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Evolution and Genetics of Functionally-Related Traits, Male Wing Pigmentation and Courtship Behavior, in the Oriental *Drosophila melanogaster* Species Group

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Abstract of the Dissertation

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The evolution of one morphological trait usually depends on or is facilitated by functionally related traits, such as physiological, behavioral, or other morphological traits. In the Oriental *Drosophila melanogaster* species group, some species possess male-specific pigmentation in the apical wing area, referred to as wing spots. Wing spots have been proposed to evolutionarily couple with frontal-wing-display in courtship. But the proposal that sexual selection shapes the evolution of wing spots is still untested. In theory, sexual selection on a wing spot display could be promoted by genetic correlations between the pigmentation and behavioral components of this complex trait. How often this scenario might occur nature is unknown. In this dissertation, the functionality, evolutionary history, and genetic architecture of wing spots and courtship behavior were explored using various approaches. First, wing spots were found to serve as a

visual stimulus via frontal wing display in behavioral essays, which support the hypothesis that sexual selection drives the evolution of wing spots and wing display behavior. Second, the association between wing pigmentation and wing display behavior was confirmed to be robust phylogenetically. This part of the study also revealed that courtship songs, another male display component, evolve rapidly and exhibit novel features in this clade. Third, genetic analysis in a pair of crossable species, one of which having lost both wing spots and wing displays, elucidated the genetic correlation of these two traits. Interestingly, the major quantitative trait locus (QTL) for wing pigmentation and one QTL for wing display behavior are located on the same region of the X chromosome, indicating either close linkage or pleiotropy. This finding provides a possible genetic mechanism for the coordinate evolution of these two traits. A central focus of modern evolutionary biology has been on the genetic underpinnings of morphological evolution. This study takes this question one step further by providing insights on the evolutionary integration of a morphological trait and the behavior by which it is utilized.

To my family

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The text of this dissertation in part is a reprint of the materials as it appears in Heredity. The co-authors listed in the publication directed and supervised the research that forms the basis for this dissertation.

I. Introduction

The spectacular diversity of morphological traits in nature has long inspired questions on how and why traits appear and diversify in certain lineages but not others, and what underlying functional and genetic mechanisms operate to build complex phenotypes (Carroll *et al.*, 2001; West-Eberhard, 2003). Many complex adaptations involve both morphological and behavioral components. Ethologists have long sought to understand the causation of animal behavior from both genetic and ecological perspectives (Tinbergen, 1989). Although correlations between behavior and morphological structures are widely observed in nature (West-Eberhard, 2003), few studies have been carried out to systematically to examine their functional, evolutionary, and genetic relationships and to address how behavior may influence the evolution of morphological structure and vice versa.

In this dissertation, I have attempted to integrate knowledge and methodology between evolutionary genetics and behavior to acquire some insights on the nature of morphological structures, behavior, and their relationship in order to understand the evolution of morphology and behavior more thoroughly. For an evolutionary genetic approach to these questions, the study system required tractable laboratory culture and crossing, as well as knowledge and tools at the molecular genetic level, along with variation in interesting and correlated morphology and behavior. The male-specific wing pigmentation and frontal wing display in the Oriental *Drosophila melanogaster* species group provide a very good system in which to attack these fundamental questions in a reasonable amount of time. Most importantly, this system provided an interfertile species

pair differing in the presence of coordinately evolving pigmentation and behavioral traits.

In the Oriental *D. melanogaster* species group, various degrees of black pigments appear on the apical region of male wings in some species (Bock and Wheeler, 1972; Kopp and True, 2002). Wing pigmentation is thought to have arisen in the common ancestor of this lineage, and subsequently the loss of the pigment occurred in some descendant taxa (Prud'homme *et al.*, 2006). Kopp and True (2002) reported that male-specific wing spots generally co-occur with frontal wing display during courtship, thus the frontal wing display has been proposed to couple with wing spots to serve as a visual signal in courtship. The aim of this dissertation is to elucidate the functionality and genetic architecture of the wing pigmentation and courtship behavior in this lineage.

Starting in Chapter 2, the function relationship between wing spot and courtship behavior was examined. The several female-choice-based tests were carried out in two wing spot bearing species, *D. elegans* and *D. biarmipes*, to determine whether wing spots are under sexual selection and how females assess this male specific wing pigmentation. The results of single choice tests on control versus wingless males and control versus spotless males are consistent with sexual selection acting on the wing display. Furthermore, I found that spotless males need to spend significantly more effort than spot bearing males, in terms of time and courtship activity, in order to gain female acceptance. These findings suggest that wing spot and wing display are functionally related in the courtship performance. Furthermore, unlike wild type females, visually compromised females of *D. biarmipes* mate with spotted and spotless males indifferently, suggesting that vision is important for females to recognize wing spots. Finally, the possible role of wing spot size in female choice was tested and

some aspects of the evolution of female preference for wing spots are discussed in light of these results.

The evolutionary history and relationship of courtship behavior and wing pigmentation are examined in Chapter 3. This chapter not only describes and compares male courtship behavior across the Oriental *D. melanogaster* species group, but also considers an additional important courtship component, acoustic signaling, that may or may not be functionally related to wing spots and displays. The qualitative results suggest that some courtship elements are specific to this lineage. The evolution of some frontal wing displays may be associated with the degree and pattern of wing pigmentation. On the other hand, I found that sine song, which appears to have been a feature of the common ancestor of the entire lineage, does not appear to be associated with wing pigmentation. Sine and pulses have been lost in the *elegans, eugracilis,* and *ficusphila* species subgroups, which is consistent with visual observations of their courtship behavior. However, the loss of courtship song does not correspond to the degree or loss of wing pigmentation.

Chapters 4 and 5 deal with the genetic aspects of coordinately evolving wing pigmentation and courtship behavior in the interfertile species pair *D. elegans*, which bears both wing spots and wing display and *D. gunungcola*, which lacks both of these traits. In Chapter 4, a basic genetic analysis was carried out with a small number of molecular markers. The reciprocal F1 male hybrids from these two species are quite distinct in the wing pigmentation but not in courtship behavior, suggesting a large X effect on the divergence of wing spots but not courtship behavior in this species pair. Moreover, a significant correlation between wing spot size and courtship behavior was found in the backcross hybrid males. These results motivated the more detailed analysis in Chapter 5.

In Chapter 5, the genetic architectures of the functionally related wing pigmentation and courtship behavior were illuminated using QTL mapping analysis in backcross hybrid males of *D. elegans* and *D. gunungcola*. Among the five genetic linkage groups, the divergence of the wing pigmentation was largely associated to *y-Moe* region on the X chromosome, but QTL on linkage groups C and D also contribute to the difference in spot intensity. On the other hand, multiple QTL with relatively uniform effects were found to underlie the species difference in courtship behavior. The results of QTL analyses in individual courtship elements, which are completely lost in one species, are generally consistent with results from the composite courtship score. Intriguingly, the *y-Moe* region is also significantly associated with of the species difference in courtship score and wing display in the *elegans* backcross. This suggests that either pleiotropic or closely linked loci may play a role in the evolution of wing pigmentation and courtship behavior in the Oriental *D. melanogaster* species group. The QTL region responsible for the divergence of both traits is still too large to make a conclusion on the possible causes of genetic correlation. However, the presence of the *yellow* gene, which has been demonstrated to play a role in wing spot evolution in these species (Prud'homme *et al.* 2006) and has well known male courtship behavior effects in *D. melanogaster* provides the exciting possibility that pleiotropy has played a major role in the coordinate evolution of pigmentation and behavior in this system.

All of the research described in this thesis was carried out primarily by S.-D. Yeh under the advisorship of John R. True, who conceived and was funded for the overall genetic framework for the project. Collaborators and their contributions to the chapters are as follows:

Chapter 2: Individual experiments on female choice of wings or wing spots in *D. elegans*, *D. gunungcola*, and *D. biarmipes* were carried out by S.-D. Yeh, R. Yukilevich, A. Khanna, and K. Dechen. These collaborators greatly assisted in fine-tuning the research methods, but S.-D. Yeh was the primary researcher in designing and overseeing these experiments, as well as in interpreting and integrating the results.

Chapter 3: S.-D. Yeh carried out the acoustic recording in the laboratory of her Ph.D. dissertation committee member Jennifer M. Gleason and collaborated with Y. Lai in the analysis of courtship song. Most of courtship behavior was videotaped and digitalized in the Stony Brook University Functional Ecology Research and Teaching Laboratory with the assistance of M. Doall as well as in the True laboratory. Most videos were recorded S.-D. Yeh. Recordings of *D. biarmipes* and *D. pulchrella* by M. Goldblatt in the True laboratory were used for supplemental observations of the courtship behavior of those two species. All other work reported in this chapter was designed and carried out by S.-D. Yeh.

Chapters 4 and 5: S.-D.Yeh undertook all of the crosses, behavior observations, phenotype analyses, and the majority of the marker development and molecular genotyping in these studies. M. Bello and S. Harry, under the direction of S.-D. Yeh, dissected and photographed the wings of backcross hybrid males and measured the wing spot size together to provide one of the two independent sets of measurement in this trait. S.-R. Liou assisted with molecular methods and development of some of the markers. S.-D. Yeh also was the primary researcher involved in statistical analysis of the results, with assistance from B. Allen, D. Moen, J. Lachance, and Z.-B. Zeng.

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II. Wing spots coupled with courtship behavior as a visual signal in *Drosophila biarmipes* and *D. elegans*

ABSTRACT

Many morphological traits are not likely to confer selective benefits unless coupled with functionally related traits, such as behaviors or physiological characters. It has been proposed that male-specific wing spots in several species of the Oriental *Drosophila melanogaster* species group are under sexual selection by coupling with a frontal wing-display courtship behavior. In this paper, several female-choice-based tests were carried out in two wing spot bearing species to test whether wing spots are under sexual selection and how females assess this male specific wing pigmentation. First, we found evidence that wing display is under sexual selection using a single female choice test with control and wingless males in *D. elegans*. Second, compared with spot bearing males, spotless males expend significantly more effort, in terms of time and courtship action, before females accept them. Third, *white* mutant females of *D. biarmipes* accept spotted and spotless males indifferently, suggesting that vision is important for females to recognize wing spots. Therefore, we conclude that wing spots functionally couple with frontal wing display to constitute a visual signal during courtship. Finally, no effect on mating success was detected when wing spots were experimentally exaggerated, suggesting that females accept males based on the features other than size of wing spots. We discuss the implications of these results on general questions regarding the nature of information females evaluate during male courtship.

INTRODUCTION

Differences between sexes in morphological characters that are not directly related to reproduction are an intriguing phenomenon in nature. Some elaborated traits in one sex are even detrimental to their survival, thus, sexual selection was proposed to explain the evolution of these traits (Darwin, 1859; 1871). However, sexually dimorphic traits may, as well, be shaped by differential ecological selection between the sexes, such as the differences of mouth parts between male and female mosquitoes (Andersson, 1994). Without further investigation, it could be misleading to assume that many sexually dimorphic traits are under sexual selection. An example of such a puzzle is the melanic pattern of adult abdominal segments in the *Drosophila melanogaster* species group. The dorsal cuticle of the fifth and sixth abdominal segments is fully pigmented in males of many species in this group, but females only exhibit narrow posterior stripes of pigment in these segments (Bock and Wheeler, 1972). This sexually dimorphic trait was adopted as a key character distinguishing the *melanogaster* species group, well before any attempts to explain the evolution of the male-specific melanin pattern. Using genetic manipulation of abdominal patterning in *D. melanogaster*, Kopp *et al.* (2000) found that some strains of wild type males strongly disfavor females with ectopic pigmentation on their fifth and sixth abdominal segments, but the discrimination disappears in *white* mutant males, which have greatly reduced visual acuity. This suggested that the lack of male-like pigmentation in females may be maintained by sexual selection through male visual preference. However, the findings of Llopart *et al.* (2000) that these results were not repeatable across *D. melanogaster* strains countered this hypothesis and left the cause of the sexual dimorphism in abdominal pigmentation in question.

Another sexually dimorphic trait present in some species in the *melanogaster* species group, male-specific wing pigmentation, has become better understood recently following the finding of Kopp and True (2002) of a phylogenetic correlation of this trait with courtship behavior. Male courtship behavior of many species in the *melanogaster* species group involves distinct movements in which the male places ('displays') his wings in front of the female head. The anterior distal ('apical') area of wings in males of most of the species showing this courtship element is black pigmented to various degrees of intensity ('wing spots'). In most of these species, there is little or no pigmentation on the female wing. Based on the distinct male courtship behavior seen in spot bearing species, many researchers have assumed that the male wing spots serve as a visual stimulus to females during courtship (Fuyama, 1977; Ewing, 1983; Singh and Chatterjee, 1987; Chatterjee and Singh, 1991; Kopp and True, 2002; Hegde *et al.*, 2005). However, only a few experiments have been carried to test for female preference on wing spots (Singh and Chatterjee, 1987; Hedge *et al.*, 2005; see Discussion). No experiment has directly demonstrated that male wing spots and wing displays work together to deliver a visual signal during male courtship. In this study, we tested both whether females in *D. elegans* and *D. biarmipes* prefer spot bearing over spotless males as well as, in *D. biarmipes*, whether female vision is used to discriminate between males bearing wing spot differences.

D. biarmipes (also called *D. rajasekari* and *D. roychaudhuri*) belongs to the *suzukii* subgroup (Wheeler, 1981). This species is widely distributed in southeast Asia, including in Cambodia, India, Thailand, and Taiwan (Bock and Wheeler, 1972; Wheeler, 1981; new collection by C. S. Ng, identified with S.-C. Tsaur, 2003). The apical area of male *D. biarmipes* wings bears dark black pigment. Variation in spot size, ranging from pigmentation from the wing margin through longitudinal vein 3 to a complete lack of pigmentation in some

individuals of some strains, has been described (Bock and Wheeler, 1972; Singh and Chatterjee, 1987; Hedge *et al.*, 2005). Males of this species hold both wings out in nearly a straight line (about 180 degrees) with wing spots facing toward the female during courtship, which we call 'two-wing display' (Singh and Chatterjee, 1987; McRobert and Jackson, 1989; Chatterjee and Singh, 1991; Hedge *et al.*, 2005; personal observation). In the work described here we used an improved experimental manipulation over previous studies to test for female preference for wing spots while controlling for male vigor, in an attempt to confirm the previous findings of Singh and Chatterjee (1987) and Hegde *et al.* (2005). Also, white-eyed females were used to test the importance of vision in female mate choices between spot bearing and spotless males.

We also addressed these questions in the species pair *D. elegans* and *D. gunungcola*, which are sibling species in the *elegans* group. *D. elegans*, with two body color morphs, is widely distributed in southeast Asia. The brown morph populations tend to be distributed near the equator (Hong Kong, Hainan, Phillipines, Indonesia) and the populations with heavily melanic morph (black morph) are found in the subtropical islands and more northern latitudes (Ryukyu Islands and Taiwan)(Bock and Wheeler, 1972; Hirai and Kimura, 1997). Male *D. elegans* possess wing spots and exhibit a two-wing display similar to male *D. biarmipes*, but lack the acoustic signals exhibited in *D. biarmipes* and other species (Yeh *et al.*, 2006; See Chapter 3). On the other hand, *D. gunungcola* has black body color, similar to black morph, and has only been found in the mid-elevations of Indonesia, where it overlaps with *D. elegans* parapatrically (Sultana *et al.*, 1999; Suwito *et al.*, 2002). In contrast to its sibling species, the wings of male *D. gunungcola* do not have spots and the two-wing display is not observed during courtship (Yeh *et al.*, 2006). We tested whether *D. elegans* females prefer

more exaggerated wing spots as well as the female response to artificial wing spots in *D. gunungcola*.

MATERIALS AND METHODS

Fly stocks and cultures: *D. biarmipes* 361.3 was obtained from the *Drosophila* Species Stock Center (http://stockcenter.ucsd.edu). The *white* mutant of *D. biarmipes* is a spontaneous mutation that was isolated from the 361.3 strain in 2002 in the True laboratory at Stony Brook University. This mutant is sex-linked and behaves similarly to the *white* mutant in *D. melanogaster*, but has low fecundity (see Discussion). We have provisionally named it *white* under the assumption that the underlying locus is the *D. biarmipes* ortholog of *D. melanogaster white*. *D. elegans* HK (brown morph strain) was founded with several females collected in Hong Kong, China, and *D. elegans* TP (black morph strain) was collected in Taipei, Taiwan (Ishii *et al*., 2001). *D. gunungcola* SK was funded from several females collected at an elevation of 1150 m in Sukarami, Indonesia (Ishii *et al*., 2002). The *D. elegans* and *D. gunungcola* strains were provided by M.T. Kimura. All stocks were cultured in corn meal-yeast extract-agar medium with insertion of folded white printer paper for a pupation substrate. Males and Females were separated under CO_2 aesthesia within 0-4 hours after eclosion and aged in vials with fly food for 4-7 days before tests. Males were treated according to specific experimental protocols, as described below.

Wing treatments: To produce wingless males, we used micro-scissors to completely excise wings from one day old males. Newly eclosed flies in the spot bearing species are lightly pigmented and the wing pigmentation gradually accumulates over the first day of adult life. Since wing melanin formation depends on precursors in the hemolymph delivered by wing veins, interruption of hemolymph circulation in male wings in newly emerged flies will prevent wing

pigmentation (True *et al.*, 1999). For the spotless treatment, we used syringe needles to make a cut in the proximal section of longitudinal vein 1 (L1; which encompasses the anterior wing margin) in the males a few minutes to two hours after eclosion. For the control treatment, we cut the similar position of L1 in one day old males after the wing spots had already formed. In the wing spot enlargement experiments, male wings were painted using ultra fine point Sharpie (Sanford L.P.) permanent markers. The spot-enlarged wings were painted from apical L1 to L5 with black color and the control wings were painted with light yellow (Fig. 1).

The *D. elegans* males that experimentally had their wings removed or their wing veins cut still performed the full suite of courtship actions (see Introduction), except wing extension was not possible in wing-removed males. Thus, the courtship behavior did not appear to be grossly affected by either type of surgery.

No choice test: One aged virgin male was aspirated into a glass vial with fly food and allowed a few minutes of acclimation before one virgin female was aspirated in. The time was recorded when each event occurred, including when the female was introduced, when the male started to orient and court the female, when copulation began (meaning that the male grasps the female's genitalia, mounts on the female's abdomen with his forelegs holding her abdomen, forces her wings to spread out, and stays in this position for more than three minutes) and when the male and female separated. Male latency was counted as the time duration from the introduction of the female to the initiation of courtship. Courtship duration was counted as the time duration from the initiation of courtship to the initiation of copulation. Copulation duration was counted as the time duration from the beginning to the end of copulation. Trials were terminated 30 minutes after females were introduced if no courtship occurred, otherwise they were terminated

at the end of copulation. Males were scored as 'failed to mate' if they did not copulate with females after 10 minutes of courtship.

Single choice test: One control and one spotless male were aspirated into a 35x10 mm petri dish (Falcon 3001) and allowed one minute of acclimation before one female was aspirated in. The male of *D. elegans* usually orients toward the female, moves to the front of female with the leading wing extended out (circling), holds both wings at 180 degrees with ventral wing surfaces toward females (two-wing display), bends his abdomen toward the female and shakes his body laterally (body shaking), circles to the back of the female, attempts to copulate, and repeats this series of actions if the attempt fails. One courtship 'bout' was recorded when the courting male exhibited a full courtship sequence, including circling, two-wing display, body shaking, circling, and copulation attempt. Incomplete courtship was also counted as a courtship bout if the courtship performance was interrupted due to female movements such as rejection (female moving away).

Multiple choice test: The multiple choice test was designed to test groups of males and females with replacement of each copulating pair with one new male and one new female (e.g. Yukilevich and True 2008). 20 five-day-old virgin males of each type were aspirated into a 150 x 75 mm glass mating chamber (KIMAX No. 23000) with a plastic cover (VWR No.25384-139, 150 x 15 vial) and allowed a few minutes of acclimation before 20 virgin females (also five days old) were introduced by aspiration. The plastic cover of the mating chamber allowed flies to be aspirated out from chamber during experiments. Unlike males of *D. melanogaster* which typically have short courtship latency, the length of courtship latency in *D. elegans* is often much longer than in *D. melanogaster* and the variation among individuals is larger than for copulation time. Thus, the copulating pairs were replaced to maintain an equal number of control and treated males. When copulation occurred, the couple was recorded and aspirated out.

Then, one virgin female and one virgin male with the same type were immediately aspirated into the mating chamber to replace the copulating pair. The experiment ran until either type of males was exhausted (See Table 1.1 for sample sizes).

Statistical analysis: Male courtship latency, courtship duration, copulation duration, and courtship bouts were analyzed using a two-tailed Mann-Whitney rank test that was performed using Vassar Stats (Richard Lowry; http://faculty.vassar.edu/lowry/VassarStats.html) because these variables deviate from the normal distribution and the regular t-test is not appropriate. The difference of mating rate form multiple choice test was tested using a χ^2 test.

RESULTS

Wings are involved in courtship behavior and mating success in D. elegans

The importance of presence of wings in mating success was examined by simultaneously introducing winged and wingless (wing-excised) males to the females in multiple choice tests. As shown in Table 2.1, the mating rate of wingless males was significantly less than intact males. Several factors may contribute to less mating success of wingless males, including the reduction of copulation ability and the absence of acoustic and visual signals. Since copulation between females and wingless males occurred in this experiment, loss of wings does not appear to strongly inhibit the copulation ability. Furthermore, male wings are not usually involved in physical contact between males and females during copulation. Thus, wing removal may be less likely to affects copulation ability compared with courtship. Wing movement during courtship is important for mating success in many *Drosophila* species because wing movements produce

acoustic and/or visual signals (Arthur and Bennet-Clark, 1968; Bennet-Clark, 1971; Fuyama, 1977; Kopp and True, 2002). In many species, acoustic signals are important to attract courted females and females may discriminate males based on acoustic signals (Ritchie *et al.*, 1998; Tomaru *et al.*, 2000; Rybak *et al.*, 2002). Removal of wings may influence both acoustic and visual signals. In *D. elegans*, however, males are mute; no wing vibration has been observed and no sound has been detected during courtship in this species (See Chapter 3). Thus, the surgical loss of wings in *D. elegans* males is most likely to affect the visual aspect of male courtship.

Because *D. elegans* is parapatric with the non-spot-bearing species *D. gunungcola*, male wing spots in *D. elegans* may serve as a factor in species recognition. Indeed, interspecific copulation between these two species was not observed in the mating tests of Ishii *et al.* (2002) and seldom occurs when males and heterospecific virgin females are kept together (S.-D. Yeh, unpublished observation), though heterospecific courtship by males is frequently observed. It is possible that females may recognize their own species males based on many courtship signals, such as cuticular hydrocarbons and body movement (see Discussion).

Spotless males mate less in D. biarmipes

The multiple choice test was employed to test the effect of wing spot loss in *D. biarmipes*. Spotless males were accepted less than the control males (spotless=21, control= 38, χ^2 test, 1 df, P=0.027). *D. biarmipes* males produce various types of sounds during courtship by wing vibration (Yeh *et al.*, in prep). Whether wing surgery affects the wing vibration was untested. But this would not seem to affect our conclusion, since the wings of control males were also cut.

Spotless males need to perform longer courtship in D. biarmipes

We also examined the course of courtship behavior in both *D. biarmipes* and *D. elegans* in no-choice tests with individual pairs (Table 2.2). In *D. biarmipes*, mating rates between spotless and control males were not significantly different (75% versus 85.7%; χ^2 test, 1df, P=0.141). This result is different from the multiple choice test but is perhaps not surprising because in no-choice tests females may accept less preferred males after a substantial amount of courtship. No significant difference in male latency was found, indicating that the wing surgery does not influence this aspect of male courtship. However, courtship duration of spotless males was significantly longer than that of control males, suggesting spotless males need to spend more time courting than control males before they are accepted by females. The results in *D. elegans* are very similar to those in *D. biarmipes*.

Blind females mate spotless and control males equally in D. biarmipes

white mutants in most species completely lack visual pigments and in *D. melanogaster* these mutants are known to have very low visual acuity (Morgan, 1941; Connolly *et al*., 1969; Wehner *et al.*, 1969; Heisenberg and Wolf, 1984). The *white* mutant in *D. biarmipes* is X chromosome inherited and the mutant allele is recessive to wild type allele, like *white* mutants in *D. melanogaster*. When the spotless and control males of *D. biarmipes* were paired with *white* mutant females, the mating rates were not significantly different (89.5% and 64.5%; χ^2 test, 1df, P=0.222). Interestingly, courtship duration between spotless and control males in these tests was also not significantly different (Table 2.2), providing addition evidence that *white* mutant females may not be able to discriminate spotless males from control males.

Spotless males expend more effort to secure matings in D. elegans

The longer courtship duration in spotless males may be due to longer breaks between courtship bouts or more courtship bouts. To test this, single choice tests were used in which the courtship bouts before the copulation were counted when one spotless and one control male were allowed to compete for one female in *D. elegans*. The mating rates between spotless and control males were not significantly different (spotless=26, control=32, χ^2 test, 1df, P=0.43). However, spotless males that successfully copulated with females performed about twice the courtship bouts on average than did control males (spotless=13.7 courtship bouts, control=6.6 courtship bouts, t-test, P=0.035; Fig. 2). Spotless males tended to spend more effort to stimulate females before copulation occurred.

On the other hand, no-choice test of spotted and spotless males in *D. elegans* did not yield conclusive results because of small sample size and high variability among males from the same type. Noticeably, the copulation time of spotless males is significantly shorter than that of spotted males (Table 2.3).

Larger wing spots do not increase male mating success

To investigate whether *D. elegans* females may prefer extreme wing spot size, we tested the mating success of males with enlarged wing spots in no-choice tests. The mating rates were the same between control males and males with enlarged wing spots (N=15 and mating rate=73% from each). No significant difference was found in mating latency, courtship duration, or copulation duration (Table 2.4). This suggests females did not discriminate between control males and males with enlarged wing spots.

Female preference of wing spots may or may not be lost in D. gunungcola

According to the phylogenetic analysis of Prud'homme *et al.* (2006), wing spots were present in the ancestor of the *D. elegans* species subgroup but lost in the *D. gunungcola*, which is closely related to and crossable with *D. elegans*. To examine the female preference of wing spots in this species, the mating performance of males with painted or control wings was tested in no-choice tests. The mating rate of males with black painted wings was higher but not significantly different from mating rate of control males (52.6% in control, 82.6% in black painted, P=0.23). The mean courtship duration of black painted and control males was similar. However, the mean copulation duration of black painted males was significantly longer (Table 2.4). These results are consistent with the possibility that *D. gunungcola* females may retain an ancestral preference for male wing spots but other explanations are possible (see Discussion).

DISCUSSION

The importance of visual signals produced by Drosophila males

Male courtship behavior in *Drosophila* usually provides four types of signals: acoustic (auditory), olfactory or gustatory (pheromone/chemical), tactile, and visual stimuli (Spieth and Ringo, 1983; Greenspan and Ferveur, 2000). Many studies have focused on intraspecific and interspecific differences and the genetics of acoustic and pheromone signals because these types of signals are thought to be the most important in species recognition systems (Kyriacou and Hall, 1982; Ritchie *et al.*, 1998; 1999; Rybak, *et al.*, 2002; Ferveur, 2005). Many species are able to mate in dark or red light conditions, thus acoustic and olfactory signals, which are independent of light, are relevant in mating success. On the other hand,

visual signals have received less attention probably because they are thought to be relatively unimportant to male-female communication in well-studied species like *D. melanogaster*.

Among the potential male courtship signals described in *D. melanogaster* mating, the only strongly visual ones would seem to be the movement of the male from the rear to the front of the female during wing vibration, which is not major stimulatory signal for females (Spieth and Ringo, 1983; Greenspan and Ferveur, 2000, but see counter argument in Barth *et al.*, 1997). In contrast, males of *D. elegans* and *D. biarmipes* spend a great deal of time in front of females engaging in the elaborate courtship wing display and signals described above. According to field observations, *D. elegans* males occupy the newly opened flowers of *Ipomea* and other plants, wait for females to visit, and mate, which is reported to occur during the day but not in the evening (Kimura and Hirai, 2001). *D. elegans* males are mute during courtship (See Chapter 3). Therefore the wing and body movements are expected to provide mainly visual and olfactory signals to females. *D. biarmipes* males, however, provide visual, olfactory, and acoustic signals before they physically contact females (McRobert and Jackson, 1989; Chatterjee and Singh, 1991; See Chapter 3). Despite of the existence of other signals, we have shown that male visual signals influence female acceptance in these two species.

Variation of wing spot size in nature has been reported in *D. biarmipes* and the correlation between the presence of wing spots and mating success has been examined previously (Prakash and Reddy, 1976; Singh and Chatterjee, 1987; Hedge *et al.*, 2005). By using single choice test, the Singh and Chatterjee (1987) found that the mating rate of males with wing spots is significantly higher than spotless males from a laboratory stock with variation in wing spot size. However, it is unknown whether there was a difference in courtship vigor between the

spotted and spotless males in that study. Hegde *et al.* (2005) took the advantage of a natural population in which 70% of males possessed wing spots and 30% of males were spotless to investigate the effect of wing spots on mating success. In a field study, they examined the proportion of spotted and spotless males in mating pairs and among surrounding males. Interestingly, about 75% of spotted males and 30% of spotless males were copulating, which is very close to the ratio of spotted and spotless males in the population. Spotted males exhibited significantly more copulation than spotless males in single choice tests under laboratory conditions, which is consistent to our results. However, this may have been due to differences in male vigor because Hegde *et al.* noted that naturally occurring spotless males exhibited significantly longer courtship latency and fewer courtship behavior actions (tapping, scissoring, vibration, two-wing display) than spot bearing males.

Our experiments found no differences in courtship vigor between spotted and spotless males. Even in single choice tests in which spotless males were less successful than spot bearing males, the spotless males vigorously courted females.

In our no-choice tests, the average courtship duration of spotless males was significantly longer than that of spot bearing males. This suggests that females are less receptive to spotless males and that these males need to spend relatively more time courting before they are accepted. However, this difference in courtship duration between spotted and spotless males was not seen in the experiments with *white* mutant females, which presumably have less visual acuity than wild type females. The apparent loss of discrimination in *white* mutant females is not likely to be due to genetic factors other than the *white* locus because this mutant line was isolated from the same stock, *D. biarmipes* 361.3, from which wild type females were taken for the other *D. biarmipes* experiments.

It has long been hypothesized that wing spots provide a visual signal in the species exhibiting male-specific black patches on the apical wings (Fuyama, 1977; Ewing, 1983; Chatterjee and Singh, 1991; Kopp and True, 2002; Hegde *et al.*, 2005). However, before our study, whether females use their vision to assess wing spots remained fairly untested. Singh and Pandey (1994) reported that *D. biarmipes* females with purple eyes are more sexually receptive than wild type females. Eye pigments function in screening and absorbing non-useful light frequencies, intensities and angles, and the various eye color mutants are widely thought to have subnormal visual acuity (Heisenberg and Wolf, 1984). Thus, vision loss is a potential explanation of why purple-eyed females become less picky. In our study, the results of mating tests in *white* mutant females strongly suggest that wild type females use their vision to assess wing spots and are consistent with the hypothesis that male wing spots coupled with the two-wing display provide a visual courtship signal.

The importance of male vision in mating success

Fly mating behavior involves interactions between females and males (Hall, 1994). Visual signals provided by females may be as important as the signals from males in the communication between sexes. Males of many *Drosophila* species do not court in the dark, including *D. subobscura*, *D. simulans*, *D. rufa*, *D. auraria* and *D. affinis* (Philip *et al.*, 1944; Rendel, 1945; Wallace and Dobzhansky, 1946; Spieth and Hsu, 1950; McRobert and Tompkins, 1987). Some of these species may occasionally mate in the dark, suggesting that males are able to court in dark but lack the stimulation to do so (Spieth and Hsu, 1950; McRobert and Tompkins, 1987). Such stimulation may come from females. One of the stimuli from females is the female locomotor activity (i.e. female movement within the male's visual field), which has been shown to be important for the

initiation of male courtship behavior in *D. melanogaster* (Tompkins *et al.*, 1982; Joiner and Griffith, 1997; also see review by Yamamoto *et al.*, 1997). Furthermore, wild type males in dark conditions and *white* mutant males in general cannot follow females well. This further suggests that vision is important to males for maintaining the courtship sequence, even if males can detect females from olfactory cues (Connolly *et al*., 1969; Sakai *et al.*, 1997).

Whether *D. biarmipes* only mate under sufficient light condition in nature is unclear, but the defects in mating propensity is observed in *white* mutant and purple eye color mutants (Singh and Pandey, 1994; personal observation) provide evidence for light dependence in this species. The *white* mutant line of *D. biarmipes*, found in our lab, has very low fecundity, which may be due to several causes. First, we and Nicolas Gompel (pers. comm.) both have tried to obtain a healthier *white* mutant line by repeatedly crossing the *white* mutant to wild type and allowing recombination to occur, but these attempts failed. Thus, the low fecundity is unlikely caused by linkage of the eye color mutation to a mutation(s) causing partial lethality or sterility unless this linkage is very close. Second, the ovipostion of *white* mutant females is usually delayed when they are crossed to *white* mutant males. Many of the eggs laid do not hatch, which may indicate that they are unfertilized. However, *white* mutant females produce normal numbers of offspring when they are crossed to wild type males. Thus, the low fecundity is probably not due to female sterility. Third, *white* mutant males seldom orient to females, initiate courtship, perform courtship behavior, or follow females (S.-D. Yeh unpublished observation). This 'behavioral sterility' in *white* mutant males may contribute to the low fecundity of the *white* mutant strain. *white* mutants of *D. melanogaster* males have problems following females but they still inseminate females at approximately normal levels (Connolly *et al*., 1969). In contrast, *white* mutant males of *D. biarmipes* are not as active as wild type males,

suggesting that males in this species may rely mainly on their vision to recognize and court females. Fourth, the *white* mutant may have a pleiotropic effect on male courtship behavior. The *white* gene in *D. melanogaster* encodes a transmembrane protein that transports guanine and tryptophan into pigment cells (Sullivan and Sullivan, 1975; Sullivan *et al.*, 1979; 1980). The induction of ubiquitous expression of *white* in *D. melanogaster* results in an increase in mating vigor, including both male-female and male-male courtship behavior (Zhang and Odenwald, 1995; Hing and Carlson, 1996; Nilson *et al.*, 2000). Campbell and Nash (2001) reported that *white* mutants have higher resistance to volatile general anesthetics, a response independent of light, and that *white* is expressed in head tissues outside of compound eyes and ocelli. These findings suggest that *white* may have a more profound role in neural function than simply in transporting pigment precursors. But whether the *white* gene product is directly involved in the neural functions associated with courtship behavior in wild type flies is still unclear.

What information do females obtain from male wing spots?

Since Charles Darwin (1859) proposed his idea of sexual selection, many explanations of why females are picky and many models of sexual selection mediated by female choice have been proposed. These hypotheses of female preference for male traits can be grouped into three categories-- (1) Sensory bias with no benefit, (2) direct benefit to immediate reproductive success, (3) indirect benefit with runaway process or indicators of genetic quality (see reviews by Kirkpatrick and Ryan, 1991; Andersson, 1994; Andersson and Iwasa, 1996; Futuyma, 1998; Ryan, 1999). Below, these hypotheses are briefly discussed in the context of our findings.

The sensory bias hypothesis suggests that males exploit the preexisting biases females have toward certain sensory cues, such as colors related to food, in order to draw female attention during courtship (Kirkpatrick and Ryan, 1991; Endler and Basolo, 1998; Fuller *et al.*, 2005). This theory seeks to explain both the origin of female preference and the exaggeration of male traits. Based on this theory, female preference on more exaggerated traits is predicted. We found that *D. elegans* females do not discriminate between control and spot-enlarged males. This suggests that sensory bias is not responsible for the maintenance of male-specific wing spots in *D. elegans*, although whether the female preference in this lineage originated from biases of the female sensory system is unclear. Similarly, the presence of a runaway process proposed by Fisher (1915) also is inconsistent with our data, although such a process may have acted on these traits in the past.

Whether females gain higher genetic quality for their offspring or any direct benefit for themselves from mating with spotted males is difficult to speculate upon until we know what kind of information females obtain from the male wing spots and display. In stalk-eyed flies, males have exaggerated eyestalk length and males with the longer eyestalk, but not larger eyes, are preferred by females (Wilkinson and Reillo, 1994). Because the length of eyestalk reflects the nutrient condition of the individual, it has been proposed as an indicator for females to evaluate male condition (David *et al.*, 1998; 2000). Similarly, it is possible that *Drosophila* wing spots are an indicator relating to male condition. The possible signal from two-wing display and wing spots may relate to wing length, which may be envisioned by the distance between two wing spots or between the wing spots and the male head. Wing length is correlated with body size and is used as an index of body size in many studies (Robertson and Reeve, 1952; Robertson, 1962; Tantawy and Rakha, 1964; Sokoloff, 1966; Cavicchi *et al.*, 1981). Also, wing length correlates to some fitness components, such as

longevity (Tantawy and Rakha, 1964; Pieragostini *et al*, 1979; Soto *et al.*, 2006), but this correlation may be due to the relation between the fitness traits and body size. Larger males in *Drosophila* are generally thought to have better reproductive success, but the consequences of this on female reproduction are controversial (Partridge and Farquhar, 1983; Partridge *et al.*, 1987; Santos *et al.*, 1992; Bangham *et al.*, 2002; Friberg and Arnqvist, 2003). Nonetheless, body size is a plastic trait resulting from both genetic and environmental factors. Females may obtain larger offspring by copulating with larger males, but females may also gain some aspects of direct benefit from healthier (which means larger) males, such as substances transferred with the sperm during copulation.

There is some evidence suggesting that females of *D. elegans* and *D. biarmipes* may preferentially mate with larger males. In a field survey, the average wing length of mating *D. biarmipes* males was significantly larger than that of non-mating males (Hegde *et al.*, 2005). In *D. elegans*, male courtship occurs in leks on flowers after which females mate and lay eggs in the flowers (Suwito *et al.*, 2002; S.-D. Yeh personal observation, 2005; Shu Fang, pers. comm.). It was found that males occupying newly opened flowers are significantly larger (using thorax length as an index) than males staying on one day old flowers (Kimura and Hirai, 2001), which may indicate that larger males are relatively more successful in holding preferred territory and accessing females. Thus, it is possible that females choose males based on the distance between wing spots or between wing spot and male head. This hypothesis would also explain the distribution of pigments on wings and why enlarged wing spots are not more attractive to females in *D. elegans*. But further experiments are required to test this hypothesis and the potential benefits of the preference.

Do D. gunungcola females retain an ancestral spot preference?

The loss of these male traits may be due to factors other than sexual selection. Thus, females may or may not retain the preference for the ancestral male traits that have been lost in their own species. The factors involved in mate choice by *D. gunungcola* females are less conspicuous and mostly unknown, compared to its sibling species, *D. elegans*. In the experiments of Ishii *et al.* (2001), some *D. elegans* males exhibited longer courtship to *D. gunungcola*, but interspecific copulation did not occur. This suggests that *D. gunungcola* females discriminate strongly against heterospecific males. Nonetheless, the subtle difference of cuticular hydrocarbon profile between male *D. elegans* and *D. gunungcola* led Ishii *et al.* (2001) to argue that females use non-olfactory signals to discriminate between conspecific and heterospecific males. *D. gunungcola* males usually follow females and attempt to copulate after they orient to females. No courtship song is produced and males do not circle in front of the female's head. Thus, the acoustic and visual signals appear to be missing in the courtship of *D. gunungcola* males. Within *D. gunungcola*, there is also little sexual dimorphism in cuticular hydrocarbon profiles. It is unclear whether cuticular hydrocarbons, pheromones, or tactile stimuli are the major cues for females to choose males, or what the strength of female choice in this species may be.

Our finding of no significant differences in courtship duration before copulation between control and artificially black spotted males suggests that there is no female preference for wing spots before copulation. But why the copulation duration was longer when females mated with black spotted males is puzzling. Variation in copulation duration has been observed in various populations of *D. elegans*. Copulation duration of the tropical brown morph is shorter than that of the more northern black morph and both sexes appear to be involved in the termination of copulation (Hirai *et al.*, 1999). Hirai and Kimura (1999) found that

female remating rate is higher if the first copulation is interrupted. Also, we observed that *D. elegans* females escape by vigorously moving their body and flipping their wings during early copulation when males have inserted their genitalia into the female. It is possible that females end the copulation if they do not like their mates and the ability to control copulation duration is probably beneficial to females. Whether *D. gunungcola* females choose males through controlling the copulation duration still awaits experimental tests. But if this mechanism is operating, then females may prefer black spotted males given our result. We can think of two possible mechanisms by which this might occur, one olfactory and the other visual. The potential olfactory explanation could be an artifact of the experimental design; females may perceive the difference between light yellow and black markers. The light yellow marker was used to control for possible organic volatiles in the marker treatment, but perhaps females may be able to distinguish subtle differences in volatiles or pigments between the yellow and black ink. The potential visually based explanation might involve species recognition. The *D. elegans* morph that overlaps with *D. gunungcola* in their geographic distribution is brown morph, which might be the ancestral body color in this species since the rest of species in the same species subgroup and the closely related species subgroups are all brown (similar to wild type *D. melanogaster* body color). The body color of *D. gunungcola* is dark black (similar to *D. melanogaster ebony* mutants), which might be a derived state. Body color may be a visual cue in the species recognition system of *D. gunungcola* females. Thus the black painted wings may increase the overall darkness of male body, and consequently may be preferred by *D. gunungcola* females. With respect to the wing spot trait, neither of these potential explanations, which are testable, would provide evidence for a remnant preference for wing spots in *D. gunungcola* females.

Sexual selection and the evolution of female preference traits

Competition between mates of the same sex, mate choice by the opposite sex, and conflicts of reproductive interest between the sexes are three major factors driving sexual selection and evolution of exaggerated sex-specific traits (Andersson, 1994; Futuyma, 1998, Arnqvist and Rowe, 2005). The wing spot trait could be involved in male-male competition, such as holding territory in a lek, or involved in preventing the escape of non-receptive females by coupling with the frontal body movement to block the female. But here we provide evidence that wing spots are a visual signal during courtship in spot bearing species. Females in *D. elegans* and *D. biarmipes* preferentially mate with spotted males, suggesting that this male-specific trait in spotted species is maintained by sexual selection. Further investigation is required to understand the origin and the reason of the female preference on wing spots, as well as the existence of variation in the presence and absence of wing spots in *D. biarmipes*.

The strong correlation between wing pigmentation and two-wing display in phylogenetic analysis and our female mating tests are consistent with wing spots and two-wing display being functionally related during courtship in the spot bearing species of the *D. melanogaster* species group. No wing spots have been reported in the *ananassae* and *montium* subgroups, the basal subgroups of *D. melanogaster* species group (Spieth, 1952; Kopp, 2006). A recent phylogenetic analysis shows the gain of wing spots in the ancestor of the oriental *D. melanogaster* species group (Prud'homme *et al.*, 2006). Thus, wing spots and two-wing display are two novel traits evolved in the oriental *D. melanogaster* species group (although the two behaviors appear to have been independently gained in two lineages of the *obscura* species group; Prud'homme *et al.* 2006). Whether such behavioral traits evolve first and influence the evolution of the functionally-related morphology or the other way around has been a highly

discussed question (West-Eberhard, 2003). Studies on the origin and evolutionary history of these two functionally-related traits will help us to elucidate their functional relationship and the genetic process by which they became coupled. The evolution of such tightly coordinated traits may result in part from the evolution of genetic correlation and aspects of physical genome structure (Feder *et al.*, 2003; Via and Hawthorne, 2005; Joron *et al.*, 2006). By taking advantage of the biology of *D. melanogaster* species group and close relationship of the well-studied *D. melanogaster*, studies on the genetics of wing spots and courtship behavior in spotted species should provide many insights on these processes.

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Table 2.1. Results of multiple-choice tests between wingless and intact *D. elegans* males.

Strain	Winged	Wingless	Total	Chi-square value[1]	P value[1]
HK	21	11	32	3.13	0.077
TP	45	25	70	5.71	0.017
Pooled[2]	66	36	102	8.82	0.003

[1] Tested using the expectation of 1:1 ratio.
[2] Summation of data from the two strains.

Table 2.2. Comparison of courtship variables between control and spotless males in *D. biarmipes* from no-choice tests.

Variables	Male type	Sample size	Mean ± SE (s)	P
D. biarmipes males paired with wild type females				
Male latency	Control	63	218.1±27.5	0.337
	Spotless	56	274.5±36.7	
Courtship duration	Control	43	129.0±25.0	0.038
	Spotless	37	169.9±28.6	
Copulation duration	Control	43	959.7±26.8	0.322
	Spotless	37	958.9±30.0	
D. biarmipes male paired with white mutant female				
Male latency	Control	31	199.2±40.9	0.194
	Spotless	19	355.3±98.5	
Courtship duration	Control	20	156.2±36.6	0.842
	Spotless	17	118.5±29.6	
Copulation duration	Control	20	961.2±33.5	0.070
	Spotless	17	1018.5±29.7	

P values from Mann-Whitney rank test (Sokal and Rohlf, 2001).

Table 2.3. Comparison of courtship variables between control and spotless males in *D. elegans* HK from no-choice tests.

Variables	Male type	Sample size	Mean ± SE (s)	P
Male latency	Control	9	372.0±361.9	0.017
	Spotless	9	1001.3±521.4	
Courtship duration	Control	7	199.6±75.4	0.197
	Spotless	6	74.5±84.5	
Copulation duration	Control	7	554.3±33.6	0.087
	Spotless	6	446.0±24.4	

P values from Mann-Whitney rank test (Sokal and Rohlf, 2001).

Table 2.4. Results of tests using spot-enlarged males in *D. elegans* HK and *D. gunungcola* from no-choice tests.

Species	Variables	Male type	Sample size	Mean ± SE (s)	P
D. elegans					
	Male latency	Control	15	240.3±88.7	1
		Enlarged	15	236.5±106.0	
	Courtship duration	Control	11	133.3±65.4	0.596
		Enlarged	11	91.9±19.7	
	Copulation duration	Control	11	493.7±44.8	0.697
		Enlarged	11	535.6±19.1	
D. gunungcola					
	Male latency	Control	17	90.8±19.4	0.529
		spotted	21	126.2±34.4	
	Courtship duration	Control	10	109.7±60.3	0.447
		spotted	19	132.5±54.9	
	Copulation duration	Control	8	2167.6±316.9	0.0096
		spotted	17	3368.8±221.3	

P value is obtained from Mann-Whitney rank test.

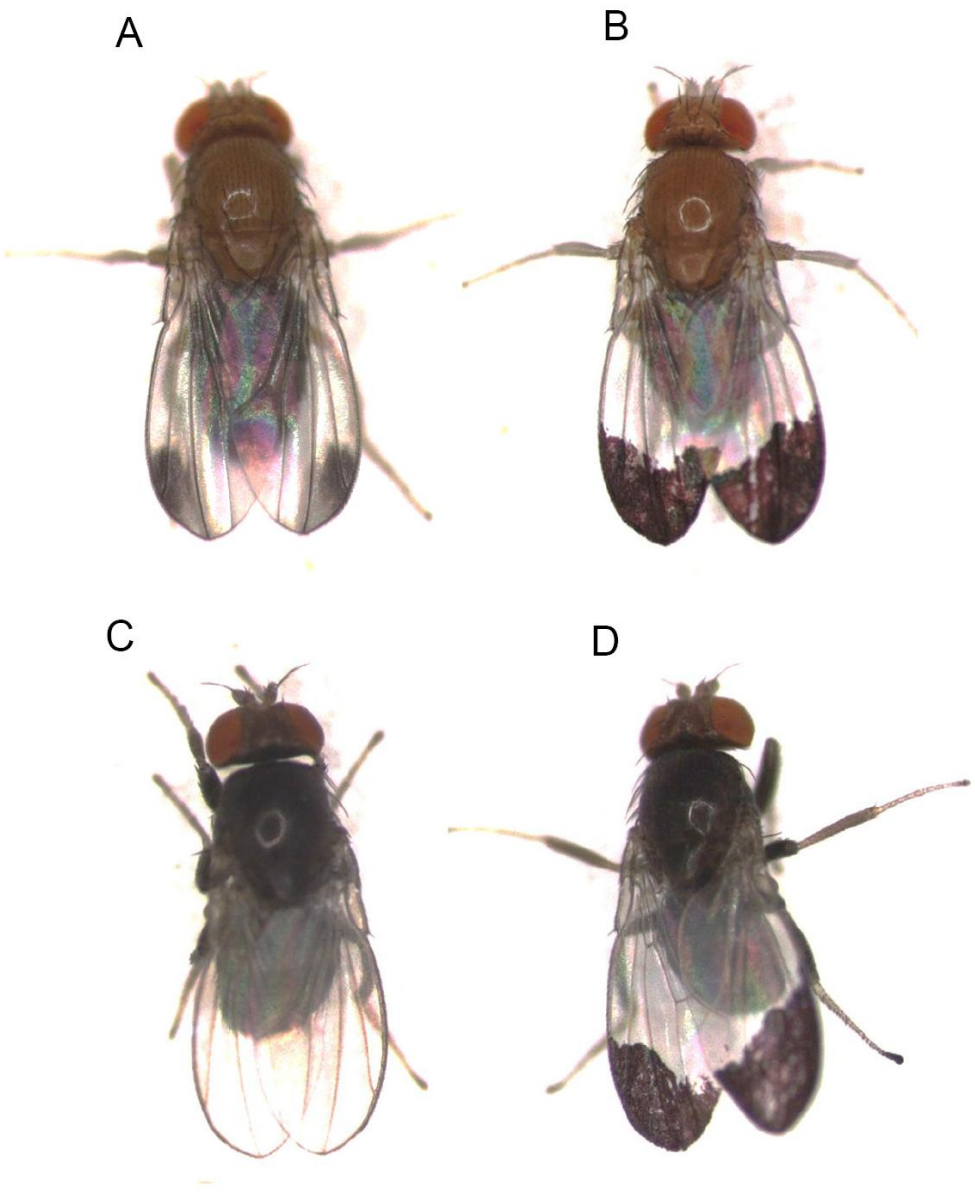

Figure 2.1. Examples of wing spot enlargement in *D. elegans* HK and *D. gunungcola*. (A) *D. elegans* HK male painted with light yellow marker; (B) *D. elegans* HK male painted with black marker; (C) *D. gunungcola* male painted with light yellow marker; and (D) *D. gunungcola* male painted with black marker.

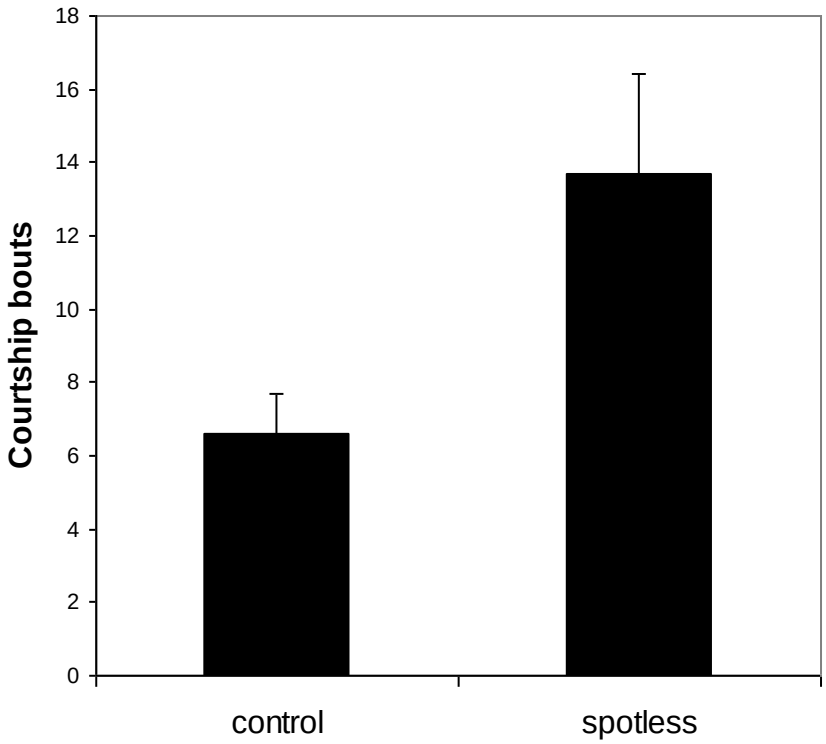

Figure 2.2. Differences in number of courtship bouts between spotless and control males of *D. elegans* HK.

