M6	78D4	****	NS	NS	*	NS	NS	****	NS	NS	****	NS	NS
eagle †	78E5-6	****	****	*	****	***	****	****	**	*	****	NS	**
CG7145	78F1	****	NS	NS	*	NS	NS	***	NS	NS	**	NS	NS
BG01362	78F4-79A1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NS	***	NS
BG01919	79A7	NA	NA	NA	NA	NA	NA	NA	NA	NA	****	*	NS
QTL 96F;97A													
taxi	96A20; 96F9	****	NS	NS	**	NS	NS	****	*	NS	****	NS	NS
l(3)rQ197	96F1-2	****	*	NS	**	NS	NS	****	NS	NS	**	NS	NS
Enhancer of split †	96F8	****	****	**	***	***	***	****	***	NS	****	***	*
BG02029	96F8	NA	NA	NA	NA	NA	NA	NA	NA	NA	***	*	NS
groucho	96F8-9	****	NS	NS	***	NS	NS	****	*	NS	****	NS	NS
Prickly	96F11-14	****	NS	NS	**	NS	NS	****	NS	NS	****	NS	NS
spindle-D	97A1-2	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
goulash	97A1; 98A2	****	NS	NS	****	NS	NS	****	NS	NS	****	NS	NS
Aldolase	97A6	***	NS	NS	*	*	NS	****	NS	NS	NS	NS	NS
BG01280	97B6; 97B9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NS	***	NS

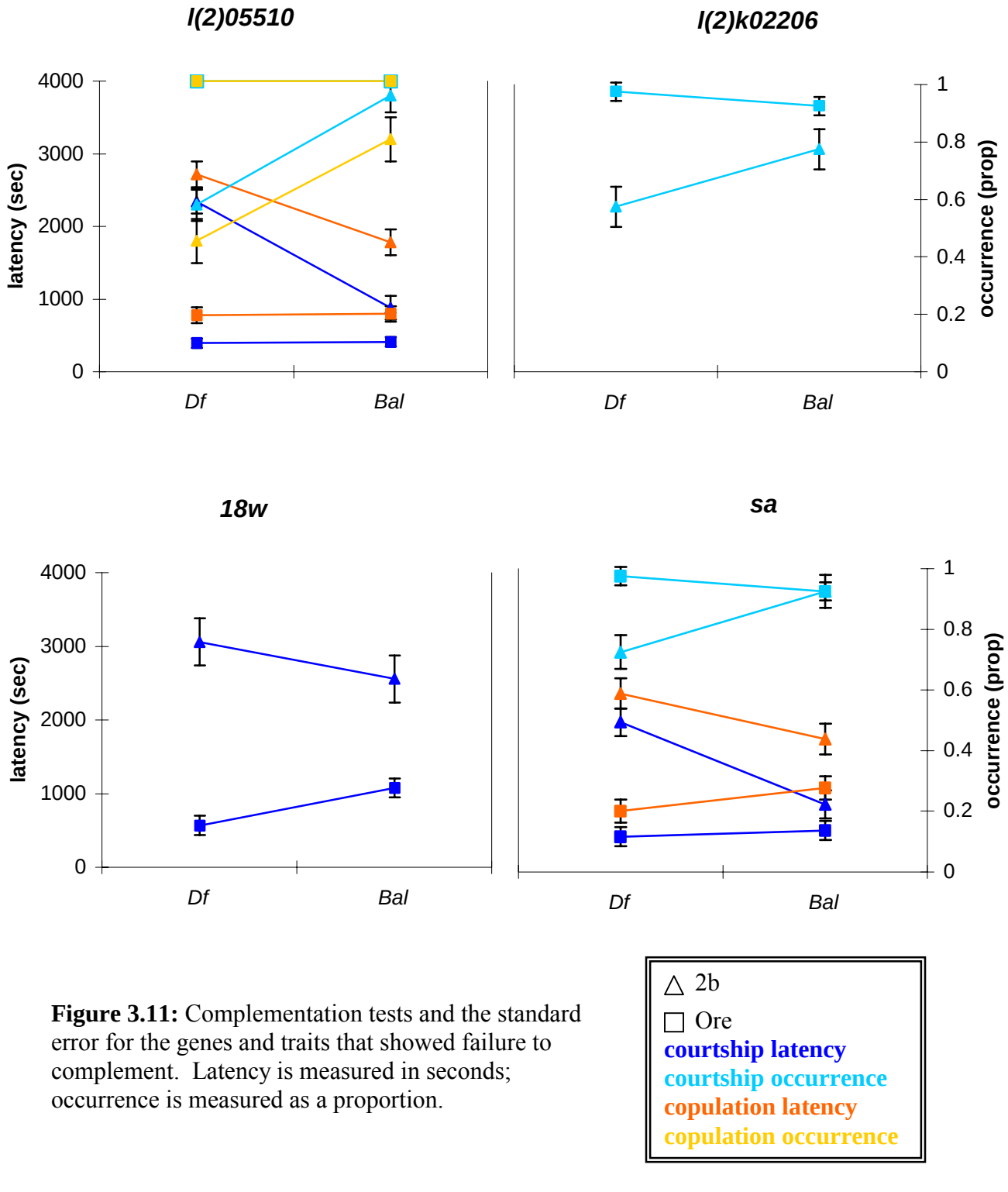


Figure 3.11: Complementation tests and the standard error for the genes and traits that showed failure to complement. Latency is measured in seconds; occurrence is measured as a proportion.

Figure 3.11 (continued)

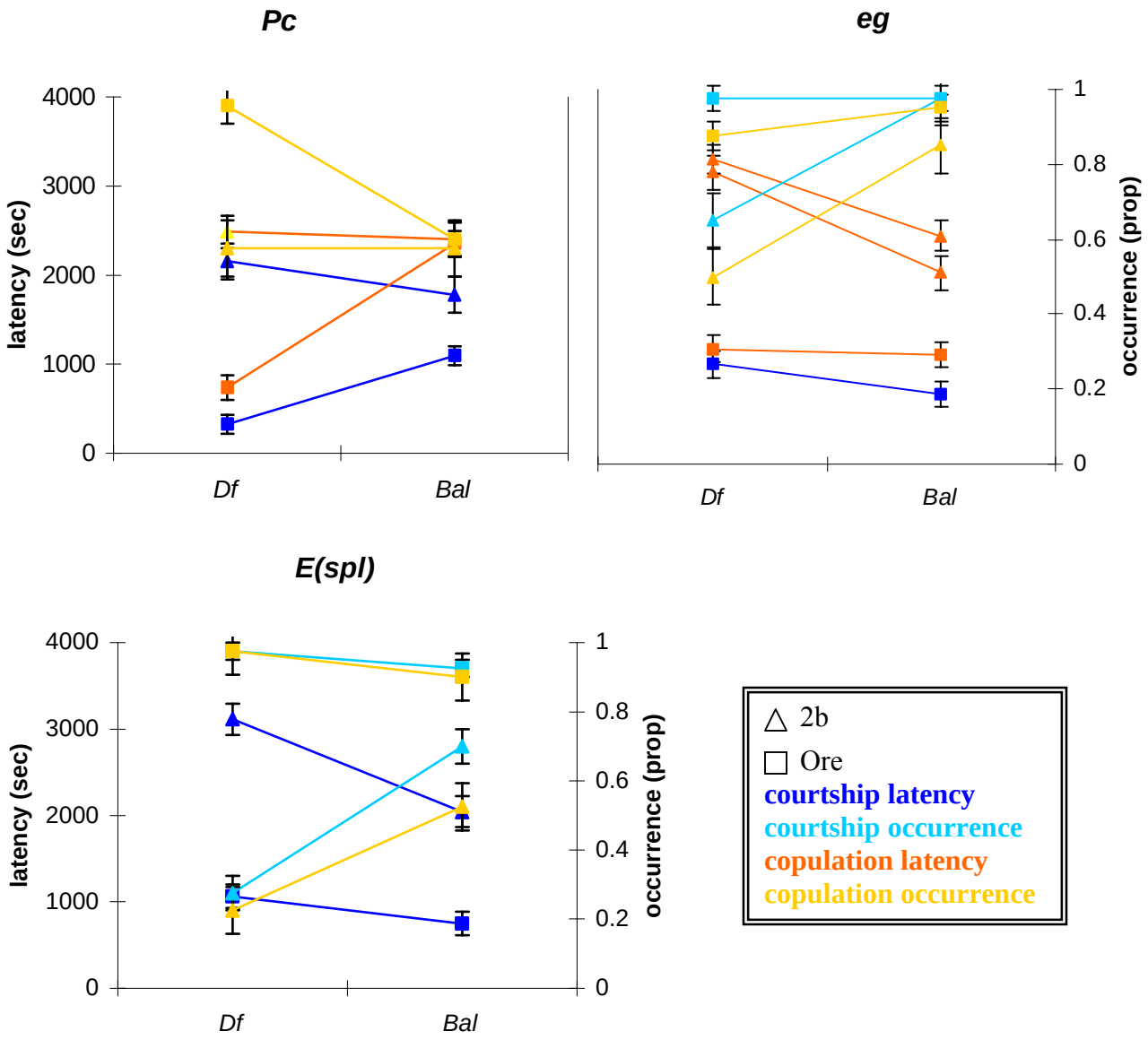


Table 3.11: *P* values from ANOVA for candidate genes that showed failure to complement. NS, not significant.

Candidate gene	courtship latency	courtship occurrence	copulation latency	copulation occurrence
l(2)05510	0.0001	<0.0001	0.0018	NS
l(2)k02206	NS	0.0300	NS	0.0008
18w	0.0177	NS	NS	NS
sa	0.0002	0.0405	0.0116	NS
Pc	0.0008	NS	<0.0001	0.0074
eg	0.0202	<0.0001	0.0138	0.0017
E(spl)	0.0011	0.0003	NS	0.0205

al., 1997). Serotonergic cells are almost entirely lacking in loss of function mutants, while hypomorphic alleles result in a dramatic reduction in the number of serotonin-producing neurons (Lundell and Hirsh, 1998). This gene is particularly interesting since several other genes involved in the catacholamine pathway, such as *Dopa decarboxylase* (Tempel *et al.*, 1984) and *pale* (Buchner, 1991), have previously been shown to affect mating behavior.

