BEHAVIOURAL AND GENETIC ANALYSIS OF SEXUAL ISOLATION BETWEEN SPECIES OF THE *DROSOPHILA AURARIA* COMPLEX

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ABSTRACT

The mechanisms of sexual isolation were behaviourally and genetically analysed in the species of the *Drosophila auraria* complex of the *montium* species subgroup in the *melanogaster* species group. There is strong reproductive isolation between sympatric species of the *D. auraria* complex, both sexual (pre-mating) isolation and hybrid male sterility (post-mating isolation). To analyse the speciation of the complex, the cause and development of sexual isolation was studied. The present study has attempted to reveal behavioural factors now maintaining sexual isolation between sympatric species and then to analyse the genetic basis of those factors.

Observation of inter- and intra-specific crosses showed that although males occasionally attempted to copulate heterospecific females, females strongly rejected courting heterospecific males. This evidence suggests that female discrimination plays a more important role than male choice.

Crossability tests were made to clarify the role of courtship song in sexual isolation. Wing (the courtship song generator) removal from males or arista (the courtship song receptor) removal from females decreased insemination frequency in conspecific crosses, suggesting that the conspecific courtship song raises female receptivity. Those operations, however, increased the insemination in heterospecific crosses, suggesting that a heterospecific courtship song inhibited female receptivity and elicited female rejection.

The courtship songs were recorded and analysed for five species of the complex. The inter-pulse interval was species-specific between sympatric species, but the other parameters - such as burst length, number of pulses per burst and intra-pulse frequency - were not. An identical inter-pulse interval was found in two allopatric species. It is suggested that inter-pulse interval is a species discriminator in females.

Three types of artificially synthesized courtship songs were given to females with wingless antennaless males of *D. biauraria*. Under the conspecific type of inter-pulse interval song copulation frequency was highest, and rejection frequency lowest. Under the heterospecific types of inter-pulse interval copulation frequencies were lower and rejection frequencies were higher. The control condition of random noise and silence showed intermediate copulation frequency and rejection frequency between conspecific and heterospecific inter-pulse interval songs. Thus inter-pulse interval appears to be the species discriminator.

The inter-pulse interval of all inter-specific hybrids between the five species except for two showed intermediate values, suggesting autosomal control of species-specific inter-pulse interval. The chromosomal analysis between *D. auraria* and *D. biauraria* revealed that the two major autosomes had quite significant effects on the determination of inter-pulse interval but the sex chromosome and cytoplasm had no effect. Since no interaction between chromosomes was detected, it is concluded that each autosome acts additively in the determination of species-specific inter-pulse interval.

This is the first report that a heterospecific courtship song elicits female rejection.

GENERAL INTRODUCTION

Understanding the mechanisms of speciation brings us important knowledge concerning biological evolution. Biological diversity requires speciation; the formation of two species from one species is the source of variation of life forms. Not only divergence within lineages but also the splitting of lineages is a most important phenomenon in evolution (Dobzhansky 1940). The mechanisms of speciation have long attracted much attention but still remains as a fundamental problem in evolutionary biology.

The biological species concept defines species as populations that are reproductively isolated from other populations (Dobzhansky 1951; Mayr 1969). Formation of reproductive isolating mechanisms which prevent gene flow between populations is a prerequisite to complete speciation (Dobzhansky 1937, 1951). After the completion of reproductive isolation, species evolve independently. Owing to reproductive isolation, divergence within lineages cannot ordinarily be disturbed by other lineages through gene flow. In the light of the concept of a biological species, the study of speciation is narrowed to that of development of reproductive isolating mechanisms.

Reproductive isolating mechanisms are categorized into two: pre-mating and post-mating isolation (Mayr 1969).

The post-mating isolation generally obeys Haldane's rule (Coyne & Orr 1989b); when one sex of a species hybrid is sterile or lethal, the sex is heterogametic. This rule found in several animal species suggests that there are some special genes or some specific mechanisms that produce hybrid sterility/inviability (Dobzhansky 1940; Coyne & Orr 1989b). Hybrid sterility and hybrid lethality should arise from the developmental discordance. Post-mating isolating mechanisms should be ultimately understood in terms of developmental biology. Now the study of post-mating isolation at the molecular level is progressing (Perez et al. 1993).

In contrast there is no general rule for pre-mating isolation. Although differences between closely related species are often morphologically and behaviourally recognized, not all of them are instrumental in their pre-mating isolation. We need to specify characteristic differences that play a significant role in pre-mating isolation.

Sexual isolation, one of the types of pre-mating isolation, is the most important mechanism preventing the gene flow between sympatric species especially in animals (Dobzhansky 1951; Bush 1975). It is the main mechanism maintaining reproductive isolation between some incipient species of *Drosophila* (Ayala 1975). When both sexes from different species meet, they will not succeed in copulation. Each sex plays its courtship in the way of its own species. The reason both sexes cannot succeed in copulation is the incompatibility of communication signals between the two species. The intersexual communication includes precise species discrimination through courtship. To understand species discrimination it is easy to divide it into two sides, the male and female. Thus the study of sexual isolation is, at first approximation, equivalent to the study of male discrimination and female discrimination.

Female discrimination is the most important behaviour that prevents inter-specific mating, especially at the early stages of speciation (von Schilcher & Dow 1977). The female acceptance/rejection of a courting male plays an important role in the establishment of sexual isolation. In order to understand the mechanisms of sexual isolation, we should specify the characteristics of rejected males.

The phenomenon of sexual isolation has been known before establishment of the biological species concept and the thought of isolating mechanisms. Females do not prefer to mate with males of different species in birds, pigeons and fowls (Darwin 1875). Darwin also pointed out that although female preference within species is not marked, a female makes a choice for mate. Choices can be looked at from two sides: acceptance and rejection (Fisher

1930). Female rejection (Fisher used 'discrimination', in his book, but I use the term, 'discrimination', in a broader sense) is favoured when both sexes encounter different populations that are in the process of fission into two species. He predicted that the rejection (discrimination, his word) and the acceptance (preference, his word) will be enhanced in such a situation. This consideration led to the emergence of the 'reinforcement model' of sexual isolation (Dobzhansky 1951). After the establishment of the biological concept, the 'reinforcement model' of sexual isolation was refined and reconstructed into the 'reinforcement model' and the 'reproductive character displacement model'. The former is the enhancement process of sexual isolation between populations 'not' completing isolating mechanisms. The latter is the enhancement process of sexual isolation between species or populations completing isolating mechanisms (Butlin 1989).

Several evolutionary processes can bring about pre-mating isolation (Endler 1989): a by-product of genetic divergence between populations, habitat choice and genetic predisposition, reinforcement, reproductive character displacement, sexual selection, and sensory drive. These processes are not mutually exclusive. Thus more than one process can operate and usually does in the establishment of sexual isolation during speciation. Pre-mating isolation between sympatric species has evolved more rapidly than that between allopatric ones in *Drosophila* (Coyne & Orr 1989a). Coyne & Orr concluded that this evolutionary phenomenon can be brought about only by a reinforcement model. Thus sexual isolation observed between sympatric species is of great interest in speciation study.

Although there is genetic differentiation between species, it does not mean that all of the genetic differences between populations reflect reproductive isolating mechanisms. The accumulation of a certain amount of genetic differences between populations does not automatically bring about reproductive isolation. For example, although reproductive isolation between the semispecies (incipient species) of *D. paulistorum* has been completed, the degree of genetic differentiation between semispecies does not significantly differ from those between non reproductively isolated subspecies (Ayala 1975). Little genetic differentiation observed between reproductively isolated populations suggests that a small number of genetic changes brought about speciation. Thus the number of genes controlling the characters of reproductive isolation is also of interest in the evolutionary genetic context.

Sexual isolation is due to the incompatibility of communication signals between both sexes. Sexual communication signals are maintained within species during speciation and they diverged between species during speciation. Thus evolution of pre-mating isolation should be also examined in the context of evolution of communication signals.

Female recognition for conspecific males has been known in several animal species. Vocal communications are well studied in several species using played back or artificially synthesized sounds. Songs were used as a sexual communication signals in birds (Searcy & Marler 1981; Searcy & Brenowitz 1988), the calling song in frogs (Ryan 1980, 1983; Ryan et al. 1990; Ryan & Rand 1993; Simmons et al. 1993), the calling song in crickets (Hoy et al. 1977; Pollack & Hoy 1979), the courtship song in lacewings (Henry 1985; Wells & Henry 1992a, 1992b) and the courtship song in *Drosophila* (Bennet-Clark & Ewing 1969; Kyriacou & Hall 1982; Crossley & Bennet-Clark 1993). These studies demonstrated that a female chooses her appropriate partner based on the vocal signal emitted by males.

Some parameter(s) of courtship song in many sympatric species of *Drosophila* are species-specific (e.g., Bennet-Clark & Ewing 1969; Ewing 1969; Miller et al. 1975; Kyriacou & Hall 1980; Crossley 1986; Ewing & Miyan 1986; Hoikkala & Lumme 1987; Hoy et al. 1988; Wheeler et al. 1988; Hoikkala et al. 1989). Courtship song is expected to be a species discriminator for females. Experiments of amputation of male wings (Sturtevant 1915; Ewing 1964; Narda 1966; Grossfield 1968; Bixler et al. 1992; Liimatainen et al. 1992; Hoikkala & Aspi 1993) and giving artificial song (Bennet-Clark & Ewing 1969; von Schilcher 1976b; Kyriacou & Hall 1982; Crossley & Bennet-Clark 1993) revealed that courtship song is one of the most important signals for mating success within a species. However, those experiments did not reveal whether females reject inappropriate males through the discrimination of courtship song.

Species of the *Drosophila auraria* complex in the *montium* species subgroup of the *melanogaster* species group are distributed in east Asia, including Japan, Korea and China. These sibling species show strong sexual isolation to each other (Kurokawa 1960; Kurokawa et al. 1982). Courtship behaviour of each species in the *D. auraria* complex essentially follows the description of *D. auraria* by Spieth (1952). When a male fly finds a female, he orients to her, and then follows and taps her body. Wing vibration was observed during following, at the time of attempted copulation and even during copulation. (Oguma et al. 1987). The courtship is frequently interrupted at a certain stage of the courtship sequences in inter-specific crosses, for example, by female rejection or by males' stopping tapping. Acoustic or pheromonal exchange might be involved in this interruption.

Sexual isolation is one of the pre-mating isolating mechanisms; potential mates meet but do not mate. Although species of the *D. auraria* complex used in this study are sympatric, they are different from each other in microhabitats (Kurokawa 1967; Kimura 1987). However these microhabitat differences alone cannot prevent inter-specific gene flow. We can collect any of two species at the same time from the same site using a bait trap. This means that if there is some suitable fermented substance in nature, flies have a chance to meet other species and hybridize. Although some hybrid males are sterile, hybrid females are fertile (Kimura 1987) and can be backcrossed (Hara & Kurokawa 1984). If there were no pre-mating isolation, introgression could be accelerated and then two species would fuse to one. Thus sexual isolation is one of the most important mechanisms that now maintain the gene pool of each species in the *D. auraria* complex. The wing vibration affects mating success within species in *D. triauraria* (Grossfield 1968), suggesting that the courtship song may play an important role in sexual isolation in the *D. auraria* complex.

Regarding the present situation mentioned above, I studied the mechanisms of sexual isolation using sympatric species of the *D. auraria* complex by behavioural and genetic approaches. In Part I, the sex which plays the important role in sexual isolation is clarified. In Part II, the essential role of courtship song in sexual isolation between sympatric species is shown. In Part III, the courtship song is described and shown to be a species discriminator. In Part IV, the species-specific inter-pulse interval of courtship song is shown to be a species discriminator. In Part V, the genetic basis of species-specific courtship song is examined. Finally in General Discussion, the evolutionary forces operating on the species discriminating system are discussed.

PART I

Courtship Behaviour in Inter- and Intra-specific Crosses

Introduction

Sexual isolation is brought about by the failure of courtship success between heterospecific pairs. Courtship is always initiated by a male; a male orients a female. The male follows her, taps her body, vibrates his wing to her, and attempts to copulate. In this behavioural sequence the female does not always play a passive role; she sometimes stops her movements, walks rapidly, and accepts/rejects him during attempted copulation. If there is sexual isolation between the sexes, the courtship generally breaks off, due to the incompatibility of communication signals between the sexes. Although communication involves both sexes, it is very likely that one sex plays a more significant role than the other in the behavioural sequence. In the early stages of speciation, female mate choice is more important than male one and the weight of mate choice will alter from females to males (von Schilcher & Dow 1977). In order to understand the process of development of sexual isolation, it is thus important to clarify which sex plays a more significant role in sexual isolation.

Observations of inter- and intra-specific matings were conducted to draw the distinction between the roles of the males and females, and to find the most critical courtship element operating in sexual isolation. All experiments were designed in the 'no choice' condition; i.e., each sex was from only one species.

Methods

Drosophila Stocks

All *Drosophila* strains originated from isofemales which have been maintained in our laboratory for more than 10 years. The strains and their collection sites were as follows: *D. biauraria* (B16 and B18, Tokyo), *D. subauraria* (ONM-29, Onuma; KT4, Kitakami), *D. triauraria* (T544, Tsukuba; T748, Miyazaki) and *D. auraria* (A662, Obihiro; A12, Tokyo). The strains were maintained in glass vials (3 cm diameter x 10.5 cm high) containing standard *Drosophila* sucrose - yeast - cornmeal medium at 24.5 ± 0.5 °C under a light dark cycle of 14(7:00 - 21:00):10 h. All flies were sexed without anaesthesia, within 12 h of their emergence. Flies were kept in groups of ten in a vial for four or five days. Flies were transferred once to new vials before observation.

Observation of Courtship

A pair of flies were introduced into a glass mating chamber (15mm in diameter x 3mm high), with an aspirator. After the introduction of flies into a mating chamber, courtship behaviour within 10 min was noted. Courtship latency (time to start courtship after introducing the flies into the mating chamber), courtship elements (orientation, tapping, wing vibration, attempted copulation, fluttering, and kicking), courtship time (time spent in all courtship elements except copulation) and copulation latency (time to copulate after introduction) were recorded. Fluttering and kicking were treated as rejection behaviour.

All strains from four species of the complex were used and twelve pairs of flies for pairs of each strain were observed. Observations were made within the first half of the light periods (8:30 - 13:00). Courtship behaviour between intra-/inter-specific crosses of all possible pairs were observed. Orderings of the observation for strain pairings were randomized.

Courtship index calculated in this study was defined as the time spent in courtship divided by the total observation time (10 min), or by copulation latency (for copulated pairs). In statistical analysis, the courtship index of pairs showing no courtship was omitted.

Results

Male courtship behaviours observed in all species/strain pairs are represented in Fig. 1. A male oriented to a female, tapped and vibrated his wings, and then attempted to copulate. Courtship elements in the inter-specific crosses did not differ from those in the intra-specific crosses. However, courtship did not always continue onto the next stage following each courtship elements. For example, orientation, tapping and wing vibration were observed even when males did not attempt to copulate. Tapping and wing vibration are the courtship elements that follow orientation. However, tapping and/or wing vibration were not observed after orientation in some pairs. The courtship index provided the quantitative information about courtship (Table I).

Males of most species courted more conspecific females than heterospecific ones. In some species (e.g., *D. auraria*), the number of courting males (orientation) in inter-specific crosses were more than those in intra-specific crosses (Fig. 1a). Courtship indices also showed that *D. auraria* males courted more heterospecific females and less conspecific females (Table I). Tapping and wing vibration were less than the orientation (Figs. 1b and 1c). The reduction was more extreme for inter-specific pairs than for the intra-specific pairs (Figs. 1b and 1c).

Attempted copulation was observed both in intra-specific crosses and in inter-specific crosses. There were more attempted copulation pairs within intra-specific pairs than between inter-specific pairs (Fig. 1d). These observations indicate that there is male discrimination, males court conspecific females more than heterospecific ones.

Table II summarizes the observation of inter- and intra-specific crossing experiments. Courtships were observed both in intra-specific crosses and in inter-specific crosses. The number of pairs with orientation did not significantly differ between intra-specific crosses and inter-specific crosses ($X_{c}{,}^{2} = 1.810$, df = 1, $P > 0.1$). However, courtship indices in intra-specific crosses were significantly higher than those in inter-specific crosses (Mann-Whitney's $U = 24888.5$, $P < 0.001$, chi-square approximation with 1 df). Many attempted copulations in intra-specific crosses and some in inter-specific crosses were observed. There were significantly more attempted copulations compared to orientation in intra-specific crosses than in inter-specific crosses ($X_{c}{,}^{2} = 70.625$, df = 1, $P < 0.001$). Although many intra-specific copulations were observed, only one inter-specific copulation was observed. The copulation frequency and frequency of attempted copulation were significantly different between intra-specific crosses and inter-specific crosses ($X_{c}{,}^{2} = 66.212$, df = 1, $P < 0.001$), indicating that there is female discrimination in inter-specific crosses.

Discussion

Sexual isolation is brought about by mate discrimination. Observations of courtship behaviour in the inter- and intra-specific crosses revealed that sexual isolation between the species of the *D. auraria* complex involves both male discrimination and female discrimination. Although a male orients to a female who is conspecific or heterospecific, he attempts to copulate less with a heterospecific female than with conspecific one. Thus male discrimination is one of the effective mechanisms of sexual isolation. Since sex pheromones alter male courtship in some *Drosophila* species (Jallon 1984; Cobb & Jallon 1990; Oguma et al. 1992), male discrimination will depend on sex pheromone in species of the *D. auraria* complex.

The female accepted a conspecific male but rejected a heterospecific one. Species-specific mating signals from the male are likely to be the species discriminator used by females. Visual, chemical or auditory signals are used as species discriminators in other species (Ewing 1983).

Morphological differences or colour patterns of the male body are possible visual signals for species identification. However, there is little male morphological differences except genitalia (Bock & Wheeler 1972; Kimura 1987) and no difference in colouration. Although some behavioural patterns can also be used as a visual signal, they are not species-specific (Oguma et al. 1987). Thus visual characteristics does not appear to be a species discriminator in this complex.

Sex pheromones identified in three *Drosophila* species are linear hydrocarbons (Jallon 1984; Oguma et al. 1992). If there is sex pheromone in the *D. auraria* complex, it will also be a hydrocarbon. Gas chromatographic analysis of hydrocarbons in the complex suggests that although there are variations between the species of the complex, those differences are not qualitative but quantitative (Oguma, unpublished observations). This fact suggests that hydrocarbons may facilitate another sex, but are not the species discriminator.

Female rejection was observed during the attempted copulation more in inter-specific crosses than in intra-specific crosses (Table II). No rejection behaviour was observed before the male attempted to copulate, suggesting that the female fly makes the decision to accept or to reject him during his attempted copulation. If so, since the male fly vibrates his wing during the attempted copulations, it is possible that the female uses an auditory signal from wing vibration as a species discriminator. A wingless male succeeded less in copulation in intra-specific cross of *D. triauraria* (Grossfield 1968), suggesting that wing vibration is an essential signal for mating success. The courtship song has species-specific parameters in many sympatric species of *Drosophila* (Bennet-Clark & Ewing 1969; Ewing 1969; Miller et al. 1975; Kyriacou & Hall 1980; Crossley 1986; Ewing & Miyan 1986; Hoikkala & Lumme 1987; Hoy et al. 1988; Wheeler et al. 1988; Hoikkala et al. 1989). Following sections reveal that wing vibration is a species discriminator in sexual isolation between species of the *D. auraria* complex.

PART II

The Role of the Courtship Song in Sexual Isolation

Introduction

Two roles of courtship song of *Drosophila* have been proposed namely, as stimuli giving rise to female receptivity, and as a species discriminator signal (Bennet-Clark et al. 1976; von Schilcher 1976b). The auditory signal is the prime candidate of species discriminator for females in the *D. auraria* complex (Part I). Since females reject heterospecific males more than conspecific ones (Part I), it is possible that courtship songs produced by heterospecific males elicit female rejection or inhibit females to accept males. If so, the male-wing removal would reduce the female discrimination; wingless (mute) heterospecific males will succeed in mating more than winged ones. Male wing removal experiments and artificial song experiments revealed that the courtship song is a stimulus that gives rise to female receptivity (Ewing 1964; Bennet-Clark & Ewing 1967, 1969; Narda 1966; Grossfield 1968; Eastwood & Burnet 1979; Kyriacou & Hall 1982; Robertson 1982; Bixler et al. 1992; Liimatainen et al. 1992; Crossley & Bennet-Clark 1993; Hoikkala & Aspi 1993). If songs play a role as a facilitating signal for females to accept courting males also in the *D. auraria* complex, wingless males will fail to copulate with conspecific females. In addition if the song is not a species discriminator, the degree of the failure in copulation of wingless conspecific males will be the same as for winged heterospecific males.

Insemination rates of the cross between female and normal conspecific males were higher than those of the cross between females and normal heterospecific males (Kurokawa 1960, Fig. 2a). Because of sexual isolation heterospecific mating hardly occurs. In the present study, crossability tests under no choice conditions were designed to clarify the role of courtship song in the *D. auraria* complex. Two crossability tests were designed to reveal whether a heterospecific courtship song elicits female rejection: Crossability Tests of Females x Wingless Males and Crossability Tests of Aristaless Females x Males. A crossability test was composed with four types of mating tests (Fig. 2b); crosses between females and normal wing conspecific males (2 wings), crosses between females and wingless conspecific males (No wing), crosses between females and wingless heterospecific males (No wing), and crosses between females and normal wing heterospecific males (2 wings). Three null hypotheses for insemination rates were made (Fig. 2b). Testing these null hypotheses provides a measure of the role of wing vibration in inter- and intra-specific crosses.

