
Incipient sexual isolation in the *nasuta-albomicans* complex of *Drosophila*: mating preference in male-, female- and multiple-choice mating experiments

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Interracial divergence is an important facet of speciation. The *nasuta-albomicans* complex of *Drosophila* with sixteen morphologically identical, karyotypically different but cross-fertile races is an excellent system to study a few dimensions of riaciation. *Drosophila nasuta nasuta*, *Drosophila nasuta albomicans*, Cytorace 1, Cytorace 2, Cytorace 3 and Cytorace 4 of this subgroup have been subjected to male-, female- and multiple-choice mating experiments. Out of 8456 crosses conducted, 7185 had successful matings. The overall impression is that mating is far from random amongst these six closely related races of the *nasuta-albomicans* complex. The males of *D. n. albomicans*, Cytorace 1 and Cytorace 4 in male-choice, the females of Cytorace 1 and Cytorace 2 in female-choice, and the males and females of *D. n. nasuta*, *D. n. albomicans*, Cytorace 1 and Cytorace 4 against the males and females of Cytorace 2 in multiple-choice experiments, had significantly more homogamic matings than expected. Thus in this study of evolutionary experimentation on riaciation under laboratory conditions, we have documented the initiation of preference for con-specific matings among closely related and independently evolving members of the *nasuta-albomicans* complex of *Drosophila*.

1. Introduction

Reproductive isolation originates as a byproduct of genetic divergence between diversifying races which effects the mate recognition system, ultimately resulting in the strains showing incipient isolation (Singh 1996). There has been a recent push to expand the understanding of the mechanisms of reproductive isolation beyond hybrid sterility and hybrid inviability to include other traits that differentiate species, such as inter-specific mate discrimination and the divergence of secondary sexual characteristics. Such a shift in focus is particularly needed as genetic information about these traits is scant, yet these traits are often as important as hybrid incompatibilities for theories of speciation, especially those involving sexual selection and reinforcement (Iwasa and Pomiankowski 1995; Kelly and Noor 1996).

Drosophila nasuta nasuta ($2n = 8$) and *Drosophila nasuta albomicans* ($2n = 6$) are allopatric, sibling, cross-

fertile chromosomal races of the *nasuta* subgroup of the *immigrans* species group of *Drosophila* (Nirmala and Krishnamurthy 1972; Ranganath and Hagele 1981; Ranganath and Ramachandra 1987). The F_1 of these races have $2n = 7$, while the F_2 and the subsequent hybrid generations are karyotypically polymorphic (Ranganath 1978; Rajasekarasetty *et al* 1979). Subsequent maintenance of these hybrid populations by inbreeding has resulted in the disappearance of karyotypic polymorphism and a population with a stable karyotype has appeared. Such karyotypically stable hybrid populations of *D. n. nasuta* and *D. n. albomicans* have been called Cytoraces and sixteen Cytoraces have been evolved. Karyotypically one Cytorace is different from the other. In each of these Cytorace, there is differential representation of the parental chromosomes of *D. n. nasuta* and *D. n. albomicans*. The assemblages of *D. n. nasuta*, *D. n. albomicans* and sixteen Cytoraces have been called *nasuta-albomicans* complex of *Drosophila* (Ramachandra and Ranganath 1996).

Keywords. *Drosophila*; incipient isolation; mating preference; *nasuta-albomicans*

Periodical analysis of these members have shown strong symptoms of interracial divergence, such as differences for parameters of fitness (Ramachandra and Ranganath 1988; Tanuja 2000), emergence of a new neo-Y chromosome (Tanuja *et al* 1999a), evolution of a new chromosomal lineage through centric fission (Tanuja *et al* 1999b), divergence in body size and bristles (Harini and Ramachandra 1999a, b, 2000). Tanuja *et al* (2001) have reported evidence of incipient reproductive isolation among parental races and four Cytoraces of the *nasuta-albomicans* complex of *Drosophila* by assessing the fraction of no-matings, mating latency and copulation duration in all possible types of homo- and heterogamic crosses. In an extension of these studies we report the very early events in the evolution of 'mating preference' among a few members of the *nasuta-albomicans* complex of *Drosophila*.

