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CYTOGENETIC RELATIONSHIPS OF THREE SIBLING SPECIES OF THE *DROSOPHILA KIKKAWAI* COMPLEX

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Hybridization tests among the available strains of three sibling species of the *D. kikkawai* complex were performed. All cases of intraspecific crosses were successful. All interspecific crosses involving *D. bocki* were completely unsuccessful; however, interspecific crosses between *D. kikkawai* and *D. leontia* in mass mating involving as many as 20-30 pairs in most cases were successful, producing variable numbers of F_1 offspring. Fertility tests of F_1 progeny revealed that the F_1 males were completely sterile while the F_1 females were fertile when backcrossed to the males of parental types, yielding considerable numbers of offspring. The data suggest that *D. bocki* is the most genetically isolated, although all are morphologically indistinguishable. *D. bocki* and *D. leontia* are found existing sympatrically. Although *D. kikkawai* and *D. leontia* are occurring sympatrically, the former species has so far not been found to coexist with *D. bocki*. *D. kikkawai* and *D. leontia* differ in gene sequences in chromosome 2L, 2R and 3L and most extensively in the X chromosome. Nevertheless, chromosomes 3R and 4 do not show any differences in gene order among the species. *D. kikkawai* is polymorphic for a sequence (3LB) which appears to be fixed in *D. leontia*, indicating that they have diverged recently from a common ancestor.

INTRODUCTION

The *Drosophila kikkawai* complex, a cluster of closely related species belonging to the *montium* subgroup of the *melanogaster* group, consists of at least three known sibling species thus far, viz. *D. kikkawai*, *D. leontia* and *D. bocki* (Burla 1954; Tsacas and David 1977; Baimai 1979). *D. kikkawai* is a subcosmopolitan species and is widespread in the Asian and Pacific areas. It is also very common in South America, while the latter two sibling species seem to be confined to the Asian areas. Cytologically, *D. kikkawai* is unusual in that it shows variation in metaphase chromosomes largely due to the different amount of heterochromatin, particularly in the 4th chromosome (Baimai 1978; Baimai and Chumchong 1980). However, *D. leontia* and *D. bocki* show

uniformly similar metaphase karyotypes; each is quite distinct from that of *D. kikkawai*. Interestingly enough, it is very difficult if not impossible to separate these sibling species morphologically. A systematic investigation of their cytogenetics has been carried out in this laboratory to define the sibling species involved in this complex group. It is hoped that the results of this study will shed some light on the mode of speciation of these Oriental *Drosophila* species.

This paper presents the results of hybridization tests among the available strains of the three species from different geographic origins. Detailed analysis of polytene chromosome differences in the F_1 hybrids is described. Phylogenetic relationships are discussed.

MATERIALS AND METHODS

All culture stocks of *D. kikkawai*, *D. leontia* and *D. bocki* were established from individual wild-caught females collected at different geographic localities by many field workers (Table 1). Most of the *D. kikkawai* culture stocks which have been maintained in laboratory for several years were made available for this study by The Genetics Foundation, University of Texas. All culture strains of *D. leontia* were obtained from different localities in Thailand (Fig. 1). Only three culture stocks of *D. bocki* were available for this study (Table 1). All culture stocks of these species have been maintained in our laboratory at 25±1°C.

RESULTS

Hybridization experiments

The first series of hybridization experiments involved various combinations of intra- and interspecific crosses among the available strains of the three sibling species (Fig. 2). In these preliminary crosses about 3-5 pairs of virgin flies of both reciprocal crosses with 3-5 replicates were tested and kept for 3 weeks. All fertile crosses producing a large number of F_1 offspring within 10-15 days were considered to indicate that the strains involved belonged to the same species. Intraspecific crosses occurred easily and produced large numbers of progeny. Crosses failing to produce F_1 progeny within the 3 week period were taken to indicate that the strains were different biological species. Females involved in the unsuccessful crosses were dissected to check for the presence or absence of sperm in seminal receptacles and spermathecae.