Wingless males may loose their courtship activity because of the injury of wing-removal in Crossability Tests of Females x Wingless Males. This possibility was tested in Crossability Tests of Aristaless Females x Males. Since female's aristae that extended from antennae were the sound receptor (Manning 1967; Burnet et al. 1971), the removal of aristae made females deaf. There is another pre-mating isolation, a mechanical isolation, where physical non-correspondence (e.g., genitalia) prevents sperm transfer. The insemination was assumed to reflect copulation in crossability tests. A mechanical isolation could not be detected under this assumption. However, it is very unlikely that there is mechanical isolation in the cross between females and normal wing males whereas there is not in the cross between females and wingless males. Thus results of null hypothesis testing will provide the efficiency of wings in sexual isolation.

Methods

Drosophila Stocks

All *Drosophila* strains originated from isofemales which have been maintained in our laboratory for more than 10 years. The strains and their collection sites were as follows: *D. biauraria* (B16 and B18, Tokyo), *D. subauraria* (ONM-29, Onuma; KT4, Kitakami), *D. triauraria* (T544, Tsukuba; T748, Miyazaki) and *D. auraria* (A662, Obihiro; A12, Tokyo). The strains were maintained in glass vials (3 cm diameter x 10.5 cm high) containing standard *Drosophila* sucrose - yeast - cornmeal medium at 24.5 ± 0.5 °C under a light dark cycle of 14(7:00 - 21:00):10 h. All flies were sexed without anaesthesia, within 12 h of their emergence. Flies were kept in groups of ten in a vial for four or five days. Flies were transferred once to new vials before crossability tests.

Surgery of Flies

Wings of male flies were removed with a pair of microscissors under carbon dioxide anaesthesia within 3 min. About half of the anaesthesised flies had their wings removed; the rest were used as 2-wing male controls. Thus there should be no anaesthesia bias on the experiments. Aristae of female flies were amputated, and preparation of control flies made, similarly.

Crossability Tests

Two crossability tests were designed: Females x Wingless Males and Aristaless Females x Males. Crossability Tests of Females x Wingless Males were constructed with four types of mating tests (Fig. 2b); females x winged conspecific males, females x wingless conspecific males, females x wingless heterospecific males, females x winged heterospecific males. These tests provide wing effects for females within a strain (null hypothesis 1), male discrimination (null hypothesis 2), and wing effects on sexual isolation between the two species/strains (null hypothesis 3). Every type of cross was made in parallel with others; five or more replications were made for each set of four types. When two replications were made simultaneously and I failed to separate sexes, the other seven vials were used for the test. Thus the sample size of one type differed from the other type of the cross in some crossability tests.

Crossability Tests of Aristaless Females x Males were constructed with four types of mating tests; 2-arista females x homotypic males, aristaless females x homotypic males, aristaless females x heterotypic males, 2-arista females x heterotypic males. Every type of cross was made in parallel with others, and six or more replications of the sets of four types were made. Since the arista is the courtship song receptor, these crosses provide the effects of the courtship song in sexual isolation. Both two crossability tests, Females x Wingless Males and Aristaless Females x Males, provide the importance of the courtship song in sexual isolation in the *D. auraria* complex.

Ten females and ten males were introduced into a food vial. After 2, 6, 24, or 48 h of confinement, these females were dissected and their spermatheca and seminal receptacles were examined for sperm. The flies with sperm were counted as inseminated. All crossability tests were carried out from 9:00 - 13:00.

Statistical Procedures

The chi-square test for contingency table was applied for detecting the difference between insemination rates of two different types of the tests in crossability tests. The chi-square test with Cochran correction for the 2 x 2 contingency table was applied for pairwise comparisons (Zar 1984). When any of the cells of expected frequency was less than 5, the Fisher exact probability test was used.

Results

Crossability Tests: Females x Wingless Males 1. Interspecific Cross

Significant differences were observed in insemination rate between the cross of females x 2-wing conspecific males and that of females x 2-wing heterospecific males in all crossability tests (Table III). All pairs of species showed sexual isolation. Statistical testing of the three null hypotheses revealed the effective role of male wing in insemination rate.

Null hypothesis 1: 2-wing males showed significantly higher insemination rate than wingless males in 22 crosses. There were 15 crosses where the insemination rate of 2-wing male and that of wingless male were not significantly different. Female species of the non-significant cross cases were *D. biauraria* and *D. subauraria*. In *D. triauraria* and *D. auraria* females, significant differences were observed in all crosses.

Wingless males showed significantly higher insemination rates than 2-wing males in the cross between *D. subauraria* KT4 and *D. triauraria* T544 in a 2h experiment.

Null hypothesis 2: Males inseminated more often in conspecific crosses than in heterospecific crosses in 21 crosses. There were 16 crosses where the insemination rate of conspecific males and that of heterospecific males were not significantly different. The cross of *D. auraria* A662 females x *D. triauraria* T748 males in 2h showed a significantly higher insemination rate in heterospecific males than in conspecific males.

Null hypothesis 3: The results of the hypothesis tested were divided into four categories. First, wingless males inseminated significantly higher than winged males; 15 crosses were in this category. The cross in this category indicates that the heterospecific courtship song elicits female rejection. Second, there were some number of inseminated females and non-significant differences were found; 10 crosses were in this category. The cross in this category indicates that the effect of heterospecific song was the same as under no song conditions. Third, wingless males showed significantly lower insemination rates than winged ones; the cross of *D. triauraria* T544 females x *D. auraria* A662 males is in this category. The cross in this category showed that a heterospecific courtship song gave rise to female receptivity. Finally there were no or nearly no inseminated females; 11 crosses were in this category. Crosses in this category provide little information for the effect of heterospecific song in testing null hypothesis 3.

Crossability Tests: Females x Wingless Males 2. Intra-specific Cross

No isolation was found in five intra-specific crosses (Table IV). Significant positive isolation was found in the cross of *D. biauraria* B18 females x B16 males and the cross of *D. triauraria* T544 females x T748 males, whereas negative isolation was also found in the cross *D. auraria* A662 females x A12 males (Table IV). No 2-wing male showed significantly lower insemination rate than wingless males in any intra-specific crosses, except for the cross of *D. subauraria* ONM-29 females x KT4 males (Table IV).

Crossability Tests: Aristaless Females x Males

If the wing removal did not affect male activity and the arista removal did not affect female activity, results of Aristaless Females x Males tests were expected to parallel those of Female x Wingless Males tests. In Aristaless Females x Males tests *D. biauraria* females and *D. subauraria* females (Table V) showed essentially the same results as the Female x Wingless Males tests (Table III). In conspecific crosses (null hypothesis 1) the arista removal did not affect the insemination rates compared to the insemination of intact arista females. Conspecific males inseminated significantly more often than heterospecific males in 4 out of 5 crosses (null hypothesis 2). Normal arista females were inseminated significantly less often than aristaless females in interspecific crosses (null hypothesis 3).

In *D. triauraria* females 2-arista females showed significantly higher insemination rates than aristaless females (null hypothesis 1). The insemination rate for the conspecific cross was significantly higher than that for the heterospecific cross in aristaless females (null hypothesis 2). Normal arista female did not show significantly different insemination rates compared to aristaless females in the heterospecific cross. In the Female x Wingless Males tests, the insemination rates from the crosses with wingless heterospecific males were significantly lower than those from the cross with winged heterospecific males (Table III).

In *D. auraria* females 2-arista females showed significantly higher insemination rates than aristaless females (null hypothesis 1). The insemination rate of the conspecific cross in aristaless females did not differ from that of the heterospecific cross in aristaless females (null hypothesis 2). In the heterospecific crosses, the insemination rates of aristaless females and that of normal arista females did not differ. In the Female x Wingless Males tests, the insemination rates from the cross with wingless heterospecific males were significantly higher than those from the crosses with winged heterospecific males (Table III).

Discussion

Results of crossability tests are summarized into four groups. Table VI schematically represents results of testing the three null hypotheses. (1) A conspecific courtship song increases insemination rates of females compared to those females under no song condition. This result came from testing null hypothesis 1. (2) Conspecific wingless males succeeded better in mating compared to heterospecific wingless males (null hypothesis 2). (3) A heterospecific courtship song decreases insemination rates of females compared to those of females under no song condition in most cases (null hypothesis 3). (4) A heterospecific song increases the insemination rate in one species pair, *D. triauraria* females x *D. auraria* males (null hypothesis 3).

Crossability tests were designed according to the three null hypotheses. Several interpretations shown to be significant by null hypothesis testing have important implications in the present study. Null hypothesis 1 stated that the insemination rate of the cross of females x conspecific 2-wing males and that of the cross of females x conspecific wingless males does not differ. Null hypothesis 2 stated that the insemination rate of the cross of females x wingless conspecific males and that of the cross of females x wingless heterospecific males does not differ. Null hypothesis 3 stated that the insemination rate of the cross of females x wingless heterospecific males and that of the cross of females x 2-wing heterospecific male does not differ.

When 2-wing conspecific males show higher insemination rates than wingless ones and null hypothesis 1 is statistically rejected, crossability tests can declare that the conspecific courtship song increases female receptivity. When wingless conspecific males show higher insemination rates than heterospecific ones and null hypothesis 2 is statistically rejected, crossability tests can declare that male flies discriminate conspecific females from heterospecific ones. When wingless heterospecific males show higher insemination rates than winged ones and null hypothesis 3 is statistically rejected, crossability tests can declare that the heterospecific courtship song elicits female rejection. When wingless heterospecific males show lower insemination rates than winged ones and null hypothesis 3 is statistically rejected, crossability tests can declare that the heterospecific courtship song increases female receptivity.

Amputation of the wing and arista may decrease male courtship activity. Crossability tests showed no influence on courtship by these operations, which can be explained by the following two reasons. First, although wingless males showed lower insemination rates than normal wing males in conspecific mating, wingless males showed higher rates than normal wing male in heterospecific matings for many cases. For example, wingless *D. triauraria* T544 males showed lower insemination rate than winged ones in conspecific crosses, while wingless ones showed higher insemination rates than winged ones in the heterospecific crosses with *D. biauraria* females (Table III). Second, Crossability Tests of Aristaless Females x Males showed similar results (Tables III and V).

In all but two conspecific matings, the insemination rate increased when the male's wings were intact compared to when the male's wings were removed (Table IV). Although there were some crosses where the insemination rates with wingless conspecific males were not significantly different from, or significantly higher than, that with winged conspecific males, pooled data for each female species indicated that the conspecific courtship song effectively increased mating in *D. biauraria*, *D. triauraria* and *D. auraria* (null hypothesis 1, Tables III, IV, and V). These results confirm several reports of intra-specific crossing experiment using wingless males; the mating frequency increased under the existence of male wing or conspecific type of artificial courtship song (Ewing 1964; Narda 1966; Grossfield 1968; Eastwood & Burnet 1979; Kyriacou & Hall 1982; Robertson 1982; Bixler et al. 1992; Liimatainen et al. 1992; Crossley & Bennet-Clark 1993; Hoikkala & Aspi 1993).

Wingless *D. biauraria* males succeeded in copulation at a very high rate (pooled, 79.8%, Table III), whereas *D. triauraria* males and *D. auraria* males succeeded less in copulation (pooled, 9.8% and 13.0% respectively, Table III). *D. subauraria* wingless males copulated at the same rate as winged ones (Table III). These differences of mating success in wingless males indicate that *D. triauraria* and *D. auraria* females need the conspecific song to accept males but that *D. biauraria* and *D. subauraria* females do not necessarily need a conspecific song in a cross of 2h or longer.

Insemination rates of the cross between females and wingless conspecific males were significantly higher than those between females and wingless heterospecific males in most cases; whereas those between females and wingless conspecific males were not significantly different from, or significantly lower than, those between females and wingless heterospecific males in some cases (null hypothesis 2, Tables III and V). Since differences between these two types of the mating tests depend on male species, the comparison of two types reveals male discrimination. Significant differences indicate that there is effective male discrimination involved in sexual isolation in the *D. auraria* complex. Male discrimination coincided with the observation of inter-specific crosses (Part I). It might be due to species differences in female sex pheromones. A male can discriminate a heterospecific female if she belongs to a certain species, but he cannot if she belongs to another species. Thus, male discrimination is not enough to maintain sexual isolation between sympatric species of the *D. auraria* complex.

Null hypothesis 3 of the crossability test provides information only when one of the cross types (wingless heterotypic male or 2-wing heterotypic male) has some inseminations. If a cross type brought no inseminated females, testing null hypothesis 3 provides little information. In other words, when both insemination rates of wingless heterotypic male and 2-wing heterotypic male are nearly zero, there is no resolution of differences in female rejection. Thus there is no information for the effect of heterospecific song in the crosses of; *D. subauraria* x wingless *D. triauraria*, *D. subauraria* x wingless *D. auraria*, *D. triauraria* x wingless *D. biauraria*, *D. auraria* x wingless *D. auraria*, and *D. auraria* x wingless *D. subauraria*. The conspecific courtship song is an effective signal for the female to accept males in *D. triauraria* and *D. auraria*, indicating that acceptance of a male with a conspecific song is also an important mechanism to maintain sexual isolation.

Females of the *D. auraria* complex reject heterospecific winged males. This evidence is the most important finding of the present study and is the first report that clearly demonstrates female rejection under the existence of a heterospecific courtship song. An artificial courtship song of the conspecific type effectively acts as a signal to elicit matings (Bennet-Clark & Ewing 1967, 1969; Kyriacou & Hall 1982; Crossley & Bennet-Clark 1993). Present results are consistent with those other studies (null hypothesis 1). Mating speed decreased under heterospecific songs but it was still faster than that under no song (Bennet-Clark & Ewing 1967, 1969) or under random song (Kyriacou & Hall 1982) conditions. Although the results of this study varied under the heterospecific condition (null hypothesis 3), results of testing null hypothesis 3 indicated active rejection of heterospecific winged males. Rejection of heterospecific winged males is stronger than wingless (no song) males. A parameter of courtship song that elicits female rejection will be estimated in Part III and will be clarified in Part IV. It is unknown whether the female rejection elicited by a heterospecific song is generally observed in *Drosophila*.

Although antennaless *D. persimilis* females accept *D. pseudoobscura* males, *D. persimilis* females with intact antennae reject *D. pseudoobscura* males (Mayr 1950). Mayr suspected that since an antennaless *D. persimilis* female cannot receive the chemical signals from males, she cannot discriminate whether courting males are conspecific, so then she accepts a heterospecific male. There are chemoreceptors on the third segment of antenna and also sound receptor as aristae, which extend from the third antennal segment, and Johnston's organ within the second antennal segment (Manning 1967). Courtship songs of *D. persimilis* differ significantly from those of *D. pseudoobscura* in inter-pulse interval and in song patterns (Waldron 1964; Ewing 1969). The removal of antenna prevents females from receiving both chemical and sound signals. Results of the *D. auraria* complex suggest that *D. persimilis* females might judge an appropriate mate from courting males with courtship song, but not with chemical signals. There is a report that male wings do not affect sexual isolation between *D. persimilis* females and *D. pseudoobscura* males (Mayr & Dobzhansky 1945). Since the male choice method was used in that study, the interaction between females or that between sexes may play a more significant role than wing effects. It is worth examining the wing effects again in the sexual isolation between *D. persimilis* and *D. pseudoobscura* under no choice conditions.

PART III

Analysis of Courtship Song

Introduction

To analyse the occurrence of pre-mating isolation, we need to specify the characteristics of the relevant isolating mechanisms. A character that has an important role in sexual isolation should be species-specific. If no male characteristics were species-specific, sexual isolation that depends on the female discrimination, especially at the early stages of speciation (von Schilcher & Dow 1977), would be ineffective.

Female rejection was an important behaviour in sexual isolation between sympatric species of the *Drosophila auraria* complex (Part I). Females rejected heterospecific winged males but accepted wingless males (Part II), indicating that the signal from male wings was a species discriminator for females. Wing vibration generated courtship songs, which are species-specific in many *Drosophila* species and each species has one or two types of song. The courtship songs probably play a role in sexual isolation between closely related species (Ewing 1983). The species differences are characterized by one or more parameters such as inter-pulse interval, fluctuation of inter-pulse intervals, intra-pulse frequency, sine song frequency, number of pulses per burst, burst length and the number of cycles per pulse (e.g., Shorey 1962; Ewing & Bennet-Clark 1968; Bennet-Clark & Ewing 1969; Ewing 1969; Miller et al. 1975; Chang & Miller 1978; Ewing 1979; Lakovaara & Hoikkala 1979; Ikeda et al. 1980; Kyriacou & Hall 1980; Robertson 1983; Crossley 1986; Ewing & Miyan 1986; Hoikkala & Lumme 1987; Hoy et al. 1988; Wheeler et al. 1988; Cobb et al. 1989; Hoikkala et al. 1989; Bernstein et al. 1992). One or more song parameters may exhibit species-specificity depending on the species.

In the present section the courtship song of sympatric and allopatric species of the *D. auraria* complex is described. Inter-pulse interval, burst length, number of pulses per burst and intra-pulse frequency were measured to determine the species-specific parameters. The species-specific parameter(s) will be a prime candidate for a species discriminator.

Methods

Preparation of Flies for Recording

All *Drosophila* strains originated from isofemales which have been maintained in our laboratory for more than 10 years. The strains and their collection sites were as follows: *D. auraria* (A541, Tsukuba; A662, Obihiro; A12, Tokyo), *D. biauraria* (B16 and B18, Tokyo; B660, Obihiro), *D. triauraria* (T544, Tsukuba; T748, Miyazaki), *D. subauraria* (ONM-29, Onuma; KT4, Kitakami), *D. quadraria* (Q, Chi Tou, Taiwan, Texas stock No. 3075.1). The strains were maintained in glass vials (3 cm diameter x 10.5 cm high) containing standard *Drosophila* sucrose - yeast - cornmeal medium at 24.5 ± 0.5 °C under a light dark cycle of 14(7:00 - 21:00):10 h.

All flies were sexed without anaesthesia, within 10 h of their emergence. Males were reared singly; females were kept in pairs in a vial for 4 days for recording the courtship song, except for four ONM-29 flies which were 5 days old. Flies were transferred once to new vials before recording. For most recordings a single pair of flies was introduced into the mating chamber with an aspirator. In some cases two or four females were introduced into the mating chamber with a single male to reduce courtship latency.

Recording Equipment

A glass mating chamber (15 mm diameter x 5 mm in deep) was placed on a Sony ECM-55B Condenser Microphone with a frequency response of 30 - 18000 Hz. The microphone was covered with stainless steel mesh and settled in a plaster recording apparatus (6.5 cm diameter, 6.5 cm high). The gap between the chamber and the apparatus was filled with dental wax to cut off extraneous noises. The recording apparatus was put in the box (27.5 x 27.5 x 27.5 cm) and was hung from a clear acrylic board to avoid vibrations from the floor. Courtship song was transmitted from the microphone to a Nihon Kohden Biophysical Amplifier AVB-11 and monitored on a Nihon Kohden Memory Oscilloscope VC-11. Amplified song was recorded on a Sony L-830 EG-HG tape using a Sony SL-HF 3000 VTR and a Sony PCM-501ES PCM Processor, the frequency response of which was almost flat from 20 to 20000 Hz. All recordings were made under 100 - 300 lx from a Nikon Fiber Optic Light Source in a temperature controlled room (24.5 ± 0.5 °C). Under recording conditions the temperature inside the box was between 24 and 25 °C for more than 2h, so the temperature in the mating chamber, which was not monitored, was estimated to be 24 - 25 °C. The behaviour of the flies was simultaneously recorded on another channel of the same tape. Each recorded song was digitized at 44100 Hz, 16 bit using an analogue to digital converter (Canopus Sound Master) and was stored on an Itec IT RL-100 hard disk. As the Nyquist frequency, a half of the sampling frequency, was higher than the frequency response of the microphone (18000 Hz), there was no loss of information when digitizing (sampling theorem). Since the digitized songs were directly processed and computed on a NEC PC-9801 RS21 personal computer, no noise mixed into the song when the songs were manipulated.

Analysis of Courtship Song

Inter-pulse interval

The inter-pulse interval was defined as the time interval from one peak of the pulse to the next. Since the peak-detecting program was too sensitive to run automatically, the peak-tops of pulses on the cathode ray tube (CRT) display were visually checked to exclude the misdetection of a peak, for example a ghost peak because of baseline noise (Fig. 3a). The mean inter-pulse interval was calculated from 30 inter-pulse intervals per male (three bursts per male).

Spectral analysis

The fast Fourier transform (FFT) spectrum was derived from each pulse. First, the digitized courtship song was processed to single pulses (Fig. 3b). The width of the processed pulse differed between species because each species has a different pulse duration. Courtship song was automatically processed to single pulses, which were a half of the pulse width for each species (Table IX) on either side of the logged pulse (Fig. 3b), using the signal of the peak-top of the detected pulse while computing the inter-pulse interval. In the program developed, the FFT was directly performed on each processed single pulse (4096 points) to obtain the frequency spectrum, after detecting the pulse and calculating the inter-pulse interval. The intra-pulse frequency was defined as the frequency showing maximum power in the spectrum, following Wheeler et al. (1988). The smoothing and differentiation method (Savitzky & Golay 1964) was used to detect the maximum power spectral peak. The range of detection of intra-pulse frequency was restricted according to the width of the pulse. The lowest frequency of the available detection in intra-pulse frequency is the inverse of the pulse width (1/pulse width). The highest detectable frequency range was 18000 Hz. When the frequency with maximum power in the spectrum was outside the detectable range, it was not treated as intra-pulse frequency and so there were fewer intra-pulse frequencies than the calculated pulses.

Statistical procedures

I used analysis of variance (ANOVA) with the reciprocal transformation to detect species differences, followed by Tukey multiple comparison testing (Zar 1984). When a Bartlett test revealed heteroscedasticity, in spite of several transformations before analysis, a Kruskal-Wallis test was applied, followed by non-parametric Tukey-type multiple comparisons. For testing species differences in inter-pulse interval, means per fly were calculated and an ANOVA was applied.

