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# Genetic basis and evolution of species-specific courtship song in the *Drosophila auraria* complex

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**Summary**

The interpulse interval (IPI) of courtship song in the *Drosophila auraria* complex is the only parameter that is consistently species-specific among the several courtship elements examined within the complex. The genetic basis of the species-specific courtship song was examined by analysing the song of interspecific hybrids and of backcross progeny. IPI of all interspecific hybrids except two showed intermediate values, suggesting autosomal control of species-specific IPI. However, significant deviation for shorter IPI from midparent was found in thirteen out of 20 crosses. The chromosomal analysis between *D. auraria* and *D. biauraria* revealed that the two major autosomes had significantly large effects on IPI, but the sex chromosome and cytoplasm had no effect. Since no interaction was detected, it is concluded that each autosome acts additively in the determination of species-specific IPI. The common ancestors of the *D. auraria* complex may also have had autosomal control of IPI, which has been conserved during speciation in the complex.

## 1. 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. 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 between *Drosophila 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).

The *D. auraria* complex consists of closely related species distributed either in sympatry or allopatry over the oriental area – Japan, Korea, China and Taiwan (Kimura, 1987). Strong reproductive isolation among the complex has been observed, brought about by pre-mating (Kurokawa, 1960; Kurokawa *et al.* 1982) and post-mating isolation (Kimura, 1987). The wing vibration affects mating success within species in *D. triauraria* (Grossfield, 1968). The interpulse interval (IPI) of the courtship song is the only parameter of the courtship elements that is consistently species-specific among the sympatric species (Tomaru & Oguma, 1993). Species-specific courtship song and failure to copulate in wingless males suggest that the courtship song may play an important role in sexual isolation in the *D. auraria* complex.

Interspecific 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 study, interspecific hybrids between species of the complex were generated and their courtship songs were analysed. The analysis of the

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backcross progeny allowed us to map the chromosomes on which the gene(s) controlling IPI differences were located.

## 2. Materials and methods

### (i) *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, Tokyo), *D. biauraria* (B16, B18, Tokyo), *D. triauraria* (T544, Tsukuba), *D. subauraria* (ONM-29, Onuma), *D. quadraria* (Q, Taiwan, Texas stock no. 3075.1). Files were maintained in cylindrical glass vials (3 cm diameter × 10·5 cm height) containing standard *Drosophila* sucrose–yeast–corn meal medium at 24·5 ± 0·5 °C under a light : dark cycle (L : D = 14 : 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.

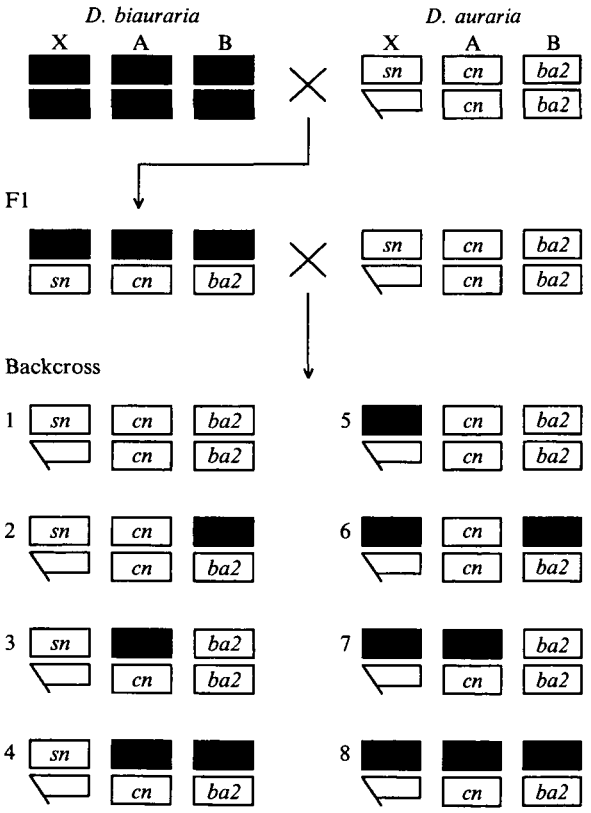


Fig. 1. Mating scheme for chromosome substitution in the cross (*D. biauraria* female × *D. auraria* male) × *D. auraria* male. Virgin flies of female *D. biauraria* were crossed to *D. auraria sn*; *cn*; *ba2* males. Hybrid females were backcrossed with *D. auraria* marker strains. For the cross (*D. auraria* female × *D. biauraria* male) × *D. auraria* male, $F_1$ hybrid females were generated from the reciprocal cross.

