

## Genetic and reproductive differentiation in *Drosophila sulfurigaster*

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### ABSTRACT

Twelve local populations of *Drosophila sulfurigaster albostrigata* from the Philippines, Palawan, Borneo, the Malay Peninsula, Thailand and Sri Lanka, were crossed to *D. s. bilimbata* and *D. s. sulfurigaster*. With respect to postmating isolation, the fertility and the sex ratio distortion in F<sub>1</sub> hybrids and the frequency of the insemination reaction causing the reaction mass were investigated. In crosses to *D. s. sulfurigaster*, the extent of postmating isolation, both prezygotic and postzygotic ones, were relatively weak. In crosses to *D. s. bilimbata*, the extent of both kinds of isolation suggested that local populations of *D. s. albostrigata* have genetically diverged among the six regions described above. In interpopulational hybridization, hybrids from crosses of marginal populations showed the hybrid breakdown in the F<sub>2</sub> or F<sub>3</sub> generation for egg-to-adult viability. On the other hand, in premating isolation to *D. s. neonasuta* estimated by no-choice experiments, significant genetic differentiation among local populations of *D. s. albostrigata* was not detected. In intersubspecific crosses, between two groups; one consists of *D. s. neonasuta* and *D. s. albostrigata* and the other *D. s. bilimbata* and *D. s. sulfurigaster*, prezygotic isolation by the insemination reaction causing the reaction mass was developed in either of the reciprocal crosses, and postzygotic isolation by F<sub>1</sub> male sterility and the sex ratio distortion in F<sub>1</sub> progeny in the other crosses. Strong one sided sexual isolation was observed between *D. s. neonasuta* and the other group including *D. s. bilimbata* and *D. s. sulfurigaster*. It is clear that isolating mechanisms to prevent the gene exchange between subspecies have developed to some extent. On the basis of the degree of genetic and reproductive differentiation estimated by hybridization experiments, the processes of subspecies formation in *D. sulfurigaster* are discussed.

### 1. INTRODUCTION

Most animal and plant species are complexes of subdivided, local populations which differ from each other genetically to some extent. These populations are sometimes named race or subspecies according to the extent of their differences between populations. In these cases, groups of populations are usually allopatric, and geographic isolation keeps the gene exchange infrequent. By contrast, species are able to maintain their genetic integrity despite of sympatric coexistence. Mayr (1942) put forward the 'geographic

speciation' model: geographic isolation of two populations is a necessary prerequisite in time to the development of reproductive isolating mechanisms. The first stage of geographic speciation is the appearance of genetic difference. This differentiation occurs if the populations are geographically separated for some time with no or little migration between them. One or several primary isolating mechanisms can be formed during this stage. The second stage takes place when these populations come into contact geographically. Isolating mechanisms will be reinforced by natural selection. Unless the genes which guarantee reproductive isolation are fixed into the genotype of each individual of the population, any genetic differentiation which may have arisen is potentially reversible. The acquisition of the substantial reproductive isolation is thus a critical point in the speciation process. In this model, how much and what kind of genetic differentiation is required for primary reproductive isolating mechanisms to arise are asked at the first stage of geographical isolation. In order to approach these questions, it is necessary to know the development of reproductive isolating mechanisms, that is, the kind of mechanisms evolved at the first stage and its underlying genetic basis. The study might best be done in an actively evolving group which includes forms at various levels and stages of diversification.

The *Drosophila nasuta* subgroup, belonging to the *Drosophila immigrans* species group, includes more than ten species which are morphologically similar distributed in Pacific-Australasian and the pan-Indian Ocean areas. A number of these populations in different continental areas and islands of the Pacific were demonstrated to have diverged to the point of being separate sibling species (Wheeler and Takada 1964; Kitagawa *et al.* 1982). One of the species, *Drosophila sulfurigaster* has the largest distribution among the *D. nasuta* subgroup, and consists of four subspecies. These subspecies are found allopatrically according to the definition of subspecies by Mayr (1970). It is very suitable for the study of the speciation process to have many groups of populations at the various levels of diversification and to be widely distributed both in continental and island areas. Wilson *et al.* (1969) surveyed three subspecies of *D. sulfurigaster*; *D. s. albostrigata*, *D. s. bilimbata* and *D. s. sulfurigaster*. They found that the three subspecies differ greatly in the number of and distribution of chromosomal inversions and in its behavior in crosses to the other subspecies. The investigation of the salivary gland chromosomes showed that *D. s. bilimbata* is monomorphic (Wilson *et al.* 1969; Matsuzaki and Kitagawa 1982). When it is used as the standard, ten heterozygous inversions were found in *D. s. sulfurigaster* and 17 in *D. s. albostrigata* among all four long euchromatic arms (Matsuzaki and Kitagawa 1982). In crosses between subspecies, the sex ratio distortion with the increase of males occurred in crosses of *D. s. albostrigata* females to *D. s. bilimbata* and *D. s. sulfurigaster* males, and  $F_1$  male sterility was observed in the former cross (Wilson *et al.*

1969). *D. s. neonasuta* was described by Nirmala and Krishnamurthy (1973) on the basis of the difference of the metaphase karyotype. When it was crossed to the other subspecies,  $F_1 \times F_1$  crosses were found to be sterile in some cases (Ranganath and Krishnamurthy 1976).

Males of the *D. nasuta* subgroup have white to silvery markings on the frons of all but one of the species. The four subspecies of *D. sulfurigaster* have pronounced whitish bands along the frontal orbits of the males. These are not precisely distinguished morphologically, but *D. s. sulfurigaster* has narrower bands than the others. *Drosophila pulaua*, another member of the *D. nasuta* subgroup, has bands of the same type which are much fainter, and is morphologically quite similar to *D. sulfurigaster*. The distribution of *D. pulaua* is very narrow, and it is sympatric with one of the subspecies of *D. sulfurigaster*, *D. s. albostrigata*. The results of hybridization experiments between them indicate that *D. pulaua* is the most closely related species to *D. sulfurigaster* (Wilson *et al.* 1969).

In the case of subspecies, or allopatric populations, the real functional deterrent to the gene exchange is a physical geographic barrier. It is never realized in nature whether the primary reproductive isolating barrier exists or not. Therefore, in order to assess the extent of the reproductive barrier between them, it is necessary to bring the populations together artificially. Laboratory investigation of these reserve mechanisms by bypassing of the earliest-acting and operational barrier, often manifests the extent of the genetic gulf between populations or subspecies, and may indicate important phases in the process of divergence. The experimental hybridization method was employed in this study to ascertain the extent of divergence between populations, subspecies and species. The isolating mechanisms involved were separately investigated for premating and postmating mechanisms. For postmating barriers, the performance of  $F_1$  hybrids from interspecific crosses were tested with regards to fertility and sex ratio. While these barriers are postzygotic ones, the prezygotic one is the insemination reaction that is well known in many species of *Drosophila* (Patterson 1946, 1947; Wheeler 1947). All possible combinations between subspecies and species were made to estimate the extent of genetic differentiation between them. Differences among local populations of *D. s. albostrigata* were also examined by crossing each population to other subspecies, and in addition to these, some components of fitness of hybrids from interpopulational crosses were measured. It is known that the fitness of hybrids between genetically diverged local populations is reduced in *D. pseudoobscura* (Vetukhiv 1953, 1954, 1956, 1957), *D. melanogaster* (Tantawy and El-Helw 1970) and other species (Wallace and Vetukhiv 1955). On the other hand, ethological isolation, on which *Drosophila* species have been widely used for observations and experiments, was investigated between subspecies and species by the no-choice method. Intrasub-

specific variation was investigated for local populations of *D. s. albostrigata* with respect to the extent of ethological isolation to *D. s. neonasuta*, which is distributed adjacent to *D. s. albostrigata*.