III. Evolution of courtship behavior, courtship song, and wing pigmentation in the Oriental *Drosophila melanogaster* species group.

ABSTRACT

Behavioral and morphological traits function together and often co-evolve. In the Oriental *Drosophila melanogaster* species group, male wing pigmentation appears to evolve in concert with frontal wing courtship displays. Another courtship element, courtship song, may also evolve non-independently from these characters, but this behavior has not been well studied in this group. Here, I conduct a survey of courtship repertoires and courtship song characteristics for all of the available species in the Oriental *melanogater* group lineages. The courtship behavior and courtship song of several species, including *D. biarmipes*, *D. elegans, D. eugracilis, D. ficuphila, D. fuyamai*, *D. gunungcola, D. lutescens*, *D. mimetica, D. napalensis, D. pulchrella, D. pseudotakahashii,* and *D. takahashii*, are first described in this study. Our preliminary results suggest that some courtship elements, such as wing rowing and frontal wing displays may be derived characters in the Oriental subgroups. The evolution of some frontal wing displays appears to be associated with the degree and pattern of wing pigmentation. On the other hand, we found that sine song, which may have been present in the ancestor of the entire assemblage, does not appear to be evolutionarily associated with wing pigmentation. Sine and pulse songs have been lost in the *elegans, eugracilis,* and *ficusphila* species subgroup, which correlates with their visually-based courtship behavior. However, the loss of courtship song does not appear to correspond overall to the degree or loss of wing pigmentation in this species group.

INTRODUCTION

"Why does the animal behaves as it does? " is the main theme in the study of ethology, as pointed out in Tinbergen's classic book on "The Study of Instinct" first published in 1951 (Tinbergen, 1986). This question can be addressed from very different aspects, such as the internal physiology, external stimuli, and historical influence. As noticed by many researchers, correlations between behavior and morphological/physiological characters are very common in nature (Tinbergen, 1989; Andersson, 1994; Futuyma, 1998; West-Eberhard, 2003). Thus, the above question can include even more complicated problems involving how these trait correlations evolve. If the functional relationship between behavior and other types of traits is subject to selection, these traits will not evolve independently. Here, we examine potential linkages between the evolution of courtship behavior and functionally related wing pigmentation in the Oriental *Drosophila melanogaster* species group.

The *D. melanogaster* species group, classified within the *Sophophora* subgenus, consists of several species subgroups. These species subgroups are further categorized in three clades, the *ananassae* subgroup, the *montium* subgroup, and the *melanogaster-elegans-eugracilis-ficusphila-rhopaloa-suzukii-takashii* species subgroups (Bock and Wheeler, 1972). With insights from recent molecular studies, this classic view of phylogenetic relationships has been generally upheld (Kopp and True, 2002a; Schawaroch, 2002; Lewis *et al.*, 2005; Da Lage *et al.*, 2007). The third clade above is known as the "Oriental" *Drosophila melanogaster* species group, reflecting the distribution of most of

these species in the Australasian region, with the exception of the *melanogaster* species subgroup, which is Afrotropical.

Male-specific wing pigmentation is found in many species in the Oriental *Drosophila melanogaster* species subgroup and is thought to be an ancestral character that has been subsequently lost in several lineages (Prud'homme *et al.*, 2006). This male specific trait has been proposed to be associated with the frontal wing display from preliminary analyses (Kopp and True, 2002b). However, many of the detailed features of wing pigmentation and courtship behavior were lacking in the previous study. In addition, data on courtship song, another important courtship component, were absent from the previous study. Courtship song is known to be associated with one wing vibration, but different acoustic signals may be produced by other types of wing movements or body movement, such as frontal wing display or body shaking. Furthermore, acoustic signals may be neglected when sexual selection appears to emphasize visual signals. In order to fully understand the evolutionary relationship of wing pigmentation and courtship behavior, the presence and details of courtship song should be taken into account.

In this study, the courtship behavior and courtship songs of several species in the group are newly described. In addition, the evolution of different elements of courtship behavior and courtship songs are examined and the potential evolutionary implications of functional relationships among these three courtship components are discussed.

MATERIALS AND METHODS

***Drosophila* strains and cultures:** The species studied are listed in Table 3.1. *D. biarmipes* (360.0, 360.2, and 360.3), *D. eugracilis*, *D. ficusphila*, *D. lucipennis*, *D. lutescens* (271.0, and 271.1), *D. prostipennis*, and *D. pseudotakahashii* were

obtained from Tucson Drosophila Species Stock Center (now located at University of California at San Diego; https://stockcenter.ucsd.edu). *D. elegans* (HK and TP), *D. gunungcola* (SK), and *D. pulchrella* were obtained from Dr. M. T. Kimura at Hokkaido University. *D. fuyamai*, *D. mimetica*, *D. nepalensis* (48 and 56), and *D. takahashii* were obtained from Dr. A. Kopp at University of California at Davis. All stocks were cultured in corn meal-yeast extract-agar medium with folded pieces of printer paper for pupation substrate. Males and females were separated under CO_2 aesthesia within 0-4 hours after eclosion and aged in vials with fly food for 4-7 days before observation. The descriptions of courtship behavior elements performed by males are listed in Table 3.2.

The species subgroup system: In this study, the species examined were grouped into 11 species subgroups following the system of Bock and Wheeler (1972). The *melanogaster-elegans-eugracilis-ficusphila-rhopala-suzukii-takahashii* species subgroup complex is referred to as the Oriental *D. melanogaster* species group, thus, the *ananassae* and *montium* species subgroups were used as outgroups for comparison. Recent phylogenetic studies suggest that the *suzukii* species subgroup is paraphyletic, with *D. mimetica* grouped in the clade of the *takahashii* species subgroup, *D. lucippennis* more closely related to the *rhopaloa* and *elegans* species subgroups, and *D. biarmipes*, *D. pulchrella*, and *D. suzukii* remaining in a monophyletic group (Kopp and True, 2002a; Schawaroch, 2002; Lewis *et al.*, 2005; Da Lage *et al.*, 2007). For convenience and to prevent possible confusion, all of these species are still listed as being in the *suzukii* species subgroup in this report.

Courtship behavior recording and observation: Several virgin males and females were aspirated into a plastic petri dish (Falcon 100 x 15mm) with fresh fly food in the bottom. The petri dishes with multiple males and females were placed under a video camera affixed to a stage apparatus and connected to a VHS

recorder. After the files were videotaped, the images were converted into digitized video files and analyzed on a computer. During the course of videotaping, the courtship behavior was also noted. The number of individuals videotaped in each species is listed in Table 3.1.

Courtship song recording and analysis: Recordings were carried out in J. M. Gleason's laboratory at the University of Kansas in the summer of 2005 and winter of 2005-2006. Virgin males were collected 0-6 hours after eclosion and aged singly in small plastic vials with fly food for 3-13 days, depending on the species. To prevent interference from the sound produced from female wings during courtship song recordings, wings of all females in trials were removed by micro-scissors during virgin female collection and then the females were aged together. Before recording, one male and one wingless female were aspirated into a fly recording cage, which is a small plastic cylinder or rectangle with mesh nets on the top and bottom (Fig.3.1). The fly recording cage then was placed on the stage of the recording chamber apparatus, which is described by Gleason and Ritchie (2004) (Fig. 3.1). The sound produced by flies was received by an "insectavox" microphone (Gorczyca and Hall, 1987) below the stage and recorded by a Marantz CP430 cassette tape recorder. Since temperature may influence interpulse interval (IPI) (Ritchie and Gleason, 1995), the temperatures at the beginning and the end of each recording were noted. The minimum, maximum, and mean temperatures in which the recording of each strain was made are listed in Table 3.3. Courtship songs were digitized and filtered in GoldWave software (www.goldwave.com), and analyzed in Praat software (www.fon.hum.uva.nl/praat/). The duration of different types of sound wave were labeled in TextGrid format, and the length of sine, IPI, and the frequency were obtained by running custom scripts. The courtship songs of four to six individuals were analyzed in each species.

RESULTS

Courtship behavior of each species

The courtship elements performed by the male of each species are summarized in Table 3.4. The details of the male courtship behavior are described below. See Appendix A for the outgroups and the *melanogaster* species subgroup.

The *elegans* species subgroup

D. elegans

The male courtship behavior of this species includes tapping, circling with one-wing extension, two-wing extension with abdomen shaking, and attempting copulation. The male taps the female after he approaches her and attempts to copulate. In copulating attempts, he attempts to put his forelegs on the female's abdomen, bends his abdomen down and toward the female's genitalia, extrudes his genitalia forward to reach the female's genitalia while simultaneously opening his wings, and then drops his wings back to resting position if the attempt fails. The male also usually moves from the rear to the front of the female with the leading wing extended out at 90°, stops at the head to face to the female, extends both wings out, holds the wings at 180° with the ventral sides of the wings facing the female, moves his body laterally back and forth with his abdomen bent toward the female, and adjusts his position according to the female's movements. Then he circles around the female with one wing extended (the wing close to her head), moves back behind the female, and initiates further copulation attempts. Vigorous wing vibration was not observed in this species.

D. gunungcola

The male courtship behavior of this species includes wing fluttering with body shaking, tapping, licking, and attempting copulation. When the male orients to the female, he slightly extends one or both wings out at about 30° to his body, slightly moves his body laterally back and forth, and drops his wings back to the resting position before he starts to move to the rear of the female. The male usually taps and licks the female before attempting copulation. The copulation attempts are similar to those of *D. elegans*. The male typically follows the female with his abdomen bent down toward her if the female rejects him and tries to run away. The male does not circle around the female to perform wing displays and *D. elegans*-like wing displays were never seen.

The *eugracilis* species subgroup

D. eugracilis

The male courtship behavior of this species includes tapping, circling, one-wing rowing, two-wing rowing, heading, and attempting copulation. After the male senses the female, he moves to the rear of female, taps her with his forelegs, curls the tip of his abdomen downward and toward the female genitalia, and attempts to copulate. When the male attempts to insert his genitalia into female's, he extends the right wing out to 90° and points it upward with his left wing slightly moving upward, then both wings return to resting position. As the wings move upward, the anterior (the longitudinal vein 1) moves up and turns back to horizontal position, following by a movement to the posterior. Thus, the motion of wings is similar to oar rowing. Sometimes when the male taps the female, the anterior of both wings may lift upward to about 60° from horizontal with the

posterior wing edges pointed downward. This rearward one wing rowing may be repeated two or more times. The female usually keeps the tip of her abdomen pointed toward the substrate or turns her abdomen laterally and upward if she rejects the male. The male often moves around on either side of the female to face her (circling). The position of the male wings varies during circling. These wing movements may include: both wings in resting position, both wings with anterior margin lifted, the leading wing extending out horizontally, or both wings extended out horizontally. After the male positions himself to the front of female, he rams the female head with his head once or twice (head butting), stops and stands in front of female face to face, then either rows both wings simultaneously two or more times (two-wing-rowing) or slightly extends both wings out with a slight back and forth motion and then back to resting position over one to two seconds (two-wing-fluttering). The sequence and frequency of head butting, two-wing rowing, and two-wing fluttering vary between each circling episode and among individuals.

The *ficusphila* species subgroup

D. ficusphila

The male courtship behavior of this species is very simple. When a male senses a female, he orients to her, moves behind her, jumps onto her back, uses his forelegs and midlegs to grasp her abdomen, forces her to open both wings, and then attempts to copulate. Sometimes leg kicking occurs between individuals (including male-female and male-male kicking) during orientation, but whether it is part of male-female courtship signaling is not clear. The male keeps his wings in resting position during the entire courtship. The female rejects the male by keeping her wings in resting position and shaking her body to force the male to dismount. The sex comb is highly developed on both the tarsi and tibia of male

forelegs in this species (Kopp and True, 2002b). Thus, *D. ficusphila* may use predominantly tactile or olfactory signals for male-female communication during courtship.

The *rhopaloa* species subgroup

D. fuyamai

The male courtship behavior of this species includes tapping, one-wing vibration, circling with one-wing extension, two-wing rowing, prolonged two-wing extension with a unique foreleg movement, and attempting copulation. The male taps the female, extends one wing out to 90°, vibrates the wing up and down rapidly, and attempts to copulate. Tapping and copulation attempts usually occur while the male vibrates one wing behind the female. The first copulation attempt is usually rejected and the female moves forward somewhat or turns her abdomen slightly to one side with the ventral side facing out laterally as a sign of rejection. The male moves from the rear to the front of the female with or without the leading wing extended at roughly 90°, stops at the head in a facing position, then performs two-wing rowing or two-wing display. In two-wing rowing, the male extends both wings out to 45° from the body (thus the wings form a roughly 90° configuration), holds the wings at that position for up to five seconds, then turns the anterior surfaces of the wings slightly up while dropping them back to the resting position. This courtship element is also observed when the male is in front of the female but not in the head-to-head position. In two-wing display, the male extends out both wings, holds them at 180°, moves his forelegs up and down in turns, apparently causing the wings to move slightly up and down in turns. The male gradually approaches the head of female during this two-wing display so that his forelegs may hit her forelegs and his antenna may touch her head or

antenna. The male also reacts to slight female movements to maintain the head-to-head position. The male may perform several rounds of two-wing rowing and two-wing display before moving back to the rear of the female and performing another copulation attempt. Copulation attempts usually involve the abdomen bending forward and the male genitalia extruding, and mounting usually occurs after the male's genitalia grasps the female's genitalia.

The *suzukii* species subgroup

D. biarmipes

McRobert and Jackson (1989) reported that the male of this species performs tapping, circling, one-wing vibration, and attempting copulation, and that these elements are largely similar to the courtship behavior of *D. melanogaster*. Unlike *D. melanogaster* however, the male of *D. biarmipes* does not lick the female abdomen. In addition to the above courtship elements, the male of *D. biarmipes* performs two-wing extension, one-wing horizontal vibration, and abdomen bobbing. In our observations, the male usually extends one wing to 90° and vibrates his wings behind the female after he orients to the female. The extended wing may move up and down or slightly back and forth to resting position during wing vibration. Unlike *D. melanogaster,* the male does not perform one wing vibration in front of the female. The male circles around the female, sometimes with both wing extended out to 180°. The male maintains both wings held out with ventral sides of the wings slightly facing toward the female after he reaches the front of the female. The male sometimes moves his body from one side of female's head to the other side during two-wing display. Wing vibration during the two-wing display was not visually observable but was recorded (see acoustic signal section below). If the female moves during two-

wing display, the male may reposition himself to keep in front of female or may interrupt his courtship behavior. Wing fluttering with both wings slightly extended was also observed when the male was in front of the female, and was usually followed by the male slightly rowing one wing before both wings dropped back to resting position. After these displays, the male moves around to behind the female via either side and then performs more one-wing vibrations and/or attempts copulation. We noticed that the wing may move back and forth to resting position during one-wing vibration, which is different from the one-wing vibration performed by *Drosophila melanogaster* males. Thus, this courtship element is referred as one-wing horizontal vibration (see Table 3.2). Due to limitations of the video equipment, we were not able to fully describe the detailed wing movements during wing vibration. As described by McRobert and Jackson (1989), the male vibrates his abdomen up and down when he approaches to the female (abdomen bobbing). Abdomen bobbing is also observed after the male reaches the female and the courtship is interrupted by female movement. We noted that one wing vibration might be combined with wing extension back and forth to the resting position. Wing semaphoring was reported in this species by Chatterjee and Singh (1991) with no definition/description. We did not observe wing semaphoring as defined by Spieth (1974) (See Table2) in this species.

D. pulchrella

The male courtship behavior of this species is similar to that of *D. biarmipes*, except for the lack of the two-wing extension, one-wing horizontal vibration, and prominent circling. After the male orients to the female, he taps the female's abdomen with his forelegs, gradually extends out both wings to about a 60° angle to his body with slight vertical wing vibration (referred to as wing stretching), then drops one wing back to resting position and keeps extending the

other wing back and forth from a 60 to 90° angle to his body (referred to as one wing horizontal vibration). The male may move from the rear to any position relative to the female with both wings slightly extended at about 10°, but this circling motion occurs irregularly and much less than in the other species in which circling has been observed. Wing stretching, which is usually followed by one-wing horizontal vibration, may be performed with the male in any position relative to the female, thus this courtship element may or may not couple with wing spots to provide a visual stimulus. Compared to *D. biarmipes*, the wing movement during one-wing horizontal vibration is slower in this species. The male usually vibrates the wings while one or both wings are extended slowly, which may contribute to the sound waves produced by this motion being distinct from the typical pulses found in other *Drosophila* species (see acoustic signal section below for more detail). During the courtship song, instead of vibrating up and down, the male of this species extends his wing outward and back to resting position. Abdomen bobbing was observed in between other courtship elements or when the female began to move away. The details of the abdomen bobbing element could not be observed because the resolution of the video images was low and lateral views of courtship behavior in this species were seldom captured. Quiet but regular sound waves were recorded when the male performed abdomen bobbing (see acoustic signal section below). This may be due to the male abdomen contacting the substrate/ground during abdomen bobbing. Fuyama (1980) reported that *D. pulchrella* males only exhibit courtship song while behind the female. However, we have not been able to confirm this because only one strain, a long term laboratory stock, was observed in our study. Variation among laboratory strains could be due to inbreeding depression, which can affect courtship behavior (Sharp, 1984; Meffert and Bryant, 1991; Miller *et al.*, 1993).

D. mimetica

The male courtship behavior of this species includes tapping, circling, one-wing vibration, one-wing rowing, wing fluttering, and wing shuffling. The male taps, extends one wing out to about 90° with the anterior wing slightly elevated, and vibrates the extended wing up and down very quickly. If a copulation attempt fails, the male moves to the front of the females, either stopping in a head-to-head position or performs arch circles,which moves his body back and forth in the front of the female, a few times. The male generally rows one wing once or flutters his wings to 5-10° when he stops in front of the female. When he circles in the front, he usually extends the wing that is opposite to the direction of his movement until he reaches one side of the female (about 30° from female anterioposterior axis), then stops and drops the wing, moving back to the other side with the other wing extended. This is usually repeated several times. This motion is referred as wing shuffling. The above courtship elements may repeat several times in various sequences before successful copulation or rejection. If the female rejects the male, she may extend both wings slightly and vibrate the wings vigorously, run away, and/or decamp.

The *takahashii* species subgroup

D. takahashii

The male courtship behavior of this species includes tapping, circling, one-wing vibration, two-wing rowing, two-wing extension, and attempting copulation. Like *D. eugracilis*, the *D. takahashii* male moves his wings outward and upward to 45° and quickly drops them to the resting position when he stops in front of the female. This two-wing rowing behavior was also described by Speith (1952). The male may extend and hold both wings in front of the female, but no

body shaking was observed. Most of time, the male chases or taps the female and when orienting to the female he vibrates his wings from behind her.

D. pseudotakahashii

The male courtship behavior of this species consists of most observed courtship elements in this species group, including tapping, circling with one-wing extension, one-wing vibration, wing scissoring, one-wing rowing, two-wing rowing, two-wing extension with body shaking, wing fluttering, and attempting copulation. The male usually taps, vibrates one wing, and attempts to copulate after he approaches the female. One-wing vibration usually starts by the male moving one wing outward and back to the resting position several times (similar to one-wing vertical vibration), and then the wing may be held out at about 90° and vibrated up and down vigorously. He circles to the front of female, stops in a head-to-head position, and performs various wing movements, including one- or two-wing rowing, two-wing display, and wing fluttering. Wing fluttering may lead to one- or two-wing rowing, or one-wing vibration to one-wing rowing, before both wings are dropped back to the resting position simultaneously. Similar to the male of *D. elegans*, the male of this species circles with the leading wing (which is the wing closer to female head) extended, holds both wings at 75-90° relative to his body , and shakes his body laterally maintaining a head-to-head position during two-wing display. Overall, the male courtship behavior of this species is quite complex in that transition between different types of wing movements is irregular and occurs rapidly.

D. lutescens

The male courtship behavior of this species includes tapping, circling with one-wing extension, one-wing vibration, two-wing vibration, and attempting copulation. The male horizontally moves one wing back and forth several times and then moves the wing up and down with vibration during wing extension behind the female. One-wing vibration is sometimes exhibited when the male circles around the female or intermittently during two-wing vibration. The male circles the front of the female with the leading wing extended out to about 45°, stopping at a head-to-head position. He then typically extends both wings at up to 150° and moves the wings up and down and slightly back and forth, a movement similar to wing fluttering. The male usually moves in response to the female and attempts to maintain the head-to-head position, even when the female is walking. Two-wing rowing with wings slightly turning upward and back is occasionally seen when the male is in front of the female.

D. nepalensis

The male courtship behavior of this species is very similar to that of *D. lutescens*, except for subtle differences in wing movement. During two wing vibration, the wings are extended and held slightly closer to the body than *in D. lutescens*.

D. paralutea

The male courtship behavior of this species includes tapping, circling with one-wing extension, one-wing vibration, two-wing rowing, wing shuffling, and attempting copulation. The male typically taps the female many times after he

reaches her. He then slowly extends one wing outward, vibrating the wing up and down slightly during this extension, then moves the wing slightly back and forth, holds the wing out at about 90° for one or two seconds, and drops the wing back to resting position. Unlike the vertical wing vibration described earlier in the *suzukii* and *takahashii* species subgroups, the back and forth wing movement in *D. paralutea* is subtle and may not produce pulses. The courtship song of this species was not thoroughly examined in this study. The rear to front semi-circle usually occurs when the male has one wing extended to about 90°. The male may keep holding or vibrating the extended wing while arch circling in the front of the female. Alternatively, he may extend and drop back the wings in turns during arch circling, which is similar to the wing shuffling observed in *D. mimetica*. The male attempts to copulate during one-wing vibration.

D. prostipennis

The male courtship behavior of this species includes tapping, one-wing vibration, circling with one-wing extension, two-wing extension, and attempting copulation. Similar to *D. pulchrella* and *D. lutescens*, the male moves one wing back and forth vertically. This vertical vibration is usually followed by rapid up and down vibration with the wing held out at about 90°.

The features of acoustic courtship signals in the Oriental *melanogaster* group species

Drosophila males produce two distinct types of sound waves with their wings during courtship song, sine and pulse waves. These usually are made by vibrations of their wing(s), but some special types that consist of more complex

waves, suggesting more complex wing movements, are also produced, depending on the species. Sine song, also referred to humming, consists of continuous sinusoidal waves. This type of sound wave is usually produced by rapidly vibrating wing(s) at low amplitude. Pulse song consists of a series of pulses, sometime with repeated sets or rhythms of pulses. The intrapulse frequency (IPF), which is the frequency of pulses, and the interpulse interval (IPI), which is the time between two pulses, are usually used as the main descriptors of pulse song. IPF and IPI vary within and among species. Furthermore, IPI differences between species have been demonstrated to be subjects of female mating preference (Tamaru *et al.*, 1995; Ritchie *et al.*, 1998; Hoikkala and Leena, 1999; Talyn and Dowse, 2004; Tamaru *et al*., 2004). Table 3.5 summarizes the types of acoustic signals produced by the male of each species in the Oriental *melanogaster* species group for which data is available either in this study or previous studies. The detailed features are described below.

The *melanogaster* species subgroup

The courtship songs of this subgroup are well studied. Cowling and Burnet (1981) compared six species in this subgroup, including *D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. yakuba*, *D. teissieri*, and *D. erecta*. The courtship songs of *D. sechellia* and *D. orena* were first described by Cobb *et al.* (1989), and that of the recently discovered species, *D. santomea*, was described by Watson *et al.* (2007). Most of species in this subgroup produce sine songs, except *D. sechellia*, *D. yakuba*, and *D. santomea*. *D. yakuba* has two types of pulse song, a ‘thud' produced by wing vibration and a ‘clack’ produced by wing scissoring (Demetriades *et al.* 1999). Compared to the ‘thud’, the IPFs of the ‘clack’ are higher and the IPIs are longer among the strains studied in Demetriades *et al.* (1999) than in the other studies. Though Cowling and Burnet (1981) did not link

wing scissoring to sound production in *D. simulans*, the arrhythmic 'clack' pulses produced by wing scissoring were also observed during my song recordings of *D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. santomea*, and *D. erecta*. Two species of this subgroup, *D. sechellia* and *D. orena*, were not available during my song recording. The 'clack' pulse is possibly produced in these two species because they perform wing scissoring during courtship. The types of courtship songs that are produced by the species in this subgroup are listed in Table 3.5.

The *elegans* species subgroup

Four strains of *D. elegans* (including CB, OH, SK and HK) and one strain of *D. gunungcola* (SK) were examined, but no detectable sound was produced during male courtship. The males of these species are therefore described as "mute".

The *eugracilis* species subgroup

D. eugracilis

The male produces polycylic pulses when he rows one wing during copulating attempts (Fig. 3.2). It is unclear whether these sound waves influence the female receptivity.

The *ficusphila* species subgroup

D. ficusphila

D. ficusphila is presumably mute because no observable male wing movement is observed during courtship. This species was not examined acoustically.

The *rhopaloa* species subgroup

D. fuyamai

The male of this species produces sine song, 'grouting' (see below), and polycyclic pulses during courtship. The duration of sine song ranged from 32 to 350 ms with a mean of 157.2 ± 99 ms. The mean frequency of sing songs was 183.1 ± 9.87 Hertz. The polycyclic pulses appear as a series of several periodic bursts of sine waves. The mean IPF of polycyclic pulses was 139. 6 ± 50.0 Hertz and the mean IPI was 56.8 ± 3.8 ms. Figure 3.3 shows an example of a burst of pulses followed by sine songs in *D. fuyamai*. 'Grouting' is a type of sine song that we have not yet quantified; an example is shown in Figure 3.4.

The *suzukii* species subgroup

D. biarmipes

Males in this species produce three types of sound waves (toot, pulse, and sine-like) during courtship. After the male orients to the female, he produces a set of high frequency sound waves, called a "toot" (shown in Fig. 3.5). The duration of toots ranged from 86 to 266 ms, and the mean frequency was 235.7 ± 3.4 Hertz. A running female will often stop after the male produces a toot, thus, this sound may function as an attention-getting signal to the female so that she may begin to

assess a potential mate. As mentioned above, the male of this species exhibits two types of wing movement during one-wing vibration-- horizontal and vertical vibration. Usually, the one-wing vibration begins with vertical movement and then changes to horizontal vibration, or switches between the two movements. The pulse frequency of vertical movement is very similar to that of horizontal vibration, and the boundary between these two types of wing movements is difficult to define (Fig. 3.6). The sine-like and polycyclic features of the pulses may reflect this complex wing movement. The mean IPF was 287 ± 32.4 Hertz. The mean IPI was 137.4 ± 8.5 ms. A histogram of the IPI data is shown in Figure 3.7. When the male exhibits two-wing extension in front of the female, quiet sine-like waves are produced (shown in Fig. 3.8).

D. pulchrella

Similar to *D. biarmipes*, *D. pulchrella* males produce toots and pulses. In addition, the pulses are produced when the male exhibits abdomen bobbing. The males produce longer and higher frequencies of toots in *D. pulchrella* versus *D. biarmipes*, with the duration in *D. pulchrella* ranging from 152 up to 413 ms (mean ± SD = 207.9 ± 21.6) and a mean frequency of 285.9 ± 21.0 Hertz. The toot is usually followed by polycyclic pulses, as shown in Figure 3.9. The mean IPF is 278±5.7 Hertz. The first IPI, with mean of 166.2 ± 18.3 ms, of a pulse train is typically longer than subsequent IPIs in a series (See Fig. 3.10 for an example). The mean IPI was 135 ± 7.8 ms. The acoustic data during abdomen bobbing are not yet quantified, because these are quiet and have been difficult to distinguish from background noise.

D. mimetica

Similar to *D. melanogaster*, the males of this species produce sines and pulses (but polycyclic). The mean frequency of sine song is 163.2 ± 43.5 and the length of sine ranges from 58 ms to 342 ms. The mean IPF was 336.6 ± 14.4 Hertz and the mean IPI was 24.7 ± 2.0 ms. An example of *D. mimetica* courtship song is shown in Figure 3.11. No songs were detected during the wing shuffling element.

The *takahashii* species subgroup

D. takahashii

The male of this species produces sine song and two types of pulses. The mean sine frequency was 121.2 ± 10.5 Hertz. The mean sine length was 251.8 ± 124.4 ms, but occasionally the male performed the sine element for up to 1000 ms. The males produced polycylic pulses with a mean IPF of 113 ± 4.77 Hertz and a mean IPI of 209.3 ± 79.3 ms. Presumably, these polycyclic pulses are produced by vibrating one wing back and forth; but the details of wing movement in this species need to be examined. Chains of polycyclic pulses were usually short, ranging from one to six pulses. The male also produced longer trains of monocyclic pulses with a mean IPF of 82.5 ± 17.2 Hertz and a mean IPI of 60.5 ± 3.8 ms. A chain of monocyclic pulses may occur alone, but the polycyclic pulses are usually companied by monocyclic pulses.

D. lutescens

The male of this species produces two types of pulses. “Frontal pulses”, which are produced by two-wing vibration in the front of the female, are polycyclic with three major peaks (Fig. 3.12). The number of pulses in a burst of

frontal pulse song ranged from one to five. The mean IPF was 157.1 ± 4.0 Hertz and the mean IPI was 131.4 ± 30.7 ms. “Rear pulse”, which is produced by one wing vibration in the rear of the female, is also polycyclic (Fig. 3.12). The mean IPF was 171.4 ± 7.4 Hertz and the mean IPI was 96.0 ± 9.4 ms.

D. nepalensis

The male of this species produces sine-like song and two types of pulses. The sine-like song is shorter than the typical sine songs of other species and is repeated fairly rapidly (Figs. 3.13 and 3.14). The length of sine-like song ranged from 47-187 ms with an average of 98.6 ± 37.1 ms. The mean frequency of sine-like song was 178.9 ± 11.0 Hertz. The male repeatedly moves one wing back and forth before he starts vibrating the wing up and down. The horizontal vibration produced pulses with a mean IPF of 196.1 ± 4.8 Hertz and a mean IPI of 168.8 ± 23.1 ms. The vertical vibration produced pulses with a mean IPF of 293.0 ± 10.8 Hertz and a mean IPI of 72.4 ± 6.7 ms. The pulses produced during frontal two-wing vibration were not quantified due to inadequate space for this movement in the recording cage and the high mobility of females under the recording conditions.

D. prostipennis

The male of this species produces very loud pulses and distinct song, referred to as “turbo”. A chain of polycyclic pulses, produced by horizontal vibration, is usually followed by turbo song (Fig. 3.15). The mean IPF of pulses was 282.6 ± 17.2 and the mean IPI was 364.6 ± 34.7. A turbo consists of various numbers of sine waves which change pitch in cycles, each cycle containing 16 to 17 peaks (Fig. 3.16). The length of turbo ranged from 321 to over 2500 ms with a

mean of 834.3 ± 692.8 ms. The mean duration of a cycle was 41.4 ± 2.5 ms and the mean of the mean frequency within a cycle was 239.4 ± 21.1 Hertz.

DISCUSSION

As observed by many researchers, courtship behavior and courtship songs are rapidly evolving traits in *Drosophila*, with quantitative variation in these two traits frequently observed within species (Connolly *et al.*, 1974; Cobb *et al.*, 1985; Ritchie and Gleason, 1995; Demetriades *et al.*, 1998; Gleason and Ritchie, 1998; Colegrave *et al.*, 2000; Yukilevich and True, 2008). The qualitative characteristics of these two traits are less likely to be variable among different populations within species (Cobb *et al.*, 1985). With substantial taxon sampling in the Oriental *D. melanogaster* species group, the evolution of these two traits is examined qualitatively in the present study.

The dynamic evolution of licking behavior

The courtship behavior of *Drosophila* consists of a number of different types of courtship elements. Some of these seem to be invariable and are assumed to be required for mating, such as orienting and attempting copulation. We do not consider these further. As shown in Table 3.4, tapping is almost invariable across species, being absent only in *D. eugracilis* and *D. ficusphila*. On the other hand, licking is only found in the *melanogaster* subgroup and in *D. gunungcola*. Licking is a subtle and very rapid action, and it is almost impossible to observe without looking at the ventral or lateral view of the flies during courtship. The licking behavior was observed in *D. gunungcola* when the flies stood on the cover of the petri dish and performed the courtship behavior in an upside down orientation.

Thus far, licking behavior has not been observed in *D. elegans*. Licking behavior has been observed in the other species subgroups not studied here, such as the *saltans* and *willistoni* species groups within the subgenus *Sophophora* (Speith, 1952). However, licking has not been reported in the *ananassae* and *montium* subgroups, which are the sister clades to the Oriental *melanogaster* species group (Fig. 3.17). Without further phylogenetic analysis, it is not clear that the licking behavior in the Oriental *melanogaster* species group reflects a synapomorphy with the same behavior in the *saltans* and *willistoni* groups or is independently evolved.

Novel two-wing vibration in the *takahashii* species subgroup

One-wing vibration is a general element in *Drosophila* male courtship behavior (Spieth, 1952) and this courtship element produces acoustic signals that are important for male mating success (see below). The lack of one-wing vibration in the *elegans*, *eugracilis*, and *ficusphila* species subgroups and the presence of this element in the other groups in this study suggests that one-wing vibration has been lost in these subgroups, and indeed these subgroups generally do not produce courtship songs, except the pulse-like sound in *D. eugracilis*. This element was originally described as an up-and-down wing movement performed behind or to the side of the female (Spieth, 1952). In our trials, we found that one-wing vibration was generally only exhibited when the male was behind the female in most of the subgroups, except the *melanogaster* subgroup. Thus, performing one-wing vibration from the side of the female is likely a male character that evolved in the ancestor of the *melanogaster* subgroup. In addition, we found that the wing can also be vibrated horizontally, a novel type of wing vibration that produces interesting sound waves in the *suzukii* and *takahashii* subgroup (see above description of courthip behavior and courtship song). This horizontal wing

vibration might have been present in the ancestor of these two subgroups, since the two subgroups form a sister clade in phylogenetic studies (Kopp and True, 2002a; Lewis *et al.*, 2005; Da Lage, 2007).

Circling and wing display may correlate to wing pigmentation

Circling occurs in most species in the Oriental *melanogaster* species subgroup. However, the precise path of circling shows many variations and combinations with wing movements in different species. Full circling, which is non-stop circling around the female for about 360°, has been observed in many distantly related *Drosophila* species. Speith (1985) listed about 34 species in *Drosophila* that exhibit full circles and hypothesized that this behavior originated for preventing the escape of non-receptive females. Semi circling, which is back-and-forth circling from the back to the front of the female, and arch circling, which is side-to side circling from one side to the other side of the front of the female, are possibly derived states that are associated with various wing movements, such as one- or two-wing vibration, wing scissoring, and wing displays (rowing, extension, shuffling). Circling has apparently been lost in *D. gunungcola, D. ficusphila*, and some species in the *montium* subgroups.

Various types of wing movements exhibited in the front of the females were found in the Oriental *D. melanogaster* species group, but not in the *ananassae* and *montium* subgroups. This suggests the frontal wing display is likely a derived characteristic in the Oriental species. Based on the phylogenetic analysis reported by Prud'homme *et al.* (2006) (Fig. 3.17), frontal wing displays might have arisen after the split of the *ficusphila* subgroup from the rest of the lineages. This evolutionary event appears to have co-occurred with the evolution of wing pigmentation (node 2 of Fig. 3.17). Several species derived after that

node exhibit a form of two-wing display. Two-wing rowing may have arisen just prior to node 2 since this courtship element is found in four out of six subgroups. This scenario would therefore involve subsequent loss of two-wing rowing in the *elegans* and *suzukii* subgroups. Whether two-wing rowing is a visual signal in male courtship is unclear, but the males in the species that only perform two-wing rowing but no two-wing extension tend to possess clear to lightly pigmented wings. Two-wing extension occurs in the *elegans* and *rhopaloa* subgroups, which are sister lineages, as well as in the *suzukii* and *takahashii* subgroups, which also are sister lineages. The species whose males perform this courtship element generally possess dark pigmentation (spots) in the apical area of wings. Two-wing vibration is only found in *D. lutescens* and *D. nepalensis*, which has been classified in the *takahashii* subgroup by morphological characteristics. This suggests that two-wing vibration is a newly derived element in the *takahashii* species subgroup. Further phylogenetic analysis would help to elucidate the evolutionary relationship between the intensity of wing pigmentation and the courtship behavior elements.

Sine song might have evolved in the common ancestor of the Oriental *D. melanogaster* species group

In general, the males in this species group are very acoustically active. Pulse songs appear to be the primary acoustic signal in *Drosophila* and are subject to sexual selection in several species (Hoikkala and Aspi, 1993; Tamaru *et al.*, 1995; Ritchie *et al.*, 1998; Hoikkala and Leena, 1999; Talyn and Dowse, 2004; Tamaru *et al*., 2004; but see Boake and Poulsen, 1997 for an interesting counter example in picture-winged species.) On the other hand, less attention has been focused on sine songs, probably for two reasons. First, no conclusive function of sine song in mating success and female preference has been demonstrated in *D.*

melanogaster (von Schilcher, 1976; Kyriacou and Hall, 1982; Crossley *et al.*, 1995; Talyn and Dowse, 2004). No effect of sine song was reported on female locomotor activity in *D. melanogaster* (Crossley *et al.* 1995). In contrast to these findings, running females of *D. biarmipes* generally ceased movement immediately after the male produced the toot element in our study. It would be very interesting to determine whether the toot element affects male mating success in *D. biarmipes*. Second, the production of sine songs was exclusively reported in the *melanogaster* species subgroup. To our knowledge, sine-like song has not been reported in the species outside of the *melanogaster* species subgroup. This study is the first discovery of sine-like songs in the *takahashii*, *suzukii*, and *rhopaloa* subgroups. Thus, the origin of sine songs may date back to node 2 of the phylogeny (Fig. 3.17).

The pulse song produced by horizontal wing vibration has not been reported in the *ananassae* and *montium* subgroups, which are the sister subgroups of the Oriental *melanogaster* species group, although this type of pulse song has been described in distantly related species. For instance, horizontal vibration has been also described in the *obscura* species group, including *D. pseudoobscura*, *D. persimilis*, *D. ambigua*, with small differences in wing movement reported during song production (Ewing and Bennet-Clark, 1968; Noor and Aquadro, 1998). Thus, it is unclear whether horizontal wing vibration is an ancestral or independently evolved character in the *melanogaster* group. As expected from the pattern of courtship behavior, *D. eugracilis*, *D. ficusphlia*, and *D. gunungcola* males do not produce courtship songs. But also, surprisingly, no sound was found during male courtship in *D. elegans*. These observations suggest that the sine and pulse songs might have been lost in the *elegans*, *eugracilis*, and *ficusphila* species subgroups. Unlike the courtship behavior, the qualitative characteristics of

courtship song have no obvious association with the wing pigmentation (Fig. 3.17).

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Table 3.1. List of species included in this study

Species subgroup (#)	Species ($)	Traits
ananassae subgroup (23)	*D. ananassae*	CB*, WP
elegans subgroup (5)	*D. elegans* (6)	CB^, CS^, WP
	D. gunungcola (13)	CB^, CS^, WP
eugracilis subgroup (1)	*D. eugracilis* (12)	CB^, CS^, WP
ficusphila subgroup (6)	*D. ficusphila* (5)	CB^, CS^, WP
melanogaster subgroup (9)	*D. erecta*	CS*, WP
	D. mauritiana	CB*, CS*, WP
	D. melanogaster	CB*, CS*, WP
	D. orena	CB*, CS*,WP
	D. santomea	CB*, CS*, WP
	D. sechellia	CB*, CS*, WP
	D. teissieri	CB*, CS*, WP
	D. simulans	CB*, CS*, WP
	D. yakuba	CB*, CS*, WP
montium subgroup (88)	*D. auraria*	CB*, WP
	D. montium	CB*, WP
	D. rufa	CB*, WP
rhopoloa subgroup (5)	*D. fuyamaii* (16)	CB^, CS^, WP
suzukii subgroup (16)	*D. biarmipes* (15)	CB*^, CS^, WP
	D. mimetic (9)	CB^, CS^, WP
	D. pulchrella (11)	CB*^, CS^, WP
	D. suzukii	CB*, WP
takahashii subgroup (13)	*D. lutescens* (12)	CB^, CS^, WP
	D. nepalensis (0)	CB^, CS^, WP
	D. paralutea (6)	CB^, CS^, WP
	D. prostipennis (3)	CB^, CS^, WP
	D. pseudotakahashii (16)	CB^, CS^, WP
	D. takahashii (3)	CB*^, CS^, WP

The number of species in the species subgroup, according to Ashburner *et al.* (2005)

$ The number of individuals in the species was videotaped for courtship behavior analysis.

CB=male courtship behavior; CS=courtship song; WP=wing pigmentation

* based on literature references; ^ newly described.

Table 3.2. List of courtship elements performed by *Drosophila* males.

Courtship elements	**Description**	**References**
Orienting	Male turns to toward another individual.	Spieth,1952
Tapping	Male lifts and straightens foreleg(s)s, then strikes legs(s) downward at another individual.	Spieth,1952, 1974
Licking	Male opens labellar lobes, extends proboscis, and licks female genitalia. Male may lick either intermittently and repeatedly or continuously for a prolonged period.	Spieth, 1952, 1974
Mounting	Male bends the tip of abdomen downward and forward, rears upward, thrusts head under female's wings or between her spread wings, grasps her body with his fore- and midlegs, and attempts intromission.	Spieth, 1974
Circling	Male faces female and moves around the female. Various types of wing movement may be performed during circling. There are three types of circling: full, semi, and arch. In full circling, male moves around female for 360˚ without stopping. In semi circling, male moves from the rear to the front of female, stops in the front, and moves back to the rear from either side of female. In arch circling, the male moves from one side of female to the other side resulting in an arch-like path. Angle of arch various from 90˚ to 180˚, depending on species.	Spieth, 1974; Cobb *et al.*, 1985; Refined definitions in this study
One wing vibration	Male extends one wing outward and moves it up and down rapidly. Degree of extension, amplitude, and speed of vibration vary among species. This action may be exhibited as the male is in the rear of a female, as the male attempts to grasp the female's abdomen, as the male faces one compound eye of the female, or as the male is copulating.	Spieth, 1952, 1974
One wing horizontal vibration	Male extends one wing outward and backward to the resting position repeatedly. Unlike wing flicking, this horizontal vibration is usually slow and may be rhythmic.	Reported in this study

Table 3.2 (continued)

Courtship elements	Description	References
Wing waving	Male extends one wing and then slowly waves it up and down. (This is like a slow motion version of the one wing vibration.)	Spieth, 1974
Wing flicking	Male flicks one wing sharply out, then back to resting position.	Spieth, 1974
Wing semaphoring	Male alternately and repeatedly flicks wings sharply outward, then back to resting position. Typically, one wing is moved outward while the other is returning to the resting position.	Spieth, 1974
Wing scissoring	Male repeatedly and rapidly extends both wings horizontally outward and back to the resting position.	Spieth, 1974
Wing rowing	Male extends out one wing or two wings simultaneously, lifts up the wing(s), and drop wing(s) back to the resting position. The wing extending angle and lifting amplitude vary among species.	Spieth, 1974; Cobb *et al.*, 1985
Wing shuffling	Male extends one wing out while circling from one side to the other side of female. Male holds the wing out when he reaches the other side and stops there, then he returns the wing to back to the resting position and then circles back to the starting side of the female with the other wing extended.	Reported in this study
Two wing vibration	Male extends both wings outward then moves them up and down. Degree of extension, amplitude, and speed of vibration vary among species. This action is usually observed when male stands in front of female and faces her.	Reported in this study
Two wing extension	Male extends both wings out and holds them in a straight line conformation. The ventral side of wings is usually turned toward to the female. Body shaking may occur during two wing extension.	Reported in this study

Table 3.2 (continued)

Courtship elements	**Description**	**References**
Wing fluttering	Male repeatedly extends both wings outward and back to resting position, increasing the angle with each new outward movement until a given angle is reached.	Reported in this study
Leg shaking	Male raises one or both midlegs and shakes midleg(s) in the front of female's eyes.	Yamada *et al.*, 2002
Head butting	Male rams the female head with his head.	Reported in this study
Arch circling	Male faces to the female, moves from one lateral side of the female to the other side, then may move back to the starting position and repeate several times	Defined in this study
Semi circling	Male faces to the female, moves from the rear to the front of the female, stops at the front, then moves back from either side of the female to the rear of the female.	Defined in this study
Full circling	Male faces to the female, moves around the female without stoping in the front of the female.	Defined in this study

Table 3.3. Temperatures at which courtship songs were recorded.

Species (strain)	Min. Temp.[1]	Max. Temp.[2]	Mean Temp. ±SD[3]
D. biarmipes (361.00)	21.4	25.9	23.69 ±1.80
D. biarmipes (361.02)	21.8	25.0	23.18±1.53
D. biarmipes (361.03)	21.5	22.3	21.87±0.31
D. fuyamai	22.1	23.5	22.94±0.62
D. lutescens (271.00)	22.4	23.1	22.66±0.26
D. lutescens (271.01)	21.1	22.0	21.60±0.35
D. mimetica	20.5	22.9	21.77±1.07
D. nepalensis (48)	21.0	23.5	22.47±1.01
D. nepalensis (56)	23.1	23.5	23.29±0.16
D. protispennis	20.8	23.7	22.32±1.31
D. pulchrella	22.5	23.4	23.08±0.31
D. takashii	22.5	23.4	22.84±0.23

[1] Lowest temperature recorded.
[2] Highest temperature recorded.
[3] Mean and standard deviation of individual mid temperatures, which is the mean of the beginning and end temperatures of each recording trial.

Table 3.4. Summary of male courtship behavior.

Species	Courtship elements						
	Taping	Licking	One-wing vibration[1]	One-wing rowing	Circling[2]	Frontal wing display[3]	Others[4]
***ananassae* subgroup**							
D. ananassae[5]	yes	no	no	no	f-ws	no	leg shaking
D. pallidosa[5]	yes	no	r	no	f	no	leg shaking
***montium* subgroup**							
D. montium	yes	no	r, v	no	f-2wl	no	-
D. auraria[6]	yes	no	r	no	no	no	-
***melanogaster* subgroup**							
D. melanogaster	yes	yes	fl, r, v	no	s	ws	-
D. simulans	yes	yes	fl, r, v	f	s, a	ws, 2wr	-
D. mauritiana	yes	yes	fl, r, v	no	s, a	ws, 2wr	-
D. sechellia	yes	yes	fl, r, v	no	s	ws, 2wr	-
D. yakuba	yes	yes	fl, r, v	no	s	ws, 2wr	-
D. santomea	yes	yes	fl, r, v	no	s	ws, 2wr	-
D. teissieri	yes	yes	fl, r, v	no	s, a	ws, 1wr, 2wr	-
D. erecta	yes	yes	fl, r, v	no	s	no	ab, ws in rear
D. orena	yes	yes	fl, r, v	no	s	no	ws in rear

Table 3.4. (continued)

Species	Courtship elements						
	Taping	Licking	One-wing vibration[1]	One-wing rowing	Circling[2]	Frontal wing display[3]	Others[4]
elegans subgroup							
D. elegans	yes	no	no	no	s-1we	2we-bs	hh
D. gunungcola	yes	yes	no	no	no	no	-
eugracilis subgroup							
D. eugracilis	no	no	no	r	s	2wr	hh, head butting
ficusphila subgroup							
D. ficusphila	no	no	no	no	no	no	direct mounting
rhopaloa subgroup							
D. fuyamai	yes	no	r , v	no	s	2we-bs, 2wr	hh
suzukii subgroup							
D. biarmipes	yes	no	r,v,h	no	s-1we	2we	hh, ab
D. pulchrella	yes	no	r,v	no	?	wf	ab
takahashii subgroup							
D. lutescens	yes	no	r,v	no	s-2we	2wv	hh
D. mimetica	yes	no	r,v	f	s, a	wsf	-
D. nepalensis	yes	no	r,v	no	s-1we	2wv	hh
D. paralutea	yes	no	r,v	no	s-1we, a	2wr, wsf, 1we	-

Species	Courtship elements						
	Taping	Licking	One-wing vibration[1]	One-wing rowing	Circling[2]	Frontal wing display[3]	Others[4]
takahashii subgroup							
D. prostipennis	yes	no	r,v,h	no	s-1we	2we	hh
D. pseudotakahashii	yes	no	r,v,h	f	s-1we	1wr,2wr,wf, 2we-bs	hh
D. takahashii	yes	no	r,v	no	s	2wr	-

[1] fl- displayed to the side of the female; r- displayed to the rear of the female; v-up and down movement; h-horizontal back and forth movement.

[2] f- full circling; s- semi circling; a- arch circling; ws- wing scissoring; 2wl- two wing lifting; lwe-one wing extension; 2we-two wing extension.

[3] ws- wing scissoring; 1wr-one wing rowing; 2wr- two wing rowing, 2we- two wing extension, bs- body shaking, wf-wing fluttering, 2wv- two wing vibration, wsf-wing shuffling

Dashes (-) are used to connect two courtship elements if the courtship elements are exhibited simultaneously.

[4] ab- abdomen bobbing, hh- head to head performance, male stands in front of female face to face (thus, bodies of male and female form a straight line) to exhibit wing display.

[5] *D. ananassae* and *D. pallidosa* males also perform leg shaking during courtship (Yamada *et al.*, 2002).

[6] *D. auraria* males also perform one wing vibration during copulation.

Table 3.5. List of courtship song types in the Oriental *D. melanogaster* species group.

Species	Sound	Sine	Pulse[1]	Special feature[2]	Ref. [3]
the *ananassae* subgroup					
D. ananassae	yes	no	v, poly	-	3,6
D. pallidosa	yes	no	v, bi	-	6
the *monitum* subgroup					
D. auraria	yes	no	v, bi	-	4
the *melanogaster* subgroup					
D. melanogaster	yes	yes	v, mono	ws	2, 3, -
D. simulans	yes	yes	v, mono	ws	2,-
D. mauritiana	yes	yes	v, mono	ws	2,-
D. sechellia	yes	no	v, mono	ws(?)	1
D. yakuba	yes	no	v, mono	ws	2, 7, -
D. santomea	yes	no	v, bi,	ws, (clack, poly)	5, -
D. teissieri	yes	yes	v, poly	ws(?), complex	2,-
D. erecta	yes	yes	v, bi	-	2,-
D. orena	yes	yes	v, poly	-	1
the *elegans* subgroup					
D. elegans	no	-	-	-	-
D. gunungcola	no	-	-	-	-
the eugracilis subgroup					
D. eugracilis	yes	no	rowing, poly	sparse	-
the *ficusphila* subgroup					
D. ficusphila	no	-	-	-	-
the *rhopaloa* subgroup					
D. fuyamai	yes	yes	v, poly	grouting	-

Table 3.5. (continued)

Species	Sound	Sine	Pulse[1]	Special feature[2]	Ref. [3]
the *suzukii* subgroup					
D. biarmipes	yes	yes	h, poly	tootie	-
D. pulchrella	yes	no	h, poly	tootie; abdomen bobing	-
the *takahashii* subgroup					
D. lutescens	yes	no	v, poly	frontal pulse, poly	-
D. mimetica	yes	yes	v, poly	frontal pulse?	-
D. nepalensis	yes	yes	h and v, poly	-	-
D. prostipennis	yes	no	v, poly	turbo	-
D. takahashii	yes	yes	poly and mono	-	-

1 v - pulses produced by up-and-down movement; h – pulses produced by back-and-forth from the resting position; mono – a pulse consists single cycle; bi – a pulse consists two cycles; poly – a pulse consists three or more cycles.
2 ws – pulses produced by wing scissoring in the rear side of the female. complex – the sine wave is a synthesis of two frequency of waves, the train of pulses is a synthesis of two type of pulses with the major pulses fall in the IPI of minor pulses.
3 Reference: 1. Cobb *et al.*, 1989. 2. Cowling and Burnet, 1981. 3. Ewing and Bennet-Clark, 1968. 4. Tomaru and Oguma, 1994. 5. Watson *et al.*, 2007. 6. Yamada *et al.*, 2002. 7. Demetriades *et al.*, 1999. -. this study.