The second series of hybridization experiments involved interspecific crosses between the standard strain of *D. kikkawai* from Samut Songkhram (SS) and the three strains of *D. leontia* from different geographic areas i.e. *D. leontia*-1, -2 and -3 from Chiangmai (CM), Songkhla (SK6) and Phuket (PK) respectively (Fig. 1). These crosses involved mass matings of 5, 10, 20 or 30 pairs of flies per bottle. A successful cross yielding F_1 progeny was scored for females and males. All cases of $F_1 \times F_1$ self-crossing failed to give hybrid progeny. F_1 male hybrids proved to be sterile as microscopic examination of their testes revealed no motile sperm.

Table 1. Stocks of the three sibling species of the *D. kikkawai* complex that have been maintained in the laboratory and used in this study

Species stock	Locality of origin	Collector (date)
D. bocki		
1. KY	Khao Yai National Park, Nakhon Nayok, 200 km North-East of Bangkok.	Baimai (1971)
2. TW7	Yun-Shui, Chia-I, Taiwan	Lin and Wang (1974)
3. LT7	Lamtakong, Nakhon Ratchasima, 200 km North-East of Bangkok.	Baimai (1977)
D. leontia		
4. CM	Chiangmai, 800 km North of Bangkok.	Baimai (1971)
5. SR	Surat Thani, 750 km South of Bangkok.	Srikiow (1971)
6. SK6	Songkhla, 1000 km South of Bangkok.	Baimai (1976)
7. PK	Phuket, 900 km South of Bangkok.	Baimai (1976)
8. KN	Kanchanaburi, 120 km North-West of Bangkok.	Baimai (1976)
9. WK	Wangtakrai, Nakhon Nayok, 120 km North-East of Bangkok.	Baimai (1977)
10. LT6	Lamtakong, Nakhon Ratchasima, 200 km North-East of Bakgkok	Baimai (1977)
D. kikkawai		
11. SS	Samut Songkhram, 60 km South-West of Bangkok.	Baimai (1971)
12. SK2	Songkhla, 1000 km South of Bangkok.	Baimai (1976)
13. PH	Luzon, Philippines	Throckmorton (1968)
14. PA	Palau, Auluptagel Is., Micronesia	Carson (1968)
15. PO	Ponape, Kolonia, Micronesia	Wasserman (1959)
16. KO	Chungju, Seoul, Korea	Kitagawa (1976)
17. CO	Leticia, Colombia	Carson (1960)
18. HW	Oahu, Hawaii, U.S.A.	Baimai (1975)
19. BS	Belavista, Mato Crosso, Brasil	Sene (1977)
20. TW	Ken-Ting, Pintung, Taiwan	Lin (1977)
21. NA	Naze, Amami-oshima, Is., Japan	Kitagawa (1976)
22. GO	Goroko, New Guinea	Carson (1961)
23. TV	Townsville, Australia	Bock (1976)
24. WU	Wau, New Guinea	Carson (1977)

Interpretation of hybridization experiments

In the first series of hybridization tests, it is clear that the 24 culture stocks comprise three distinct reproductively isolated groups (Fig. 2). These results agree with the evidence from metaphase figures, particularly those of *D. kikkawai*, which obviously differ from those of its two sibling species (Baimai and Chumchong 1980).

In the second series of crosses, an extensive study of interspecific matings yielded very interesting results as shown in Table 2. All interspecific crosses involving *D. bocki* (nos. 1-4) completely failed to produce hybrid offspring even though a large number of parents (up to 30 pairs) were employed in the mass matings. Thus *D. bocki* is completely reproductively isolated from *D. leontia* and *D. kikkawai*. Micro-