The purpose of this study is to clarify the state of the reproductive isolation, and to infer the subspeciation process in *D. sulfurigaster*.

## 2. MATERIALS AND METHODS

The materials used in the study consisted of four subspecies of *D. sulfurigaster* and *D. pulaua*. *D. s. bilimbata* stocks from two localities were supplied by Dr. M. R. Wheeler at University of Texas, and *D. s. neonasuta* stock by Dr. N. B. Krishnamurthy at Mysore University. All other stocks were from collections at various localities in southeast Asia and Papua New Guinea, as indicated in Figure 1. In all collections, each wild-caught inseminated female was isolated and cultured to establish an iso-female line. These stocks were maintained at 20°C at Tokyo Metropolitan University. All the cultures were maintained on Brent and Oster (1974) medium, which was slightly modified.

The experiments were conducted at 25°C, ca. 70% relative humidity and under a 24hr light regime. For hybridization experiments, virgin females and males were collected without etherization within three hours of eclosion and aged for five to six days before mating.

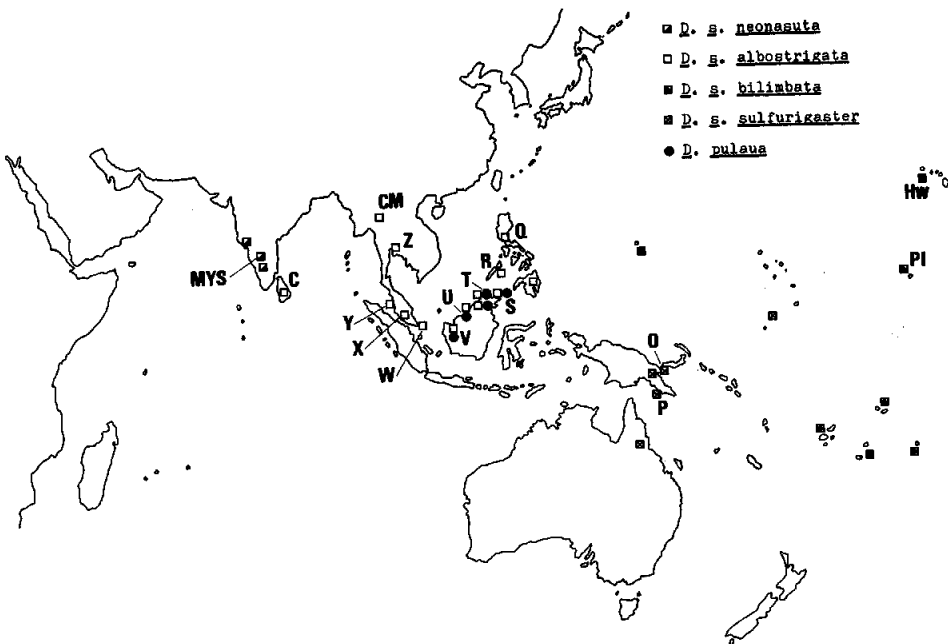


Fig. 1. Distribution of four subspecies of *Drosophila sulfurigaster* and *Drosophila pulaua*.

*Fertility and the sex ratio distortion in F<sub>1</sub> hybrids:* Reciprocal crosses were made, in two replicates, between species and subspecies. For each locality of *D. s. albostrigata*, three iso-female lines were randomly chosen and one or two iso-female lines for other subspecies and species. Fifteen females and 20 males were put together in a vial with medium and transferred to fresh medium after five days, then discarded after another five days. When F<sub>1</sub> flies were obtained, the sex ratio was examined and F<sub>1</sub> females × F<sub>1</sub> males crosses were made to test for fertility. Flies were transferred to fresh food every five days and were followed for up to 20 days. In the cases when the F<sub>1</sub> failed to produce progeny, the F<sub>1</sub> flies were backcrossed to both parental strains to determine if either F<sub>1</sub> males or F<sub>1</sub> females were sterile.

*Insemination reaction:* Reciprocal crosses were made between species and subspecies using one iso-female line as a representative of that locality for each subspecies and species. Ten females and 15 males were mated for five days in a vial with medium. Then the females were dissected in Shen's solution and examined for the presence or absence of the reaction mass in the uterus and sperm in the spermathecae and ventral receptacles. Females having either the reaction mass or sperm, or both, were regarded as having been inseminated. Over 20 inseminated females were examined for each cross.

*No-choice experiments:* Twenty females from one strain were placed together with 25 males from the same or different strain in a vial with medium. After 48 hours, females were dissected to check for the presence or absence of sperm in the manner described above. More than 50 females were dissected for each combination. For each species and subspecies, one iso-female line was selected from each locality.

*Productivity of interpopulational hybrids:* For six localities, Los Baños (designated as Q in Figures and Tables), Miri(U), Singapore(W), Panang(Y), Nakhon Nayok(Z), Sri Lanka(C), experimental populations were established by mixing ten iso-female lines of each population of *D. s. albostrigata*. After five generations, these mixed populations were used to assess fitness in interpopulational hybrids. This procedure was conducted twice using the same iso-female lines.

A diallel cross was made for each set, and each cross consisted of two vials containing 20 females and 20 males. F<sub>1</sub> flies were collected within 24 hours after eclosion and the fixed number of flies, ten of each sex for the first set and five for the second set, were put into vials. Four or five replicates were made for each set. Five days after the cross, all flies were transferred to fresh food, thereafter they were transferred to vial containing fresh food every three days, and allowed to lay eggs for six days. F<sub>2</sub> flies to be tested

for their productivity were obtained from  $F_1 \times F_1$  crosses and the same procedure was applied for  $F_3$  flies. Productivity was measured by the number of progeny per day per female.

*Viability of interpopulational hybrids:* As for the viability test, another test medium was used, consisting of agar, brewer's yeast and sugar. Experimental populations were established and diallel crosses were made in the same manner as the productivity tests. In each generation, from the  $F_1$  to  $F_3$ , eggs were laid on the test medium and sampled on the 3rd and 4th days after the cross. A small batch containing 50 eggs was transferred to the new test medium in a small vial (2 cm  $\times$  10 cm). Two series including five replications were carried out for each set. Viability was expressed as the number of adults emerged/number of eggs sampled in percentage, and an arcsin transformation was applied for the analysis.