Genes of the *Enhancer of split* [*E(spl)*] complex, located at 96F10, act as a functional unit composed of redundant genes which can partially substitute for each other. *E(spl)* encodes an RNA Polymerase II transcription factor whose product is thought to function as a receptor rather than serving as a signal (Technau and Campos-Ortega, 1987). It is involved in mesoderm development (Corbin *et al*., 1991) and differentiation of the neural ectoderm into epidermoblasts and neuroblasts (Knust et al., 1987). Increased levels of E(spl) product favor epidermal differentiation, whereas decreased levels favor neuronal differentiation. Mutants have no ventral cuticle and display hypertrophy of the central nervous system, whereby there is an increase in the size rather than the number of neuron cells (Corbin *et al*., 1991).

DISCUSSION

We have identified putative candidate genes affecting variation in mating behavior within a species using a three-step protocol. A whole genome scan for QTL identified relatively large genomic regions harboring one or more genes affecting mating behavior. Quantitative complementation to deficiencies further defined the QTL locations, and complementation tests to positional candidate genes revealed seven novel

candidate loci associated with variation in mating behavior between Oregon and 2b. Identification of the genes *18 wheeler*, *l(2)k02206*, *l(2)05510*, *spermatocyte arrest*, *eagle* and *Enhancer of split* as those that underlie the genetic basis of variation in mating behavior in these two lines of *Drosophila melanogaster* provides a framework with which to understand the genetic basis of naturally-occurring variation in mating behavior. These genes have not been implicated through *P*-element or chemical mutagenesis as affecting mating behavior. The most likely reason for this is that these genes had simply never been tested previously for mating behavior.

The genes that previously had been identified through mutagenesis as influencing the production of mating behavior, such as *fruitless*, *period* and *transformer*, do not show significant variation for mating behavior in our lines. Although mutational analysis has been crucial in the identification of the factors necessary in order for mating behavior to be produced, these loci seem to have little effect on the genetic basis of variation in mating behavior, at least in the two lines that we tested. Perhaps it is due to their critical role in development of the proper sexual orientation that these genes do not vary in nature, for selection would act against any changes which would greatly decrease overall reproductive success.

The identification of novel genes affecting behavior using QTL mapping reinforces the utility of this technique. Null mutations in many genes have an embryonic, larval or adult-lethal phenotype as homozygotes, precluding the characterization of their behavioral effects. Alternatively, mutations at many genes may not have any visible effect on a particular trait, and would have to be tested for every trait of interest before the function of the gene would become clear. However, the use of QTL mapping relies

upon naturally-occurring variation in order to identify the genetic basis of a trait, enabling the characterization of gene functions for which a mutant would be lethal. It also greatly narrows the number of genes that are candidates for a trait, allowing for the testing of a reasonable number individual candidate genes.

At least seven candidate genes have been identified here as influencing variation in mating behavior, with the strong certainty that there are many additional genes which contribute to variation in behavior overall. These seven genes do not overlap those found when the same recombinant inbred lines were analyzed for variation in sex comb tooth number (which are used by the male to grip the female during copulation) (Nuzhdin and Reiwitch, 2000), and variation in courtship song (Gleason *et al.*, 2002), demonstrating either that the variation in these lines for sex comb and courtship song do not contribute significantly to the initiation of courtship and copulation or that variation in these modalities alone do not affect the overall success of courtship in these lines.

QTL mapping is a powerful tool for detecting the genes underlying variation in a trait, but is limited in power by the sample size used. While we were able to locate multiple QTL for mating behavior, it seems quite likely that there are additional genes with smaller effects that could be identified by increasing the number of RI lines and the number of individuals tested per line. Furthermore, we were only able to test candidate genes within our mapped regions for which a mutant stock was available, and it is possible for there to be additional genes within these regions that contribute to variation in mating behavior that we were unable to test due to the unavailability of a mutant stock.

The candidate genes affecting variation for mating behavior between Oregon-R and 2b can be compared to those found to cause partial reproductive isolation of the

Zimbabwe subpopulation of *D. melanogaster* with other populations. The Zimbabwe subpopulation exhibits strong prezygotic reproductive isolation from the other African populations as well as all of the other continental *D. melanogaster* strains that have been tested, whereby Zimbabwe females do not mate readily with non-Zimbabwe males (Wu *et al.*, 1995). A comparison of genes found to contribute to isolation of the Zimbabwe population from other populations with those for variation of mating behavior within a species will allow us to address whether genes underlying variation within a species are the same as those that cause incipient reproductive isolation.

By performing mating behavior assays on a cosmopolitan line into which Zimbabwe chromosomes had been substituted, it was found that each of the major chromosomes had an effect on the ability of a non-Zimbabwe male to mate with a Zimbabwe female (Hollocher *et al.*, 1997). A later study used recombinant lines containing portions of the Zimbabwe third chromosome to map for loci contributing to partial reproductive isolation (Ting *et al.*, 2001). Although it is impossible to tell the precise point of recombination with the eight markers that were used, it is possible from their results for all of the third chromosome genes found in this study (*sa*, *Pc*, *eg* and *E(spl)*) to also be involved in the divergent behavior seen with the Zimbabwe subpopulation. It would be very interesting to use linkage disequilibrium mapping to test whether these specific genes are having an affect on the reproductive behavior of the Zimbabwe population.

Although it is not a direct comparison, it is interesting to contemplate whether these genes also affect variation in mating behavior between Drosophila species. Recombination mapping of courtship song differences between D. pseudoobscura and D.

persimilis found a cytological region surrounding the equivalent of 93-98, which overlaps the region we found for courtship and copulatory behavior at 96F-99A (Williams et al., 2001). In a recent study which used introgression lines to identify QTL for divergence between D. simulans and its sibling species D. sechellia (Civetta and Cantor, 2004), cytological region 84A-86B was found to be the only region on the third chromosome to significantly contribute to courtship latency and copulation latency. This region was not significant for variation in mating behavior within the two lines of D. melanogaster that we tested, which could indicate that genes responsible for variation within a species are not the same as those causing divergence between species. However, it is possible that the two lines that we used for this experiment are not representative of the larger population of D. melanogaster, or, more plausibly, that the genes causing divergence between D. simulans and D. sechellia are different than those that cause divergence between D. melanogaster and one of its sibling species.

Understanding the genetic basis for behavior in Drosophila, particularly understanding the contributions of QTLs, can open the door for behavioral genetics research in a multitude of other species, including humans. Mutagenesis studies have identified single genes affecting mating behavior, providing the genetic framework by which the formation of sexual orientation, neural processing of external stimuli, and manifestation of response are built. The isolation of QTLs for mating behavior will further characterize the components responsible for the variation seen within and among species in the courtship ritual, enhancing the picture of how genes and environment interact to produce the behavior that is expressed. This is a critical step in understanding the genetic basis of evolution since it is only upon variation that selection acts to create

shifts in gene frequencies, leading to the divergent evolution of two populations, and ultimately, speciation.

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CHAPTER 4

Quantitative Trait Loci For Sexual Isolation Between *Drosophila simulans* and *D. mauritiana*

Amanda J. Moehring*, Jian Li*, Malcolm D. Schug†, Shelly G. Smith†,
Matthew deAngelis‡, Trudy F. C. Mackay* and Jerry A. Coyne‡

* *Department of Genetics, North Carolina State University, Raleigh, NC 27695, USA*
† *Department of Biology, University of North Carolina, Greensboro, Greensboro, NC 27402, USA*
‡ *Department of Ecology and Evolution, University of Chicago, Chicago, IL 60637, USA*

This chapter consists of a manuscript that will be submitted for publication to *Nature*. I performed all DNA extractions. I developed and genotyped all SNP markers. I performed all data analysis except for genetic map construction and logistic CIM, which were performed by Jian Li. I wrote the manuscript. Shelly Smith genotyped the microsatellite markers while under the supervision of Malcolm Schug. Matt deAngelis created the backcross flies and scored behavior while under the supervision of Jerry Coyne. Trudy Mackay supervised my portion of the research project and provided extensive suggestions on the organization and content of the manuscript.

ABSTRACT

Speciation is characterized by the evolution of prezygotic isolation (ethological barriers to interspecific mating) and postzygotic isolation (reduced viability and fertility of interspecific hybrids). Although recent progress has been made towards understanding the genetic basis of postzygotic isolation (Wittbrodt *et al.*, 1989; Ting *et al.*, 1998; Barbash *et al.*, 2003; Presgraves *et al.*, 2003), little is known of the genetic architecture of sexual isolation – arguably the most important form of reproductive isolation in animals (Mayr, 1963; Coyne and Orr, 1997, 1998). Previous studies of prezygotic isolation in Drosophila have localized genes affecting sexual isolation to whole chromosomes (Zouros, 1981; Coyne, 1989; 1993; 1996; Noor, 1997). Here, we map quantitative trait loci (QTL) contributing to pre-zygotic reproductive isolation between *Drosophila simulans* and *D. mauritiana*. We mapped at least seven QTL affecting discrimination of *D. mauritiana* females against *D. simulans* males, three QTL affecting *D. simulans* male traits against which *D. mauritiana* females discriminate, and six QTL affecting *D. mauritiana* male traits against which *D. simulans* females discriminate. QTL affecting sexual isolation are largely different in males and females, and are not disproportionately concentrated on the *X* chromosome. The observation of a few QTL with moderate to large effects will facilitate positional cloning of genes underlying sexual isolation. In contrast to results for postzygotic isolation (Coyne, 1996a; Davis *et al.*, 1994; Hollocher *et al.*, 1997; Presgraves, 2003; Tao *et al.*, 2003), no epistasis was detected between QTL for prezygotic isolation.

INTRODUCTION

D. simulans and *D. mauritiana* are sibling species in the *D. melanogaster* subgroup. *D. simulans* is a cosmopolitan species largely commensal with humans, while *D. mauritiana* is restricted to the island of Mauritius, on which *D. simulans* does not occur. *D. mauritiana* probably arose after colonization by a *D. simulans*-like ancestor approximately 250,000 years ago (Kliman *et al.*, 2000). This species pair is separated by male-limited hybrid sterility and three traits involved in pre-zygotic reproductive isolation. The sexual isolation is asymmetrical – in the laboratory, *D. mauritiana* females rarely mate with courting *D. simulans* males, but the reciprocal cross occurs readily. The sexual isolation is thus based on a species difference in female mating preference (*D. mauritiana* females discriminate against *D. simulans* males, but *D. simulans* females do not discriminate against *D. mauritiana* males) as well as a difference in an unknown male trait (or traits) against which the females discriminate (Coyne, 1989). Although *D. simulans* females readily copulate with a courting *D. mauritiana* male, the copulations are often abnormally short, with many terminating prior to the time needed for adequate sperm transfer (Coyne, 1993). The shortened copulations are thus a form of post-mating, prezygotic isolation.