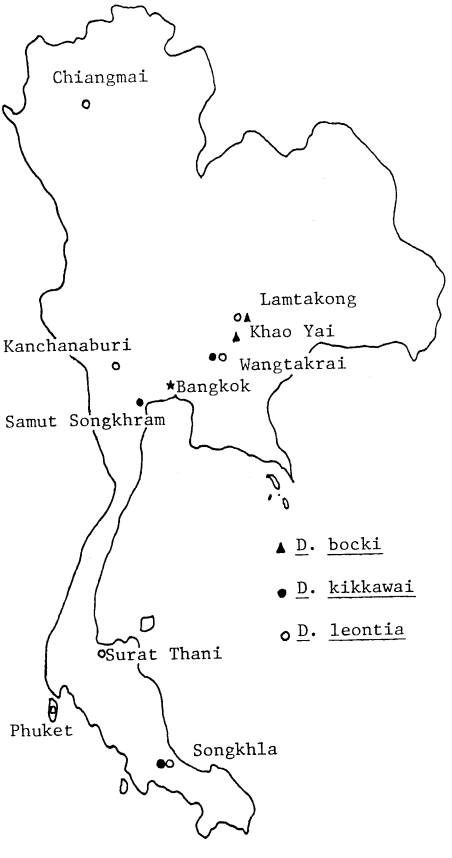

Fig. 1. Map of nine locations in Thailand where the strains of the three sibling species used in hybridization experiments were collected.

scopic examination of seminal receptacles of the parental females involved in these interspecific crosses showed no motile sperm. Thus, *D. bocki* has evidently been reproductively isolated to a greater extent than the other two sibling species. In particular, behavioural isolation is seemingly a strong premating mechanism. In fact, *D. bocki* exhibits obviously different courtship behaviour from the other two sibling species (unpublished observations). *D. bocki* has not been found in sympatry in nature with *D. kikkawai*. Should *D. bocki* exist in sympatry with *D. kikkawai*, we would not expect to find natural hybridization. In fact, there has been no case of natural hybridization in the small samples of *D. bocki* and *D. leontia* from Lamtakong where the two sibling species coexist.

On the other hand, some interspecific crosses between the standard stock of *D. kikkawai* and the three geographic strains of *D. leontia* were fairly successful in producing F_1 hybrid offspring, although none produced as many progeny as their respective control crosses. Two points may be noted from Table 2. First, those crosses

Female \ Male		*D. bocki*			*D. leontia*							*D. kikkawai*													
		KY	TW7	LT7	CM	SR	SK6	PK	KN	WK	LT6	SS	SK2	PH	PA	PO	KO	CO	HW	BS	TW1	NA	GK	TV	WU
D. bocki	KY		+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	TW7	+		+	−	−				−	−										−				
	LT7	+	+		−	−					−										−				
D. leontia	CM	−	−	−		+	+	+	+	+	+	+*	+*								+*				
	SR	−	−	−	+		+	+	+	+	+	+*									+*				
	SK6	−	−		+	+		+	+	+	+	+*	+*								+*				+*
	PK	−	−		+	+	+		+	+	+	+*	+*												
	KN	−	−		+	+	+	+		+	+														
	WK	−	−		+	+	+	+	+		+														
	LT6	−	−	−	+	+	+	+	+	+											+*				
D. kikkawai	SS	−			+*	+*	+*	+*					+	+	+	+	+	+	+		+	+	+	+	
	SK2	−			+*		+*	+*				+		+							+		+		
	PH	−										+	+		+	+	+	+	+		+			+	
	PA	−										+		+		+	+	+	+		+				
	PO	−										+		+	+		+	+	+		+			+	
	KO	−										+		+	+	+		+	+	+	+	+		+	
	CO	−										+		+	+	+	+		+		+	+		+	
	HW	−										+		+	+	+	+	+			+	+		+	
	BS	−															+							+	
	TW1	−	−	−	+*	+*	+*				+*	+	+	+	+	+	+	+	+			+		+	+
	NA	−										+					+	+	+		+			+	
	GK	−										+	+											+	+
	TV	−										+		+		+	+	+	+	+	+	+	+		
	WU	−					+*														+		+		

Fig. 2. Summary of results from the hybridization experiments among the strains within and between species of the *D. kikkawai* complex. Abbreviations of the strains are the same as in Table 1. +=successful crosses; −=unsuccessful crosses; *=mass mating involving 10-30 pairs of parents per bottle.