### 3. RESULTS

#### I. *Differentiation in local populations of D. s. albostrigata.*

##### (I-1) *Postmating barriers to D. s. bilimbata and D. s. sulfurigaster*

Twelve local populations of *D. s. albostrigata* were examined as to the sex ratio distortion in  $F_1$  progeny and their fertility in crosses to *D. s. bilimbata* and *D. s. sulfurigaster*. The sex ratio distortion was observed only in crosses with females of *D. s. albostrigata*, and the progeny of their reciprocal crosses showed all the normal fertility and sex ratio. In crosses to *D. s. sulfurigaster* males, given in Table 1, the ratio of males in  $F_1$  progeny, which were all fertile, ranged from about 50 to 70%. The higher values were found with the Port Moresby (P33) strain than with the Lae (O14) strain, but no correlation was found between the values with the P33 and O14 strains. In some strains of the Q, U, Z and C populations, normal or nearly normal sex ratios were indicated with the O14 strain. The analysis of variance, however, showed no significant differences among local populations. By contrast, in crosses to *D. s. bilimbata* males, statistically significant differences at the 5% level were observed among populations. All those crosses resulted in sterile male progeny. Table 2 shows that the Hawaii (Hw) and Palmyra (Pl) strains differed greatly in the extent of the distortion. In the case of the Hw strain, about 70 to 80% of the  $F_1$  progeny were males, on the average, this being a greater number than in crosses to *D. s. sulfurigaster*. With the Pl strain, however, the Q, U and W populations showed a significant distortion, but the values were much lower than that with the Hw strain. In the Y and Chiang-mai (CM) populations, conversely, significant increases of females were observed. It appears that the ratio of males increases in Borneo to the Malay

Table 1. Sex ratio distortion in  $F_1$  hybrids from crosses between *D. s. albostrigata* females and *D. s. sulfurigaster* males

Locality	Male strain	P33 (Port Moresby)			O14 (Lae)		
		No. of $F_1$ ♀♀	No. of $F_1$ ♂♂	% ♂♂ of $F_1$ progeny	No. of $F_1$ ♀♀	No. of $F_1$ ♂♂	% ♂♂ of $F_1$ progeny
Los Baños	Q 1	237	308	56.5**	179	195	52.1
	47	339	603	64.0***	230	236	50.6
	60	176	494	73.7***	113	311	73.3***
Palawan	R 24	349	731	67.7***	442	634	58.9***
	42	415	784	65.4***	453	675	59.8***
	67	247	395	61.5***	145	215	59.7***
Sandakan	S 3	307	538	63.7***	288	423	59.5***
	32	295	598	67.0***	436	666	60.4***
	43	195	298	60.4***	176	343	66.1***
Kota Kinabalu	T 33	176	233	57.0***	380	457	54.6**
	53	153	358	70.1***	161	261	61.8***
	66	224	469	67.7***	265	365	57.9***
Miri	U 1	100	287	74.2***	291	347	54.4*
	28	200	323	61.8***	240	299	55.5*
	94	152	368	70.8***	232	290	55.5*
Kuching	V 11	525	843	61.6***	404	584	59.1***
	28	206	350	62.9***	168	487	74.4***
	47	370	594	61.6***	382	659	63.3***
Singapore	W 3	295	595	66.9***	290	330	53.2
	54	203	422	67.5***	192	284	59.7***
	99	367	495	57.4***	227	375	62.3***
Kuala Lumpur	X 24	170	348	67.2***	213	512	70.6***
	36	220	503	69.6***	269	555	67.4***
	65	82	190	69.9***	171	397	69.9***
Penang	Y 24	305	375	55.1**	172	242	58.5***
	34	264	664	71.6***	287	437	62.1***
	64	274	526	65.8***	466	553	54.3**
Nakhon Nayok	Z 15	312	508	62.0***	533	621	51.6
	26	180	259	59.0***	194	275	58.6**
	27	266	406	60.4***	242	275	53.2
Chiangmai	C M 26	306	645	67.8***	378	838	63.9***
	63	531	727	57.8***	376	509	57.5***
	76	204	520	71.8***	247	510	67.4***
Kandy	C 10	122	292	70.5***	288	296	50.7
	34	152	331	68.5***	453	600	57.0***
	96	291	514	63.9***	373	523	58.4***

\*: Significant at the 5% level.  
 \*\*: Significant at the 1% level.  
 \*\*\*: Significant at the 0.1% level.

Table 2. Sex ratio distortion in  $F_1$  hybrids from crosses between *D. s. albostrigata* females and *D. s. bilimbata* males

Locality	Male		Hawaii			Palmyra		
	Female strain	No. of $F_1$ ♀♀	No. of $F_1$ ♂♂	% ♂♂ of $F_1$ progeny	No. of $F_1$ ♀♀	No. of $F_1$ ♂♂	% ♂♂ of $F_1$ progeny	
Los Baños	Q 1	120	248	67.4***	271	305	53.0	
	47	218	612	73.7***	317	515	61.9***	
	60	47	263	84.8***	102	145	58.7**	
Palawan	R 24	363	881	70.8***	490	451	47.9	
	42	246	622	71.6***	398	288	42.0***	
	67	274	431	61.1***	166	141	45.9	
Sandakan	S 3	130	190	59.4***	270	253	48.4	
	32	248	993	80.0***	601	572	48.8	
	43	186	656	77.9***	387	349	47.4	
Kota Kinabalu	T 33	225	375	62.5***	518	471	47.6	
	53	238	339	58.8***	328	271	45.2*	
	66	293	660	69.3***	548	556	50.4	
Miri	U 1	189	700	78.7***	239	234	54.3*	
	28	291	464	61.5***	209	327	61.0***	
	94	75	318	80.9***	228	333	59.4***	
Kuching	V 11	362	652	64.3***	312	265	45.9	
	28	208	803	79.4***	353	328	48.2	
	47	274	900	76.7***	350	296	45.8*	
Singapore	W 3	118	426	78.3***	129	177	57.8**	
	54	123	388	75.9***	208	283	57.6***	
	99	84	584	87.4***	82	210	71.9***	
Kuala Lumpur	X 24	105	426	80.2***	202	176	46.6	
	36	35	387	91.7***	394	391	49.8	
	65	100	345	77.5***	232	236	50.4	
Penang	Y 24	110	289	72.4***	343	274	44.4**	
	34	100	483	82.8***	188	197	51.2	
	64	83	304	78.6***	458	381	45.4**	
Nakhon Nayok	Z 15	83	292	77.9***	218	187	46.8	
	26	64	365	85.1***	283	256	47.5	
	27	294	456	60.8***	288	274	48.8	
Chaingmai	C M 26	214	756	77.9***	491	421	46.2*	
	63	357	887	71.3***	596	489	45.1**	
	76	282	732	72.2***	366	355	49.2	
Kandy	C 10	226	521	69.7***	343	336	49.5	
	34	133	312	70.1***	155	146	48.5	
	96	217	558	72.0***	420	459	52.2	

\*: Significant at the 5% level.

\*\*: Significant at the 1% level.

\*\*\*: Significant at the 0.1% level.