Because F_1 females are fertile and can be readily crossed to *D. mauritiana* males to produce segregating backcross (BC) progeny, we can map the genes contributing to species divergence in female mate choice, the male traits that are discriminated against, and the attenuated period of copulation. Such data enable us to address longstanding questions regarding the genetic basis of pre-zygotic isolation: Do few or many genes contribute to reproductive isolating mechanisms? What are the relative magnitudes of

their effects? How are these loci distributed throughout the genome? Do they interact epistatically? Do the same loci contribute to reproductive isolation in males and females, and in the two species?

In this study we generated three groups of approximately 1000 backcross (BC) individuals and paired them singly with pure-species individuals. For each BC individual we recorded whether or not copulation occurred, and copulation latency and copulation duration for those flies that did copulate. All BC individuals were genotyped for 32 evenly spaced molecular markers fixed for alternate alleles in the two pure species stocks (Table 1 – markers and cytological and genetic map positions). We performed genome scans for quantitative trait loci (QTL) affecting reproductive isolation in each of the BC populations, using composite interval mapping (CIM) (Zeng, 1994). Copulation occurrence is a binary trait (mated or not mated). We analyzed these binary data using standard CIM and also using a logistic regression model (Falconer and Mackay, 1996; Xu and Atchley, 1996; Tao *et al.*, 2003).

RESULTS AND DISCUSSION

QTL affecting the discrimination of *D. mauritiana* females against *D. simulans* males were mapped by pairing BC females with *D. simulans* males. 239 of the 1005 BC females tested mated (23.8%). At least seven QTL, all with large effects, affect female mate choice: two on the *X*, two on the second and three on the third chromosome (**Figure 4.1A**, **Table 4.2**). One additional QTL was identified with the logistic model on the third chromosome. The magnitudes of effects are consistent with a previous study mapping at least one factor associated with female sexual isolation to all major chromosomes, with

Table 4.1: Molecular markers and map positions. *r* is the recombination rate between two adjacent markers. The genetic distance *d* was inferred from *r* using the Kosambi map function, $d = 1/4 \ln[(1+2r)/(1-2r)]$.

Marker number	Marker names	Cytological location	*r*	Genetic distance (cM)
***X* Chromosome**				
1	*ewg*	1A	0.0000	0.00
2	*Dmu56661*	4F	0.1663	17.29
3	*Deltex*	6B	0.0851	8.59
4	*AC004114*	8E	0.1437	14.79
5	*V*	9F	0.0691	6.95
6	*DroExo2*	13F	0.2563	28.31
7	*DroMariadne*	16F	0.1893	19.92
8	*run*	19E	0.1311	13.42
Chromosome *2*				
9	*DroExpand*	21C	0.0000	0.00
10	*Gpdh*	26A	0.2537	27.96
11	*AC005889*	30A	0.1561	16.15
12	*prd*	33C	0.1660	17.25
13	*AC002474*	38D	0.3451	42.42
14	*DucC*	42C	0.2828	32.05
15	*eve*	46C	0.0854	8.62
16	*Su(z)*	49E	0.1219	12.44
17	*sli*	52D	0.1341	13.75
18	*AC004365*	58A	0.2571	28.42
19	*twi*	59C	0.1030	10.45
Chromosome *3*				
20	*ve*	62A	0.0000	0.00
21	*h*	66D	0.2148	22.97
22	*CycA*	68E	0.1379	14.16
23	*Eip71CD*	71D	0.1143	11.64
24	*rdgC*	77B	0.1485	15.31
25	*5-HT2*	82C	0.0714	7.19
26	*Antp*	84B	0.0575	5.78
27	*DroHoxNK4*	93D	0.0490	4.92
28	*DroTrxIII3*	88B	0.2840	32.23
29	*Dmtf125*	95C	0.3667	46.80
30	*Ald*	97A	0.1523	15.73
31	*DroRough*	97D	0.0793	8.00
32	*Efld2*	100E	0.2060	21.90

Figure 4.1: QTL affecting prezygotic reproductive isolation between *D. simulans* and *D. mauritiana*. (**A**) BC females paired with *D. simulans* males. This group identifies the QTL in *D. mauritiana* females that affect their discrimination against *D. simulans* males. (**B**) BC males paired with *D. mauritiana* females. This group identifies the QTL in *D. simulans* males that *D. mauritiana* females discriminate against. (**C**) BC males paired with *D. simulans* females. This group identifies the QTL in *D. mauritiana* males that *D. simulans* females discriminate against. Molecular markers are indicated as black triangles on *x*-axis. Plots are likelihood ratio (LR) test statistics for copulation occurrence (dark blue = copulation occurrence, CIM; light blue = copulation occurrence, logistic model); copulation latency (yellow) and copulation duration (purple) as determined by composite interval mapping. Significance thresholds for each trait were determined by permutation and are denoted by dashed lines with the same color code as the traits.

Figure 4.1

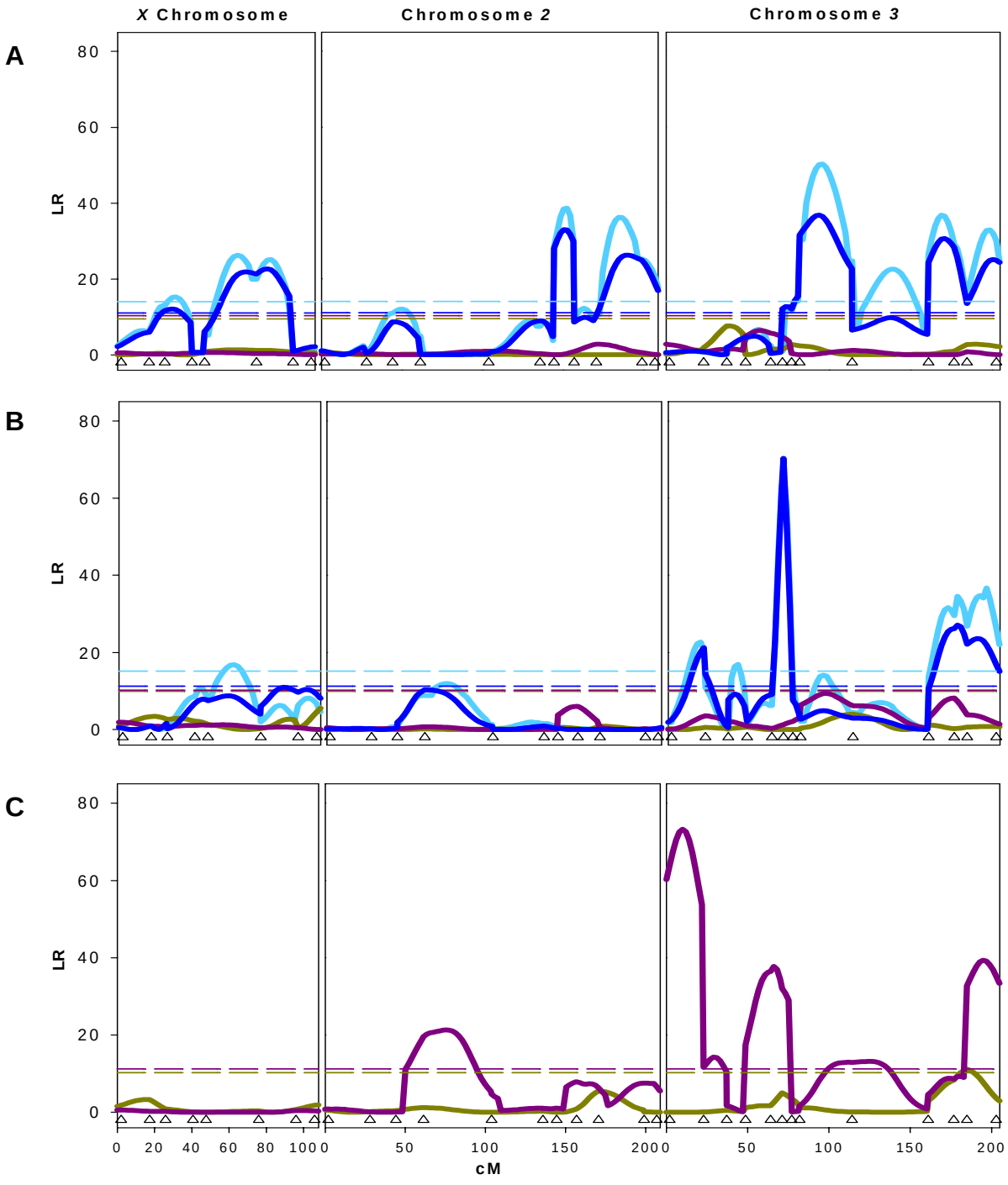
X Chromosome
Chromosome 2
Chromosome 3
A
B
C
LR
0
20
40
60
80
0 20 40 60 80 100
0 50 100 150 200
0 50 100 150 200
cM

Table 4.2: QTL affecting prezygotic reproductive isolation. QTL regions are estimated from 2 LOD support intervals ($P \leq 0.05$). When evaluating the regions, note that there is a large inversion from 84F-93F in relation to *D. melanogaster*. The peak is the cytological location with the highest likelihood ratio (LR). QTL effects are in phenotypic standard deviation units.