Results

Courtship Songs

Courtship behaviour of each species in the *D. auraria* complex essentially follows the description of *D. auraria* by Spieth (1952). When a male finds a female, he orients to her, then follows and taps her body. Wing vibration was observed during following in most cases; this behaviour produces courtship song in the form of bursts of sound pulses (Fig. 4). All male flies vibrated a wing during attempted copulation and copulation. Males produced courtship song less before copulation and at attempted copulation, and more during copulation. Although wing-amputated males can produce almost normal courtship song in some species (Waldron 1964; Miller et al. 1975), courtship song in the *D. auraria* complex is produced by male wing movement during wing vibration and transmitted through the air; no courtship song could be detected when wingless males were tested for recording.

Each species produced only one sort of song, 'pulse song'. Figure 4 shows the typical wave patterns of the courtship songs. The pulses in each species consisted of two or three cycles. Other sorts of song, for example, sine song (*D. melanogaster*), were not observed. Although the wave pattern of courtship song changes together with the behaviour of the male in some species (Ikeda et al. 1980; Crossley 1986; Cobb et al. 1989), this was not observed in species of the *D. auraria* complex. Only pulse song was observed during following, attempted copulation and copulation.

Inter-pulse Interval

The frequency distributions of inter-pulse intervals (Fig. 5) did not differ between before copulation/during attempted copulation and during copulation. The inter-pulse interval did not alter during courtship (Fig. 5).

Inter-pulse intervals significantly differed between strains (ANOVA, $F_{10,\ 64} = 165.37$, $P < 0.001$; Table VII). Multiple comparison revealed no significant differences within the species and significant differences between species except for *D. triauraria* and *D. quadraria,* the only allopatric species (Table VII).

Burst Length and Pulses per Burst

Courtship song consisted of several bursts of pulse trains. The burst length did not differ significantly between species (Table VIII). The number of pulses per burst before copulation and during attempted copulation also did not differ significantly between species, but there were more pulses per burst during copulation in *D. biauraria* than in the other three species (Table VIII). However, the number of pulses per burst in *D. biauraria* is unlikely to be species-specific because before copulation and during attempted copulation they showed the smallest value of all the species (Table VIII).

Spectral Analysis

Frequency spectra were derived from a single pulse (Figs 3 and 6). All spectra of each species were L-shaped and their peaks lay from 50 to 1000 Hz. High frequency signals were not observed.

The spectrum of each species had several peaks (Fig. 6). Some were harmonics of the main peak and others were ghosts that cannot be excluded in the spectral analysis, because measurement over an infinite observation time is impossible. The power of harmonics cannot ever exceed that of the main peak. Background noise superimposed on each spectrum were weaker than the maximum powers in absolute units of the spectra from the song. Intra-pulse frequency should not be altered even if the shape of spectrum might be affected by noise and harmonics.

Intra-pulse frequency was determined from the frequency spectrum derived from single pulses (Fig. 6). Average intra-pulse frequencies differed between strains (Table IX; Kruskal-Wallis test, $H = 362.60$, $P < 0.001$, assuming chi-square distribution with 10 df). Non-parametric multiple comparisons showed that the intra-pulse frequency of *D. subauraria* ONM-29 was significantly larger than in the other strains and that the other strains were overlapping (Table IX). There is no species specificity in intra-pulse frequency, and significant intra-specific variation was observed.

Discussion

Inter-pulse interval was the only parameter that consistently shows significant differences between the sympatric species of the *D. auraria* complex; the correlated inter-pulse interval parameters (burst length and number of pulses per burst) were not species-specific. The other parameters of courtship behaviour do not show qualitative or quantitative differences (Oguma et al. 1987). Although inter-pulse intervals of some species overlap or have a large coefficient of variation (e.g., Miller et al. 1975; Cowling & Burnet 1981; Crossley 1986; Ewing & Miyan 1986), inter-pulse interval of each species of the *D. auraria* complex shows less overlap and little variation (maximum coefficient of variation was 10.5%). This evidence strongly suggests that female flies use inter-pulse interval as a species discriminator. This will be clarified in Part IV.

Sexual isolation between *D. triauraria* and *D. quadraria* is very weak (Kurokawa et al. 1982). Since inter-pulse intervals of these two species are not significantly different, female flies might not be able to detect whether the courting males are homotypic. Asymmetrical mating observed in inter-specific crosses (Kurokawa 1960; Kurokawa et al. 1982) cannot be explained on the basis of inter-pulse interval, since the difference of inter-pulse interval does not correlate with mating success. Though there is a strong pre-mating isolation between the species of the *D. auraria* complex, inter-specific hybrids can be produced in the laboratory. Since intra-pulse frequencies of species within the complex were similar (Table IX) and the range of inter-pulse intervals of the species overlapped (Fig. 5), a female fly may not be able to detect a homospecific male in a confined vial.

Many winged males mated with females in intra-specific crosses; wingless ones did not mate so often (Part II, Grossfield 1968). Thus courtship song also has a role as a sexual stimulus in the *D. auraria* complex. The courtship song has two functions as a sexual stimulus and a species discriminator, and these functions are shared by different wave patterns in some species (von Schilcher 1976b; Ikeda et al. 1981; Ewing & Miyan 1986). Since the courtship song of the *D. auraria* complex shows only one kind of song (Fig. 4), the pulse song of the *D. auraria* complex might play a role as a sexual stimulus and as a species discriminator.

The inter-pulse intervals of *D. triauraria* and *D. quadraria* did not differ significantly (Table VII). The morphological evidence (Bock & Wheeler 1972) and the study of protein electrophoresis and of isozymes (Ohnishi & Watanabe 1984) show that *D. triauraria* is the most closely related species to *D. quadraria*. I conclude that the time of divergence between *D. triauraria* and *D. quadraria* is not long enough for their inter-pulse intervals to have diverged.

The arista (a bristle-like extension of the third antennal segment) is considered to be a sound receptor, which responds to frequencies less than 450 Hz in *D. funebris* (Ewing 1978). Intra-pulse frequency in the *D. auraria* complex (Table IX) lies within the response frequency of aristae. Since intra-pulse frequency was not species-specific in the *D. auraria* complex (Table IX), it might play a less important role in species recognition. The courtship song of the *D. auraria* complex did not contain high frequency sounds (Fig. 6) which supports the view that only some of the Hawaiian *Drosophila* species developed the means to produce high frequency songs after colonizing the Hawaiian Islands (Hoy et al. 1988; Hoikkala et al. 1989). Wheeler et al. (1988) used spectral peak width as one of the parameters showing species-specificity. Since in the system used here the pulse width differed with the same sampling frequency, the peak width depended on pulse width. The wider the pulse, the sharper the spectral peak. Thus, peak width cannot be a parameter of courtship song.

The X chromosome is responsible for determination of species-specific inter-pulse interval in some *Drosophila* species (e.g., Hoikkala & Lumme 1987), and the autosomes are responsible in other species (e.g., Ewing 1969; Cowling & Burnet 1981; Kawanishi & Watanabe 1981). The underlying genetic control of species differences in the *D. auraria* complex will be revealed in Part V.

PART IV

Artificial Song Experiments

Introduction

Previous sections (Parts I - III) reported three main results: first, a female discrimination plays an important role in sexual isolation between sympatric species of the *D. auraria* complex (Part I), second, the courtship song is a species discriminator (Part II), and third, a species-specific parameter of courtship song is the inter-pulse interval (Part III). In Part II a simple but a quite powerful approach to detect the role of courtship song was developed: Crossability Tests between Females x Wingless Males. These clarified that female rejection is the most important mechanisms of sexual isolation. These lines of evidence suggest that the species-specific inter-pulse interval is a species discriminator; when a female receives the heterospecific type of inter-pulse interval, she rejects courting males.

An artificial song experiment must be one of the most direct methods to prove whether a heterospecific type of inter-pulse interval elicits female rejection. When females are given heterospecific types of songs synthesized artificially, they will reject courting wingless males; when females are given conspecific types of songs, they will easily accept males. In the present Part, two kinds of experiments were performed.

The first one is to test the same framework of crossability tests of Part II in a shorter crossing time: "Observation of Female x Wingless Male in 30 Min." Since it is difficult to perform long-time observation under an artificial song, a female rejection that can be observed in a shorter period is a prerequisite for artificial song experiments.

The second one is artificial song experiments. Artificially synthesized songs were given to a female with a wingless antennaless male. Four different types of song, conspecific type inter-pulse interval, longer inter-pulse interval, shorter inter-pulse interval and random noise, were synthesized. A random noise song and silence (no song) were used as controls. Artificial song experiments can prove whether the inter-pulse interval is a species discriminator.

Methods

Drosophila Stocks

D. biauraria B16 (Tokyo) was used as the tester female strain, because this strain was inseminated by heterospecific wingless males but rarely by heterospecific winged ones (Part II), and the inter-pulse interval of *D. biauraria* is the intermediate value (12 - 13 ms) within the complex (Part III). In crossability tests in 30 min (Females x Wingless Males in 30 Min), *D. triauraria* T544 (Tsukuba) was used as a heterospecific male strain. Wingless males of this strain inseminated well with *D. biauraria* B16 females within 2 h (Part II). The strains were maintained in glass vials (3 cm diameter x 10.5 cm high) containing standard *Drosophila* sucrose - yeast - cornmeal medium at 24.5 ± 0.5 °C under a light dark cycle of 14(7:00 - 21:00):10 h. All flies were sexed without anaesthesia, within 12 h of their emergence. Flies were kept in groups of ten in a vial for four or five days. Flies were transferred once to new vials before observation or artificial song experiments.

Surgery of Flies

Wings of male flies were removed with microscissors under carbon dioxide anaesthesia within 3 min. About half of anaesthesised flies had their wings removed; and the rest were used as 2-wing male controls. Thus there might be no anaesthesia bias on the experiments. In artificial song experiments antennae of flies were also removed with forceps.

Observation of Female x Wingless Male in 30 Min

A pair of flies were introduced into a glass mating chamber (15mm in diameter x 3mm high), with an aspirator. After the introduction of flies into a mating chamber, courtship behaviour was observed over a 30 min period. Courtship elements (orientation and attempted copulation) and copulation latency (time to copulate after introduction) were recorded. Observations were made within the first half of the light periods (9:00 - 13:00). Four types of crosses were made; crosses with winged *D. biauraria* males, with wingless *D. biauraria* males, with wingless *D. triauraria* males and with winged *D. triauraria* males. The framework of these crosses were identical to that in Part II. The same number of pairs were always observed simultaneously for each four cross types within the same day.

Artificial Song Experiments

Equipment to produce artificial song

Mating chambers (15 mm inside diameter x 6.5 mm height) for the artificial song experiments were made with acoustically transparent nylon mesh floors and roofs (Fig. 7a). Recorded artificial songs were played back by a Sony cassette tape recorder WM-R707 and transmitted to a loudspeaker (90 mm diameter) via a power amplifier. Six mating chambers were settled in a circle (Fig. 7b) on a stainless steel mesh placed 25 mm above the loudspeaker (Fig. 7b). Artificial songs were played back at a sound pressure level of 120 dB above 2×10^{-5} Pa.

Synthesized artificial songs

A burst of artificial song was synthesized as a train of single sine waves (Fig. 8a). The frequency of the sine wave was 110.2 Hz and burst length was 400 ms; both parameters lay within the range of the *D. auraria* complex

(Part III). Artificial songs consisted of regularly repeated bursts with 50 ms interval (Fig. 8c). Three types of pulse song were synthesized (Table X). The inter-pulse intervals of each pulse song were 13 ms, 16 ms and 11ms, which correspond to mean values of *D. biauraria*, *D. triauraria* and *D. subauraria*, respectively. Random noise song was synthesized as a burst of data points randomly distributed along the time axis (Fig. 8a). A song synthesized on a MS-DOS machine was transmitted to a Sony cassette tape recorder WM-R707 via a Canops digital to analogue converter Sound Master, and was recorded on a Hitachi Maxell US II tape. Oscillographic patterns of played back artificial songs are shown in Figs. 8b and 8c. There were some additional pulses with the main pulses (Fig. 8b), inter-pulse intervals of played back artificial song were not altered (Table X).

Observation under artificial song

A pair of flies were introduced into the mating chamber for artificial song experiments, with an aspirator. Immediately after the introduction of flies into a mating chamber, artificial songs were given to the pairs and courtship behaviour was observed for during 30 min. Courtship elements (orientation, attempted copulation, fluttering and kicking) and copulation latency were recorded. Fluttering and kicking were treated as rejection behaviour. Observations were made during the light periods (9:00 - 16:30). Since the equipment to produce artificial song can produce only one kind of song at the same time, not only song conditions but also the time of experiment may affect the results. To avoid this, five types of song experiments were made in the same day and orderings of song types were randomized by three different 5 x 5 Latin squares.

Results

Observation of Female x Wingless Male in 30 Min

Numbers of pairs showing orientation, attempted copulation and copulation are shown in Table XI. Chi-square analyses of 2 x 4 contingency table were performed to reveal heterogeneity between four male types. The numbers of pairs with orientation and without orientation across four male types were significantly heterogeneous ($X^2 = 9.81$, df = 3, $P < 0.025$). The numbers of pairs with attempted copulation and without attempted copulation (but showing orientation) across male types were also significantly heterogeneous ($X^2 = 10.21$, df = 3, $P <$

0.025). The male discrimination may cause these significant heterogeneities in orientation and in attempted copulation. To diminish male discriminations and to extract female responses for male wings, data of pairs without attempted copulation were omitted.

Mating frequencies of different cross types were summarized in Fig. 9a. No heterospecific (*D. triauraria*) winged males copulated with a *D. biauraria* female, whereas more than 60% of *D. triauraria* wingless males showing attempted copulation had successful matings. Numbers of pairs with copulation and without copulation (but showing attempted copulation) were significantly heterogeneous ($X^2 = 71.81$, df = 3, $P < 0.001$). Pairwise comparison (Table XII) revealed the significant excess in mating frequency for conspecific winged, conspecific wingless and heterospecific wingless males compared to heterospecific winged males. This means that sexual isolation between *D. biauraria* females and *D. triauraria* males were established by intersexual communication mediated by wing vibration. It also indicates that observation time of 30 min is sufficient to detect female rejection.

Artificial Song Experiments

In artificial song experiments, it is expected that the female behaviour will change under different songs. Different song conditions may affect not only female behaviour but also male behaviour. When a male courts a female, he alters his behaviour according to the female behaviour. For example in normal courtship, a male follows a female moving around the mating chamber. When she stops her movements for a long time, he interrupts and abandons his courtship. The numbers of pairs showing several courtship elements are shown in Table XIII. Chi-square analysis of 2 x 5 contingency table indicated that the number of pairs showing orientation and those without orientation was significantly heterogeneous across the song types ($X^2 = 15.18$, df = 4, $P < 0.005$). The numbers of pairs with attempted copulation and that without attempted copulation (but with orientation) was also significantly heterogeneous across five song types ($X^2 = 18.72$, df = 4, $P < 0.001$). Pairwise comparison revealed that the number of pairs with attempted copulation in silence was significantly more than the other song types (Table XIV). Significant heterogeneities observed in orientation and in attempted copulation suggest that the male behaviour was altered under different types of artificial song.

Mating frequencies under various song conditions are summarized in Fig. 9b. The chi-square analysis comparing the number of mated females and unmated ones (though some attempted copulations were observed) across the five experimental conditions was significant ($X^2 = 20.61$, df = 4, $P < 0.001$). Pairwise comparison between any of two conditions (Table XVI) revealed that mating frequency under 13 ms song conditions is significantly higher than that under all the other conditions except under random song. Mating frequencies under random song did not significantly differ from those under the other four types of song. This fact indicates that mating frequency under random song was no greater than that under 13 ms and also no lower than under 16 ms or 11 ms song.

Longer confinement with heterospecific winged males brought some heterospecific matings (1.8% with *D. triauraria* T544 males, Table III in Part II), whereas this was not observed over a short time (Fig. 9a). This evidence suggests that although a female rejects a heterospecific winged male at first, she accepts him after experiencing many courtships. Thus female responses during the first attempted copulation will provide more precise information for the effects of each song. Female responses during attempted copulation were categorized into three classes: copulation, rejection and failure without rejection. The class of copulation may include behaviour such that a female first responds with fluttering (one of rejection behaviour) but at last she accepts a courting male. Pairs of failure without rejection were those where females showed no rejection behaviour (fluttering or kicking), but pairs did not copulate.

As mentioned above, significant heterogeneity was observed in the number of pairs between attempted copulation and not attempted copulation across song types. The relative frequency of three types of female responses per number of attempted copulations during first attempted copulation was shown in Fig. 10. Differential responses were found in the first 5 min and continued to the end of 30 min observation. The number of female responses during the first attempted copulation were shown in Table XIII. Pairwise comparisons between copulation and non-copulation and those between rejection and non-rejection were performed (Tables XVI and XVII). A 13 ms song interval was significant compared to the other types of song condition, except compared to random song in copulation. The other of song type pairs were not significant.

Discussion

Heterospecific wingless males never copulated but heterospecific wingless male were able to copulate within 30 min. If females reject males under heterospecific conditions, mating frequency under heterospecific song will decrease compared to under conspecific song, random noise or silence in 30 min. Observation of courtship will provide more information than testing insemination in Part II. Behavioural responses for artificial song revealed how females reject courting heterospecific males. This is the female responses that actually play an important role in sexual isolation.

The inter-pulse interval was shown to be a species discriminator. Artificial song experiments demonstrate that (1) a conspecific type inter-pulse interval increases mating frequency, and (2) a heterospecific type inter-pulse interval decreases mating frequency and elicits female rejection. A female received artificially synthesized courtship songs and accepted a courting male depending on the inter-pulse interval.

Since a heterospecific type of inter-pulse interval elicits female rejection, the inter-pulse interval functions as a species discriminator; and since successful copulations are more common under a conspecific type of inter-pulse interval than under a random song or no song, the conspecific type of inter-pulse interval functions to increase female receptivity. Under random noise and silence, frequencies of female responses (copulation and rejection) were intermediate between frequencies under conspecific and heterospecific types (Figs. 7b and 8). Assuming that female responses observed under random song and under silence represent initial responses without any stimuli, a conspecific type of inter-pulse interval inhibits rejection, and a heterospecific type of inter-pulse interval accelerates rejection. The courtship song has two functions as a sexual stimulus and as a species discriminator and these functions are shared by different wave patterns in some species (von Schilcher 1976b; Ikeda et al. 1981; Ewing & Miyan 1986). Artificial song experiments demonstrate that the pulse song of the *D. auraria* complex plays a role as a sexual stimulus and also as a species discriminator, and the critical parameter is the inter-pulse interval.

When a male attempts to copulate, a female spreads her wings in some *Drosophila* species (Spieth 1952). This behaviour was thought to be a receptive posture which is a stimuli to inform a male that she is now receptive (Spieth 1952). Receptive postures of females were induced by male wing vibration in *D. mercatorum* in a double cell experiment (Ikeda et al. 1981). When a male vibrates in the isolated inner cell with two females, some females in the outer cell stop movement and spread their wings in 'receptive postures'. Ikeda et al. (1981) showed that conspecific song elicits a female receptive posture without a male. Artificial songs could not elicit such a female receptive posture in *D. biauraria*.

Artificial song experiments were carried out in *D. melanogaster* and *D. simulans*. Bennet-Clark & Ewing (1969) revealed that the inter-pulse interval is the critical parameter. Kyriacou & Hall (1982) claimed that the inter-pulse interval itself is not important but the rhythmic fluctuation of inter-pulse intervals is critical. Those two reports were inconsistent in the indicated female reception factor. Although synthesized courtship song in this study had altered inter-pulse interval, they had a constant value of inter-pulse interval for each song. Inter-pulse intervals were less variable in the *D. auraria* complex (Part III), suggesting that there are no rhythmic fluctuations.

Although heterospecific winged male did not copulate within 30 min, many heterospecific wingless males could copulate (Fig. 7a). Since songs from intact wings inhibited inter-specific copulation completely within 30 min, it was expected that heterospecific types of artificial song also inhibit copulation. However, in more than 20% of pairs, copulations were observed under heterospecific types of artificial song (Fig. 7b). The inhibition of heterospecific types of artificial song were less than that of real song produced by heterospecific winged males. There are several reasons why the artificial song could not inhibit mating completely. Sound or electric noises will make females receptive. Rejection was less observed under random noise than silence (Table XIII). Some common noise superimposed on a synthesized song may affect female receptivity. Secondly, the artificial song may fail to reach the female at the appropriate time. Although artificial songs were constantly repeated in bursts of pulse song, songs produced by males were not constantly produced: songs were produced repeatedly during attempted copulation but seldom during the other courtships. Hence, different timings allow wingless males to copulate even under the heterospecific type of inter-pulse interval.

Thirdly, the sound level may have been insufficient; too large or too small to completely inhibit copulation. When various sound levels of artificial song are examined in *D. parabipectinata*, male movements and courtship interactions between males were observed under the sound level between 66 and 120 dB (Crossley & Bennet-Clark 1993). Successful copulations of wingless males were observed under sound levels of 84 and 100 dB in *D. melanogaster*, *D. simulans* and *D. parabipectinata* (Bennet-Clark & Ewing 1969; Kyriacou & Hall 1982; Crossley &

Bennet-Clark 1993). Estimated sound pressure of real courtship song received by female is 119 dB in *D. melanogaster* (Bennet-Clark & Ewing 1969). Although artificial song experiments with altered sound level are needed to examine the effective level in *D. biauraria*, sound level of artificial song was almost sufficient to test the effectiveness of inter-pulse interval.