Table 3. Percentage of the formation of the reaction mass in the insemination reaction in crosses between *D. s. albostrigata* and *D. s. sulfurigaster* or *D. s. bilimbata*

<i>s. a.</i> strain	<i>s. s.</i> ♀ × <i>s. a.</i> ♂		<i>s. a.</i> ♀ × <i>s. s.</i> ♂		<i>s. b.</i> ♀ × <i>s. a.</i> ♂		<i>s. a.</i> ♀ × <i>s. b.</i> ♂	
	P33	O14	P33	O14	Hw	Pl	Hw	Pl
Q 1	4.3	6.9	25.0	34.8	25.0	23.8	23.8	12.5
R 67	0.0	6.7	25.9	11.8	6.6	7.4	0.0	9.1
S 43	8.7	11.1	4.5	17.2	30.8	56.0	0.0	0.0
T 66	14.3	12.5	3.6	4.8	65.4	53.3	7.7	4.8
U 1	6.3	6.3	6.3	18.5	40.7	73.3	3.4	4.2
V 28	9.1	6.9	11.5	6.9	32.0	40.5	9.1	12.1
W 54	4.2	4.5	5.0	0.0	42.3	42.3	18.2	4.5
X 36	10.3	18.2	12.5	0.0	68.2	14.3	0.0	0.0
Y 24	10.5	10.3	9.1	9.4	33.3	17.2	7.7	7.4
Z 27	19.2	14.8	17.9	3.6	75.0	68.2	3.6	0.0
CM 63	0.0	3.2	22.7	20.8	69.6	24.2	8.7	6.7
C 34	29.2	0.0	17.4	7.7	28.6	11.4	4.8	4.8

*s. a.*: *s. albostrigata*, *s. b.*: *s. bilimbata*, *s. s.*: *s. sulfurigaster*.

Peninsula, and decreases in Thailand and Sri Lanka.

The results of the insemination reaction, the prezygotic barrier, are given in Table 3. Using *D. s. sulfurigaster* as the tester, four kinds of crosses between *D. s. albostrigata* and the tester strains did not differ significantly: the values pooled over 12 strains were 9.8% with crosses to the P33 females, 8.8% with the O14 females, 13.7% with the P33 males and 10.9% with the O14 males. A significant difference among populations was not revealed by the analysis of variance. Using *D. s. bilimbata*, however, a highly significant difference at the 0.1% level was found between female and male testers. The pooled values in crosses to the tester males were 6.8% with the Hw and 5.8% with the Pl, while to the tester females 45.3% with the Hw and 35.7% with the Pl strains. In addition, in the case of the female testers, the difference among strains of *D. s. albostrigata* was statistically significant at the 1% level. The insemination reaction causing the reaction mass occurred at the high frequency in the Thailand populations and at the low frequency in the Q, Palawan(R) and C populations that are marginal ones, and that the occurrence rate decreases slightly from Borneo to the Malay Peninsula.

#### (I-2) *Fitness of interpopulational hybrids*

Table 4 gives the fitness of six local populations with respect to productivity and egg-to-adult viability. The difference among populations was statistically significant by the analysis of variance. In the productivity test, the

Table 4. Means of productivity and viability of six local populations of *D. s. albostrigata*

	Productivity	Viability	
		arcsin $\sqrt{\%$	%
LOB (Q)	18.16±0.78	38.47±0.92	78.9
MY Y (U)	16.58±1.12	40.34±1.18	80.5
S I N (W)	18.32±1.04	46.41±0.95	85.1
P E N (Y)	23.26±1.26	42.20±0.81	82.0
NN Y (Z)	22.26±1.44	49.15±0.82	87.0
K D Y (C)	21.76±0.96	49.25±0.86	87.0

number of pairs per vial was ten in the first set and five in the second set. As no significant interaction was found between combinations of parents; populations and the density of adults, analyses were carried out with the first and second set pooled. The number of replications were, on the average, 4.5 in the first set and 4.3 in the second. To compare the performance of hybrids with the parental one, each productivity was standardized by the mid-parent value, and the average deviations from the mid-parent values were presented in Figure 2, with a *t*-test applied. The deviations were small in crosses among the Q, U and W populations and among the Y, Z and C populations. Some tendencies of hybrid breakdown were observed;  $F_1$  hybrids from crosses of the U females to males of all populations except the Q population and  $F_2$  hybrids from crosses of the Z females to the Y and C males, showed lower productivities than the mid-parent values.

With respect to the viability tests, two series of experiments in each set were regarded as independent experiments to be analysed. The average number of replications was 27.7 for each of the crosses, which resulted in about 1400 eggs being sampled. As in the case of the productivity test, Figure 3 gives the average deviations from the mid-parent values for each cross. The hybrid breakdown was observed more clearly in viability than in productivity. The Q population, both females and males, gave the hybrid breakdown in the  $F_2$  generation when crossed to more distant populations. The  $F_1$  heterosis in crosses with the Q males probably resulted from the low viability of the Q population itself. The hybrids between the W, Y and Z populations, which are all distributed in continental area, showed similar patterns from the  $F_1$  to  $F_2$  generations. When the C population was crossed to these populations, however, striking breakdown in the  $F_2$  generation were found for the C males. The U population in Borneo tended to show the breakdown when the U males were crossed to the others.

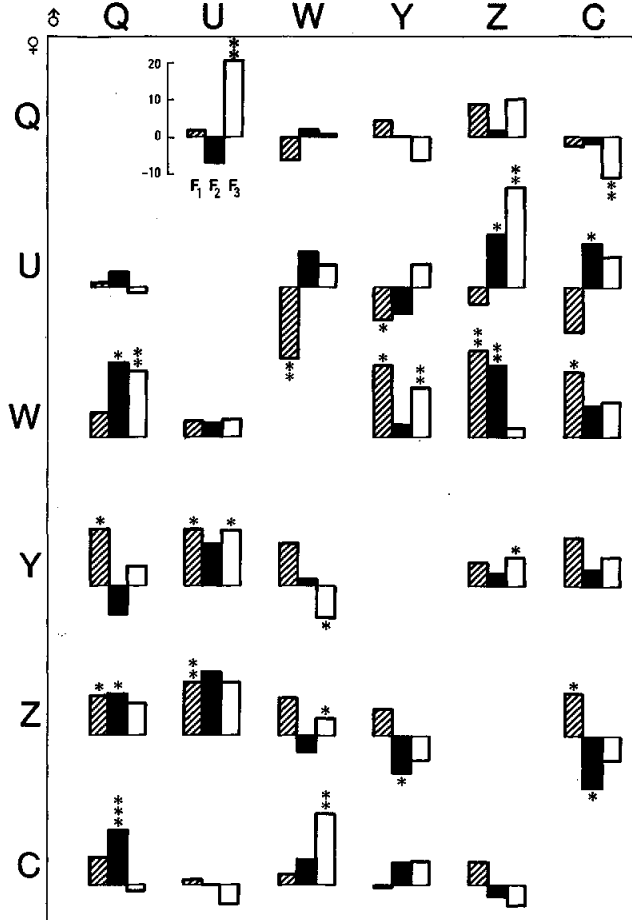


Fig. 2. The average deviations from the mid-parent values for productivity of hybrids between local populations of *D. s. albostrigata*.