		CIM				*Logistic CIM*			
Cross	**Trait**	**Region**	**Peak**	**LR**	**Effect**	**Region**	**Peak**	**LR**	**Effect**
BC fem. × *D. sim.* males	Copulation occurrence	5E-7F	6E	12.11	6.13	5E-8C	7B	15.32	0.709
		10F-16F	15A	22.71	8.59	9D-16F	12D	26.31	0.948
		46C-49E	47F	32.99	10.90	46C-49E	48C	38.64	1.188
		52F-59C	56B	26.25	10.88	53B-59C	55B	36.26	1.242
		88B-93F	91C	36.79	13.39	88F-93D	91B	50.26	1.382
		-	-	-	-	87B-94E	85E	22.56	0.978
		95C-97D	96D	30.67	9.70	95C-97D	96B	36.80	1.077
		97D-100E	99F	25.12	8.83	97D-100E	99E	32.92	0.977
BC males × *D. mau.* fem.	Copulation occurrence	-	-	-	-	10D-12F	11D	16.84	-0.725
		64D-67A	66C	21.19	−12.19	64A-66D	65F	22.67	-0.983
		-	-	-	-	69A-71B	70C	16.87	0.958
		81B-83E	82C	70.31	−17.55	79E-83E	82C	66.07	–1.349
		95D-100E	97B	27.06	−9.51	95C-100E	99C	36.70	-0.870
BC males × *D. sim.* fem.	Copulation duration	30A-36F	34D	21.33	0.333	N/A			
		62A-66D	64A	73.29	0.583				
		66D-68E	67D	14.28	0.306				
		71D-84B	79B	37.73	0.398				
		85F-90E	87A	13.19	0.277				
		97D-100E	99A	39.37	0.401				
	Copulation latency	97C-97F	97D	11.19	0.213				

the effects of the autosomes much greater than that of the *X* chromosome (Coyne, 1989). No QTL for copulation latency or duration were detected.

We mapped QTL associated with traits of male *D. simulans* against which female *D. mauritiana* discriminate by pairing *D. mauritiana* females with BC males. 459 of the 1002 flies tested copulated (45.8%). At least three QTL with large effects, all on chromosome *3*, contribute to differences between males causing sexual isolation (**Figure 4.1B, Table 4.2**). Two additional QTL with small effects were detected by the logistic model, one each on the *X* and third chromosomes. These data are consistent with a previous study in which at least one gene on each of the *X* and third chromosomes affected sexual isolation of male BC hybrids (Coyne, 1996b). No QTL for copulation latency or duration were detected.

The genetic basis of shortened copulation between *D. simulans* females and *D. mauritiana* males was studied by pairing *D. simulans* females with BC males. The duration of copulation ranged from 0.58 – 49.35 minutes (SD = 7.30 minutes). At least six autosomal QTL (one on the second and five on the third chromosome) with moderate effects are associated with male *D. mauritiana* traits used by *D. simulans* females to discriminate against these males after copulation (**Figure 4.1C, Table 4.2**). A single QTL for copulation latency mapped to the tip of chromosome *3* (**Figure 4.1C, Table 4.2**). Again, these results are consistent with a previous study of shortened copulation in this interspecific cross, which mapped at least one gene with large effects to the second and third chromosomes, with a marginally significant contribution from the *X* chromosome (Coyne, 1993).

The only diagnostic morphological difference between these species is the posterior lobe of the male genital arch, which is long and thin in *D. mauritiana* and broad and helmet-shaped in *D. simulans* (Coyne, 1993). It is possible that the *D. simulans* females sense the aberrant shape of the smaller *D. mauritiana* arch and use this as a cue to terminate copulation prematurely (Coyne, 1993). Under this hypothesis, we expect QTL affecting copulation duration and those affecting the size and shape of the genital arch in the same inter-specific backcross to co-localize. A minimum of eight QTL affect the difference in morphology of the male genital arch in backcrosses of F1 females to *D. mauritiana* males; two on the *X* chromosome, two on the second chromosome, and four on the third chromosome (Zeng *et al.*, 2000). The third chromosome QTL at 64BC-65E and 97AB-100E affecting this morphological trait do indeed overlap the QTL we detected for copulation duration at 62A-65E and 97D-100E (**Table 4.2**), but the remaining six QTL affecting genital morphology and three QTL affecting behavior map to different genomic locations. Thus, differences in the size and shape between the genital arches of *D. mauritiana* and *D. simulans* males may indeed provide some cue for *D. simulans* females to cease copulation with *D. mauritiana* males prematurely, but cannot be the major cue.

We also assessed whether the markers exhibited segregation distortion in the backcross hybrids, as would be expected if they were associated with differences in viability. The ratio of heterozygotes to homozygotes is shown for each marker in **Figure 4.2**. We expected a decrease in the number of heterozygous individuals at loci that are linked to *D. simulans* genes that decrease viability when present in a *D. mauritiana* genetic background. This was observed for marker *AC002474* at cytological location

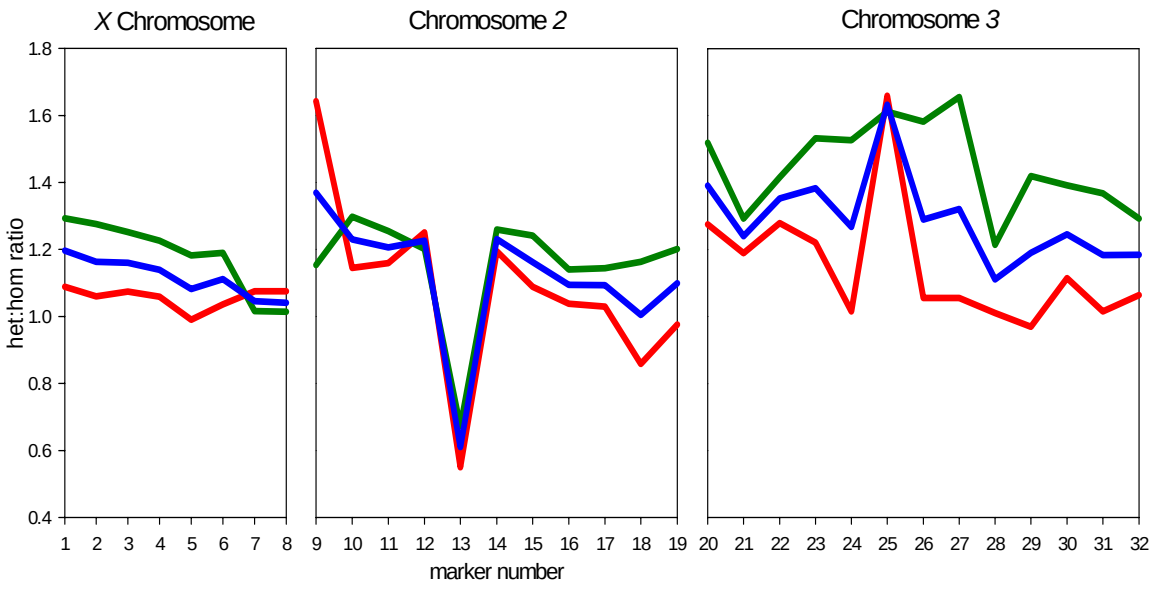

Figure 4.2: The ratio of heterozygotes to homozygotes for BC females crossed to *D. simulans* males (red), BC males crossed to *D. mauritiana* females (green) and BC males crossed to *D. simulans* females (blue).

38D, implicating factors affecting viability near this locus. Remarkably, however, there was a consistently greater number of heterozygotes than homozygotes for all other genotypes. This implies heterosis for viability in interspecific hybrids, which seems counterintuitive. The most parsimonious explanation is that inbreeding depression for viability occurred independently in the *D. simulans* and *D. mauritiana* stocks during their long-term laboratory culture, and the observed heterosis in the interspecific crosses is not related to speciation.

Our results indicate that relatively few QTL (from 3-7 or 5-8, depending on the model used) with moderate to large effects contribute to pre-zygotic reproductive isolation between these species. This statement must be tempered by the usual caveat that these are minimum numbers, as larger samples and a more dense marker map would have greater power to detect QTL with smaller effects and separate any linked genes located within significant QTL regions. However, our sample sizes of approximately 1000 BC individuals in each experiment are uncommonly large, and the statistical support for the mapped QTL is generally very high. It is therefore possible that a few genes with large effects account for the pre-zygotic isolation between this species pair. The effects of all QTL were in the same direction as the difference in behavior between the pure species, but we detected too few QTL in each experiment to apply a formal statistical test for positive selection (Orr, 1998).

We tested for epistatic interactions for each significant QTL within each mapping population. The only significant interaction observed was in the BC female X *D. simulans* male cross between the markers at the tip of the third chromosome (*Ald* and haplotype *DROROUGH/Efld2*, $P<0.0007$), but we attribute this to linkage rather than

epistasis. This is in sharp contrast to QTL affecting postzygotic isolation (Coyne, 1996a; Davis *et al.*, 1994; Hollocher *et al.*, 1997; Presgraves, 2003; Tao *et al.*, 2003), among which substantial epistasis occurrs.

While most of the significant regions containing QTLs do not contain any obvious candidate genes due to the broad range of factors that could influence mate choice, one candidate has emerged at locus 82C. The marker 5-Hydroxytryptamine 2 (5-HT2) was highly significant for the occurrence of mating when BC males were paired with *D. mauritiana* females (**Figure 4.1B**), yet the flanking markers were not significant. It is possible that polymorphisms at 5-HT2, or a gene closely linked to it, cause the difference in interspecific mating behavior attributable to this QTL. The 5-HT2 gene codes for a serotonin receptor that is expressed in the central nervous system and is part of the large family of receptors that interact with G proteins. The product of 5-HT2 acts on phospholipase C (Saudou, F. and R. Hen, 1994), encoded by *no receptor potential A* (*norpA*), which been shown to affect visual and circadian behaviors as well as neurophysiology (Inoue, H. *et al*, 1985; Kaneko, M. *et al*, 2000). This gene is of particular interest since several other genes involved in the catacholamine pathway, such as *Dopa decarboxylase* (Tempel *et al.*, 1984) and *pale* (Buchner, 1991), have been shown to affect mating behavior.

It is a prediction of some models of sexual isolation via sexual selection that genes involved in sexual isolation should accumulate preferentially on the *X* chromosome. Sex chromosomes facilitate the evolution of sexually antagonistic phenotypes that are selectively favored in one sex but disadvantageous in the other (Rice, 1984), and also the accumulation of recessive alleles that are advantageous in males (Charlesworth *et al*.,

1987). In contrast to this prediction, we found that autosomal loci had the greatest effects for all traits involved in pre-zygotic isolation of this species pair. A similar result was noted in previous studies of sexual isolation between these two species (Coyne, 1989; 1993; 1996), between *D. simulans* and *D. sechellia* (Coyne, 1992), and between *D. pseudoobscura* and *D. persimilis* (Noor, 1997).