2. Materials and methods

2.1 Fly stocks

The following six chromosomal races of the *nasuta-albomicans* complex have been used for present investigations.

- (i) *Drosophila nasuta nasuta* ($2n = 8$; Coorg strain, India)
- (ii) *Drosophila nasuta albomicans* ($2n = 6$, Okinawa strain, Texas collection, USA 3045.11)
- (iii) Cytorace 1 ($2n = 7$ in males; $2n = 6$ in females; Ramachandra and Ranganath 1986)
- (iv) Cytorace 2 ($2n = 6$; Ramachandra and Ranganath 1986)
- (v) Cytorace 3 ($2n = 8$; Ramachandra and Ranganath 1990)
- (vi) Cytorace 4 ($2n = 7$ in males; $2n = 8$ in females; Ramachandra and Ranganath 1990)

2.2 Mating choice experiments

By adopting the protocols of Spieth (1968) and Parsons (1973) following types of mating choice experiments have been carried out for the above six races. In each setup the extent of homogamic and heterogamic matings have been recorded.

2.2a Male-choice experiments (1 male : 2 females): The males of a race are given a choice between two different females, one of their own race and another from a different race in equal proportion. Therefore the sex-ratio was 1 male : 2 females.

2.2b Female-choice experiments (1 female : 2 males): The females of one type are confined with both conspecific and non-conspecific males in equal proportion. The sex ratio was 1 female : 2 males.

2.2c Multiple-choice experiments (2 males : 2 females): Males and females of two races in equal ratio are confined together. Here, both males and females will have a choice. The sex ratio was 1 male : 1 female for each race.

From each race fifty eggs were collected in fresh quarter-pint-milk bottles with wheat cream agar medium to avoid larval competition. They were maintained under a constant temperature of 22°C. Once the flies start emerging from the bottles, virgin females and males from each of these bottles were isolated and transferred to fresh media vials within 4 h of their eclosion and maintained separately at 22°C. These flies were aged for 5 days. One day before the onset of the experiment, the flies were marked, as they are morphologically identical. In male-choice, one of the female, in female-choice one of the male and in multiple-choice one of the male and female is marked with a fine marker on the thorax. The flies were aspirated into the empty vials, to avoid etherization and the vials are plugged with cotton. These experiments were done at room temperature approximately under normal laboratory light conditions from 7 a.m. to 12 noon, which is for about 5 h. Observations were made without disturbing the vials after introducing the flies. Each vial contains three flies in both male- and female-choice experiments and four flies in multiple-choice experiments. Except the copulating pair, all other flies were aspirated out to avoid males and females from mating for the second time.

2.3 Statistical analysis

To know the differences between homogamic and heterogamic matings, the data is subjected to χ^2 test. Also isolation index if any was measured to know the degree of sexual isolation as per the formula of Merrel (1950).

$$\text{Isolation index} = \frac{\% \text{ homogamic matings} - \% \text{ heterogamic matings}}{\text{Total matings}}$$

If isolation index is 'zero', there is no isolation, + 1 when isolation is complete, and - ve when heterogamic matings are more than homogamic ones.

3. Results

In the present experiment, out of 8456 crosses established, 7185 had successful matings. The male-choice setup with

30 combinations established 2693 successful matings; similarly 30 combinations of female-choice had 2787 successful matings, while 15 combinations of multiple choice registered 1705 successful matings. None of the pairs of races under study showed complete sexual isolation. Virtually in every experiment mating was observed, but with different degrees of preference for conspecific/non-conspecific matings. The findings of these experiments have been analysed hierarchically by comparing the homo- and heterogamic matings recorded under different choice experiments.

At the first level, the data was pooled from all the experiments and it revealed that out of 7185 successful matings, 54% were of homogamic type and this is significantly more than that of heterogamic category (table 1).