involving *D. kikkawai* females × *D. leontia* males proceeded relatively more easily than the respective reciprocal crosses in all cases. For example, in the cross (no. 5) between *D. kikkawai* females × *D. leontia*-1 males of the Chiangmai stock, mass mating produced an average of 6.52 F_1 hybrid offspring per female, whereas the reciprocal cross (no. 6) gave an average of only 1.08 F_1 hybrids per female. The difference is statistically significant ($P<0.001$). Similar results were obtained when *D. leontia*-2 and *D. leontia*-3 were involved in hybridization tests with the standard *D. kikkawai* stock i.e. cross nos. 7-8 and 9-10. Student's t-tests on the average numbers of F_1 offspring per female show highly significant differences ($P<0.001$) between all interspecific crosses

Table 2. Combinations of interspecific crosses among the three sibling species of the *D. kikkawai* complex

Female	Male	No. of replicates	Total no. of pairs in mass mating	No. of F_1 offspring		Average no. of F_1 per female
				Female	Male	
Interspecific cross						
1. *kikkawai*	× *bocki*	5	150	—	—	—
2. *bocki*	× *kikkawai*	5	150	—	—	—
3. *leontia*-1	× *bocki*	5	150	—	—	—
4. *bocki*	× *leontia*-1	5	150	—	—	—
5. *kikkawai*	× *leontia*-1	10	150	532	446	6.52
6. *leontia*-1	× *kikkawai*	10	250	164	106	1.08*
7. *kikkawai*	× *leontia*-2	8	110	811	703	13.76
8. *leontia*-2	× *kikkawai*	6	160	89	67	0.98*
9. *kikkawai*	× *leontia*-3	8	60	1032	919	32.52
10. *leontia*-3	× *kikkawai*	8	190	109	87	1.03*
Control cross						
11. *bocki*	× *bocki*	5	5	327	242	113.80
12. *kikkawai*	× *kikkawai*	5	5	430	400	166.00
13. *leontia*-1	× *leontia*-1	5	5	590	400	198.00
14. *leontia*-2	× *leontia*-2	5	5	523	511	206.80
15. *leontia*-3	× *leontia*-3	5	5	485	479	192.80

The three strains of *D. leontia* used in hybridization experiments were from Chiangmai (1), Songkhla (2) and Phuket (3). *D. kikkawai* and *D. bocki* used were from Samut Songkhram (standard stock) and Khao Yai National Park, respectively.

* Student t-test among these crosses show no significant differences, $P>0.05$.

except that crosses 6, 8 and 10 do not differ significantly from one another (Table 2).

Secondly, the degree of incompatibility between *D. kikkawai* females and *D. leontia* males depended on the stock of *D. leontia* males used. The productivity of crosses with *D. leontia*-3 (Phuket) males was highest, with 32.52 F_1 hybrids per female (with only 60 pairs of parents) and lowest with *D. leontia*-1 (Chiangmai) males yielding an average of only 6.52 F_1 hybrids per female. The crosses involving *D. leontia*-2 (Songkhla) males were intermediate, with 13.76 F_1 hybrids per female; the differences among the three strains of males were highly significant ($P<0.001$). Interestingly, reciprocal crosses (*D. leontia* females × *D. kikkawai* males) produced few offspring regardless of the stock of *D. leontia* females used (Table 2). The results suggest that the northern stock of *D. leontia* has developed a greater degree of premating isolation from *D. kikkawai* than the southern ones, and that selection has affected the two sexes to different degrees. The three rather widely separated populations of. *D. leontia* might have undergone independent genetic differentiation to some extent.

Self-crosses between the F_1 hybrid females and males from each interspecific cross in each case failed. Microscopic examination of testes of F_1 males revealed no motile sperm. The testes examined showed only debris of cells or bundles of non-motile sperm; hence, the F_1 males were completely sterile in all cases.

Backcrosses

F_1 female hybrids were backcrossed to their respective parental males. Each backcross involved 10 pairs in mass mating and was kept for three weeks. Hybrids were then scored for females and males.

The less compatible *D. kikkawai* × *D. leontia* combinations also result in less fertile female progeny in backcrosses, as might be expected. The first set of backcrosses involving F_1 female hybrids (derived from a *D. kikkawai* female × *D. leontia* male cross, i.e. nos. 5, 7 and 9) and males of either of the parental types gave large numbers of progeny, ranging from about 48 to 129 offspring per female (in 2500–4000 hybrid flies scored per cross). Moreover, the average number of offspring per female in backcrosses involving *D. kikkawai* males was signigicantly larger than in those involving *D. leontia* males in all cases.