Comparison of the map locations of QTL for the discrimination of *D. mauritiana* females against *D. simulans* males and QTL affecting male *D. simulans* traits against which female *D. mauritiana* discriminate allows us to directly address the question of whether female discrimination has the same genetic basis as the male traits that are being discriminated against. The answer is clearly negative, as only one QTL region, at the tip of the third chromosome (95D-100E) appears in both analyses (**Figure 4.1A, 4.1B; Table 4.2**). (The region from 10D-12F is also implicated by the logistic analysis). Further, co-localization of QTL does not imply the same genes affect reproductive isolation in both males and females, as there may be linked genes separately affecting the sexes within a common QTL. Independent genetic architectures of female preference and preferred male traits have also been demonstrated in previous analyses of these species pairs (Coyne, 1989; 1993; 1996), between *D. arizonensis* and *D. mojavensis* (Zouros, 1981), and between two 'races' (Wu *et al.*, 1995) of *D. melanogaster* (Ting *et al.*, 2001). Third, the genetic data do not support suggestions that the same genes may cause behavioral isolation in both sexes (i.e., that the same genes affect trait and preference). Genes causing behavioral isolation in males and females usually map to different chromosomes (as in *Ostrina nubialis*) or to different regions of the same chromosomes (Ritchie, 1998; Butlin and Ritchie, 1989). This result is not unexpected; it

would be surprising if interspecific differences in traits such as morphology, song, behavior or chemistry were based on the same genes used to perceive and evaluate those traits.

We mapped three QTL affecting *D. simulans* male traits against which *D. mauritiana* females discriminate prior to copulation, and six QTL affecting *D. mauritiana* male traits against which *D. simulans* females discriminate after copulation. QTL mapped to the third chromosome in both experiments, with regions of overlap at 64D-67A, 81B-83E and 97D-100E (**Figure 4.1B, 4.1C; Table 4.2**). Additional regions on the second (34C-35F) and third (90C-90E) chromosomes showed overlap with the logistic analysis. To assess whether this overlap in QTL locations between the two experiments was more than expected by chance, we computed the fractions of the third chromosome encompassed in total by the QTL mapped in each experiment, assuming for simplicity that each of the 240 cytological subdivisions on the third chromosome is the same physical size. The four QTL affecting *D. simulans* male traits together covered 25.4% of chromosome *3* and the four QTL affecting *D. mauritiana* male traits together covered 68.8% of chromosome *3*. We therefore expect $25.4 \times 68.8 = 17.5\%$ overlap in QTL locations between the two experiments by chance. Since the observed regions of overlap uncovered 20.4% of the third chromosome, we conclude that it is statistically possible as well as biologically plausible that the genetic basis of these two reproductive isolating mechanisms is not the same.

Civetta and Cantor (2003) mapped a single QTL affecting differences in copulation duration between *D. simulans* and *D. sechellia* to 84A-86B on chromosome *3*, which overlaps the QTL affecting copulation duration we detected between *D. simulans*

and *D. mauritiana* from 85F-86B. Similar calculations to those described above indicate that the amount of QTL overlap is less than expected by chance. Thus, it does not appear that mutations at a common set of genes affect reproductive isolation between *D. simulans* and these two sibling species.

Our observation that relatively few QTL with moderate to large effects underlie prezygotic reproductive isolation between *D. simulans* and *D. mauritiana* is a favorable scenario for future positional cloning of genes responsible for behavioral reproductive isolation.

MATERIALS AND METHODS

Drosophila strains

All flies were maintained in 8 dram vials containing standard cornmeal-agar-Karo media on a 12h:2h light:dark cycle at 24°C. *D. simulans* Florida City (FC) is an isofemale line collected in Florida City, Florida in 1985 (Coyne 1989; 1993; 1996). *D. mauritiana* synthetic (SYN) is derived from 6 isofemale lines collected in Mauritius in 1981 and combined in 1983 (Coyne 1989; 1993; 1996). Backcross hybrids were produced by crossing four-day-old virgin *D. simulans* FC females to virgin *D. mauritiana* SYN males, then crossing four-day-old virgin F_1 females to virgin *D. mauritiana* SYN males. At any one locus, each hybrid is either homozygous *D. mauritiana* or heterozygous *D. mauritiana/simulans*. Three separate groups of backcross hybrids were produced: (A) 1005 BC females, (B) 1002 BC males and (C) 1002 BC males, described in **Figure 4.1**.

Courtship behavior

Three sets of 'no-choice' mating assays were conducted, in which single BC individuals were paired with single pure-species individuals: (A) BC females and *D. simulans* males, (B) *D. mauritiana* females and BC males, and (C) *D. simulans* females and BC males. BC flies were collected as virgins and sorted by sex using brief exposure to CO_2 and kept in uncrowded vials for 4 days prior to use in experiments. Four-day-old virgin BC and pure species flies were transferred by aspiration to vials containing standard cornmeal-agar-Karo media within 1.5 h of 'lights on'. Experiments were conducted at room temperature, which varied from 21-23°C. For experiments (A) and (B) we recorded whether or not copulation occurred within 45 minutes, and, for those flies that did copulate, copulation latency (time to copulation) and copulation duration. We recorded copulation latency and copulation duration for 60 minutes for experiment (C), in which all flies mated.

Molecular markers

We tested 10 *D. simulans* FC and 10 *D. mauritiana* SYN individuals for polymorphism at 53 microsatellite markers using *D. melanogaster* primers (Schug *et al.*, 1998), and 45 single nucleotide polymorphisms (SNPs) previously developed for inbred lines of these species (Liu *et al.*, 1996; Zeng *et al.*, 2000). The parental lines were fixed for different alleles of 13 microsatellite and 6 SNP variants. An additional 12 informative markers were developed utilizing PCR primers described in Zeng *et al*. (2000) and digesting PCR products with a battery of restriction enzymes. One marker (at *Su(z)*) was developed *de novo* utilizing *D. melanogaster* sequence to design primers to amplify the homologous

sequence from *D. simulans* and *D. mauritiana*, and identifying an informative insertion/deletion variant by direct sequencing. The 32 markers, their cytological locations and conditions for genotyping are listed in **Table 4.3**.

All BC flies from the mating behavior assays were aspirated into 0.5 ml eppendorf tubes and stored at -80°C. DNA was extracted from each BC individual using the Puregene (Gentra Systems) single fly DNA extraction protocol, with the minor revisions of increased centrifugation times and pipette transfer of supernatant rather than pouring. The genotypes of the ~3000 backcross hybrids were determined for all 32 markers (i.e. ~96,000 genotypes). The marker map was constructed using MapMaker.

QTL Mapping

QTL for copulation latency and copulation duration were mapped in each backcross population using composite interval mapping (CIM, Zeng, 1994), implemented using QTL Cartographer software (Basten *et al.*, 1999; <ftp://esssjp.stat.ncsu.edu/pub/qtlcart/>). CIM tests whether an interval between two markers contains a QTL affecting the trait while simultaneously controlling for the effect of QTL located outside the interval using multiple regression on marker co-factors. Marker co-factors were chosen by forward selection – backward elimination stepwise regression. The likelihood ratio (LR) test statistic is $-2\ln(L_0/L_1)$, where L_0/L_1 is the ratio of the likelihood under the null hypothesis (there is no QTL in the test interval) to the alternative (there is QTL in the test interval). LR test statistics were computed every 2 cM with marker co-factors 10 cM or more from

Table 4.3: Molecular polymorphisms discriminating *D. simulans* FC and *D. mauritiana* SYN. Cytological locations are given based on *D. melanogaster* cytology (FlyBase, 2003). The marker type is m, microsatellite; s, SNP; id, insertion/deletion. The PCR protocol for all microsatellites is: 1 cycle 95°C 5m; 5 cycles 95°C 45s, 68°C 5m, 72°C 1m, decreasing the T_A each cycle by 2°C; 4 cycles 95°C 45s, 58°C 2m, 72 °C 1m, decreasing the T_A each cycle by 2°C; 27 cycles 95°C 45s, 50°C 2m, 72°C 1m; 1 cycle 72°C 5m. The PCR protocol for all other markers is: 1 cycle 94°C 5m; 30 cycles 94°C 1m, T_A°C 45s, 72°C 1m; 1 cycle 72°C 5m; where T_A is listed in the table. Primers are listed from the 5' end. Microsatellite markers were run on a 6% polyacrylamide gel and imaged with a LICOR Gene Readir 4200 DNA Analyzer. PCR fragments containing SNPs were digested with a restriction endonuclease and then run on a 3% agarose gel and manually genotyped. N/A, not applicable.

Marker name	Cytological location	Type	Primers	PCR T_A	Restriction endonuclease
ewg	1A	s	ATAACAGCAACCAGCGGCGG GGGCATCCATCCTCACATTGG	64°C	*Acc*I
DMU56661	4F	m	TATTTCGCTAACAAACCGGC AACGCGATCACAAACATCAA	N/A	N/A
DELTEX	6B	m	ACGCAATAAGTTGGCGTA AATCAGGATAATGCCTAAT	N/A	N/A
AC004114	8E	m	TTTTATTCCAGCCATCAGGC TGCGGTCCTTTACCATAAGC	N/A	N/A
V	9F	s	TGTCCCTATGCAGGAAACGG TGAACAGATGCTCATCGTGC	52°C	*Taq*I
DROEXO2	13F	m	TGCAGGGCACCTTCTCTCCA GAACGCTTGATTTAGATTTGGG	N/A	N/A
DMARIADNE	16F	m	AACACTGTCCCCATCCACAT TCTGTTCAACTCCTTCGGCT	N/A	N/A
run	19E	s	AGTGCATACCGAGAATCCGC ATTGATGGCGATTGCGGAGG	53°C	*Bsi*EI
DROEXPAND	21C	m	GTGATCGATCCCGCTGTC TCCGGTTTCCAATTAGCTTG	N/A	N/A
Gpdh	26A	s	CCCCTGTTCACGGCTATTC CTGGTGATTTGATCTATGCGG	60°C	*Hin*fI
AC005889	30A	m	GCGTGGCTGGCATATAG TAAGCCCCCTCGTGTAATTG	N/A	N/A
prd	33C	s	GATGCAAGGTGAGTGTCTATC GCCATGGGATACACGTAGCT	52°C	*Tsp*509I
AC002474	38D	m	GATGCTGTCCTTCGGACTTC AACAACAAAGCCCATTCTGC	N/A	N/A
DucC	42C	s	AAGAGGCCACAGAGCAGC TTACCCGAGAAGATGATGGC	65°C	*Alu*I
eve	46C	s	TTGTGGACCTCTTGGCCACC AACTCCTTCTCCAAGCGACC	63°C	*Dra*I
Su(z)	49E	id	GTTACAACTGGAGCCGGGTA CACAATTGGATTGGGTTTCC	62°C	N/A
sli	52D	s	TTACCAGCTTTAAGGGCTGC CATTTGTTCTCCAGGCAAGG	50°C	*Aci*I

