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CROSSING EXPERIMENTS WITHIN AND BETWEEN SPECIES OF THE NASUTA COMPLEX OF DROSOPHILA

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Reciprocal group mating crosses were used to investigate the evolutionary status of iso-♀ lines of 2 species of the *nasuta* complex, *D. sulfurigaster albostrigata* and *D. kohkoa*, both collected from Continental Malaysia and Cebu. Assortative mating between iso-♀ lines of these and iso-♀ lines of 2 other species of the same complex, *D. albomicans* and *D. kepulauana* was also investigated using reciprocal group mating crosses. Evidence is presented of a speciation trend between populations of *D. sulfurigaster albostrigata* from Continental Malaysia and Cebu (Philippines). In contrast populations of *D. kohkoa* from these same regions show no signs of speciation. Divergence between the mate recognition systems of different species was found to be very high, however hybrids were obtained from some crosses. F_1 from *D. albomicans* and *D. kepulauana* crosses were found to produce fertile offspring, suggesting a close evolutionary relationship. However, other data show that the mate recognition systems of the 2 species have diverged to such an extent that their specific identity cannot be in doubt.

Introduction

Bock & Wheeler (1972) have commented '... the species group in *Drosophila* represents the results of a major burst of speciation within a subgenus in a particular geographic region'. The *nasuta* complex agrees well with this definition. A subgroup of the *immigrans* species group, the *nasuta* complex contains 9 species which are morphologically very similar and have different, though relatable, polytene chromosome sequences. Polytenes of hybrid larvae of some crosses have been used to analyse phylogenetic relationships within the subgroup (Lambert 1975). The group is confined mostly to the South East Asian region but members have been recorded from Africa and Hawaii. The species occurrence on islands has no doubt facilitated this burst of speciation by providing the necessary geographic separation. Evidence for the comparable age of the species is provided by the fact that some of the species share a number of floating chromosomal inversions (Lambert 1976 a,b). It is generally accepted that inversions have a limited phylogenetic life and hence the species that share floating inversions have evolved specific status in a length of time which is less than the phylogenetic life of the inversions.

The genetical concept of a species is one of a population of sexually reproducing animals that exchange genes only with other members of the species. The biological limits of the species is set by the system of recognition of conspecific mates. The lack of hybrids between species found in nature is evidence for the effectiveness of this mechanism. According to the allopatric model of speciation, divergence in the mate recognition systems (MRS's) occur in allopatry. This leads to positive assortative mating in areas of secondary overlap.

It was the purpose of this study to investigate the divergence of these mate recognition systems within

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and between 4 species of the *nasuta* complex using cross-mating experiments without choice.

Material and methods

Intraspecific crosses between flies from the same collection sites

All iso-♀ lines (cultures derived from single inseminated females caught in the wild) were collected from tropical rainforest using fermenting banana as bait. For each species one iso-♀ line from each of the 3 collection sites (Fig. 1) was chosen as a representative of that species from that region. The process for doing this was as follows: firstly one iso-♀ line was selected based on the pattern of naturally occurring chromosomal inversions present in the culture. This was always a culture which had commonly occurring inversion polymorphisms. Reciprocal group mating crosses were then made between this culture and all other cultures of the same species from that collection site e.g. in the case of *D. s. albostrigata* culture KT_1 was crossed to the other 15 iso-♀ lines of that species collected from Kota Tinggi (see Tab. 1). The procedure was as follows: 10 virgin females of an iso-female line aged from 7-10 days were placed in a single vial containing culture media with 10 males of a different iso-female line aged approximately 7 days. After 5 days the adults were removed and the females were dissected in 0.7% NaCl solution and examined for the presence or absence of sperm in the spermathecae and central receptacles. The F_1 progeny were allowed to emerge, and at regular intervals of 2-3 days the adults were taken from the vials and the number and sex of the individuals recorded. This procedure was continued until flies no longer emerged.