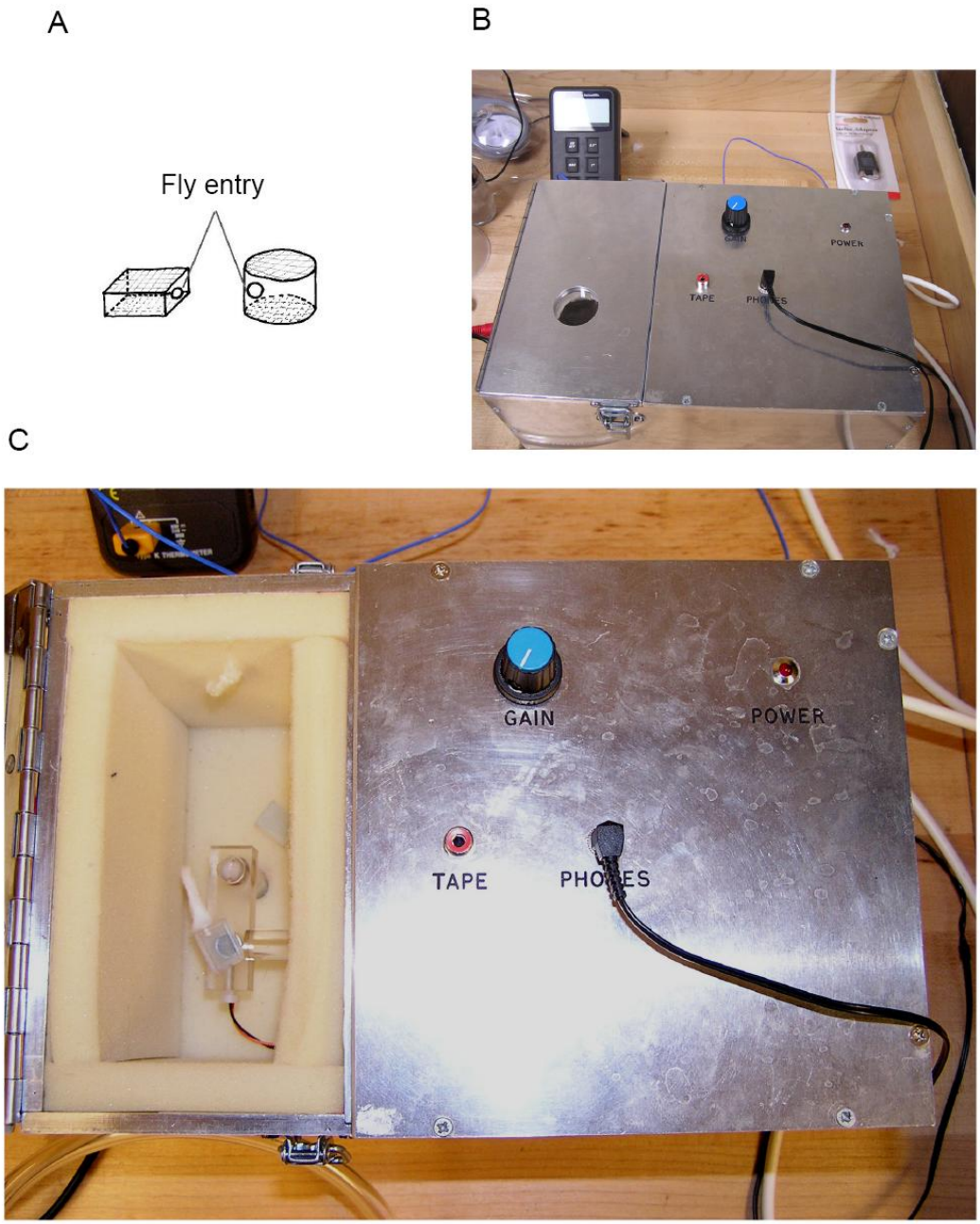


Figure 3.1. Apparatus for recording courtship songs. A. Fly recording cage. B. Recording chamber. C. Inside of view of recording chamber.

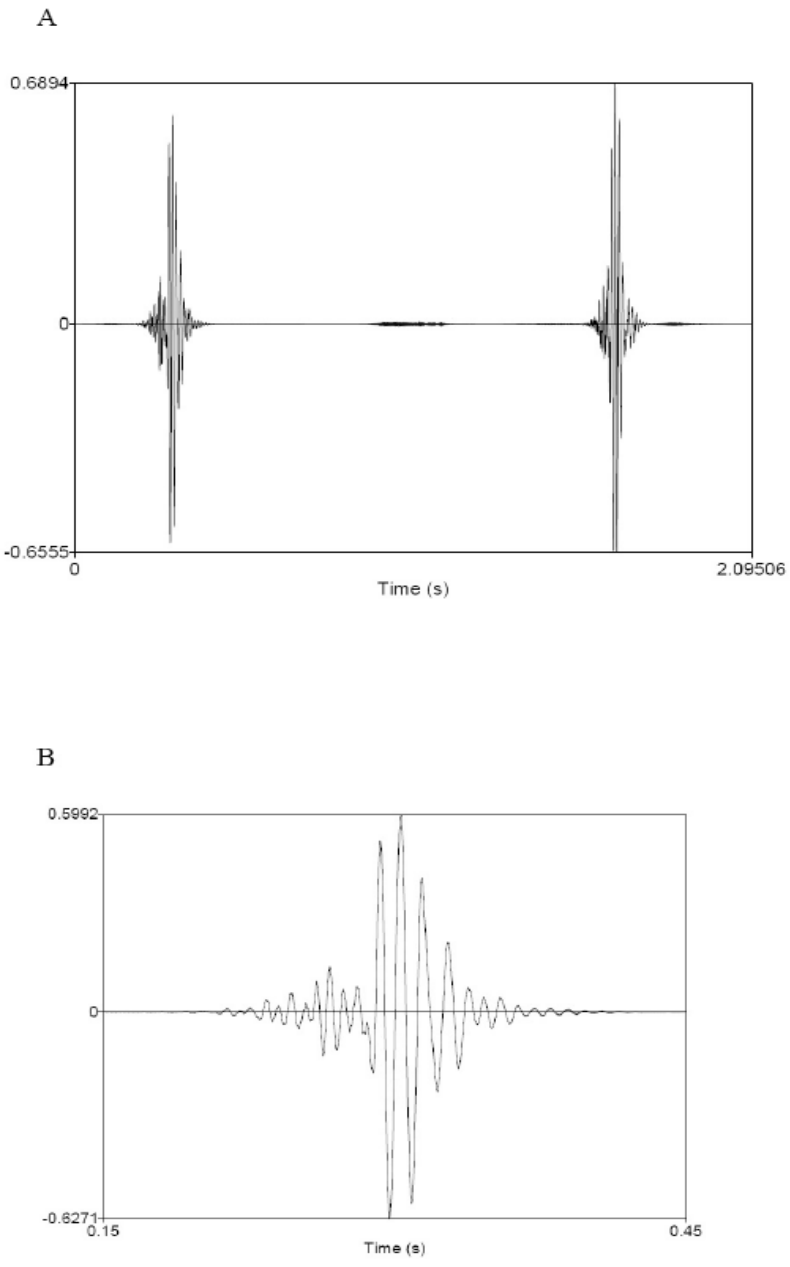


Figure 3.2. The polycylic pulses produced by *D. eugracilis* male. A. two constitutive pulse, B. the waveform of a pulse.

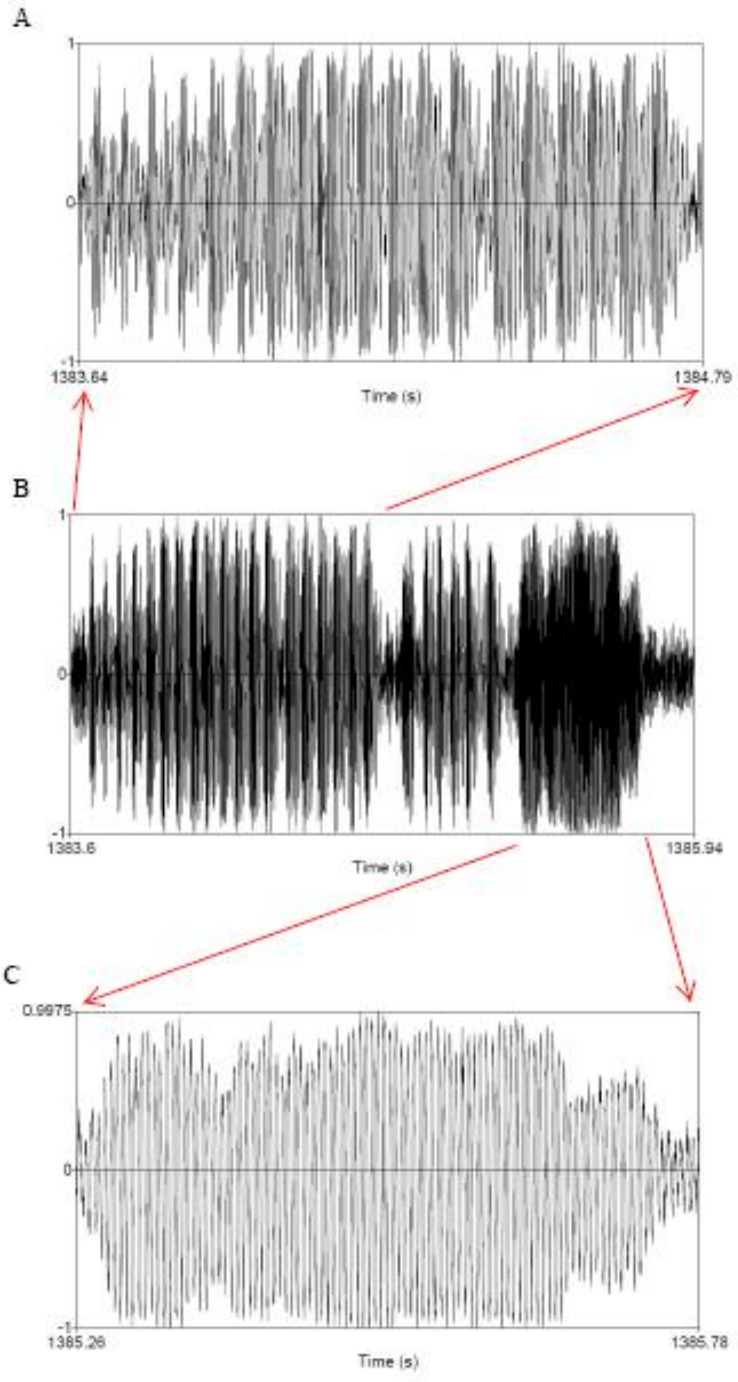


Figure 3.3. A burst of pulse and sine songs in *D. fuyamai*. A. Polycyclic waves of pulses. B. Entire burst. C. Sine song.

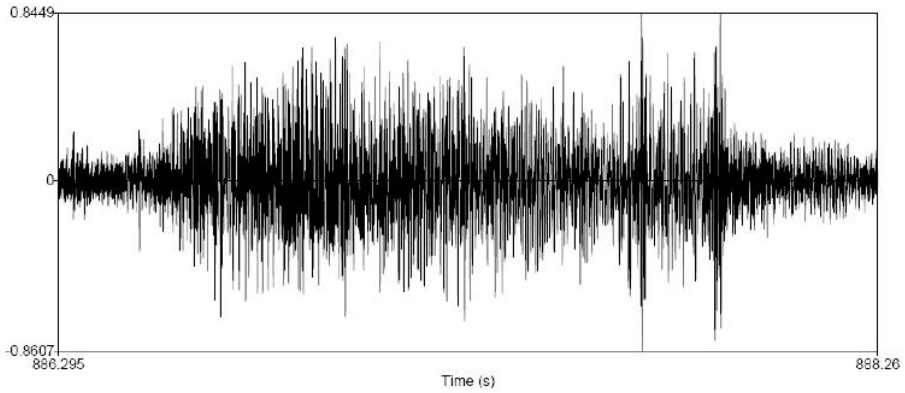
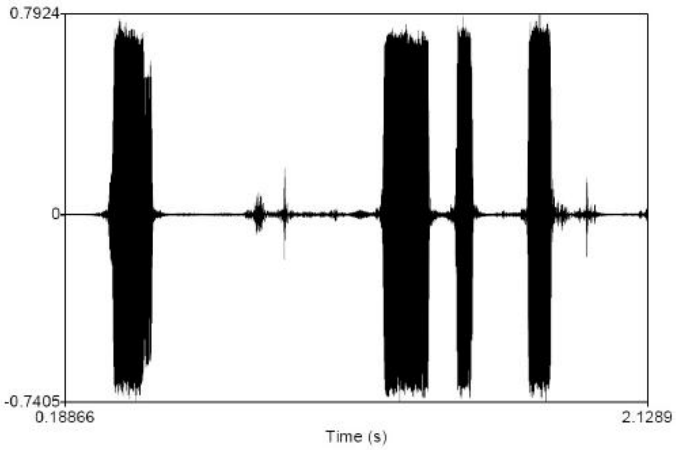


Figure 3.4. The grouting sound of *D. fuyamai*.

A



B

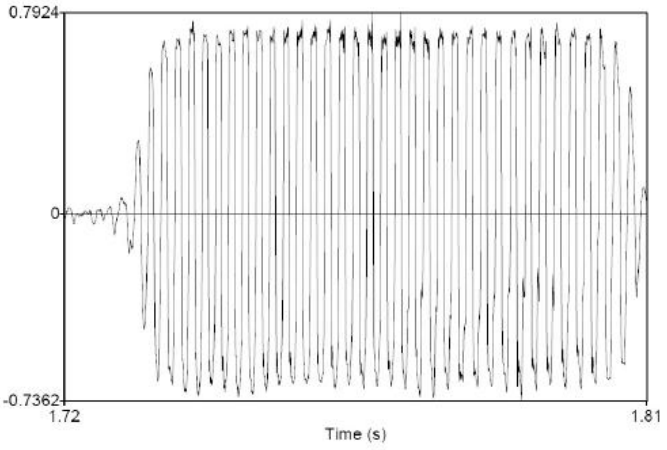
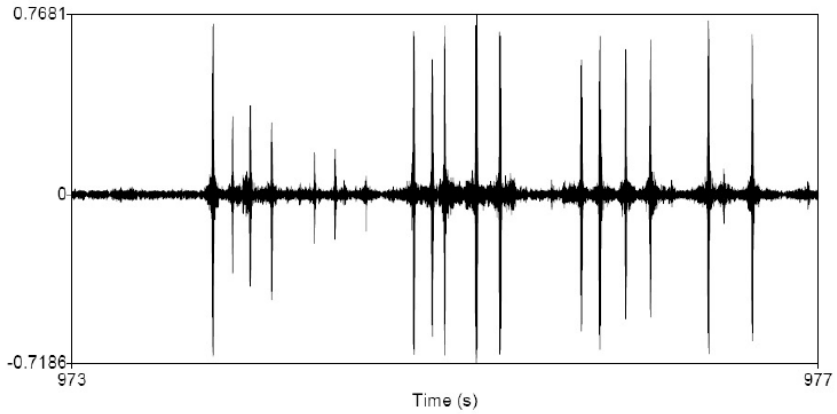


Figure 3.5. The toot element in *D. biarmipes*. A. A group of toots. B. A typical toot.

A



B

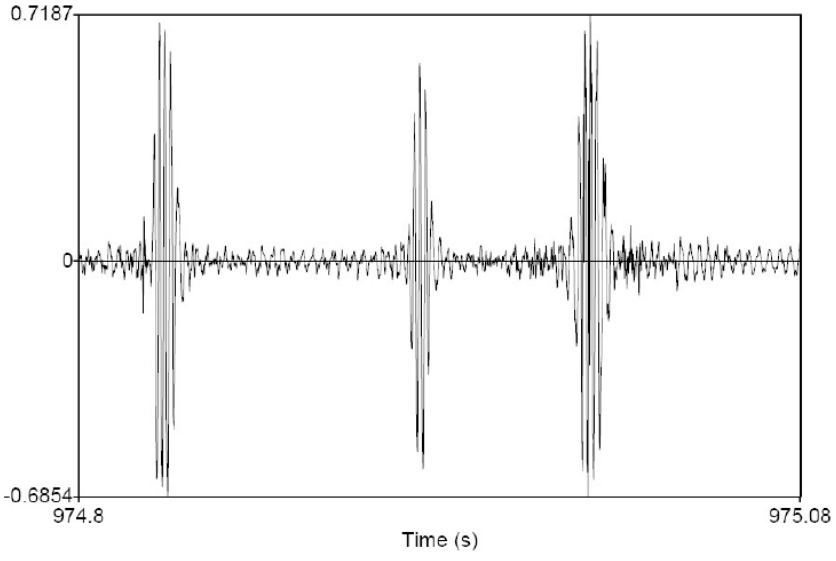


Figure 3.6. Pulse song in *D. biarmipes*. A. A typical chain of pulses. B. Polycyclic pulse.

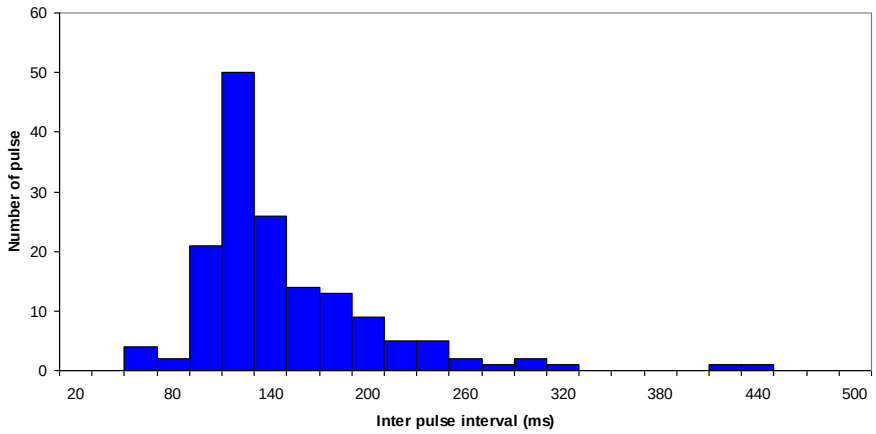
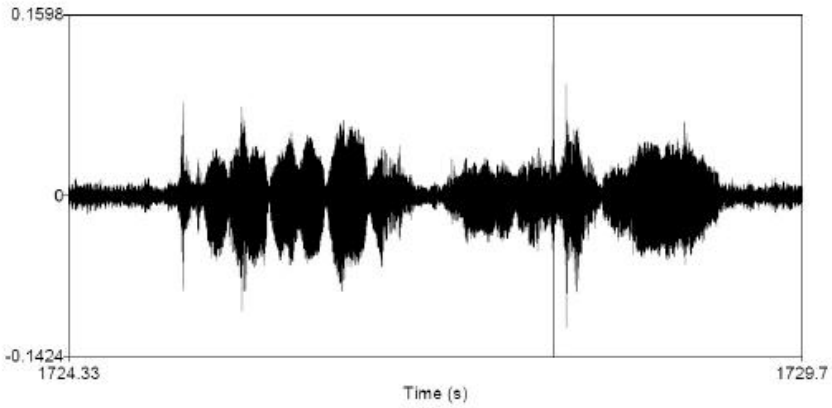


Figure 3.7. Histogram of inter pulse intervals in *D. biarmipes*.

A



B

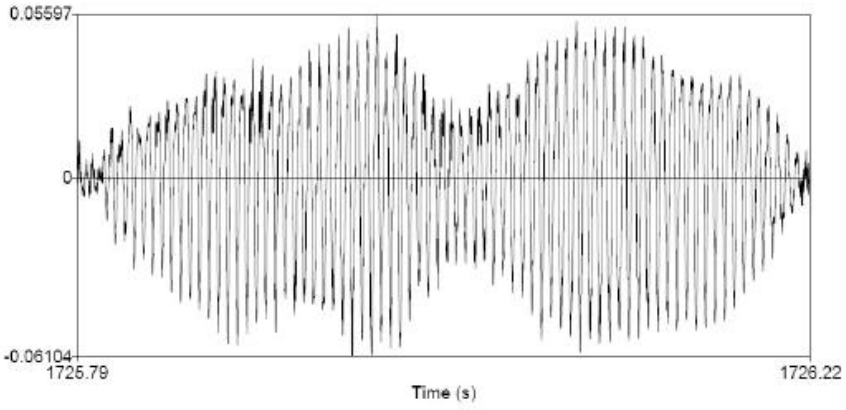


Figure 3.8. Sine song produced during two wing extensions in *D. biarmipes*. A. A burst of sine song. B. Part of the burst.

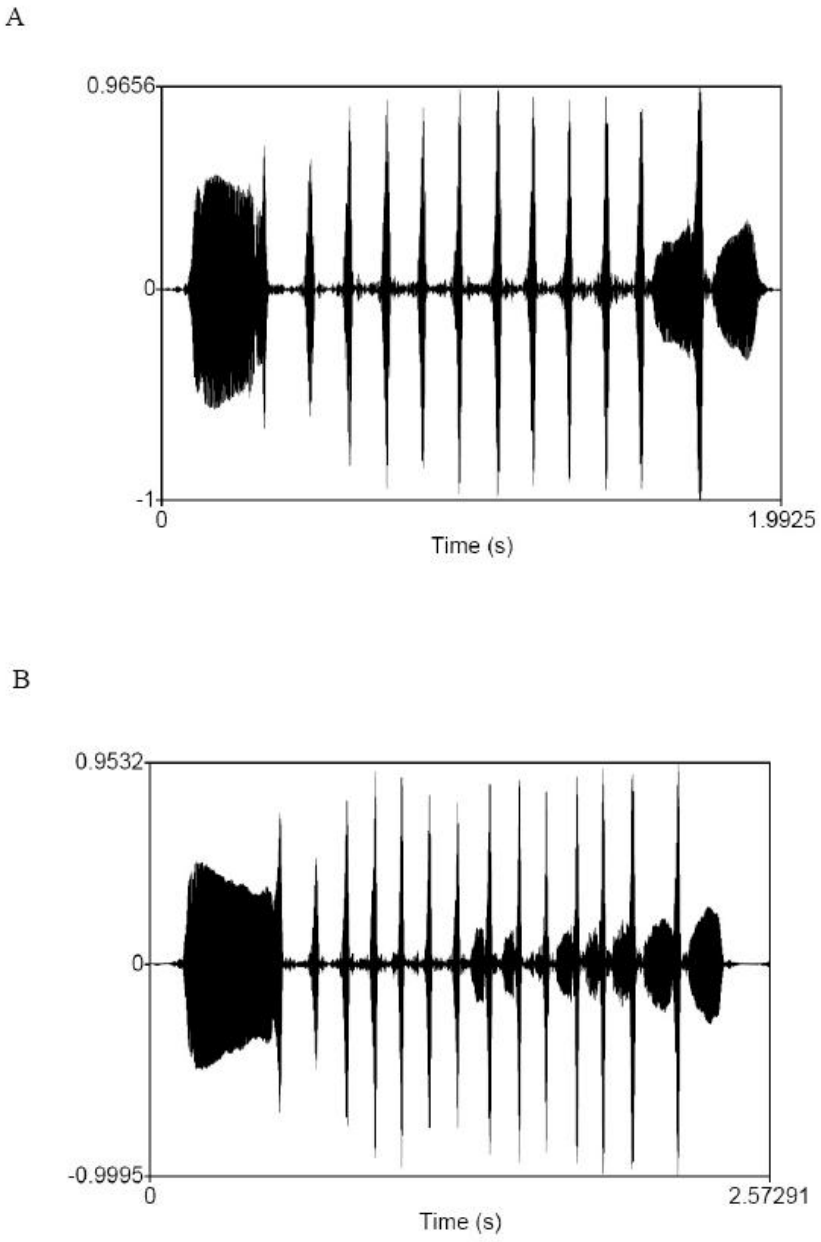


Figure 3.9. Two examples of courtship songs in *D. pulchrella*. Toots are usually followed by a chain of pulses. Toot-like sound waves with lower amplitude are sometimes produced between pulses.

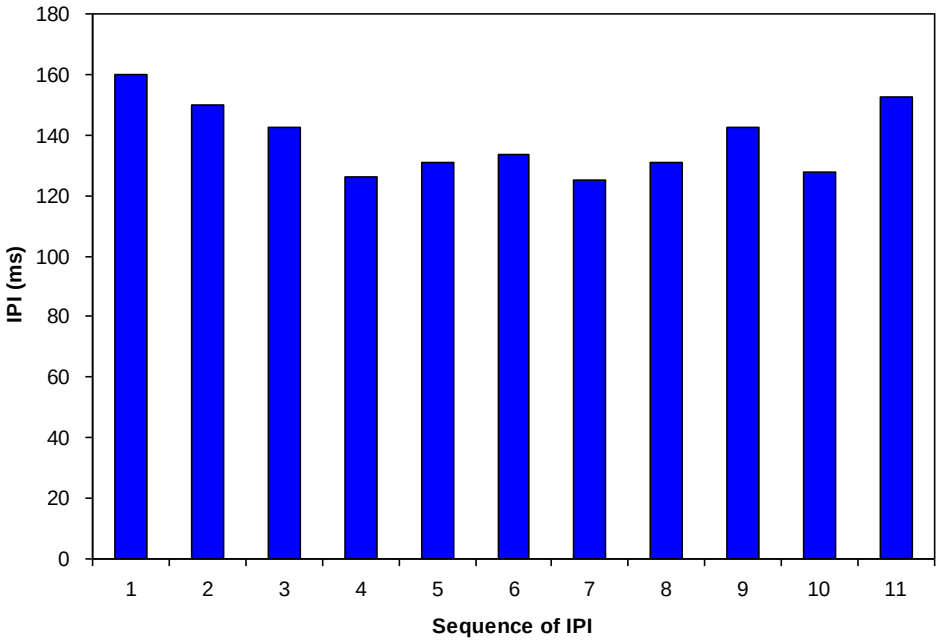


Figure 3.10. An example of IPIs during a chain of pulses in *D. pulchrella*.

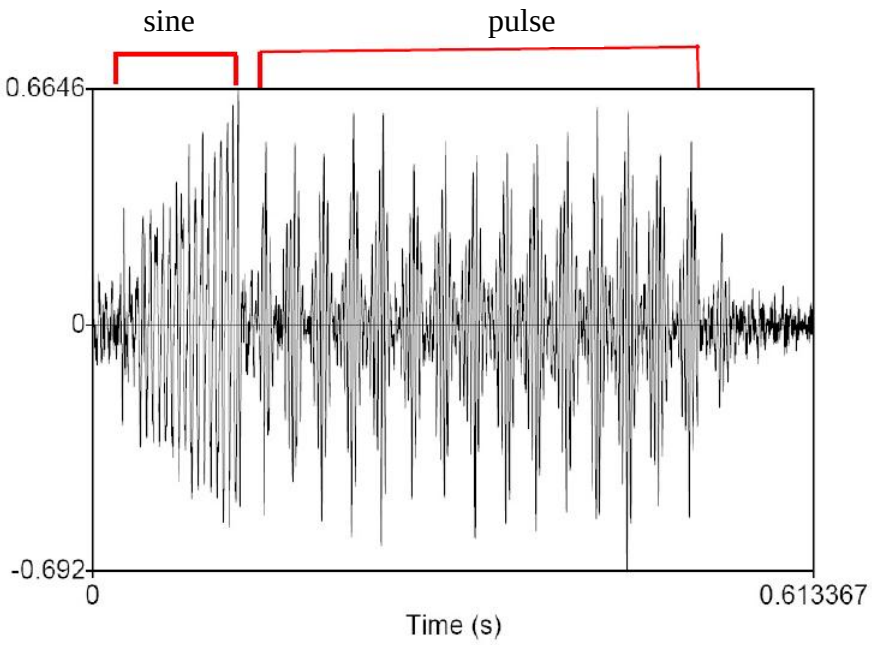


Figure 3.11. Sine and pulse songs in *D. mimetica.*

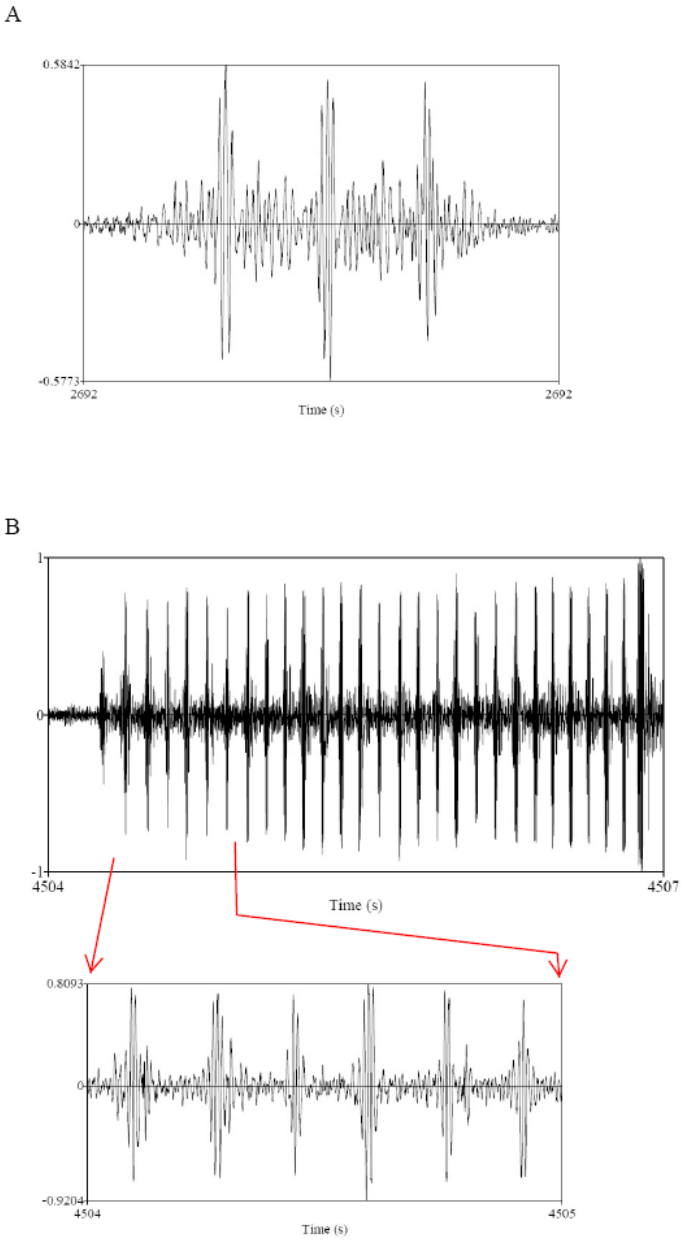


Figure 3.12. Courtship songs in *D. lutescens*. A. Frontal pulse produced by two-wing vibration. B. A chain of pulses produced by one-wing vibration.

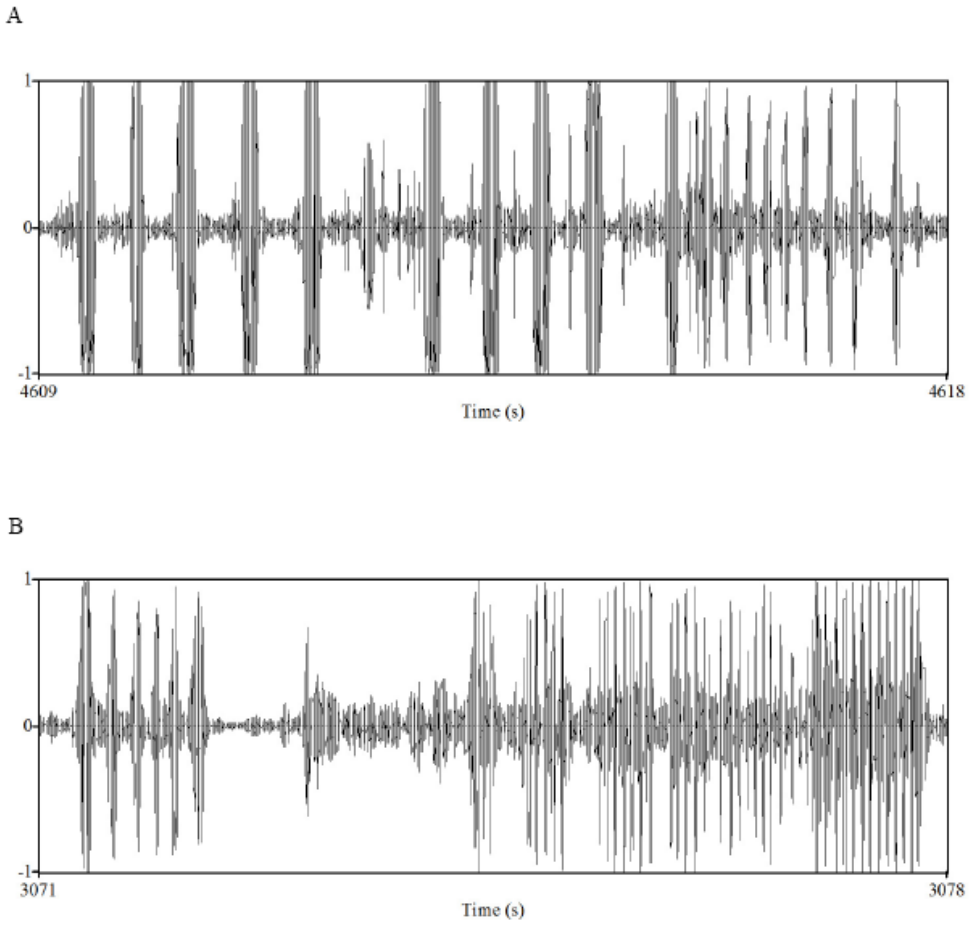


Figure 3.13. Courtship songs in *D. nepalensis*. A. A series of sine-like songs followed by horizontal vibration. B. A chain of horizontal vibration followed by vertical vibration.

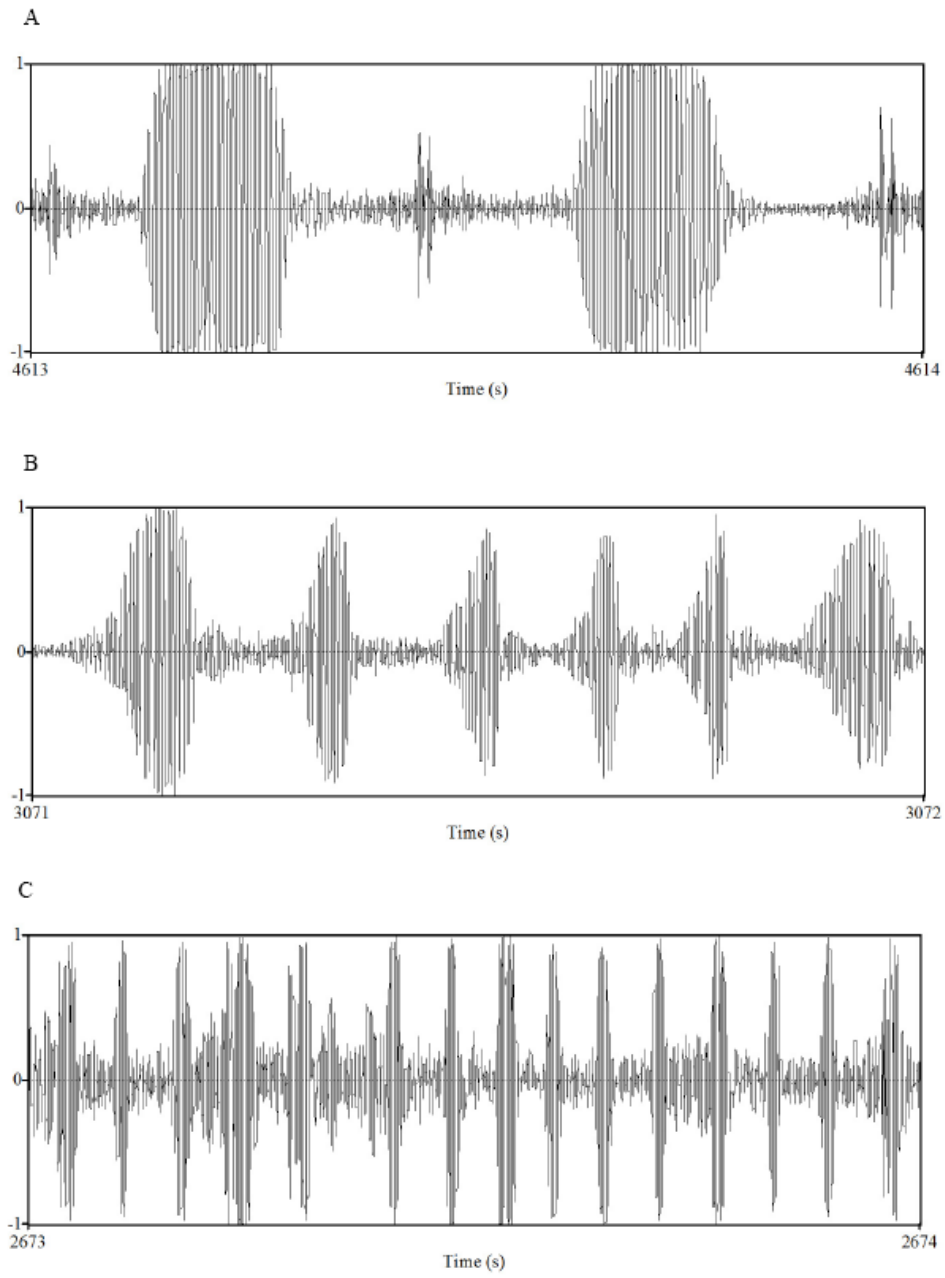


Figure 3.14. Waves of courtship songs in *D. nepalensis*. A. sine-like songs. B. horizontal vibration. C. vertical vibration.

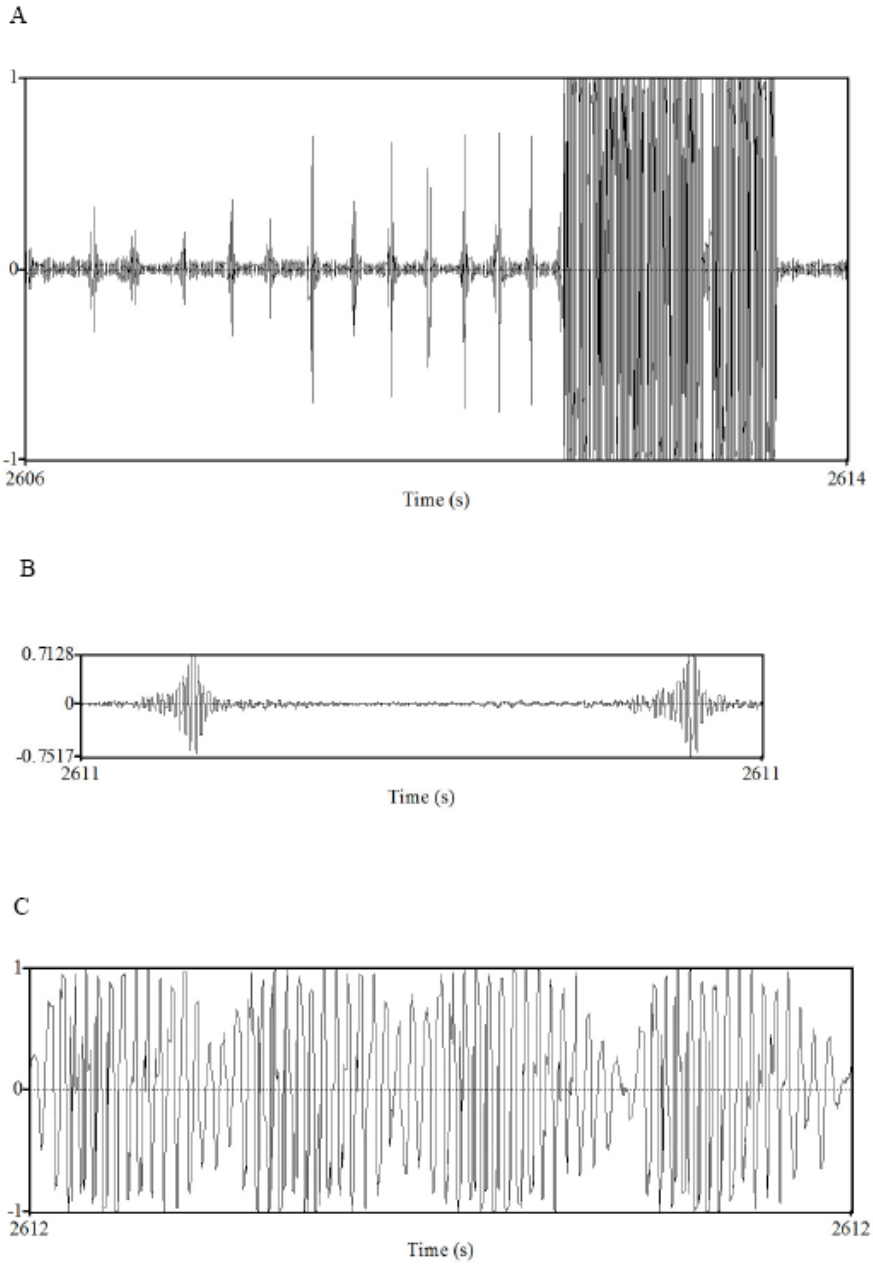


Figure 3.15. Courtship songs in *D. prostipennis*. A. A chain of pulses followed by a turbo. B. A pulse. C. Part of a turbo. Note the amplitudes of waves changing in cycles.

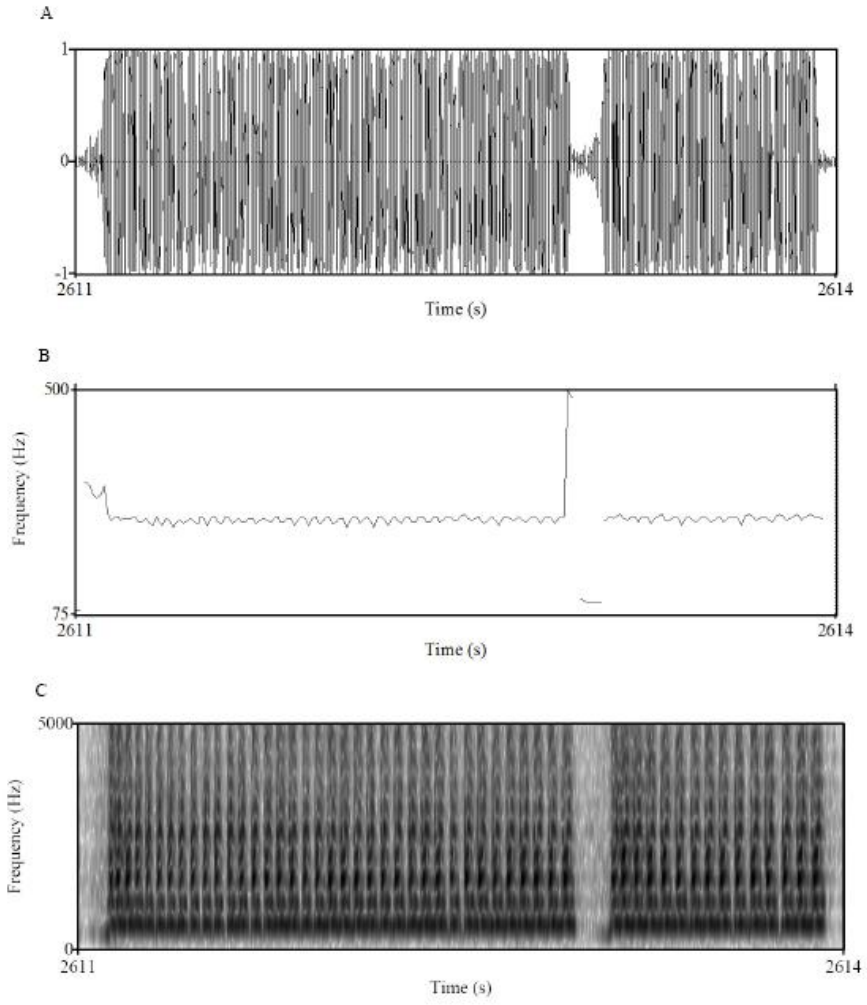


Figure 3.16. The features of a turbo in *D. prostipennis*. A. wave. B. pitch. C. spectrum.

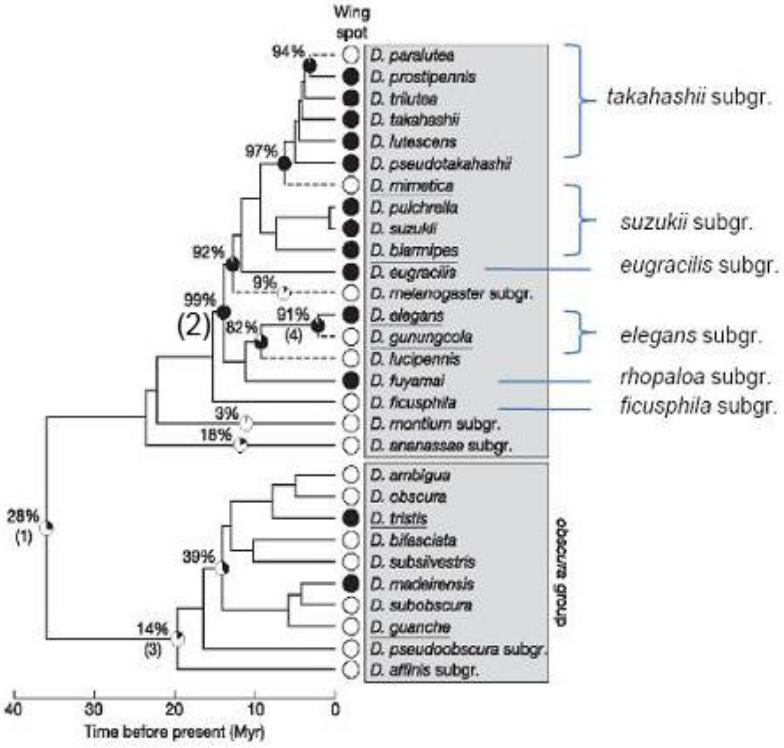


Figure 3.17. Phylogenetic tree of the *D. melanogaster* species group (modified from Prud'homme *et al.*, 2006). The *D. obscura* species group is the closest relative to this radiation, inferred from the studies of Lewis *et al.*(2005) and De Lage *et al.*(2007). Filled circles indicate the presence and open circles indicate the absence of male wing spots. In the pie charts on the key nodes, the black portion and the percentages indicate the posterior probability of the presence of male wing spots in that ancestor. Dotted branches indicate inferred losses of the wing spot. *D. nepalensis* was not included in this phylogeny, but presumably this species should fall into the *takahashii* subgroup, based on the morphological and behavioral data (Okada, 1955; Dwivedi, 1982; Parkash *et al.*, 1994).

IV. Genetics of divergence in male wing pigmentation and courtship behavior between *Drosophila elegans* and *D. gunungcola*.

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ABSTRACT

Many sex-specific traits involved in mating consist of functionally coordinated morphologies and behaviors. How the components of these complex traits evolve and become coordinated during evolution is unknown. In order to understand how such trait complexes evolve and diversify, we must decipher the genetic underpinnings of their components. In this study, we begin to elucidate the genetic architecture underlying differences in functionally related male pigmentation and behavior between two Asian *Drosophila melanogaster* group species, *D. elegans* and *D. gunungcola*. *D. elegans* possesses a male-specific wing melanin spot and a stereotypical wing display element in male courtship, whereas *D. gunungcola* lacks both of these traits. Using reciprocal F1 male hybrids, we demonstrate that the X chromosome contains a major locus or loci required for wing spot formation and that autosomal loci largely determine the male courtship display. Using phenotypic and genetic analysis of backcross progeny, we further demonstrate that both the wing spot and courtship differences between the two species are polygenic and both depend at least in small part on genetic factors on both the X and the autosomes. Finally, we find that male wing spot size and courtship wing display are highly correlated in backcross progeny, suggesting that linkage or pleiotropy may have been involved in their coordinate evolution.

INTRODUCTION

In animals, many obvious species differences consist of complex combinations of male-specific ornamentation and behavioral characters presumed to have evolved via sexual selection (Andersson, 1994). These traits are often proposed to be involved in sexual isolation during speciation (Lande, 1981; West-Eberhard, 1983; Kaneshiro and Boake, 1997; Higashi *et al.*, 1999; Kaneshiro, 2000; Panhuis *et al.*, 2001). Selection promoting combinations of visible traits and behavior presumably requires both components to be present. For example, both transverse wing bands and wing flicking behavior are required for *Z. vittigera* flies to escape from the predation of salticid spiders (Greene *et al*., 1987; Whitman *et al*., 1988). Therefore, the traits are expected to evolve in concert (e.g. Prum 1990, but see Wiens, 2000 for a counter example).

Little is known about the genetic mechanisms by which natural and sexual selection construct such functionally related trait combinations. Are independent sets of genes involved in each component trait? Are the genes involved in separate components, for example a novel behavior and a novel morphology, linked in the genome and is this linkage important for the long-term evolution of the adaptation? Do some genes play roles in multiple traits? Geneticists have recently begun to address these questions. For example, Hawthorne and Via (2001) used quantitative trait locus (QTL) mapping to examine the architecture of genetic correlations between a behavioral trait, host choice, and a physiological trait, performance on the host, in the aphid *Acyrthosiphon pisum*. They found that the QTL effects underlying these two distinct traits were not distributed randomly in the genome. QTLs tended to be clustered, with each cluster containing QTL

effects consistent with adaptation to one of the two hosts. More empirical studies are needed in order to determine whether such functionally related QTL clusters, which represent linked or pleiotropic loci, are a general feature of adaptive evolution.

In this study, we begin to decipher the genetic architecture of coordinately evolving male pigmentation and behavioral traits in the sibling species pair *Drosophila elegans* and *D. gunungcola*, which are members of the *elegans* subgroup of the *melanogaster* species group (Hirai and Kimura, 1997; Kimura and Hirai, 2001). *D. elegans* is distributed throughout Southeast Asia, including Malaysia, Philippines, Hong Kong, Taiwan, and Okinawa. *D. gunungcola* is found in Indonesia and its range overlaps at least partially with *D. elegans* (Hirai and Kimura, 1997; Sultana *et al.*, 1999; Ishii *et al*., 2002; Suwito *et al*., 2002). *D. elegans* and *D. gunungcola* court each other in the laboratory and interspecific courtships have been reported in the field (Ishii *et al.*, 2002). However, interspecific copulation or production of hybrids has not been reported.

The Oriental *melanogaster* group species vary in a number of male secondary sexual traits (Kopp and True, 2002a), the most obvious of which is the presence of a male-specific wing melanin spot, which takes on various shapes and intensities in several Oriental species (Fig. 1A-C). The Oriental species also differ strikingly in male courtship. All of these species exhibit standard elements of *Drosophila* male courtship, including orientation and following, wing extension, and licking (Greenspan and Ferveur, 2000). However, species with male-specific wing spots, including *D. elegans*, exhibit a qualitative difference in courtship in comparison to non-spot-bearing species. In the spot-bearing species, the male exhibits a conspicuous wing display behavior in which he circles to the front of the female and faces her, then repeatedly waves his wings and shakes his body. *D. elegans* males may also extend their wings while competing with other

males for territories on flowers (Kimura and Hirai, 2001). Species lacking wing spots, including *D. gunungcola*, may face the females during courtship, but they do not perform the conspicuous wing display of the spot-bearing species. The strong association of wing spots with wing displays in this species group (Fig. 1A) suggests that the two traits are maintained for a joint function, and that sexual selection involving female choice has been important in maintaining these two traits. The sibling species pair *D. elegans* and *D. gunungcola* provide an excellent opportunity to study the genetics of pigmentation and courtship evolution because they are the interfertile pair of species that differ qualitatively in the presence of male wing spots and wing displays. Here, we present an initial analysis of the genetic basis of male wing spot and courtship divergence between *D. elegans* and *D. gunungcola*, using reciprocal F1 crosses and backcrosses with molecular marker genotyping.