\*: Significant at the 5% level.

\*\*: Significant at the 1% level.

\*\*\*: Significant at the 0.1% level.

(I-3) Premating barrier to *D. s. neonasuta*

Table 5 shows the percentages of successful matings between *D. s. albostrigata* and *D. s. neonasuta*. The percentage of successful matings in intrastain crosses was 92.0%, on the average, in *D. s. albostrigata* and 85.8% in *D. s. neonasuta*. *D. s. albostrigata* males inseminated *D. s. neonasuta* females as well as females of their own strains, and no mating preference was observed. Between *D. s. albostrigata* females and *D. s. neonasuta* males, however, the percentage of successful matings was lower than their reciprocal crosses. Pooled over 12 strains, the percentage of successful matings was 91.6% in

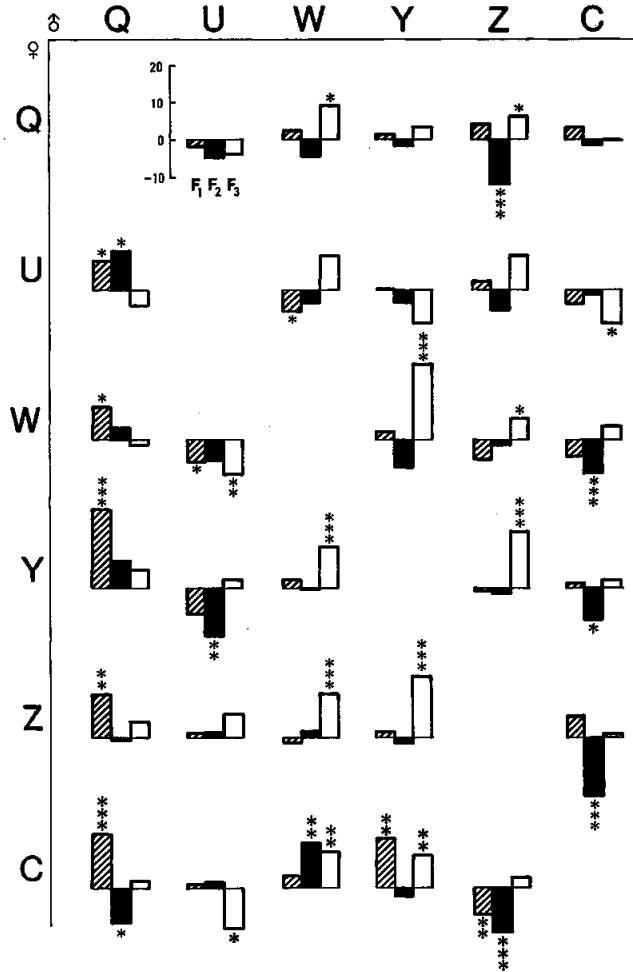


Fig. 3. The average deviations from the mid-parent values for viability of hybrids between local populations of *D. s. albostrigata*.

crosses to *D. s. neonasuta* females, which is equivalent to that found in intrastrain crosses. In contrast to this, in crosses to *D. s. albostrigata* females, 77.7% of females were inseminated by *D. s. neonasuta* males. Cases of remarkable asymmetry were found in the Q, Kota Kinabalu(T), Kuching(V) and Kuala Lumpur(X) strains, and there appeared no relationship between the extent of premating isolation to *D. s. neonasuta* and the geographical location of the population of *D. s. albostrigata*.

Table 5. Percentage of successful matings between *D. s. albostrigata* and *D. s. neonasuta*

<i>s. albostrigata</i> strain	<i>s. neonasuta</i> ♀ × <i>s. albostrigata</i> ♂			<i>s. albostrigata</i> ♀ × <i>s. neonasuta</i> ♂		
	A	B	B/A (%)	A	B	B/A (%)
Q 1	76	75	98.7	76	58	76.3
R 67	76	70	92.1	64	52	81.3
S 32	77	65	84.4	75	59	78.7
T 66	57	48	84.2	77	43	55.8
U 1	73	66	90.4	72	62	86.1
V 28	84	75	89.3	73	51	69.9
W 3	79	76	96.2	83	69	83.1
X 36	78	72	92.3	81	47	58.0
Y 24	72	64	88.9	80	72	90.0
Z 27	71	67	94.4	77	69	89.6
CM 63	82	75	91.5	76	63	82.9
C 34	80	76	95.0	75	61	81.3

A: No. of females dissected.

B: No. of females inseminated.

## II. Differentiation between the four subspecies of *D. sulfurigaster* and *D. pulaua*.

### (II-1) Postmating barriers

The results of diallel crosses are given in Table 6, using the Mysore(MYS) strain of *D. s. neonasuta*, the T66 and Z27 strains of *D. s. albostrigata*, the Hw and Pl strains of *D. s. bilimbata*, the P33 and O14 strains of *D. s. sulfurigaster* and the T2 strain of *D. pulaua*. Cases of the sex ratio imbalance are given in Table 7 and parts of Table 1 and 2. In intersubspecific crosses, F<sub>1</sub> hybrids between *D. s. neonasuta* and *D. s. albostrigata* and between *D. s. bilimbata* and *D. s. sulfurigaster* were fertile and normal sex ratios were observed. While crosses between males of *D. s. neonasuta* or *D. s. albostrigata* and females of *D. s. bilimbata* and *D. s. sulfurigaster* produced F<sub>1</sub> hybrids which showed normal fertility and sex ratio, some of their reciprocal crosses resulted in abnormal F<sub>1</sub> hybrids, that is, a sex ratio distortion and F<sub>1</sub> male sterility. The extent of the distortion in the sex ratio was clearly higher in crosses with *D. s. bilimbata* males than in crosses with *D. s. sulfurigaster* males to *D. s. albostrigata* females. In crosses of *D. s. neonasuta* females to *D. s. bilimbata* males, the extent of the distortion was relatively high, too. All intersubspecific crosses except for a cross of *D. s. neonasuta* females to *D. s. sulfurigaster* males were easily made and produced a number of progeny. The cross between these two subspecies was slightly fertile in

Table 6. Results of crosses between subspecies of *D. sulfurigaster* and *D. pulaua*

♀ \ ♂	<i>s. n.</i>	<i>s. a.</i>	<i>s. b.</i>	<i>s. s.</i>	<i>pulaua</i>
<i>s. neonasuta</i>		F	f*	F	F
<i>s. albostrigata</i>	F		f*	F*	F*
<i>s. bilimbata</i>	F	F		F	F
<i>s. sulfurigaster</i>	F	F	F		F
<i>pulaua</i>	F	F	F	F	

F: F<sub>1</sub> ♀♂ fertile.f: F<sub>1</sub> ♀ fertile, F<sub>1</sub> ♂ sterile.\*: sex ratio distortion in F<sub>1</sub> hybrids.Table 7. Sex ratio distortion in F<sub>1</sub> hybrids from crosses between subspecies of *D. sulfurigaster* and *D. pulaua*

	No. of F <sub>1</sub> ♀♀	No. of F <sub>1</sub> ♂♂	% ♂♂ of F <sub>1</sub> progeny
<i>s. neonasuta</i> ♀♀ × <i>s. bilimbata</i> ♂♂			
MYS × Hw	257	987	79.3***
MYS × PI	586	756	56.3***
<i>s. albostrigata</i> ♀♀ × <i>s. pulaua</i> ♂♂			
T66 × T2	11	88	88.9***
Z27 × T2	27	104	79.4***

the parental generation, but no sex ratio distortion was detected.