Synthesized artificial songs were fixed for burst length and inter-burst interval (50 ms). Thus the number of pulses given to females differed from each other; 11 ms songs included 40% more pulses than 16 ms songs did (Table X). A female may reject a courting male because the number of pulses received by her were too small. This hypothesis cannot explain the fact that the increased female receptivity is greatest with the intermediate inter-pulse interval (13 ms), whereas longer (16 ms) or shorter (11 ms) inter-pulse intervals elicit female rejection. The number of pulses per burst is also detectable. However, since it is not a species-specific parameter (Part III), it cannot act as a species discriminator.

Copulation frequencies of wingless *D. biauraria* male in "Female x Wingless Male in 30 Min" did not differ from that under silent conditions ($X_c{}^2 = 0.778$, df = 1, $P > 0.25$). Although male flies had intact antennae in "Observation of Female x Wingless Male in 30 Min," they did not in artificial song experiments. Male's antennae did not affect female acceptance and rejection. Although size of the mating chamber in "Observation of Female x Wingless Male in 30 Min" differed from that in artificial song experiment, chamber size may not affect behaviours of both sexes.

Since conspecific types of artificial song alter male behaviour in *D. melanogaster* (von Schilcher 1976a) and *D. parabipectinata* (Crossley & Bennet-Clark 1993), this may also be observed in *D. biauraria*. Sound pressure of courtship song sways the arista, which transmits information of air vibration to Johnston's organ mechanistically (Manning 1967). The Johnston's organ is the mechano-receptor that responds to vibration (Manning 1967). Males used in artificial experiments had their antennae removed from the base; the second segments including Johnston's organ inside and the aristae were completely removed from males. It is unlikely that a male heard the artificial song and that he altered his behaviour by receipt of the song. In addition, data of pairs showing attempted copulation were analysed and those not showing attempted copulation were neglected. Analysed data were already corrected by omission of pairs not showing attempted copulation. Thus results of artificial song experiments show female responses for differential song conditions.

The number of pairs showing attempted copulation was significantly more in no song conditions than in the other four artificial song conditions (Table XIV). It is plausible that female behaviour under artificial song conditions includes signals to enhance or to inhibit males' attempting copulation. Light dependency of matings suggests that the visual signals are one of the important stimuli for mating success (Grossfield 1971; Oguma et al. 1987). Female movement is one of the important stimuli to elicit male courtship in the *D. auraria* complex (Oguma et al. in preparation). Female behaviour may change under the artificial song. Female behaviour influences male behaviour in several ways (Ewing 1983). All females moved around during the 30 min observation periods. Although the quantitative data of female behaviour was not recorded, the number (and possibly also quality) of female movements changed under the artificial song. A female with less movement may be less courted by a male. Artificial songs may reduce female movements.

PART V

Genetic Basis of Species-Specific Inter-pulse Interval

Introduction

Genetic analysis of species-specific characters can reveal the evolutionary significance of species differences. If a species-specific characteristic involved reproductive isolation, the process of speciation should be traceable by means of genetic analysis. The inter-pulse interval of the courtship song is the only parameter of the courtship elements that is consistently species-specific between the sympatric species of the *Drosophila auraria* complex (Part III). The species-specific inter-pulse interval was shown to be a species-discriminator in *D. biauraria* females (Part IV), suggesting that it plays an important role in sexual isolation in *D. auraria* complex.

Hybrid and chromosomal (backcross) analyses were used to clarify the genetic basis of species-specific courtship. Zouros (1981) observed that the species-specific courtship behaviour of different sexes in *D. arizonensis* and *D. mojavensis* were controlled by different chromosomes; male behaviour was affected by the Y chromosome and one autosome whereas female behaviour was affected by two other chromosomes. In *D. melanogaster* and *D. simulans*, the X chromosome (von Schilcher & Manning 1975) or the autosomes (Kawanishi & Watanabe 1981; Cowling & Burnet 1981; Kyriacou & Hall 1986) play a significant role in the determination of courtship song, and the X chromosome influences the mating success of females (Kawanishi & Watanabe 1981). Inter-specific genetic control of courtship song was also studied in species of the *D. virilis* group (Hoikkala & Lumme 1984, 1987, 1990; Hoikkala 1985).

Inter-specific hybrids are viable for both sexes, and all courtship elements - that is, orientation, following, tapping, vibration, attempted copulation and copulation - are also observed in male hybrids. Although male hybrids are sterile, females can be backcrossed with the male parental strain. The chromosomes of the *D. auraria* complex consist of an acrocentric X chromosome, two metacentric autosomes, a dot-like fourth chromosome, and the Y chromosome. At least one marker mutation is found on each major chromosome in *D. auraria*.

In the present part, inter-specific hybrids between species of the complex were generated and their courtship songs were analysed. The analysis of the backcross progeny allowed us to map the chromosomes on which the gene(s) controlling the differences in inter-pulse interval were located.

Methods

Fly Stocks and Crosses

The *Drosophila* strains used as parents in the hybrid analysis and their collection sites were as follows: *D. auraria* (A541, Tsukuba; A662, Obihiro), *D. biauraria* (B16 and B18, Tokyo), *D. triauraria* (T544, Tsukuba), *D. subauraria* (ONM-29, Onuma), *D. quadraria* (Q, Chi Tou, Taiwan, Texas stock No. 3075.1). The strains were maintained in glass vials (3 cm diameter x 10.5 cm high) containing standard *Drosophila* sucrose - yeast - cornmeal medium at 24.5 ± 0.5 ^{o}C under a light dark cycle of 14(7:00 - 21:00):10 h. Ten to twenty virgin flies which were either several hours old or several days old were mated freely. Flies were transferred to new vials after five or ten days. All hybrid flies were sexed without anaesthesia within 10 h after their emergence, with an aspirator. Three to seven day-old flies were used for recordings.

The marker stock *sn; cn; ba2* of *D. auraria* was used in the chromosomal analysis. The recessive mutant markers used in the present study were as follows: *sn* (*singed*, twisted, short bristles) for the X chromosome; *cn* (*cinnabar*, eye colour) for the A chromosome; and *ba2* (*balloon2*, blistered wings) for the B chromosome. Reciprocal hybrids between wild type strain A541 and mutant *sn; cn; ba2* were used to examine the effects of markers. To examine the effects of the wing mutation, flies carrying only *ba2* or *cu* (*curled*, wing curved upward), located on the B chromosome, were also analysed. The hybrid females from a cross between female *D. biauraria* B16 and male *D. auraria sn; cn; ba2* were backcrossed to male *D. auraria sn; cn; ba2* (Fig. 11). Since reciprocal hybrids (female *D. auraria sn; cn; ba2* x male *D. biauraria* B16) in the parental cross were not easily obtained, only a few backcross progeny were obtained. The progeny contained eight different combinations of the major chromosomes, and thus the courtship song of the males offered information on the contributions of the X chromosome and different autosomes. Since the cytoplasm shows the maternal inheritance, a reciprocal parental cross enables comparison of the cytoplasmic differences between *D. auraria* and *D. biauraria*. No crossing-over between the chromosomes of the two species in hybrid females has been detected (Hara & Kurokawa 1984). Flies from this cross were sexed and separated into each genotype under carbon dioxide anaesthesia. Flies showing phenotypically weak *ba2* mutation were used in mutant strains and backcross progeny. Three to ten day-old males were recorded.

Song Recording and Analysis

The detailed methods of recording and analysing the courtship song can be found in Part III. Males of each species in the *D. auraria* complex produce less courtship song before copulation but much during copulation. Since the inter-pulse intervals produced during attempted copulation and during copulation were identical (Part III), the songs produced during copulation were analysed. Females of the same genotype were used as partners for each hybrid. In the chromosomal analysis *D. auraria* females were used. The single pair of copulating flies was reared in a glass mating chamber (15 mm diameter, 5 mm in depth) and placed on a Sony ECM-55B Condenser Microphone. Courtship song was transmitted from the microphone to a Nihon Kohden Biophysical Amplifier AVB-11 and was recorded on a Sony L-830 EG-HG tape using a Sony SL-HF 3000 VTR and a Sony PCM-501ES PCM Processor or on a TEAC CT-90 tape using a TEAC R-60 Cassette Data Recorder. All flies were recorded during the light period (L) in a room constantly regulated at 24.5 ± 0.5 °C. Each recorded song was digitized at 44100 Hz, 16 bit, using an analogue to digital converter (Canopus Sound Master) and was stored on an Itec IT RL-100 hard disk. Inter-pulse interval, which was defined as the time interval from one peak of the pulse to the next, was measured on a MS-DOS machine with our song analysis program (Part III).

Intra-pulse frequencies of marker mutants were calculated using fast Fourier transformation (FFT) after pulse detection (Part III). Intra-pulse frequency was defined as the frequency giving the maximum FFT power spectrum. Although intra-pulse frequency is not a species-specific parameter in the *D. auraria* complex (Part III), wing mutation may affect sound frequency of the song.

Statistical Procedures

Three bursts of song for each fly and 10 inter-pulse intervals and 11 intra-pulse frequencies from each burst were analysed. The mean inter-pulse interval and mean intra-pulse frequency of each fly were used in the statistical tests. The one-sample *t* test was applied for testing the departure of the song of the inter-specific hybrids from the midparents. The effect of X/Y chromosome and/or cytoplasm was also tested as the difference between reciprocal hybrids by a two-tailed *t* test. The parent strain data were from Part III. The three way analysis of variance (ANOVA) was used to test the effect of each chromosome and of their interactions (Snedecor & Cochran 1989). Reciprocal parental crosses were constructed as the blocks in the ANOVA, which estimates the cytoplasmic effects.

Results

Hybrids

Before testing hybrid songs, the power of the hybrid analysis to detect the differences in genetic basis should be examined. The one-sample *t* test was applied for analysis of the difference between inter-pulse interval of each parent strain and the midparent. The inter-pulse interval of all parent strains but two were significantly different from the midparent inter-pulse interval ($P < 0.005$). Non-significant differences were found in *D. triauraria* and the midparent (*D. triauraria* and *D. quadraria*, $t_6 = 1.043$, $P > 0.2$), and in *D. quadraria* and the midparent (*D. triauraria* and *D. quadraria*, $t_4 = 0.957$, $P > 0.2$). The inter-pulse interval of *D. triauraria* and of *D. quadraria* did not significantly differ ($t_{10} = 1.394$, $P > 0.05$). The differences between hybrid and midparent or between hybrid and parent can be detected by *t* tests.

Inter-specific hybrids were obtained from 20 crosses. All hybrids except two showed an intermediate inter-pulse interval between the parental values (Table XVIII). However, inter-pulse interval was significantly shorter than the midparent in thirteen out of 20 crosses. In the other four crosses the shorter interval was not statistically significant compared to the midparent value (Table XVIII). Only in the two crosses, *D. auraria* A662 female x *D. biauraria* B18 male and *D. quadraria* female x *D. triauraria* male, did hybrid songs have significantly longer inter-pulse interval than the midparent value (Table XVIII). The difference between inter-pulse interval of inter-specific hybrids and parent strains was tested by *t* test. Songs did not significantly differ between hybrids from *D. quadraria* female x *D. triauraria* male and parent *D. triauraria*, and between hybrids from *D. subauraria* female x *D. biauraria* male and parent *D. subauraria*, ($t_{11} = 1.409$, $P > 0.1$ and $t_{13} = 1.490$, $P > 0.1$, respectively). Significant differences were found in the other hybrid-parent pairs.

The effects of the X/Y chromosomal and/or cytoplasmic factors were measured by comparing reciprocal crosses. Five out of eight pairings showed significantly different inter-pulse interval between the reciprocal crosses (Table XVIII). Four of these showed a deviation towards the paternal species and one deviated towards the maternal species. The other three pairs did not reveal significant differences between the reciprocal crosses.

Chromosomal Analysis

The wing mutant markers may influence the courtship song. Inter-pulse interval of the mutant male and reciprocal hybrids between *sn; cn; ba2* and wild type A541 were measured (Table XIX). Since all mean inter-pulse intervals lay within the range of *D. auraria* (18.9 - 21.4 ms, Table VII, Part III), the chromosomes carrying marker mutations showed no influence on inter-pulse interval. The reason intra-pulse frequency was also calculated to compare wild type and the wing mutants, *ba2* and *cu*, was that different wing morphology may affect the sound frequency. Although intra-pulse frequencies of *ba2* did not lie within the range of the *D. auraria* complex (98.9 - 184.1 Hz, Table IX, Part III), the other two strains were within the range.

Eight genotypes of flies, containing at least half of the *D. auraria sn; cn; ba2* chromosomes, and with some chromosomes substituted with *D. biauraria* ones, showed that the inter-pulse interval shortened with increased number of *D. biauraria* chromosomes (Fig. 12). The analysis of variance was performed without transformation because of homogeneity of variance between the eight genotypes (Bartlett test, $P > 0.4$). The two autosomes had significant effects on inter-pulse interval determination, but the X chromosome had a non-significant effect (Table XX). The cytoplasmic factor and the interaction were not detected. The factorial effect mean of cytoplasm, each chromosome, and each interaction was calculated (Table XX).

Discussion

Inter-specific hybrid songs between the *D. auraria* complex showed intermediate inter-pulse interval between that of their parents (Table XVIII), suggesting that the autosomes play major roles in the determination of inter-pulse interval. Autosomal control of inter-pulse interval has been found in several species (Ewing 1969; Ikeda et al. 1980; Cowling 1980; Cowling & Burnet 1981; Kawanishi & Watanabe 1981; Kyriacou & Hall 1986; Hoikkala & Lumme 1987). Although the inter-pulse interval of the hybrid from the cross between *D. subauraria* females and *D. biauraria* males (inter-pulse interval = 11.5 ms) lay between the inter-pulse interval of their parents (*D. subauraria* ONM-29, 11.1 ms; *D. biauraria* B16, 12.9 ms), it did not differ significantly from the inter-pulse interval of mother *D. subauraria*.

The only exceptions in the hybrid analysis are the hybrids between *D. triauraria* and *D. quadraria*. Since the inter-pulse interval of the two parent species did not differ significantly (*D. triauraria* T544, 16.1 ms; *D. quadraria* Q, 15.6 ms), it was expected that reciprocal hybrids would also show the same value. However, the inter-pulse interval of the reciprocal hybrids significantly differed from each other (14.9 ms and 16.5 ms, Table XVIII). One of the hybrids, the cross between *D. triauraria* female and *D. quadraria* male, also showed significantly different inter-pulse interval from their parent strains.

Significantly shorter inter-pulse interval compared to the midparent values was found in thirteen out of 20 crosses in the *D. auraria* complex (Table XVIII). The direction of deviation observed was significantly more for short than for long (Sign test, $n = 15$, $P < 0.005$). Dominance for shorter inter-pulse interval was also reported in the *virilis* phylad (Hoikkala & Lumme 1987).

The chromosomal analysis revealed that the two major autosomes had significant effects on inter-pulse interval determination but the X chromosome did not (Table XX). Since no interaction was detected, the two autosomes act additively. This confirmed the autosomal control result from the hybrid analysis, which showed the hybrid song was the intermediate inter-pulse interval between that of parents (Table XVIII). Species differences between *D. auraria* and *D. biauraria* were controlled by the two major autosomes but not the X chromosome. Although cytoplasmic factors did not significantly affect inter-pulse interval in the chromosomal analysis (Table XX), reciprocal hybrids, from the cross between *D. auraria* A662 x *D. biauraria* B18, showed significant X/Y and cytoplasmic effects (Table XVIII). These effects may arise from the differences in the strains used. Factors observed in every strain are probably more important for speciation studies than those not observed in some strains. Since the cytoplasmic effect varied between the strains, it may not provide important information to trace speciation.

The flies of genotype 8 of backcross progeny (*sn/Y; cn/+; ba2/+*) produced longer inter-pulse interval than *D. biauraria* - *D. auraria* hybrids (Fig. 12 and Table XVIII). In spite of the identical genotype, different values were obtained from the hybrid analysis and the chromosomal analysis. Cytoplasmic factors cannot explain the differences, because *sn/Y; cn/+; ba2/+* flies from the backcross with the *D. biauraria* cytoplasm showed longer inter-pulse interval than hybrid flies with the same genotype and the same cytoplasm. The design of the present experiment might have some bias in that only songs generated during copulation were recorded. Since a male fly copulating with a female was chosen for recording, no songs produced by males that failed to copulate could be recorded. There was some variation between the flies within the same genotype (Table XVIII, Fig. 12). Female preferences can influence the result of recordings. Although in recording hybrids' song, the female flies used as partners had the same genotype as the corresponding male hybrid, virgin *D. auraria* females were used in the chromosomal analysis. In *D. biauraria* females, preferences for its own species value of inter-pulse interval was revealed in Part IV. Although there is no empirical data about the female preferences for inter-pulse interval variation in species hybrids, it is possible that the preferences of *D. auraria* female and that of hybrid females are not identical. If so, *D. auraria* females chose the males whose songs were more similar to those of *D. auraria*, and then, these copulating pairs were chosen for recording. The data may have some bias due to female preferences. The other possibility is that the fourth chromosome with no marker mutation has some influence on female preference.

Post-mating isolation generally obeys Haldane's rule, which states that when one sex shows hybrid sterility or inviability it is heterogametic, suggesting that there are some special genes or some specific mechanisms that produce hybrid sterility/inviability (Dobzhansky 1940; Coyne & Orr 1989b). For pre-mating isolation, however, general rules have not yet emerged. In the *D. virilis* group, the *montana* phylad species have longer inter-pulse interval than the *virilis* phylad species, and the differences depend on the X chromosome. The species in the *montana* phylad shared the major changes in the X chromosome during the separation of the two phylads (Hoikkala & Lumme 1987). It is likely that the genetic elements on the X chromosome are conserved during speciation in the *montana* phylad. For species differences of inter-pulse interval between *D. melanogaster* and *D. simulans*, there are contrasting reports of X chromosomal control (von Schilcher & Manning 1975) and autosomal control (Cowling & Burnet 1981; Kawanishi & Watanabe 1981; Kyriacou & Hall 1986). If we accept autosomal control (see discussion in Cowling & Burnet 1981; Kawanishi & Watanabe 1981; Kyriacou & Hall 1986), we must infer that the autosomes affect the species differences between the species in the *D. melanogaster* species subgroup (Cowling & Burnet 1981). It is likely that no genetic elements are on the X chromosome; they are probably conserved during speciation in the *D. melanogaster* species subgroup.

In the *D. auraria* complex, hybrid and backcross analyses showed autosomal control in species differences. This is the case observed in the *D. melanogaster* species subgroup. The common ancestors of the species in the *D. auraria* complex shared the characteristics of non-X-chromosomal control in inter-pulse interval. Inter-pulse interval diverged to be species-specific during speciation, but there are no effective genes on the X chromosome. The chromosome influencing inter-pulse interval may be constrained by the ancestor species. Since the chromosomes controlling species-specific inter-pulse interval are shared by closely related species but not always by distant species in the *D. virilis* group (Hoikkala & Lumme 1987), the *D. melanogaster* species subgroup (Cowling & Burnet 1981; Kawanishi & Watanabe 1981; Kyriacou & Hall 1986) and the *D. auraria* complex, it is difficult to conclude that the genetic systems involved in song parameters are the same in various species. However, it is plausible that the genetic elements for species differences are shared by members of this group of closely related species.

GENERAL DISCUSSION

The present study revealed that female discrimination plays an important role in sexual isolation between species of the *Drosophila auraria* complex (Part I). The inter-pulse interval of the courtship song was species-specific between species of the complex (Part III). A female rejected a courting male, when she received a heterospecific type of inter-pulse interval (Parts II and IV). The species-specific inter-pulse interval (Part III) were controlled by autosomes (Part V). The present study revealed a significant role of female rejection in sexual isolation. The species discriminator is the species-specific inter-pulse interval.

Here I reported that females reject males with heterospecific courtship song more strongly than males without song (wing). This will provide a good viewpoint to clarify evolution of the species discrimination system in *Drosophila*.

Criteria of Female Rejection

Choice experiments are affected by several factors such as variation in female preferences (Crossley &

McDonald 1979), male-male competition (Partridge & Farquhar 1983) and the replacement of the male by another male after the female has indicated her willingness to mate (Eastwood & Burnet 1979). The present study attempted to reveal the role of each sex (Part I) and to clarify the role of male ornaments (Parts II and IV) in the sexual isolation. Any interaction between different species of the same sex will make the role of sex less clear. No choice experiments cannot detect whether females use relative mate choice criterion, and are also less sensitive to detect the importance of male traits than choice experiments (Hoikkala & Aspi 1993).

The heterospecific type of inter-pulse interval elicited female rejection in the no choice situation, suggesting that females use absolute criteria when they reject courting heterospecific males. Courtship and mating occur on/near the natural food place where many flies come together. A female encounters a chance to obtain information from several male species within a short period. Females of *D. melanogaster* can remember the received stimuli from courting males for some minutes (Kyriacou & Hall 1984). Females are able to use both absolute and relative criteria for mate choice in nature. It is very likely that female discrimination for courting males in absolute and relative criteria is more precise than that in only an absolute criterion (Hoikkala & Aspi

1993). Although female rejection detected in this study is a minimum estimate, it was the most important behaviour observed in the laboratory. Female discrimination and rejection will play a significant role in maintaining the sexual isolation between sympatric species of the *D. auraria* complex in nature.

Decision Making and Integration of Received Songs

The sound frequency of courtship song that can be received by female antennae is less than 450 Hz in *D. funebris* (Ewing 1978). The electrophysiological study revealed that an antennal nerve showed action potentials in phase to the signal (Ewing 1978). Although action potentials are compound rather than a simple form, their intervals correspond to the simulated pulse song. Although the mean inter-pulse interval of *D. funebris* is 8.3 ms, antennal nerves respond to inter-pulse intervals of 6 ms, 25 ms and 50 ms. This evidence suggests that the inter-pulse intervals of courtship song are sent to the central nervous system even if the intervals of the song are heterospecific.