MATERIALS AND METHODS

***Drosophila* strains and cultures:** *D. elegans* HK (collected in Hong Kong), a brown morph strain and *D. gunungcola* SK (collected in Sukarami, Indonesia), were kindly provided by Dr. Masahito T. Kimura. Cultures were kept on standard fly food (corn meal, yeast extract, and agar) with folded paper inserted into the media for pupation.

***Drosophila* crosses:** For all crosses, virgin females and males were collected within six hours after eclosion and housed separately in food vials for three to five days. For hybrid crosses, five to ten virgin females from one species and five to ten virgin males from the other species were put in a vial with food and transferred into a new vial every week for several weeks until all the females were dead. After eclosion, hybrid progeny were sorted and stored in separate vials by

sex for three to five days. The F1 hybrid females were then backcrossed to either parental species males while the F1 hybrid males and backcross progeny were either preserved in 70% ethanol or frozen after their courtship behavior was observed.

Genotyping methods: Genomic DNA of male backcross progeny was isolated by grinding individual flies in 0.2 mg/ml proteinase K in homogenization buffer (50mM Tris-HCl pH7.5, 20 mM EDTA, 0.5% SDS) followed by incubation at 55^{o}C for 2 hr. NaCl was then added to 400mM final concentration and preps were centrifuged at 14K rpm for 10 min. DNA in supernatant was then ethanol-precipitated. Pellets were air dried and resuspended in 50 μl 10mM Tris-HCl pH 8.5. Preps were diluted 100-fold and 1-10 μl of dilution was used as template in each PCR reaction.

The primers used to amplify target loci were designed in conserved regions determined by comparison of the *D. melanogaster*, *D. simulans*, *D. marutiana*, *D. yakuba*, and *D. pseudoobscura* genomes. More specific primers for genotype diagnosis in male backcross progeny were designed based on DNA sequences of *D. elegans* and *D. gunungcola* (see Table 1). Initially, primers for five genes, *yellow* (*y*), *aristaless* (*al*), *Ecdysone Receptor* (*EcR*), *aracaun* (*ara*), and *ebony* (*e*), were developed, providing markers corresponding to each major chromosome arm of *D. melanogaster* (the effect of tiny fourth chromosome, which constitutes about 2% of the genome, was not studied). These five markers were all unlinked in *D. elegans/D. gunungcola* (data not shown), which is consistent with their positions on the linkage group obtained later (see Chapter V). Two further markers, *Moesin* (*Moe*), which is close to pigmentation candidate gene *tan* on the *D. melanogaster* X chromosome and *Transcription Factor II A-L* (*TfIIA-L*) on the right arm of the *D. melanogaster* third chromosome, close to *ebony*, were also developed. *D. elegans* HK and *D. gunungcola* SK-specific

alleles were defined either by single nucleotide polymorphisms (SNPs), genotyped using restriction enzyme digestion followed by agarose gel electrophoresis, or insertion/deletion differences, genotyped by gel electrophoresis alone. Methods and primers used for each marker locus are summarized in Table 4.1.

The lack of obvious inversion loops in the salivary gland chromosomes of female *D. elegans* HK/*D. gunungcola* SK F1 hybrid larvae (J.R. True, unpublished) indicates that *D. elegans* and *D. gunungcola* are homokaryotypic on a gross scale. Small inversion differences between *D. elegans* and *D. gunungcola*, constituting less than half of a chromosome arm, cannot be ruled out at this time, but such differences, if present, would not affect the conclusions of this study.

Although the cytological map of genes in the *D. elegans* species subgroup is not available, the polytene chromosomal structure of these two species is very similar to *D. melanogaster*, with five major chromosome arms (J.R. True unpublished). In this study we use genetic markers presumed to be homologous to *D. melanogaster* loci. Results are depicted using the approximate physical positions of the *D. melanogaster* homologues, although this has been established only for the X-linked markers, *yellow* and *Moe* (See Chapter 5 for further analysis in linkage group map). *yellow* is allelic to a spontaneous mutant in *D. elegans* that is null for Yellow protein expression in 60-75 hour old pupae and bears light yellow body pigmentation similar to *D. melanogaster yellow* (J.R.T and S.-D. Y. unpublished data). Chromosome arm assignments of the autosomal markers are tentative, but because they are all unlinked, their coverage of the *D. elegans* and *D. gunungcola* genomes is approximately the same as one marker per major chromosome arm.

Behavioral and pigmentation assessment in male F1 and backcross progeny: Male backcross progeny were obtained by backcrossing the F1 hybrid females to

males of either parental species. Virgin females and males were collected using light CO_2 anesthetization and stored separately (in groups of 5-20) in food vials three to five days after eclosion in a 25^{o}C incubator with a 12h:12h light:dark cycle before behavioral observation. Flies were then individually separated into food vials, again using light CO_2 anesthetization at least 24 but no more than 72 hours before the courtship observations were performed. The courtship behavior of individual males was observed by placing the male in a food vial containing one *D. elegans* HK 3-5d old virgin female and one *D. gunungcola* SK 3-5d old virgin female in a vial without anesthesia. The courtship behavior of each male was observed until copulation occurred or one hour elapsed. In order to make sure male behavior was thoroughly tested, each male was observed on the next day with one new virgin female of each species. Males differing in courtship score over the two days were generally rare and were assigned the more *D. elegans*-like of the two scores (higher score; see below). This is because we were interested primarily in the ability of individual males to perform particular courtship elements, rather than the frequency at which they performed them. *D. elegans* courtship is more elaborate than *D. gunungcola* (i.e. it consists of more elements). The ability of backcross males to perform these elements is expected to be determined by the presence of *D. elegans*-specific alleles and the lack of ability to perform these elements is expected to be determined by the presence of *D. gunungcola*-specific alleles. The body color of the backcross males was scored under a Leica stereomicroscope and these males were preserved in 1.5 ml microcentrifuge tubes individually and frozen at -20^{o}C freezer prior to dissection and DNA isolation.

The courtship score of each backcross male was determined by observing the courtship elements that each male exhibited using a scale of 1 to 4. The specific criteria for each courtship score are listed in Table 4.2. The presence or

absence of three *D. elegans*-specific courtship elements (Table 4.3) were also recorded for each backcross male and analyzed separately. Variation in courtship intensity was not examined in this study.

Wing dissection and imaging: The right wings (except for a few individuals in which the right wing was damaged) of male backcross progeny were mounted in glycerol with 10% ethanol on glass slides and pictures of the wings were taken using a Zeiss Axiocam HRC digital camera attached to a Leica MZ7.5 dissecting microscope. All wings were imaged on the same day with the same lighting. Wing spot size was measured in Image J 1.31v by two different workers. Values were divided by wing area (wing length x wing width) to control for body size effects and then averaged between the two workers.

Statistical analyses: Genotype-phenotype data were analyzed using JMP version 4.04 software (SAS Institute, Cary, NC). For analysis of individual courtship elements, individual male backcross progeny were classified as either exhibiting or not exhibiting each of four courtship elements. These data were coded nominally and tested for associations with genotype at each marker separately using a nominal logistic test in JMP. Trait correlations in backcross progeny were assessed using the non-parametric Kendall's coefficient of rank-correlation (τ; Sokal and Rohlf, 1995) in JMP.

RESULTS

Fertility and morphology of D. elegans/D. gunungcola F1 hybrids

D. elegans HK was crossed with *D. gunungcola* SK in both directions (see Materials and Methods). F1 hybrid females from both reciprocal crosses did not differ morphologically from females of either parental strain and were typically fertile, although fertility was not quantified. F1 males from both reciprocal

crosses, however, were completely sterile (inferred from their failure to sire progeny in matings with either parental species). Partially or fully deleted abdominal tergites were seen in approximately 50% of F1 hybrid males with *D. elegans* mothers but F1 males of the reciprocal cross were morphologically normal.

Male wing pigmentation in F1 hybrids

Hybrid males with a *D. gunungcola* mother had clear wings with no apical melanin patch (N=33) (Fig. 4.1D), but hybrid males with a *D. elegans* mother invariably exhibited an apical wing patch, which had a smaller size on average than pure *D. elegans* males (Fig. 4.1E) (*D. elegans/D. gunungcola* F1 males: N=40, mean wing spot size corrected for wing size = 0.056 mm^2/mm^2; pure *D. elegans* HK males: N=26, mean wing spot size corrected for wing size = 0.090 mm^2/mm^2). The mean spot size difference between *D. elegans/D. gunungcola* F1 and pure *D. elegans* males was significant (one-tailed t-test, $P<0.0001$).

Wing spots of *D. elegans* HK/*D. gunungcola* SK F1 males were typically less intensely melanized than pure *D. elegans* HK males (compare Figs. 4.1E and 4.1B) but we did not attempt to quantify this difference. F1 male spots typically had a slightly yellowish tint to their spots whereas pure *D. elegans* HK and other strains tend to be darker brown to black (not shown).

Genetic analysis of male backcross progeny

F1 female progeny from the cross of *D. elegans* females to *D. gunungcola* males were used to breed backcross progeny because these were easier to produce than the reciprocal F1 females. A total of 269 male backcross progeny were genotyped at seven loci representing the five major chromosome arms. Wing spot

sizes were scored for 125 *elegans* backcross males (progeny of *D. elegans/D. gunungcola* F1 females x *D. elegans* HK males) and 99 *gunungcola* backcross males (progeny of *D. elegans/D. gunungcola* F1 females x *D. gunungcola* SK males). Courtship was scored for 131 *elegans* backcross and 95 *gunungcola* backcross males. Linkage was found only for two pairs of loci, *y* and *Moe* (2.7 cM apart) on the X chromosome and *e* and *TfIIA-L* (7.5 cM apart) on the presumptive third-chromosome right arm (3R). This linkage is as expected from the *D. melanogaster* genome and suggests that the relative positions of these genes are similar to their homologues in *D. melanogaster*. However, these recombination rates are much lower than expected from *D. melanogaster* (27.5 cM and 21.8 cM, respectively).

Wing spot size in backcross populations

Overall, the wing spot size of backcross males was continuously distributed, varying from no spot to a spot very close to pure *D. elegans* in size. The two backcross populations differed significantly in mean wing spot size (Mann-Whitney U test, $P<0.0001$). The predominant effect of the *D. elegans* X chromosome was apparent; around half of all backcross males lack wing spots (see below). This corresponds to inheritance of an intact or nearly intact *D. gunungcola* X chromosome. The continuous distribution of wing spot size among males possessing wing spots suggests that the species difference is polygenic, involving at least one autosomal modifying locus in addition to one or more loci of collectively large effect on the X chromosome.

Mean spot sizes for each recovered genotypic class in the backcross populations are shown in Figure 4.2. In the *elegans* backcross population (Fig. 4.2A), only three genotypic classes lacking the *D. elegans* X chromosome, marked by genotype at the *yellow* locus, possessed wing spots of any size, and all

three of these classes carried the *D. elegans* homologue of the *D. melanogaster* right arm of the chromosome III (3R), marked by *ebony*. However, carrying the *D. elegans* homologue of 3R was not sufficient to confer a wing spot as several genotypic classes carrying this element did not exhibit wing spots. In the *gunungcola* backcross population (Fig. 4.2B), no genotypes lacking the *D. elegans* X chromosome exhibited wing spots. Overall, wing spots in the *gunungcola* backcross population were smaller on average than those in the *elegans* backcross population.

In the F1 hybrid background the *D. elegans* X chromosome was necessary for production of wing spots. The *D. elegans* backcross data (Fig. 4.2A) are largely consistent with the necessity of the *D. elegans* X chromosome for spot production. Among individuals bearing wing spots and carrying the *D. gunungcola* allele at the X-linked *y* marker, a total of four individuals also carried the *D. gunungcola* allele at the other X-linked marker, *Moe*. Nevertheless, because these two markers may not cover the entire X chromosome (they span only about 42% of the X. in *D. melanogaster*), we cannot rule out the possibility that these individuals were recombinants that still possessed some part of the *D. elegans* X chromosome. In the *gunungcola* backcross population (Fig. 4.2B), the *D. elegans* X-chromosome, or at least that portion linked to *y* and *Moe*, appeared to be necessary for production of a wing spot. However, one genotypic class with the *D. elegans y* allele, represented by two individuals, had no wing spots. One of these two individuals also had the *D. elegans* allele at *Moe* and the other had the *D. gunungcola Moe* allele.

The potential sufficiency of the *D. elegans* X chromosome for spot production could in principle be addressed by examining the *D. gunungcola* backcross population. Two individuals from this backcross possessed the *D. elegans* allele at both X-linked markers and yet had no wing spots (not shown).

However, one of these two individuals carried the *D. elegans* allele at the *al* and *ara* markers. The other carried the *D. elegans* allele at all of the autosomal marker loci except *ara*. We also cannot rule out possession by these non-spot-bearing males of some fraction of the *D. elegans* X chromosome outside the *y-Moe* interval.

Marker association tests for wing spot size

Two types of tests were used to determine whether marker genotypes were associated with wing spot size in the backcross populations (Fig. 4.3). A single marker association analysis was performed by using a t-test to test the hypothesis of equal means of different genotypes at the same locus, pooled irrespective of genotypes at the other marker loci. Also, a factor analysis was performed by analysis of variance (ANOVA) with each of the seven markers as an effect in the model. The results of these two tests were fairly similar. In both backcross populations, *yellow* (*y)* was significantly associated with wing spot size in both tests but *Moesin* (*Moe*) showed a significant effect on wing spot size only in the single marker test. Since *y* and *Moe* are on the X-chromosome, this might suggest that the X-linked locus (or loci) responsible for wing spot are closer to *y* than to *Moe*, but the power of the ANOVA test is low due to small sample sizes for each genotypic class. The presumptive 2L marker *aristaless* (*al)* showed a significant effect in wing spot size, but only in the *gunungcola* backcross (Fig. 4.3B), which implies dominance of *D. elegans* allele(s) affecting wing spot size on 2L. *ebony* (*e*) showed a significant effect in the ANOVA test in the *elegans* (Fig. 3A) but not the *gunungcola* (Fig. 4.3B) backcross population, suggesting the presence of a dominant *D. gunungcola* allele reducing wing spot size.

Courtship of *D. elegans*

D. elegans male courtship begins with orientation of the male toward the female, followed by tapping of the female abdomen with his forelegs. Then, if the male maintains interest, he circles from the back of the female to her front, facing her head, with one wing extended. While circling, the male's head faces the female and the wing on the side closest to the female head is fully extended and maintained in extended position. After he moves to her head, the male shakes his body with both wings opened, which is referred to as the wing display. While shaking his body, the male opens and steadily holds both wings at approximately a 180^{o} angle (perpendicular to the anterior-posterior axis of his body), while turning the ventral surfaces of the wings forward, lifting his head and thorax, keeping his abdomen close to the substrate or bending the tip of his abdomen toward the female. (Body shaking can also occur without wing extension, but we have only seen this in backcross progeny of *D. elegans/D. gunungcola* hybrids.) During a bout of courtship, the male typically tries to stay directly in front of the female while displaying, often adjusting his position to maintain this orientation in response to female movement. After one or more wing displays, the male moves behind the female, bends the tip of his abdomen toward the female genitalia, grasps her posterior abdomen with his forelegs, and attempts to copulate with her. When he is attempting to copulate, one of his wings is typically extended. If the copulation attempt is not successful, the male may remain behind the female with one wing extended for several seconds and then attempt to copulate again. Alternatively, he may repeat circling and frontal wing display.

Courtship of D. gunungcola

Like *D. elegans*, *D. gunungcola* males also tap the female abdomen with their forelegs prior to courting the female. Courtship consists of the male

extending one or both wings to about a 30^{o} angle to his body and then back over his abdomen repeatedly and rapidly. Then the male attempts to copulate with the female. Unlike *D. elegans* males, *D. gunungcola* males remain behind or to the side of the females during courtship. They do not circle to the front of the females, and they do not hold their wings perpendicular to their body at any time.

Courtship of *D. elegans/D. gunungcola* F1 hybrid males

Hybrid males from both reciprocal crosses showed wing display and male circling in their courtship. However, the courtships of hybrid males from the reciprocal crosses were not identical, and neither F1 genotype was the same as pure *D. elegans* males. Shaking of the body during the frontal wing display was much less pronounced than pure *D. elegans* and often the wings were not held fully out during the display. Furthermore, in F1 males with *D. gunungcola* mothers (N=33), wings were usually held out one by one, not simultaneously, during the frontal display. F1 males with *D. elegans* mothers (N=40) usually held their wings out simultaneously during the frontal display but this was seen less frequently in F1 males from the reciprocal cross. The hybrid males from both reciprocal crosses showed less frequent wing display than pure *D. elegans* males. Courtship of both reciprocal F1 male genotypes can be classified as category 3 in our numerical classification system (see below and Table 4.2).

Courtship in backcross populations

Distributions of courtship scores in the *elegans* and *gunungcola* backcross populations are shown in Figure 4.4. In both backcrosses, a continuous distribution of scores was seen, with the *elegans* backcross males showing higher scores than the *gunungcola* backcross males, as expected from the parental

species differences. The two backcross populations differed significantly in mean courtship score (Mann-Whitney U test, $P<0.0001$).

The mean courtship scores of genotypic classes are depicted for the *elegans* backcross population in Figure 4.5A and the *gunungcola* backcross population in Figure 4.5B. The consistently higher scores in the *elegans* backcross population compared to the *gunungcola* backcross population indicate that the autosomal backgrounds differ at one or more loci affecting the courtship difference between species. The X chromosome appeared to have a subtle effect in the *elegans* backcross population (*cf.* genotypes carrying and lacking the *elegans* X chromosome in Fig. 4.5A and see below).

Marker association tests for courtship score

As with the wing spot data, single marker association and factor analysis (ANOVA) tests were was performed on the courtship data. Results are shown in Figure 4.6. In both backcrosses, *y* and *Moe* showed significant associations with courtship in the single marker analysis (but not in the ANOVA), suggesting the existence of an X-linked locus or loci affecting the wing display difference between species. Few other markers showed strong effects on courtship score, although most of the markers showed significant effects in the *gunungcola* backcross (Fig. 4.6B).

Genetics of divergence in courtship elements

The genetic basis of courtship divergence between *D. elegans* and *D. gunungcola* was studied in more detail by examining the presence or absence of three *D. elegans*-specific elements (Table 4.3) in the backcross progeny. These

were: the presence or absence of a display utilizing both wings (wing display), circling, and body shaking.

Results of marker association tests for each of these elements are shown in Table 3. Interestingly, the different courtship elements showed evidence of separable genetic architectures controlling the species differences. The two backcross populations gave different results, suggesting dominance of some of the alleles underlying species differences. In the *elegans* backcross population (Table 4.4, top), wing display showed significant influences of X-chromosome markers. These associations were not seen in the *gunungcola* backcross population (Table 4.4, bottom), suggesting that *D. elegans* alleles at these loci are recessive to *D. gunungcola* alleles. In the *gunungcola* backcross population, *al* showed a significant association with the wing display element. This suggests that a *D. elegans* allele in the vicinity of this marker has a dominant effect on the species difference in wing display. Only markers on the second chromosome were associated with circling behavior, with *al* showing association in the *elegans* backcross and *Ecdysone receptor* (*EcR*) showing an association in the *gunungcola* backcross. Finally, body shaking showed associations with the linked markers *ebony* (*e*) and *TfIIA-L* in both backcrosses with a significant effect of *EcR* in the *gunungcola* backcross.

Correlations among male traits in backcross progeny

In both backcross populations, male wing spot size was highly significantly correlated with courtship score (*elegans* backcross: Kendall's τ = 0.242, $P<0.0001$; *gunungcola* backcross: $\tau = 0.348$, $P<0.0001$). These correlations may be due either to linkage of loci responsible for these two traits or

low recombination rates in F1 hybrid females (see above). Alternatively, one or more genetic factors may be pleiotropic, affecting more than one of the traits.

DISCUSSION

This study represents an initial, low-resolution analysis of the genetic architecture of divergent male pigmentation and courtship behavior between *D. elegans* and *D. gunungcola*. Before discussing the biological significance of our findings, we must first mention several caveats to this analysis. First, the genetic resolution is very low. Only one marker or one linked pair of markers was used per chromosome arm and the fourth chromosome (2% of the genome in *D. melanogaster*) was not studied. Moreover, the positions of genetic factors affecting species differences within chromosomes and the magnitudes of their effects cannot be estimated with the current data. Although the number of chromosome arm elements is conserved throughout the *D. melanogaster* species group (Ashburner, 1989), we cannot be sure at this time that all chromosome arms were represented in this study, since genomic rearrangements, including translocations, may have taken place since the common ancestor of *D. melanogaster* and *D. elegans/D. gunungcola*. Nevertheless, our markers do not show evidence of linkage other than between the linked pair *y* and *Moe*, both of which are known to be on the *D. elegans* X chromosome, and the tightly linked pair *e* and *TfIIA-L*, which are known to be linked on 3R in *D. melanogaster*. Thus, the genomic coverage provided by our markers is the same as one marker per chromosome arm.

Another caveat involves the simplification of male courtship behavior into an ordinal score. This score doubtless misses many subtleties of courtship in the backcross hybrid males. However, we believe this scoring system captured a

substantial amount of the variation in the backcross populations. Our analysis of individual courtship elements was consistent with the results from the ordinal scoring system.

Large X-effect on male wing pigmentation

Both male wing spots and courtship behavior have polygenic architectures controlling the species differences between *D. elegans* and *D. gunungcola*. Most strikingly, the *D. elegans* X chromosome appears to confer a very large effect on wing spot size. The presence of at least two factors promoting wing spot formation/size is suggested by the approximately continuous distribution of wing spot sizes in the backcross progeny. F1 hybrid males carrying the *D. elegans* X had, on average, wing spots that were about 62% the size of pure *D. elegans* spots and the F1 spots are typically not as dark as pure *D. elegans* spots. This indicates that one or more genes in the *D. elegans* autosomal background with apparently smaller effects than the X-linked gene(s) are also required for production of spots with full size and intensity.

Large X-effects have been found in the genetic basis of postzygotic reproductive isolation in many *Drosophila* species pairs (Coyne and Orr, 1989), the most thoroughly studied of which is *D. simulans/D. mauritiana* (Hollocher and Wu, 1996; True *et al*., 1996). This is potentially related to a larger pattern among animals in which intra- and interspecific differences in sex-specific characters tend to show large X-effects in their genetic architecture (Prowell, 1998; Reinhold, 1998; Paallysaho *et al*., 2003). These effects are also consistent with recent applications of sexual selection theory, including Fisher's runaway and "good genes" models (Kirkpatrick and Hall, 2004ab). Large X-effects on sexually selected traits are also predicted consequences of sexual antagonism by genes selectively favored in one sex but disadvantageous in the other (Rice, 1984),

the "dominance" theory of hybrid incompatibility genes (reviewed by Turelli and Orr, 2000), and the greater exposure of X-linked recessive alleles to selection in males in comparison with autosomal recessive alleles (Charlesworth *et al*., 1987). However, theoretical work by Reeve and Pfennig (2003) argued that large X-effects (Z-effects) are more likely to underlie divergence in male secondary sexual traits in male homogametic species than female homogametic species such as *Drosophila*. It should also be pointed out that the X chromosome constitutes roughly one fifth of the genome in the *melanogaster* species group, a large proportion relative to other *Drosophila* lineages (Patterson and Stone, 1952; Ashburner, 1989), so on the basis of chance alone large X-effects underlying *D. elegans/gunungcola* trait differences might not be too surprising.

We have found that one potential component of prezygotic reproductive isolation, male wing spots, exhibits a strong X-effect but that another component, male courtship, does not. Large X-effects in traits involved in sexual isolation are far from universal. Many studies on various traits involved in male courtship, especially courtship song, have failed to find evidence for large X-chromosome effects (Cowling and Burnet, 1981; Kawanishi and Watanabe, 1981; Kyriacou and Hall, 1986; Tomaru and Oguma, 1994; Pugh and Ritchie, 1996; Noor, 1997; Colegrave *et al.*, 2000; Gleason *et al*., 2002; Gleason and Ritchie, 2004; Huttunen *et al*., 2004; Moehring and Mackay, 2004). In the present case, the explanation for the large X-effect on male wing spots could be that the most important variable pigmentation genes, possibly *yellow* and *tan*, happen to reside on the X chromosome. Therefore, the role of the X chromosome in species divergence could depend critically on genomic history. This would predict that species closely related to *D. elegans* and *D. gunungcola* should also show strong X-effects on pigmentation differences between species but that this may not apply to distantly related lineages.

Modern adaptation theory (Kimura, 1983; Orr, 1998; 2001; 2005) posits that selection toward a phenotypic optimum should involve early large steps and later steps of progressively smaller effect ("fine-tuning" or "modifying" factors). In the case of male-specific traits that are the product of sexual selection, such modifiers may be favored by stabilizing sexual selection if a particular (i.e. intermediate) size of the male ornament is favored, as opposed to an ever increasing ornament size that would be favored by runaway sexual selection. Thus, one possible trajectory for the evolution (or loss) of male wing spots may have been to first substitute alleles of major effect at the X-linked loci, followed by accumulation of modifier alleles at the autosomal loci.

Genetic basis of courtship differences between species

Our analysis of the genetics of male courtship divergence is one of the first attempts to genetically dissect a discrete courtship difference between closely related species. Intermediate behavioral phenotypes are common in species hybrids and have been found for a variety of traits, including migratory behavior in birds (Helbig, 1991) and courtship behavior in birds (Ficken and Ficken, 1968) and spiders (Stratton and Uetz, 1986). Pioneering work by Shaw (1996; 2000) characterized the quantitative genetic basis of differences in male courtship song and female preferences among species in the cricket genus *Laupala*. Both traits were found to be polygenic, suggestive of gradual, incremental divergence (Shaw, 2000). By contrast, Doi *et al.* (2001) mapped a single locus or linked gene complex near the autosomal gene *Delta* that largely controls female preferences for male courtship song in the *Drosophila ananassae/D. pallidosa* species pair.

The observation that reciprocal F1 hybrid males differ only subtly in their courtship suggests that the major loci controlling courtship differences are on the autosomes. However, evidence of one or more X-linked factors is provided by

the backcross progeny analysis. Intra-locus dominance and differential genetic background effects are apparent, as most of the markers had effects in only one of the two backcross populations. Another intriguing finding was that the genetic architectures of specific courtship elements that differ between the species appear to be at least partially separable. In particular, wing display appeared to be largely influenced by X chromosomal genotypes whereas body shaking was more strongly dependent on third chromosome genotypes. The only marker effects on circling behavior were found for second chromosome markers. Such a genetically separable pattern of elements composing a complex behavioral trait suggests that the more complex courtship in *D. elegans*, or its loss in *D. gunungcola*, may have occurred by a stepwise or incremental accumulation of elements involving movement of distinct anatomical parts.

Correlations among male wing spots and courtship behavior

Although the genetics of the male wing spot and display can be somewhat separated, as evidenced by reciprocal F1 male phenotypes, nevertheless we have also found evidence of overlaps in the genetic architectures of wing spot and courtship divergence between *D. elegans* and *D. gunungcola*. In particular, the X chromosome largely determines the presence or absence of wing spots and also has highly significant effects in the backcross populations on wing display.

More detailed QTL analysis is needed to resolve whether linked or possibly pleiotropic loci underlie divergence in pigmentation and behavior in this species pair. Close physical linkage of pigmentation and behavior genes with major effects could constrain trajectories of short term evolution by promoting persistent linkage disequilibrium between alleles conferring particular pigmentation and behavioral states. Single loci underlying variation in pigmentation and behavioral traits could similarly bias the types of pigmentation-

behavior correlations that evolve, depending on the degree of independence of their regulation.

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Table 4.1. Molecular marker primer sets, annealing temperatures, and alleles used in this study.

Locus	Forward primer sequence	Reverse primer sequence	Anneal temp.	Allelic difference between *D. elegans* HK and *D. gunungcola* SK
yellow	5'-CCCAGCCCATACCCTTTCAAAAATG-3'	5'-AATCCTCTTCTGTGGACCGTGGCGC-3'	60°C	indel: 43 bp deletion in *D. elegans* HK
Moesin	5'-CCGKAAYACATTCAAGTATGG-3'	5'-AGATCCTGTTTCAGGGCCTGAA-3'	56°C	indel: 121 bp deletion in *D. elegans* HK
aristaless	5'-GAGAATTCAGGGGCTCCAAGCTG-3'	5'-AACTGACCGGGCATGTAATGAC-3'	66°C	indel: 15 bp deletion in *D. gunungcola* SK
Ecdysone Receptor	5'-AGAGGATCTCAGGCGTATAATG-3'	5'-CMGCCATTCCGGCCATTTTGTA-3'	56°C	RFLP: *MluI*
araucan	5'-GYGAGAAGATYATGCTGGCCAT-3'	5'-ATGGCATCCTCCTCCTCTTTGG-3'	55°C	RFLP: *ScrF1*
ebony	5'-AAGTGCATGCAGGCGATGTTCTCG-3'	5'-GGTGGCCAGTAACCAGACTTGATTCT-3'	57°C	RFLP: *ScaI*
TfIIA-l	5'-CGCATTCTTGTGCCATTTGTATG-3'	5'-ATGGCTTTACCTTGGTGCTCTG-3'	56°C	RFLP: *MslI*

Table 4.2. Courtship categories in *D. elegans/D. gunungcola* backcross hybrids.

Score	Description
1	Male stays beside or behind female at all times during courtship. No circling is seen, and no double wing extensions over 45^{o} are performed, like a *D. gunungcola* male.
2	Male moves from the back of the female to the side or nearly (but not directly) in front of the female several times. Wing waving, in which one or both wings are moved by flexing of wing hinges, occurs and wing may be raised above body instead of spread outward. Body shaking occurs during wing movements. Note that wing waving is distinct from wing display (see Table 2b).
3	Circling, wing display, and body shaking are performed during courtship, but wings tend to be extended in turns, not held steady.
4	Circling, full wing display (wings held steady), and body shaking are performed vigorously during courtship, like a *D. elegans* male.

Table 4.3. Courtship elements investigated in this study.

Element	Description
Circling	Male circles from behind to the front of the female, facing her as he moves. Male may move back and forth in a 180^{o} arc or a complete 360^{o} circle. In *D. elegans*, male usually pauses in front of female, performs wing displays (see below), and then returns to the back and the sequence is repeated multiple times.
Wing display	While in front of female, male faces female and extends both wings fully so that they form a 180^{o} angle. Male then holds wings steady (without flexing wing hinges) while shaking body (see below), causing wings to wave repeatedly in front of female.
Body shaking	Rapid shaking of body in left/right movement while wings are held out. Body pivots on legs during this movement.

Table 4.4. Effects of marker genotype on divergent male courtship elements scored in backcross progeny.

Marker (arm)	Wing display	Circling	Body shaking
elegans backcross population			
y (X)	**0.0110**	NS	NS
Moe (X)	**0.0070**	NS	M
al (2L)	NS	**0.0015**	NS
EcR (2R)	M	NS	NS
ara (3L)	NS	NS	NS
e (3R)	NS	NS	**0.0167**
TfIIA (3R)	NS	NS	**0.0055**
gunungcola backcross population			
y (X)	NS	NS	**0.0239**
Moe (X)	NS	NS	NS
al (2L)	**0.0199**	NS	NS
EcR (2R)	NS	**0.0032**	**0.0280**
ara (3L)	NS	NS	NS
e (3R)	NS	NS	**0.0059**
TfIIA (3R)	M	M	**0.0041**

Arm refers to chromosome arm in *D. melanogaster*. Arm location has been confirmed for X-linked markers in *D. elegans* and *D. gunungcola* but not autosomal markers. Numbers indicate P values of nominal logistic regressions in JMP.
Significant P values are indicated. NS: not significant. M: marginal significance; $0.10 > P > 0.05$.

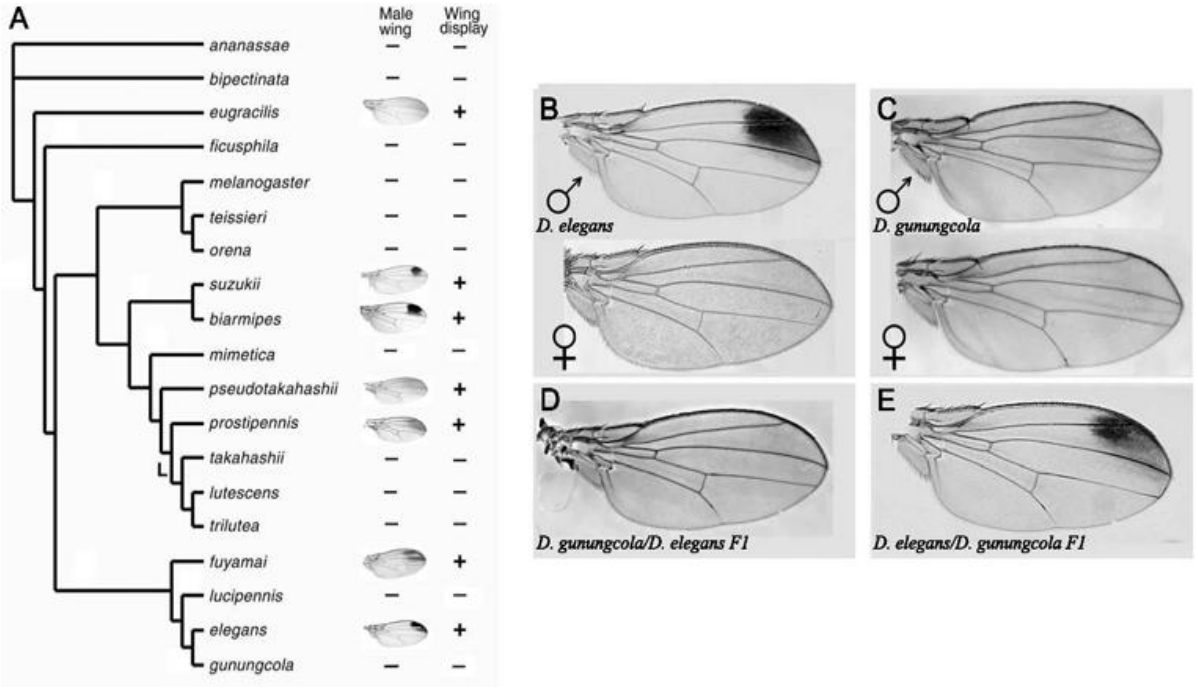

Figure 4.1. A. Phylogeny of Oriental *Drosophila melanogaster* species group (after Kopp and True, 2002 ab) showing typical male wing of species bearing male wing spots. In all species except *D. eugracilis*, female wings have no pigmentation. In *D. eugracilis*, both male and female wings have slight anterior-distal melanization (male shown). Plus signs indicate species possessing wing displays. Dashes indicate species lacking wing spots and/or wing displays. B. *D. elegans* male and female wings. C. *D. gunungcola* male and female wings. D. Typical wing of a *D. gunungcola/D. elegans* F1 male hybrid carrying a *D. gunungcola* X chromosome and a *D. elegans* Y chromosome. E. Typical wing of a *D. elegans/D. gunungcola* F1 male hybrid carrying a *D. elegans* X chromosome and a *D. gunungcola* Y chromosome.

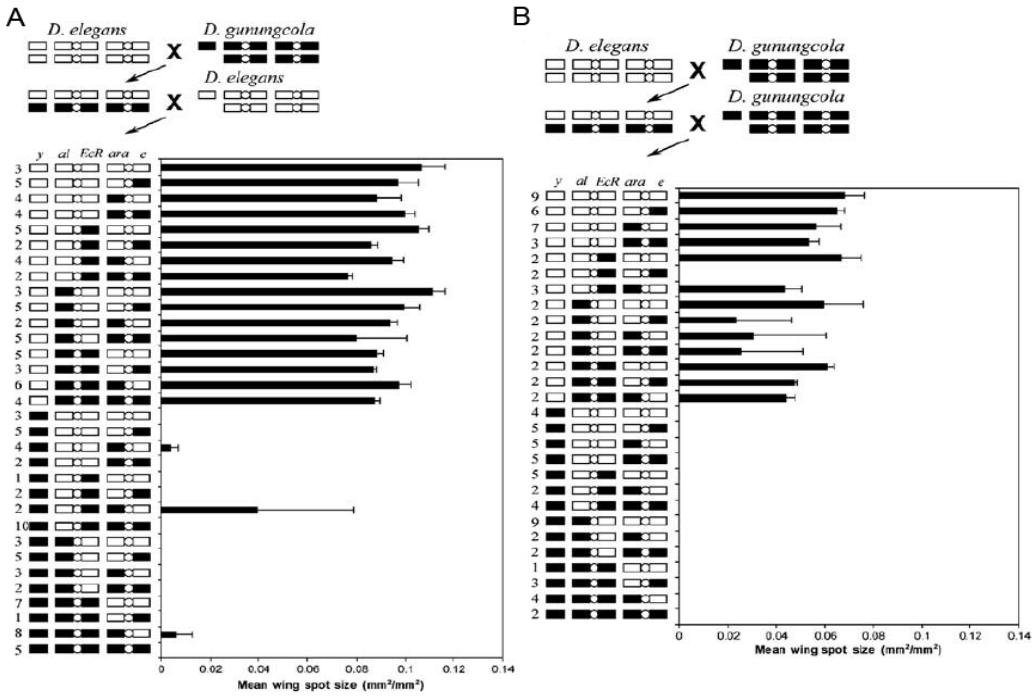

Figure 4.2. Genetic analysis of wing spot size in *elegans* and *gunungcola* backcross populations. A. *elegans* backcross males. B. *gunungcola* backcross males. Backcross is shown at top of each panel. White and black rectangles represent marked *D. elegans* and *D. gunungcola* genome segments, respectively. X, left and right arms of second chromosome, and left and right arms of third chromosome, from left to right, are indicated (Y and fourth chromosomes are not shown and were not analyzed in this study). For backcross progeny at left, the invariable haplotype inherited from the male backcross parent is not shown. Numbers on left indicate sample sizes of each genotype. Error bars represent one standard error. Genotypes with no error bars had either no variance or sample size of one. Some genotypes were not present in the backcross populations. The X is represented by *yellow* and the presumptive third chromosome right arm (3R) is represented by *ebony*.

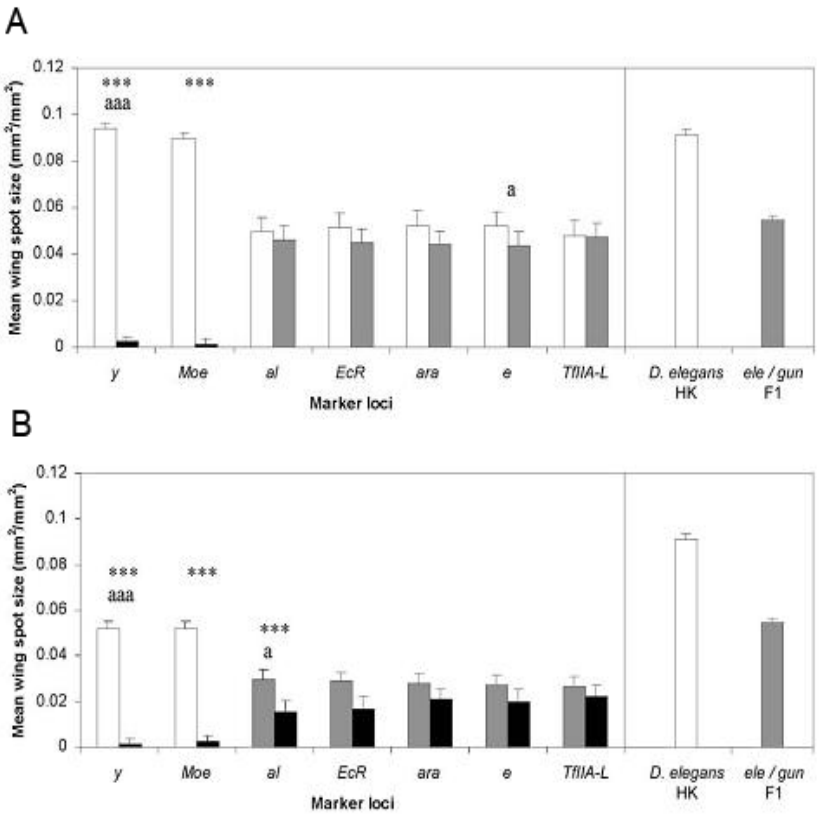

Figure 4.3. Wing spot size of genotypic classes at each marker locus. A. *elegans* backcross males. B. *gunungcola* backcross males. White bars are hemizygous or homozygous for *elegans* alleles. Black bars are hemizygous or homozygous for *gunungcola* alleles. Gray bars are heterozygous for *elegans* and *gunungcola* alleles. For t-test on one marker, *** indicates P<0.0001. For ANOVA test, aaa indicates P<0.0001. a indicates P<0.05. Error bars indicate one standard error. On right of both panels, mean spot sizes of pure *D. elegans* HK and *D. elegans/D. gunungcola* F1 hybrids (with *D. elegans* X chromosome) are shown.

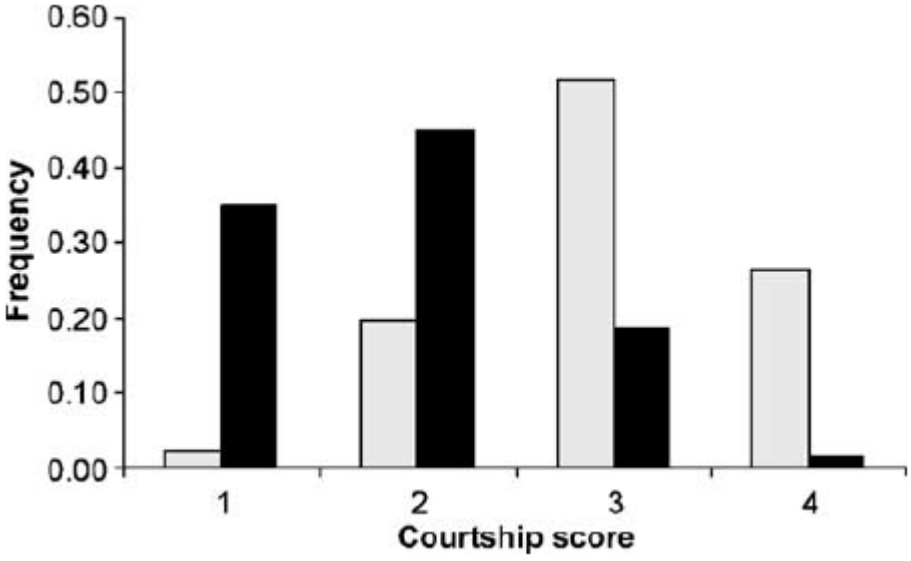

Figure 4.4. Distribution of male courtship score in backcross populations. White bars indicate *elegans* backcross progeny. Black bars represent *gunungcola* backcross progeny. Pure *D. elegans* males have a courtship score of 4. Pure *D. gunungcola* males have a courtship score of 1. F1 males of both reciprocal genotypes have courtship scores of 3 (see text).

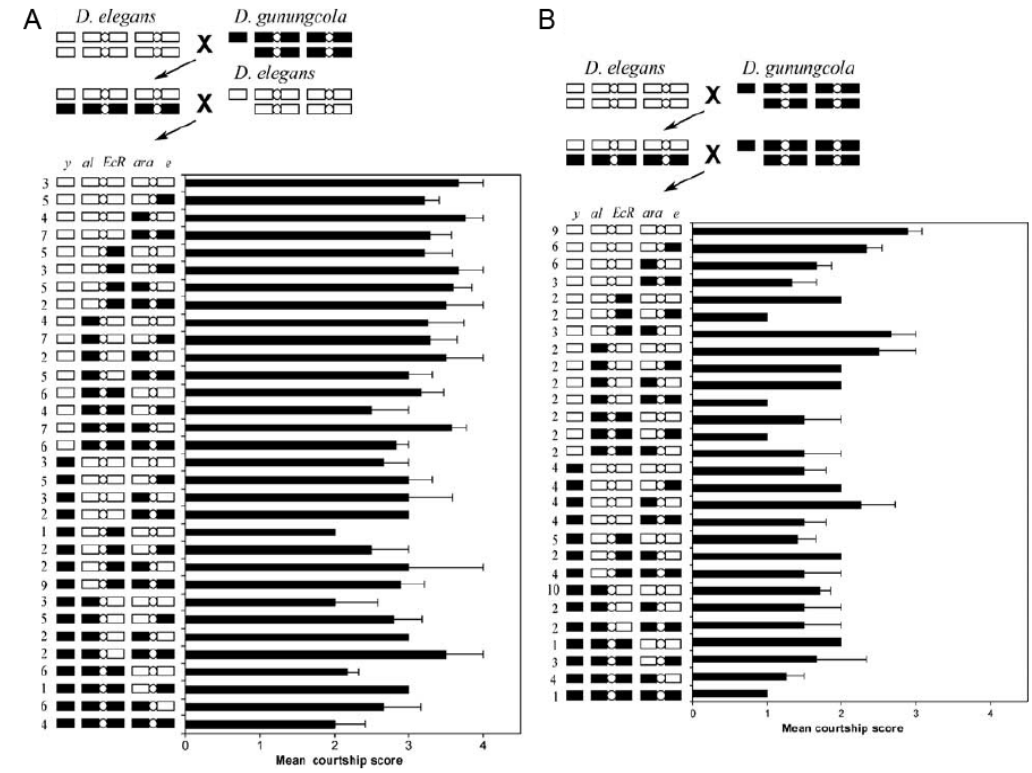

Figure 4.5. Genetic analysis of male courtship score in *elegans* and *gunungcola* backcross populations. A. *elegans* backcross males. B. *gunungcola* backcross males. Backcross is shown at top of each panel. White and black rectangles represent marked *D. elegans* and *D. gunungcola* genome segments, respectively. X, left and right arms of second chromosome, and left and right arms of third chromosome, from left to right, are indicated (Y and fourth chromosomes are not shown and were not analyzed in this study). For backcross progeny at left, the invariable haplotype inherited from the male backcross parent is not shown. Numbers on left indicate sample sizes of each genotype. Error bars represent one standard error. Genotypes with no error bars had either no variance or sample size of one. Some genotypes were not present in the backcross populations. The X is represented by *yellow* and the presumptive third chromosome right arm (3R) is represented by *ebony*.

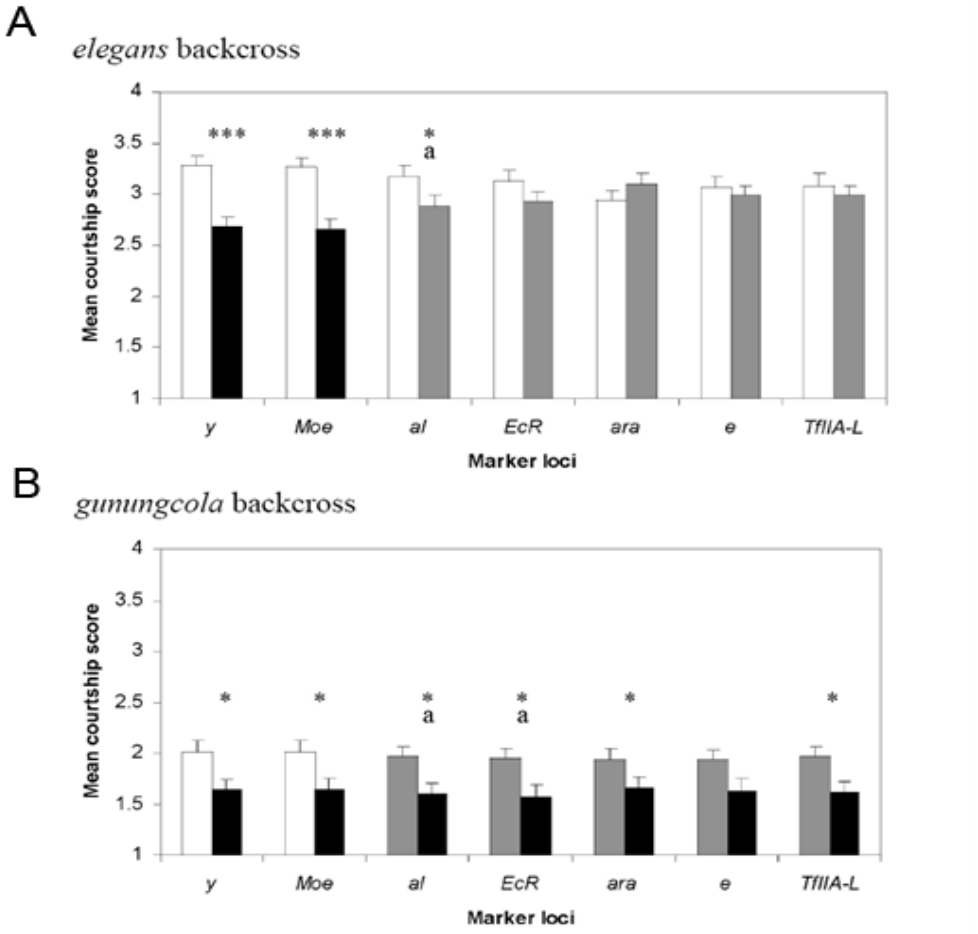

Figure 4.6. Courtship score of genotypic classes at each marker locus. A. *elegans* backcross males. B. *gunungcola* backcross males. White bars are hemizygous or homozygous for *elegans* alleles. Black bars are hemizygous or homozygous for *gunungcola* alleles. Gray bars are heterozygous for *elegans* and *gunungcola* alleles. For t-test on one marker, *** indicates $P<0.0001$. * indicates $P<0.05$. For ANOVA test, a indicates $P<0.05$. Error bars indicate one standard error.

V. The genetic basis of divergence in coordinately evolving male wing pigmentation and courtship behavior in *Drosophila elegans* and *D. gunungcola*.

ABSTRACT

Many adaptive phenotypes consist of complexes of simpler traits that act in combination, including morphologies and the behaviors that utilize those morphologies. Genetic correlations between components of such combinatorial traits, in the form of pleiotropic or tightly linked genes, could in principle promote the evolution and maintenance of these traits but this idea is largely untested. In the Oriental *Drosophila melanogaster* species group, male wing pigmentation shows phylogenetic correlations with male courtship behavior; species with male-specific apical wing melanin spots also exhibit male visual wing displays whereas species lacking these spots generally lack the displays. In this study, we investigated the quantitative genetic basis of divergence in male wing spots and displays between *D. elegans*, which possesses both traits, and its sibling species *D. gunungcola*, which lacks them. We found that divergence in both wing spot size and male courtship score is determined by at least five quantitative trait loci (QTL). On the autosomes, QTL locations for pigmentation and behavior were generally separate but on the X chromosome two clusters of QTL were found affecting both wing pigmentation and courtship behavior. One of these, with the largest effect on wing spots, contains the *yellow* gene, which in *D. melanogaster* is required for dark pigmentation and normal male mating success and has been previously shown to be regulated differently in these two species. We also examined the genetic basis of divergence in three components of male courtship, wing display, circling, and body shaking. Each of these showed a distinct genetic architecture, with some QTL mapping to similar positions as QTL for overall

courtship score. Pairwise tests for interactions between marker loci revealed evidence of epistasis between putative QTL for wing pigmentation but not those for courtship behavior. The co-occurrence of X-linked QTL for male pigmentation and behavior is consistent with the coordinate evolution of these traits and motivates fine scale mapping studies to elucidate the nature of the contributing genetic factors in these intervals.