As to interspecific crosses, fertile F<sub>1</sub> hybrids were obtained when *D. pulaua* males were crossed to females of all four subspecies of *D. sulfurigaster*. Despite of the low successful mating, it was relatively easy to obtain F<sub>1</sub> hybrids, while in the sympatric case, *D. s. albostrigata* and *D. pulaua*, the cross showed extremely low fertility in the parental generation and the sex ratio distortion was found in F<sub>1</sub> hybrids. It showed the highest extent of the distortion of all crosses. On the other hand, in crosses with *D. pulaua* females, it was difficult to obtain F<sub>1</sub> hybrids because of the low successful matings and the high frequency of the insemination reaction causing the reaction mass described below. The F<sub>1</sub> progeny obtained showed normal fertility and normal sex ratio.

Table 8. Percentage of the formation of the reaction mass in the insemination reaction in crosses between subspecies of *D. sulfurigaster* and *D. pulaua*

♀	♂	s. n.		s. a.		s. b.		s. s.		<i>pulaua</i>
		MYS	T66	Z27	Hw	Pl	P33	O14		
<i>s. neonasuta</i>	MYS	7.9	8.6	7.1	0.0	0.0	18.5	3.1	9.5	
<i>s. albostrigata</i>	T66	3.7	4.2	12.5	7.7	4.8	3.6	4.8	0.0	
	Z27	3.3	7.4	12.5	3.6	0.0	17.9	3.6	5.0	
<i>s. bilimbata</i>	Hw	27.6	65.4	75.0	3.2	12.0	11.1	23.8	0.0	
	Pl	10.0	53.3	68.2	3.6	7.9	20.8	8.7	0.0	
<i>s. sulfurigaster</i>	P33	8.0	14.3	19.2	0.0	0.0	3.0	6.1	4.0	
	O14	8.0	12.5	14.8	7.1	0.0	3.1	2.3	0.0	
<i>pulaua</i>	T2	56.5	66.7	47.4	60.0	19.2	33.3	85.7	4.2	

Table 8 gives the occurrence rate with which the insemination reaction causing the reaction mass in crosses between eight strains. The frequencies in crosses of intrasubspecies and of intrasubspecies ranged from 0.0 to 12.5%, which indicated that the reaction masses were observed even in homogamic matings at the 5th day after matings to a certain extent. Heterogamic matings gave higher frequencies, which ranged from 0.0 to 75.0%. *D. s. bilimbata* was most liable to have the insemination reaction with the reaction mass. While males of this subspecies induced the reaction mass in low frequency, females reacted to a much greater extent when crossed to other subspecies, and the highest frequency, 63.8% on the average, was obtained in crosses to *D. s. albostrigata* males. They also caused a significant reaction with females of *D. s. sulfurigaster*.

In interspecific crosses, using *D. pulaua* as the male parent, the reaction mass was in similar frequencies as in homogamic matings. In contrast to this, in crosses with *D. pulaua* females, higher frequencies were observed in all heterogamic matings than in intersubspecific crosses, although a large variation among strains was found. For all interspecific crosses, young females, one or two days old, were used because of low frequencies of successful matings.

#### (II-2) Premating barrier

Using one strain of each species or subspecies, the percentages of successful matings between them were given in Table 9. The values between *D. pulaua* and the four subspecies of *D. sulfurigaster* were considered low. Amongst these, the highest frequency was found for *D. s. sulfurigaster* (18.5

Table 9. *Percentage of successful matings between subspecies of D. sulfurigaster and D. pulaua*

Female	Male	No. of ♀♀ dissected	No. of ♀♀ inseminated	Percent of suc- cessful matings
<i>s. n.</i>	<i>s. n.</i>	127	109	85.8
<i>s. a.</i>	<i>s. a.</i>	91	78	85.7
<i>s. b.</i>	<i>s. b.</i>	124	111	89.5
<i>s. s.</i>	<i>s. s.</i>	119	113	95.0
<i>pulaua</i>	<i>pulaua</i>	74	58	78.4
<i>s. n.</i>	<i>s. a.</i>	57	48	84.2
<i>s. a.</i>	<i>s. n.</i>	77	43	55.8
<i>s. n.</i>	<i>s. b.</i>	53	36	67.9
<i>s. b.</i>	<i>s. n.</i>	101	3	3.0
<i>s. n.</i>	<i>s. s.</i>	61	60	98.4
<i>s. s.</i>	<i>s. n.</i>	82	6	7.3
<i>s. n.</i>	<i>pulaua</i>	101	3	3.0
<i>pulaua</i>	<i>s. n.</i>	76	9	11.8
<i>s. a.</i>	<i>s. b.</i>	77	32	41.6
<i>s. b.</i>	<i>s. a.</i>	79	46	58.2
<i>s. a.</i>	<i>s. s.</i>	59	42	71.2
<i>s. s.</i>	<i>s. a.</i>	62	53	85.5
<i>s. a.</i>	<i>pulaua</i>	68	0	0.0
<i>pulaua</i>	<i>s. a.</i>	79	1	1.3
<i>s. b.</i>	<i>s. s.</i>	57	51	89.5
<i>s. s.</i>	<i>s. b.</i>	61	47	77.0
<i>s. b.</i>	<i>pulaua</i>	78	7	9.0
<i>pulaua</i>	<i>s. b.</i>	72	3	4.2
<i>s. s.</i>	<i>pulaua</i>	103	19	18.5
<i>pulaua</i>	<i>s. s.</i>	75	14	18.7

*s. n.*: *s. neonasuta*, *s. a.*: *s. albostrigata*,  
*s. b.*: *s. bilimbata*, *s. s.*: *s. sulfurigaster*.

and 18.7% in each of reciprocal crosses) and the lowest for *D. s. albostrigata* (0.0 and 1.3%), which is sympatric with *D. pulaua*.

Between subspecies, the degree of the isolation ranged from the local population level to the species level. The strongest isolation was obtained in crosses between *D. s. neonasuta* males and *D. s. sulfurigaster* females, which indicated these are at the species level for premating isolation. *D. s. neonasuta* males discriminated against *D. s. albostrigata* females to some extent, as given in Table 5. The difference in reciprocal crosses was greater in crosses for *D. s. neonasuta*, and those differences were not found in other crosses between subspecies. While *D. s. albostrigata* and *D. s. bilimbata* showed isolation to the extent that the percentage of successful matings was about 50%



in both of reciprocal crosses, significant sexual isolation was not detected between *D. s. albostrigata* and *D. s. sulfurigaster*, nor between *D. s. bilimbata* and *D. s. sulfurigaster*.