The intra-pulse frequencies of each species of the *D. auraria* complex lay between 98.9 and 184.1 Hz (Table IX, Part III), which is the response range of the sound receptor. If the fundamental characteristics of the responses of antennae of females in the *D. auraria* complex do not differ from those of *D. funebris*, the inter-pulse intervals of courtship song are received by female antennae and sent to the central nervous system. Since the sound frequency of the courtship song did not differ from each other, it is very likely that the inter-pulse interval of heterospecific type is also sent to the central nervous system.

When the female received a heterospecific song, she rejected a male. But when she received a conspecific song, she accepted a male (Parts II and IV). A female hears the male's song and judges whether she accepts him or not. Information of inter-pulse interval will be sent to the central nervous system and then the decision to accept or reject him will be made. Thus rejection/acceptance of males should be determined at the integration level of neural circuits not in the sound receptor.

Female Response to Courtship Song

The insemination rate of *D. triauraria* females in the cross with winged *D. auraria* males is higher than that with wingless *D. auraria* males (Table III, Part II), suggesting that *D. auraria* song includes some appropriate signals for *D. triauraria* females. In contrast *D. triauraria* females reject 2-wing *D. subauraria* males more than wingless ones. Females of *D. triauraria* showed not only the rejection of males with heterospecific (*D. subauraria*) song, but also the acceptance of males with heterospecific (*D. auraria*) one. These results can be explained by the assumption that female responses for song differ in each species (Ryan & Rand 1993). The artificial song experiments with songs of slightly different inter-pulse interval (e.g., 1 ms differences) will clarify how females of each species respond for differences of inter-pulse interval.

Asymmetric matings, differential insemination rates between reciprocal crosses, were observed in the *D. auraria* complex (Table III, Part II). The insemination rates of the cross with wingless heterospecific males were in the range between 41.7% and 53.3% in experiments of 2h or 48h crossing time (Table III, Part II). In these crosses *D. biauraria* females were seldom inseminated by *D. subauraria* males (2 or 7%) whereas *D. subauraria* females were more inseminated by *D. biauraria* males (15 or 20%). This means that rejection of *D. subauraria* males by *D. biauraria* females is stronger than that of *D. biauraria* males by *D. subauraria* females. The asymmetry between *D. triauraria* and *D. auraria* is explained similarly. The rejection of *D. auraria* females is stronger than that of *D. triauraria* females: *D. auraria* A662 females rejected winged *D. triauraria* T748 males significantly more than wingless males, whereas *D. triauraria* T544 females accepted winged *D. auraria* A662 males significantly more than wingless males (Table III, Part II). The asymmetric mating preference is explained by the loss of male trait in new species (Kanashiro 1976) or by the gain of new male trait in new species (Watanabe & Kawanishi 1979). Species differences of inter-pulse interval of courtship song are quantitative but not qualitative. The loss/gain of male trait should not be simply applied to explain the differences in the *D. auraria* complex. It is plausible that metric character of males and responses of females have changed together in a continuous fashion.

Evolution of Female Rejection

Female rejection is one of the most important components of the mechanisms of pre-mating isolation in the *D. auraria* complex and must have developed during speciation. Female rejection and acceptance predicted by Fisher (1930) and Sved (1981a, 1981b) are clearly demonstrated. Fisher thought it is impossible to specify the sensory stimuli that determine differences in female response. This study showed that the inter-pulse interval is the sensory stimulus used as a species discriminator.

The process of development of female rejection will be explained in the context of evolutionary causes of reproductive isolation. Endler (1989) listed six processes that can favour the evolution of pre-mating isolation: a by-product of genetic divergence between populations, microhabitat or habitat choice and genetic predisposition, reinforcement of pre-mating isolation by post-mating isolation, reproductive character displacement, sexual selection, and sensory drive. The reconstructed speciation history based on habitat differences, morphology and molecular phylogeny (Kimura 1987) tells us that five species of the *D. auraria* complex have been formed by four successive splits of the lineage (Fig. 13). The ancestral species split into two: the ancestor of *D. biauraria* and *D. subauraria* and the ancestor of *D. auraria, D. triauraria* and *D. quadraria*. Then *D. biauraria* and *D. subauraria* were split from their ancestor. The other lineage split into *D. auraria* and the ancestor of *D. triauraria* and *D. quadraria*. Finally the lineage of *D. triauraria* and *D. quadraria* split allopatrically.

It is likely that two incipient species met and made some interactions each other. If so, the speciation scenario of the *D. auraria* complex will essentially follow the 'reinforcement model' (Dobzhansky 1951; Fisher 1930; Sved 1981a, 1981b; Coyne & Orr 1989a). A female who rejects a heterospecific male must have been selected for. The interaction between incipient species is probably the most important selective force. This is the reinforcement, if there were some post-mating isolation. Or, if the two incipient species have species status, it is reproductive character displacement. These interactions would lead female discriminatory criteria in the opposite direction to the other species; while criteria for female discrimination and male character had altered unidirectionally. Sexual selection has been mediated by reinforcement or reproductive character displacement. The direction of divergence may be initiated by a by-product of genetic divergence, a genetic predisposition through (micro-) habitat choice, or a sensory drive.

The inter-pulse interval of the courtship song is the species discriminator used by females. The direction of female preference will be explainable as the evolution of female response for the inter-pulse interval. Since the rejection of males producing heterospecific song was found in all four sympatric species, it is very likely that the ancestral state was also rejection of males producing heterospecific song. Outgroup comparisons might clarify how this rejection emerged in ancestors of the *D. auraria* complex.

In the first speciation the inter-pulse interval diverged, becoming shorter in the ancestor of *D. biauraria* and *D. subauraria* and longer in the ancestor of *D. triauraria, D. quadraria* and *D. auraria*. In Crossability Tests between Females x Wingless Males, *D. biauraria* and *D. subauraria* females accepted conspecific wingless males whereas *D. auraria* and *D. triauraria* females seldom accepted (Part II). Thus two trends - rejection and acceptance - were accelerated in the two lineages after the first divergence. Females of ancestors with shorter-inter-pulse interval tended to reject while females of ancestors with longer-inter-pulse interval tended to accept conspecifics. The present study cannot specify which of six evolutionary forces acted at this splitting. Then the shorter-inter-pulse interval-ancestor split to longer (*D. biauraria*) and shorter (*D. subauraria*). Trends in female discrimination in *D. biauraria* have reversed from shorter to longer. This led *D. biauraria* female to reject shorter inter-pulse interval but not to reject longer inter-pulse interval males. Evolutionary trends of longer-inter-pulse interval-ancestor suggest the acceptance of longer inter-pulse interval and might be conserved after the splitting of *D. triauraria* and *D. auraria*. Then although *D. triauraria* have had the preference for longer inter-pulse interval, the most preferable inter-pulse interval for *D. triauraria* female is the intermediate one; *D. auraria* females have conserved a preference for longer-inter-pulse interval. Sexual selection mediated by reinforcement/reproductive character displacement might be the evolutionary force. Since several length of inter-pulse interval can be synthesized, the artificial song experiments can clarify whether sexual selection is now operating on the variation of inter-pulse interval.

The female response has evolved to accept a conspecific male but to reject a heterospecific male. The response of the present may be asymmetrically distributed. The evolutionary changes were made not in receptor but in neural circuits. These behaviour and decision making are genetically determined. Thus the differences of female response between species may be detected by methods of electrophysiology or by methods of developmental biology as differences in neurogenesis in future.

Genetic Basis of Sexual Isolation

There have been some genetic analyses of male characteristics playing a role in sexual isolation reported. Although there are some debates on its existence (Crossley 1988, 1989; Ewing 1988, 1989; Kyriacou

& Hall 1988; Logan & Rosenberg 1989; Bennet-Clark 1990), the rhythmic fluctuation of inter-pulse interval of courtship song in *D. melanogaster* and *D. simulans* is the critical parameter that affects mating success (Kyriacou & Hall 1982). Species-specific length of fluctuation is determined by the *period* gene (Kyriacou & Hall 1980, 1989; Kyriacou 1990; Kyriacou et al. 1990) of particular sequences within the gene (Wheeler et al. 1991).

The species-specific discrimination system in females has been evolving together with a male character. Not only males' characteristics but also female discrimination is important for the evolution of sexual isolation (Kyriacou et al. 1992). Genetic analysis revealed that the species-specific discrimination for fluctuation of inter-pulse interval, that is determined by *period*, is not determined by the *period* gene (Greenacre 1993). Genetic evidence of preference for inter-pulse interval fluctuation is unknown.

A *D. arizonensis* female is courted by a *D. mojavensis* male, but she rejects him. Genetic elements involved in female sexual isolation between the two species are on two autosomes (Zouros 1981). A *D. simulans* male courts a *D. mauritiana* female, but he is rejected by her. Genes affecting rejection in *D. mauritiana* females are on both arms of the second chromosome and on the third chromosome (Coyne 1989, 1992). Although these studies clearly show that several genes control female mate discrimination, no characteristic courtship behaviour stands out.

Quantitating behaviour is the first and preliminary step to detect and isolate behavioural mutants and further analysis (Benzer 1973). In addition, although the origin of species differences must be mutations in a certain species, these mutations are now playing 'normal' alleles of each species. This means that the mutation causing abnormal behaviour is not always the allele of the gene causing species differences. Female rejection of males with heterospecific song will be a good criterion to analyse female discrimination.

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TABLES

Table I. Courtship indices of inter- and intra-specific cross in 10 min

	Male							
	D. biauraria		*D. subauraria*		*D. triauraria*		*D. auraria*	
Female	B16	B18	ONM-29	KT4	T544	T748	A662	A12
D. ***biauraria***								
B16	24.5 ± 8.49 (10)	16.8 ± 7.11 (9)	3.2 (1)	1.7 ± 0.72 (5)	5.3 ± 1.35 (10)	2.2 ± 0.85 (4)	2.5 ± 0.98 (4)	1.8 ± 1.50 (2)
B18	37.3 ± 8.77 (11)	21.6 ± 6.28 (11)	8.3 ± 4.04 (6)	1.0 (1)	7.1 ± 1.49 (12)	2.6 ± 0.84 (6)	24.6 ± 7.55 (10)	3.4 ± 1.05 (4)
D. ***subauraria***								
ONM-30	19.2 ± 5.57 (10)	23.6 ± 5.49 (10)	6.0 ± 2.22 (8)	5.3 ± 2.79 (4)	11.1 ± 4.97 (12)	6.2 ± 2.71 (4)	27.4 ± 10.92 (9)	0.9 ± 0.49 (3)
KT5	3.8 ± 1.73 (8)	15.0 ± 3.70 (10)	1.4 ± 0.77 (4)	1.5 (1)	12.7 ± 4.93 (12)	15.3 (1)	7.2 ± 3.47 (11)	22.8 ± 22.50 (2)
D. ***triauraria***								
T544	3.8 ± 1.63 (10)	6.1 ± 2.20 (10)	0.0 ± 0.00 (2)	7.3 ± 3.93 (8)	47.7 ± 8.45 (12)	28.9 ± 8.88 (11)	6.0 ± 4.72 (6)	2.1 ± 0.77 (10)
T748	14.5 ± 8.33 (10)	5.9 ± 3.10 (6)	0.9 ± 0.14 (6)	5.6 ± 3.70 (6)	16.6 ± 7.21 (12)	28.3 ± 8.91 (9)	18.4 ± 6.04 (7)	23.8 (1)
D. ***auraria***								
A662	6.4 ± 2.30 (11)	5.9 ± 1.54 (9)	1.4 ± 0.95 (3)	2.8 ± 0.86 (6)	6.9 ± 2.75 (8)	1.2 ± 1.00 (2)	7.3 ± 5.56 (5)	4.0 ± 3.67 (2)
A12	5.0 ± 2.42 (6)	7.8 ± 2.14 (11)	0.8 ± 0.32 (8)	1.0 ± 0.61 (4)	19.8 ± 5.05 (9)	1.3 ± 0.67 (2)	4.0 ± 1.98 (6)	6.1 ± 5.25 (2)

Mean(%) ± SE (N).

Table II.
Summary of the observation of inter- and intra-specific crosses

Courtship element [a]	Conspecific cross	Heterospecific cross
Orientation (*N*)	60.9 (192)	55.2 (576)
Attempted copulation (Orientation)	53.0 (117)	13.5 (318)
Copulation (Attempted copulation)	83.9 (62)	2.3 (43)
Rejection (Attempted copulation)	32.3 (62)	93.0 (43)
Courtship index [b]	22.8 ± 2.37	9.1 ± 0.78

N: Number of observed pairs.
a: Per cent frequencies compared to the courtship elements in the parentheses.
b: Mean ± SE.

Table III.
Insemination rate (%) of the Crossability Tests of Females x Wingless Males in interspecific pairs

Female	Heterospecific male	Crossing time (h)	Conspecific cross		Heterospecific cross		Chi-square [a]			
							Sexual isolation [c]	Null hypothesis [b]		
			2 wings	No wing	No wing	2 wings		1	2	3
D. biauraria										
B16	*D. subauraria* ONM-29	2	91.7 (48)	82.8 (58)	52.5 (59)	6.9 (58)	72.79 ***	1.33	11.26 ***	27.56 ***
	D. subauraria KT4	2	88.3 (60)	81.7 (60)	10.0 (60)	3.3 (60)	87.31 ***	1.05	62.06 ***	*P* = 0.139
	D. triauraria T544	2	81.4 (59)	82.8 (58)	62.1 (58)	1.8 (56)	72.06 ***	0.00	6.21 *	46.27 ***
	D. triauraria T748	2	86.0 (57)	67.2 (58)	52.6 (57)	8.5 (59)	67.10 ***	4.84 *	2.32	25.58 ***
	D. auraria A662	2	91.4 (58)	71.2 (59)	43.1 (58)	5.5 (55)	81.62 ***	6.77 **	8.94 **	20.95 ***
	D. auraria A12	2	86.2 (58)	81.4 (59)	81.4 (59)	6.8 (59)	72.78 ***	0.25	0.00	66.56 ***
B18	*D. subauraria* ONM-29	2	96.5 (57)	88.3 (60)	53.3 (60)	5.1 (59)	96.89 ***	*P* = 0.163	17.79 ***	31.74 ***
	D. triauraria T544	2	86.7 (60)	81.4 (59)	30.4 (56)	13.8 (58)	59.83 ***	0.56	29.78 ***	4.15 *
	D. triauraria T748	2	91.4 (70)	91.2 (68)	25.3 (79)	28.4 (67)	56.54 ***	0.00	63.90 ***	0.14
	D. auraria A662	2	88.4 (69)	69.6 (69)	10.4 (67)	15.9 (69)	72.60 ***	7.38 **	48.85 ***	0.58
Pooled[d]		2	88.8 (596)	79.8 (608)	41.1 (613)	10.0 (600)	738.97 ***	18.22 ***	190.65 ***	152.53 ***
B16	*D. subauraria* KT4	48	100.0 (60)	96.6 (59)	46.6 (58)	3.3 (60)	112.26 ***	*P* = 0.244	35.50 ***	28.58 ***
D. subauraria										
ONM-29	*D. biauraria* B16	2	68.1 (69)	71.0 (69)	49.3 (69)	15.7 (70)	38.35 ***	0.14	6.80 **	17.38 ***
	D. biauraria B18	2	68.3 (60)	56.7 (60)	41.7 (60)	20.0 (60)	28.42 ***	1.74	2.70	6.60 *
	D. triauraria T544	2	83.6 (55)	80.0 (60)	0.0 (50)	0.0 (50)	71.70 ***	0.23	68.91 ***	-
	D. auraria A662	2	75.9 (58)	66.7 (60)	0.0 (59)	0.0 (59)	68.54 ***	1.03	57.28 ***	-
KT4	*D. biauraria* B16	2	48.3 (60)	53.3 (60)	0.0 (60)	0.0 (60)	38.24 ***	0.30	43.64 ***	-
	D. triauraria T544	2	59.3 (59)	85.1 (67)	0.0 (68)	0.0 (69)	53.39 ***	10.35 **	96.67 ***	-
	D. auraria A662	2	50.0 (60)	48.3 (60)	0.0 (60)	0.0 (60)	40.00 ***	0.03	38.24 ***	-
Pooled		2	64.6 (421)	66.3 (436)	13.8 (426)	5.4 (428)	327.31 ***	0.25	244.00 ***	17.48 ***
ONM-29	*D. triauraria* T544	48	100.0 (60)	98.3 (60)	1.7 (60)	0.0 (60)	120.00 ***	1.01	112.13 ***	*P* = 0.500

Parenthesis: Number of females dissected.
a: Chi-square value with Cochran correction. When one of the expected values is less than 5, the Fisher exact probability is given as '*P* ='.
b: Chi-square test between 2-wing conspecific males and 2-wing heterospecific males.
c: Chi-square tests for three null hypotheses. See text for detailed expalnation.
d: Pooled for each female species.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table III. Continued.

							Chi-square			
	Heterospecific	Crossing	Conspecific cross		Heterospecific cross		Sexual	Null hypothesis		
Female	male	time (h)	2 wings	No wing	No wing	2 wings	isolation	1	2	3
*D. **triauraria***										
T544	*D. biauraria* B16	2	67.2 (61)	6.9 (58)	0.0 (58)	0.0 (60)	59.03 ***	43.80 ***	P = 0.059	-
	D. biauraria B18	2	86.4 (59)	7.0 (57)	0.0 (58)	0.0 (57)	87.51 ***	73.18 ***	P = 0.057	-
	D. subauraria ONM-29	2	81.3 (48)	12.5 (56)	3.3 (60)	0.0 (54)	67.64 ***	48.04 ***	P = 0.680	P = 0.497
	D. auraria A662	2	79.7 (59)	6.7 (60)	5.9 (68)	19.0 (79)	47.86 ***	63.45 ***	P = 1	4.92 *
	D. auraria A12	2	82.9 (76)	15.0 (80)	10.1 (89)	14.8 (88)	74.58 ***	69.48 ***	0.87	0.83
T748	*D. biauraria* B16	2	70.0 (60)	6.7 (60)	1.7 (60)	0.0 (59)	61.86 ***	50.90 ***	P = 0.210	P = 1.000
	D. auraria A662	2	88.1 (59)	10.0 (60)	1.8 (57)	8.8 (57)	73.01 ***	71.18 ***	P = 0.115	P = 0.117
	D. auraria A12	2	74.1 (58)	11.9 (59)	4.0 (50)	6.7 (60)	53.80 ***	45.27 ***	P = 0.175	P = 0.687
Pooled		2	78.8 (480)	9.8 (490)	3.8 (500)	7.2 (514)	521.95 ***	466.98 ***	13.46 ***	5.46 *
T544	*D. subauraria* ONM-29	24	75.9 (79)	52.5 (80)	27.0 (89)	13.5 (89)	65.96 ***	8.86 **	10.99 ***	5.01 *
	D. subauraria KT4	48	94.9 (59)	70.4 (54)	15.3 (59)	1.7 (60)	101.87 ***	10.71 **	35.08 ***	P = 0.008
*D. **auraria***										
A662	*D. biauraria* B16	2	49.1 (57)	1.7 (60)	0.0 (59)	0.0 (49)	30.51 ***	33.44 ***	P = 1	-
	D. biauraria B18	2	51.7 (60)	6.8 (59)	3.3 (60)	0.0 (60)	41.80 ***	27.36 ***	P = 0.346	P = 0.248
	D. subauraria ONM-29	2	63.8 (58)	0.0 (60)	1.4 (69)	0.0 (70)	59.75 ***	52.84 ***	P = 1	P = 0.496
	D. triauraria T544	2	63.8 (58)	1.7 (59)	5.2 (58)	0.0 (58)	54.33 ***	50.51 ***	P = 0.364	P = 0.122
	D. triauraria T748	2	63.3 (60)	6.7 (60)	25.0 (60)	5.0 (60)	45.38 ***	42.34 ***	7.57 **	9.41 **
A12	*D. biauraria* B16	2	95.0 (60)	26.7 (60)	0.0 (60)	0.0 (57)	103.55 ***	58.79 ***	18.46 ***	-
	D. subauraria ONM-29	2	58.6 (58)	28.8 (59)	0.0 (58)	0.0 (60)	46.59 ***	10.05 **	17.62 ***	-
	D. triauraria T544	2	79.7 (59)	32.2 (59)	5.0 (60)	0.0 (60)	75.70 ***	26.96 ***	14.28 **	P = 0.122
Pooled		2	65.7 (470)	13.0 (476)	5.0 (484)	0.6 (474)	451.22 ***	274.96 ***	18.44 ***	15.25 ***
A662	*D. triauraria* T544	6	64.4 (59)	17.2 (58)	23.3 (60)	5.0 (60)	45.59 ***	25.75 ***	0.47	8.29 **

Table IV.
Insemination rate (%) of the cross-mating test of Females x Wingless Males in intraspecific pairs