On the other hand, the second set of backcrosses between F_1 female hybrids (*D. leontia* female × *D. kikkawai* male, i.e. nos. 6, 8 and 10) and males of either species produced significantly smaller average numbers of progeny (only about 21–32 F_1 offspring per female in 700–1500 hybrid flies scored in each backcross) when compared with the first set of backcrosses. However, it did not make a great deal of difference whether *kikkawai* or *leontia* males were used in the backcrosses in these cases.

Salivary gland chromosomes of F_1 hybrids

F_1 hybrids of interspecific crosses between *D. kikkawai* and *D. leontia* were

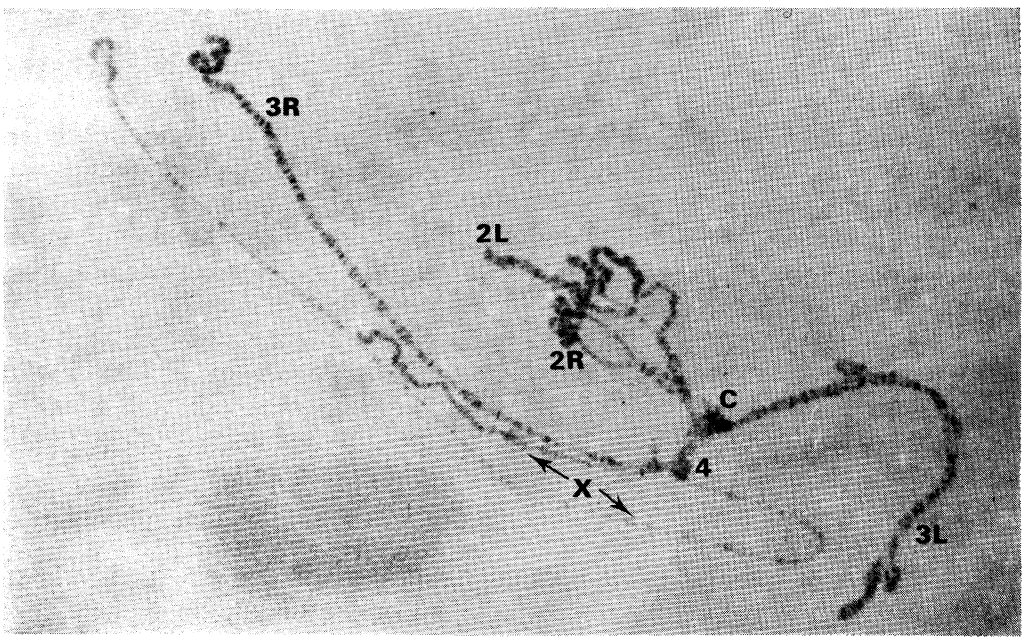

Fig. 3. The salivary gland chromosome complement of an F_1 female hybrid larva (*D. kikkawai* × *D. leontia*). The X chromosomes are completely asynapsed while the synapsis in chromosome 3R and the 4th chromosome is complete throughout the chromosome length. Differences in gene sequences in chromosomes 2L, 2R and 3L are shown. C=chromocenter.

confirmed by mitotic metaphase chromosomes. Larval salivary gland chromosomes of the F_1 hybrids were prepared to determine the synapsis and any differences in the banding patterns and gene sequences between these two species. Cytological techniques used in this study were adopted from the methods described by Baimai (1977).

Analysis of salivary gland chromosomes of the F_1 hybrid larvae of interspecific crosses between *D. kikkawai* (standard stock) and the *D. leontia* strains revealed that these two sibling species differ to some extent in gene sequences. The X chromosome differed greatly in gene sequences, as they were completely asynapsed in all preparations examined (Fig. 3).