Table 4.3 (continued)

Marker name	Cytological location	Type	Primers	PCR T_A	Restriction endonuclease
AC004365	58A	m	GCTTTATCAATGCAGCCTCC GGCCCCAATATGTCCTCGCC	N/A	N/A
twi	59C	s	TCCCTGCAGCAGATCATCCC ATCACTCGAGCTGAGCATGC	63°C	*Hin*fI
ve	62A	s	GAGAACCCAACGCAGAATGT ATATCCTCCGACTCCGGAAG	52°C	*Pst*I
h	66D	s	ACTCAAGACTCTGATTCTGG TGTCTTCTCCAGAATGTCGG	55°C	*Dra*I
CycA	68E	s	ATTTCGCCGTGCTCAATG ACGTCATGGTTCTCTTTGTCG	57°C	*Hin*fI
Eip71CD	71D	s	CCTGTATGGAGCCACCCG GGGGCTGAGATTTAGCGATG	55°C	*Bsm*AI
rdgC	77B	id	CAAAGACATCGACTCAGCTACG CGAACTCTCCACGATGCC	62°C	N/A
5-HT2	82C	s	TGACGATTCCCCTCCTCC CGCCCACTGATAGGAATTTG	52°C	*Hin*fI
Antp	84B	s	ACGGACGTTGGAGTTCCCGA ACATGCCCATGTTGTGATGG	60°C	*Mse*I
DROHOXNK4	93D	m	CTGAAGTTGAAGTCCGAGCC TACATGTGCTGCATCTGTTGC	N/A	N/A
DROTRXIII3	88B	m	GACCGTTTGTTTGCCTTGAT TGCCTGTACAAGTCTGACCG	N/A	N/A
DMTF125	95C	m	CTCGAGCGGGCCATACAAGA TGATTGAAGAGGCCACTCAA	N/A	N/A
Ald	97A	s	ATGGGCCCTCACCTTCTC GTGGTCATCCACATGCAAAG	52°C	*Xmn*I
DROROUGH	97D	m	AAGCAATGCCACACAATGAG CGGTTATTTTTTTCCTTGGC	N/A	N/A
Efld2	100E	s	GACTGGTCTCCTCAAGCCAG AGCCTCGTGGTGCATCTC	62°C	*Sfc*I

the test location. Appropriate significance thresholds that take into account the multiple tests performed and correlations among markers were determined by permutation. Trait and marker data were permuted 1000 times, and the maximum LR statistic across all intervals was recorded for each permutation. LR statistics calculated from the original data that exceed the 50^{th} greatest LR statistic from the permuted data are significant at the experiment-wise 5% level under the null hypothesis (Churchill and Doerge, 1994; Doerge and Churchill, 1996). The approximate boundaries of regions containing QTL were determined by taking 2 LOD intervals (9.22 LR) surrounding the point of greatest significance and extrapolating the cytological location of the interval based on the amount of recombination between flanking markers.

Two methods were used to map QTL for copulation occurrence in experiments (A) and (B). First, CIM as described above was used for the binary data, where each individual that mated was assigned a value of 1 and those that did not mate were scored as 0. Second, an extension of CIM based on logistic regression (Xu and Atchley, 1996) was implemented, which assumes that the binary trait is connected to its continuous underlying liability by a threshold model (Falconer and Mackay, 1996). The same window size (10) and marker co-factors used for CIM were also used in the logistic model.

Estimates of QTL effects were determined as the difference between heterozygous *simulans/mauritiana* genotypes and homozygous *mauritiana* genotypes at the peak LR, and scaled by the phenotypic standard deviation.

Pairwise epistatic interactions between all significant QTL within each experiment were evaluated using either the marker positioned at the highest LR of each

QTL peak or the haplotype of the two markers flanking the QTL peak. Tests for epistasis were calculated for the binary data with a log linear model using PROC CATMOD, and for copulation duration with an ANOVA using PROC GLM, using SAS 8.2 software. Significance thresholds were determined via a Bonferroni correction.

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APPENDICES

Appendix 1: *roo* transposable element genotypes for the 98 RI lines. 0=OreR, 1=2b, h=heterozygote (treated as missing data in analysis).

	cytological location																
line	**1 B**	**3 E**	**4 F**	**5 D**	**6 E**	**7 D**	**7 E**	**9 A**	**10 D**	**11 C**	**11 D**	**12 E**	**14 C**	**15 A**	**16 D**	**17 C**	**19 A**
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	0	0
3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
7	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
11	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0
12	1	1	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
18	1	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0
19	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
20	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0
22	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
25	0	1	h	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
1R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3R	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0
5R	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6R	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
9R	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10R	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0
11R	1	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0
12R	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
13R	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
14R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15R	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0
17R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix 1 (continued)

line	1 B	3 E	4 F	5 D	6 E	7 D	7 E	9 A	10 D	11 C	11 D	12 E	14 C	15 A	16 D	17 C	19 A
19R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22R	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25R	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
27R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29R	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
31R	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
35R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
36R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
37R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38R	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
42R	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0
43R	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0
44R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45R	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
46R	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0
47R	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0
48R	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50R	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51R	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1
52R	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1
53R	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
54R	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
55R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56R	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
57R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58R	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0
60R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
65R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
67R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
68R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
70R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
71R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
73R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
74R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
75R	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1
76R	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
77R	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	1
78R	1	1	0	0	0	0	0	h	0	0	0	0	0	0	0	0	0

Appendix 1 (continued)

line	1 B	3 E	4 F	5 D	6 E	7 D	7 E	9 A	10 D	11 C	11 D	12 E	14 C	15 A	16 D	17 C	19 A
79R	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
80R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
81R	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
84R	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	1
85R	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0
86R	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
87R	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1
88R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
89R	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0
90R	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
91R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
92R	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
93R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
94R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
95R	h	1	0	0	0	h	h	0	0	0	0	0	0	0	0	0	0
97R	1	1	0	0	0	0	0	0	0	0	0	0	h	h	h	h	h
99R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix 1 (continued)

line	cytological location																
	21 E	**22 F**	**27 B**	**29 F**	**30 AB**	**30D**	**33 E**	**34 EF**	**35 B**	**38 A**	**38 E**	**43 A**	**43 E**	**46 A**	**46 C**	**48 D**	**49 D**
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
5	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
6	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
13	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
15	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0
17	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
24	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3R	0	0	1	h	h	0	1	1	1	1	h	1	h	h	0	0	0
5R	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6R	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9R	0	0	0	0	0	0	1	1	1	h	0	h	0	h	0	0	0
10R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13R	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
14R	0	0	0	1	1	1	1	1	1	1	1	1	h	1	1	1	0
15R	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0
17R	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19R	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
22R	0	0	0	0	0	0	h	h	h	h	h	h	h	0	0	0	0

Appendix 1 (continued)

line	21 E	22 F	27 B	29 F	30 AB	30D	33 E	34 EF	35 B	38 A	38 E	43 A	43 E	46 A	46 C	48 D	49 D
25R	0	0	1	0	h	0	h	h	h	h	0	h	0	h	0	1	1
27R	h	h	h	h	h	h	1	h	h	h	h	1	h	1	h	h	0
28R	h	h	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
29R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31R	1	1	1	0	0	0	0	0	0	h	0	0	0	0	0	0	0
34R	1	1	1	0	0	0	h	h	h	h	0	h	0	h	0	0	0
35R	1	1	0	0	0	0	1	1	1	h	h	h	h	h	h	h	h
36R	1	1	1	0	0	0	0	0	0	h	0	h	0	h	0	0	0
37R	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1
38R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40R	1	1	1	0	0	0	h	0	0	0	0	0	0	0	0	h	h
42R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43R	1	1	0	0	0	0	h	h	h	h	0	h	0	h	h	1	1
44R	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1
45R	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0
46R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
47R	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
48R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50R	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
51R	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
52R	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
53R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54R	1	1	0	0	0	0	1	0	0	1	h	h	h	h	h	0	0
55R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0
56R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
57R	0	0	h	1	1	1	1	1	1	1	1	1	1	1	1	0	0
58R	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
60R	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
65R	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0
67R	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
68R	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
70R	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
71R	0	0	0	0	0	0	0	0	0	1	h	h	h	h	h	h	0
72R	h	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0
73R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
74R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
75R	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
76R	0	0	0	0	0	0	0	0	0	0	h	1	h	1	h	0	0
77R	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
78R	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
79R	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0

Appendix 1 (continued)

line	21 E	22 F	27 B	29 F	30 AB	30D	33 E	34 EF	35 B	38 A	38 E	43 A	43 E	46 A	46 C	48 D	49 D
80R	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
81R	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
84R	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
85R	0	0	0	0	h	0	1	1	1	1	h	1	h	1	0	0	0
86R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
87R	1	h	h	1	1	h	1	0	0	0	0	1	0	1	0	0	0
88R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
89R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
90R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
91R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
92R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
93R	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
94R	0	0	h	1	1	1	h	h	h	h	0	h	0	h	0	0	0
95R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
97R	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
99R	0	0	0	1	1	1	h	h	h	h	0	h	0	h	0	0	0
100R	1	1	1	1	1	1	0	0	0	h	h	h	h	h	h	0	0

Appendix 1 (continued)

line	cytological location																
	50 B	**50 D**	**50 F**	**57 C**	**57 F**	**60 E**	**61 A**	**63 A**	**65 A**	**65 D**	**67 D**	**68 B**	**68 C**	**69 D**	**70 C**	**71 E**	**72 A**
1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0
3	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1
5	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1	1	1
6	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0
7	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1
8	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
9	0	0	0	0	0	1	1	0	0	0	0	0	1	1	1	1	1
10	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1
11	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0
12	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1
13	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0
14	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
15	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
17	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	1
19	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0
21	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
22	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	1
23	0	0	0	1	1	1	1	1	0	0	0	0	0	0	h	1	h
24	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0
25	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1R	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	h	0
3R	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
5R	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
6R	0	1	1	1	1	1	0	1	1	0	0	0	0	0	1	1	1
9R	0	0	h	h	h	1	0	0	0	0	0	0	1	1	0	0	0
10R	0	0	0	1	1	0	0	1	1	1	1	0	1	1	1	1	1
11R	0	0	0	0	1	1	1	0	0	1	1	1	1	1	0	h	0
12R	0	0	0	0	1	1	0	1	1	1	1	0	0	0	0	0	0
13R	0	0	0	0	1	1	1	0	1	0	1	1	1	1	0	0	1
14R	0	0	0	0	0	0	1	1	1	0	0	1	1	0	0	0	0
15R	0	0	h	1	1	0	0	0	1	1	1	0	0	0	0	0	0
17R	0	0	0	h	1	0	0	0	1	1	1	0	0	0	0	0	0
19R	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0	0	0
22R	0	0	0	1	1	0	0	1	1	1	0	1	1	1	1	1	1