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. 1). Since reciprocal hybrids (female *D. auraria sn*; *cn*; *ba2* × male *D. biauraria* B16) in the parental cross were not easily obtained, numbers of backcross progeny from the cross were small. 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.

### (ii) *Song recording and analysis*

The detailed methods of recording the courtship song can be found elsewhere (Tomaru & Oguma, 1993). Males of each species in the *D. auraria* complex produce less courtship song before copulation, and much more during copulation. Since the IPIs produced during attempted copulation and during copulation were identical (Tomaru & Oguma, 1993), 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

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Table 1. *Interpulse intervals (ms) of interspecific hybrids*

| Parent[a] | | | Hybrid from a cross[b] | | |
|---|---|---|---|---|---|
| 1 | 2 | Midparent | 1 (female) × 2 (male) | 2 (female) × 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 interpulse intervals are presented first. IPI of parent strains except *sn*; *cn*; *ba2* were from Tomaru & Oguma (1993). Mean IPI of each strain was as follows (ms): A541 (*D. auraria*), 21·4; A662 (*D. auraria*), 18·9; *sn*; *cn*; *ba2* (*D. auraria*) 19·9 (Table 2); T544 (*D. triauraria*), 16·1; Q (*D. quadraria*), 15·6; B16 (*D. biauraria*), 12·9; B18 (*D. biauraria*), 12·4; ONM-29 (*D. subauraria*), 11·1.
[b] Mean ± S.D. (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.

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. IPI, which was defined as the time interval from one peak of the pulse to the next, was measured on an MS-DOS machine with our song analysis program (Tomaru & Oguma, 1993).

Intrapulse frequencies (IPFs) of marker mutants were calculated using fast Fourier transformation (FFT) after pulse detection (Tomaru & Oguma, 1993). IPF was defined as the frequency giving the maximum FFT power spectrum. Although IPF is not a species-specific parameter in the *D. auraria* complex (Tomaru & Oguma, 1993), wing mutation may affect sound frequency of the song.

### (iii) *Statistical analysis*

Three bursts of song for each fly and 10 IPIs and 11 IPFs from each burst were analysed. The mean IPI and mean IPF 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 interspecific 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 Tomaru & Oguma (1993). 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.

## 3. Results

### (i) *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 IPI of each parent strain and the midparent. IPI of all but two parent strains were significantly different from midparent IPI ($P < 0·005$). Non-significant differences were found in *D. triauraria* and the midparent (*D. triauraria* and *D. quadraria*, $t_6 = 1·043$, $0·2 < P < 0·5$), and in *D. quadraria* and the midparent (*D. triauraria* and *D. quadraria*, $t_4 = 0·957$, $0·2 < P < 0·5$). The IPI of *D. triauraria* and of *D. quadraria* did not significantly differ ($t_{10} = 1·394$, $0·05 < P < 0·1$). The differences between hybrid and midparent or between hybrid and parent can be detected by *t* tests.

Interspecific hybrids were obtained from 20 crosses. All hybrids except two showed an intermediate IPI between the parental values (Table 1). However, IPI was significantly shorter than the midparent in thirteen out of 20 crosses, and the other four crosses also showed shorter but statistically not significantly different IPI than the midparent value (Table 1). Only in two crosses, *D. auraria* A662 female × *D. biauraria* B18 male and *D. quadraria* female × *D. triauraria* male, did hybrid songs have significantly longer IPI than the midparent value (Table 1). The difference between IPI of interspecific hybrids and parent strains was tested by *t* test. Songs did not significantly differ between hybrids from *D. quadraria* female × *D. triauraria* male and parent *D. triauraria*, and between

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hybrids from *D. subauraria* female × *D. biauraria* male and parent *D. subauraria* ($t_{11} = 1{\cdot}409$, $0{\cdot}1 < P < 0{\cdot}2$ and $t_{13} = 1{\cdot}490$, $0{\cdot}1 < P < 0{\cdot}2$ 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 IPI between the reciprocal crosses (Table 1). 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.

Table 2. *Song parameters of mutant strains*

| Strain | Mean (±S.D.) | *N* |
|---|---|---|
| Interpulse interval (ms) | | |
| *ba2* | 20·3 (0·54) | 8 |
| *cu* | 19·4 (0·61) | 10 |
| *sn*; *cn*; *ba2* | 19·9 (0·72) | 11 |
| A541 × *sn*; *cn*; *ba2* | 21·2 (1·05) | 9 |
| *sn*; *cn*; *ba2* × 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.