Chromosome 2L of the F_1 hybrid showed only one simple inversion, with break points at 25C and 37G, with respect to the standard chromosome map of *D. kikkawai* (Fig. 4a). This chromosome arm showed complete synapsis throughout except for the area between 29C and 30A, which occasionally showed asynapsis. Chromosome 2R of the F_1 hybrids also exhibited one simple inversion with break points at 47 and 58C

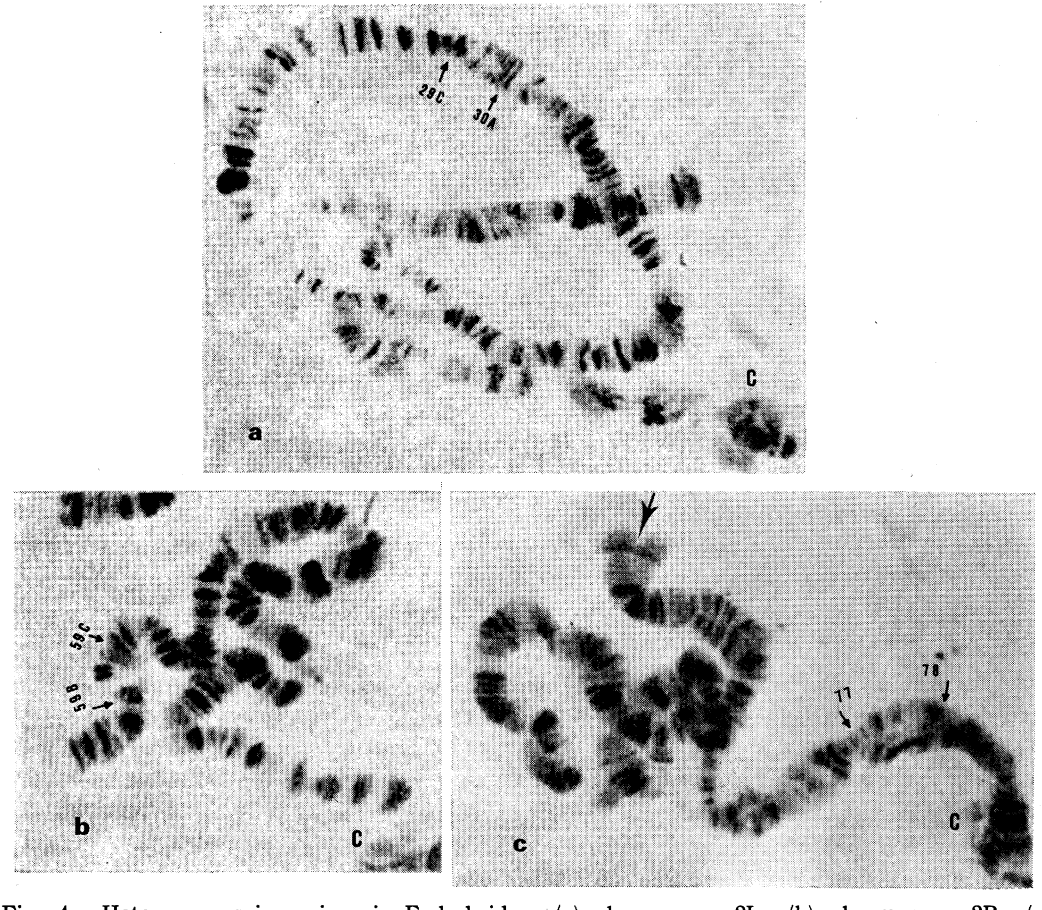

Fig. 4. Heterozygous inversions in F_1 hybrids: (a) chromosome 2L; (b) chromosome 2R; (c) chromosome 3L, with asynapsed area at the free end (arrow). The centromeric ends are indicated by C.

(Fig. 4b). Apart from this simple inversion, synapsis in polytene chromosome 2R was complete except for the region 59B-59C, where asynapsis was observed in some preparations.

Chromosome 3L of F_1 hybrids exhibited two included inversions (Fig. 4c). The longer inversion had break points at 64A and 74C. The short inversion is exactly the same as inversion 3LB found in *D. kikkawai* (Kitthawee and Baimai, 1979). These two inversions have been consistently observed in all cases of F_1 hybrid larvae. This seems to indicate that these two sibling species shared the same inversion 3LB which had become fixed in *D. leontia*. However, inversion 3LB is still polymorphic in *D. kikkawai*, which is a surprising situation in a widespread species of *Drosophila*. Furthermore, chromosome 3L consistently showed an asynapsed area at the tip, and an incomplete synapsis in the area 77-78. This is suggestive of genetic difference at the submicroscopic level between the two species. Surprisingly, chromosome 3R showed similar gene sequences in both species. Thus this polytene chromosome exhibited complete synapsis along its length (Fig. 3).