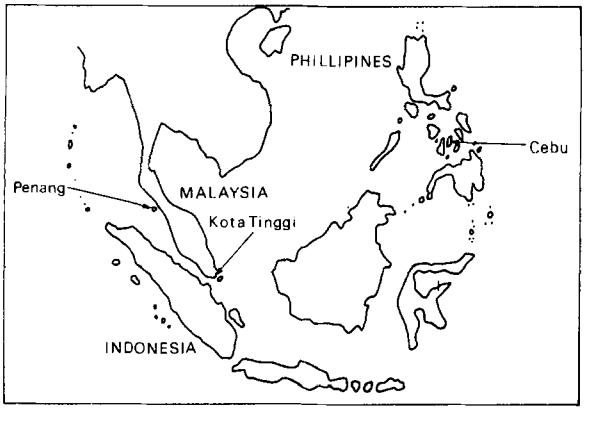

Fig. 1. Sites of collection of iso-♀ lines used in this study.

The ratio of the number of females inseminated to the number of females not inseminated in each of the intraregion crosses of *D. s. albostrigata* and *D. kohkoa* was compared with a control cross. The control cross was a mating of males and females of the representative culture e.g. $KT_{1}♂ \times KT_{1}♀$. The ratio of male to female F_1 was compared with a 1 : 1 ratio.

Using this method it was shown for example that crosses between culture KT_1 and the other iso-♀ lines of *D. s. albostrigata* did not significantly differ from the control cross of $KT_{1}♂ \times KT_{1}♀$ with respect to the percentage of females inseminated and there was no significant difference between the observed and ex-

Table 1

Details of cultures collected and representative cultures chosen for intra-and interspecific crosses

Collection sites	Species							
	D.s.albostrigata		*D kohkoa*		*D.albomicans*		*D.kepulauana*	
	No. of cultures collected	Culture chosen	No. of cultures collected	Culture chosen	No. of cultures collected	Culture chosen	No. of cultures collected	Culture chosen
Kota Tinggi (Malaysia)	16	KT_1	2	KT_{11}	–	–	1	KT_7
Penang (Malaysia	5	P_6	–	–	1	P_4	–	–
Cebu (Philippines)	20	C_4	2	C_{19}	–	–	–	–

pected 1 : 1 ratio of F_1 sexes. Table 1 shows the cultures of each species from each collection site which were chosed for intraspecific and interspecific crosses using this method. Since in the case of *D. albomicans* and *D. kepulauana* only 1 iso-♀ line was collected this procedure was not necessary.

Intraspecific crosses between flies from different collection sites

These crosses were carried out in the case of *D. s. albostrigata* and *D. kohkoa* only, since *D. albomicans* and *D. kepulauana* were not obtained from more than 1 collection site. The procedure was as outlined for intraregion crosses and the representative cultures were used in each case.

Interspecific crosses

Four iso-♀ lines, one from each species, were selected for these crosses. The same iso-♀ lines were used in interspecific crosses as were used in intraspecific crosses. Cultures KT_1, C_{19}, KT_7 and P_4 were selected to represent species *D. s. albostrigata, D. kohkoa, D. kepulauana* and *D. albomicans* respectively. The procedure was as detailed for intraspecific crosses except that crosses were allowed to continue for 28 days before discarding of parents.

Results

The results of interregion crosses between selected iso-♀ lines of *D. s. albostrigata* are given in Table 2. The ratio of the number of females inseminated to the number of females not inseminated in these crosses was compared with both control crosses by a Chi Square Test e.g. in crosses between Penang and Cebu selected iso-♀ lines of *D. s. albostrigata* the results were compared with the results of crosses $P_{6}♂ \times P_{6}♀$ and $C_{5}♂ \times C_{5}♀$. These results are presented in Table 3 and shown diagramatically in Figure 2. Details of a comparison of the ratio of male and female F_1 obtained from interregion crosses with the expected 1 : 1 F_1 sex ratio are given in Table 4. These results are presented diagramatically in Figure 3.