Female	Heterotypic male	Homotypic cross		Heterotypic cross		Chi-square[a]			
						Sexual[b] isolation	Null hypothesis[c]		
		2 wings	No wing	No wing	2 wings		1	2	3
D. ***biauraria***									
B16	B18	83.1 (59)	70.0 (60)	87.5 (48)	93.3 (60)	2.91	2.29	4.39 *	1.00
B18	B16	90.0 (60)	91.4 (58)	58.6 (58)	72.9 (59)	5.58 *	0.00	16.60 ***	2.43
Pooled[d]		86.6 (119)	80.5 (118)	71.7 (106)	83.2 (119)	0.52	1.50	2.01	3.72
D. ***subauraira***									
ONM-29	KT4	59.3 (59)	80.0 (60)	54.2 (59)	59.3 (59)	0.00	5.74 *	8.52 **	0.31
KT4	ONM-29	71.2 (59)	67.8 (59)	65.0 (60)	72.9 (59)	0.04	0.16	0.04	0.63
Pooled		65.3 (118)	73.9 (119)	59.7 (119)	66.1 (118)	0.02	1.99	5.48 *	0.89
D. ***triauraria***									
T544	T748	87.7 (57)	5.0 (60)	8.6 (58)	64.4 (59)	7.96 **	79.52 ***	0.54	37.65 ***
T748	T544	78.0 (59)	7.1 (56)	10.5 (57)	74.6 (59)	0.19	56.65 ***	0.11	48.14 ***
Pooled		82.8 (116)	6.0 (116)	9.6 (115)	69.5 (118)	5.28 *	138.31 **	0.96	85.21 ***
D. ***auraria***									
A662	A12	65.0 (60)	5.1 (59)	6.7 (60)	83.3 (60)	5.26 *	45.08 ***	$P = 1.000$	71.25 ***
A12	A662	76.3 (59)	8.3 (60)	3.3 (60)	71.7 (60)	0.17	55.19 ***	$P = 0.226$	59.77
Pooled		70.6 (119)	6.7 (119)	5.0 (120)	77.5 (120)	1.39	102.34 ***	0.30	130.14 ***

Parenthesis: Number of females dissected.

a: Chi-square value with Cochran correction. When one of the expected values is less than 5, the Fisher exact probability is given as 'P ='.

b: Chi-square test between 2-wing homotypic males and 2-wing heterotypic males.

c: Chi-square tests for three null hypotheses. See text for detailed expalnation.

d: Pooled for each female species.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table V.
Insemination rate (%) of the cross-mating test of Aristaless Females x Males

							Chi-square [a]			
	Heterospecific	Crossing	Conspecific cross		Heterospecific cross		Sexual [b]	Null hypothesis [c]		
Female	male	time (h)	2 aristae	No arista	No arista	2 aristae	isolation	1	2	3
D. ***biauraria***										
B16	*D. auraria* A662	2	86.2 (58)	86.0 (57)	29.1 (55)	3.6 (55)	75.43 ***	0.00	35.24 ***	13.02 ***
B16	*D. subauraria* ONM-29	2	91.7 (60)	84.5 (58)	70.0 (60)	1.7 (60)	97.63 ***	1.30	3.07	60.92 ***
B16	*D. triauraria* T544	2	79.3 (58)	70.7 (58)	46.7 (60)	1.8 (56)	70.11 ***	1.15	6.84 **	31.12 ***
Pooled [d]		2	85.8 (176)	80.3 (173)	49.1 (175)	2.3 (171)	241.83 ***	1.65	36.67 ***	96.12 ***
D. ***subauraria***										
ONM-29	*D. biauraria* B16	2	88.3 (60)	75.9 (58)	46.7 (60)	5.0 (60)	83.71 ***	2.84	10.30 **	27.18 ***
D. ***triauraria***										
T544	*D. auraria* A662	2	72.2 (97)	32.3 (99)	9.1 (99)	17.6 (108)	50.29 ***	24.71 ***	17.70 ***	3.65
D. ***auraria***										
A662	*D. triauraria* T544	48	72.9 (59)	6.8 (59)	11.7 (60)	11.7 (60)	44.71 ***	53.78 ***	0.40	0.00

Parenthesis: Number of females dissected.
a: Chi-square value with Cochran correction.
b: Chi-square test between 2-arista conspecific females and 2-arista heterospecific females.
c: Chi-square tests for three null hypotheses. See text for detailed explanation.
d: Pooled for *D. biauraria* females.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table VI.
Schematic representation of results of crossability tests.[a] Significant tests are found in Tables III and IV

	Male							
	D. biauraria		*D. subauraria*		*D. triauraria*		*D. auraria*	
Female	B16	B18	ONM-29	KT4	T544	T748	A662	A12
D. biauraria								
B16		= < =	= > >	= > >[b]	= > >	> = >	> > >	= = >
B18	= > =		= > >		= > >	= > =	> > =	
D. subauraria								
ONM-29	= > >	= = >		< > =	= > ?		= > ?	
KT4	= > ?		= = =		< > ?		= > ?	
D. triauraria								
T544	> = ?	> = ?	> > >[b]	> > >[c]		> = <	> = <	> = =
T748	> = ?				> = <		> = =	> = =
D. auraria								
A662	> ? ?	> = =	> ? ?		> = =	> < >		> = <
A12	> > ?		> > ?		> > =		> = <	

<,>: Significantly different insemination rates.
=: Non significant insemination rates.
?: No information because the number of inseminated females in both cross types was one or zero.
a: Results of significant testing for null hypotheses. Left, null hypothesis 1; Middle, null hypothesis 2; Right, null hypothesis 3.
b: Significant tests based on data of crossing time of 48h.
c: Significant tests based on data of crossing time of 24h.

Table VII.
Inter-pulse interval of the *D. auraria* complex

Species and strain	Mean ± SD (ms)	*N*	*n*
D. auraria			
A541	21.4 ± 1.90 ^{a}	6	180
A662	18.9 ± 1.98 ^{a}	8	240
A12	20.9 ± 0.87 ^{a}	4	120
D. biauraria			
B16	12.9 ± 0.91 ^{c}	10	300
B18	12.4 ± 0.76 ^{c}	9	270
B660	13.2 ± 0.72 ^{c}	6	180
D. triauraria			
T544	16.1 ± 0.96 ^{b}	7	210
T748	15.4 ± 0.75 ^{b}	5	150
D. subauraria			
OMN-29	11.1 ± 0.64 ^{d}	10	258
KT4	11.6 ± 0.50 ^{d}	5	150
D. quadraria			
Q	15.6 ± 0.68 ^{b}	5	150

N: Number of flies recorded.

n: Number of inter-pulse intervals.

Values with the same superscript letter are not significantly different (Tukey multiple comparisons, $P > 0.05$).

Table VIII.
Burst length and number of pulses per burst in the *D. auraria* complex

	Before copulation/during attempted copulation				During copulation			
Species	Mean	±	SD	*N*	Mean	±	SD	*N*
Burst length (ms)								
D. auraria A541	526.3	±	305.71	14	427.2	±	425.59^{a}	37
D. biauraria B16	304.2	±	173.86	50	415.4	±	283.69^{a}	206
D. triauraria T544	458.0	±	414.66	66	328.9	±	160.42^{a}	309
D. quadraria Q	411.5	±	556.59	14	310.8	±	196.79^{a}	144
	ANOVA, $F_{3,140} = 1.716$, $P > 0.2$				Kruskal-Wallis, $H = 12.32$, df = 3, $P < 0.001$			
Number of pulses per burst								
D. auraria A541	24.9	±	13.69	14	19.8	±	16.63^{b}	37
D. biauraria B16	24.7	±	13.45	50	32.5	±	21.20^{a}	206
D. triauraria T544	29.7	±	14.38	66	21.7	±	9.94^{b}	309
D. quadraria Q	26.9	±	35.10	14	20.9	±	12.69^{b}	144
	ANOVA, $F_{3,140} = 1.236$, $P > 0.2$				Kruskal-Wallis, $H = 42.93$, df = 3, $P < 0.001$			

N: Number of bursts.
Values with the same superscript letter is not significantly different (non-parametric Tukey-type multiple comparisons, $P > 0.05$).

Table IX.

Intra-pulse frequency of the *D. auraria* complex

Species and strain	Mean ± SD (Hz)	*N*	Range (Hz)	Least (Hz)	Width (ms)
D. ***auraria***				44.1	22.68
A541	99.9 ± 42.42 ^{e}	160	53.8 - 323.0		
A662	98.9 ± 21.04 ^{de}	216	53.8 - 269.2		
A12	101.2 ± 32.91 ^{de}	101	53.8 - 236.9		
D. ***biauraria***				67.8	14.74
B16	163.2 ± 86.98 ^{b}	199	75.4 - 484.5		
B18	112.9 ± 60.48 ^{de}	237	70.0 - 430.7		
B660	132.4 ± 73.41 ^{bcd}	37	75.4 - 376.8		
D. ***triauraria***				55.1	18.14
T544	127.6 ± 51.20 ^{bc}	126	64.6 - 366.1		
T748	106.2 ± 26.89 ^{cde}	103	64.6 - 247.6		
D. ***subauraria***				88.2	11.34
ONM-29	184.1 ± 53.60 ^{a}	128	96.9 - 430.7		
KT4	107.0 ± 22.40 ^{cde}	49	91.5 - 177.6		
D. ***quadraria***				55.1	18.14
Q	128.9 ± 64.89 ^{bc}	124	64.6 - 495.3		

N: Number of intra-pulse frequencies.

Least: Least value of the detectable range of intra-pulse frequencies (1000 / Width(ms)).

Values with the same superscript letter is not significantly different (non-parametric Tukey-type multiple comparisons, $P > 0.05$).

Table X.
Parameters of artificial song sampled from a burst for each song type

Song type	Synthesized[a]			Played back[b]		
	Number of pulses per burst	Inter-pulse interval[c]	Burst length (ms)	Number of pulses per burst	Inter-pulse interval[c]	Burst length (ms)
13ms	32	12.99 ± 0.000	402.79	32	12.93 ± 0.047	400.86
16ms	26	15.99 ± 0.000	399.66	26	16.06 ± 0.057	401.47
11ms	37	11.00 ± 0.000	395.92	37	10.98 ± 0.036	395.37

a: Synthesized artificial song.
b: Synthesized songs were transmitted to cassette tape recorder via a digital to analogue converter and recorded on cassette tapes. Recorded songs were played back and emitted via a loudspeaker. These sounds were received by a microphone and analysed.
c: Mean ± SD (ms).

Table XI.
Number of pairs showing several courtship elements in "Observation of Female x Wingless Male in 30 Min"

Male type	Orientation	Attempted Copulation	Copulation
Conspecific winged	55	49	43
Conspecific wingless	55	47	36
Heterospecific wingless	47	36	23
Heterospecific winged	50	33	0

N (Observed pairs) = 57.

Table XII.
Chi-squre values with Cochran correction in pairwise comparison for mating frequencies in "Observation of Female x Wingless Male in 30 Min"

Male type	Conspecific wingless	Heterospecific wingless	Heterospecific winged
Conspecific winged	1.79 *	5.62	58.76 ***
Conspecific wingless		1.49	43.82 ***
Heterospecific wingless			31.63 ***

* *P* < 0.05, *** *P* < 0.001.

Table XIII.
Number of pairs showing several courtship elements in artificial song experiments with *D. biauraria* female

				During first attempted copulation	
Song type	Orientation	Attempted copulation	Copulation	Copulation	Rejection
13 ms	86	54	36	32	16
16 ms	75	48	15	12	32
11 ms	83	53	16	13	36
Random	83	42	20	18	22
Silence	88	72	37	21	46

N (observed pairs) = 90.

Table XIV.
Chi-square values with Cochran correction in pairwise comparison for attempted copulation in artificial song experiments

Song type	16 ms	11 ms	Random	Silence
13 ms	0.00	0.00	2.41	7.37 **
16 ms		0.00	2.59	6.23 *
11 ms			2.98	6.70 **
Random				17.80 ***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table XV.
Chi-square values with Cochran correction in pairwise comparison for copulation frequencies in artificial song experiments

Song type	16 ms	11 ms	Random	Silence
13 ms	12.75 ***	13.51 ***	37.21 ***	2.69
16 ms		0.00	8.81 **	4.28 *
11 ms			8.41 **	4.83 *
Random				24.12 ***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table XVI.
Chi-square values with Cochran correction in pairwise comparison for mating frequencies during first attempted copulation in artificial song experiments

Song type	16 ms	11 ms	Random	Silence
13 ms	11.59 ***	12.43 ***	2.08	10.77 **
16 ms		0.00	3.21	0.17
11 ms			3.11	0.17
Random				2.05

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table XVII.
Chi-square values with Cochran correction in pairwise comparison for female rejection during first attempted copulation in artificial song experiments

Song type	16 ms	11 ms	Random	Silence
13 ms	12.79 ***	14.97 ***	4.42 *	14.30 ***
16 ms		0.00	1.67	0.04
11 ms			2.20	0.15
Random				1.41

* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Table XVIII.
Inter-pulse intervals (ms) of inter-specific hybrids

Parent[a]			Hybrid from a cross[b]						
1	2	Midparent	1 (female) x 2 (male)			2 (female) x 1 (male)			X/Y, cytoplasm[c]
A541	B16	17.15	15.7 ± 0.52	(22)	***[d,e]	16.0 ± 0.50	(15)	***[d,e]	NS
A541	B18	16.90	15.2 ± 1.31	(12)	***[e]	16.2 ± 0.42	(6)	**[e]	NS
A662	B18	15.65	16.6 ± 0.37	(5)	**	15.8 ± 0.49	(6)	NS	*
sn; cn; ba2	B16	16.40				16.3 ± 0.60	(27)	NS[e]	Not available
A541	T544	18.45	17.3 ± 0.43	(8)	***[e]	18.1 ± 0.39	(10)	***[e]	***
A541	Q	18.50	17.1 ± 0.41	(9)	***[e]	17.6 ± 0.26	(6)	***[e]	*
A541	ONM-29	16.25	14.3 ± 0.40	(3)	***[e]				Not available
T544	Q	15.85	14.9 ± 0.37	(9)	***[e]	16.5 ± 0.59	(6)	*	***
T544	B16	14.50				14.3 ± 0.60	(6)	NS[e]	Not available
Q	B16	14.25	13.4 ± 0.27	(10)	***[e]	13.9 ± 0.28	(5)	*[e]	**
Q	ONM-29	13.35	13.3 ± 0.52	(14)	NS[e]				Not available
B16	ONM-29	12.00	11.8 ± 0.36	(8)	NS[e]	11.5 ± 0.51	(8)	*[e]	NS

a: Strains with longer inter-pulse intervals are presented first. Inter-pulse intervals of parent strains were from Tables VII and XVII.

b: Mean ± SD (number of flies).

c: *t* test between reciprocal crosses.

d: *t* test for departure from midparent.

e: Shorter than midparent.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS Not significant.

Table XIX.
Song parameters of mutant strains

Strain	Mean	±	SD	*N*
Inter-pulse interval (ms)				
ba2	20.3	±	0.54	8
cu	19.4	±	0.61	10
sn; cn; ba2	19.9	±	0.72	11
A541 x *sn; cn; ba2*	21.2	±	1.05	9
sn; cn; ba2 x A541	21.9	±	2.01	6
Intrapulse frequency (Hz)				
ba2	93.0	±	6.92	8
cu	134.9	±	34.23	10
sn; cn; ba2	100.5	±	11.40	11

N: Number of flies.

Table XX.
Analysis of variance for effects of three major chromosomes on the determination of inter-pulse interval

Source [a]	df	Mean square	*F*	Effect (ms) [b]
Cytoplasm [c]	1	1.417	0.881	-0.112
X	1	1.775	1.104	0.119
A	1	26.474	16.457 ***	0.458
B	1	39.201	24.370 ***	0.556
XA	1	0.021	0.013	0.013
XB	1	0.099	0.061	-0.028
AB	1	0.474	0.295	0.061
XAB	1	0.032	0.020	0.016
Error	131	1.609		

a: Each chromosome.

b: Factorial effect mean.

c: Cytoplasmic factor.

*** $P < 0.001$.

FIGURES

Figure 1. The number of pairs where females or males showed various courtship elements in intra- and inter-specific crosses in 10 minutes observation. Vertical axis represents the number of pairs for each element. The intra-specific crosses were shaded and the inter-specific crosses blank.

(a) Orientation (males oriented to females).

(b) Tapping (males tapped females).

(c) Wing vibration (males vibrated their wings).

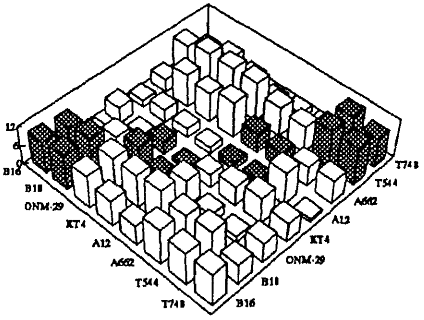
(d) Attempted copulation (males attempted to copulate).

(e) Copulation.

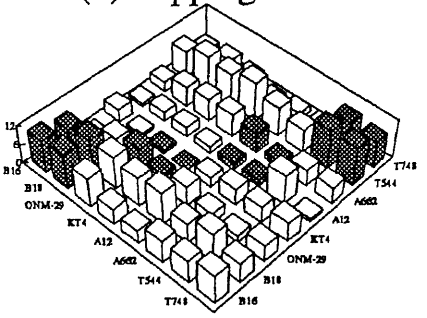
(f) Rejection (females rejected males' attempted copulation by fluttering and/or kicking.

Since some males of the 'Rejection' pairs copulated after several attempted copulations, total number of copulations and rejections exceeded the observation number ($N = 12$) in some strain pairs.

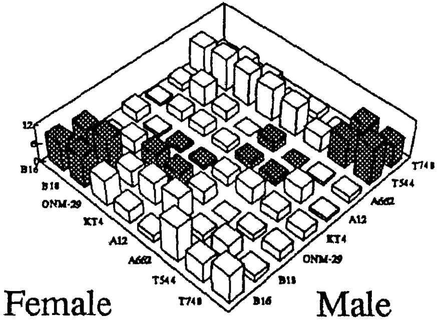
(a) Orientation



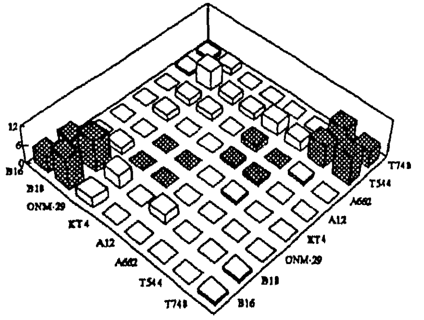
(b) Tapping



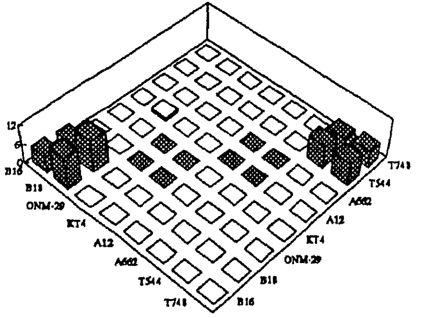
(c) Wing Vibration



(d) Attempted Copulation



(e) Copulation



(f) Rejection

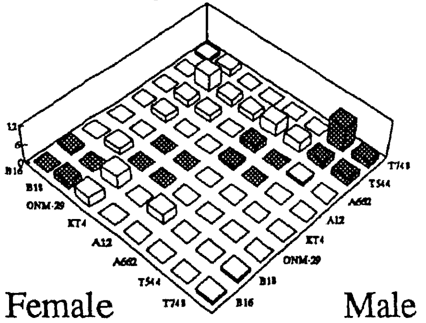


Figure 2. The underlying framework of crossability tests.

(a) Differences in insemination rates when sexual isolation exists. The insemination rate from the conspecific crosses are higher than from the heterospecific crosses, since there is sexual isolation between two species.

(b) Null hypotheses to detect wing effects on sexual isolation. Insemination rates from the crosses of males with 2 wings or without wings are compared to each other. Three equal signs represent three null hypotheses. Testing null hypothesis 1 tests wing effects on mating in the conspecific cross. Testing null hypothesis 2 looks at male discrimination. Testing null hypothesis 3 measures wing effects on sexual isolation.

(a) Sexual Isolation

Conspecific cross **Heterospecific cross**
2 wings > 2 wings

(b) Null Hypotheses

Conspecific cross **Heterospecific cross**
2 wings $\underset{1}{=}$ No wing $\underset{2}{=}$ No wing $\underset{3}{=}$ 2 wings

Figure 3. Analysis of courtship song. An example of *D. triauraria*.

(a) CRT display hardcopy of wave pattern. Vertical bars indicate pulses identified. The baseline was waved to make a ghost pulse (arrow head) that the program could not discriminate. When the program identified the second/third peak of the pulse as a main peak, it was judged to be a ghost peak and excluded.

(b) An automatically processed pulse, marked with an asterisk on (a).

(c) Power spectrum of the pulse in (b).