Appendix 1 (continued)

line	50 B	50 D	50 F	57 C	57 F	60 E	61 A	63 A	65 A	65 D	67 D	68 B	68 C	69 D	70 C	71 E	72 A
25R	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1
27R	0	0	0	h	h	0	h	0	h	h	h	h	h	h	0	h	h
28R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29R	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1	1	1
31R	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1	1	1
34R	0	0	h	h	1	1	0	0	1	1	1	1	1	1	1	1	1
35R	0	0	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1
36R	0	0	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1
37R	1	1	1	1	1	1	0	0	h	0	0	0	0	0	0	0	0
38R	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1	1	1
40R	0	0	0	0	1	h	0	0	1	0	1	1	1	1	1	1	1
42R	0	0	0	1	1	1	1	0	0	1	1	1	1	1	0	0	0
43R	0	0	0	0	0	0	h	0	h	h	1	1	h	1	h	h	0
44R	1	1	1	1	1	1	0	0	0	0	h	0	h	1	h	1	h
45R	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1
46R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	h	h	h
47R	0	0	0	1	1	1	1	1	1	0	0	0	0	0	1	1	1
48R	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1
50R	0	0	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1
51R	0	0	0	1	1	0	1	h	1	0	0	0	0	0	1	1	1
52R	0	0	0	1	1	0	1	1	1	1	1	0	0	0	0	1	0
53R	0	0	h	1	1	1	1	1	1	1	1	1	0	0	1	1	1
54R	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1
55R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
56R	0	0	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1
57R	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
58R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
60R	0	0	0	1	1	1	1	1	h	0	1	1	1	1	1	1	1
65R	0	0	0	1	1	1	1	1	1	h	1	1	1	1	1	1	1
67R	0	0	0	1	1	1	1	1	h	0	0	0	0	0	0	0	0
68R	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0
70R	0	0	0	1	1	1	1	1	h	0	0	0	0	0	0	0	0
71R	h	h	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
72R	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1
73R	0	0	0	1	1	1	1	0	0	0	0	0	0	1	1	1	1
74R	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0
75R	0	0	0	1	1	1	1	1	0	0	0	0	0	1	1	1	1
76R	0	0	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0
77R	0	0	0	1	1	1	1	1	1	0	0	0	0	1	1	1	1
78R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
79R	0	0	h	1	1	1	1	1	1	0	0	0	0	0	0	0	0
80R	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1

Appendix 1 (continued)

line	50 B	50 D	50 F	57 C	57 F	60 E	61 A	63 A	65 A	65 D	67 D	68 B	68 C	69 D	70 C	71 E	72 A
81R	1	1	1	0	0	0	1	1	1	1	1	h	h	1	h	h	h
84R	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
85R	0	0	1	1	1	1	h	h	1	1	1	1	1	1	1	1	1
86R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
87R	0	0	0	1	1	0	1	1	1	1	0	0	0	0	0	0	0
88R	0	0	0	1	1	0	0	0	0	1	1	1	1	1	0	0	0
89R	1	1	1	0	1	1	1	1	1	1	0	0	0	0	h	1	h
90R	0	0	0	1	1	1	1	0	h	0	0	0	1	1	1	1	1
91R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
92R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
93R	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1
94R	0	0	h	h	h	0	0	0	1	1	1	1	1	1	1	1	1
95R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
97R	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1
99R	0	0	h	1	1	1	h	0	0	1	0	0	0	h	0	h	0
100R	0	0	0	0	h	0	0	0	0	1	1	1	1	1	1	1	1

Appendix 1 (continued)

	cytological location																
line	**73 D**	**76 A**	**76 B**	**77 A**	**77 E**	**78 D**	**82 D**	**85 A**	**85 F**	**87 B**	**87 E**	**87 F**	**88 E**	**89 B**	**91 A**	**91 D**	**92 A**
1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
14	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	h	1	h	h	1	h	1	1	1	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
25	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1R	h	h	0	0	h	0	h	h	h	1	h	1	1	1	h	h	1
3R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	h	h	h
6R	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9R	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
10R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11R	0	h	h	0	h	0	h	h	h	h	h	1	h	h	h	h	h
12R	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
13R	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
14R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15R	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
17R	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
19R	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
22R	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0

Appendix 1 (continued)

line	73 D	76 A	76 B	77 A	77 E	78 D	82 D	85 A	85 F	87 B	87 E	87 F	88 E	89 B	91 A	91 D	92 A
25R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
27R	h	h	h	h	h	h	h	h	h	h	0	0	0	0	0	0	0
28R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	h	h	h
29R	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
31R	1	1	1	1	1	1	1	1	1	1	1	1	h	1	1	1	1
34R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
35R	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
36R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
37R	0	h	0	0	h	0	h	0	0	h	0	h	h	h	h	h	h
38R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
40R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
42R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
43R	0	h	0	0	h	0	h	0	0	h	0	h	h	h	h	0	h
44R	1	1	1	1	1	h	1	1	1	1	1	1	1	1	1	1	1
45R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
46R	h	h	h	h	h	h	h	h	h	h	h	h	h	h	1	1	1
47R	0	h	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
48R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
50R	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
51R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
52R	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0
53R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
54R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
55R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
56R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	h	h
57R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
58R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
60R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
65R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
67R	0	h	0	0	h	0	h	0	0	0	0	h	h	h	0	0	0
68R	0	0	0	1	1	1	1	1	h	1	h	1	1	1	h	h	h
70R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0
71R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
73R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
74R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	h	0	0
75R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
76R	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
77R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
78R	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
79R	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
80R	1	1	1	h	1	1	1	1	1	1	1	1	0	0	0	0	0

Appendix 1 (continued)

line	73 D	76 A	76 B	77 A	77 E	78 D	82 D	85 A	85 F	87 B	87 E	87 F	88 E	89 B	91 A	91 D	92 A
81R	h	1	0	0	1	0	h	0	0	h	0	h	h	h	0	1	1
84R	0	0	0	0	0	0	0	0	0	h	h	1	1	1	1	1	1
85R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
86R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
87R	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
88R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
89R	h	1	0	0	h	0	1	0	h	0	0	h	h	0	0	0	0
90R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
91R	1	1	1	1	1	1	1	1	1	1	1	1	1	h	1	0	0
92R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	h	0	0
93R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
94R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
95R	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0
97R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
99R	0	h	0	0	h	0	h	0	0	0	0	h	0	0	0	0	0
100R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Appendix 1 (continued)

line	cytological location												
	93 A	**93 B**	**94 D**	**96 A**	**96 F**	**97 D**	**97 E**	**98 A**	**99 A**	**99 B**	**99 E**	**100 A**	**SPA**
1	1	1	1	0	0	0	0	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	0	0	0	0	1
3	1	1	1	1	1	1	1	1	0	0	0	0	1
5	1	1	1	1	1	0	0	0	0	0	0	0	1
6	0	0	0	0	0	0	0	0	0	0	0	0	1
7	1	1	0	0	1	1	1	1	1	1	1	1	1
8	0	0	0	0	0	0	0	0	0	0	0	0	1
9	1	1	1	0	0	0	0	0	0	0	0	0	1
10	0	0	1	1	1	0	0	0	0	1	1	1	1
11	0	0	1	1	h	0	0	0	0	0	0	0	1
12	1	1	1	1	1	1	1	1	0	0	0	0	1
13	1	1	1	1	1	1	1	1	1	1	1	1	1
14	1	1	0	1	1	1	1	1	0	0	0	0	1
15	1	1	1	1	0	0	0	0	0	0	0	0	1
16	0	0	0	0	0	0	0	0	0	0	0	0	1
17	1	1	1	1	1	1	1	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1	1	1	1	1
19	1	1	1	1	1	1	1	1	1	1	1	1	1
20	0	0	1	1	1	1	1	1	1	1	1	1	1
21	1	1	1	0	0	0	0	0	0	0	0	0	1
22	1	1	1	0	0	0	0	0	0	0	0	0	1
23	0	0	1	1	1	1	1	1	0	0	1	1	1
24	1	1	1	1	1	h	h	h	h	h	1	1	1
25	1	1	1	0	0	0	0	1	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1	1	1	1	1
27	0	0	1	1	1	1	1	1	1	1	1	1	1
1R	1	1	1	1	1	1	1	1	1	1	1	1	1
3R	1	1	h	1	1	1	1	1	1	1	1	1	1
5R	1	1	1	1	1	1	1	1	0	1	1	1	1
6R	1	1	1	1	1	1	1	1	1	1	1	1	1
9R	1	1	1	1	0	0	0	0	0	0	0	0	1
10R	1	1	1	1	1	1	1	1	1	1	1	1	1
11R	0	0	0	0	0	0	0	0	0	0	0	0	1
12R	1	1	1	1	1	1	1	1	h	h	h	1	0
13R	0	0	0	0	0	0	0	0	0	0	0	0	1
14R	0	0	0	0	0	1	1	1	1	1	1	1	1
15R	1	1	1	1	1	1	1	0	0	0	0	0	0
17R	1	1	1	1	1	1	1	1	h	h	1	1	0
19R	1	1	1	1	0	0	0	1	1	1	1	1	1
22R	0	0	0	1	1	1	1	1	1	1	1	1	1