The results of interregion crosses of iso-♀ lines of *D. kohkoa* are given in Table 2. Details of a comparison of the ratio of the number of females inseminated

Table 2

Results of group mating crosses between continental malaysian and Cebu population of *D. s. albostrigata* and *D. kohkoa* (P = Penang, KT = Kota Tinggi, C = Cebu)

Cross	No. of ♀♀ insem. / No. of ♀♀ not insem.	No. of ♂♂ F_1 / No. of ♀♀ F_1
D.s. albostrigata		
P♂ x KT♀	130/1	528/512
P♀ x KT♂	100/3	469/423
P♂ x C♀	147/11	385/304
P♀ x C♂	150/6	558/456
KT♂ x C♀	48/21	271/226
KT♀ x C♂	92/10	343/349
D. kohkoa		
C♂ x KT♀	91/2	412/384
C♀ x KT♂	87/2	429/416

Table 3

Comparison of $\frac{\text{No. of ♀♀ insem.}}{\text{No. of ♀♀ not insem.}}$ in interregion crosses of *D. s. albostrigata* and *D. kohkoa* compared with control crosses

Experimental cross	Penang control cross	Kota Tinggi control cross	Cebu control cross
D. s. albostrigata			
P♂ x KT♀	0.036 ($P \geq 0.05$)	0.174 ($P \geq 0.05$)	–
P♀ x KT♂	0.197 ($P \geq 0.05$)	0.098 ($P \geq 0.05$)	–
P♂ x C♀	5.017 ($P \geq 0.05$)	–	4.887 ($P \geq 0.05$)
P♀ x C♂	2.116 ($P \geq 0.05$)	–	2.052 ($P \geq 0.05$)
KT♂ x C♀	–	29.068 ($P \geq 0.05$)	28.347 ($P \geq 0.05$)
KT♀ x C♂	–	4.772 ($P \geq 0.05$)	7.247 ($P \geq 0.05$)
D. kohkoa			
C♂ x KT♀	–	0.105 ($P \geq 0.05$)	0.161 ($P \geq 0.05$)
C♀ x KT♂	–	0.143 ($P \geq 0.05$)	0.118 ($P \geq 0.05$)

with control crossers are given in Table 3. A comparison of the sex ratio of F_1 of interregion crosses with the expected 1 : 1 F_1 sex ratio is given in Table 4.

Details of the results of the 6 possible interspecific hybridization tests are given in Table 5.

Discussion

Since Continental Malaysian and Cebu populations of *D. s. albostrigata* are geographically isolated the evidence presented here of a speciation trend between them is not unexpected. Figure 2 shows that in 3 of the 4 interregion crosses within *D. s. albostrigata* there has been a divergence in the mate recognition systems of mainland and island populations, i.e. in the ability of males of either Continental Malaysia or Cebu to inseminate females from the other region. Figure 3 shows that there is evidence of genetic incompatibility between the Malaysian and Cebu populations in that there is a deviation from the expected 1 : 1 sex ratio in 3 of the 4 interregion crosses.

In contrast to populations of *D. s. albostrigata* there is no indication of divergence between Con-

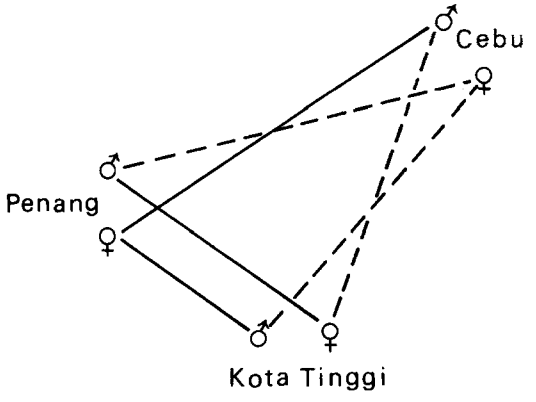

Fig. 2. Comparison of the ratio of females inseminated in crosses of *D. s. albostrigata* shown with both control crosses. Dotted lines represent a significant difference and continuous lines represent no significant difference.