(ii) *Chromosomal analysis*

The wing mutant markers may influence the courtship song. IPI of the mutant male and reciprocal hybrids between *sn*; *cn*; *ba2* and wild-type A541 were measured (Table 2). Since all mean IPIs lay within the range of *D. auraria* (18·9–21·4 ms, Tomaru & Oguma, 1993), the chromosomes carrying marker mutations showed no influence on IPI. The reason IPF 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 IPFs of *ba2* did not lie within the range of the *D. auraria* complex (98·9–184·1 Hz, Tomaru & Oguma, 1993), 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 IPI shortened with increased number of *D. biauraria* chromosomes (Fig. 2). The analysis of variance was performed without transformation because of homogeneity of variance among the eight genotypes (Bartlett test, $P > 0{\cdot}4$). The two autosomes had significant effects on IPI determination, but the X chromosome had a non-significant effect (Table 3). The cytoplasmic factor and the interaction were not detected. The factorial effect mean of cytoplasm, each chromosome and each interaction was calculated (Table 3).

## 4. Discussion

Interspecific hybrid songs among the *Drosophila auraria* complex show intermediate interpulse interval (IPI) between that of their parents (Table 1), suggest-

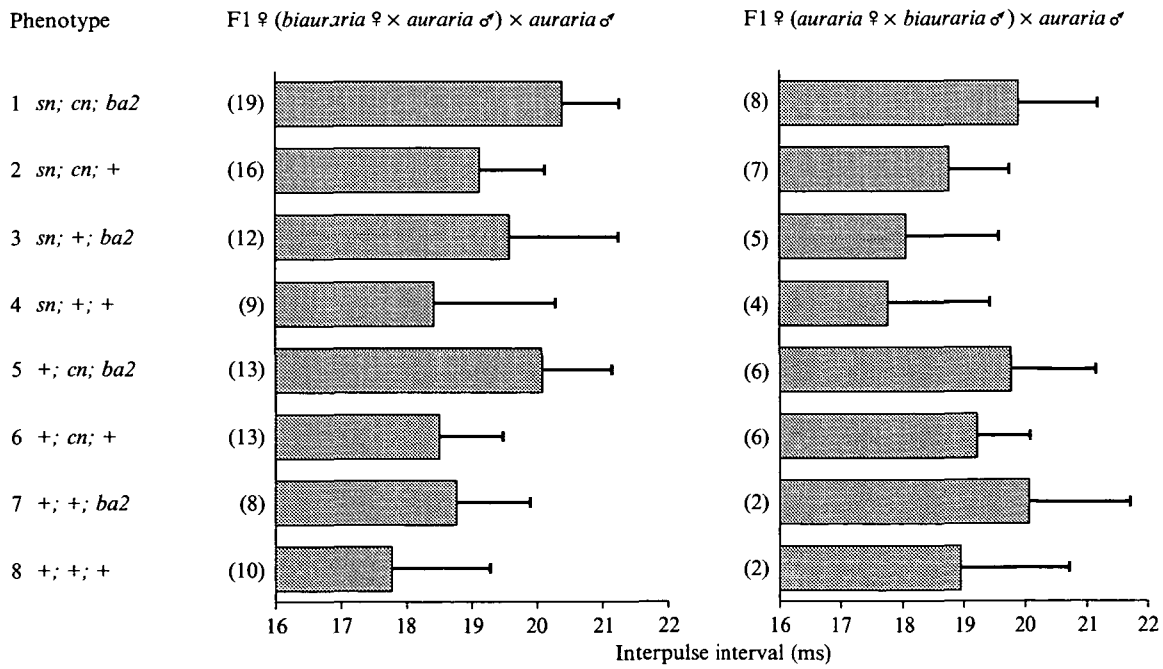


Fig. 2. Mean interpulse interval of each phenotype of backcross progeny of the cross (*D. biauraria* female × *D. auraria* male) female × *D. auraria* male, and of the cross (*D. auraria* female × *D. biauraria* male) female × *D. auraria* male. Error bar: standard deviation. Parentheses: number of flies.

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Table 3. *Analysis of variance for effects of three major chromosomes on the determination of interpulse interval*

| Source[a] | D.F. | 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.
*** $P < 0·001$.

ing that the autosomes play major roles in the determination of IPI. Autosomal control of IPI 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 IPI of the hybrid from the cross between *D. subauraria* female and *D. biauraria* male (SB hybrids, IPI = 11·5 ms) lay between IPI of their parents (*D. subauraria* ONM-29, 11·1 ms; *D. biauraria* B16, 12·9 ms), it did not significantly differ from IPI of mother *D. subauraria*. Since IPI of SB hybrids did not significantly differ from that of the hybrids of the reciprocal cross (Table 1), it is possible to commit a Type II statistical error; that is, the difference in IPI between SB hybrids and *D. subauraria* may exist, but the *t* test did not detect it.