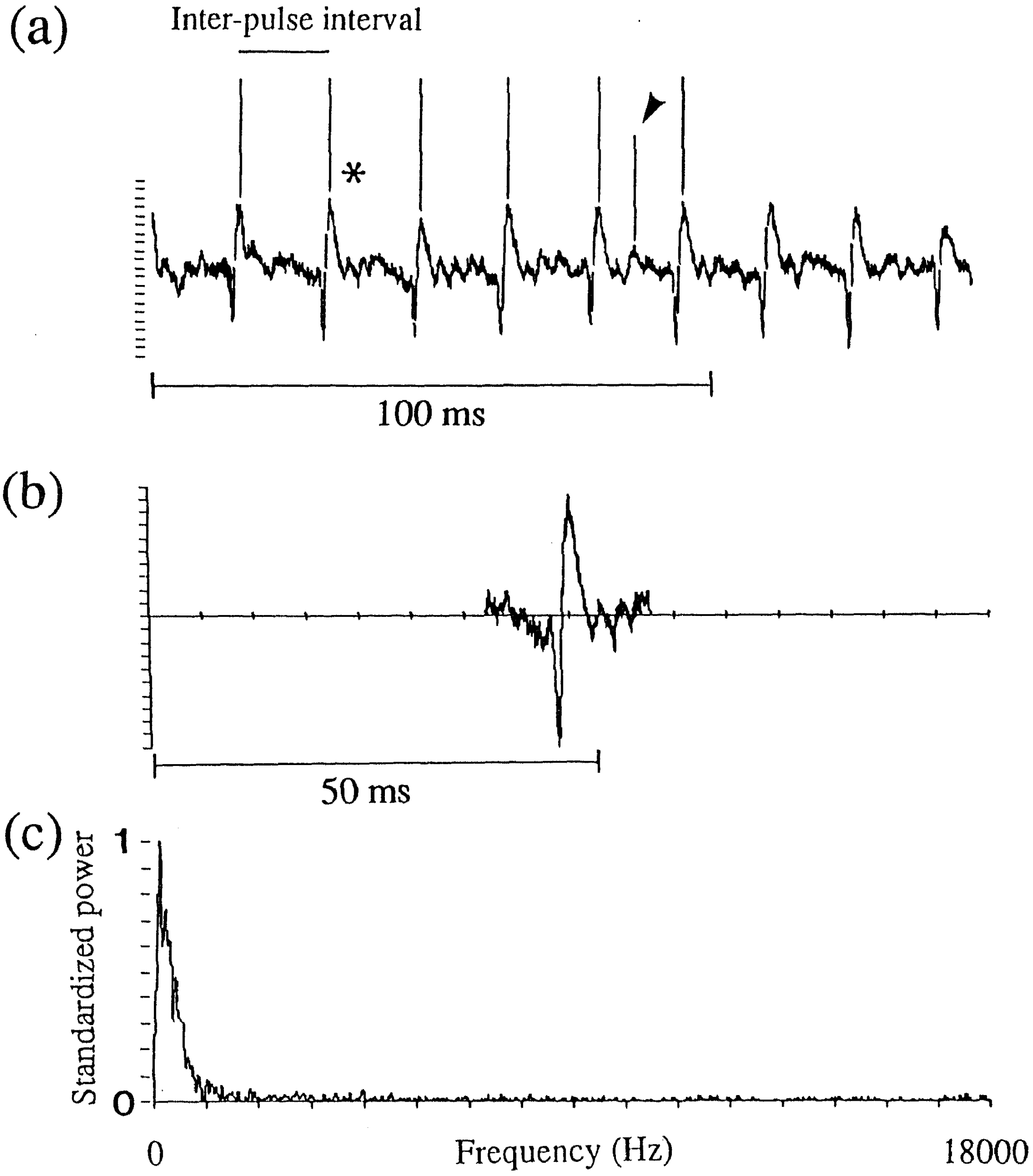

(a)
Inter-pulse interval
*
100 ms
(b)
50 ms
(c)
1
0
Standardized power
0
Frequency (Hz)
18000

Figure 4. Oscillogram of courtship songs in the *Drosophila auraria* complex.

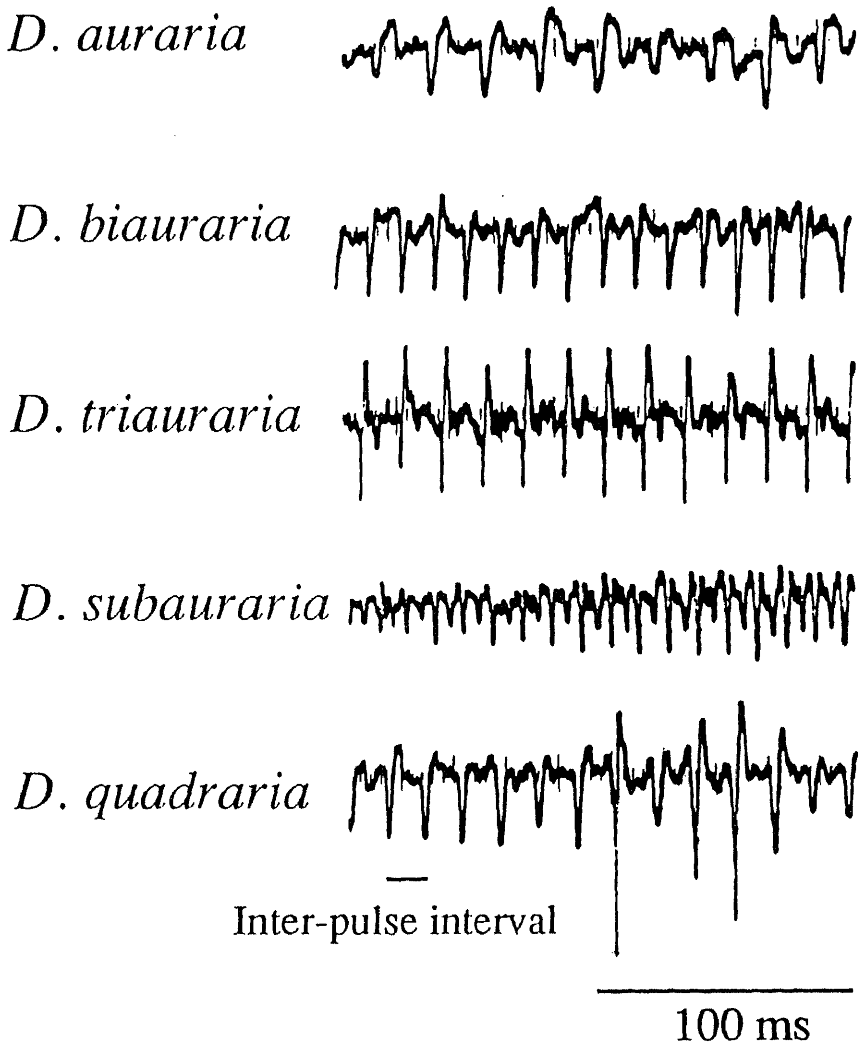

D. auraria
D. biauraria
D. triauraria
D. subauraria
D. quadraria
Inter-pulse interval
100 ms

Figure 5. Frequency distribution of inter-pulse intervals measured from all songs produced by males.

Closed squares: before copulation and during attempted copulation.

Open squares: during copulation.

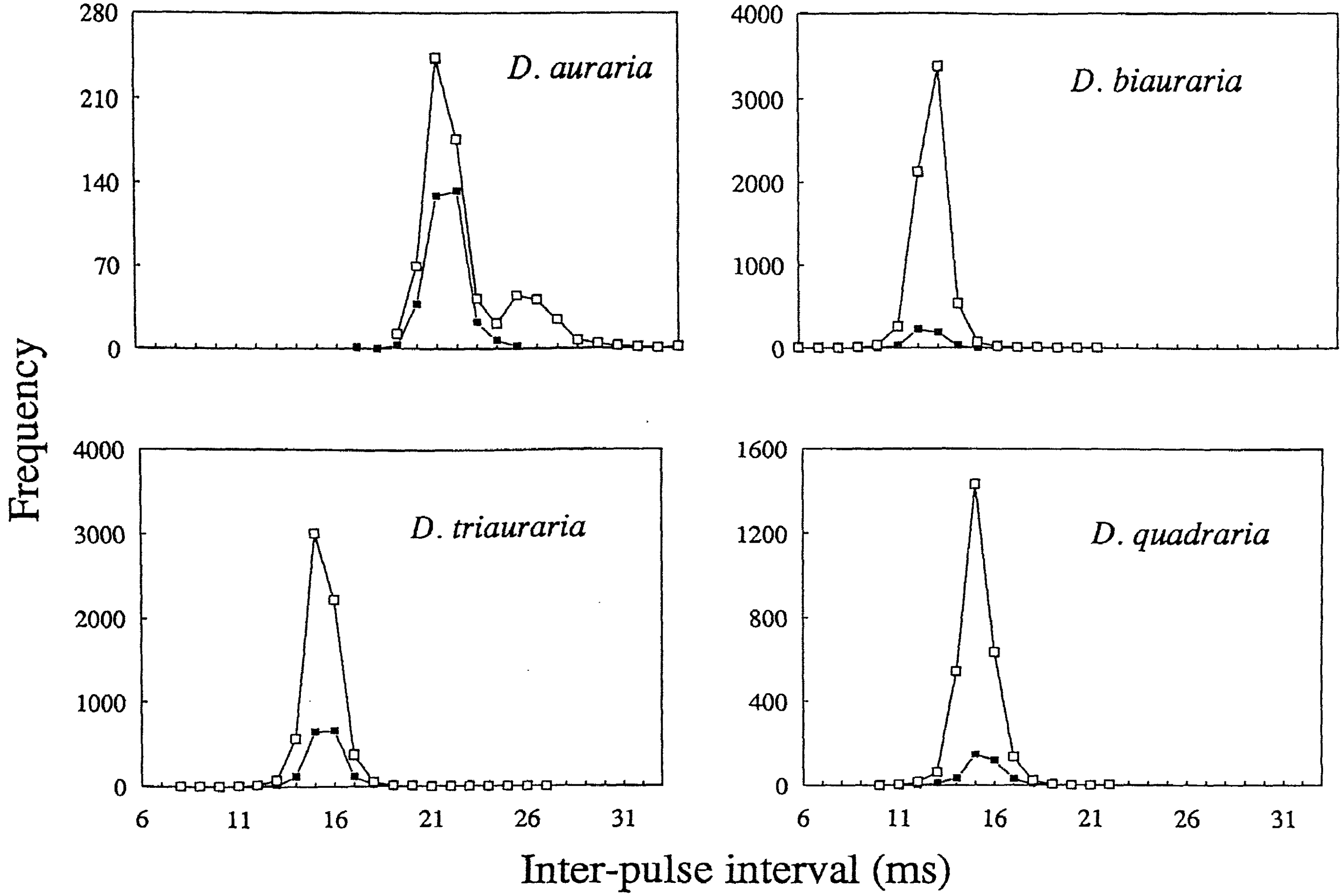

D. auraria
D. biauraria
D. triauraria
D. quadraria
Frequency
280
210
140
70
0
4000
3000
2000
1000
0
4000
3000
2000
1000
0
1600
1200
800
400
0
6
11
16
21
26
31
6
11
16
21
26
31
Inter-pulse interval (ms)

Figure 6. Power spectra from single pulses of courtship song.

(a) Power spectra of the detectable range of the system (less than 18000 Hz).

(b) Power spectra for less than 2000 Hz of the spectra in (a). Vertical bars indicate spectral peaks. The frequency of the highest peak was taken to be the intra-pulse frequency.

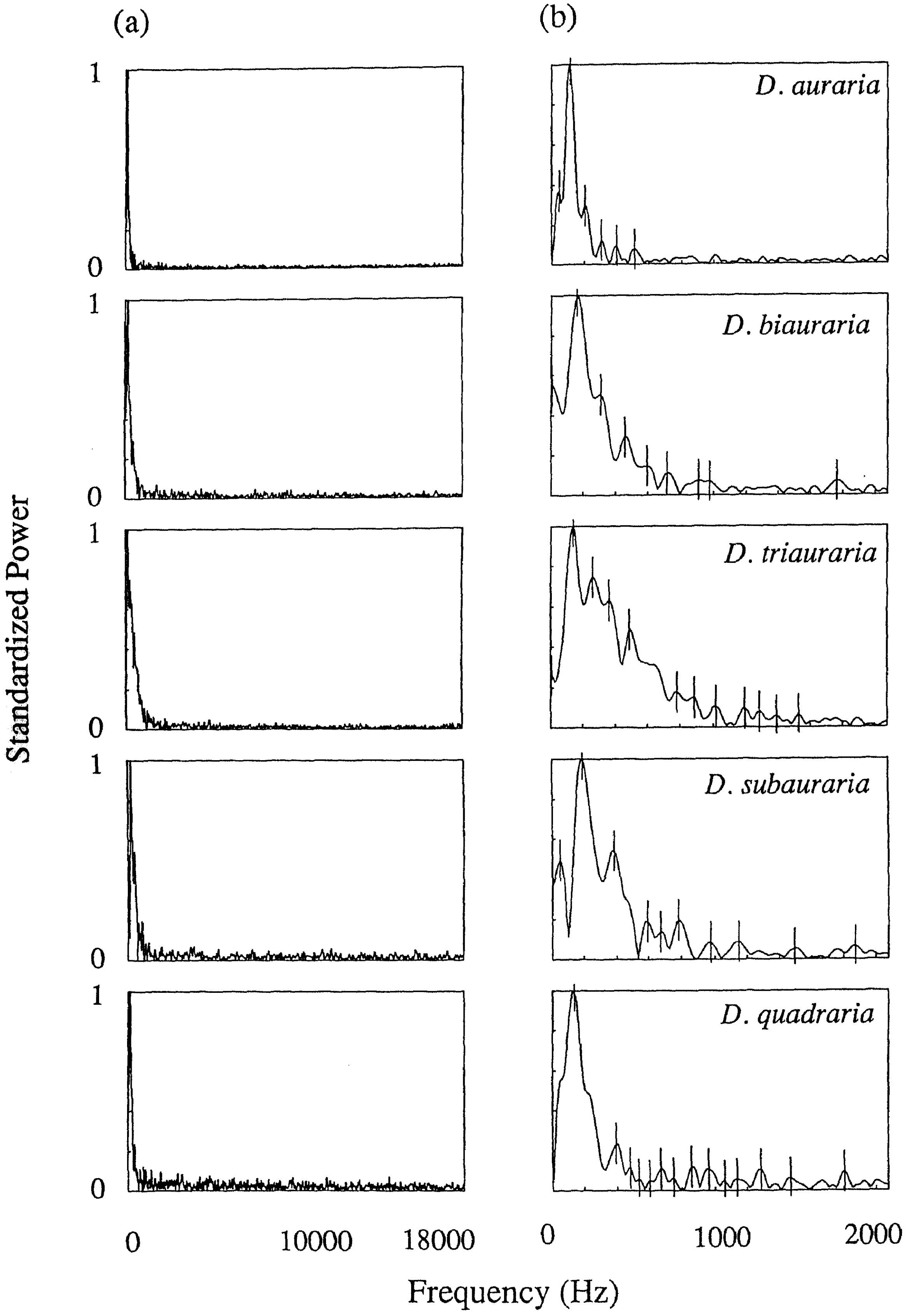

(a)
(b)
Standardized Power
1
0
D. auraria
D. biauraria
D. triauraria
D. subauraria
D. quadraria
0
10000
18000
0
1000
2000
Frequency (Hz)

Figure 7. Schematic diagram of equipment for artificial song experiments.

(a) Mating chamber.

(b) Experimental settings. Six mating chambers were arranged in circle above a loudspeaker.

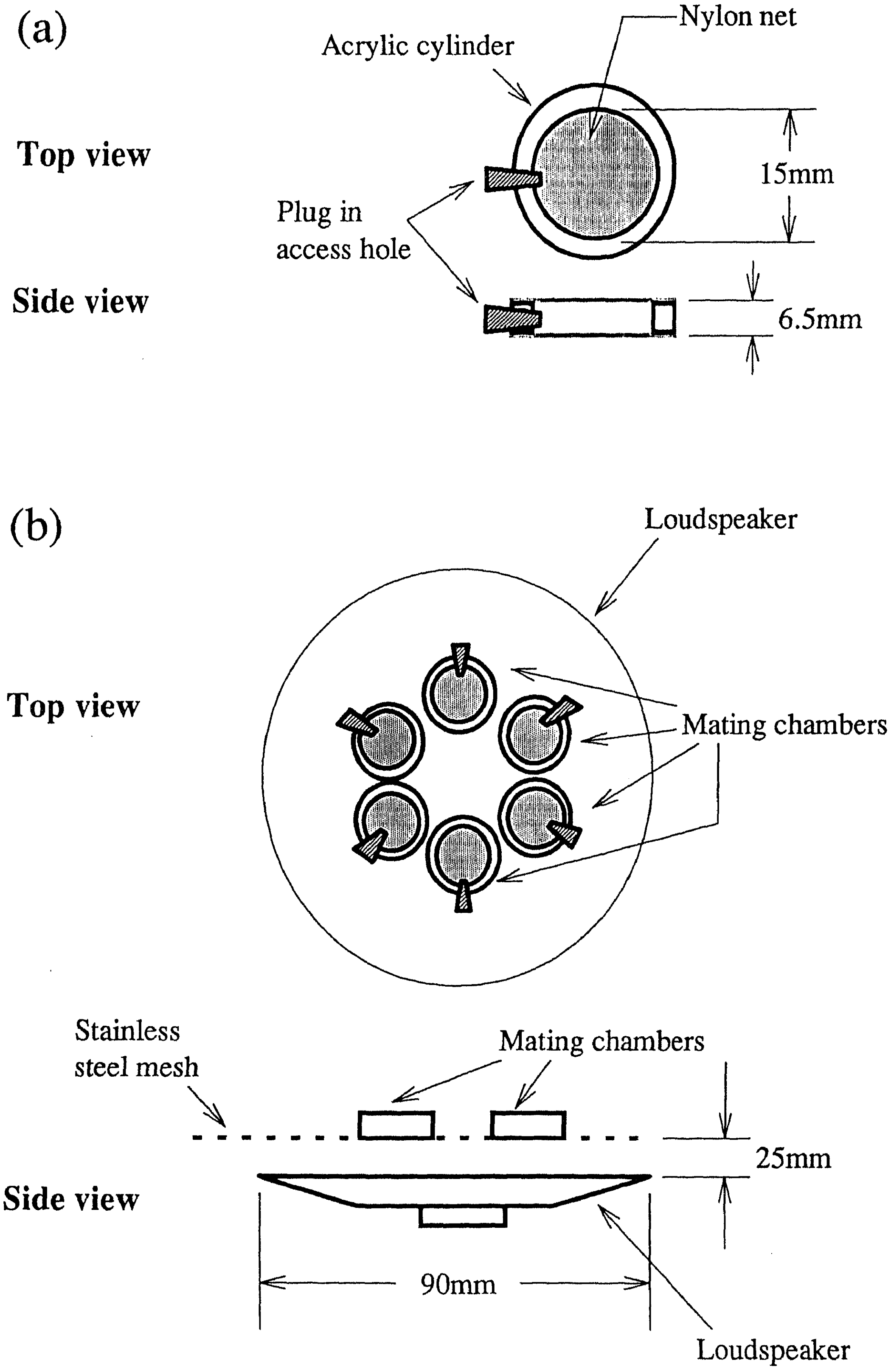

(a)
Top view
Acrylic cylinder
Nylon net
15mm
Plug in access hole
Side view
6.5mm
(b)
Loudspeaker
Top view
Mating chambers
Stainless steel mesh
Mating chambers
25mm
Side view
90mm
Loudspeaker

Figure 8. Oscillogram of artificial songs.

(a) Synthesized artificial song before transmitted to a tape recorder.

(b) Artificial song played back.

(c) Burst trains of artificial song played back.

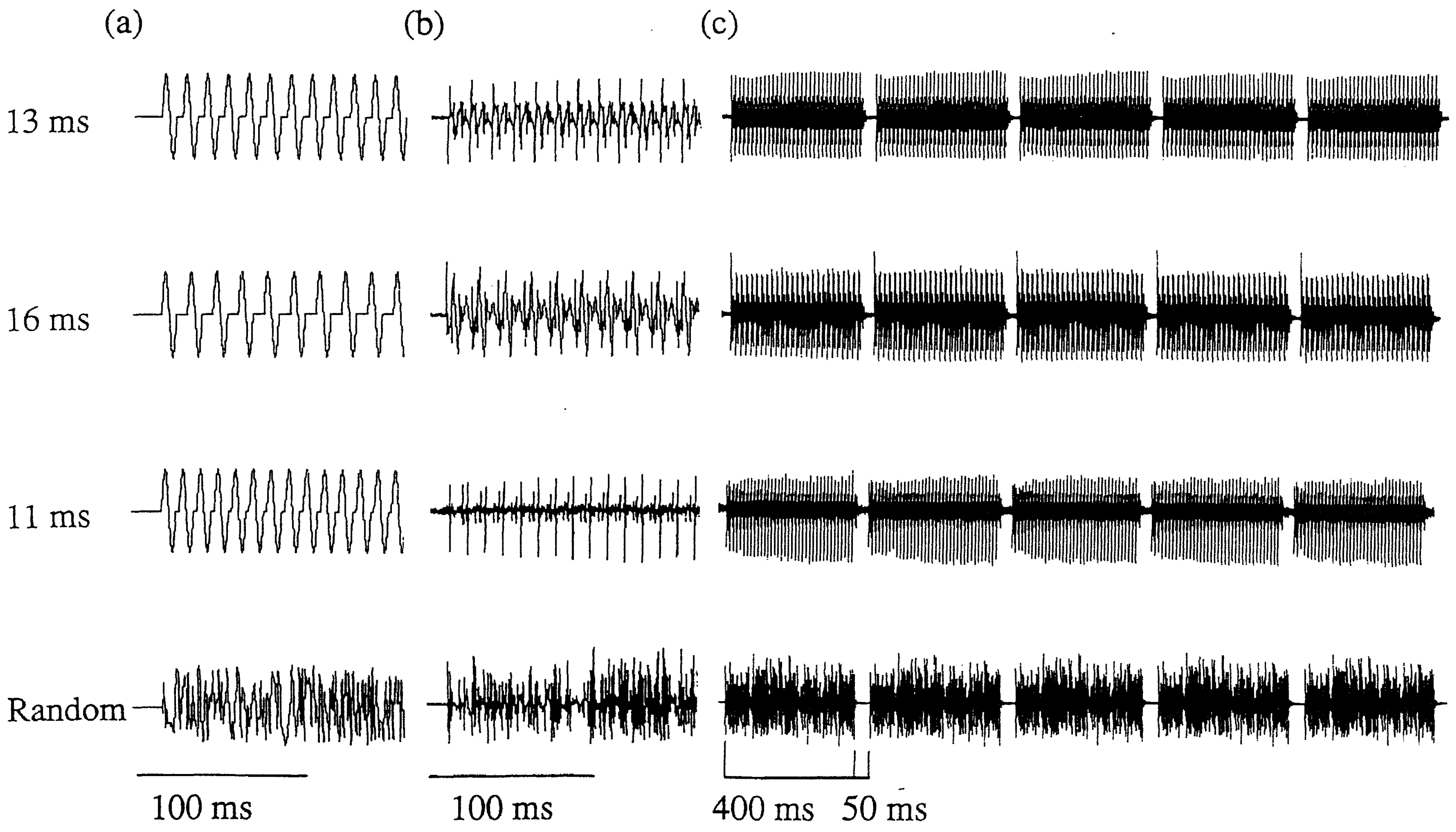

(a)
(b)
(c)
13 ms
16 ms
11 ms
Random
100 ms
100 ms
400 ms
50 ms

Figure 9. Cumulative mating frequency in 30 min.