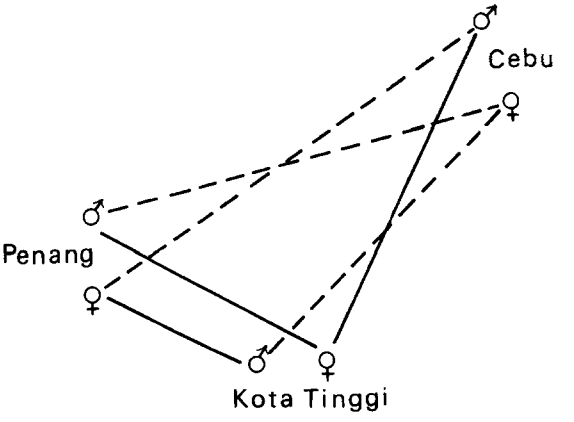

Fig. 3. Comparison of the sex ratio of F_1 in crosses of *D. s. albostrigata* shown compared with both control crosses. Dotted lines represent a significant difference and continuous lines represent no significant difference.

Table 4

Comparison of $\frac{\text{No. of } ♂♂ \text{ } F_1}{\text{No. of } ♀♀ \text{ } F_1}$ in interregion crosses of *D. s. albostrigata* and *D. kohkoa* compared with expected 1 : 1 ratio

Cross	Chi square	Probability
D. s. albostrigata		
P♂ x KT♀	0.246	> 0.05
P♀ x KT♂	2.372	> 0.05
P♂ x C♀	9.522	< 0.01
P♀ x C♂	10.260	< 0.01
KT♂ x C♀	4.074	< 0.05
KT♀ x C♂	0.052	> 0.05
D. kohkoa		
C♂ x KT♀	1.700	> 0.05
C♀ x KT♂	0.200	> 0.05

Table 5

Results of interspecific crosses
albos = *D. s. albostrigata*, koh = *D. kohkoa*, kep = *D. kepulauna*, albom = *D. albomicans*

	Ratio of insemination	% of insemination
albos ♂ x koh ♀	0/101	0.5
albos ♀ x koh ♂	1/101	
albos ♂ x kep ♀	0/88	2.2
albos ♀ x kep ♂	4/97	
albos ♂ x albom ♀	0/70	0.0
albos ♀ x albom ♂	0/83	
koh ♂ x kep ♀	9/178	3.5
koh ♀ x kep ♂	1/110	
albom ♂ x kep ♀	6/85	5.9
albom ♀ x kep ♂	6/117	
koh ♂ x albom ♀	0/123	0.00
koh ♀ x albom ♂	0/79	

tinental Malaysian and Cebu populations of *D. kohkoa*. Tables 2 and 3 show that males exhibit no significant difference in their ability to inseminate females from the same region compared with females from the other region. Table 4 shows that in all interregion crosses of *D. kohkoa* there is no significant deviation from the expected 1 : 1 F_1 sex ratio.

It has previously been shown (Mather et al., 1975; Mather, Clyde & Lambert, 1975; Lambert, 1976b) that *D. s. albostrigata* is highly polymorphic for chromosomal inversions and that although Continental Malaysian and Cebu populations of this species differ in their inversion compositions they share 4 common inversions. It has also been shown that *D. kohkoa* is highly polymorphic, however populations of *D. s. albostrigata* collected from the same areas show no common inversions. *D. s. albostrigata* may have colonised Cebu from a mainland region earlier than did *D. kohkoa*. A longer period in isolation would account for the divergence in MRS's of the two populations.