Similarly, in each of the male, female and multiple choice experiments, the incidence of homogamic mating of 55.22%, 53.35% and 53.20% respectively was more than that of heterogamic matings in the respective category (table 1).

In male- and female-choice experiments, six combinations are possible. Of these six combinations, in the male-choice experiments, the males of *D. n. albomicans*, Cytorace 1 and of Cytorace 4 showed significantly more of homogamic matings. Particularly the males of *D. n. albomicans* had nearly 80% of homogamic matings with an isolation index of 0.61 (table 2). On the other hand, the males of *D. n. nasuta*, Cytorace 2 and Cytorace 3 had random matings. In the female-choice experiments, of the six races, only the females of Cytorace 1 and Cytorace 2

Table 1. Comparisons between homogamic and heterogamic matings of the entire experiment and also of different choice experiments involving a few races of the *nasuta-albomicans* complex of *Drosophila*.

Total crosses	Total No. of matings	Homogamic matings (%)	Heterogamic matings (%)	Σc^2	<i>P</i> -value
Entire experiment					
8456	7185	3881 (54.00)	3308 (46.00)	61.62	< 0.05
Choice-experiments					
Male-choice					
3311	2693	1487 (55.22)	1206 (44.78)	29.32	< 0.05
Female-choice					
3345	2787	1487 (53.35)	1300 (46.65)	12.54	< 0.05
Multiple-choice					
1800	1705	907 (53.20)	798 (46.80)	6.96	< 0.05

Table 2. Comparisons between homogamic and heterogamic matings of male-, female- and multiple-choice experiments among a few races of the *nasuta-albomicans* complex of *Drosophila*. In each choice experiment, only those sets which showed significant differences are listed here.

Races	Total crosses	Total No. of matings	Homogamic matings (%)	Heterogamic matings (%)	Σc^2	Isolation index
Male-choice experiments						
Males						
A	527	445	358 (80.45)	87 (19.55)	165.04*	0.61
C 1	614	501	309 (61.68)	192 (38.32)	27.32*	0.23
C 4	500	467	255 (54.60)	212 (45.40)	3.96*	0.09
Female-choice experiments						
Females						
C 1	609	540	376 (69.63)	164 (30.37)	83.22*	0.39
C 2	673	494	298 (60.32)	196 (39.68)	21.06*	0.21
Multiple-choice experiments						
Males and females						
C 2	650	603	345 (57.21)	258 (42.79)	12.56*	0.14

**P* < 0.05.

showed significant preference for conspecific males, while the females of *D. n. nasuta*, *D. n. albomicans*, Cytorace 3 and Cytorace 4 had more of the heterogamic matings. In the multiple-choice experiments, among 15 combinations, more of homogamic matings than heterogamic matings was observed only in one case involving the individuals of Cytorace 2 (table 2).

In the next level of analysis, within the male-choice experiments, the matings of *D. n. albomicans*, Cytorace 1 and Cytorace 4 races with other females have been analysed (table 3). The males of *D. n. albomicans*, in all the five male-choice set ups had more of homogamic

matings. It was extra-ordinarily high, against the females of Cytorace 2 with an isolation index of 0.82 and similarly against the females of *D. n. nasuta* with an isolation index of 0.68. With regard to the males of Cytorace 1, it had significantly more of homogamic matings against the females of *D. n. nasuta*, Cytorace 2 and Cytorace 3 only. On the other hand, the males of Cytorace 4 significantly preferred homogamic matings only against the females of *D. n. nasuta* and Cytorace 2. Table 4 provides information about the matings of females of Cytorace 1 and Cytorace 2 in female-choice experiments. The females of Cytorace 1 had a significant bias for its own males against

Table 3. Male-choice experiments: Comparisons between homogamic and heterogamic matings of the males of *D. n. albomicans*, Cytorace 1 and Cytorace 4, with the females of other races.