(a) Female x Wingless Male experiments.

Closed square: conspecific winged males.

Open circle: conspecific wingless males.

Open square: heterospecific wingless males.

Closed triangle: heterospecific winged males.

(b) Artificial song experiments.

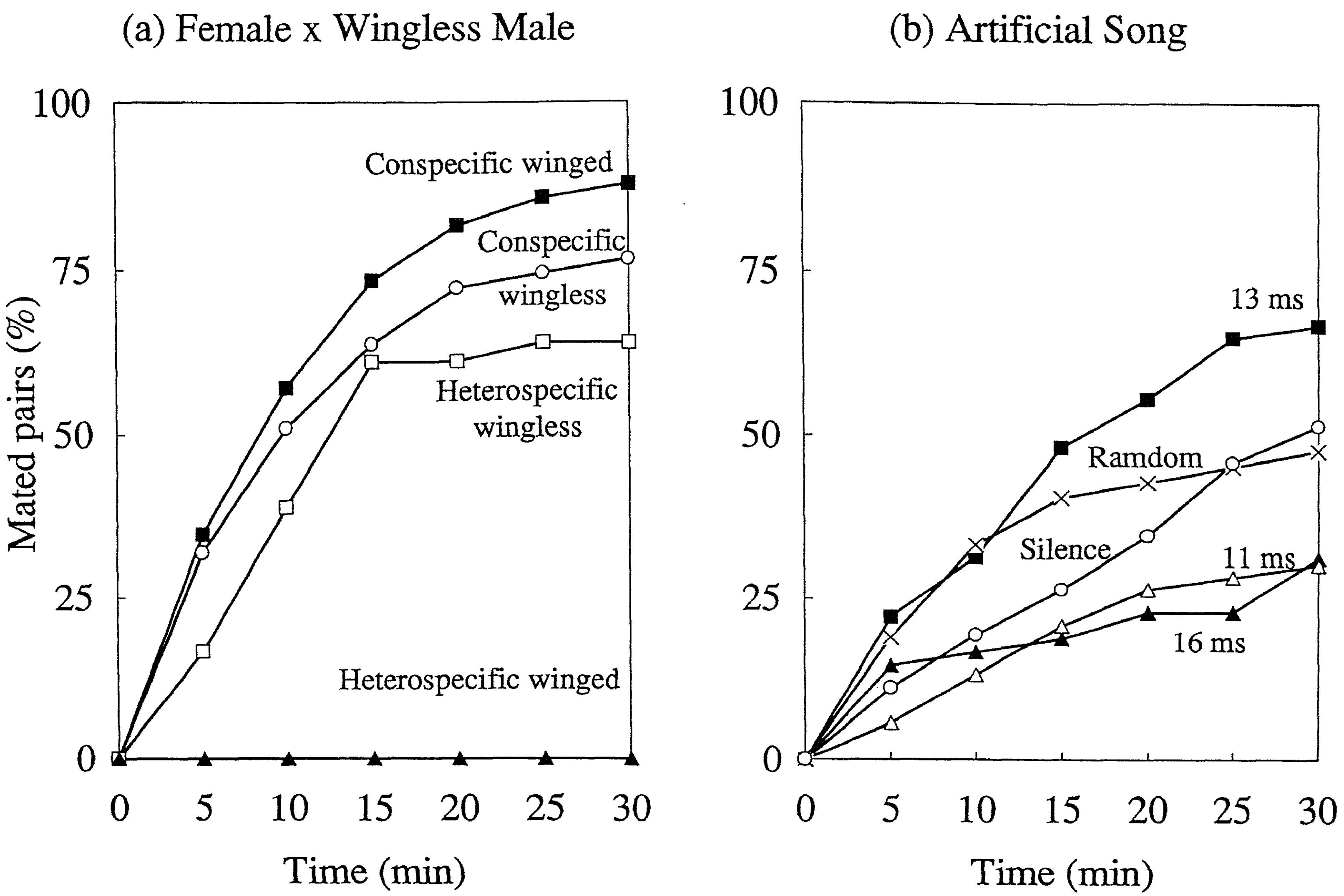
Closed square: 13 ms.

Open circle: silence.

Cross: random noise.

Closed triangle: 16 ms.

Open triangle: 11 ms.



(a) Female x Wingless Male

(b) Artificial Song

Figure 10. Cumulative frequencies of female responses at first attempted copulation in artificial song experiments.

Diagonal: copulation.

Blank: rejection (fluttering and kicking).

Cross: failure to copulation without rejection.

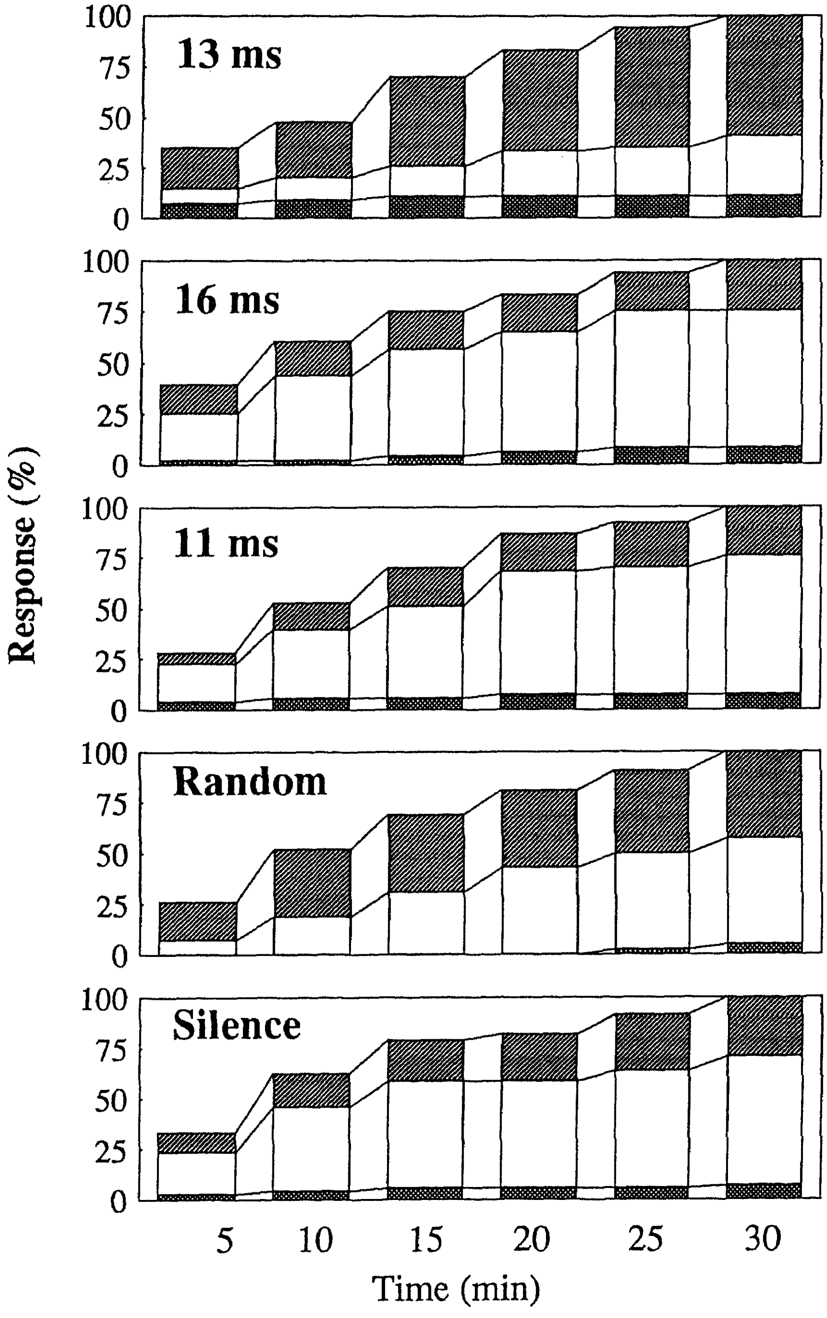

Response (%)
13 ms
16 ms
11 ms
Random
Silence
100
75
50
25
0
5
10
15
20
25
30
Time (min)

Figure 11. Mating scheme for chromosome substitution in the cross, (*D. biauraria* female x *D. auraria* male) x *D. auraria* male. Virgin flies of female *D. biauraria* were crossed to *D. auraria sn; cn; ba2* males. Hybrid females were backcrossed with *D. auraria* marker strains. For the cross (*D. auraria* female x *D. biauraria* male) x *D. auraria* male, F_1 hybrid females were generated from the reciprocal cross.

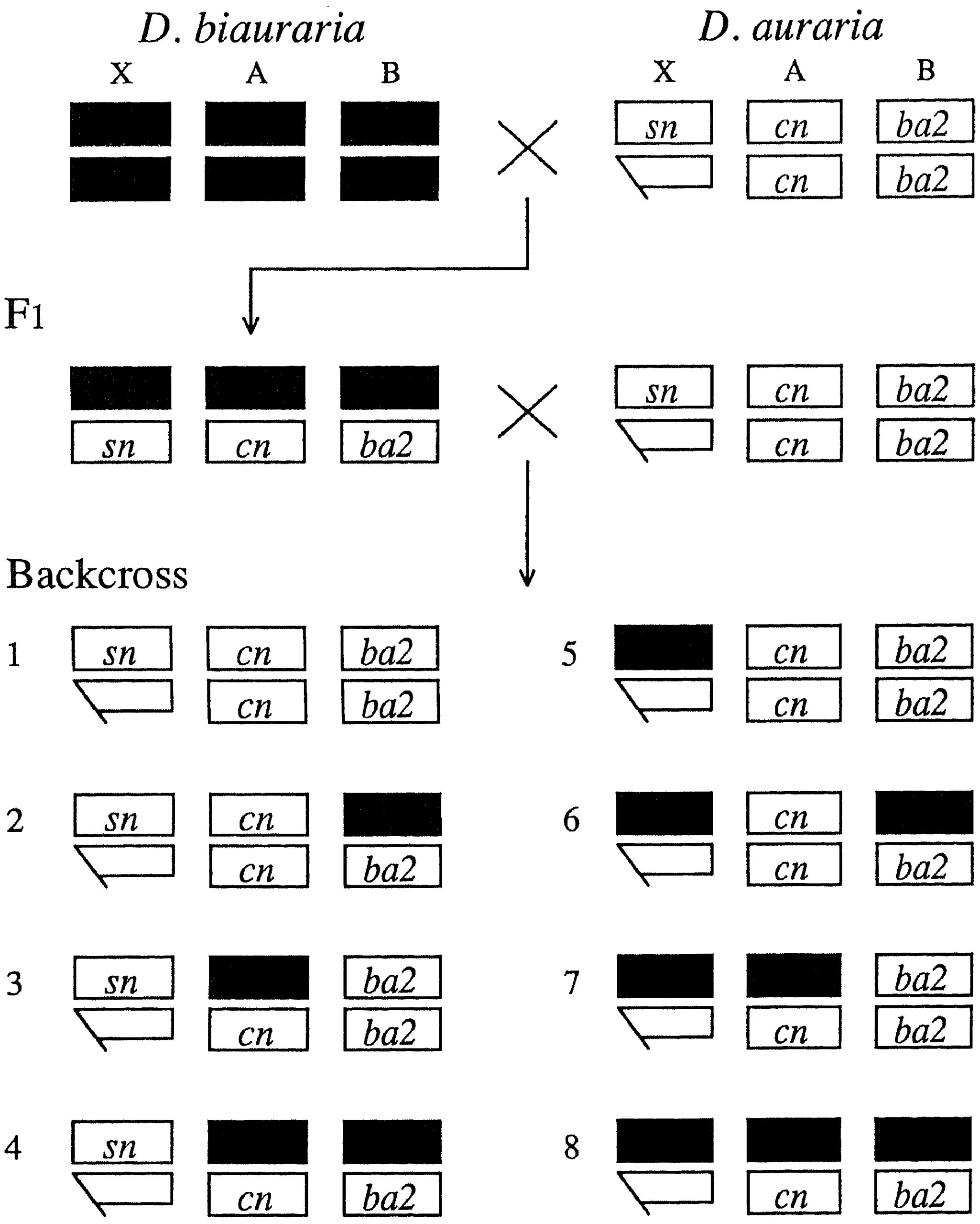

D. biauraria
D. auraria
X
A
B
X
A
B
sn
cn
ba2
cn
ba2
F1
sn
cn
ba2
sn
cn
ba2
cn
ba2
Backcross
1
sn
cn
ba2
cn
ba2
2
sn
cn
cn
ba2
3
sn
ba2
cn
ba2
4
sn
cn
ba2
5
cn
ba2
cn
ba2
6
cn
cn
ba2
7
ba2
cn
ba2
8
cn
ba2

Figure 12. Mean inter-pulse interval of each phenotype of backcross progeny of the cross (*D. biauraria* female x *D. auraria* male) female x *D. auraria* male, and of the cross (*D. auraria* female x *D. biauraria* male) female x *D. auraria* male.

Error bar: standard deviation.

Parentheses: number of flies.

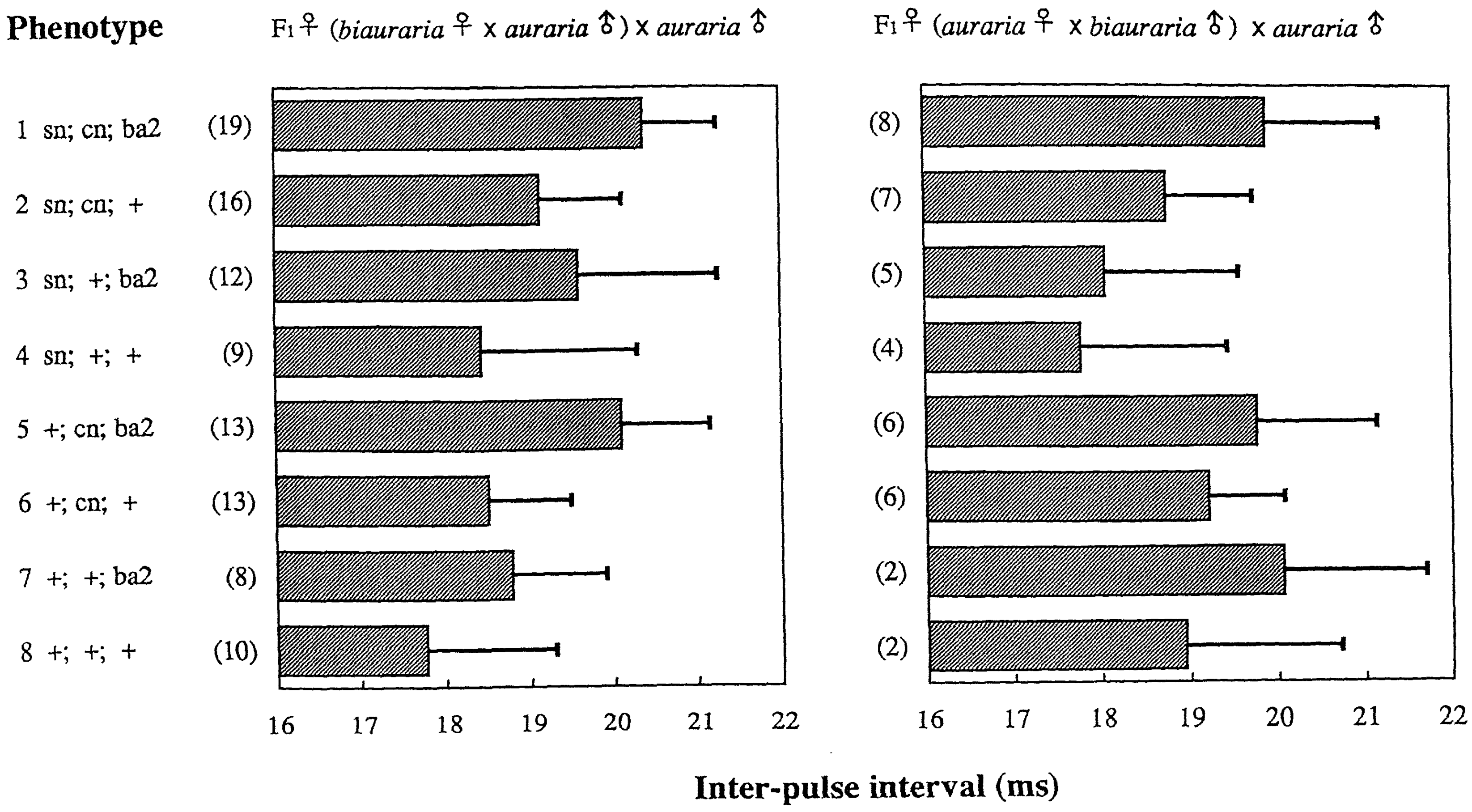
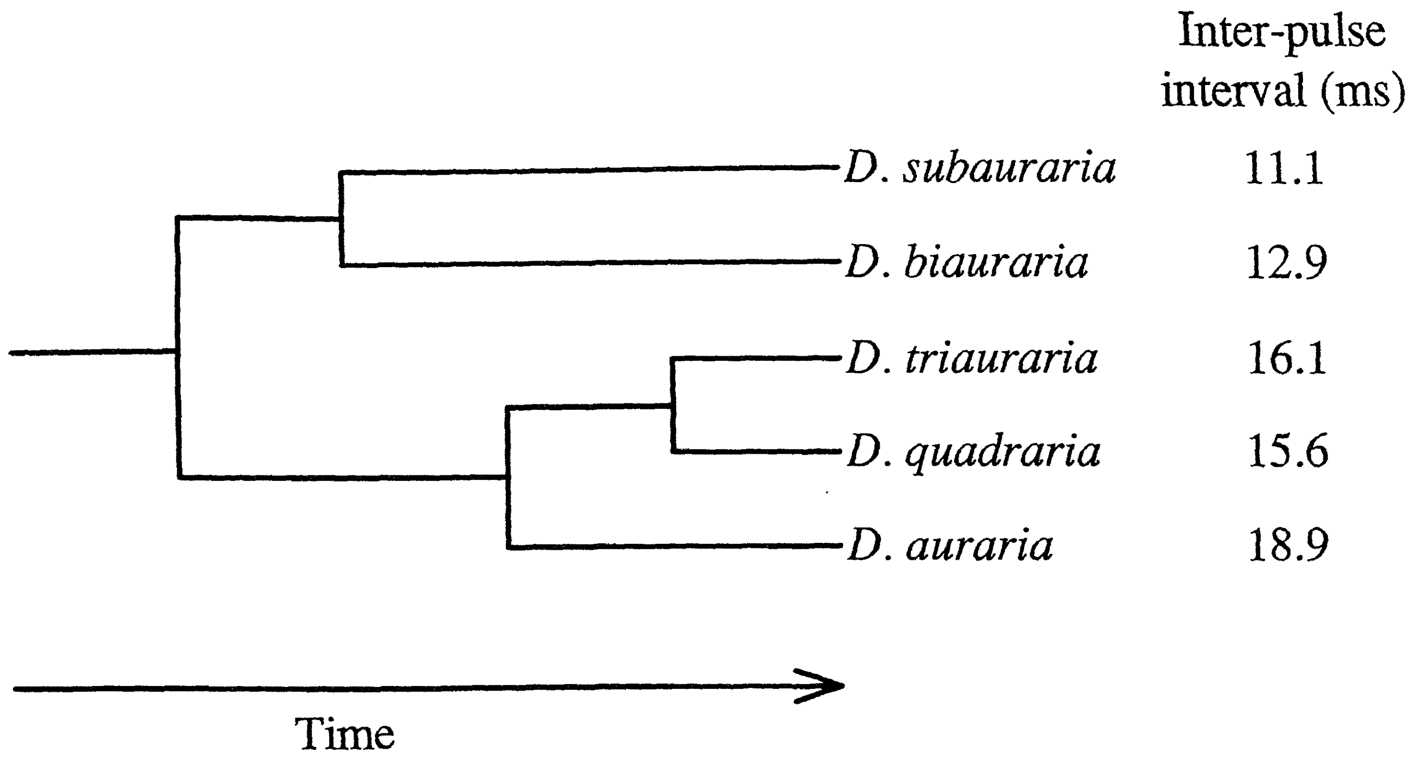

Phenotype
F1♀ (biauraria ♀ x auraria ♂) x auraria ♂
F1♀ (auraria ♀ x biauraria ♂) x auraria ♂
1 sn; cn; ba2
2 sn; cn; +
3 sn; +; ba2
4 sn; +; +
5 +; cn; ba2
6 +; cn; +
7 +; +; ba2
8 +; +; +
(19)
(16)
(12)
(9)
(13)
(13)
(8)
(10)
(8)
(7)
(5)
(4)
(6)
(6)
(2)
(2)
16
17
18
19
20
21
22
Inter-pulse interval (ms)

Figure 13. Inter-pulse intervals and speciation history in the *D. auraria* complex. The phylogenetic relationship is reconstructed after Kimura (1987). Five species were formed after the four successive splittings.


Inter-pulse interval (ms)
D. subauraria 11.1
D. biauraria 12.9
D. triauraria 16.1
D. quadraria 15.6
D. auraria 18.9
Time