Details of crosses among the 4 species *D. s. albostrigata, D. kohkoa, D. albomicans* and *D. kepulauana* are given in Table 7. Since each sample of 10 virgin females was confined with 10 males for 28 days, it is apparent that the possibility of an interspecific cross occurring between any of these species in the wild is negligible. It has recently been suggested (Sajjan & Krishnamurthy, 1972; Ranganath & Krishnamurthy, 1975; Ranganath et al., 1974) that the Indian species *D. nasuta* should be considered a subspecies of *D. albomicans* and that *D. kepulauana* should be considered a semispecies of *D. nasuta*. Data presented here show that *D. albomicans* and *D. kepulauana* are good species and could not exchange genes in the wild. The suggestion by Sajjan & Krishnamurthy (1972) that *D. nasuta* and *D. albomicans* should be considered subspecies is based on the fact that F_1, F_2 and F_3 from such a cross, in the laboratory, are fertile. However no data are presented concerning the ability of males of either species to inseminate females of the other i.e. no evidence is presented concerning divergence in the system by which conspecific individuals of opposite sex recognise each other. In this study it has been shown that although *D. albomicans* and *D. kepulauana* produce fertile F_1 their MRS's have diverged to such a degree that they can coexist and retain their specific identity. Wilson et al. (1969) have crossed *D. albomicans* from Okinawa and *D. kepulauana* from Palawan and showed that $P_1 \times P_1$ and $F_1 \times F_1$ were fertile. However all interspecific crosses in this study were performed using 300 to 1000 pairs. The results obtained then give no indication of the ability of males of one species to inseminate females of the other. It should be remembered that many studies on a great variety of animals have shown that '... in nearly every case where the production of fertile hybrids is possible in the laboratory, potent isolating mechanisms operate to prevent such gene exchanges in nature' (Wharton, 1944). The proof of sterility or partial sterility between populations is definite evidence for the specific status of the populations. However the reverse does not necessarily follow.

Data presented here support the allopatric model of speciation. The Continental Malaysian and Cebu populations of *D. s. albostrigata* have been shown to have significantly distinct MRS's which are constant within each population. This supports the theory that speciation occurs via small isolated populations which subsequently expand their ranges. This argument explains the existence of identical systems of mate recognition of a species over a large area. The spreading through a population of genes (which have a pleiotropic effect of change in the MRS) is much more feasible in small than large populations. *D. pallidifrons* of this complex is an interesting example. This species is restricted, as far as is known, to one island (Panape) of the Caroline group. Presumably the island was colonised by a very small number of ancestors (perhaps one inseminated female) and has diverged pleiotrophically. Spieth (1969) has shown that the species has a quite distinct courtship pattern. Any supposition of repeated colonization by members of the ancestral population in order to reinforce differences would be a breach of the principle of parsimony.

The basis of the divergence in MRS's between these *nasuta* complex species is certainly, at least largely, a behavioural one. Divergent courtship patterns within the group have been studied (Spieth, 1969; Lambert, 1975) and it has been shown that for *D. albomicans* and *D. kepulauana* behavioural evidence is in support of crossing and chromosome data (Lambert, 1976a) and they appear to be closely related. Details of these behavioural studies will be presented in a later paper.

It should be emphasised that laboratory tests al-

ways underestimate the differences between the MRS's of any 2 populations, since by isolating members of these populations together at least a number of steps have been bypassed. It may also happen that 2 members of different populations may never exchange genes because they are never in the same microhabitat at the same reproductively synchronised time. Laboratory crossing experiments bypass genetically programmed ecological characteristics of the species. Choice experiments are preferable in the examination of within species variation in courtship and should be restricted to that situation. For between species studies choice experiments are too sensitive to test for such large differences and, in this case, the no choice test is preferable. However, no choice tests can be used successfully, as in this study, for the detection of within species differences in mate recognition. It would be expected however, that choice experiments would magnify any differences and produce a statistically more significant result.

The results presented here relate to the need in evolutionary studies for an emphasis in the distinction of the taxonomic and genetical species. Spurway (1954, in reference to Harland, 1941) remarked 'constancy of phenotype implies constancy of some selection pressure. It need not imply constancy of the genotype producing it'. In the study of many animal groups it is not possible to make decisions regarding their genetical status as species and this should be acknowledged. The fact that morphological and genetical species do not always correspond has been amply demonstrated in a number of groups and these studies are of particular significance to the evolutionist. The decisions which have been made in this work relate to the genetical species concept: their significance in taxonomy was only then considered.

In summary this study has shown the existence of divergent MRS's in Continental Malaysian and Cebu populations of *D. s. albostrigata*. No significant divergence can be detected between corresponding populations of *D. kohkoa*. Evidence from group mating crosses testifies to the specific status of *D. albomicans* and *D. kepulauana*. It is suggested that these species have separated more recently than the others investigated.

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