INTRODUCTION

The evolution of many morphological traits often depends on or is facilitated by functionally related traits, including behavioral, physiological, or other morphological traits. A famous example is the elaborate male fan plumage of peacocks, which seems so contradictory to natural selection-based explanations that Darwin (1859) proposed a second explanation, sexual selection. The exaggerated plumage and the spread and vibration of the plumage during peacock courtship need to be coordinated in order for the males to deliver a visual courtship signal to females, otherwise natural selection would have prevented the fan from evolving. Thus, lack of one of a pair or set of functionally-related traits (e.g. a behavior) may render the other (e.g. a morphology) useless or even detrimental to fitness. Such functionally related trait complexes, especially co-evolving morphologies and behaviors, exist throughout nature, for example in mimetic behavior, diet shifts, habitat adaptation, and courtship behavior (Dewitt *et al.,* 1999; Grant and Grant, 1996; Greene *et al.,* 1987; Losos, 1990; Marroig and Cheverud, 2001; Via and Hawthorne, 2002; Wiens, 2000).

Natural selection on multiple traits simultaneously is expected to produce genetic correlations between them through linkage disequilibrium (Futuyma, 1998,

Ch. 14). Moreover, quantitative genetic theory predicts that traits that are genetically correlated, either due to pleiotropy or linkage, will evolve together (Falconer and Mackay, 1996). Many genetic correlations have been documented between morphological traits and functionally related behaviors (Bell, 2005; Brodie, 1989; Cheverud, 1982; Cheverud, 1996; Marroig and Cheverud, 2001; Sih *et al*,. 2004). In theory, linkage disequilibrium lasting longer than a few generations may be due to close physical linkage of underlying loci or strong selection on unlinked loci. However, it has been proposed that over longer evolutionary times phenotypic integration of adaptive trait complexes should be promoted by evolution of genetic integration via selection on pleiotropic loci responsible for the development of multiple component traits (Cheverud, 1996; Wagner and Altenberg, 1996).

Testing this notion requires empirical evidence. In a recent meta-scale analysis of published intraspecific quantitative trait mapping studies in which the genetic architectures of divergence in correlated traits were reported, Gardner and Latta (2007) found that about one quarter of correlated trait pairs appeared to involve at least one quantitative trait locus (QTL) that mapped to the same position. In general, the proportion of shared QTL increased with the magnitude of the genetic correlation, although there was a great deal of scatter around the regression. This analysis was consistent with shared genetic architectures underlying coordinately selected traits, but the resolution of most QTL studies usually cannot distinguish between pleiotropy and close-linkage of loci. Moreover, the majority of the studies in the Gardner and Latta (2007) analysis were of domesticated plants, with few animal studies or cases of apparent natural selection fitting the study criteria.

Some QTL mapping studies of functionally related morphological traits have reported the presence of shared QTL underlying the component traits.

Fishman *et al.* (2002) reported that among 24 QTL affecting floral trait divergence between two monkeyflower species, *Mimulus guttatus* and *M. nasutus,* the majority affected more than one component of flower morphology. In a QTL study of morphological variation within *M. guttatus*, (Hall *et al.*, 2006) similarly found that the vast majority of 28 detected QTL affected more than one trait. Joron *et al.* (2006) demonstrated that the *Yb* genetic locus in *Heliconius* butterflies controls the expression of a geographically variable yellow pigment stripe in *H. melpomene* and corresponds in genomic location to *Cr*, a locus controlling the same phenotype in the co-mimic species *H. erato*. The homologous genomic region also appears to direct the expression of multiple color patterns in the related species *H. numata.* It makes sense that these studies uncovered overlapping genetic architectures among distinct but developmentally related traits such as morphology and color patterning.

Much less is known about whether shared genetic architecture is important for the evolution of developmentally or physiologically distinct components of complex adaptations. In a study of genetically correlated differences between host strains of aphids (*Acyrthosiphon pisum*), Hawthorne and Via (2001) found that QTL underlying choice of clover versus alfalfa host plants tended to cluster with those underlying physiological performance on those plants. Nonetheless, the choice of host plant and the physiological performance (growth) in aphids may or may be not under the control of the same mechanism (i.e. choice of host plant may be related the feeding behavior which is also associated with growth rate). Recently, Mori *et al.* (2008) found that QTL for autogeny (initial female reproduction without a blood meal) in the mosquito *Aedes albopictus* clustered tightly with those for wing length, a proxy for body size, providing evidence for pleiotropy or linkage as contributing factors to this adaptive trait correlation.

In this study, we address whether similar or distinct genetic architecture underlies coordinately evolving components of male courtship display in two Oriental *Drosophila melanogaster* group species, *D. elegans* and *D. gunungcola*. In this species group, various degrees of black pigmentation appear on the apical region of male wings. Kopp and True (2002) reported that the presence of male-specific wing spots is tightly phylogenetically correlated with frontal wing display during courtship. Frontal wing displays, coupled with the wing spots, have been proposed to serve as a visual mating in two wing spot bearing species within the *suzukii* subgroup, which is one subgroup of the Oriental *melanogaster* species group (Fuyama, 1977; Hegde *et al.*, 2005). Male mating success in the laboratory is affected by the presence of wing spots in *D. elegans* and *D. biarmipes*, two Oriental *melanogaster* group species (see Chapter III.). *D. elegans*, which possesses both male wing spots and a male frontal wing courtship display, is hybridizable with its sibling species, *D. gunungcola*, which lacks both of these characters. The F1 females of this interspecies cross are fertile, providing a powerful system in which to dissect the genetics of these two traits and compare their architectures.

D. elegans and *D. gunungcola*, are members of the *elegans* species subgroup of the Oriental *melanogaster* species group. *D. elegans* is widely distributed in southeast Asia with two body color morphs: the dark form in populations in the northern part of the range (Ryukyu Islands and Taiwan) and the brown form in more southern populations (Hong Kong, Hainan, Philippines, and Indonesia) (Bock and Wheeler, 1972; Hirai and Kimura, 1997). In contrast, *D. gunungcola* has only been reported in mid-high elevations of Indonesia (Sultana *et al*,. 1999; Suwito *et al.*, 2002). As described previously (Kopp and True, 2002; Yeh *et al.*, 2006), *D. elegans* males exhibit a complex series of actions during courtship, first orienting toward females, circling in front of them while facing

them and extending the leading wing 90˚ outward ('Circling'), then engaging in a frontal two-wing display ('Wing Display') during which they hold both wings out with ventral sides facing the female while moving their body laterally with the abdomen bent toward the female ('Body Shaking'), followed by tapping the female with their front legs, and finally attempting copulation. In contrast, *D. gunungcola* males perform relatively simple actions, consisting of orienting toward females, tapping them with their front legs, and then attempting copulation.

In our previous study (Yeh *et al.,* 2006; see also Chapter IV), we used a small number molecular markers in backcross progeny to begin to uncover the genetic architectures of these two divergent male traits. We found that that both wing spot size and courtship behavior are polygenic and that wing spot size is strongly influenced by the X chromosome whereas courtship score shows a smaller X chromosome influence. A comparative developmental genetic analysis by Prud'homme *et al.* (2006) demonstrated that the X-linked gene *yellow* is expressed in the male wing spot pattern in pupae in *D. elegans* but not *D. gunungcola*. The *yellow* gene product is an extracellular protein with an as yet uncharacterized function in dopa melanin formation or sequestration and its late pupal epidermal expression correlates strongly with melanin patterns in diverse *Drosophila* species (Walter *et al.*, 1991; Wittkopp *et al.*, 2002a; Wittkopp *et al.*, 2002b). The gene expression difference mapped to a handful of nucleotide substitutions in a 775 bp cis-regulatory element ($spot^{ele}$) located in an anciently evolved wing cis-regulatory element present throughout *Drosophila*.

Here, we employ 41 molecular markers to perform a genome wide QTL mapping analysis in backcross populations of the *D. elegans* x *D. gunguncola* hybrid cross, providing the first comprehensive mapping of the genetic architecture of coordinately evolving male wing pigmentation and courtship display traits. At least five QTL were found for both wing spot size and courtship

score. Importantly, two regions of the X were associated with QTL clusters for both wing spots and courtship, one of which contains the *yellow* gene. We also describe a genome-wide test of epistasis underlying wing spot size and courtship scores, as well as an analysis of the genetic architecture of individual courtship elements.

MATERIALS AND METHODS

***Drosophila* strains and cultures:** *Drosophila elegans HK* and *D. gunungcola* SK originated from several females collected in Hong Kong, China, and Sukarami, Indonesia, respectively (Ishii *et al.*, 2002). These flies were kindly provided by Dr. M.T. Kimura and maintained on standard fly media (corn meal, yeast extract, and agar) in a 25°C incubator. The karyotyping procedure was modified from Sullivan *et al.* (2000).

***Drosophila* crosses:** The crosses used in this study have been described by Yeh *et al.*, (2006). Briefly, reciprocal interspecific crosses were performed by keeping 5 to 10 virgin females from one species and males from the other species in a fresh vial and transferring into a new vial every week for several weeks. The F1 hybrid females were backcrossed to males of either parental species. Four types of backcross progeny sired from F1 hybrid females were collected for QTL mapping analysis. These are: backcross progeny with *D. elegans HK* grand-maternity and paternity, backcross progeny with *D. gunungcola SK* grand-maternity and *D. elegans HK* paternity, backcross progeny with *D. elegans HK* grand-maternity and *D. gunungcola SK* paternity, and backcross progeny with *D. gunungcola SK* grand-maternity and paternity. In our data analysis, we pooled the backcross progeny with *D. elegans* paternity and refer to these as *elegans* (*ele*) backcross progeny. We also pooled the backcross progeny with *D. gunungcola* paternity

and refer to these as *gunungcola* (*gun*) backcross progeny. From the *ele* backcross set, 209 females and 153 males were genotyped to produce the dataset used in linkage map analysis (see below). These 153 males were also phenotyped (see below) for the QTL mapping analysis. From the *gun* backcross set, 112 males were genotyped and phenotyped for QTL mapping analysis.

Behavioral scoring of males: Virgin females from the parental species and male backcross progeny were collected under light CO_2 anesthetization and aged in food vials for three to five days in groups of 5-20 after eclosion in a 25°C incubator with a 12:12 light:dark cycle. At least 24 hours before the observation of courtship behavior, flies were anesthetized by light CO_2 and individually separated into food vials. In the courtship behavior assay, one three to five day old female from each species was tapped into a food vial with one male backcross progeny. Three courtship elements, wing display, circling, and body shaking (described by Yeh *et al.,* 2006) were recorded until copulation occurred or 1 hour elapsed. Observations of courtship behavior were repeated by adding one female from each species the next day to make sure the male behavior was thoroughly tested. Male backcross progeny then were assigned a courtship score (ranging from 1 to 4) based on the courtship elements they performed (see Yeh *et al.*, 2006 for details). For the individual courtship elements, the presence of the behavior in either of the two trials was taken as evidence of the presence of the element. For two-wing-display analysis, males were categorized into two groups: (1) those exhibiting steady wing extensions or (2) those exhibiting any other types of wing movements or no wing movement at all. A different binary analysis of the presence and absence of two-wing movements did not yield any significant QTL. For the circling element analysis, males were categorized into three states: (1) those that moved to the head-to-head position, (2) those that moved only to the side of the female, or (3) no circling. For the body shaking element analysis, four

categories were applied. (1) vigorous body shaking, like *D. elegans* males, (2) slow body shaking. (3) subtle body shaking, as occasionally exhibited by *D. gunungcola*, or (4) no body shaking. This courtship element was recorded independently of wing display, although wing movement sometimes makes the body movement more conspicuous. After the courtship assays, males were preserved in 1.5ml microcentrifuge tubes individually at -20 °C.

Wing spot size measurements: The right wings of male backcross progeny were mounted in glycerol with 10% ethanol on glass slides and photographed with a Zeiss Axiocam HRC digital camera under a Leica MZ7.5 dissecting microscope connected to a Dell PC using Zeiss AxioVision (Rel 4.3) software. The entire wing dataset was imaged on the same day with the same settings. Wing spot size, which is the area with visible melanin, was measured in Image J 1.31v software by two different workers and divided by wing area (wing length ×wing width). The standardized values obtained from the two workers were then averaged for use in QTL analysis.

Molecular marker genotyping: Genomic DNA was isolated using a single fly preparation protocol (see Yeh *et al.,* 2006). Single fly genomic DNA of parental species was used to acquire the DNA sequences of marker loci and these sequences were checked for allelic monomorphism within the parental species/strains and differences between species were noted. We chose a set of 41 loci covering approximately the entire genome (Table 5.1). Many of these genes are candidate genes for either behavior or pigmentation based on their functions in *D. melanogaster*. For initial sequencing of *D. elegans HK* and *D. gunungcola SK*, primer sequences were designed based on conserved regions among the genomes of the *D. melanogaster* species subgroup, *D. ananassae*, and *D. pseudoobscura*. The specific primers for genotype diagnosis in backcross progeny were then designed based on the DNA sequence of *D. elegans HK* and *D. gunungcola SK*.

Before examining the genotype of each locus in backcross progeny, the allelic difference between species and allelic variation within species were confirmed by applying the diagnostic method in the genomic DNA of five females and four males from each species.

One of three diagnostic methods was applied to determine marker genotypes of backcross progeny, depending on the DNA sequence difference between parental species: (1) Genotypes were examined by running horizontal agarose gel electrophoresis directly after target fragments were amplified if an electrophoretically visible insertion/deletion exists between species. (2) Genotypes were determined using an ABI 3130 capillary autosequencer (ABI3130) and analyzed in ABI GeneMapper (Version 3.7) software after target fragments were amplified with fluorescent-labeled primers. This method was used when small insertions/deletions could not be separated by horizontal gel electrophoresis. (3) Genotypes were determined by running horizontal agarose gel electrophoresis after amplified fragments were incubated with species-diagnostic restriction enzymes. dCAPS Finder 2.0 (Neff *et al.,* 2002), was used to search for diagnostic restriction sites. This method was used when the two species exhibited single nucleotide polymorphisms but not insertion-deletion differences. PCR amplifications were run as follows: 95°C 5 min to denature genomic DNA, then 40 cycles in 95°C 30 sec, annealing temp 30 sec, and 72°C from 20 sec to 1min, 30 sec depending on the amplified fragment length (see Table 5.1 for the specific method used for each marker). DNA sequencing and fragment analysis were carried out in the MEAD Laboratory in the Stony Brook University Department of Ecology and Evolution.

The DNA sequences that were used to design the specific primers for genotype diagnosis and develop diagnostic method were submitted to GenBank (http://www.ncbi.nlm.nih.gov) and the accession numbers of the corresponding

sequences are listed in Appendix B. The trait values and genotypes of each backcross male are listed in Appendix G and H, respectively.

Linkage mapping: The genotypes of 209 females and 153 males obtained from *elegans* backcross were used to construct the hybrid linkage map. The linkage mapping analysis was performed using MapMaker 3.0 (Lander *et al.* 1987). Linkage groups were determined by using the "group" command with a threshold of LOD 3 and 50 cM maximum distance. The relative positions of markers were explored by multiple point analysis and the Haldane map function was employed to compute the genetic distances.

QTL analysis: Two statistical methods, interval mapping (IM; Zeng, 1993) and composite interval mapping (CIM; Zeng, 1993; Zeng, 1994) were employed in our QTL analysis. We used IM for preliminary mapping of continuous traits and final mapping of binary traits, such as Spot Presence and the individual courtship elements. IM, a likelihood ratio test method, tests the likelihood of QTL regions by using one marker interval at time to construct a putative QTL and testing every position in the interval (Lander and Botstein, 1989). The genome scan interval, called walk speed, was 1cM in our analyses, since a walk speed less than 1cM in the IM method was beyond the memory capacity of our computer. Zeng (1993) reported that the IM method may be biased when multiple QTL are located in the same linkage group. Therefore, CIM was used to refine the QTL region of the continuous traits Spot Size 1, Spot Size 2, and Courtship Score. CIM tests the significance of candidate QTL by combining IM with multiple regression analysis. When the putative QTL in an interval is tested, the rest of the markers can be used as covariates to control for QTL other than the putative one. By doing this, the residual variance is reduced (Kao *et al.*, 1999). We chose five random markers as controls, 10 cM window size as the control range, and 0.5 cM walk speed for our CIM analyses. The threshold values of LR statistics for testing QTL were

determined by permutation tests, as proposed by Doerge and Churchill (1996). In each analysis, the data were permuted 1000 times, and the 50^{th} largest LR value was set to the experimentwise significance level of P=0.05. Multiple interval mapping (Jiang and Zeng ,1995; Kao *et al.,* 1999) showed to have very little statistical power for our datasets, possibly because of the difference in the form of wing spot versus courtship phenotypic scores, and thus was not used in our study.

Traits for QTL analysis: As described by (Yeh *et al.,* 2006), about half of the backcross progeny males lack wing spots. As a result, the backcross distributions of wing spot size measurements skew from the normal distribution. Normality of trait value distribution is a basic assumption of continuous variables in most QTL mapping methods. Since we could not transform the backcross progeny spot size distributions to normal distributions, we analyzed the wing spot trait in three ways, which we refer to as Spot Presence, Spot Size 1, and Spot Size 2. In Spot Presence, the wing spot trait was scored as present or absent, regardless of size of the spot, and categorical IM was performed. In Spot Size 1, the full backcross spot size datasets were used, including individuals with no wing spot pigmentation. In Spot Size 2, only individuals with pigment in the wing spot area were used. Both IM and CIM were performed on the Spot Size 1 and Spot Size 2 backcross datasets.

The courtship score is a numerical system with 0 referring to *D. gunungcola*-like courtship and 4 referring to *D. elegans*-like courtship (see Yeh *et al.,* 2006), but the interspecific difference of courtship behavior can also be dissected into several discrete elements. Therefore, we also scored the presence or absence of three courtship elements, Wing Display, Circling, and Body Shaking in backcross males and analyzed them using categorical IM. These courtship elements were described in detail by Yeh *et al.,* (2006). IM and CIM

QTL analyses were performed using Window QTL cartographer 2.5 (Wang *et al.*, 2005).

Epistatic interaction analysis: Pairwise interactions between markers were exhaustively tested using ANOVA. We used three way contingency table tests for Spot Presence, least square tests for Spot Size 1, and logistic regression for Courtship Score. Bonferroni corrections for multiple tests were applied by dividing P values by 820 (the number of interaction tests performed for each backcross dataset). Pairwise marker interaction tests were performed using R version 2.8.1 (http://www.r-project.org).

RESULTS

Karyotypes and linkage map of *D. elegans/D. gunungcola*

Figure 5.1 shows the 41-marker linkage map obtained from pooling both reciprocal backcross populations of progeny from *D. elegans/D. gunungcola* F1 females. The linkage groups in this map correspond to the six Muller/Sturtevant/Novitsky elements A-E (Powell 1997) with A corresponding to the X chromosome. Hereafter, linkage group A is referred to as X and the autosomes are referred to by the letters B through F. Many rearrangement differences from *D. melanogaster* are apparent (for example *y* is near the tip of the X in *D. melanogaster* but in the center of the X in *D. elegans/D. gunungcola*). Although the length of our *D. elegans/D. gunungcola* map is likely to be underestimated due to incomplete coverage of the chromosome ends, the total length of the X and major autosomes (261.0 cM) is very similar to *D. melanogaster* (258.9 cM) and this similarity also appears to apply at the level of the arms (see Fig. 1 of True *et al.* 1996).

The linkage map is consistent with the mitotic karyotype of *D. elegans* and *D. gunungcola*, which consists of 4 pairs of rods, one pair of dot-like, and a pair of sex chromosomes (Appendix C; see also Deng *et al*,. 2007). Because all chromosomes in the *D. elegans/D. gunungcola* genome are acrocentric, the centromere-telomere orientation of the chromosomes is unknown.

At least five QTL underlie wing spot divergence

The male backcross datasets of wing spots were scored in three different ways: a binary score of spot presence, full data set (Spot Size 1), and only males with wing spots (of any size; Spot Size 2) and both interval mapping (IM and composite interval mapping (CIM) methods were used (see Materials and Methods). Putative QTL with their positions and effects are listed in Table 2. Figure 5.2 A,B shows the CIM maps of the *elegans* (*ele*) and *gunungcola* (*gun*) backcrosses respectively for Spot Size 1. Figure 2 C,D shows the CIM maps for the *ele* and *gun* backcrosses, respectively for Spot Size 2. The spot presence dataset gave similar results to Spot Size 1. Many of the QTL positions seem to be detected in more than one analyses and/or in both backcrosses. In the right hand column of Table 5.2, we have given these QTL the same name to reflect the hypothesis that in these cases one QTL underlies the effect that appears in multiple analyses or in both backcrosses.

All analyses support a major QTL, SP1, on the X chromosome in the *y-Moe* interval. A QTL exceeding the significance threshold is found in this interval all in wing spot analyses except Spot Size 2 in the *gun* backcross (Fig. 5.2D), which shows a subthreshold peak in this interval. On the right end of the X there is also evidence of a peak in both backcrosses for Spot Size 2 but the peak only reaches the threshold in the *ele* backcross. We have designated this QTL as SP2. CIM mapping of Spot Size 2 shows a QTL in the left end of linkage group

C, SP3, in both backcrosses as well as two marginally significant QTL peaks on linkage group D in the *gun* backcross. Since these two latter peaks are adjacent, we have designated them as a single QTL, SP4. Finally, IM but not CIM mapping (see Table 5.2 and Appendix D) shows a peak in the *cac-dy* interval of the *gun* backcross, which we have designated as SP5. Taken together these analyses, at least five QTL determine the wing spot difference between *D. elegans* and *D. gunungcola*. The two autosomal QTL, SP3 and SP4, only appear in the Spot Size 2 analysis, which excludes backcross males without wing spots. This suggests that SP3 and SP4 may be associated only with size (or intensity) of the wing spot area whereas the X-linked QTL may be involved in determining both wing spot size and wing spot formation.

The R^2 values in Table 5.2, which are computed for the traits scored as continuously variable, indicate the relative contributions of the detected QTL. For the wing spot analyses, SP1 consistently shows the largest effect (R^2=0.384-0.947) and in some cases is the only QTL detected. SP5 also has a large effect in IM of the *ele* backcross (R^2=0.899) but this is not detected in CIM of this backcross or in any of the other analyses. Since SP5 is in a very wide interval on the linkage map, encompassing over 20cM, more markers in this region need to be developed in order to confirm the presence of this QTL. SP2, which was detected only in the CIM analysis of the *ele* backcross has a relatively minor effect (R^2=0.137). SP3 on linkage group C, which was detected in both backcrosses in the Spot Size 2 analysis, is associated with intermediate R^2 values (R^2=0.248-0.385) while SP4 on linkage group D, which was only detected in CIM mapping of the *gun* backcross, has a small effect (R^2=0.125).

At least five QTL underlie the species difference in courtship score

CIM QTL maps of courtship score are shown in Figure 5.2 E and F for the *ele* and *gun* backcrosses, respectively, and positions and effect sizes of QTL for all analyses are listed in Table 5.2. The two backcross datasets both provide support for a major QTL on the X chromosome, but the QTL peaks were in non-adjacent intervals between the two backcross, *y-Moe* in the *ele* backcross and *cac-dy* in the gun backcross. We provisionally interpret this as evidence for two distinct X-linked QTL. The *y-Moe* peak we designate as CS1 and the *cac-dy* peak we designate as CS3. Both backcross analyses support a QTL toward the left end of linkage group B, CS2. The *gun* backcross analysis indicates an additional significant peak on the right end of linkage group C, CS4. Finally, in the IM but not the CIM analysis of the *gun* backcross, there is a significant peak in the *yellow c-Ddc* interval of linkage group B, which we have designated as CS5. Both backcrosses provide evidence for two additional subthreshold peaks located medially in linkage groups D and E, but we have provisionally not designated these as QTL. These peaks may indicate loci of minor effect, which we will leave to future analyses.

Unlike the wing spot analyses, the QTL detected in analysis of male courtship score generally had small and fairly uniform R^2 values. CS2 consistently exhibited the strongest effect (R^2=0.129-0.152). The effects of the other Courtship Score QTL were fairly uniform and ranged from R^2=0.010-0.141. Overall, the courtship score analysis suggests that the species difference in male courtship behavior results from the contributions of at least five loci, each with moderate to small individual effects.

Individual courtship elements show distinct QTL architectures

In addition to scoring overall courtship, we scored the backcross populations for the presence and absence of three male courtship elements which are exhibited by *D. elegans* but not *D. gunungcola*: wing display, circling, and body shaking (see Materials and Methods and Yeh *et al.* 2006 for descriptions of these elements). Because these traits were each scored as ordinal characters (see Materials and Methods), only IM analysis was possible. IM profiles for these traits are shown in Figure 5.3 and putative QTL positions are summarized in Table 5.2. For Wing Display, two QTL were uncovered, both in the *ele* but not the *gun* backcross. WD1 is in the *y-Moe* interval on the X and thus may contribute to the effect of this interval on Courtship Score (CS1). WD2 is in the *aaNAT2-yellow c* interval on linkage group B and thus may contribute to Courtship Score effect of that interval (CS2). Three QTL were detected for Circling behavior, all in the *gun* but not the *ele* backcross. CI1 is in the *cac-dy* interval of the X and thus may contribute to the Courtship Score QTL effect (CS3). CI2 is located medially on linkage group C where, interestingly, it potentially forms a cluster with SP3, and CI3 maps broadly between *ple* and *bab1* on linkage group D. Finally for Body Shaking, we also detected three QTL, two of which are in similar positions to Courtship Score QTL on the X. BS1 is in the *cac-dy* interval and may contribute to the effect of CS3. BS2 is in the *y-Moe* interval and may contribute to CS1. BS3 maps medially on linkage group E and is the only significant QTL to be detected on that linkage group in our study.

Evidence for epistatic genetic architecture in wing spots but not courtship score

In order to examine whether epistasis among loci contributes to the genetic architecture of wing spot and courtship behavior divergence, we

performed two-way ANOVA for each pair of markers, analyzing the backcrosses separately. This was done for the Spot Presence, Spot Size 1, and Courtship Score datasets. Figure 5.4 shows the results for Spot Size 1. Several significant interactions were found after a highly conservative Bonferroni correction for the large number of tests. First, the *sl-crl* region on the X, which corresponds to the SP5 QTL, showed an interaction with *CG2658*, which is linked to the SP2 QTL. Also, the *dy-crl* interval on X showed a significant interaction with the *Bc-EcR* region on linkage on linkage group C, which approximately corresponds to the SP3 QTL. The *crl* marker interacted significantly with the left end of linkage group E and the *dy* marker interacted significantly with both *DopR1* on linkage group E and *ci*, the linkage group F marker (the presumed homolog of the *D. melanogaster* fourth chromosome). The autosomal components of these latter interactions do not correspond to any significant wing spot QTL detected in this study. In contrast to the Spot Size 1 analysis, neither the Spot Presence analysis (Appendix E) nor the Courtship Score analysis (Appendix F) provided evidence for significant pairwise interactions among markers. This may suggest either that the scoring of these traits is not conducive to detecting epistatic interactions in the analyses we used, or in the case of Courtship Score, that the genetic architecture of the species difference is principally additive.

DISCUSSION

Genetic architecture of divergence in wing spot and courtship behavior

We have found that the genetic architecture of coordinately evolving male wing spots and wing courtship displays in *D. elegans* and *D. gunungcola* reflects a polygenic basis for both pigmentation and behavioral components. At least five QTL underlie species divergence in both wing spot size, scored in various ways,

and male courtship. Relatively little evidence for epistasis among loci was found underlying these traits, although the Spot Size 1 analysis (Fig. 5.4) provided some evidence that epistatic interactions may exist among three of the wing spot QTLs (SP2, SP3, and SP5). Another general hallmark of epistasis or dominance (intralocus epistasis) is the observation of QTL effects in one but not both backcrosses. Of the five wing spot QTL, the two with the highest LR peaks, SP1 and SP3, are found in both backcrosses but the three less significant QTL are only found in one backcross. Even more interestingly, all three courtship component trait analyses (Fig. 5.3) uncovered QTL sets only in one backcross. It is not immediately clear how this could be the result of an artifact in scoring or analysis. If this result is not an artifact, then it suggests that the genes that underlie these courtship elements are strongly influenced by genetic background and that the elements have different degrees of dominance. In particular, the Circling QTL were the only courtship element factors to be detected in the *gun* backcross, suggesting dominance of the underlying *D. elegans* alleles. In this case, it is peculiar that these QTL were not detected in the *ele* backcross, although subthreshold IM peaks were detected for two of them (CI1 and CI2; see Fig. 5.3 C,D). However, since these are male traits, QTL on the X chromosome that were only found in one backcross, but not the other, cannot be explained by simple dominance. Instead, this result may imply a particular kind of epistatic interaction between X and autosomal factors. It is possible that some autosomal QTL or undetected factors act upstream of the effect of X chromosomal QTL, which would then not be expressed in the absence of these autosomal factors.

These results corroborate and extend our previous lower resolution study (Yeh *et al.,* 2006) and complement the study of Prud'homme *et al.* (2006) on the molecular genetic basis of differences between *D. elegans* and *D. gunungcola* in male wing expression of the *yellow*. Yellow protein is required for dark

pigmentation in diverse *Drosophila* species (e.g. Walter *et al.*, 1991; Walter *et al.*, 1996; Wittkopp *et al.*, 2002b), including *D. elegans* (Prud'homme *et al.*, 2006 and our unpublished data). However, ectopic *yellow* expression alone is insufficient to produce novel pigment patterns (Wittkopp *et al.*, 2002a). Consistent with this, we find that in addition to a major QTL in the genomic interval containing *yellow*, which must reflect at least in part the functional molecular differences mapped to *yellow* by Prud'homme *et al.* (2006), at least four other QTL contribute to interspecific wing spot divergence. Presumably one or more of the genes underlying these QTL would be sufficient to cause novel pigmentation if expressed alone or co-expressed with *yellow*.

There has been a great deal of recent interest and research into the genetics of melanin pattern diversity in both insect (True, 2003; Wittkopp *et al.*, 2003) and vertebrate models (Kelsh, 2004; Majerus and Mundy, 2003). In particular, evolutionary geneticists would like to determine whether independent evolution of similar melanin patterns (or losses of patterns) involves genetic changes at the same gene or genes or whether a large menu of possible genetic avenues are available for pigment pattern evolution. In mammals and birds, many cases of Mendelian melanism are caused by amino acid substitutions in the *Melanocortin 1 receptor* (*Mc1r*), which controls the shift from pheomelanin to eumelanin production in melanocytes (Majerus and Mundy, 2003), although cases in which *Mc1r* is not implicated have been reported (see e.g. Steiner *et al.*, 2009).

In *Drosophila*, at least two genes in addition to *yellow* have been implicated in independent cases of melanin pattern evolution or intraspecific variation. *ebony*, which encodes an enzyme that converts the melanin precursor dopamine to the precursor of yellowish, non-melanized sclerton, n-β-alanyl dopamine (NBAD), has been found to underlie variation in body melanization within *D. melanogaster* (Pool and Aquadro, 2007; Takahashi *et al.*, 2007). *ebony*

has also been shown to be expressed in the non-melanized wing areas and down-regulated in wing-spot regions in wing-spot-bearing species (Wittkopp *et al.*, 2002a). Regulatory evolution of *tan*, which encodes the enzyme catalyzing the reverse of the Ebony step (True *et al.*, 2005) has been shown to underlie a major QTL contributing to abdominal pigmentation divergence between *D. yakuba* and *D. santomea* (Carbone *et al.*, 2005; Jeong *et al.*, 2008). Finally, Ng *et al.* (2008) found that variation in male-specific abdominal pigmentation within *D. malerokotliana* is due largely to three QTL which do not correspond positionally to any known *Drosophila* pigmentation genes. Our results are consistent with the previously known role of *yellow* but do not appear to implicate *ebony* as a genetic factor in pigmentation divergence in this species pair. The wing spot QTL SP2, which was found only in the *D. elegans* backcross analysis (see Fig. 5.2C) may correspond to *tan*. Nevertheless, it seems clear that multiple genetic paths exist in *Drosophila*, and possibly other insects, for natural selection to act upon to alter melanin patterns. Furthermore, since many of the QTL in these studies do not correspond to known melanin patterning genes, a number of important functional loci are likely yet to be discovered.

Species divergence in Courtship Score was found to be caused by at least five QTL. When the genetics of divergence in three individual courtship characters were examined, three of the five Courtship Score QTL (CS1, CS2, and CS3) mapped closely to QTL for individual courtship components. A fourth courtship score QTL, CS4 in the *Dat-EcR* interval on linkage group C, also mapped fairly closely to CI3, a QTL for the Circling component. These results are consistent with our earlier study (Yeh *et al.*, 2006) and illustrate how courtship is itself a composite of genetically distinct sub-behaviors that likely evolved sequentially. Future analyses should concentrate on individual courtship

components to avoid possible artifacts of studying composite behavioral traits like the Courtship Score composite used in this study.

Various methods have been used to test whether apparent clustering of QTL for different traits is significant compared to results expected by chance alone. For example, Hawthorne and Via (2001) used a model developed by Orr (1998) to provide statistical support for clustering of physiological and behavioral QTL in their aphid study. Fishman *et al.* (2002) and Hall *et al.* (2006) used multiple interval mapping (MIM; Jiang and Zeng, 1995; Kao *et al.*, 1999). The MIM approach unfortunately did not provide enough statistical power to produce interpretable results with our dataset, possibly owing to the different types of phenotypic scoring between pigmentation and behavioral traits. An alternative method to determine whether there is significant overlap between the QTL positions for two traits is to use a Fisher's Exact Test with a 2x2 contingency table (Sokal and Rohlf, 1995). Given that there are 261 1-cM intervals in the genome, in the *ele* backcross 185 of these have no QTL (defined as having a CIM or IM profile above the significance threshold) for either wing spots or courtship traits, 33 have QTL for courtship only, 24 have QTL for wing spots only, and 19 have QTL for both wing spots and courtship traits. A one-tailed Fisher's exact test gives $P<0.0001$. For the *gun* backcross, on the other hand, these numbers are 147, 78, 34, and 2, which actually indicates highly significantly less overlap than expected (right-tail $P=0.9999$). For the combined data (with intervals positive for QTL if they had a QTL in either backcross), the numbers are 139, 80, 22, 21, which gives a marginal P-value of 0.1111. Therefore, we can conclude that in the *ele* backcross, there is evidence of overlap of QTL for wing spot and courtship traits.

In the case of the QTL cluster in the *yellow* region on the X chromosome, containing the QTL BS2, SP1, CS1, and WD1, the *yellow* gene itself a very strong candidate gene for male courtship behavior divergence between species. In *D. melanogaster, yellow* mutants exhibit well known deficiencies in male mating success that appear to be due to a defects in wing extension during courtship (Bastock, 1956; Burnet *et al.*, 1973; Drapeau *et al.*, 2003). Recent studies by Drapeau and colleagues has shown that sexually dimorphic expression of Yellow in a small subset of brain cells is associated with the wing extension defect in *yellow* males and both the expression and phenotype can be rescued by supplementary expression of yellow. The brain expression of *yellow* is downstream of the sex-determination transcription factor Fruitless (Drapeau *et al,.* 2003) and transcriptional activation of the *yellow* pattern occurs through a 300 bp *cis*-regulatory element upstream of the *yellow* promoter (Drapeau *et al.,* 2006). Fine scale mapping of the X-linked QTL in *D. elegans/D. gunungcola* are underway in our laboratory to determine whether divergence of *yellow* or other genes underlies evolutionary genetic correlations between male pigmentation and courtship behavior.

Genetic scenarios of wing spot loss in the *elegans* species subgroup

Our results suggest that for wing spots, epistatic interactions may be involved in determining the effects of divergent QTL loci on the phenotype. Two different types of gene functions could be envisioned as part of such a mechanism. One type of locus, a "regulator", might determine whether pigment could be deposited in the wing spot area, the other type, a "modifier", would control the intensity (including the darkness and size) of pigmentation in wing spot area. Given such a functional division, the loss of wing spots in the *D. gunungcola* lineage might occur by two different scenarios of genetic changes: "regulators

first" or "modifiers first". In the "regulators first" scenario, changes of upstream regulators might occur first to knock out the expression of melanin pathway genes in the wing spot area. Mutations in wing-spot-specific regulatory regions of modifiers would subsequently accumulate over time, due to the relaxation of selective pressure or genetic drift. Alternatively, in the "modifiers first" scenario, loss of modifier gene expression in the wing spot region might have occurred and been fixed in the population before the changes of regulators. In the former scenario, the pigmentation in wing spot area might be expected to disappear before the fixation of any changes in pigmentation genes. Selection pressure would directly act on the changes of regulators, but not modifiers. In the latter scenario, the pigmentation intensity might have been lost gradually before major changes in regulators occurred (although minor changes affecting spatial patterns or expression levels could occur).

Ascertaining which of these scenarios occurred during the divergence of these two species would involve weighing evidence on the function of the traits. Wing spots in the Oriental *melanogaster* species group appear to be a sexually-selected trait maintained by female preference (Fuyama, 1977, 1979; Kopp and True, 2002; Hedge *et al*., 2005). Thus, how the females assess wing spots would influence the evolution of this trait. Artificial increase of wing spot size does not affect male mating speed in *D. elegans*, though the absence of wing spots prolongs female acceptance (See Chapter II). The consequences of smaller wing spots have not yet been tested, but we would predict that changes in wing spot size may incur different selective pressures than the complete loss of pigmentation in the wing spot area. For example, if the loss of wing spots in *D. gunungcola* resulted from intensive selection (sexual or natural), the individuals with complete absence of wing spots would have been selectively favored during the initial course of evolution. Thus, the changes of "regulators" would be more

likely fixed before the evolution of modifiers. But if wing spots became a neutral trait in *D. gunungcola* due to the loss of sexual selection, then changes in regulators and modifiers would be equally likely. These scenarios could be tested by examining the genetic variation in *D. gunungcola*. In the former scenario, we would expect to find modifier genes would show little or no evidence for selection, at least in sequences important for wing spot expression but that the regulatory gene(s) would show evidence of strong selection.

Many evolutionary developmental biologists have argued that the evolution of morphological novelties can often occur through changes in the expression of single "master control" genes, which consequently regulate the expression of the conserved genetic cascades (Sordino *et al.*, 1995; Shubin *et al*., 1997; Brakefield and French, 1999; Carroll *et al.*, 2001; Pichaud *et al.*, 2001; True and Carroll, 2002; Mann and Carroll, 2002; Cubas, 2004; Albertson *et al.*, 2005). One of the best known examples is the convergent evolution of trichome patterns in *Drosophila* larvae, where the regulatory changes of *shavenbaby*/ovo were found to responbsible for the loss of trichome in *Drosophila schellia* and the expression patterns were found to associate with the divergence of trichome patterns in *D. virilis* species group (Sucena and Stern, 2000; Sucena *et al.*, 2003). In *Heliconius*, the major loci were found to associate with the divergence of parallel mimetic patterns by carrying out classic crossing experiments and later by QTL mapping analyses (Sheppard *et al*., 1985; Joron *et al*. 2006). Futhermore, the change of master gene expression has also been found to associate with the loss of traits, such as limbs in snakes and lizards, and armor and pelvic structures in fish (Cohn and Tickle, 1999; Shapiro *et al.*, 2003; Shapiro *et al.*, 2004; Tanaka *et al.*, 2005; Shapiro *et al.*, 2006). Strikingly, the same locus, *Pitx 1*, has been found to be responsible for the recurring loss of pelvic structure in threespine

sticklebacks, ninespine sticklebacks, pufferfish, and possibly in other vertebrates (Shapiro *et al.*, 2004; Tanaka *et al.*, 2005; Shapiro *et al.*, 2006).

Our finding of a QTL of apparent large effect contributing to the species difference in wing spots bears on the related question of the number of factors involved in adapation and their magnitude of effects. The development of a trait may require many loci, but variations within a species or across several species likely involves a subset of these loci, perhaps one major locus with few minor modifier or alternatively several loci with moderate to small effects. The latter mode was found in the genetic architectures of the loss of body color in *D. santomea* and the variation of male-specific abdominal pigmentation among populations in *D. malerkotliana* (Carbone *et al.*, 2005; Ng *et al.*, 2008). In contrast, we found the former mode responsible for the loss of wing pigmentation in *D. gunungcola*. Though the loss of pigmentation is the common phenotypic change in these three species, there is a fundamental difference in pigmentation loss. The pigmentation level in the abdomen is reduced in *D. santomea* and *D. malerkotliana*, but the pigment is completely absent in wing spot area of *D. gunungcola*. Thus, the developmental mechanism underlying the loss of wing spots is more similar to the loss of denticle trichomes in *D. sechellia*, and the loss of armor plates and pelvic structures in threepine sticklebacks(Sucena and Stern, 2000; Colosimo *et al*., 2004; Shapiro *et al.*, 2004). The genetic mode underlying these trait losses is also similar to our QTL mapping results. Taken together, these studies suggest that the reduction of the degree of a trait may be more likely due to changes in several loci with small effects, whereas the complete loss of a trait or structure may more likely involve evolution of a major gene.

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Table 5.1. The divergence of molecular markers between *D. elegans* HK and *D. gunungcola* SK.

Locus	Abbreviation	Cytological Position [1]	Primer sequence (5' to 3')	T_A [2] (°C)	Allelic difference
X					
yellow	*y*	1A5	CCCAGCCCATACCCTTTCAAAAATG AATCCTCTTCTGTGGACCGTGGCGCGC	60	Indel: 43 bp deletion in *D. elegans* HK
CG2658	CG2658	3E1-2	AGCTCCTGGTCGAGATGGATGG AGGATGTGGCGATCGAAGCGAC	60	Indel: 2 bp deletion in *D. gunungcola* SK
Tyramine beta hydroxylase	*Tbh*	7D2	AGCAGGACTGCGAGGTCTTC TACATGGTGCCCTCCTGGAA	60	Indel: 7 bp deletion in *D. elegans* HK
Moesin	*Moe*	8B4-6	CCGKAAYACATTCAAGTATGG AGATCCTGTTTCAGGGCCTGAA	56	Indel: 121 bp deletion in *D. elegans* HK
tan	*t*	8D1	TTGTTCGTACATATCAAATATGCCTT ACATACGCAATATAAGTGCTTTACAC	54	Indel: 9 bp deletion in *D. gunungcola* SK
dusky	*dy*	10E2	AAGGGATTCCATWAGAYTCCATACTACAG AAGAGCACATCACAATGGATTAAGG	58	Indel: two alleles, about 150 and 250 bp insertion in *D. gunungcola* SK(a)
cacophony	*cac*	10F8-11A1	ATCCTCGCCTTAGGGCTTGTTCTG TTGCACGGCGTGGTTGACAT	54	Indel: 6 bp deletion in *D. elegans* HK
upheld	*up*	12A7	CATCGTAYCMATCGATTTCC TACGTGCGGTACAAACGTGG	54	Indel: 8 bp deletion in *D. gunungcola* SK
small wing	*sl*	14B15-17	AACGTGCAGAGGGACAACTCG CGAATATCTCAATGGAGTCGGTTC	64	Indel: about 200 bp deletion in *D. elegans* HK
courtless	*crl*	14F1	CCTGTTCACTTGCCCATTGATCTTG ACTCTCATCGTTGGGTTCTGTTGG	54	Indel: two alleles, 17 and 23 bp deletions in *D. elegans* HK (b)

Table 5.1. (continued)

Locus	Abbreviation	Cytological Position [1]	Primer sequence (5' to 3')	T_A [2] (°C)	Allelic difference
outstretched	*os*	17A5	GCAACTGGATCGACTATCGCAACT ACTTACCGTGGAATTGGGCTTGAG	54	Indel: two alleles, 14 and 20 bp insertions in *D. elegans* HK(b)
CG11943	CG11943	18F4	ACTCGGAGAGCATTGTGAACACG TGTCGCCCGGTCTCGTGTAG	56	Indel: 12 bp deletion in *D. elegans* HK
2L					
aristaless	*al*	21C3	GAGAATTCAGGGGCTCCAAGCTG AACTGACCGGGCATGTAATGAC	66	Indel: 15 bp deletion in *D. gunungcola* SK
timeless	*tim*	23F6	ATGGCGACTACGAGGATCAG TTCAAGGGTTCAGAGGCTGC	62	Indel: about 400bp deletion in *D. elegans* HK (a)
echinoid	*ed*	24D4-6	ATGGAGCTCACATGCAGCAG ATTGGTGGGCTGATCCTTGG	62	Indel: 4 bp deletion in *D. gunungcola* SK
arylalkylamine N-acetyltransferase 2	*aaNAT2*	26C1	ACGAGCCGCTGATGCTGATCC CTGTCCACGCCGAGCATGTAG	58	RFLP: *Hph I*
Btk family kinase at 29A	*Btk29A*	29A1-3	CCACAGTCGCATGTGAAGCACTA CCATGCTTCATGTACTCGGTGAC	54	RFLP: *Alu I*
black	*b*	34D1-3	ATCTCGGAGCTGTGCAAGAAG CCACAGTCGCATGTGAAGCACTA	62	Indel: 2 bp deletion in *D. gunungcola* SK
yellow c	*yellwow_c*	35B8	TGGAGCGCTGGCAAACAGAATC GGTTTCCACTCTTGACGCTCGATG	58	Indel: 7 bp deletion in *D. gunungcola* SK
Dopa decarbosylase	*Ddc*	37C1	AAGGCAGTTTAAGCGACCTTCC TCAGATACCCGGGCTTCACTTC	54	Indel: 39 bp deletion in *D. elegans* HK
2R					
Ecdysone Receptor	*EcR*	42A6-9	AGAGGATCTCAGGCGTATAATG CMGCCATTCCGGCCATTTTGTA	56	RFLP: *Mlu I*
spinster	*spin*	52E5-8	CAGAACAATAACAACCCGTACAATGG TGACGATGACAATCTCCAAGTGC	58	Indel: two alleles, 26 and 31 bp insertions in *D. elegans* HK (b)

Table 5.1. (continued)

Locus	Abbreviation	Cytological Position [1]	Primer sequence (5' to 3')	T_A [2] (°C)	Allelic difference
Dopamine Transporter	*DAT*	53C7-8	ATTGAAGTCCCTGGATTGCT AATATCATCACAACCCGTTCG	54	Indel: 6 bp deletion in *D. elegans* HK
Black cells	*Bc*	54F6	ATCTGCCCGAAAGTGGATGAG GGTTTCGCACTCTTGACATTCC	57	Indel: 296 bp deletion in *D. elegans* HK
Dopamine N acetyltransferase	*aaNAT1*	60B12-C1	CAAGGCGGTCAACAAGAAGG ACATCATCGGGGGACTGTTG	60	Indel: 117 bp deletion in *D. gunungcola* SK
3L					
bric a brac 1	*bab1*	61E2-F1	CTAAATCGCAGCATTGGTCTTAC TCACTTGTATTGTAAGGCAGGGA	46	Indel: 11 bp deletion in *D. gunungcola* SK
yellow g	*y-g*	62D5	CCCAARATCGTKGCCATMAACAC TTGTTKCCSGTGATGTCGTANAC	57	Indel: about 400bp deletion in *D. gunungcola* SK (a)
pale	*ple*	65C3	AAGCTCTTTGTCCCAGCCTAATTGC GCAGAGGAGAACGAACGCTTGTT	56	Indel: 12 bp deletion in *D. elegans* HK
Clock	*Clk*	66A12	CCGCAATTCTGAAACAAAACAAC TATGCGGAGTCTTGGGATTATTG	54	Indel: 135 bp deletion in *D. elegans* HK
aracaun	*ara*	69C8-69C10	GYGAGAAGATYATGCTGGCCAT ATGGCATCCTCCTCCTCTTTGG	55	RFLP: *ScrF I*
yellow k	*yellow_k*	71D4	ACTCTGATCGAAGCGCAGTG CCAGTCTATCCACGGTGCTG	60	RFLP: *Dra II*
Baldspot	*Baldspot*	73B4-5	GCAAAGCAAGAACTAGTCAATAGCAC CTGGTGTATTCCGTGTAGCTG	59.5	Indel: 56 bp deletion in *D. elegans* HK
3R					
yellow e	*yellow_e*	87E10	CAGCCCTTGGCCACTGATAG AACCAACATTAACCTGGCATTGAA	54	Indel: 23 bp deletion in *D. gunungcola* SK
Dopamine Receptor (1)	*DopR*	88A10-12	TCAGTTTCTACTTCCCCTGTGTGG GAAGAGATTGCATTTATGCCTCCAG	58	Indel: two alleles, 19 bp deletion or 2 bp insertion in *D. gunungcola* SK (b)

Table 5.1 (continued)

Locus	Abbreviation	Cytological Position [1]	Primer sequence (5' to 3')	T_A [2] (°C)	Allelic difference
fruitless	*fru*	91A7-B3	GGAGAGAGGGTAAAGGGGATATAG TAGAACGGAAAAGGGTTACAGG	54	Indel: 4 bp deletion in *D. elegans* HK
Hairless	*H*	92F3	TAACACATGGGACTCCGGTTC GAGCTGTTGTCATCCGAAACTG	58	Indel: 27 bp deletion in *D. elegans* HK
ebony	*e*	93C7-93D1	AAGTGCATGCAGGCGATGTTCTCG GGTGGCAGTAACCAGACTTGATTCT	57	RFLP: *Sca I*
torso-like	*tsl*	93F9-10	AAGGAGCCCACGAGGAACATTTAC TTTCSCCACAATCTGGTTARCCAG	56	Indel: 10 bp deletion in *D. gunungcola* SK
Transcription factor II A-L	*TFIIA-L*	97E11-97E11	CGCATTCTTGTGCCATTTGTATG ATGGCTTTACCTTGGTGCTCTG	56	RFLP: *Ms I*
Dopamine Receptor2	*DopR2*	99B5-6	TCATCCGCAGCCACTGACAT ACTCATACGCCTTGTAGCCACAT	59	Indel: 16 bp delection in *D. gunungcola* SK
4 *cubitus interruptus*	*ci*	102A1-A3	GTATTCGTGCCAGCATTAGC CTGTAAACGTGGTGCCAATG	60	RFLP: *Alu I*

[1]Cytological position in *D. melanogaster* is obtained from Flybase (http://flybase.org/). [2]PCR program, 1 cycle at 94°C 5 s, 40 cycles at 94°C 30 s, T_A 30s, 72°C 20-70s, 1 cycle at 72°C 3 min.

Table 5.2. List of detected QTL associated with divergence of male wing spots and courtship behavior between *D. elegans* and *D. gunungcola*. CIM maps for Spot Size 1, Spot Size 2, and Courtship Score are shown in Figure 2. IM maps for Wing Display, Circling, and Body Shaking are shown in Figure 3.