#### 4. DISCUSSION

##### I. *Differentiation at the local population level*

In this study, the differentiation among local populations was detected as the differences of the extent of isolation to the tester subspecies. While the sex ratio distortion in  $F_1$  hybrids between subspecies of *D. sulfurigaster* has been known (Wilson *et al.* 1969), nothing has been discussed about the genetic basis of the distortion. In the case of *D. sulfurigaster*, the distortion is due to the preponderance of males, the heterozygous sex, in  $F_1$  hybrids. It is opposed to Haldane's rule (1922) that the sex with heterozygous sex chromosome is absent, rare or sterile in the  $F_1$  offspring of two different animal races. On the other hand, the sterility in male hybrids agrees with the rule. Therefore the different genetic basis may be suggested about the sterility and the distortion. The incompatibility between X chromosomes from both parent or between X chromosome from the male and the female cytoplasm might cause the imbalance, and if postmating isolation is developed as a by-product, in parallel with the genetic differentiation between local populations, the sex ratio might be affected by the genetic differentiation between local populations and tester subspecies.

The results showed that *D. s. albostrigata* females crossed to *D. s. sulfurigaster* males yielded fertile  $F_1$  hybrids and that those crossed to *D. s. bilimbata* males yielded fertile  $F_1$  females and sterile  $F_1$  males. For both tester subspecies, as shown in Tables 1 and 2, the sex ratio distortion was found in all of 12 populations of *D. s. albostrigata*. These results are consistent with those by Wilson *et al.* (1969). Only in crosses to *D. s. bilimbata*, populations of *D. s. albostrigata* differed significantly with the extent of the distortion from one another. Twelve populations can be subdivided into five groups: the Philippines (Q and R), Borneo (S, T, U and V), the Malay Peninsula (W, X and Y), Thailand (Z and CM) and Sri Lanka (C). The behavior of the U population was characteristic: that is, when crossed to *D. s. sulfurigaster*, the extent of the distortion that the U showed with the O14 strain was lower than that of other populations, although the U was similar to others in crosses to the P33 strain. When crossed to the P1 strain of *D. s. bilimbata*, the U was one of three populations which showed the significant distortion of excess of males.

The insemination reaction as a prezygotic isolating mechanism, is a very interesting phenomenon even though its nature remains to be discussed. The reaction might be determined by two factors: the protein or protein-like

material and the hypersensitiveness of the membrane of the uterus to it. The variation in the occurrence rate of the reaction mass is statistically significant in crosses to *D. s. bilimbata*, and the difference among subdivisions of *D. s. albostrigata*, which are roughly in accordance with those of postzygotic isolation described above, is clearer than that in postzygotic isolation. In Borneo, the values were decreased continuously from the Sandakan (S) to the V and nearly constant in the Malay Peninsula.

These facts showed the differentiation among local populations of *D. s. albostrigata* in the extent of postmating isolation to *D. s. bilimbata*. The Q, R and S populations were different in prezygotic isolation though their distribution are geographically very close. Populations in Borneo and Singapore showed similar extent for both post- and prezygotic isolation, and the W population differed particularly from other populations in the Malay Peninsula in the extent of prezygotic isolation. In populations from Thailand, a high level of insemination reaction causing the reaction mass was found. The C population was similar to the Y as to postmating isolation.

On the other hand, interpopulational hybridization between six populations was made to assess the fitness of hybrids in the  $F_1$ ,  $F_2$  and  $F_3$  generations. In productivity, hybrids with low fitness were obtained in crosses using the U, Z and C populations, but the hybrid breakdown observed was not so clear as in egg-to-adult viability in crosses concerning the Q, Z and C populations. Productivity is a character composed of fecundity and viability in the next generation, and that possibly made the results obscure in productivity. Inoue and Kitagawa (1974, 1975) found that the hybrid breakdown occurred not in fecundity but in larva-to-adult viability in the  $F_2$  generation in interpopulational hybrids of *D. albomicans*. In *D. s. albostrigata*, marginal populations, the Q and C, were indicated to be differentiated from other populations. The hybrid breakdown also occurred using the U and Z populations, which are not inconsistent with those suggested from postmating isolation to *D. s. bilimbata*.

In contrast with postmating barriers to *D. s. sulfurigaster* and *D. s. bilimbata*, the premating barrier of *D. s. albostrigata* to *D. s. neonasuta* was not very strong, although it was asymmetric. The populations that showed relatively strong isolation to *D. s. neonasuta* seemed not to be related to their localities. This was contrary to the expectation that the C population, located adjacent to *D. s. neonasuta*, would show strong premating isolation to it. *D. s. neonasuta* may be regarded as a local population of *D. s. albostrigata*, which will be discussed later.

On the basis of allelic frequencies at 26 loci, genetic distances by Nei (1972) were calculated by Ayala *et al.* (1981). Distances between the C and other populations ranged from 0.0426 to 0.0623, and between the Z and other from 0.0344 to 0.0623. Among four populations in Borneo, genetic distances are

small,  $D=0.0109\sim 0.0211$ . The relationship between populations in Borneo suggested by genetic distances, however, did not necessarily agree with that of their geographical distances.

Matsuzaki and Kitagawa (1981, 1982) investigated the inversion polymorphism in local populations of *D. s. albostrigata* and in 14 species of the *D. nasuta* subgroup including three undescribed taxa, and discussed the phylogenetic relationship of them. They showed that *D. s. albostrigata* was subdivided into five groups on the basis of inversion polymorphism, which agree quite with those from postmating isolation clarified in this study. That is, for postmating isolation, the differentiation determined from the extent of reproductive isolation to other subspecies is parallel to the genic differentiation based on isozyme variation and to the chromosomal divergence in inversion polymorphisms. It is considered that the postmating barrier as a primary isolating mechanism arises not from the appearance of genes controlling isolating mechanisms at specific loci, but as a by-product of genetic differentiation of gene blocks or the genome, which has been postulated by Dobzhansky (1970). On the other hand, the differentiation among populations obtained from the premating barrier to *D. s. neonasuta*, was not necessarily comparable to the differentiation indicated above, which suggests that premating barriers have different genetic basis from postmating barriers.

## II. Differentiation at the subspecies and species level.

The genetic differentiation among subspecies was investigated by the experimental hybridization test, which showed that *D. sulfurigaster* was subdivided into two groups consisting of two subspecies each. Reciprocal crosses between *D. s. neonasuta* and *D. s. albostrigata* and between *D. s. bilimbata* and *D. s. sulfurigaster* yielded fertile  $F_1$  hybrids, which indicates two subspecies are very closely related in each group. Males of *D. s. bilimbata* caused  $F_1$  male sterility and the sex ratio distortion in crosses to females of *D. s. neonasuta* and *D. s. albostrigata*, and males of *D. s. sulfurigaster* caused the sex ratio distortion in crosses to *D. s. albostrigata* females. Judging from the extent of this postzygotic isolation, *D. s. sulfurigaster* is considered to be genetically closer to the other group than *D. s. bilimbata*. On the other hand, as to prezygotic isolation estimated by the occurrence rate of the formation of the reaction mass, some distinctive features were discovered. High frequencies of the insemination reaction causing the reaction mass were observed in crosses between the two groups of subspecies described above. It is noted that the reaction mass arose only in crosses in which postzygotic isolation was not detected. Especially, females of *D. s. bilimbata* gave rise to high frequency of the reaction mass in crosses to males of other subspecies, and males of this subspecies caused  $F_1$  male sterility and the sex ratio distortion when crossed to other subspecies. It is postulated that *D. s. bilimbata* has

developed substantial postmating isolation to the other subspecies.