There is no evidence of any differences in gene order in the 4th chromosome (microchromosome) as observed in the F_1 hybrid larvae (Fig. 3).

DISCUSSION

Mayr (1963) pointed out that individuals of the same species could interbreed and produce normal offspring but that they would not naturally cross with members of different species. Nevertheless, interspecific crosses may occur if the isolating mechanisms are not extensive. The closely related species *D. kikkawai*, *D. leontia* and *D. bocki* are not readily separated morphologically, even using details of the male genitalia, which are generally important taxonomically. However, the last two species can be separated from the first by metaphase chromosome figures. *D. leontia* and *D. bocki*, however, are indistinguishable morphologically as well as in metaphase karyotypes (Baimai and Chumchong 1980). These three sibling species exhibit reproductive isolation to varying degrees; *D. bocki* completely fails to cross with both *D. leontia* and *D. kikkawai*, while *D. leontia* and *D. kikkawai* can be hybridized under crowed mass-mating conditions, producing sterile F_1 male hybrids. The most important isolating mechanism in this species complex appears to consist of conspicuous differences in mating behaviour (unpublished observation).

D. leontia and *D. kikkawai* have been found in mixed natural populations at Songkhla and Wangtakrai in Thailand and *D. bocki* has been found sympatrically with *D. leontia* at Lamtakong. Further, in the sympatric populations they have been found in different frequencies. This may reflect some ecological differences between these species, permitting them to coexist, or it may reflect shifting competition. Detailed investigation into ecological habitats and requirements should prove interesting. The data from the present study suggest that *D. bocki* has undergone more complete speciation than its two sibling species.

The data in this study are in accordance with the results obtained by David *et al.*

(1978) in that *D. kikkawai* and *D. leontia* could be forced to cross under laboratory conditions. Moreover, our data indicate that hybridization capabilities of different geographic strains of *D. leontia* vary to some extent. It is possible that allopatric populations of *D. leontia* have undergone genetic differentiation to varying degrees.

As a rule, natural hybridization between full-fledged species is rare in animals. Only a few cases of naturally occurring hybrids have been recorded in *Drosophila* (reviewed by Dobzhansky 1970; Kaneshiro and Val 1977). The present results suggest that hybridization between *D. kikkawai* and *D. leontia* should not be expected to occur naturally at Songkhla or Wangtakrai where they have been found in sympatry because of the strong premating isolation between them. Should natural hybridization between them occur it must be very rare; the hybrids would be recognised by inversions in salivary gland chromosomes.

D. kikkawai and *D. leontia* are very different in gene orders in the X chromosome, while chromosome 2L, 2R and 3L exhibit marked differences in gene arrangement. Chromosome 3R and the microchromosome manifest no differences in gene sequences. It is interesting to note that *D. kikkawai* is polymorphic for a sequence (3LB) which appears to be fixed in *D. leontia*. The phenomenon is not uncommon in certain groups of homosequential species of Hawaiian *Drosophila* (Carson *et al.* 1970; Carson 1970; Carson and Kaneshiro 1976).

From the present data, there are some indications that genetic and/or chromosomal differentiation is involved in the process of species divergence in the *D. kikkawai* complex. Since *D. leontia* and *D. bocki* are apparently restricted to the Southeast Asian region while *D. kikkawai* is widespread, the speciation process is likely to have taken place in this part of the world. This situation is comparable to the *D. willistoni* group of the South American continent (Dobzhansky 1957; Ayala *et al.* 1972). However, the situation in the *D. kikkawai* complex is especially interesting because *D. kikkawai* itself manifests a remarkable metaphase karyotype variation (Baimai and Chumchong 1980). *D. kikkawai* is thus a potentially good candidate for further cytogenetic investigation of the mechanism involved in the process of speciation.

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