Trait	Backcross	Method	QTL interval¶	QTL peak £	LR†	R^2‡	Presumed QTL[æ]
Spot Presence	*elegans*	IM	X (*sl-crl*)	1.0	52.0	NA	SP1,SP2
	gunungcola	IM	X (Entire)	34.0	24.5	NA	SP1,SP2
Spot Size 1*	*elegans*	IM	X (Entire)	37.7	380.1	0.947	SP1
		CIM	X(*crl-up*)	37.7	400.3	0.924	SP1
	gunungcola	IM	X(Entire)	16.7	115.8	0.899	SP1, SP5
		CIM	X(d*y-up*)	37.7	127.4	0.632	SP1
Spot Size 2§	*elegans*	IM	X(*crl-Tbh*)	39.9	33.2	0.388	SP1
		CIM	X(*crl-Tbh*)	39.9	46.6	0.384	SP1
		CIM	X(*up-CG2658*)	51.4	12.5	0.137	SP2
		CIM	C(*aaNAT1-DAT*)	13.6	34.7	0.283	SP3
	gunungcola	IM	C(*aaNAT1-DAT*)	0.0	12.2	0.248	SP3
		CIM	C(*aaNAT1-Bc*)	11.0	26.7	0.385	SP3
		CIM	D(*ara-bab1*)	55.6	11.1	0.125	SP4
Courtship Score	*elegans*	IM	X(*cac-t*)	38.7	15.1	0.104	CS1, CS3
		IM	B(*aaNAT2-yellow c*)	13.0	15.4	0.143	CS2
		CIM	X(*crl-CG2658*)	37.2	20.9	0.113	CS1
		CIM	B(*aaNAT2-yellow c*)	12.5	20.1	0.152	CS2
	gunungcola	IM	X(*cac-crl*)	25.7	12.6	0.120	CS3
		IM	B(*aaNAT2-b*)	22.0, 38.4	12.4	0.149	CS2, CS5
		IM	C (Entire)	26.5	10.9	0.115	CS4
		CIM	X(*cac-crl*)	22.7	14.7	0.010	CS3
		CIM	B(*aaNAT2-Ddc*)	21.5	15.5	0.129	CS2

Table 5.2. (Continued)

Trait	Backcross	Method	QTL interval¶	QTL peak £	LR†	R^2‡	Presumed QTL[æ]
Courtship Score	*gunungcola*	CIM	C(*DAT-EcR*)	30.0	10.6	0.074	CS4
Wing Display	*elegans*	IM	X(*y-Moe*)	39.0	11.0	NA	WD1
		IM	B(*aaNAT2-yellow c*)	18.0	10.1	NA	WD2
	gunungcola	IM	None	-	-	-	-
Circling	*elegans*	IM	None	-	-	-	-
	gunungcola	IM	X(*sl-dy*)	11.0	10.7	NA	CI1
		IM	C(*Bc-EcR*)	18.0	11.0	NA	CI2
		IM	D(*ple-bab1*)	41.0	12.8	NA	CI3
Body Shaking	*elegans*	IM	X(*cac-crl*)	25.0	12.5	NA	BS1
		IM	X(*y-Moe*)	39.0	13.1	NA	BS2
		IM	E(*yellow e-DopR1*)	24.0	11.1	NA	BS3
	gunungcola	IM	None	-	-	-	-

¶ QTL intervals given as linkage group (marker inveral within linkage group). Linkage groups are named after the five conserved Muller elements, A-E with X corresponding to A. 'Entire' indicates that the IM profile exceeded the significant threshold across the entire linkage group.

£ QTL peak indicates the position in cM on the linkage map of the peak IM or CIM value in the interval corresponding to that QTL.

† LR=Likelihood ratio.

‡R^2 represents the proportion of variance explained by the QTL, computed as $R2 = (s^2_0 - s^2_1)/s^2$; s^2 = trait variance, s^2_0 = sample variance of residuals, s^2_1 = variance of residuals (BASTEN et al. 1999). R^2 is not applicable (NA) to binary data.

* Spot set 1 consists of all individuals (see Materials and Methods).

§ Spot set 2 consists of only individuals with wing spots of any size (see Materials and Methods).

[æ] See Figures 1-3 for positions of QTL peaks.

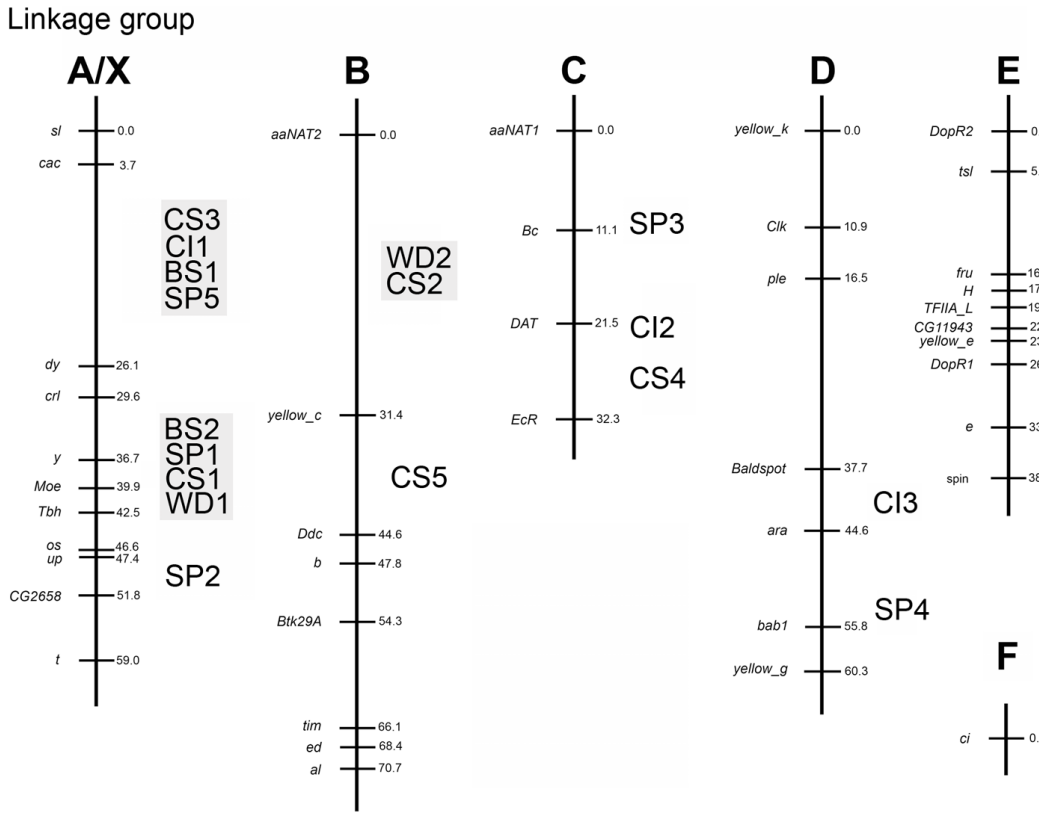

Figure 5.1. Linkage map of *D. elegans/D. gunungcola* F1 hybrids with approximate QTL locations for traits in this study. Designations A through E correspond to Muller's elements. Marker loci (on left of linkage groups) are named using the same names as the presumed orthologous *D. melanogaster* gene sequences used to develop the *D. elegans/D. gunungcola* markers. Approximate positions of putative QTL are indicated on right of linkage groups (see also Table 5.2 and Figs. 5.3, 5.4). See Figure 5.2 for CIM maps of Spot Size 1, Spot Size 2, and Courtship Score datasets. See Figure 5.3 for IM maps of Wing Display, Circling, and Body Shaking datasets. Clusters of QTL with very similar peak positions are shown in gray boxes, but the order in which QTL within the clusters are listed does not imply the linear order of their positions. Note that SP5 and CS5 were found in IM but not CIM analyses.

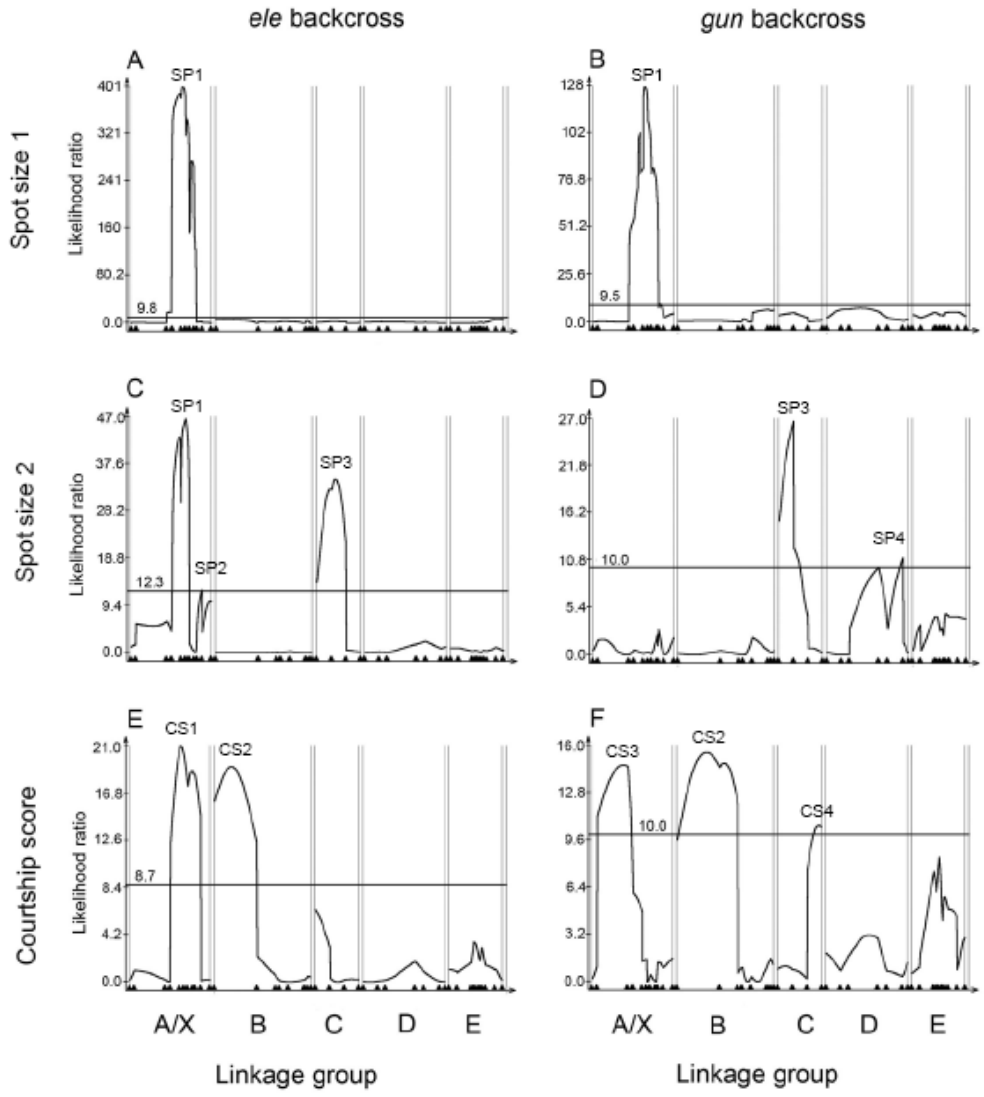

Figure 5.2. Composite interval maps (CIM) for *elegans* (left) and *gunungcola* (right) backcross populations. A., B. Spot Size 1, C., D. Spot Size 2, E., F. Courtship Score. Horizontal lines in each plot indicate LR significance thresholds (see Materials and Methods).

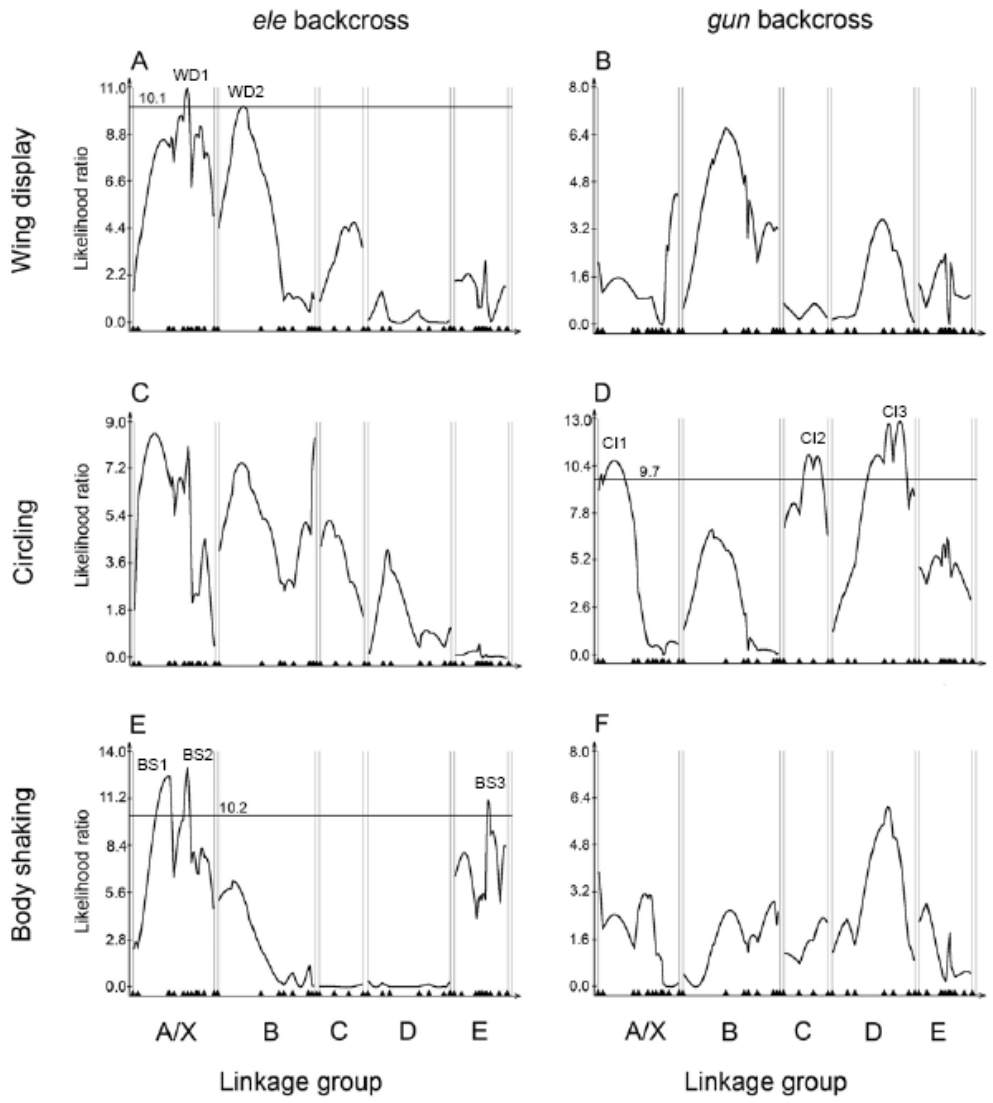

Figure 5.3. IM profiles for individual courtship elements in *elegans* (left) and *gunungcola* (right) backcross populations. A., B. Wing Display, C., D. Circling, E., F. Body Shaking. Horizontal lines in each plot indicate LR significance thresholds (see Materials and Methods). For B, C, and F, thresholds fell above all IM peaks. These thresholds were 9.00, 9.26, and 9.90, respectively.

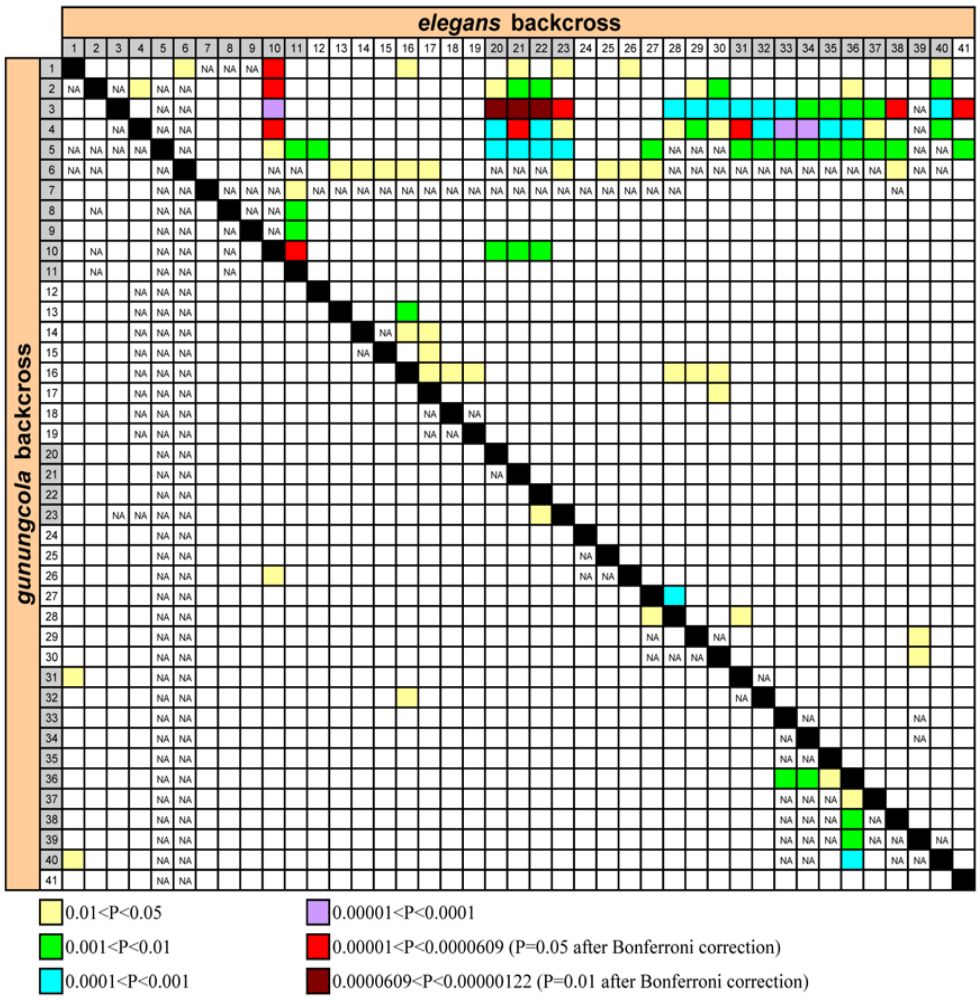

Figure 5.4. Pairwise marker interaction significance levels (see Materials and Methods) for Spot Size 1. *gunungcola* backcross results are in the lower left and *elegans* backcross results are in the upper right. NA indicates not enough data present for the analysis (non-zero wing spot size datapoints are needed, which are rare for some marker combinations in the *gun* backcross).

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Appendix A. Courtship behavior of the *melanogaster* , *ananassae*, and *montium* species subgroups

The *melanogaster* species subgroup

The male courtship behavior of this subgroup is well-studied. Cobb *et al.* (1985, 1986) described and compared seven species. Male courtship behavior in *D. sechellia* is described by Cobb *et al.* (1989) and that of the recently described species, *D. santomea*, is briefly mentioned by Watson *et al.* (2007). Thus, the descriptions below are all based on the available references. Generally, males of this species subgroup tap, vibrate one wing close to one side of female compound eyes or in the rear of females, circle around the females, exhibit wing scissoring in various circumstances, lick the female's abdomen, and slightly spread both wings during copulation attempts.

D. melanogaster

The male courtship behavior of *D. melanogaster* has been described several times (Sturtevant, 1915; Spieth, 1952; Bastock and Manning, 1955; Cobb *et al.*, 1985, 1986; Welbergen *et al.*, 1987; Hall, 1994). Since this is a cosmopolitan species, variation of courtship behavior among populations is often observed. The male courtship elements in this species include tapping, circling, one-wing vibration, wing scissoring, licking, and attempting copulation. Typically a male orients to a female, taps the female with his forelegs, moves to the rear of the female, extrudes his proboscis to lick females (licking), and attempts to copulate by curling his tip of his abdomen down and toward the female genitalia. Sometimes the male positions himself to one side of the female's compound eyes with the one wing extending out at 90 degrees and vigorously vibrates his wings when the courted female is stationary. One-wing vibration is usually observed when a male follows a mobile female. Wing scissoring is occasionally observed when the male moves to the rear of the female. (Cobb *et al.*, 1985, 1986).

D. simulans

Male courtship behavior in this species is similar to that of *D. melanogaster*, with some exceptions and two new courtship elements. When the male circles the female, he often stops either directly in front of her or to one side of her compound eyes and rapidly scissors both wings, gradually increasing the amplitude of the wings to 70-90˚ and holds the wings briefly in this position, then drops the wings back to resting position and moves to the rear of female. The male sometimes extends both wings to 45˚ and raises them up about 30-40˚, then simultaneously refolds and drops them to resting position. This two-wing rowing action is very similar to that of *D. eugracilis*, but with different amplitude. One-

wing rowing with slight extension of the other wing is also observed when the male stands close to the female's head. Frontal semi-circling, in which the male moves his body back and forth between the female's compound eyes, usually occurs during wing scissoring or two-wing rowing. One-wing vibration is usually seen when the male is in the rear of female, instead of the front as in *D. melanogaster*.

D. mauritiana

The male courtship behavior elements of this species include tapping, licking, one-wing extension, two-wing extension, one-wing vibration, frontal semi-circling, two-wing rowing, and wing scissoring (Robertson, 1983; Cobb *et al.*, 1985, 1986). Like in *D. simulans*, the male moves from one side to the other side of the female while performing wing rowing or scissoring. The male may extend one wing singly to 75˚ without vibration or while extending the non-vibrating wing to 20-30˚. Two-wing extension was reported by Cobb *et al.* (1985), but no information of the relative position between the male and the female was described. By investigating the hybrid males from the reciprocal crosses between *D. simulans* and *D. mauritiana*, Cobb *et al.* (1988) found that the genetic control of wing extension is X-linked. But the kind of wing extension investigated was not defined in their paper. Thus, the frequency and role of two wing extension are unclear.

D. sechellia

The male courtship behavior of this species is similar to *D. melanogaster*, with the courtship elements of tapping, circling, one-wing vibration, wing scissoring, licking, and attempting copulation. Like *D. melanogaster*, wing scissoring usually occurs during circling from the rear to one side of the female's head or in the opposite direction. The male also rows one wing when he is close to the female's head, as in *D. simulans.*

D. yakuba

The male courtship behavior elements of this species include tapping, one-wing extension, one-wing vibration, and two-wing rowing. Like in *D. melanogaster*, one-wing vibration occurs when the male follows a female or stands to one side of female's head, but the wing may be extended out in different angles during one-wing vibration. One-wing extension occurs before or after one-wing vibration and usually involves the wing nearest the female's head. Two-wing rowing usually occurs during the movement of the male to the rear of the female.

Appendix A (continued)

D. santomea

D. santaomea is very closely related to *D. yakuba* (Lachaise *et al.*, 2000). As noted by Watson *et al.* (2007) and in our observations, male courtship behavior is very similar to *D. yakuba*, but a detailed quantitative analysis of the difference between these two species has yet been done.

D. teissieri

The male courtship behavior elements of this species include tapping, licking, frontal semi-circling, two-wing extension, one-wing or two-wing vibration, wing scissoring, and two-wing rowing. The male exhibits two-wing vibration, wing scissoring, and two-wing rowing during circling, but he also exhibits two-wing rowing without moving his body. Two wing rowing is also observed in *D. eugracilis*.

D. erecta

The male courtship behavior elements of this species include tapping, licking, circling, one- or two-wing extension, one-wing vibration, wing scissoring, and abdomen bobbing. Wing extension and one-wing vibration usually occur during male movement to the rear of the female. In a unique element, the male rapidly dips his abdomen downward and returns it to the horizontal position when he moves to the rear of female or follows the female. This abdomen bobbing element is only observed in *D. erecta* among the *melanogaster* subgroup species, but similar elements have been observed in other subgroups.

D. orena

The courtship behavior of this species is similar to that of *D. erecta*, but two-wing extension and abdomen bobbing appear to be absent. The male courtship behavior elements of this species include tapping, licking, circling, one-wing extension, and one-wing vibration. Like *D. erecta*, one-wing extension and one-wing vibration usually occur during circling. Unlike *D. simulans*, wing scissoring in this species usually occurs when the male is behind the female.

The *ananassae* species subgroup

D. ananassae and *D. pallidosa*

The male courtship behavior of *D. ananassae* and *D. pallidosa* are very similar, except for wing movement (Speith1952, 1966; Futch,1973; Yamada *et al.* 2002). The male courtship behavior of *D. ananassae* includes tapping, circling

with wing scissoring, two-wing vibration, and attempting copulation. But the male of *D. pallidosa* performs one-wing vibration, instead of two-wing vibration.

The male taps the female, bends his abdomen toward the female abdomen, use his legs to grasp her abdomen, pushes her wings with his head, and attempts to grasp her genitalia while extruding his genitalia. He circles around the female with wing scissoring if he is rejected, then moves back behind the female, extends both wings out a few degrees in *D. ananassae* but only one wing in *D. pallidosa*, vibrates the wing(s) up and down very rapidly, and then engages in more copulation attempts.

The *montium* species subgroup

According to Speith (1952) and Oguma *et al.* (1984, 1987), *D. auraria*, *D. rufa*, and *D. montium* males have very similar courtship behavior, including tapping, one-wing vibration, and attempting copulation. The male taps the female's wing(s), legs, thorax or abdomen, moves to the rear of the female, follows her, extends one wing out and vibrates the wing, and attempts to copulate during one wing vibration. In addition, in *D. montium* it has been reported that after the female rejects the male, he circles around the female with both wings extended and lifted to about 45˚ (Speith, 1952).

Appendix B List of accession numbers of DNA sequences in this study.

Locus	Species	Accession number
X		
yellow	*D. elegans*	FJ889358
	D. gunungcola	FJ889359
CG2658	*D. elegans*	FJ889360
	D. gunungcola	FJ889361
Tyramine beta hydroxylase	*D. elegans*	FJ889362
	D. gunungcola	FJ889363
Moesin	*D. elegans*	FJ889364
	D. gunungcola	FJ889365
tan	*D. elegans*	FJ889366
	D. gunungcola	FJ889367
*dusky(L)**	*D. elegans*	FJ889368
	D. gunungcola	FJ889369
*dusky(R)**	*D. elegans*	FJ889370
	D. gunungcola	FJ889371
cacophony	*D. elegans*	FJ889372
	D. gunungcola	FJ889373
upheld	*D. elegans*	FJ889374
	D. gunungcola	FJ889375
small wing	*D. elegans*	FJ889376
	D. gunungcola	FJ889377
courtless	*D. elegans*	FJ889378
	D. gunungcola	FJ889379
outstretched	*D. elegans*	FJ889380
	D. gunungcola	FJ889381
CG11943	*D. elegans*	FJ889382
	D. gunungcola	FJ889383
2L		
aristaless	*D. elegans*	FJ889384
	D. gunungcola	FJ889385
timeless	*D. elegans*	FJ889386
	D. gunungcola	FJ889387
echinoid	*D. elegans*	FJ889388
	D. gunungcola	FJ889389

Appendix B (Continued)

Locus	Species	Accession number
arylalkylamine N-acetyltransferase 2	*D. elegans*	FJ889390
	D. gunungcola	FJ889391
Btk family kinase at 29A	*D. elegans*	FJ889392
	D. gunungcola	FJ889393
black	*D. elegans*	FJ889394
	D. gunungcola	FJ889395
yellow c	*D. elegans*	FJ889396
	D. gunungcola	FJ889397
Dopa decarbosylase	*D. elegans*	FJ889398
	D. gunungcola	FJ889399
2R		
Ecdysone Receptor	*D. elegans*	FJ889400
	D. gunungcola	FJ889401
spinster	*D. elegans*	FJ889402
	D. gunungcola	FJ889403
Dopamine Transporter	*D. elegans*	FJ889404
	D. gunungcola	FJ889405
Black cells	*D. elegans*	FJ889406
	D. gunungcola	FJ889407
Dopamine N acetyltransferase	*D. elegans*	FJ889408
	D. gunungcola	FJ889409
3L		
bric a brac 1	*D. elegans*	FJ889410
	D. gunungcola	FJ889411
yellow g	*D. elegans*	FJ889412
	D. gunungcola	FJ889413
pale	*D. elegans*	FJ889414
	D. gunungcola	FJ889415
Clock	*D. elegans*	FJ889416
	D. gunungcola	FJ889417
aracaun	*D. elegans*	FJ889418
	D. gunungcola	FJ889419
yellow k	*D. elegans*	FJ889420
	D. gunungcola	FJ889421

Appendix B (Continued)

Locus	Species	Accession number
Baldspot	*D. elegans*	FJ889422
	D. gunungcola	FJ889423
3R		
yellow e	*D. elegans*	FJ889424
	D. gunungcola	FJ889425
Dopamine Receptor (1)	*D. elegans*	FJ889426
	D. gunungcola	FJ889427
fruitless	*D. elegans*	FJ889428
	D. gunungcola	FJ889429
Hairless	*D. elegans*	FJ889430
	D. gunungcola	FJ889431
ebony	*D. elegans*	FJ889432
	D. gunungcola	FJ889433
torso-like	*D. elegans*	FJ889434
	D. gunungcola	FJ889435
TfIIA-L	*D. elegans*	FJ889436
	D. gunungcola	FJ889437
Dopamine Receptor2	*D. elegans*	FJ889438
	D. gunungcola	FJ889439
4		
cubitus interruptus	*D. elegans*	FJ889440
	D. gunungcola	FJ889441

*The DNA sequence between left and right primers of *dusky* is incomplete. Thus, the sequence for designing the primer set of *dusky* is submitted separately.

Appendix C. Karyotypes of *D. elegans* and *D. gunungcola* and their evolutionary context.

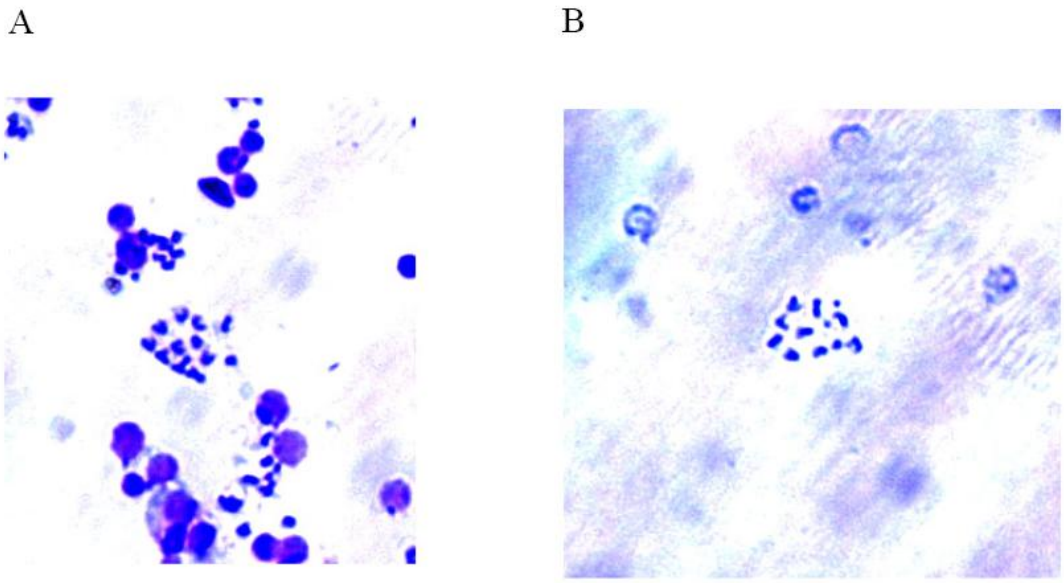

Figure A.1 Mitotic karyotypes prepared from larval brain. A. *D. elegans*. B. *D. gunungcola*.

Based on the observation of mutants with similar phenotypes located on the same linkage groups, the system of Muller/Sturtevant/Novitski elements with A to F elements corresponding to each chromosomal arm in *D. melanogaster* was proposed to name the chromosome arms in early nineteenth century (Muller, 1940; Sturtevant and Novitski, 1941). The ancestral karyotype of the *Drosophila* genus is thought to be six chromosome pairs, consisting five pair of acrocentric chromosomes and one pair of dot chromosomes (Powell, 1997). But various rearrangements, including fusion and fission among elements and inversions, have resulted in karyotypic variation within the *Sophophora* subgenus. Two large metacentric chromosomes in the *ananassae* subgroup (i.e. *D. ananassae* and *D. malerkotliana*) and in the *melanogaster* subgroup result from the centromeric fusions between B and C elements and between D and E elements (Powell, 1997; Schaeffer *et al.*, 2008; Ng *et al.*, 2008). The most parsimonious explanation of

karyotype evolution is that the ancestors of the *D. melanogaster* species group carry B+C and D+E fusion chromosomes. However, the discovery of six chromosomal pairs in the *elegans* subgroup (Figure A1 and Deng et al. 2007) suggests retention of an ancestral karyotype in this lineage. Deng *et al.* (2007) asserted that the karyotype of *D. elegans* is a primitive karyotype in the *D. melanogaster* species group. However, the *D. elegans* subgroup is consistently placed in oriental *D. melangaster* species group with the *ananassae* and *bipectinata* subgroups split earlier in *D. melanogaster* species group, based on several independent phylogenetic analyses (Kopp and True, 2002; Yang *et al.*, 2004; Kopp, 2006). The karyotype of the *elegans* subgroup is thus more likely a derived state according to currently available phylogeny, though chromosomal fissions are thought to be very rare.

That the system of Muller/Sturtevant/Novitski elements widely applies to the species within *Drosophila* genus also implies the evolutionary conservation of linkage groups. Small linkage groups and synteny are even found in the genomic comparison of *D. melanogaster* and *Anopheles gambiae*, which have diverged about 250 Myr (Bolshakov *et al.*, 2002). Though there is a conservation in gene order among *D. melanogaster* species complex, the inversions and translocations disrupt the large scale of synteny between *D. melanogaster* and distant-related species. The more extensive gene shuffling within Muller elements tends to appear between the genomes of the more distant-related species (*Drosophila* 12 Genomes Consortium, 2007). The comparison of our putative linkage map and the genome of *D. melanogaster* showed that gene translocations occur in two of our markers, with CG11943 and *spin* moved between linkage group E and X chromosome and 2R, respectively. The conservation in gene order between the genomes of *D. melanogaster* and the *elegans* subgroup is substantial, except for A and part of the B element. Since the whole genomic sequence is not available in the *elegans* subgroup currently, this information may help in choosing and designing new markers near putative QTL in future fine-scale mapping. The candidate gene approach can be also employed to examine the association between loci and phenotypic variation. However, the most interesting QTL, *y-Moe* region, found in this study maps to the least conserved linkage group in terms of gene order, the X chromosome. It is hard to predict what known genes reside in this chromosomal region in the *elegans* subgroup by using the available genomes in the *melanogaster* subgroup since multiple inversions might have occurred in *y-Moe* region during the divergence of these two subgroups.

Appendix C (continued)

LITERATURE CITED

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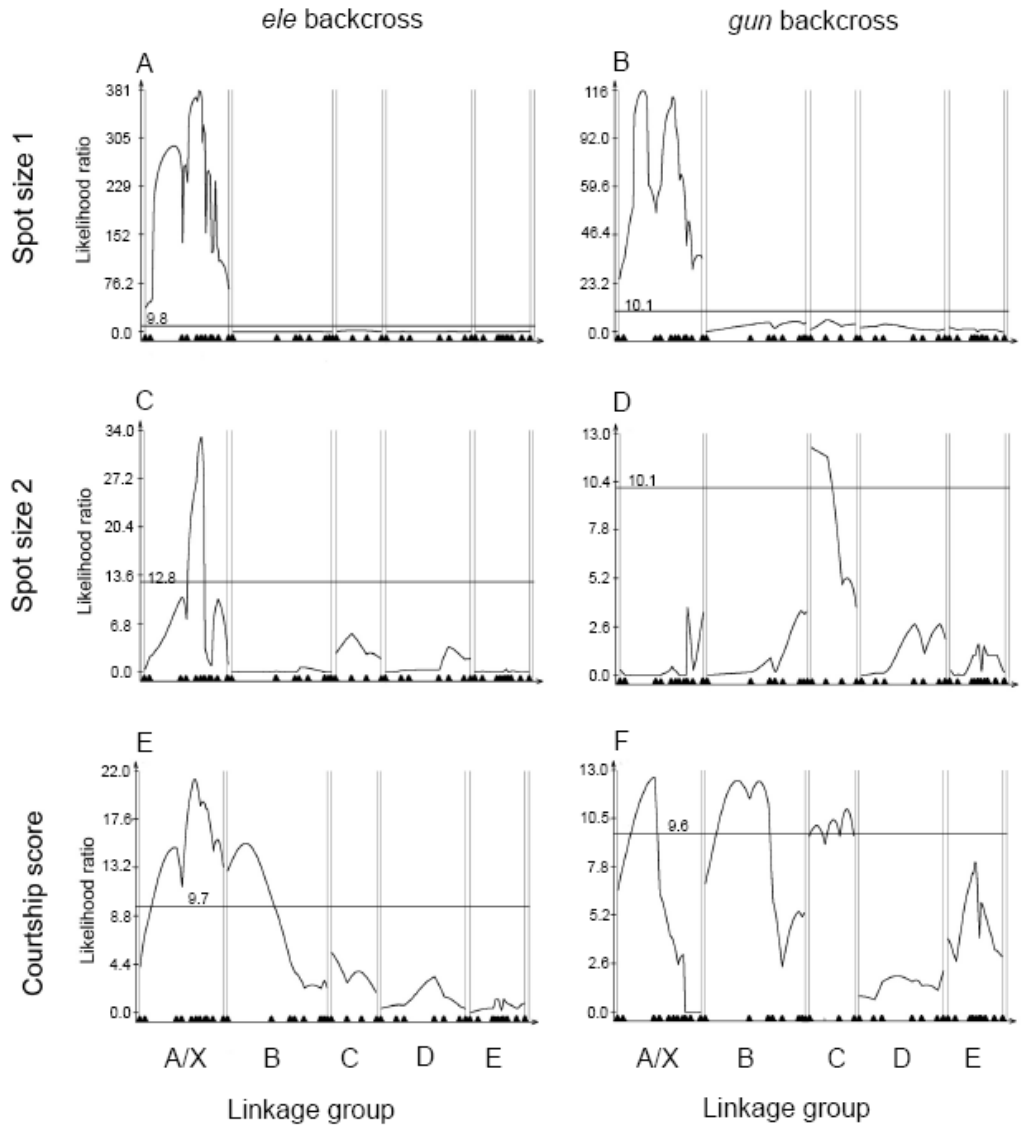
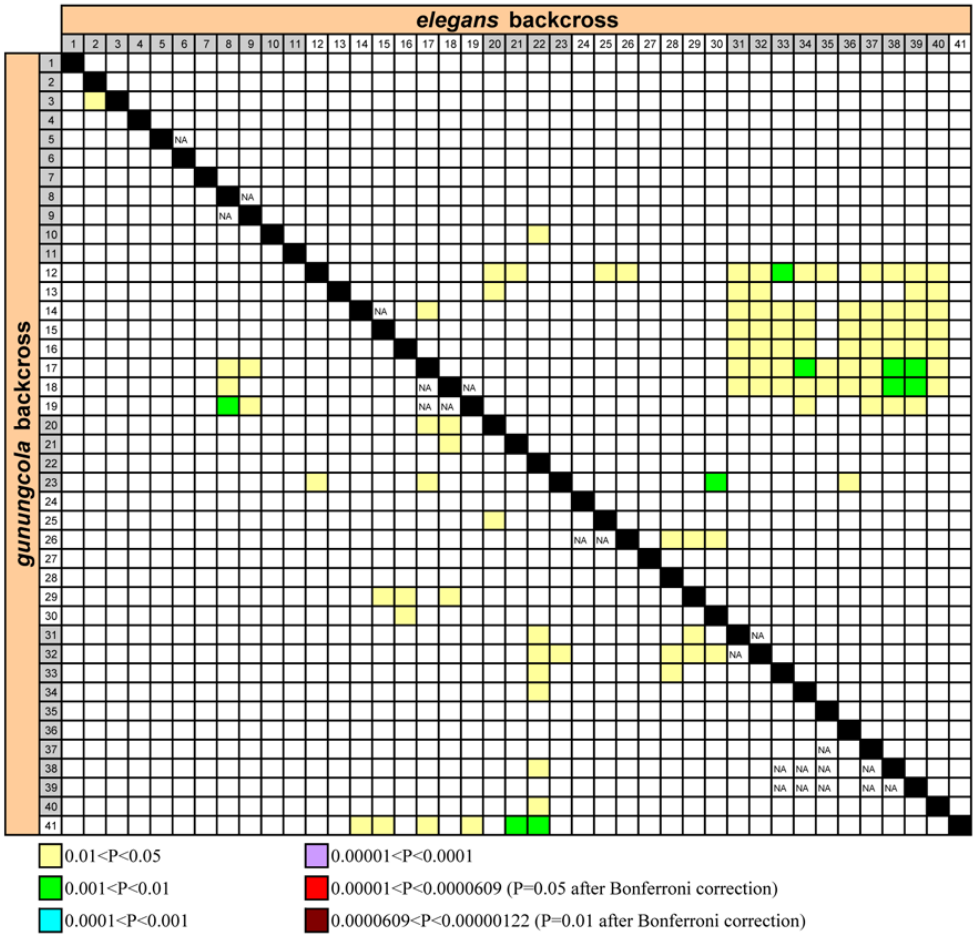
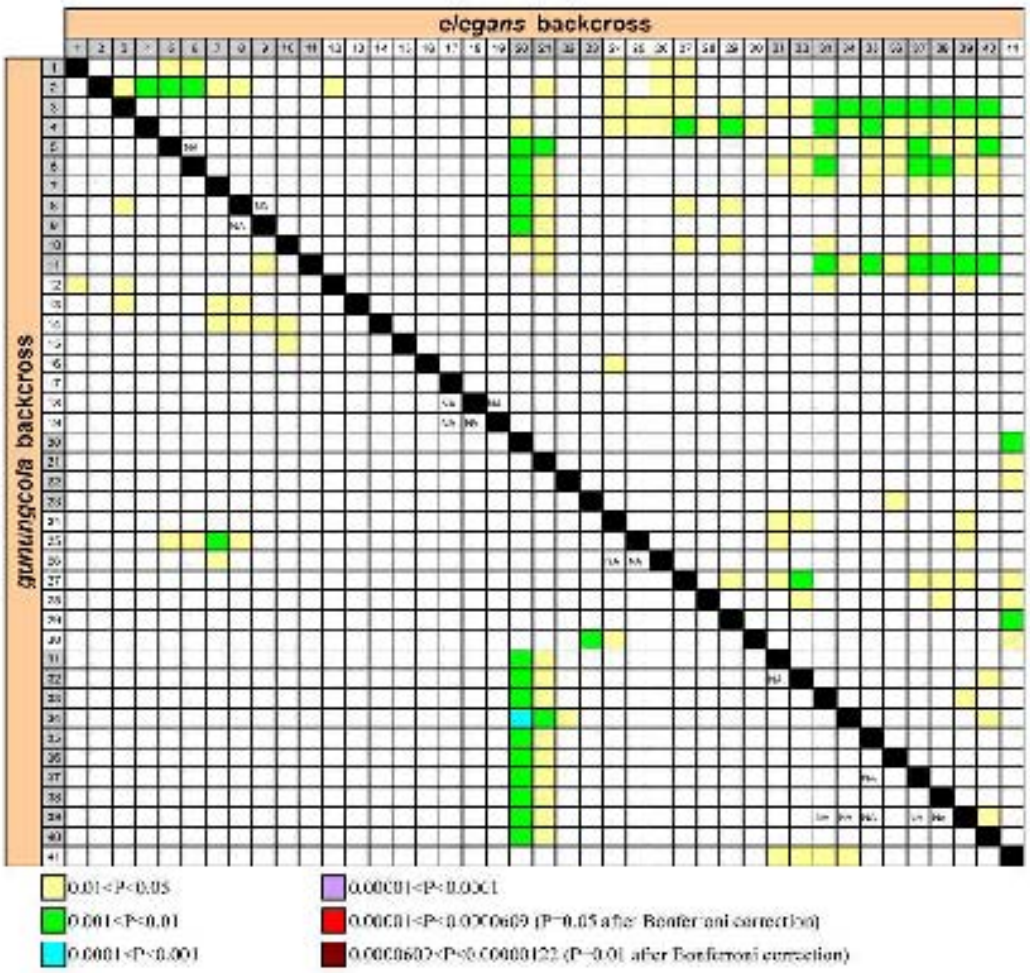
Appendix D. Interval maps (IM) for *elegans* (left) and *gunungcola* (right) backcross populations. A., B. Spot Size 1, C., D. Spot Size 2, E., F. Courtship Score. Horizontal lines in each plot indicate LR significance thresholds (see Materials and Methods).



Appendix E Pairwise marker interaction significance levels (see Materials and Methods) for Spot Presence. *gunungcola* backcross results are in the lower left and *elegans* backcross results are in the upper right.



Appendix F Pairwise marker interaction significance levels (see Materials and Methods) for Courtship Score. *gunungcola* backcross results are in the lower left half and *elegans* backcross results are in the upper right.



Appendix G Trait values of backcross males.

No	Courtship score	Wing spot	Wing display	Circling	Body shaking
elegans backcross					
HKGHK1M01	4	0.0749	Y	Y	Y
HKGHK1M02	4	0.0000	Y	Y	Y
HKGHK1M03	4	0.0983	Y	Y	Y
HKGHK1M04	4	0.1087	Y	Y	Y
HKGHK1M05	3	0.0000	N	Y	Y
HKGHK1M06	3	0.0000	N	Y	Y
HKGHK1M07	2	0.0000	Y	S	Y
HKGHK1M08	-	0.0916	.	.	.
HKGHK1M09	4	0.0875	Y	Y	Y
HKGHK1M10	3	0.0000	Y	Y	Y
HKGHK1M11	-	0.0000	.	.	.
HKGHK1M12	3	0.1080	Y	Y	L
HKGHK1M13	3	0.0845	Y	Y	L
HKGHK1M14	4	0.0000	Y	Y	Y
HKGHK1M15	4	0.0000	Y	Y	Y
HKGHK1M16	2	0.0000	N	S	S
HKGHK1M17	4	0.0000	Y	Y	Y
HKGHK1M18	4	0.0000	Y	Y	Y
HKGHK1M19	3	-	Y	Y	Y
HKGHK1M20	2	0.0000	N	Y	Y
HKGHK1M21	-	0.0000	.	.	.
HKGHK1M22	-	0.0140	.	.	.
HKGHK1M23	4	-	Y	Y	Y
HKGHK1M24	4	-	Y	Y	Y
HKGHK1M25	4	-	Y	Y	Y
HKGHK1M26	2	0.0000	Y	N	S
HKGHK1M27	3	0.0810	N	Y	S
HKGHK1M28	3	0.0785	N	Y	Y
HKGHK1M29	3	0.0000	N	Y	Y
HKGHK1M30	3	0.0878	N	S	Y
HKGHK1M31	4	0.0855	Y	Y	Y
HKGHK1M32	1	0.0000	N	N	N
HKGHK1M33	4	-	Y	Y	Y
HKGHK1M34	3	-	Y	Y	S
HKGHK1M35	2	-	N	Y	Y

Appendix G (*elegans backcross* continued)

No	Courtship score	Wing spot	Wing display	Circling	Body shaking
HKGHK1M36	3	0.0000	N	Y	S
HKGHK1M37	2	0.1039	N	Y	Y
HKGHK1M38	3	0.0000	Y	S	S
HKGHK1M39	4	-	Y	Y	Y
HKGHK1M40	3	0.1224	N	Y	Y
HKGHK1M41	3	0.0000	N	Y	L
HKGHK1M42	-	0.0000	.	.	.
HKGHK1M43	3	0.0000	Y	Y	S
HKGHK1M45	3	0.0000	Y	Y	L
HKGHK1M46	3	0.1197	Y	Y	S
HKGHK1M47	3	0.1048	Y	Y	S
HKGHK1M48	2	0.0000	N	Y	L
HKGHK1M49	4	-	Y	Y	Y
HKGHK1M50	3	0.0000	Y	Y	S
HKGHK1M51	3	0.0830	Y	S	S
HKGHK2M04	3	-	Y	S	Y
HKGHK2M05	2	-	N	N	N
HKGHK2M06	2	-	N	S	N
HKGHK2M07	3	0.0730	Y	Y	L
HKGHK2M08	4	0.1028	Y	Y	Y
HKGHK2M09	-	0.0000	.	.	.
HKGHK2M10	3	0.1048	Y	Y	S
HKGHK2M12	3	0.0903	Y	S	Y
HKGHK2M13	3	0.1048	Y	Y	S
HKGHK2M15	3	0.1039	N	Y	Y
HKGHK2M16	4	0.0880	Y	Y	Y
HKGHK2M18	2	0.0000	N	S	S
HKGHK2M19	1	0.0000	N	N	Y
HKGHK2M20	3	0.0000	Y	Y	S
HKGHK2M21	2	0.0000	N	N	Y
HKGHK2M22	4	0.0000	Y	Y	Y
HKGHK2M23	4	0.0886	Y	Y	Y
HKGHK2M24	3	0.0000	Y	Y	L
HKGHK2M25	4	0.0936	Y	Y	Y
HKGHK2M26	2	0.0000	N	Y	S
HKGHK2M27	3	0.0000	Y	Y	S

Appendix G (*elegans backcross* continued)

No	Courtship score	Wing spot	Wing display	Circling	Body shaking
HKGHK2M28	3	0.0000	Y	Y	S
HKGHK2M29	3	0.0000	Y	Y	S
HKGHK2M30	3	0.0805	Y	Y	S
HKGHK2M31	4	-	Y	Y	Y
HKGHK2M32	3	0.0000	Y	Y	S
HKGHK2M33	3	-	Y	Y	S
HKGHK2M34	3	-	Y	Y	L
HKGHK2M35	3	-	N	Y	L
HKGHK2M36	3	0.0000	Y	Y	S
HKGHK2M37	2	-	N	Y	S
HKGHK2M38	3	0.0928	Y	Y	S
HKGHK2M39	4	0.1079	Y	Y	Y
HKGHK2M40	3	0.1203	Y	S	Y
HKGHK2M41	-	0.0000	.	.	.
HKGHK2M42	3	-	Y	Y	Y
HKGHK2M43	2	0.1214	N	Y	Y
HKGHK2M45	3	0.0000	Y	Y	S
HKGHK2M46	2	0.1105	N	Y	N
HKGHK2M47	-	0.0000	.	.	.
HKGHK2M48	2	0.0000	N	Y	L
HKGHK2M49	3	0.1160	Y	Y	S
GHKHK1M01	4	0.1066	Y	Y	Y
GHKHK1M02	4	0.1035	Y	Y	Y
GHKHK1M03	4	0.1124	Y	Y	Y
GHKHK1M04	4	0.0000	Y	Y	Y
GHKHK1M05	2	0.0000	N	Y	Y
GHKHK1M06	2	0.0000	N	Y	Y
GHKHK1M07	2	0.0000	N	Y	S
GHKHK1M08	4	0.1149	Y	Y	S
GHKHK1M09	3	0.0000	Y	Y	Y
GHKHK1M10	4	0.0000	Y	Y	Y
GHKHK1M11	3	0.0892	N	N	Y
GHKHK1M12	2	0.0845	N	Y	Y
GHKHK1M13	4	0.1023	Y	N	Y
GHKHK1M14	2	0.0000	Y	Y	L
GHKHK1M15	4	0.0839	Y	Y	Y

Appendix G (*elegans backcross* continued)

No	Courtship score	Wing spot	Wing display	Circling	Body shaking
GHKHK1M16	4	0.0893	Y	Y	Y
GHKHK1M17	2	0.0000	N	Y	Y
GHKHK1M18	4	0.0609	Y	Y	Y
GHKHK1M19	-	0.0506	.	.	.
GHKHK1M20	4	0.0960	Y	Y	Y
GHKHK1M21	3	0.0926	N	Y	S
GHKHK1M22	2	0.1127	N	Y	Y
GHKHK1M23	2	0.0000	N	Y	Y
GHKHK1M24	3	0.0874	N	Y	Y
GHKHK1M25	2	0.0000	N	S	L
GHKHK1M26	.	0.0000	.	.	.
GHKHK1M27	4	-	Y	Y	S
GHKHK1M28	3	0.1034	N	Y	Y
GHKHK1M29	4	0.0968	Y	Y	Y
GHKHK1M30	4	0.0826	Y	Y	Y
GHKHK1M31	2	0.0787	N	Y	Y
GHKHK1M32	3	0.0886	N	Y	S
GHKHK1M33	3	0.0977	N	Y	L
GHKHK1M34	2	0.0889	N	Y	S
GHKHK1M35	4	0.1045	Y	Y	Y
GHKHK1M36	3	0.0000	Y	Y	L
GHKHK1M37	2	0.0000	N	Y	Y
GHKHK1M38	3	0.0957	Y	Y	Y
GHKHK1M39	4	0.0000	Y	Y	Y
GHKHK1M40	1	0.0000	N	N	N
GHKHK1M41	2	0.0000	N	Y	L
GHKHK1M42	4	0.0765	Y	Y	Y
GHKHK1M43	2	0.0830	N	Y	Y
GHKHK1M44	1	0.0000	N	N	N
GHKHK1M45	3	0.0000	N	Y	S
GHKHK1M46	4	-	Y	Y	Y
GHKHK1M47	3	0.0000	N	Y	S
GHKHK1M48	3	-	Y	Y	S
GHKHK1M49	3	0.0874	Y	S	Y
GHKHK1M50	2	0.0000	N	S	N
GHKHK1M51	3	0.0000	N	Y	Y

Appendix G (*elegans backcross* continued)

No	Courtship score	Wing spot	Wing display	Circling	Body shaking
GHKHK1M53	4	0.0953	Y	Y	Y
GHKHK1M54	3	0.1073	N	Y	S
GHKHK1M55	2	0.0880	N	S	S
GHKHK1M57	3	0.0000	Y	Y	S
GHKHK1M58	4	-	Y	Y	Y
GHKHK1M59	3	-	Y	Y	L
GHKHK1M60	3	0.0873	Y	Y	S
GHKHK1M61	3	0.0000	Y	Y	L
HKGG1M01	4	0.0801	N	Y	S
HKGG1M02	3	0.0936	N	Y	S
gunungcola backcross					
HKGG1M03	1	0.0000	N	N	N
HKGG1M04	1	0.0000	N	N	N
HKGG1M05	1	0.0000	N	N	N
HKGG1M06	1	0.0000	N	N	N
HKGG1M07	2	0.0515	N	N	L
HKGG1M08	3	0.0609	Y	Y	Y
HKGG1M09	2	0.0560	N	N	Y
HKGG1M10	2	0.0580	N	S	Y
HKGG1M11	1	0.0000	N	N	N
HKGG1M12	2	0.0586	N	S	S
HKGG1M13	1	0.0000	N	N	S
HKGG1M14	3	0.0197	N	Y	L
HKGG1M15	2	0.0748	N	S	S
HKGG1M16	2	0.0606	N	S	N
HKGG1M17	1	0.0598	N	N	N
HKGG1M18	2	0.0000	N	S	L
HKGG1M19	2	0.0477	N	Y	L
HKGG1M20	3	0.0000	N	Y	L
HKGG1M21	1	0.0000	N	N	N
HKGG1M22	2	0.0000	N	S	N
HKGG1M23	2	0.0379	Y	S	L
HKGG1M24	3	0.0353	Y	Y	S
HKGG1M25	2	0.0000	N	Y	S
HKGG1M27	2	0.0669	N	N	S

Appendix G (*gunungcola backcross* continued)

No	Courtship score	Wing spot	Wing display	Circling	Body shaking
HKGG1M28	2	0.0000	N	N	S
HKGG1M29	3	0.0576	N	Y	Y
HKGG1M30	2	0.0000	N	S	L
HKGG1M31	-	0.0000	.	.	.
HKGG1M32	-	0.0000	.	.	.
HKGG1M33	3	0.0694	Y	Y	S
HKGG1M34	2	0.0737	N	S	L
HKGG1M35	2	0.0000	N	Y	S
HKGG1M36	2	0.0000	N	Y	S
HKGG1M37	-	0.0000	.	.	.
HKGG1M38	3	0.0436	N	Y	S
HKGG1M39	1	0.0457	N	N	N
HKGG2M01	2	0.0000	N	Y	S
HKGG2M02	-	0.0000	.	.	.
HKGG2M03	-	0.0743	.	.	.
HKGG2M04	3	0.0800	Y	Y	S
HKGG2M05	1	0.0000	N	N	N
HKGG2M06	1	0.0511	N	N	N
HKGG2M08	1	0.0000	N	N	N
HKGG2M09	2	0.0000	N	S	L
HKGG2M10	-	0.0000	.	.	.
HKGG2M11	3	0.0000	Y	S	L
HKGG2M12	2	0.0000	N	Y	L
HKGG2M13	2	0.0000	N	S	N
HKGG2M14	2	0.0000	N	S	L
HKGG2M15	1	-	N	.	N
HKGG2M16	1	0.0000	N	N	N
HKGG2M17	1	0.0460	N	N	N
HKGG2M18	2	0.0000	N	S	N
HKGG2M19	2	0.0000	N	Y	L
HKGG2M20	2	0.0546	N	S	L
HKGG2M21	1	0.0000	N	N	N
HKGG2M22	1	0.0000	N	N	N
HKGG2M23	1	0.0000	N	N	N
HKGG2M24	2	0.0000	N	N	L
HKGG2M25	1	0.0486	N	N	N

Appendix G (*gunungcola backcross* continued)

No	Courtship score	Wing spot	Wing display	Circling	Body shaking
HKGG2M26	1	0.0637	N	N	N
HKGG2M27	3	0.0605	Y	Y	S
HKGG2M28	1	0.0572	N	S	N
HKGG2M29	2	0.0000	N	Y	L
HKGG2M30	3	0.0000	Y	.	L
HKGG2M31	1	0.0000	N	N	N
HKGG2M32	2	0.0000	N	S	L
HKGG2M33	2	0.0000	N	Y	L
HKGG2M34	2	0.0000	N	Y	L
HKGG2M35	1	0.0000	Y	.	L
HKGG2M36	2	0.0755	N	S	L
GHKG1M01	1	0.0000	N	N	N
GHKG1M02	1	0.0000	N	N	N
GHKG1M03	1	0.0000	N	N	N
GHKG1M04	2	0.0000	N	N	L
GHKG1M05	3	0.0990	Y	Y	L
GHKG1M06	2	0.0000	N	N	N
GHKG1M07	1	-	N	N	N
GHKG1M08	4	0.0829	Y	Y	Y
GHKG1M09	3	0.0000	Y	Y	L
GHKG1M10	1	-	N	N	N
GHKG1M11	2.5	-	Y	S	N
GHKG1M12	2	0.0000	N	S	L
GHKG1M13	2	0.0718	N	Y	N
GHKG1M14	1	-	N	N	N
GHKG1M15	1	0.0396	N	N	N
GHKG1M16	2	0.0882	N	S	L
GHKG1M17	2	0.0760	N	Y	L
GHKG1M18	2	0.0000	N	Y	Y
GHKG1M19	2	0.0681	N	Y	L
GHKG1M20	2	0.0000	N	N	L
GHKG1M21	1	0.0000	N	N	N
GHKG1M22	1.5	0.0000	N	S	L
GHKG1M23	2	0.0591	N	Y	S
GHKG1M24	3	0.0617	N	Y	Y
GHKG1M25	1.5	0.0000	N	S	L

Appendix G (*gunungcola backcross* continued)

No	Courtship score	Wing spot	Wing display	Circling	Body shaking
GHKG1M27	1	0.0000	N	N	L
GHKG1M28	1	0.0000	N	N	N
GHKG1M29	2	0.0000	N	Y	L
GHKG1M30	3	0.0398	Y	S	S
GHKG1M31	1	0.0000	N	Y	N
GHKG1M32	1	0.0000	N	N	N
GHKG1M33	2	0.0000	N	Y	L
GHKG1M34	2	-	N	Y	L
GHKG1M35	1	0.0000	N	N	N
GHKG1M36	2	0.0000	Y	Y	L
GHKG1M37	2	0.0544	N	N	N
GHKG1M38	2	0.0462	N	S	L
GHKG1M39	2	0.0000	N	Y	L
HKGHK2M01	-	0.0000	.	.	.
HKGHK2M02	2	0.0488	N	N	N
HKGHK2M03	2	0.0398	N	S	N

Appendix H The genotypes of each backcross male.