The only exceptions in the hybrid analysis are the hybrids between *D. triauraria* and *D. quadraria*. Since IPI of the two parent species did not significantly differ (*D. triauraria* T544, 16·1 ms; *D. quadraria* Q, 15·6 ms), it is expected that reciprocal hybrids also show the same value. However, IPI of the reciprocal hybrids significantly differs from each other (14·9 and 16·5 ms, Table 1). One of the hybrids, the cross between *D. triauraria* female and *D. quadraria* male, also shows significantly different IPI from their parent strains.

Significant deviation from the midparent values for shorter IPI was found in thirteen out of 20 crosses in the *D. auraria* complex (Table 1). The direction of deviation is observed significantly more for short than for long (Sign test, $n = 15$, $0·001 < P < 0·005$). Dominance for shorter IPI was also reported in the *virilis* phylad (Hoikkala & Lumme, 1987).

The chromosomal analysis revealed that the two major autosomes had significant effects on IPI determination but the X chromosome did not (Table 3). Since no interaction was detected, the two autosomes act additively. This confirmed the autosomal control result from the hybrid analysis, which showed that the hybrid song was the intermediate IPI between that of parents (Table 1). 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 IPI in the chromosomal analysis (Table 3), reciprocal hybrids, from the cross between *D. auraria* A662 × *D. biauraria* B18, showed significant X/Y and cytoplasmic effects (Table 1). 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 varies among 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 IPI than *D. biauraria–D. auraria* hybrids (Fig. 2 and Table 1). 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 IPI than hybrid flies with the same genotype and the same cytoplasm. The design of the present experiment might have some bias 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 is some variation among the flies within the same genotype (Table 1, Fig. 2). Female preferences can influence the result of recordings. Although in recording hybrids' song, female flies used as partners were the same genotype as the corresponding male hybrid, virgin *D. auraria* females were used in the chromosomal analysis. Although there is no empirical data about the female preferences for IPI variation in the *D. auraria* complex, it is possible that the preferences of *D. auraria* female and that of hybrid females are not identical. If it is 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 some special genes or some specific mechanisms produce hybrid sterility/inviability (Dobzhansky, 1940; Coyne & Orr, 1989). For pre-mating isolation, however, general rules have not yet emerged. In the *D. virilis* group the *montana* phylad species have longer IPI 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 IPI 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 among the species in the *D. melanogaster* species subgroup (Cowling & Burnet, 1981). That no genetic elements are on the X chromosome is likely; they are probably conserved during speciation in the *D. melanogaster* species subgroup.

In the *D. auraria* complex, hybrid and backcross analyses show 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 IPI. IPI diverged to be species-specific during speciation, but there are no effective genes on the X chromosome. The chromosome influencing IPI may be constrained by the ancestor species. Since the chromosomes controlling species-specific IPI 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 in the group of closely related species.

The male wing-removal experiments and simulated-song experiments show that the courtship song is an effective signal for intraspecific copulation in several *Drosophila* species (Ewing, 1964; Narda, 1966; Grossfield, 1968; Bixler *et al.* 1992; Liimatainen *et al.* 1992; Crossley & Bennet-Clark, 1993) and that the IPI is one of the most important song parameters (Bennet-Clark & Ewing, 1969; Kyriacou & Hall, 1982; Kyriacou *et al.* 1992). Particularly when a preferred IPI with a species-specific IPI rhythm was given to females, copulation frequencies increased in *D. melanogaster* and *D. simulans* (Kyriacou & Hall, 1982). This implies that the species-specific discrimination system in females has been evolving, together with a male character. The evolution of females' mate discrimination is now an interesting subject to reveal the development of sexual isolation (Kyriacou *et al.* 1992). 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). These studies clearly show that several genes control female mate discrimination. It is possible that female mate discrimination is due to song detection. In the *D. auraria* complex a male attempts to copulate with a heterospecific female, but he is rejected by her and fails to copulate (our unpublished observations). The wing vibration is an effective signal for mating success (Grossfield, 1968) and IPI is a species-specific song parameter (Tomaru & Oguma, 1993). The female song discrimination seems to be the basis of sexual isolation in the *D. auraria* complex. Genetic studies on song detection of females are now in progress in our laboratory.

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