Appendix 1 (continued)

line	93 A	93 B	94 D	96 A	96 F	97 D	97 E	98 A	99 A	99 B	99 E	100 A	SPA
25R	1	1	1	1	1	0	0	0	1	1	1	1	1
27R	0	h	h	h	h	h	h	h	h	h	h	h	1
28R	1	1	1	1	1	1	1	1	1	1	1	1	1
29R	0	0	0	1	1	1	1	1	1	1	0	0	1
31R	1	1	1	1	1	1	1	1	1	1	1	1	1
34R	1	1	1	1	1	1	1	1	1	1	1	1	1
35R	0	0	0	0	1	1	1	1	0	0	1	0	1
36R	1	1	1	1	1	1	1	1	1	1	1	1	1
37R	1	h	0	0	0	0	0	0	0	0	0	0	1
38R	1	1	1	1	1	1	1	1	1	1	1	1	1
40R	1	1	1	0	0	0	0	1	1	1	1	1	1
42R	1	1	1	1	1	1	1	1	0	0	0	0	1
43R	1	1	1	1	1	1	1	1	0	0	0	0	1
44R	1	1	1	1	1	0	0	0	0	0	0	0	1
45R	0	0	0	0	0	0	0	0	0	0	0	0	1
46R	1	1	0	0	1	1	1	1	1	1	1	1	1
47R	0	0	0	0	0	0	0	0	0	0	0	0	1
48R	1	1	1	0	0	0	0	0	0	0	0	0	1
50R	0	0	1	1	1	0	0	0	0	1	1	1	1
51R	0	0	1	1	h	0	0	0	0	0	0	0	1
52R	1	1	1	1	1	1	1	1	0	0	0	0	1
53R	1	1	1	1	1	1	1	1	1	1	1	1	1
54R	1	1	0	1	1	1	1	1	0	0	0	0	1
55R	1	1	1	1	0	0	0	0	0	0	0	0	1
56R	0	0	0	0	0	0	0	0	0	0	0	0	1
57R	1	1	1	1	1	1	1	1	1	1	1	1	1
58R	1	1	1	1	1	1	1	1	1	1	1	1	1
60R	1	1	1	1	1	1	1	1	1	1	1	1	1
65R	0	0	1	1	1	1	1	1	1	1	1	1	1
67R	1	1	1	0	0	0	0	0	0	0	0	0	1
68R	1	1	1	0	0	0	0	0	0	0	0	0	1
70R	0	0	1	1	1	1	1	1	0	0	1	1	1
71R	1	1	1	1	1	h	h	h	h	h	1	1	1
72R	1	1	1	0	0	0	0	1	1	1	1	1	1
73R	1	1	1	1	1	1	1	1	1	1	1	1	1
74R	0	0	1	1	1	1	1	1	1	1	1	1	1
75R	1	1	1	1	1	1	1	1	1	1	1	1	1
76R	1	1	h	1	1	1	1	1	1	1	1	1	1
77R	1	1	1	1	1	1	1	1	0	1	1	1	1
78R	1	1	1	1	1	1	1	1	1	1	1	1	1
79R	1	1	1	1	0	0	0	0	0	0	0	0	1

Appendix 1 (continued)

line	93 A	93 B	94 D	96 A	96 F	97 D	97 E	98 A	99 A	99 B	99 E	100 A	SPA
80R	1	1	1	1	1	1	1	1	1	1	1	1	1
81R	0	0	0	0	0	0	0	0	0	0	0	0	1
84R	1	1	1	1	1	1	1	1	h	h	h	1	0
85R	0	0	0	0	0	0	0	0	0	0	0	0	1
86R	0	0	0	0	0	1	1	1	1	1	1	1	1
87R	1	1	1	1	1	1	1	0	0	0	0	0	0
88R	1	1	1	1	1	1	1	1	h	h	1	1	0
89R	1	1	1	1	0	0	0	1	1	1	1	1	1
90R	0	0	0	1	1	1	1	1	1	1	1	1	1
91R	1	1	1	1	1	0	0	0	1	1	1	1	1
92R	0	h	h	h	h	h	h	h	h	h	h	h	1
93R	1	1	1	1	1	1	1	1	1	1	1	1	1
94R	0	0	0	1	1	1	1	1	1	1	0	0	1
95R	1	1	1	1	1	1	1	1	1	1	1	1	1
97R	1	1	1	1	1	1	1	1	1	1	1	1	1
99R	0	0	0	0	1	1	1	1	0	0	1	0	1
100R	1	1	1	1	1	1	1	1	1	1	1	1	1

Appendix 2: Line means for the 98 RI lines for each of the traits measured.

line	courtship latency	courtship occurrence	copulation latency	copulation occurrence	copulation duration
1	2231.5	0.6	3451.65	0.1	2847.857
2	741.3	1	2157.5	0.75	1926
3	1387.9	0.9	2734.95	0.45	2634.5
5	2236	0.75	3028.5	0.5	2951
6	269.4	1	890.55	1	528.2273
7	934.8	0.9	2667.4	0.65	1905.9
8	304.2	1	1029.35	1	928.4545
9	1188	0.9	2813.55	0.5	1967.818
10	800.9	0.95	2271.95	0.7	1856.833
11	716.6	0.8	2069.05	0.7	1235.636
12	526.4	0.95	2181.1	0.9	2064.818
13	460.5	1	1332.7	0.9	774.5
14	345	1	2391.5	0.65	2190.091
15	549	1	2370.65	0.65	1917.833
16	800	0.95	2549.25	0.7	2253.25
17	406.3	0.8	2214.2	0.6	1774.833
18	506.9	0.9	2324.85	0.6	1753.583
19	2463.6	0.6	3192.45	0.35	2015
20	379	1	1729.75	0.8	1568.769
21	464.6	1	2488.65	0.6	2059.636
22	1420.3	0.95	2233.65	0.8	1604.3
23	899.3	0.9	3225.9	0.2	3046
24	365.8	0.95	1317.35	0.9	1001.833
25	2690.6	0.35	3343.7	0.1	3000.4
26	1573.1	0.85	2068.15	0.75	1368.4
27	1246.6	0.85	2453.9	0.7	1588.1
1R	2397.9	0.5	3476.05	0.1	2497
3R	2211.3	0.75	2887.5	0.35	3601
5R	1372.2	0.65	2429.95	0.5	1489.25
6R	1006.9	0.7	2597.25	0.45	1654.364
9R	478.2	0.95	1875.9	0.75	1874.167
10R	2124.7	0.65	3139.25	0.35	2035.714
11R	873.3	0.9	1276.35	0.9	567.6
12R	842.9	0.9	1745.45	0.85	1103.25
13R	977.6	1	2186.85	0.8	1612.364
14R	1341.6	0.85	2023.8	0.7	1212.556
15R	1414.3	0.8	2786.8	0.5	2294.333
17R	1064	0.95	2909.25	0.4	2576.545
19R	785.7	0.9	1979.7	0.75	1465.8

Appendix 2 (continued)

line	courtship latency	courtship occurrence	copulation latency	copulation occurrence	copulation duration
22R	1485.6	0.75	3104.25	0.3	1829.091
25R	1938.2	0.5	3004.7	0.25	2005.444
27R	1022.8	0.8	2697.05	0.5	1768
28R	352.7	0.95	1426.8	0.9	1074.333
29R	2673.2	0.5	3360.1	0.15	2577.167
31R	1693.6	0.65	3501.15	0.15	3375.111
34R	3119.6	0.45	3332.8	0.25	1692.4
35R	1712.4	0.8	3077.4	0.4	3072
36R	1459.9	0.55	3092.8	0.25	2486.444
37R	873.5	0.9	3211.65	0.25	3214.3
38R	1763.9	0.6	3155.1	0.4	2411.333
40R	1140.1	0.95	3096.75	0.35	2937.3
42R	1900.3	0.9	3108.05	0.3	3393.091
43R	2302.3	0.45	2972.6	0.25	1712.4
44R	1504	0.9	3471.75	0.1	2946.273
45R	2135.2	0.75	2900.1	0.3	2816.375
46R	2467.5	0.5	2633.9	0.45	1022.5
47R	1564.4	0.8	2476.85	0.55	1738.875
48R	2645.7	0.4	3432.55	0.1	3021.2
50R	1908.1	0.6	3138.35	0.25	2013
51R	2383.3	0.5	3079.3	0.3	2076.667
52R	2228.6	0.65	3152.8	0.25	2748.167
53R	1044.1	1	2901.25	0.45	2548.375
54R	1692.1	0.75	3360.55	0.2	2554.2
55R	1944.2	0.6	2847.4	0.55	1270.5
56R	1508.1	0.75	2952.4	0.45	1762.2
57R	1016.5	0.95	2311	0.75	1753.167
58R	1962.9	0.75	2641.85	0.4	1799.909
60R	3298.5	0.25	3388.95	0.2	1607
65R	2159.8	0.75	2861.55	0.45	1892.444
67R	2098.4	0.5	3222.25	0.2	2680.667
68R	3005.1	0.25	3448.3	0.05	2750.25
70R	982.5	0.8	2740.5	0.55	2139.7
71R	2388	0.65	2916.3	0.35	2339.5
72R	2194.2	0.5	2874.15	0.35	1753.286
73R	785.3	0.85	2459.15	0.75	1473.9
74R	1896	0.7	2994.85	0.4	2279.625
75R	3114.6	0.35	3311.05	0.2	3105
76R	689.8	0.95	1796.7	0.85	1113
77R	1070.3	0.8	2174.8	0.75	935.3
78R	417.7	1	3203.1	0.35	3267.636

Appendix 2 (continued)

line	courtship latency	courtship occurrence	copulation latency	copulation occurrence	copulation duration
79R	1543.5	0.85	2067.55	0.7	1025.167
80R	1484	0.8	3524.45	0.1	3601
81R	1084.3	0.85	3049.7	0.3	2850.3
84R	355	1	1967.75	0.85	1561.364
85R	2390.1	0.65	2514.4	0.55	935
86R	1309.4	0.95	1755.8	0.8	691
87R	651.2	1	2175.85	0.7	933.7273
88R	1892.6	0.7	3215.55	0.2	2606.375
89R	265.4	1	1925	0.8	1283.958
90R	439.9	1	2847.95	0.4	2507
91R	593.6	1	1849.05	0.85	1874.091
92R	951	0.95	2071.45	0.75	1322.667
93R	1260	0.8	2499	0.5	1689.111
94R	2325.3	0.7	3093.5	0.3	2303.857
95R	833.9	1	2555.95	0.75	2115.583
97R	2038.6	0.7	2793.25	0.45	2006.333
99R	2654.2	0.5	3280.75	0.25	1644.2
100R	1801.8	0.6	3365.25	0.1	3243.125