	Locus					
No	*y*	*Moe*	*dy*	*sl*	*Tbh*	*cac*
elegans backcross						
HKGHK1M01	e	e	e	e	e	e
HKGHK1M02	g	g	g	g	g	g
HKGHK1M03	e	e	e	e	e	e
HKGHK1M04	e	e	e	g	e	g
HKGHK1M05	g	g	g	g	g	g
HKGHK1M06	g	g	e	e	g	e
HKGHK1M07	g	g	g	e	g	g
HKGHK1M08	e	e	g	g	e	g
HKGHK1M09	e	e	e	g	e	g
HKGHK1M10	g	e	g	g	e	g
HKGHK1M11	g	g	g	g	g	g
HKGHK1M12	e	e	e	e	e	e
HKGHK1M13	e	e	e	e	e	e
HKGHK1M14	g	g	e	e	g	e
HKGHK1M15	g	g	g	g	g	g
HKGHK1M16	g	g	g	g	g	g
HKGHK1M17	g	g	g	e	g	e
HKGHK1M18	g	g	g	e	g	e
HKGHK1M19	e	e	e	e	e	e
HKGHK1M20	g	g	g	g	g	g
HKGHK1M21	g	g	g	g	g	g
HKGHK1M22	g	g	g	g	e	g
HKGHK1M23	e	e	e	e	e	e
HKGHK1M24	e	e	e	e	e	e
HKGHK1M25	e	e	e	g	e	g
HKGHK1M26	g	.	.	.	g	g
HKGHK1M27	e	e	e	e	e	e
HKGHK1M28	e	e	e	e	e	e
HKGHK1M29	g	g	g	e	g	e
HKGHK1M30	e	e	e	e	e	e
HKGHK1M31	e	e	e	e	e	e

Appendix H (continued)

	Locus					
No	*y*	*Moe*	*dy*	*sl*	*Tbh*	*cac*
elegans backcross						
HKGHK1M32	g	g	e	e	g	e
HKGHK1M33	e	e	e	e	e	e
HKGHK1M34	e	e	e	e	e	e
HKGHK1M35	e	e	e	e	e	e
HKGHK1M36	g	g	e	g	g	g
HKGHK1M37	e	e	e	e	e	e
HKGHK1M38	g	g	e	g	g	g
HKGHK1M39	e	e	e	g	e	g
HKGHK1M40	e	e	e	e	e	e
HKGHK1M41	g	g	e	g	g	g
HKGHK1M42	g	g	e	e	g	e
HKGHK1M43	g	g	e	g	g	g
HKGHK1M45	g	g	e	g	g	g
HKGHK1M46	e	e	e	e	e	e
HKGHK1M47	e	e	e	e	e	e
HKGHK1M48	g	g	e	g	g	g
HKGHK1M49	e	e	e	e	e	e
HKGHK1M50	g	g	e	g	e	e
HKGHK1M51	e	e	e	e	e	e
HKGHK2M04	e	e	e	g	e	g
HKGHK2M05	e	e	e	g	e	g
HKGHK2M06	e	e	e	e	e	e
HKGHK2M07	e	e	e	e	e	e
HKGHK2M08	e	e	e	g	e	g
HKGHK2M09	e	e	e	e	e	e
HKGHK2M10	e	e	e	e	e	e
HKGHK2M12	e	e	e	e	e	e
HKGHK2M13	e	e	e	e	e	e
HKGHK2M15	e	e	e	e	e	e
HKGHK2M16	e	e	e	e	e	e
HKGHK2M18	g	g	e	g	e	g

Appendix H (continued)

	Locus					
No	*y*	*Moe*	*dy*	*sl*	*Tbh*	*cac*
elegans backcross						
HKGHK2M19	g	g	e	g	g	g
HKGHK2M20	g	g	e	g	g	g
HKGHK2M21	g	g	e	g	g	g
HKGHK2M22	g	g	e	g	g	g
HKGHK2M23	e	e	e	e	e	e
HKGHK2M24	g	g	e	g	g	e
HKGHK2M25	e	e	e	e	e	e
HKGHK2M26	g	g	e	g	g	g
HKGHK2M27	g	e	e	e	e	g
HKGHK2M28	g	g	e	e	g	e
HKGHK2M29	g	g	e	e	g	e
HKGHK2M30	e	e	e	e	e	e
HKGHK2M31	e	e	e	g	e	g
HKGHK2M32	g	g	e	g	g	g
HKGHK2M33	e	e	e	e	e	e
HKGHK2M34	e	e	e	e	e	e
HKGHK2M35	e	e	e	e	e	e
HKGHK2M36	g	g	e	g	g	g
HKGHK2M37	.	e	e	e	e	e
HKGHK2M38	e	e	e	g	e	e
HKGHK2M39	e	e	e	g	e	g
HKGHK2M40	e	e	e	e	e	e
HKGHK2M41	g	g	e	e	e	e
HKGHK2M42	e	e	e	e	e	e
HKGHK2M43	e	e	e	e	e	e
HKGHK2M45	g	g	e	g	g	g
HKGHK2M46	e	e	e	e	e	e
HKGHK2M47	g	g	e	g	g	g
HKGHK2M48	g	g	e	g	g	g
HKGHK2M49	e	e	e	g	e	g
GHKHK1M01	e	e	e	e	e	e

Appendix H (continued)

	Locus					
No	*y*	*Moe*	*dy*	*sl*	*Tbh*	*cac*
elegans backcross						
GHKHK1M02	e	e	e	e	e	e
GHKHK1M03	e	e	e	e	e	e
GHKHK1M04	g	g	e	g	g	g
GHKHK1M05	g	g	e	g	g	g
GHKHK1M06	g	g	e	g	g	g
GHKHK1M07	g	g	e	g	g	g
GHKHK1M08	e	e	e	e	e	e
GHKHK1M09	g	g	e	e	g	e
GHKHK1M10	g	g	e	e	g	e
GHKHK1M11	e	e	e	e	e	e
GHKHK1M12	e	e	e	e	e	e
GHKHK1M13	e	e	e	e	e	e
GHKHK1M14	g	g	e	g	g	g
GHKHK1M15	e	e	e	e	e	e
GHKHK1M16	e	e	e	e	e	e
GHKHK1M17	g	g	e	g	g	g
GHKHK1M18	e	e	e	e	e	e
GHKHK1M19	g	g	e	e	g	g
GHKHK1M20	e	e	e	g	e	g
GHKHK1M21	e	e	e	e	g	e
GHKHK1M22	e	e	e	e	e	e
GHKHK1M23	g	g	e	g	g	g
GHKHK1M24	e	e	e	e	e	e
GHKHK1M25	g	g	e	g	g	g
GHKHK1M26	g	g	e	g	e	g
GHKHK1M27	e	e	e	e	e	e
GHKHK1M28	e	e	e	e	e	e
GHKHK1M29	e	e	e	e	e	e
GHKHK1M30	e	e	e	e	e	e
GHKHK1M31	g	e	e	g	e	g
GHKHK1M32	e	e	e	e	e	e

Appendix H (continued)

	Locus					
No	*y*	*Moe*	*dy*	*sl*	*Tbh*	*cac*
elegans backcross						
GHKHK1M33	e	e	e	e	e	e
GHKHK1M34	e	e	e	e	e	e
GHKHK1M35	e	e	e	e	e	e
GHKHK1M36	g	g	e	e	g	e
GHKHK1M37	g	g	e	g	g	g
GHKHK1M38	e	e	e	e	e	e
GHKHK1M39	g	e	e	g	e	g
GHKHK1M40	g	g	e	g	g	g
GHKHK1M41	g	g	e	e	g	e
GHKHK1M42	e	e	e	e	e	e
GHKHK1M43	e	e	e	e	e	e
GHKHK1M44	g	g	e	e	g	g
GHKHK1M45	g	g	e	g	g	g
GHKHK1M46	e	e	e	e	e	e
GHKHK1M47	g	g	e	g	g	g
GHKHK1M48	e	e	e	e	e	e
GHKHK1M49	e	e	e	e	e	e
GHKHK1M50	g	g	e	e	g	e
GHKHK1M51	g	g	e	e	g	e
GHKHK1M53	e	e	e	g	e	g
GHKHK1M54	e	e	e	e	e	e
GHKHK1M55	e	e	e	e	e	e
GHKHK1M57	g	g	e	g	g	g
GHKHK1M58	e	e	e	e	e	e
GHKHK1M59	e	.	e	g	.	e
GHKHK1M60	e	e	e	e	e	e
GHKHK1M61	g	g	e	e	g	g
HKGG1M01	e	e	e	e	e	e
HKGG1M02	e	e	e	e	e	e

Appendix H (continued)

No	Locus					
	y	*Moe*	*dy*	*sl*	*Tbh*	*cac*
gunungcola backcross						
HKGG1M03	g	g	g	g	g	g
HKGG1M04	g	g	g	g	g	g
HKGG1M05	g	g	g	g	g	g
HKGG1M06	e	e	e	e	e	e
HKGG1M07	e	e	.	e	e	e
HKGG1M08	e	e	e	e	e	e
HKGG1M09	e	e	e	g	e	g
HKGG1M10	e	g	e	e	g	e
HKGG1M11	g	g	g	g	g	g
HKGG1M12	e	e	e	g	e	g
HKGG1M13	g	g	e	e	g	e
HKGG1M14	e	e	e	e	.	e
HKGG1M15	e	e	.	e	e	e
HKGG1M16	e	e	g	g	g	e
HKGG1M17	e	e	g	e	e	e
HKGG1M18	e	e	e	g	e	g
HKGG1M19	e	e	e	e	e	e
HKGG1M20	g	g	e	e	g	e
HKGG1M21	g	g	g	g	g	g
HKGG1M22	g	g	g	g	g	g
HKGG1M23	e	e	e	e	e	e
HKGG1M24	e	e	e	e	e	e
HKGG1M25	g	g	g	g	g	g
HKGG1M27	e	e	e	g	e	g
HKGG1M28	g	g	g	e	g	g
HKGG1M29	e	e	e	e	e	e
HKGG1M30	g	g	g	g	g	g
HKGG1M31	g	g	g	g	g	g
HKGG1M32	e	e	e	e	e	e
HKGG1M33	g	g	g	e	g	e
HKGG1M34	e	e	e	e	e	e

Appendix H (continued)

No	Locus					
	y	*Moe*	*dy*	*sl*	*Tbh*	*cac*
gunungcola backcross						
HKGG1M35	g	g	g	e	g	e
HKGG1M36	g	g	g	g	g	g
HKGG1M37	g	g	g	g	g	g
HKGG1M38	e	e	e	e	e	e
HKGG1M39	e	e	g	g	e	g
HKGG2M01	g	g	e	e	g	e
HKGG2M02	g	.	g	e	g	e
HKGG2M03	e	e	e	g	e	e
HKGG2M04	e	e	e	e	e	e
HKGG2M05	g	g	g	g	g	g
HKGG2M06	e	e	e	e	e	e
HKGG2M08	g	g	g	g	g	g
HKGG2M09	e	e	e	e	e	e
HKGG2M10	g	g	g	g	g	g
HKGG2M11	g	g	g	g	g	g
HKGG2M12	g	g	g	g	g	g
HKGG2M13	g	g	g	g	g	g
HKGG2M14	g	g	g	g	g	g
HKGG2M15	e	e	g	g	e	g
HKGG2M16	g	g	g	g	g	g
HKGG2M17	e	e	e	e	e	e
HKGG2M18	g	g	g	e	g	e
HKGG2M19	g	g	g	g	g	g
HKGG2M20	e	e	e	e	e	e
HKGG2M21	g	g	g	g	g	g
HKGG2M22	g	g	g	g	g	g
HKGG2M23	g	g	g	g	g	g
HKGG2M24	g	g	g	g	g	g
HKGG2M25	e	e	e	e	e	e
HKGG2M26	e	e	e	e	e	e
HKGG2M27	e	e	e	e	g	e

Appendix H (continued)

No	Locus					
	y	*Moe*	*dy*	*sl*	*Tbh*	*cac*
gunungcola backcross						
HKGG2M28	e	e	e	e	e	e
HKGG2M29	g	g	e	e	g	e
HKGG2M30	g	g	g	g	g	g
HKGG2M31	g	g	g	g	g	g
HKGG2M32	g	g	g	g	g	g
HKGG2M33	g	g	g	g	g	g
HKGG2M34	g	g	g	g	g	g
HKGG2M35	g	g	g	g	g	g
HKGG2M36	e	e	e	e	e	e
GHKG1M01	g	g	g	g	g	g
GHKG1M02	g	e	g	g	e	g
GHKG1M03	g	g	g	g	g	g
GHKG1M04	g	g	g	g	g	g
GHKG1M05	e	e	e	g	e	g
GHKG1M06	g	g	g	g	g	g
GHKG1M07	e	e	g	g	e	g
GHKG1M08	e	e	e	e	e	e
GHKG1M09	g	g	g	g	g	g
GHKG1M10	.	e	g	g	e	g
GHKG1M11	e	e	e	e	e	e
GHKG1M12	g	g	g	g	g	g
GHKG1M13	e	e	e	e	e	e
GHKG1M14	.	e	.	e	g	g
GHKG1M15	e	e	e	e	e	e
GHKG1M16	e	e	e	e	e	e
GHKG1M17	e	e	e	e	e	e
GHKG1M18	g	g	g	g	g	g
GHKG1M19	e	e	e	e	e	e
GHKG1M20	g	g	g	g	g	g
GHKG1M21	e	e	e	e	e	e
GHKG1M22	g	g	g	e	e	e

Appendix H (continued)

No	Locus					
	y	*Moe*	*dy*	*sl*	*Tbh*	*cac*
gunungcola backcross						
GHKG1M23	e	e	e	e	e	e
GHKG1M24	e	e	e	e	e	e
GHKG1M25	g	g	g	g	g	g
GHKG1M27	e	g	e	e	g	e
GHKG1M28	g	g	g	g	g	g
GHKG1M29	g	g	g	g	g	g
GHKG1M30	e	e	e	e	e	e
GHKG1M31	g	g	g	g	g	g
GHKG1M32	g	g	g	g	g	g
GHKG1M33	g	g	g	g	g	g
GHKG1M34	.	e	.	e	e	e
GHKG1M35	g	g	g	e	g	e
GHKG1M36	g	g	g	g	g	g
GHKG1M37	e	e	g	g	e	g
GHKG1M38	e	e	e	g	e	g
GHKG1M39	g	g	e	e	g	e
HKGHK2M01	e	e	e	g	e	g
HKGHK2M02	e	e	e	g	g	g
HKGHK2M03	e	e	e	e	e	e

Appendix H (continued)

No	Locus					
	os	*CG2658*	*t*	*up*	*CG11943*	*ple*
elegans backcross						
HKGHK1M01	e	e	e	e	e/g	e/g
HKGHK1M02	g	g	g	g	e/g	e/g
HKGHK1M03	e	e	e	e	e/g	e
HKGHK1M04	e	e	e	e	e/g	e/g
HKGHK1M05	g	g	g	g	e	e/g
HKGHK1M06	g	g	g	g	e	e/g
HKGHK1M07	g	g	g	g	e	e
HKGHK1M08	e	e	e	e	e	e
HKGHK1M09	e	e	g	e	e/g	e
HKGHK1M10	e	e	e	e	e/g	e
HKGHK1M11	g	g	g	g	e	e
HKGHK1M12	e	e	e	e	e/g	e/g
HKGHK1M13	e	e	e	e	e/g	e
HKGHK1M14	g	g	g	g	e/g	e
HKGHK1M15	g	g	g	g	e/g	e/g
HKGHK1M16	g	g	g	g	e	e/g
HKGHK1M17	g	.	g	g	e/g	e
HKGHK1M18	g	g	g	g	e/g	e/g
HKGHK1M19	e	e	e	e	e/g	e/g
HKGHK1M20	g	g	g	g	e	e
HKGHK1M21	g	g	g	g	e/g	e/g
HKGHK1M22	e	g	e	e	e	e
HKGHK1M23	e	e	e	e	e	e/g
HKGHK1M24	e	e	e	e	e	e/g
HKGHK1M25	e	e	e	e	e	e/g
HKGHK1M26	g	g	e	g	e/g	e
HKGHK1M27	e	e	e	e	e	e/g
HKGHK1M28	e	e	e	e	e/g	e
HKGHK1M29	g	g	g	g	e	e
HKGHK1M30	e	e	e	e	e/g	e/g
HKGHK1M31	e	e	e	e	e	e/g

Appendix H (continued)

No	Locus					
	os	*CG2658*	*t*	*up*	*CG11943*	*ple*
elegans backcross						
HKGHK1M32	g	g	g	g	e/g	e/g
HKGHK1M33	e	e	e	e	e/g	e
HKGHK1M34	e	e	e	e	e/g	e/g
HKGHK1M35	e	e	e	e	e/g	e
HKGHK1M36	g	g	g	g	e/g	e
HKGHK1M37	e	e	e	e	e	e
HKGHK1M38	g	g	g	g	e/g	e
HKGHK1M39	e	e	e	e	e	e
HKGHK1M40	e	e	e	e	e	e
HKGHK1M41	e	e	e	e	e/g	e/g
HKGHK1M42	g	g	g	g	e/g	e/g
HKGHK1M43	g	g	g	g	e/g	e
HKGHK1M45	g	g	g	g	e	e
HKGHK1M46	e	e	e	e	e/g	e
HKGHK1M47	e	e	e	e	e/g	e/g
HKGHK1M48	g	g	g	g	e	e/g
HKGHK1M49	e	e	e	e	e	e
HKGHK1M50	e	g	e	e	e/g	e
HKGHK1M51	e	e	.	e	e/g	e/g
HKGHK2M04	e	e	e	e	e/g	e
HKGHK2M05	e	e	e	e	e/g	e/g
HKGHK2M06	e	e	e	e	e/g	e/g
HKGHK2M07	e	g	g	e	e/g	e
HKGHK2M08	e	e	e	e	e	e/g
HKGHK2M09	e	e	e	e	e/g	e/g
HKGHK2M10	e	e	e	e	e	e
HKGHK2M12	e	g	g	.	e/g	e/g
HKGHK2M13	e	e	e	e	e/g	e/g
HKGHK2M15	g	g	g	g	e	e
HKGHK2M16	e	e	e	e	e	e
HKGHK2M18	e	e	e	e	e	e/g

Appendix H (continued)

No	Locus					
	os	*CG2658*	*t*	*up*	*CG11943*	*ple*
elegans backcross						
HKGHK2M19	g	g	g	g	e	e/g
HKGHK2M20	g	g	g	g	e/g	e
HKGHK2M21	g	g	g	g	e	e
HKGHK2M22	g	g	g	g	e	e/g
HKGHK2M23	e	e	e	e	e	e
HKGHK2M24	g	e	e	g	e/g	e
HKGHK2M25	e	e	e	e	e/g	e
HKGHK2M26	g	g	g	g	e/g	e
HKGHK2M27	e	e	e	e	e	e/g
HKGHK2M28	g	g	g	g	e/g	e/g
HKGHK2M29	g	g	g	g	e/g	e
HKGHK2M30	e	e	e	e	e	e/g
HKGHK2M31	e	e	e	e	e	e/g
HKGHK2M32	g	g	g	g	e	e/g
HKGHK2M33	e	e	e	e	e/g	e
HKGHK2M34	e	e	e	e	e/g	e
HKGHK2M35	g	g	g	g	e	e/g
HKGHK2M36	g	g	g	g	e/g	e/g
HKGHK2M37	e	e	e	e	e/g	e
HKGHK2M38	e	e	e	e	e/g	e/g
HKGHK2M39	e	e	e	e	e/g	e/g
HKGHK2M40	e	e	e	e	e/g	e/g
HKGHK2M41	g	g	e	g	e/g	e
HKGHK2M42	e	e	e	e	e	e/g
HKGHK2M43	e	e	e	e	e	e
HKGHK2M45	g	g	g	g	e/g	e
HKGHK2M46	e	e	e	e	e/g	e
HKGHK2M47	g	g	g	g	e	e
HKGHK2M48	g	g	g	g	e	e
HKGHK2M49	e	e	e	e	e/g	e
GHKHK1M01	e	e	e	e	e/g	e

Appendix H (continued)

	Locus					
No	*os*	*CG2658*	*t*	*up*	*CG11943*	*ple*
elegans backcross						
GHKHK1M02	e	e	e	e	e/g	e
GHKHK1M03	e	e	e	e	e	e
GHKHK1M04	g	g	g	g	e	e/g
GHKHK1M05	g	g	g	g	e	e
GHKHK1M06	g	e	e	g	e/g	e
GHKHK1M07	g	g	g	g	e/g	e
GHKHK1M08	e	e	e	e	e	e
GHKHK1M09	g	g	g	g	e	e/g
GHKHK1M10	g	g	e	g	e	e/g
GHKHK1M11	e	e	e	e	e	e/g
GHKHK1M12	g	g	g	g	e/g	e
GHKHK1M13	e	e	e	e	e/g	e/g
GHKHK1M14	g	g	g	g	e/g	e
GHKHK1M15	e	e	e	e	e/g	e
GHKHK1M16	e	e	e	e	e	e
GHKHK1M17	g	g	g	g	e	e/g
GHKHK1M18	e	e	e	e	e	e/g
GHKHK1M19	g	g	e	g	e	e/g
GHKHK1M20	e	e	e	e	e	e
GHKHK1M21	g	g	g	g	e/g	e/g
GHKHK1M22	e	e	e	e	e/g	e
GHKHK1M23	g	g	e	g	e/g	e/g
GHKHK1M24	e	e	e	e	e/g	e/g
GHKHK1M25	g	g	e	g	e/g	e
GHKHK1M26	e	e	e	e	e	e/g
GHKHK1M27	e	e	g	e	e/g	e
GHKHK1M28	e	e	e	e	e	e/g
GHKHK1M29	g	g	g	g	e	e/g
GHKHK1M30	e	e	e	e	e/g	e
GHKHK1M31	e	e	g	e	e/g	e
GHKHK1M32	e	e	e	e	e/g	e

Appendix H (continued)

No	Locus					
	os	*CG2658*	*t*	*up*	*CG11943*	*ple*
elegans backcross						
GHKHK1M33	e	e	e	e	e/g	e
GHKHK1M34	e	e	g	e	e/g	e/g
GHKHK1M35	e	e	e	e	e/g	e
GHKHK1M36	g	g	g	g	e/g	e/g
GHKHK1M37	g	g	g	g	e/g	e
GHKHK1M38	e	e	e	e	e	e
GHKHK1M39	e	g	e	e	e/g	e/g
GHKHK1M40	g	g	g	g	e	e
GHKHK1M41	g	g	g	g	e	e
GHKHK1M42	e	e	e	e	e/g	e
GHKHK1M43	e	e	e	e	e	e
GHKHK1M44	g	g	g	g	e/g	e/g
GHKHK1M45	g	g	g	g	e/g	e/g
GHKHK1M46	e	e	e	e	e/g	e/g
GHKHK1M47	g	g	g	g	e	e/g
GHKHK1M48	e	e	e	.	e/g	e/g
GHKHK1M49	e	e	e	e	e	e/g
GHKHK1M50	g	g	g	g	e/g	e/g
GHKHK1M51	g	g	g	g	e	e
GHKHK1M53	e	e	e	e	e	e/g
GHKHK1M54	e	e	e	e	e	e/g
GHKHK1M55	.	e	e	e	e/g	e/g
GHKHK1M57	g	e	g	g	e/g	e
GHKHK1M58	e	e	e	e	e	e
GHKHK1M59	.	g	g	.	e/g	.
GHKHK1M60	e	e	e	e	e/g	e
GHKHK1M61	g	g	g	g	e/g	e
HKGG1M01	e	e	e	e	e/g	e/g
HKGG1M02	e	e	e	e	e/g	e/g

Appendix H (continued)

No	Locus					
	os	*CG2658*	*t*	*up*	*CG11943*	*ple*
gunungcola backcross						
HKGG1M03	g	g	g	g	e/g	g
HKGG1M04	g	g	g	g	e/g	e/g
HKGG1M05	g	g	g	g	e/g	e/g
HKGG1M06	g	g	g	g	g	g
HKGG1M07	e	e	e	.	e/g	g
HKGG1M08	e	e	e	g	g	e/g
HKGG1M09	e	e	e	e	e/g	g
HKGG1M10	g	g	g	g	e/g	e/g
HKGG1M11	g	g	g	g	e/g	e/g
HKGG1M12	e	e	e	g	e/g	e/g
HKGG1M13	g	g	g	g	e/g	g
HKGG1M14	e	g	g	g	e/g	e/g
HKGG1M15	e	e	e	.	e/g	e/g
HKGG1M16	g	g	g	g	e/g	e/g
HKGG1M17	e	e	e	e	g	e/g
HKGG1M18	e	g	g	e	e/g	g
HKGG1M19	.	e	e	e	e/g	e/g
HKGG1M20	g	g	g	g	g	e/g
HKGG1M21	e	e	e	e	g	e/g
HKGG1M22	g	g	g	g	e/g	g
HKGG1M23	e	e	e	e	e/g	e/g
HKGG1M24	e	e	e	g	e/g	g
HKGG1M25	g	g	g	g	e/g	e/g
HKGG1M27	e	e	e	g	e/g	e/g
HKGG1M28	g	g	g	g	e/g	e/g
HKGG1M29	e	e	e	g	e/g	e/g
HKGG1M30	g	g	g	g	e/g	g
HKGG1M31	g	e	e	g	e/g	e/g
HKGG1M32	e	e	e	g	e/g	g
HKGG1M33	g	g	g	g	e/g	g
HKGG1M34	e	e	e	g	g	e/g

Appendix H (continued)

No	Locus					
	os	*CG2658*	*t*	*up*	*CG11943*	*ple*
gunungcola backcross						
HKGG1M35	g	e	e	g	e/g	e/g
HKGG1M36	g	g	e	g	g	g
HKGG1M37	g	e	e	g	g	g
HKGG1M38	e	g	g	g	e/g	e/g
HKGG1M39	e	e	e	g	g	g
HKGG2M01	g	g	g	g	e/g	e/g
HKGG2M02	g	e	.	g	e/g	e/g
HKGG2M03	e	g	e	e	e/g	g
HKGG2M04	e	g	e	e	e/g	e/g
HKGG2M05	g	e	e	g	g	g
HKGG2M06	e	e	e	e	g	e/g
HKGG2M08	g	g	g	g	g	g
HKGG2M09	e	e	e	e	g	e/g
HKGG2M10	e	e	e	e	g	g
HKGG2M11	g	g	g	g	e/g	g
HKGG2M12	g	g	g	g	e/g	e/g
HKGG2M13	g	g	g	g	g	g
HKGG2M14	g	g	g	g	e/g	g
HKGG2M15	e	e	e	e	e/g	g
HKGG2M16	g	e	g	g	g	e/g
HKGG2M17	e	e	e	e	g	e/g
HKGG2M18	g	g	g	g	g	g
HKGG2M19	g	g	g	g	g	e/g
HKGG2M20	e	e	e	e	g	g
HKGG2M21	g	g	g	g	g	e/g
HKGG2M22	g	g	g	g	e/g	e/g
HKGG2M23	g	g	g	g	e/g	g
HKGG2M24	g	g	g	g	e/g	e/g
HKGG2M25	e	e	e	e	g	e/g
HKGG2M26	e	e	e	e	e/g	g
HKGG2M27	g	g	g	g	g	g

Appendix H (continued)

No	Locus					
	os	*CG2658*	*t*	*up*	*CG11943*	*ple*
gunungcola backcross						
HKGG2M28	e	e	e	e	e/g	g
HKGG2M29	g	g	g	g	e/g	e/g
HKGG2M30	g	g	g	g	g	g
HKGG2M31	g	g	g	g	g	e/g
HKGG2M32	g	g	g	g	g	g
HKGG2M33	g	g	g	g	e/g	e/g
HKGG2M34	g	g	g	g	g	g
HKGG2M35	g	g	g	g	g	e/g
HKGG2M36	e	e	e	e	e/g	e/g
GHKG1M01	g	g	g	g	e/g	g
GHKG1M02	e	e	e	e	g	g
GHKG1M03	g	g	g	g	e/g	g
GHKG1M04	g	g	g	g	g	e/g
GHKG1M05	e	e	e	e	g	e/g
GHKG1M06	g	g	g	g	e/g	e/g
GHKG1M07	e	e	e	e	e/g	g
GHKG1M08	e	e	e	e	e/g	e/g
GHKG1M09	g	g	g	g	e/g	g
GHKG1M10	e	e	e	e	g	e/g
GHKG1M11	e	g	g	g	g	e/g
GHKG1M12	e	e	e	e	e/g	e/g
GHKG1M13	e	e	e	e	e/g	e/g
GHKG1M14	e	g	e	g	g	e/g
GHKG1M15	g	g	g	g	e/g	e/g
GHKG1M16	e	e	e	e	e/g	g
GHKG1M17	e	e	e	e	e/g	e/g
GHKG1M18	e	g	g	g	e/g	e/g
GHKG1M19	e	e	e	e	g	e/g
GHKG1M20	g	g	g	g	e/g	e/g
GHKG1M21	e	e	e	e	g	g
GHKG1M22	e	e	e	e	e/g	e/g

Appendix H (continued)

No	Locus					
	os	*CG2658*	*t*	*up*	*CG11943*	*ple*
gunungcola backcross						
GHKG1M23	e	e	e	e	e/g	e/g
GHKG1M24	e	e	g	e	e/g	e/g
GHKG1M25	g	g	g	g	g	g
GHKG1M27	g	g	g	g	g	e/g
GHKG1M28	g	g	g	g	g	g
GHKG1M29	g	g	g	g	g	e/g
GHKG1M30	e	e	g	e	e/g	e/g
GHKG1M31	g	e	e	g	e/g	e/g
GHKG1M32	g	g	g	g	e/g	g
GHKG1M33	g	e	e	g	e/g	e/g
GHKG1M34	e	g	e	e	e/g	g
GHKG1M35	g	g	g	g	g	g
GHKG1M36	g	g	g	g	e/g	g
GHKG1M37	e	e	e	e	g	e/g
GHKG1M38	e	e	e	e	g	e/g
GHKG1M39	g	g	g	g	e/g	g
HKGHK2M01	e	e	e	g	g	g
HKGHK2M02	e	e	e	e	g	g
HKGHK2M03	e	e	e	g	g	g

Appendix H (continued)

No	Locus					
	Clk	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
elegans backcross						
HKGHK1M01	e/g	e/g	e	e/g	e	e/g
HKGHK1M02	e/g	e	e/g	e	e/g	e/g
HKGHK1M03	e	e	e	e	e	e/g
HKGHK1M04	e/g	e	e	e	e	e
HKGHK1M05	e/g	e/g	e/g	e/g	e/g	e
HKGHK1M06	e/g	e	e/g	e	e/g	e
HKGHK1M07	e	e/g	e/g	e/g	e/g	e
HKGHK1M08	e	e/g	e	e/g	e	e
HKGHK1M09	e	e	e/g	e	e	e/g
HKGHK1M10	e	e	e	e	e/g	e/g
HKGHK1M11	e/g	e	e/g	e	e/g	e/g
HKGHK1M12	e/g	e	e	e	e	e/g
HKGHK1M13	e	e/g	e	e/g	e	e/g
HKGHK1M14	e	e	e	e	e	e/g
HKGHK1M15	e/g	e/g	e	e/g	e	e/g
HKGHK1M16	e/g	e	e/g	e	e/g	e
HKGHK1M17	e	e	e/g	e	e/g	e/g
HKGHK1M18	e/g	e	e	e/g	e	e/g
HKGHK1M19	e/g	e	e	e	e	e/g
HKGHK1M20	e	e/g	e	e/g	e	e
HKGHK1M21	e	e/g	e	e/g	e	e/g
HKGHK1M22	e	e	e	e	e	e
HKGHK1M23	e/g	e/g	e/g	e/g	e/g	e
HKGHK1M24	e/g	e/g	e	e/g	e	e
HKGHK1M25	e/g	e/g	e	e/g	e	e
HKGHK1M26	e	e	e	e/g	e	e/g
HKGHK1M27	e/g	e/g	e/g	e/g	e/g	e
HKGHK1M28	e/g	e/g	e	e/g	e	e/g
HKGHK1M29	e	e	e/g	e	e/g	e
HKGHK1M30	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M31	e/g	e/g	e	e/g	e	e

Appendix H (continued)

	Locus					
No	*Clk*	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
elegans backcross						
HKGHK1M32	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M33	e	e	e	e	e/g	e/g
HKGHK1M34	e	e	e/g	e	e/g	e/g
HKGHK1M35	e	e/g	e/g	e/g	e/g	e/g
HKGHK1M36	e	e	e	e	e	e/g
HKGHK1M37	e	e	e	e/g	e	e
HKGHK1M38	e	e/g	e/g	e/g	e/g	e/g
HKGHK1M39	e	e/g	e/g	e/g	e/g	e
HKGHK1M40	e	e	e	e	e	e
HKGHK1M41	e/g	e/g	e/g	e/g	e	e/g
HKGHK1M42	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M43	e	e	e	e	e	e/g
HKGHK1M45	e	e	e/g	e	e/g	e
HKGHK1M46	e	e	e	e	e	e/g
HKGHK1M47	e/g	e	e	e	e	e/g
HKGHK1M48	e/g	e/g	e/g	e/g	e/g	e
HKGHK1M49	e	e	e/g	e	e/g	e
HKGHK1M50	e	e	e/g	e	e	e/g
HKGHK1M51	e	e/g	e	e/g	e/g	e/g
HKGHK2M04	e	e/g	e/g	e	e/g	e/g
HKGHK2M05	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M06	e/g	e	e/g	e	e	e/g
HKGHK2M07	e	e	e/g	e	e	e/g
HKGHK2M08	e/g	e	e	e	e/g	e
HKGHK2M09	.	e	e/g	e	e/g	e/g
HKGHK2M10	e	e	e/g	e	e/g	e
HKGHK2M12	e	e	e/g	e	e/g	e/g
HKGHK2M13	e/g	e	e/g	e	e/g	e/g
HKGHK2M15	e	e/g	e	e/g	e	e
HKGHK2M16	e	e/g	e	e	e	e
HKGHK2M18	e/g	e/g	e/g	e/g	e/g	e

Appendix H (continued)

	Locus					
No	*Clk*	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
elegans backcross						
HKGHK2M19	e/g	e/g	e/g	e/g	e/g	e
HKGHK2M20	e	e	e	e	e	e/g
HKGHK2M21	e	e/g	e/g	e/g	e/g	e
HKGHK2M22	e/g	e/g	e/g	e/g	e/g	e
HKGHK2M23	e	e/g	e/g	e/g	e/g	e
HKGHK2M24	e	e/g	e/g	e	e/g	e/g
HKGHK2M25	e	e/g	e/g	e	e/g	e/g
HKGHK2M26	e	e/g	e/g	e/g	e/g	e/g
HKGHK2M27	e/g	e	e/g	e	e/g	e
HKGHK2M28	e/g	e	e/g	e	e/g	e/g
HKGHK2M29	e	e/g	e	e/g	e	e/g
HKGHK2M30	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M31	e/g	e/g	e/g	e/g	e/g	e
HKGHK2M32	e/g	e/g	e/g	e/g	e/g	e
HKGHK2M33	e	e/g	e	e/g	e	e/g
HKGHK2M34	e	e	e/g	e	e/g	e/g
HKGHK2M35	e/g	e	e/g	e	e	e
HKGHK2M36	e/g	e	e/g	e	e	e/g
HKGHK2M37	e	e/g	e/g	e/g	e	e/g
HKGHK2M38	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M39	e	e	e/g	e	e	e/g
HKGHK2M40	e/g	e	e	e	e/g	e/g
HKGHK2M41	e/g	e	e/g	e	e/g	e/g
HKGHK2M42	e/g	e	e/g	e	e/g	e
HKGHK2M43	e	e	e	e	e/g	e
HKGHK2M45	e	e	e	e	e/g	e/g
HKGHK2M46	e	e	e	e	e/g	e/g
HKGHK2M47	e	e/g	e/g	e/g	e/g	e
HKGHK2M48	e	e/g	e	e/g	e/g	e
HKGHK2M49	e	e/g	e/g	e/g	e	e/g
GHKHK1M01	e	e	e/g	e	e/g	e/g

Appendix H (continued)

No	Locus					
	Clk	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
elegans backcross						
GHKHK1M02	e	e	e/g	e	e	e
GHKHK1M03	e	e	e	e	e	e
GHKHK1M04	e/g	e	e/g	e/g	e/g	e
GHKHK1M05	e	e/g	e/g	e/g	e/g	e
GHKHK1M06	e	e	e/g	e	e/g	e/g
GHKHK1M07	e	e/g	e	e/g	e	e/g
GHKHK1M08	e/g	e	e/g	e/g	e/g	e
GHKHK1M09	e/g	e/g	e/g	e/g	e/g	e
GHKHK1M10	e/g	e/g	e	e	e	e
GHKHK1M11	e/g	e/g	e	e/g	e	e
GHKHK1M12	e	e	e/g	e	e/g	e/g
GHKHK1M13	e/g	e	e	e	e/g	e/g
GHKHK1M14	e	e/g	e	e	e	e/g
GHKHK1M15	e	e	e/g	e	e/g	e/g
GHKHK1M16	e	e/g	e/g	e/g	e/g	e
GHKHK1M17	e/g	e	e	e	e	e
GHKHK1M18	e/g	e	e	e	e	e
GHKHK1M19	e/g	e	e/g	e	e/g	e
GHKHK1M20	e	e/g	e	e/g	e	e
GHKHK1M21	e/g	e	e/g	e	e/g	e/g
GHKHK1M22	e	e	e/g	e	e/g	e/g
GHKHK1M23	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M24	e/g	e/g	e	e/g	e	e/g
GHKHK1M25	e	e/g	e/g	e/g	e/g	e/g
GHKHK1M26	e/g	e/g	e	e/g	e/g	e
GHKHK1M27	e	e/g	e	e/g	e	e/g
GHKHK1M28	e/g	e	e	e	e/g	e
GHKHK1M29	e/g	e	e/g	e	e/g	e
GHKHK1M30	e	e/g	e	e/g	e	e/g
GHKHK1M31	e	e/g	e	e/g	e	e/g
GHKHK1M32	e	e/g	e	e/g	e	e/g

Appendix H (continued)

No	Locus					
	Clk	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
elegans backcross						
GHKHK1M33	e	e	e/g	e	e	e/g
GHKHK1M34	e/g	e	e/g	e	e/g	e/g
GHKHK1M35	e	e	e	e	e	e
GHKHK1M36	e/g	e/g	e	e/g	e	e/g
GHKHK1M37	e	e	e	e/g	e	e/g
GHKHK1M38	e	e	e/g	e/g	e/g	e
GHKHK1M39	e/g	e	e	e/g	e	e/g
GHKHK1M40	e	e/g	e/g	e/g	e/g	e
GHKHK1M41	e	e	e/g	e	e/g	e
GHKHK1M42	e	e/g	e/g	e/g	e/g	e/g
GHKHK1M43	e	e/g	e/g	e/g	e/g	e
GHKHK1M44	e/g	e/g	e	e/g	e	e/g
GHKHK1M45	e/g	e	e	e/g	e	e/g
GHKHK1M46	e/g	e	e	e	e	e/g
GHKHK1M47	e/g	e	e	e	e	e
GHKHK1M48	.	e	e	e/g	e	e/g
GHKHK1M49	e/g	e/g	e/g	e/g	e/g	e
GHKHK1M50	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M51	e	e/g	e	e/g	e	e
GHKHK1M53	e/g	e/g	e/g	e/g	e/g	e
GHKHK1M54	e/g	e	e	e/g	e	e
GHKHK1M55	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M57	e	e	e	e	e	e/g
GHKHK1M58	e	e/g	e/g	e/g	e/g	e
GHKHK1M59	.	e	e	e	e	e/g
GHKHK1M60	e	e	e/g	e/g	e/g	e/g
GHKHK1M61	e	e/g	e	e/g	e	e/g
HKGG1M01	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M02	e/g	e/g	e/g	e/g	e/g	e/g

Appendix H (continued)

	Locus					
No	*Clk*	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
gunungcola backcross						
HKGG1M03	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M04	e/g	g	g	g	g	e/g
HKGG1M05	e/g	e/g	g	e/g	g	e/g
HKGG1M06	g	g	e/g	g	e/g	g
HKGG1M07	.	e/g	e/g	e/g	e/g	e/g
HKGG1M08	e/g	e/g	e/g	e/g	e/g	g
HKGG1M09	g	e/g	e/g	e/g	e/g	e/g
HKGG1M10	e/g	g	g	g	g	e/g
HKGG1M11	e/g	g	e/g	e/g	e/g	e/g
HKGG1M12	e/g	e/g	e/g	g	e/g	e/g
HKGG1M13	e/g	e/g	g	e/g	g	e/g
HKGG1M14	e/g	g	e/g	g	e/g	e/g
HKGG1M15	e/g	e/g	e/g	g	e/g	e/g
HKGG1M16	e/g	e/g	g	e/g	g	e/g
HKGG1M17	e/g	e/g	e/g	e/g	e/g	g
HKGG1M18	.	e/g	g	e/g	g	e/g
HKGG1M19	e/g	g	g	g	g	g
HKGG1M20	e/g	e/g	g	g	g	g
HKGG1M21	e/g	g	e/g	g	e/g	g
HKGG1M22	g	g	e/g	e/g	e/g	e/g
HKGG1M23	e/g	g	e/g	g	e/g	e/g
HKGG1M24	g	g	e/g	e/g	e/g	e/g
HKGG1M25	e/g	e/g	g	e/g	g	e/g
HKGG1M27	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M28	e/g	g	e/g	g	e/g	e/g
HKGG1M29	e/g	g	e/g	g	e/g	e/g
HKGG1M30	g	e/g	e/g	e/g	e/g	e/g
HKGG1M31	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M32	g	e/g	e/g	e/g	e/g	e/g
HKGG1M33	g	e/g	e/g	e/g	e/g	e/g
HKGG1M34	e/g	e/g	e/g	e/g	e/g	g

Appendix H (continued)

No	Locus					
	Clk	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
gunungcola backcross						
HKGG1M35	e/g	e/g	e/g	g	e/g	e/g
HKGG1M36	.	e/g	e/g	e/g	e/g	g
HKGG1M37	g	g	e/g	g	e/g	g
HKGG1M38	e/g	e/g	g	e/g	g	e/g
HKGG1M39	g	e/g	g	e/g	e/g	g
HKGG2M01	e/g	e/g	g	e/g	g	e/g
HKGG2M02	e/g	g	g	g	g	e/g
HKGG2M03	g	e/g	e/g	e/g	e/g	e/g
HKGG2M04	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M05	g	g	e/g	g	e/g	g
HKGG2M06	e/g	e/g	g	e/g	g	g
HKGG2M08	g	g	g	g	g	g
HKGG2M09	e/g	e/g	g	e/g	g	g
HKGG2M10	.	e/g	g	e/g	g	g
HKGG2M11	g	e/g	e/g	e/g	e/g	e/g
HKGG2M12	e/g	e/g	g	e/g	g	e/g
HKGG2M13	g	e/g	e/g	e/g	e/g	g
HKGG2M14	g	e/g	e/g	e/g	e/g	e/g
HKGG2M15	g	e/g	e/g	e/g	e/g	e/g
HKGG2M16	e/g	g	g	g	g	g
HKGG2M17	e/g	g	g	g	g	g
HKGG2M18	g	e/g	e/g	e/g	e/g	g
HKGG2M19	e/g	e/g	g	e/g	e/g	g
HKGG2M20	g	e/g	e/g	e/g	e/g	g
HKGG2M21	e/g	e/g	e/g	e/g	e/g	g
HKGG2M22	e/g	g	g	g	e/g	e/g
HKGG2M23	g	g	e/g	g	e/g	e/g
HKGG2M24	e/g	g	g	g	g	e/g
HKGG2M25	e/g	g	g	g	g	g
HKGG2M26	g	e/g	g	g	g	e/g
HKGG2M27	g	e/g	e/g	e/g	e/g	g

Appendix H (continued)