*D. s. neonasuta* was not isolated from *D. s. albostrigata* by postmating barriers such as  $F_1$  sterility, the sex ratio distortion and the insemination reaction. The distribution of these two subspecies are very close to each other, and it can be assumed that *D. s. neonasuta* originated from one local population of *D. s. albostrigata*, which are highly diverged. At the genic level, *D. s. neonasuta*, the MYS strain, showed much differentiation from other *D. s. albostrigata* populations, and it may apparently be classified as a subspecies. In postmating barriers to others, however, the extent of the sex ratio distortion in  $F_1$  hybrids or the frequency of the reaction mass formation was not so different from those of *D. s. albostrigata*, and even seemed to be similar to the C population. The isolating mechanism developed in *D. s. neonasuta*, or the MYS strain, was revealed in premating isolation as shown in Table 9. *D. s. neonasuta* males were extremely difficult to mate to females of *D. s. bilimbata* and *D. s. sulfurigaster*. They also discriminated against *D. s. albostrigata* females to some extent. Therefore, *D. s. neonasuta* may be differentiated from other members of *D. sulfurigaster* by some genetic changes concerning premating barriers, and the mode of subspecies formation may be different from those prevailing in the other three subspecies.

Reproductive isolation between *D. pulaua* and *D. sulfurigaster* has attained clearly a higher level than the subspecies level. Successful matings were few in reciprocal crosses, and the extent corresponded to the level of postmating isolation. Isolation to *D. s. albostrigata*, sympatric with *D. pulaua*, was the strongest, in accordance with Dobzhansky *et al.* (1969) who have pointed out that stronger isolation exists between sympatric species than between allopatric species. In interspecific crosses, females of all four subspecies of *D. sulfurigaster* produced fertile  $F_1$  hybrids. In contrast to this, the insemination reaction in their reciprocal crosses effectively prevented the formation of zygotes, although this was not complete. Because the isolating mechanism acting before the formation of zygotes, premating and prezygotic isolation, is very effective and natural selection probably does not act to reinforce postzygotic isolation between them.

These facts agree well with the relationship estimated by genetic distances on the basis of allelic frequencies at 26 loci by Ayala *et al.* (1981). Moreover, quantitative characters were analysed by principal component analysis in the *D. nasuta* subgroup (Kitagawa *et al.* 1982). As with *D. pulaua* and subspecies of *D. sulfurigaster*, *D. pulaua* and *D. s. albostrigata* were shown to be very similar, and *D. s. bilimbata* differed a little from other subspecies morphologically. Some characteristics that indicate genetic divergence between subspecies or species mostly suggest the close relationship between *D. pulaua* and *D. sulfurigaster* and the notable differentiation of *D. s. bilimbata* among subspecies of *D. sulfurigaster*.

### III. Genetic and reproductive differentiation in *D. sulfurigaster*.

The results found in this study indicate that the four subspecies in *D. sulfurigaster* have differentiated from one another and that isolating mechanisms to prevent the gene exchange have developed to some extent between them. It is significant for the understanding of the speciation processes to clarify how the four subspecies have genetically diverged from one another. One of the ways to assess ancestral and derived species is using banding patterns of salivary gland chromosomes. After Wilson *et al.* (1969), Matsuzaki and Kitagawa (1981, 1982) found that *D. s. albostrigata* is highly polymorphic for chromosomal inversions and that *D. s. neonasuta* and *D. s. sulfurigaster* are less polymorphic. Total numbers of inversions found in each subspecies are 17, 4 and 10 for *D. s. albostrigata*, *D. s. neonasuta* and *D. s. sulfurigaster*, respectively. *D. s. bilimbata* is monomorphic. The fact indicates that the level of inversion polymorphism is high in the center of the population distribution of the species and that marginal populations are chromosomally homozygous. Among local populations of *D. s. albostrigata*, each chromosome arm is highly polymorphic in Borneo and the Malay Peninsula, and that region is considered the origin of the distribution of *D. s. albostrigata*. In this region, five species belonging to the *D. nasuta* subgroup are found sympatrically. Thus, many species of this subgroup may originate here.

Recently the direction of evolution has been discussed relating to the interpretation of asymmetric isolation. Kaneshiro (1976, 1980) insisted that males of derived species are discriminated by females of ancestral species. Watanabe and Kawanishi (1980), contrary to this, said that females of derived species discriminate males of ancestral one. Further, Wasserman and Koepfer (1980) explained the evolution in the *mulleri* group by character displacement for sexual isolation. In *D. sulfurigaster*, the direction of evolution is *D. s. albostrigata*→*D. s. sulfurigaster*→*D. s. bilimbata*→*D. s. neonasuta* by Kaneshiro's hypothesis, and the opposite direction by Watanabe and Kawanishi's. Considering the inversion polymorphism, it is appropriate to postulate *D. s. albostrigata* as the ancestral subspecies, which agree with Kaneshiro's hypothesis. In addition, taking into consideration the extent of pre- and postmating isolation between subspecies in *D. sulfurigaster*, it might be postulated that one direction of subspeciation proceed from *D. s. albostrigata* to *D. s. sulfurigaster* then to *D. s. bilimbata*, and another from *D. s. albostrigata* to *D. s. neonasuta*.

Divergence from *D. s. albostrigata* to *D. s. sulfurigaster* and *D. s. bilimbata* may occur by postmating isolation as a secondary event of genetic divergence in the same way as in other allopatric subspecies. The populations of *D. s. albostrigata* in the Philippines, Palawan, Thailand and Sri Lanka, showed high differentiation, such that hybrids between them show low fitness in

some cases. Differentiation in postmating isolation to other subspecies was well consistent with those from isozyme variation and the inversion polymorphism. Thus, it is clear that genetic differentiation arises among local populations of *D. s. albostrigata*. It is conceivable that *D. s. sulfurigaster* was originally a local population of *D. s. albostrigata*. Populations going south-eastward might greatly diverge from the ancestral population before showing any kind of reproductive difference, while populations going northward did not differentiate enough to develop reproductive isolation. Perhaps local populations of *D. s. bilimbata*, distributed on many islands in the wide range, may have accumulated considerable genetic differences. Divergence from *D. s. albostrigata* to *D. s. neonasuta*, however, is different from the other direction of subspeciation. It is suggested that this differentiation depends not on postmating isolation but on premating reproductive isolation. Whether *D. s. neonasuta* is a local population of *D. s. albostrigata* or not, the differentiation between *D. s. albostrigata* and *D. s. neonasuta* may not be preparatory to postmating isolation. Nevertheless, more experimental evidence is necessary to determine the evolutionary status of *D. s. neonasuta*.

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