No	Locus					
	Clk	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
gunungcola backcross						
HKGG2M28	g	e/g	e/g	e/g	e/g	e/g
HKGG2M29	.	g	e/g	g	.	e/g
HKGG2M30	g	g	e/g	g	e/g	g
HKGG2M31	e/g	g	g	g	g	g
HKGG2M32	g	e/g	e/g	e/g	e/g	g
HKGG2M33	e/g	e/g	g	e/g	g	e/g
HKGG2M34	g	e/g	g	e/g	g	e/g
HKGG2M35	e/g	e/g	g	e/g	g	e/g
HKGG2M36	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M01	g	e/g	g	e/g	g	e/g
GHKG1M02	g	g	g	g	g	g
GHKG1M03	g	g	e/g	g	e/g	g
GHKG1M04	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M05	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M06	e/g	g	g	g	e/g	g
GHKG1M07	g	g	e/g	g	e/g	g
GHKG1M08	e/g	e/g	g	e/g	e/g	e/g
GHKG1M09	g	e/g	g	e/g	e/g	e/g
GHKG1M10	e/g	g	g	g	g	g
GHKG1M11	e/g	g	e/g	g	e/g	e/g
GHKG1M12	e/g	e/g	g	e/g	g	g
GHKG1M13	e/g	e/g	e/g	e/g	e/g	g
GHKG1M14	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M15	e/g	g	e/g	g	g	e/g
GHKG1M16	g	e/g	e/g	e/g	e/g	e/g
GHKG1M17	e/g	e/g	g	e/g	g	e/g
GHKG1M18	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M19	e/g	e/g	e/g	e/g	e/g	g
GHKG1M20	e/g	e/g	g	e/g	g	e/g
GHKG1M21	g	g	g	g	g	g
GHKG1M22	e/g	e/g	g	e/g	g	e/g

Appendix H (continued)

No	Locus					
	Clk	*Bc*	*Btk29A*	*DAT*	*ed*	*yellow_e*
gunungcola backcross						
GHKG1M23	e/g	g	e/g	e/g	g	e/g
GHKG1M24	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M25	g	e/g	e/g	e/g	e/g	g
GHKG1M27	e/g	g	e/g	g	e/g	g
GHKG1M28	g	g	e/g	g	e/g	g
GHKG1M29	e/g	e/g	e/g	e/g	e/g	g
GHKG1M30	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M31	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M32	g	g	g	g	g	e/g
GHKG1M33	e/g	g	e/g	e/g	g	e/g
GHKG1M34	.	g	g	e/g	g	e/g
GHKG1M35	g	g	e/g	g	e/g	g
GHKG1M36	g	e/g	e/g	e/g	g	e/g
GHKG1M37	e/g	g	e/g	g	e/g	g
GHKG1M38	e/g	e/g	g	e/g	g	g
GHKG1M39	g	e/g	g	e/g	g	e/g
HKGHK2M01	g	e/g	g	e/g	g	g
HKGHK2M02	g	e/g	e/g	e/g	e/g	g
HKGHK2M03	g	e/g	g	e/g	g	g

Appendix H (continued)

	Locus					
No	*tsl*	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
elegans backcross						
HKGHK1M01	e/g	e/g	e	e/g	e/g	e/g
HKGHK1M02	e/g	e/g	e	e	e/g	e/g
HKGHK1M03	e/g	e/g	e/g	e	e/g	e/g
HKGHK1M04	e	e	e	e	e	e
HKGHK1M05	e	e	e/g	e/g	e	e
HKGHK1M06	e	e	e/g	e	e/g	e/g
HKGHK1M07	e	e	e/g	e/g	e	e
HKGHK1M08	e	e	e	e/g	e	e
HKGHK1M09	e/g	e/g	e	e/g	e	e
HKGHK1M10	e/g	e/g	e	e	e	e/g
HKGHK1M11	e/g	e	e	e	e/g	e
HKGHK1M12	e/g	e/g	e	e/g	e	e
HKGHK1M13	e/g	e/g	e	e/g	e/g	e/g
HKGHK1M14	e/g	e/g	e	e	e	e
HKGHK1M15	e/g	e/g	e	e/g	e/g	e/g
HKGHK1M16	e	e	e/g	e	e	e/g
HKGHK1M17	e/g	e/g	e/g	e	e	e
HKGHK1M18	e/g	e/g	e	e	e/g	e/g
HKGHK1M19	e/g	e/g	e	e	e/g	e/g
HKGHK1M20	e/g	e/g	e	e/g	e	e
HKGHK1M21	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M22	e	e	e	e	e	e/g
HKGHK1M23	e	e	e/g	e/g	e/g	e/g
HKGHK1M24	e	e	e	e/g	e	e
HKGHK1M25	e	e	e	e/g	e/g	e/g
HKGHK1M26	e/g	e/g	e	e/g	e	e
HKGHK1M27	e/g	e	e/g	e/g	e	e
HKGHK1M28	e/g	e/g	e	e/g	e/g	e/g
HKGHK1M29	e	e	e/g	e	e	e
HKGHK1M30	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M31	e	e	e	e/g	e/g	e/g

Appendix H (continued)

	Locus					
No	*tsl*	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
elegans backcross						
HKGHK1M32	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M33	e/g	e/g	e	e	e	e
HKGHK1M34	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M35	e/g	e/g	e	e/g	e	e/g
HKGHK1M36	e/g	e/g	e	e	e/g	e/g
HKGHK1M37	e	e	e	e/g	e	e
HKGHK1M38	e/g	e/g	e/g	e/g	e	e
HKGHK1M39	e/g	e	e	e/g	e	e
HKGHK1M40	e	e	e/g	e	e	e
HKGHK1M41	e/g	e/g	e	e/g	e/g	e/g
HKGHK1M42	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M43	e/g	e/g	e	e	e/g	e/g
HKGHK1M45	e/g	e	e	e	e	e
HKGHK1M46	e/g	e/g	e/g	e/g	e	e
HKGHK1M47	e/g	e/g	e	e	e/g	e/g
HKGHK1M48	e	e	e/g	e/g	e/g	e/g
HKGHK1M49	e	e	e	e	e	e
HKGHK1M50	e/g	e/g	e	e	e	e
HKGHK1M51	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M04	e/g	e/g	e	e/g	e	e
HKGHK2M05	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M06	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M07	e/g	e/g	e/g	e	e	e
HKGHK2M08	e	e	e	e	e/g	e
HKGHK2M09	e/g	e/g	e	e	e/g	e/g
HKGHK2M10	e	.	e/g	e	e	e
HKGHK2M12	e	e/g	e/g	e/g	e/g	e/g
HKGHK2M13	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M15	e	e	e	e/g	e	e
HKGHK2M16	e/g	e	e	e/g	e	e
HKGHK2M18	e	e	e/g	e/g	e/g	e/g

Appendix H (continued)

	Locus					
No	*tsl*	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
elegans backcross						
HKGHK2M19	e	e	e/g	e/g	e	e/g
HKGHK2M20	e/g	e/g	e/g	e	e	e
HKGHK2M21	e/g	e	e/g	e/g	e	e
HKGHK2M22	e	e	e/g	e/g	e/g	e/g
HKGHK2M23	e/g	e/g	e	e/g	e	e
HKGHK2M24	e/g	e/g	e	e/g	e	e
HKGHK2M25	e/g	e/g	e/g	e/g	e	e
HKGHK2M26	e/g	e/g	e/g	e/g	e	e
HKGHK2M27	e	e	e	e	e/g	e/g
HKGHK2M28	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M29	e/g	e/g	e	e/g	e/g	e/g
HKGHK2M30	e	e	e	e/g	e/g	e
HKGHK2M31	e	e	e	e/g	e/g	e/g
HKGHK2M32	e	e	e/g	e/g	e/g	e/g
HKGHK2M33	e/g	e/g	e	e/g	e	e/g
HKGHK2M34	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M35	e	e	e/g	e	e/g	e/g
HKGHK2M36	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M37	e/g	e/g	e/g	e/g	e	e
HKGHK2M38	e/g	e/g	e	e/g	e	e
HKGHK2M39	e/g	e/g	e	e	e/g	e/g
HKGHK2M40	e/g	e/g	e	e	e/g	e/g
HKGHK2M41	e/g	e/g	e/g	e	e	e
HKGHK2M42	e/g	e	e/g	e	e/g	e/g
HKGHK2M43	e	e	e/g	e	e	e
HKGHK2M45	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M46	e/g	e/g	e/g	e	e	e/g
HKGHK2M47	e	e	e/g	e/g	e	e
HKGHK2M48	e	e	e/g	e/g	e	e
HKGHK2M49	e/g	e/g	e	e/g	e	e
GHKHK1M01	e	e	e	e	e	e

Appendix H (continued)

	Locus					
No	*tsl*	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
elegans backcross						
GHKHK1M02	e	e	e	e	e/g	e/g
GHKHK1M03	e/g	e	e	e	e	e
GHKHK1M04	e	e	e	e	e/g	e/g
GHKHK1M05	e	e	e	e/g	e	e
GHKHK1M06	e	e/g	e	e/g	e	e
GHKHK1M07	e/g	e/g	e	e/g	e	e/g
GHKHK1M08	e	e	e/g	e	e/g	e/g
GHKHK1M09	e	e	e/g	e/g	e/g	e/g
GHKHK1M10	e	e	e	e	e/g	e/g
GHKHK1M11	e	e	e	e/g	e/g	e/g
GHKHK1M12	e/g	e/g	e	e	e	e
GHKHK1M13	e	e/g	e	e	e/g	e/g
GHKHK1M14	e/g	e/g	e	e/g	e	e
GHKHK1M15	e/g	e/g	e/g	e	e/g	e/g
GHKHK1M16	e	e	e/g	e/g	e	e
GHKHK1M17	e	e	e/g	e	e/g	e/g
GHKHK1M18	e	e	e	e	e/g	e/g
GHKHK1M19	e	e	e/g	e	e/g	e/g
GHKHK1M20	e	e	e	e	e/g	e/g
GHKHK1M21	e/g	e/g	e/g	e	e/g	e
GHKHK1M22	e	.	e/g	e	e	e/g
GHKHK1M23	e	e/g	e	e/g	e/g	e/g
GHKHK1M24	e/g	e/g	e	e/g	e/g	e/g
GHKHK1M25	e/g	e/g	e/g	e/g	e	e
GHKHK1M26	e	e	e	e/g	e/g	e/g
GHKHK1M27	e/g	e/g	e	e/g	e	e
GHKHK1M28	e	e	e	e	e/g	e/g
GHKHK1M29	e	e	e/g	e	e/g	e/g
GHKHK1M30	e/g	e/g	e	e/g	e	e
GHKHK1M31	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M32	e/g	e/g	e/g	e	e	e

Appendix H (continued)

	Locus					
No	*tsl*	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
elegans backcross						
GHKHK1M33	e/g	e/g	e/g	e	e	e
GHKHK1M34	e/g	e/g	e	e	e/g	e/g
GHKHK1M35	e	e/g	e	e	e/g	e/g
GHKHK1M36	e/g	e/g	e	e/g	e/g	e/g
GHKHK1M37	e/g	e/g	e	e	e/g	e/g
GHKHK1M38	e/g	e	e/g	e	e	e
GHKHK1M39	e/g	e/g	e	e	e/g	e/g
GHKHK1M40	e	e	e/g	e/g	e	e
GHKHK1M41	e	e	e/g	e	e	e
GHKHK1M42	e/g	e/g	e/g	e/g	e	e
GHKHK1M43	e	e	e/g	e/g	e	e
GHKHK1M44	e/g	e	e/g	e/g	e/g	e
GHKHK1M45	e/g	e/g	e	e	e/g	e
GHKHK1M46	e/g	e/g	e	e	e/g	e/g
GHKHK1M47	e	e	e	e	e/g	e/g
GHKHK1M48	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M49	e	e	e	e/g	e/g	e/g
GHKHK1M50	e/g	e/g	e	e/g	e/g	e/g
GHKHK1M51	e	e	e	e/g	e	e
GHKHK1M53	e	e	e	e/g	e/g	e/g
GHKHK1M54	e	e	e	e	e/g	e/g
GHKHK1M55	e/g	e/g	e	e/g	.	e
GHKHK1M57	e/g	e/g	e	e	e	e
GHKHK1M58	e	e	e/g	e/g	e	e
GHKHK1M59	e	e	.	e	e/g	e/g
GHKHK1M60	e/g	e/g	e/g	e	e/g	e/g
GHKHK1M61	e/g	e/g	e	e/g	e	e
HKGG1M01	e/g	e/g	e	e/g	e	e/g
HKGG1M02	e/g	e/g	e	e/g	e/g	e/g

Appendix H (continued)

	Locus					
No	*tsl*	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
gunungcola backcross						
HKGG1M03	e/g	e/g	e/g	e/g	g	e/g
HKGG1M04	g	e/g	e/g	g	e/g	g
HKGG1M05	e/g	e/g	g	e/g	e/g	e/g
HKGG1M06	g	g	e/g	g	e/g	e/g
HKGG1M07	e/g	e/g	g	g	.	g
HKGG1M08	g	g	g	e/g	e/g	e/g
HKGG1M09	e/g	e/g	g	e/g	g	g
HKGG1M10	e/g	e/g	g	g	e/g	e/g
HKGG1M11	e/g	e/g	e/g	g	e/g	e/g
HKGG1M12	e/g	e/g	g	e/g	e/g	g
HKGG1M13	e/g	e/g	g	e/g	g	g
HKGG1M14	e/g	e/g	e/g	g	g	g
HKGG1M15	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M16	e/g	e/g	g	g	g	g
HKGG1M17	g	g	e/g	e/g	g	g
HKGG1M18	e/g	e/g	g	e/g	g	g
HKGG1M19	e/g	e/g	e/g	g	e/g	e/g
HKGG1M20	g	g	e/g	e/g	e/g	g
HKGG1M21	g	g	e/g	g	e/g	e/g
HKGG1M22	e/g	e/g	g	g	e/g	e/g
HKGG1M23	e/g	e/g	e/g	g	g	g
HKGG1M24	e/g	e/g	e/g	g	g	g
HKGG1M25	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M27	e/g	e/g	e/g	e/g	g	g
HKGG1M28	e/g	e/g	e/g	g	e/g	e/g
HKGG1M29	e/g	e/g	e/g	g	e/g	g
HKGG1M30	e/g	e/g	e/g	e/g	g	g
HKGG1M31	e/g	e/g	g	e/g	g	g
HKGG1M32	e/g	e/g	e/g	e/g	g	e/g
HKGG1M33	e/g	e/g	e/g	e/g	g	g
HKGG1M34	g	g	e/g	e/g	e/g	e/g

Appendix H (continued)

No	Locus					
	tsl	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
gunungcola backcross						
HKGG1M35	e/g	e/g	g	e/g	e/g	e/g
HKGG1M36	g	g	g	e/g	g	e/g
HKGG1M37	g	g	e/g	g	g	e/g
HKGG1M38	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M39	g	g	e/g	e/g	g	g
HKGG2M01	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M02	e/g	e/g	e/g	g	g	e/g
HKGG2M03	e/g	e/g	e/g	e/g	g	g
HKGG2M04	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M05	g	g	g	e/g	g	g
HKGG2M06	e/g	g	g	g	g	g
HKGG2M08	e/g	g	e/g	g	g	g
HKGG2M09	g	g	e/g	e/g	e/g	e/g
HKGG2M10	g	g	e/g	e/g	g	g
HKGG2M11	e/g	e/g	e/g	e/g	g	g
HKGG2M12	e/g	e/g	g	e/g	e/g	e/g
HKGG2M13	g	g	e/g	e/g	g	g
HKGG2M14	g	e/g	g	e/g	g	g
HKGG2M15	e/g	e/g	e/g	e/g	g	g
HKGG2M16	g	g	e/g	g	e/g	e/g
HKGG2M17	g	g	g	g	e/g	e/g
HKGG2M18	g	g	.	e/g	e/g	e/g
HKGG2M19	g	g	g	e/g	e/g	e/g
HKGG2M20	g	g	e/g	e/g	g	g
HKGG2M21	g	g	g	e/g	g	g
HKGG2M22	g	e/g	e/g	e/g	e/g	g
HKGG2M23	e/g	e/g	g	g	e/g	e/g
HKGG2M24	g	e/g	e/g	g	e/g	g
HKGG2M25	g	g	g	g	e/g	e/g
HKGG2M26	e/g	e/g	g	e/g	e/g	e/g
HKGG2M27	g	g	e/g	e/g	e/g	e

Appendix H (continued)

No	Locus					
	tsl	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
gunungcola backcross						
HKGG2M28	e/g	e/g	e/g	e/g	g	g
HKGG2M29	e/g	.	e/g	g	e/g	g
HKGG2M30	g	g	e/g	e/g	g	g
HKGG2M31	g	g	e/g	g	e/g	g
HKGG2M32	g	g	e/g	e/g	g	g
HKGG2M33	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M34	g	e/g	e/g	e/g	g	e
HKGG2M35	e/g	e/g	g	e/g	e/g	e/g
HKGG2M36	g	e/g	e/g	e/g	e/g	e/g
GHKG1M01	e/g	g	e/g	e/g	g	g
GHKG1M02	e/g	g	g	g	g	g
GHKG1M03	g	g	e/g	g	g	g
GHKG1M04	g	e/g	g	e/g	e/g	e/g
GHKG1M05	g	e/g	e/g	e/g	e/g	e/g
GHKG1M06	g	g	e/g	g	e/g	e/g
GHKG1M07	g	g	g	g	e/g	e/g
GHKG1M08	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M09	e/g	e/g	e/g	e/g	g	g
GHKG1M10	g	.	e/g	g	e/g	g
GHKG1M11	g	e/g	e/g	g	e/g	e/g
GHKG1M12	g	g	e/g	e/g	g	g
GHKG1M13	g	g	g	e/g	e/g	e/g
GHKG1M14	g	e/g	g	e/g	e/g	e/g
GHKG1M15	e/g	e/g	e/g	g	g	g
GHKG1M16	e/g	e/g	e/g	e/g	g	g
GHKG1M17	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M18	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M19	g	g	g	e/g	e/g	e/g
GHKG1M20	e/g	e/g	g	e/g	e/g	g
GHKG1M21	g	g	g	g	g	g
GHKG1M22	e/g	e/g	g	e/g	e/g	e/g

Appendix H (continued)

No	Locus					
	tsl	*fru*	*aaNAT2*	*aaNAT1*	*Baldspot*	*bab1*
gunungcola backcross						
GHKG1M23	e/g	e/g	e/g	g	e/g	e/g
GHKG1M24	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M25	g	g	e/g	e/g	g	e/g
GHKG1M27	g	g	g	g	e/g	e/g
GHKG1M28	g	g	e/g	g	g	g
GHKG1M29	g	g	e/g	e/g	e/g	e/g
GHKG1M30	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M31	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M32	e/g	e/g	g	g	g	g
GHKG1M33	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M34	e/g	g	g	g	g	g
GHKG1M35	g	g	e/g	g	g	g
GHKG1M36	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M37	g	g	e/g	g	e/g	e/g
GHKG1M38	g	g	e/g	e/g	e/g	e/g
GHKG1M39	e/g	e/g	e/g	e/g	g	g
HKGHK2M01	g	g	e/g	e/g	g	g
HKGHK2M02	g	g	e/g	e/g	g	g
HKGHK2M03	g	g	e/g	e/g	e/g	e/g

Appendix H (continued)

No	Locus					
	yellow_c	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
elegans backcross						
HKGHK1M01	e	e/g	e/g	e/g	e/g	e/g
HKGHK1M02	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M03	e	e/g	e/g	e/g	e	e/g
HKGHK1M04	e/g	e	e	e	e/g	e
HKGHK1M05	e/g	e/g	e	e	e/g	e
HKGHK1M06	e/g	e	e	e	e/g	e/g
HKGHK1M07	e/g	e	e	e	e	e
HKGHK1M08	e	e/g	e	e	e	e
HKGHK1M09	e/g	e/g	e/g	e/g	e	e
HKGHK1M10	e	e/g	e/g	e/g	e	e
HKGHK1M11	e/g	e	e/g	e	e/g	e/g
HKGHK1M12	e	e/g	e/g	e/g	e/g	e
HKGHK1M13	e	e/g	e/g	e/g	e	e/g
HKGHK1M14	e	e/g	e/g	e/g	e	e
HKGHK1M15	e	e/g	e/g	e/g	e/g	e/g
HKGHK1M16	e/g	e	e	e	e/g	e
HKGHK1M17	e/g	e/g	e/g	e/g	e	e
HKGHK1M18	e	e/g	e/g	e/g	e/g	e/g
HKGHK1M19	e	e/g	e/g	e/g	e/g	e/g
HKGHK1M20	e	e	e/g	e/g	e	e
HKGHK1M21	e/g	e/g	e/g	e/g	e	e/g
HKGHK1M22	e	e	e	e	e	e/g
HKGHK1M23	e/g	e	e	e	e/g	e/g
HKGHK1M24	e	e/g	e	e	e/g	e
HKGHK1M25	e	e	e	e	e/g	e/g
HKGHK1M26	e	e/g	e/g	e/g	e	e
HKGHK1M27	e/g	e	e/g	e	e/g	e
HKGHK1M28	e	e/g	e/g	e/g	e/g	e/g
HKGHK1M29	e/g	e	e	e	e	e
HKGHK1M30	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M31	e	e	e	e	e/g	e/g

Appendix H (continued)

No	Locus					
	yellow_c	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
elegans backcross						
HKGHK1M32	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M33	e/g	e/g	e/g	e/g	e	e
HKGHK1M34	e/g	e/g	e/g	e/g	e	e/g
HKGHK1M35	e/g	e/g	e/g	e/g	e	e
HKGHK1M36	e	e/g	e/g	e/g	e	e/g
HKGHK1M37	e	e	e	e	e/g	e
HKGHK1M38	e/g	e/g	e/g	e	e	e
HKGHK1M39	e/g	e	e/g	.	e	e
HKGHK1M40	e/g	e	e	e	e	e
HKGHK1M41	e	e/g	e/g	.	e/g	e/g
HKGHK1M42	e/g	e/g	e/g	e/g	e	e/g
HKGHK1M43	e	e/g	e/g	e/g	e	e/g
HKGHK1M45	e	e	e/g	e	e	e
HKGHK1M46	e	e/g	e/g	e/g	e	e
HKGHK1M47	e	e/g	e/g	e/g	e/g	e/g
HKGHK1M48	e/g	e	e	e	e/g	e/g
HKGHK1M49	e	e	e	e	e	e
HKGHK1M50	e	e/g	e/g	e/g	e	e
HKGHK1M51	e	e/g	e/g	e/g	e	e/g
HKGHK2M04	e	e	e/g	e/g	e	e
HKGHK2M05	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M06	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M07	e/g	e/g	e/g	e/g	e	e
HKGHK2M08	e	e	e	e	e/g	e/g
HKGHK2M09	e/g	e/g	e/g	e/g	.	e/g
HKGHK2M10	e	e	e	e	e	e
HKGHK2M12	e/g	e/g	e	e	.	e/g
HKGHK2M13	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M15	e	e	e	e	e	e
HKGHK2M16	e	e	e/g	e	e	e
HKGHK2M18	e/g	e	e	e	e	e/g

Appendix H (continued)

	Locus					
No	*yellow_c*	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
elegans backcross						
HKGHK2M19	e/g	e	e	e	e/g	e/g
HKGHK2M20	e	e/g	e/g	e/g	e	e
HKGHK2M21	e/g	e	e/g	e	e	e
HKGHK2M22	e/g	e	e	e	e/g	e/g
HKGHK2M23	e/g	e	e/g	e/g	e	e
HKGHK2M24	e/g	e/g	e/g	e/g	e	e
HKGHK2M25	e/g	e/g	e/g	e/g	e	e
HKGHK2M26	e/g	e/g	e/g	e/g	e	e
HKGHK2M27	e/g	e	e	e	e/g	e/g
HKGHK2M28	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M29	e	e/g	e/g	e/g	e	e/g
HKGHK2M30	e/g	e	e	e	e	e/g
HKGHK2M31	e	e	e	e	e	e/g
HKGHK2M32	e/g	e	e	e	e	e/g
HKGHK2M33	e	e/g	e/g	e/g	e	e
HKGHK2M34	e/g	e/g	e/g	e/g	e	e/g
HKGHK2M35	e	e	e	e	e/g	e/g
HKGHK2M36	e	e/g	e/g	e/g	e/g	e/g
HKGHK2M37	e/g	e/g	e/g	e/g	e	e
HKGHK2M38	e/g	e/g	e/g	e/g	e/g	e
HKGHK2M39	e	e/g	e/g	e/g	e	e/g
HKGHK2M40	e	e/g	e/g	e/g	e/g	e/g
HKGHK2M41	e/g	e/g	e/g	e/g	e	e
HKGHK2M42	e/g	e	e/g	e	e/g	e/g
HKGHK2M43	e/g	e	e	e	e	e
HKGHK2M45	e/g	e/g	e/g	e/g	e	e/g
HKGHK2M46	e/g	e/g	e/g	e/g	e	e
HKGHK2M47	e/g	e	e	e	e	e
HKGHK2M48	e/g	e	e	e	e	e
HKGHK2M49	e	e/g	e/g	e/g	e	e
GHKHK1M01	e/g	e/g	e	e	e	e

Appendix H (continued)

	Locus					
No	*yellow_c*	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
elegans backcross						
GHKHK1M02	e	e	e	e	e	e/g
GHKHK1M03	e	e	e/g	e	e	e
GHKHK1M04	e	e	e	e	e/g	e/g
GHKHK1M05	e/g	e	e	e	e	e
GHKHK1M06	e/g	e/g	e	e/g	e	e
GHKHK1M07	e	e/g	e/g	e/g	e	e
GHKHK1M08	e/g	e	e	e	e/g	e/g
GHKHK1M09	e/g	e	e	e	e/g	e/g
GHKHK1M10	e	e	e	e	e/g	e/g
GHKHK1M11	e	e	e	e	e	e/g
GHKHK1M12	e/g	e/g	e/g	e/g	e	e
GHKHK1M13	e	e/g	e	e/g	e/g	e
GHKHK1M14	e	e/g	e/g	e/g	e	e
GHKHK1M15	e/g	e/g	e/g	e/g	e	e/g
GHKHK1M16	e/g	e	e	e	e	e
GHKHK1M17	e	e	e	e	e/g	e/g
GHKHK1M18	e	e	e	e	e/g	e/g
GHKHK1M19	e/g	e/g	e	e	e/g	e/g
GHKHK1M20	e	e	e	e	e/g	e/g
GHKHK1M21	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M22	e/g	e/g	e	e/g	e	e
GHKHK1M23	e/g	e/g	e	e/g	e/g	e/g
GHKHK1M24	e	e/g	e/g	e/g	e/g	e/g
GHKHK1M25	e/g	e/g	e/g	e/g	e	e
GHKHK1M26	e	e	e	e	.	e/g
GHKHK1M27	e	e/g	e/g	e/g	e	e
GHKHK1M28	e	e	e	e	e/g	e/g
GHKHK1M29	e/g	e	e	e	e/g	e/g
GHKHK1M30	e	e/g	e/g	e/g	e	e
GHKHK1M31	e	e/g	e/g	e/g	e	e/g
GHKHK1M32	e/g	e/g	e/g	e/g	e	e

Appendix H (continued)

	Locus					
No	*yellow_c*	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
elegans backcross						
GHKHK1M33	e/g	e/g	e/g	e/g	e	e
GHKHK1M34	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M35	e	e	e/g	e/g	e	e/g
GHKHK1M36	e	e/g	e/g	e/g	e/g	e/g
GHKHK1M37	e	e/g	e/g	e/g	e	e/g
GHKHK1M38	e/g	e	e/g	e	e/g	e
GHKHK1M39	e	e/g	e/g	e/g	e/g	e/g
GHKHK1M40	e/g	e	e	e	e	e
GHKHK1M41	e/g	e	e	e	e	e
GHKHK1M42	e/g	e/g	e/g	e/g	e	e
GHKHK1M43	e/g	e	e	e	e	e
GHKHK1M44	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M45	e	e/g	e/g	e/g	e/g	e/g
GHKHK1M46	e	e/g	e/g	e/g	e/g	e/g
GHKHK1M47	e	e/g	e	e	e/g	e/g
GHKHK1M48	e/g	e/g	e	e/g	e/g	.
GHKHK1M49	e/g	e	e	e	e/g	e/g
GHKHK1M50	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M51	e	e	e	e	e	e
GHKHK1M53	e/g	e	e	e	e/g	e/g
GHKHK1M54	e	e	e	e	e/g	e/g
GHKHK1M55	e/g	e/g	e/g	e/g	e/g	e
GHKHK1M57	e	e/g	e/g	e/g	e	e
GHKHK1M58	e/g	e	e	e	e	e
GHKHK1M59	e/g	e	e	e	e/g	.
GHKHK1M60	e/g	e/g	e/g	e/g	e	e/g
GHKHK1M61	e	e/g	e/g	e/g	e	e
HKGG1M01	e/g	e/g	e/g	e/g	e	e
HKGG1M02	e	e/g	e/g	e/g	e/g	e/g

Appendix H (continued)

No	Locus					
	yellow_c	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
gunungcola backcross						
HKGG1M03	g	e/g	e/g	e/g	g	e/g
HKGG1M04	g	e/g	g	g	e/g	g
HKGG1M05	g	e/g	e/g	e/g	e/g	e/g
HKGG1M06	e/g	g	g	g	g	e/g
HKGG1M07	g	e/g	e/g	e/g	g	g
HKGG1M08	e/g	g	g	g	e/g	e/g
HKGG1M09	e/g	e/g	e/g	e/g	g	g
HKGG1M10	g	e/g	e/g	e/g	e/g	e/g
HKGG1M11	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M12	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M13	g	e/g	e/g	e/g	e/g	g
HKGG1M14	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M15	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M16	g	e/g	e/g	e/g	e/g	g
HKGG1M17	e/g	g/g	g	g	e/g	g
HKGG1M18	g	e/g	e/g	e/g	g	g
HKGG1M19	e/g	e/g	e/g	e/g	e/g	g
HKGG1M20	g	g/g	g	g	e/g	e/g
HKGG1M21	e/g	g/g	g	g	e/g	.
HKGG1M22	g	e/g	e/g	e/g	g	e/g
HKGG1M23	e/g	e/g	e/g	e/g	e/g	g
HKGG1M24	e/g	e/g	e/g	e/g	g	g
HKGG1M25	g	e/g	e/g	e/g	e/g	e/g
HKGG1M27	e/g	e/g	e/g	e/g	e/g	g
HKGG1M28	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M29	e/g	e/g	e/g	e/g	e/g	g
HKGG1M30	e/g	e/g	e/g	e/g	g	g
HKGG1M31	g	e/g	e/g	e/g	e/g	g
HKGG1M32	e/g	e/g	e/g	e/g	e/g	g
HKGG1M33	e/g	e/g	e/g	e/g	g	g
HKGG1M34	e/g	g	g	g	e/g	e/g

Appendix H (continued)

No	Locus					
	yellow_c	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
gunungcola backcross						
HKGG1M35	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M36	e/g	g	g	g	g	e/g
HKGG1M37	e/g	g	g	g	g	g
HKGG1M38	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M39	e/g	g	g	g	e/g	g
HKGG2M01	g	e/g	e/g	e/g	e/g	e/g
HKGG2M02	g	e/g	e/g	e/g	e/g	g
HKGG2M03	e/g	e/g	e/g	e/g	g	g
HKGG2M04	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M05	e/g	g	g	g	g	g
HKGG2M06	g	g/g	e/g	g	e/g	g
HKGG2M08	g	g	e/g	g	g	g
HKGG2M09	e/g	g/g	g	g	e/g	e/g
HKGG2M10	g	g	g	g	g	g
HKGG2M11	e/g	e/g	e/g	e/g	g	g
HKGG2M12	g	e/g	e/g	e/g	e/g	e/g
HKGG2M13	e/g	g/g	g	g	g	g
HKGG2M14	e/g	e/g	g	e/g	g	g
HKGG2M15	e/g	e/g	e/g	e/g	g	g
HKGG2M16	g	g/g	g	g	e/g	e/g
HKGG2M17	g	g	g	g	e/g	e/g
HKGG2M18	g	g	g	g	g	e/g
HKGG2M19	g	g/g	g	g	e/g	e/g
HKGG2M20	e/g	g/g	g	g	g	g
HKGG2M21	e/g	g	g	g	e/g	g
HKGG2M22	e/g	e/g	g	e/g	e/g	e/g
HKGG2M23	e/g	e/g	e/g	e/g	g	e/g
HKGG2M24	g	e/g	g	e/g	e/g	e/g
HKGG2M25	g	g/g	g	g	e/g	e/g
HKGG2M26	g	e/g	e/g	e/g	g	e/g
HKGG2M27	e/g	g/g	g	g	g	e/g

Appendix H (continued)

No	Locus					
	yellow_c	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
gunungcola backcross						
HKGG2M28	g	e/g	e/g	e/g	g	g
HKGG2M29	e/g	e/g	e/g	e/g	e/g	g
HKGG2M30	e/g	g/g	g	g	e/g	g
HKGG2M31	g	g/g	g	g	e/g	e/g
HKGG2M32	e/g	g/g	g	g	g	g
HKGG2M33	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M34	e/g	e/g	g	e/g	g	g
HKGG2M35	g	e/g	e/g	e/g	e/g	e/g
HKGG2M36	g	e/g	g	e/g	e/g	e/g
GHKG1M01	e/g	e/g	e/g	e/g	g	g
GHKG1M02	g	g/g	e/g	g	g	g
GHKG1M03	e/g	g	g	g	g	g
GHKG1M04	e/g	e/g	g	e/g	e/g	e/g
GHKG1M05	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M06	e/g	g	g	g	e/g	g
GHKG1M07	g	g/g	g	g	g	e/g
GHKG1M08	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M09	e/g	e/g	e/g	e/g	g	g
GHKG1M10	g	g/g	g	g	e/g	g
GHKG1M11	e/g	g/g	g	e/g	e/g	g
GHKG1M12	g	g/g	g	g	e/g	g
GHKG1M13	e/g	g/g	g	g	e/g	e/g
GHKG1M14	e/g	e/g	g	e/g	e/g	e/g
GHKG1M15	e/g	e/g	e/g	e/g	e/g	g
GHKG1M16	e/g	e/g	e/g	e/g	g	g
GHKG1M17	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M18	e/g	e/g	.	e/g	e/g	e/g
GHKG1M19	g	g/g	g	g	e/g	e/g
GHKG1M20	g	e/g	e/g	e/g	e/g	e/g
GHKG1M21	g	g/g	g	g	e/g	g
GHKG1M22	g	e/g	e/g	e/g	e/g	e/g

Appendix H (continued)

No	Locus					
	yellow_c	*DopR1*	*DopR2*	*H*	*yellow_k*	*ara*
gunungcola backcross						
GHKG1M23	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M24	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M25	e/g	g/g	g	g	g	g
GHKG1M27	g	g/g	g	g	e/g	e/g
GHKG1M28	e/g	g/g	g	g	g	g
GHKG1M29	e/g	g	g	g	e/g	e/g
GHKG1M30	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M31	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M32	g	e/g	e/g	e/g	g	g
GHKG1M33	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M34	g	e/g	e/g	e/g	g	g
GHKG1M35	e/g	g	g	g	g	g
GHKG1M36	e/g	e/g	e/g	e/g	g	e/g
GHKG1M37	e/g	g/g	g	g	e/g	e/g
GHKG1M38	g	g/g	g	g	e/g	e/g
GHKG1M39	e/g	e/g	e/g	e/g	g	g
HKGHK2M01	g	g	g	g	g	g
HKGHK2M02	e/g	g	g	g	g	g
HKGHK2M03	e/g	g	g	g	g	e/g

Appendix H (continued)

	Locus					
No	*e*	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
elegans backcross						
HKGHK1M01	e/g	e/g	e	e/g	e	e/g
HKGHK1M02	e/g	e/g	e/g	e	e/g	e
HKGHK1M03	e/g	e/g	e	e	e	e
HKGHK1M04	e	e	e	e	e	e/g
HKGHK1M05	e	e	e/g	e/g	e/g	e
HKGHK1M06	e	e	e/g	e	e/g	e
HKGHK1M07	e	e	e/g	e/g	e/g	e/g
HKGHK1M08	e	e	e	e/g	e	e/g
HKGHK1M09	e/g	e/g	e	e	e/g	e/g
HKGHK1M10	e/g	e/g	e/g	e	e/g	e
HKGHK1M11	e	e	e/g	e	e/g	e/g
HKGHK1M12	e/g	e/g	e	e	e	e/g
HKGHK1M13	e	e/g	e	e	e	e
HKGHK1M14	e/g	e/g	e	e	e	e/g
HKGHK1M15	e/g	e/g	e	e/g	e	e/g
HKGHK1M16	e	e	e/g	e	e/g	e/g
HKGHK1M17	e/g	e/g	e/g	e	e/g	e
HKGHK1M18	e/g	e/g	e	e/g	e	e
HKGHK1M19	e/g	e/g	e	e	e	e
HKGHK1M20	e	e	e	e/g	e	e/g
HKGHK1M21	e/g	e/g	e	e/g	e	e
HKGHK1M22	e	e	e	e	e	e
HKGHK1M23	e	e	e/g	e/g	e/g	e
HKGHK1M24	e	e	e	e/g	e	e/g
HKGHK1M25	e	e	e	e/g	e	e
HKGHK1M26	e/g	e/g	e	e/g	e	e/g
HKGHK1M27	e	e	e/g	e/g	e/g	e/g
HKGHK1M28	e/g	e/g	e	e/g	e	e
HKGHK1M29	e	e	e	e	e/g	e/g
HKGHK1M30	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M31	e	e	e	e/g	e	e/g

Appendix H (continued)

	Locus					
No	*e*	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
elegans backcross						
HKGHK1M32	e/g	e/g	e/g	e/g	e/g	e
HKGHK1M33	e/g	e/g	e/g	e	e/g	e/g
HKGHK1M34	e/g	e/g	e/g	e	e/g	e/g
HKGHK1M35	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK1M36	e/g	e/g	e	e	e	e/g
HKGHK1M37	e	e	e	e/g	e	e
HKGHK1M38	e/g	e/g	e/g	e/g	e/g	e
HKGHK1M39	e	e	e/g	e/g	e/g	e/g
HKGHK1M40	e	e	e	e	e	e/g
HKGHK1M41	e/g	e/g	e	e/g	e	e
HKGHK1M42	e/g	e/g	e/g	e/g	e/g	e
HKGHK1M43	e/g	e/g	e	e	e	e/g
HKGHK1M45	e	e	e/g	e	e/g	e/g
HKGHK1M46	e/g	e/g	e	e	e	e
HKGHK1M47	e/g	e/g	e	e	e	e/g
HKGHK1M48	e	e	e/g	e/g	e/g	e
HKGHK1M49	e	e	e/g	e	e/g	e/g
HKGHK1M50	e/g	e/g	e	e	e	e
HKGHK1M51	e/g	e/g	e/g	e/g	e/g	e
HKGHK2M04	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M05	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M06	e/g	e/g	e	e	e	e/g
HKGHK2M07	e/g	e/g	e	e	e	e/g
HKGHK2M08	e	e	e/g	e/g	e/g	e/g
HKGHK2M09	e/g	e/g	e/g	e	e	e
HKGHK2M10	e	e	e/g	e	e	e/g
HKGHK2M12	e	e/g	e/g	e	.	e/g
HKGHK2M13	e/g	e/g	e/g	e	e	e/g
HKGHK2M15	e	e	e	e/g	e	e/g
HKGHK2M16	e	e	e	e	e	e
HKGHK2M18	e	e	e/g	e/g	e/g	e/g

Appendix H (continued)

	Locus					
No	*e*	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
elegans backcross						
HKGHK2M19	e	e	e/g	e/g	e/g	e
HKGHK2M20	e/g	e/g	e	e	e	e/g
HKGHK2M21	e	e	e/g	e/g	e/g	e
HKGHK2M22	e	e	e/g	e/g	e/g	e/g
HKGHK2M23	e/g	e	e/g	e/g	e/g	e/g
HKGHK2M24	e/g	e/g	e/g	e	e/g	e
HKGHK2M25	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M26	e	e/g	e/g	e/g	e/g	e
HKGHK2M27	e	e	e/g	e	e/g	e/g
HKGHK2M28	e/g	e/g	e/g	e/g	e/g	e/g
HKGHK2M29	e/g	e/g	e	e/g	e	e
HKGHK2M30	e	e	e/g	e/g	e/g	e
HKGHK2M31	.	e	e/g	e/g	e/g	e/g
HKGHK2M32	.	e	e/g	e/g	e/g	e
HKGHK2M33	.	e/g	e	e/g	e	e/g
HKGHK2M34	.	e/g	e/g	e	e/g	e
HKGHK2M35	.	e	e	e	e	e
HKGHK2M36	e/g	e/g	e	e/g	e	e/g
HKGHK2M37	e	e/g	e	e/g	e	e
HKGHK2M38	e	e/g	e/g	e/g	e/g	e
HKGHK2M39	e/g	e/g	e	e	e	e
HKGHK2M40	e/g	e/g	e/g	e	e/g	e
HKGHK2M41	.	e/g	e/g	e	e/g	e/g
HKGHK2M42	e/g	e	e/g	e/g	e/g	e
HKGHK2M43	e	e	e/g	e	e/g	e
HKGHK2M45	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M46	e/g	e/g	e/g	e	e/g	e/g
HKGHK2M47	e	e	e/g	e/g	e/g	e/g
HKGHK2M48	e	e	e/g	e/g	e/g	e
HKGHK2M49	e	e/g	e	e/g	e	e/g
GHKHK1M01	e	e	e/g	e	e/g	e/g

Appendix H (continued)

	Locus					
No	*e*	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
elegans backcross						
GHKHK1M02	e	e	e	e	e	e
GHKHK1M03	e	e	e	e/g	e	e
GHKHK1M04	e	e	e/g	e/g	e/g	e
GHKHK1M05	e	e	e/g	e/g	e/g	e
GHKHK1M06	e/g	e/g	e/g	e	e/g	e/g
GHKHK1M07	e/g	e/g	e	e/g	e	e
GHKHK1M08	e	e	e/g	e/g	e/g	e/g
GHKHK1M09	e	e	e/g	e/g	e/g	e/g
GHKHK1M10	e	e	e	e	e	e
GHKHK1M11	e	e	e	e/g	e	e
GHKHK1M12	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M13	e/g	e/g	e/g	e	e/g	e/g
GHKHK1M14	e/g	e/g	e	e	e	e
GHKHK1M15	e/g	e/g	e/g	e	e/g	e
GHKHK1M16	e	e	e/g	e/g	e/g	e
GHKHK1M17	e	e	e	e	e	e
GHKHK1M18	e	e	e	e	e	e
GHKHK1M19	e	e	e/g	e/g	e/g	e/g
GHKHK1M20	e	e	e	e/g	e	e
GHKHK1M21	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M22	e/g	e/g	e/g	e	e/g	e
GHKHK1M23	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M24	e/g	e/g	e	e	e	e
GHKHK1M25	e/g	e/g	e/g	e	e/g	e
GHKHK1M26	e	e	e/g	e/g	e	e
GHKHK1M27	e/g	e/g	e	e/g	e	e/g
GHKHK1M28	e	e/g	e/g	e/g	e/g	e
GHKHK1M29	e	e	e/g	e	e/g	e
GHKHK1M30	e/g	e/g	e	e/g	e	e/g
GHKHK1M31	e	e/g	e	e/g	e	e/g
GHKHK1M32	e/g	e/g	e	e/g	e	e

Appendix H (continued)

	Locus					
No	*e*	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
elegans backcross						
GHKHK1M33	e/g	e/g	e	e	e	e/g
GHKHK1M34	e/g		e/g	e	e/g	e
GHKHK1M35	e	e/g	e	e	e	e
GHKHK1M36	e/g	e/g	e	e/g	e	e
GHKHK1M37	e/g	e/g	e	e/g	e	e
GHKHK1M38	e	e	e/g	e/g	e/g	e
GHKHK1M39	e	e/g	e	e/g	e	e/g
GHKHK1M40	e	e	e/g	e	e/g	e
GHKHK1M41	e	e	e	e	e/g	e
GHKHK1M42	e/g	e/g	e/g	e	e/g	e
GHKHK1M43	e	e	e/g	e/g	e/g	e
GHKHK1M44	e/g	e/g	e	e/g	e	e
GHKHK1M45	e/g	e/g	e	e/g	e	e
GHKHK1M46	e/g	e/g	e	e	e	e/g
GHKHK1M47	e	e	e	e	e	e/g
GHKHK1M48	.	e/g	e	e/g	e	e/g
GHKHK1M49	e	e	e/g	e/g	e/g	e/g
GHKHK1M50	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M51	e	e	e	e	e	e
GHKHK1M53	e	e	e/g	e/g	e/g	e/g
GHKHK1M54	e	e	e	e/g	e	e/g
GHKHK1M55	e/g	e/g	e/g	e/g	e/g	e
GHKHK1M57	e/g	e	e	e	e	e/g
GHKHK1M58	.	e	e/g	e/g	e/g	e/g
GHKHK1M59	.	e	e	e	e	e/g
GHKHK1M60	e/g	e/g	e/g	e/g	e/g	e/g
GHKHK1M61	e/g	e/g	e	e/g	e	e/g
HKGG1M01	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M02	e/g	e/g	e/g	e/g	e/g	e

Appendix H (continued)

No	Locus					
	e	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
gunungcola backcross						
HKGG1M03	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M04	e/g	e/g	g	g	g	g
HKGG1M05	e/g	e/g	g	e/g	g	g
HKGG1M06	g	g	e/g	g	e/g	e/g
HKGG1M07	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M08	g	g	e/g	e/g	e/g	e/g
HKGG1M09	e/g	e/g	e/g	e/g	e/g	g
HKGG1M10	e/g	e/g	g	g	g	g
HKGG1M11	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M12	e/g	e/g	e/g	g	e/g	g
HKGG1M13	e/g	e/g	g	g	g	g
HKGG1M14	e/g	e/g	e/g	e/g	e/g	g
HKGG1M15	e/g	g	e/g	g	e/g	g
HKGG1M16	e/g	e/g	g	e/g	g	e/g
HKGG1M17	g	g	e/g	e/g	e/g	g
HKGG1M18	e/g	e/g	g	e/g	g	g
HKGG1M19	e/g	g	g	g	g	g
HKGG1M20	g	g	g	g	g	e/g
HKGG1M21	g	g	e/g	g	e/g	e/g
HKGG1M22	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M23	e/g	e/g	e/g	g	e/g	g
HKGG1M24	e/g	e/g	e/g	g	e/g	g
HKGG1M25	e/g	e/g	g	e/g	g	g
HKGG1M27	e/g	e/g	e/g	e/g	e/g	g
HKGG1M28	e/g	e/g	e/g	g	e/g	g
HKGG1M29	e/g	e/g	e/g	g	e/g	g
HKGG1M30	e/g	e/g	e/g	g	e/g	g
HKGG1M31	e/g	e/g	e/g	e/g	e/g	e/g
HKGG1M32	e/g	e/g	e/g	e/g	e/g	g
HKGG1M33	e/g	e/g	.	e/g	e/g	g
HKGG1M34	g	g	e/g	e/g	e/g	g

Appendix H (continued)

No	Locus					
	e	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
gunungcola backcross						
HKGG1M35	e/g	e/g	e/g	g	e/g	g
HKGG1M36	g	g	e/g	e/g	e/g	g
HKGG1M37	g	g	e/g	e/g	e/g	g
HKGG1M38	e/g	e/g	g	e/g	g	e/g
HKGG1M39	g	g	e/g	e/g	e/g	g
HKGG2M01	e/g	e/g	g	e/g	g	g
HKGG2M02	e/g	e/g	g	g	g	g
HKGG2M03	e/g	e/g	e/g	e/g	e/g	g
HKGG2M04	e/g	e/g	e/g	e/g	e/g	g
HKGG2M05	g	g	e/g	g	e/g	e/g
HKGG2M06	g	g	g	e/g	g	e/g
HKGG2M08	g	g	g	g	g	e/g
HKGG2M09	g	g	g	e/g	g	e/g
HKGG2M10	g	g	g	g	g	g
HKGG2M11	e/g	e/g	e/g	e/g	e/g	g
HKGG2M12	e/g	e/g	g	e/g	g	e/g
HKGG2M13	g	g	e/g	e/g	e/g	e/g
HKGG2M14	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M15	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M16	g	g	g	g	g	e/g
HKGG2M17	g	g	g	g	g	g
HKGG2M18	g	g	e/g	e/g	e/g	e/g
HKGG2M19	g	g	e/g	e/g	e/g	g
HKGG2M20	g	g	e/g	e/g	e/g	e/g
HKGG2M21	g	g	e/g	e/g	e/g	g
HKGG2M22	e/g	e/g	e/g	g	e/g	e/g
HKGG2M23	e/g	e/g	e/g	g	e/g	e/g
HKGG2M24	e/g	e/g	g	g	g	e/g
HKGG2M25	g	g	g	g	g	g
HKGG2M26	e/g	e/g	g	g	g	e/g
HKGG2M27	g	g	e/g	e/g	e/g	e/g

Appendix H (continued)

No	Locus					
	e	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
gunungcola backcross						
HKGG2M28	e/g	e/g	e/g	e/g	e/g	e/g
HKGG2M29	e/g	e/g	e/g	g	e/g	e/g
HKGG2M30	g	g	e/g	g	e/g	e/g
HKGG2M31	g	g	g	g	g	e/g
HKGG2M32	g	g	e/g	e/g	e/g	g
HKGG2M33	e/g	e/g	g	e/g	g	g
HKGG2M34	e/g	e/g	g	g	g	g
HKGG2M35	e/g	e/g	g	e/g	g	e/g
HKGG2M36	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M01	e/g	e/g	g	e/g	g	g
GHKG1M02	g	g	g	e/g	g	g
GHKG1M03	e/g	g	e/g	e/g	e/g	e/g
GHKG1M04	.	e/g	e/g	e/g	e/g	g
GHKG1M05	e/g	e/g	e/g	e/g	e/g	g
GHKG1M06	.	g	e/g	g	e/g	e/g
GHKG1M07	.	g	e/g	g	e/g	e/g
GHKG1M08	e/g	e/g	e/g	e/g	e/g	g
GHKG1M09	e/g	e/g	e/g	e/g	e/g	g
GHKG1M10	e/g	g	g	g	g	g
GHKG1M11	.	e/g	e/g	g	e/g	e/g
GHKG1M12	g	g	g	e/g	g	g
GHKG1M13	g	g	e/g	e/g	e/g	.
GHKG1M14	.	g	e/g	g	e/g	g
GHKG1M15	e/g	g	g	g	g	e/g
GHKG1M16	e/g	e/g	e/g	e/g	e/g	g
GHKG1M17	e/g	e/g	g	e/g	g	g
GHKG1M18	e/g	e/g	e/g	e/g	e/g	g
GHKG1M19	g	g	e/g	e/g	e/g	g
GHKG1M20	e/g	e/g	g	e/g	g	g
GHKG1M21	g	g	g	e/g	g	e/g
GHKG1M22	e/g	e/g	g	e/g	g	g

Appendix H (continued)

No	Locus					
	e	*TFIIA-L*	*al*	*EcR*	*tim*	*ci*
gunungcola backcross						
GHKG1M23	e/g	e/g	e/g	e/g	e/g	g
GHKG1M24	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M25	g	g	e/g	e/g	e/g	g
GHKG1M27	g	g	e/g	g	e/g	g
GHKG1M28	g	g	e/g	g	e/g	e/g
GHKG1M29	g	g	e/g	e/g	e/g	e/g
GHKG1M30	e/g	e/g	e/g	e/g	e/g	e/g
GHKG1M31	e/g	e/g	e/g	g	e/g	g
GHKG1M32	e/g	e/g	g	g	g	g
GHKG1M33	e/g	e/g	g	e/g	g	e/g
GHKG1M34	e/g	e/g	g	e/g	g	e/g
GHKG1M35	g	g	e/g	g	e/g	g
GHKG1M36	e/g	e/g	g	e/g	g	g
GHKG1M37	g	g	e/g	e/g	e/g	g
GHKG1M38	g	g	g	e/g	g	e/g
GHKG1M39	e/g	e/g	g	e/g	g	g
HKGHK2M01	g	g	g	e/g	g	e/g
HKGHK2M02	g	g	e/g	e/g	e/g	g
HKGHK2M03	g	g	g	e/g	g	e/g

e and g represent *elegans* and *gunungcola* alleles, respectively.