Crosses			No. of crosses made	No. of matings recorded N (%)	Homogamic matings N (%)	Heterogamic matings N (%)	Σc^2	Isolation index
Males	Females							
A	A	N	100	87 (87.00)	73 (83.91)	14 (16.09)	38.66*	0.68
A	A	C 1	101	87 (86.14)	64 (73.56)	23 (24.44)	18.38*	0.49
A	A	C 2	126	101 (80.16)	92 (91.09)	09 (08.91)	66.58*	0.82
A	A	C 3	100	80 (80.00)	59 (73.75)	21 (26.25)	17.12*	0.47
A	A	C 4	100	90 (90.00)	70 (77.78)	20 (22.22)	26.68*	0.56
C 1	C 1	N	152	104 (68.42)	74 (71.15)	30 (28.85)	17.78*	0.42
C 1	C 1	A	112	102 (91.07)	40 (39.22)	62 (60.78)	4.32*	-0.22
C 1	C 1	C 2	150	106 (70.66)	72 (67.92)	34 (32.08)	12.92*	0.36
C 1	C 1	C 3	100	92 (92.00)	72 (78.26)	20 (21.74)	28.26*	0.56
C 1	C 1	C 4	100	97 (97.00)	51 (52.58)	46 (47.42)	0.16	0.05
C 4	C 4	N	100	89 (89.00)	64 (71.91)	25 (28.09)	16.22*	0.44
C 4	C 4	A	100	96 (96.00)	28 (29.17)	68 (70.83)	15.84*	-0.42
C 4	C 4	C 1	100	95 (95.00)	45 (47.37)	50 (52.63)	0.16	-0.05
C 4	C 4	C 2	100	91 (91.00)	74 (81.32)	17 (18.68)	34.46*	0.63
C 4	C 4	C 3	100	96 (96.00)	44 (45.83)	52 (54.17)	0.52	-0.08

* $P < 0.05$.

Table 4. Female-choice experiments: Comparisons between homogamic and heterogamic matings of the females of Cytorace 1 and Cytorace 2, with the males of other races.

Crosses			No. of crosses made	No. of matings recorded N (%)	Homogamic matings N (%)	Heterogamic matings N (%)	Σc^2	Isolation index
Females	Males							
C 1	C 1	N	118	99 (83.89)	71 (71.72)	28 (28.28)	17.82*	0.43
C 1	C 1	A	115	100 (86.96)	72 (72.00)	28 (28.00)	18.49*	0.44
C 1	C 1	C 2	176	153 (86.93)	98 (64.05)	55 (35.95)	11.52*	0.28
C 1	C 1	C 3	100	95 (95.00)	77 (81.05)	18 (18.95)	35.42*	0.62
C 1	C 1	C 4	100	93 (93.00)	58 (62.37)	35 (37.63)	5.20*	0.25
C 2	C 2	N	148	86 (58.11)	63 (73.26)	23 (26.74)	17.68*	0.46
C 2	C 2	A	145	104 (71.72)	66 (63.46)	38 (36.54)	7.00*	0.27
C 2	C 2	C 1	183	140 (76.50)	68 (48.57)	72 (51.43)	0.06	-0.03
C 2	C 2	C 3	100	76 (76.00)	57 (75.00)	19 (25.00)	18.02*	0.50
C 2	C 2	C 4	100	88 (88.00)	44 (50.00)	44 (50.00)	0.00	0.00

* $P < 0.05$

the males of all the other races under study namely *D. n. nasuta*, *D. n. albomicans*, Cytorace 2, Cytorace 3 and Cytorace 4. On the other hand, the females of Cytorace 2, had more of conspecific matings with its males only against those of *D. n. nasuta*, *D. n. albomicans* and Cytorace 3.

An interesting situation is seen in multiple-choice experiments. In the multiple-choice experiments, in each set up, two types of homogamic and two types of heterogamic matings are possible. The details of the matings in five multiple-choice experiments involving Cytorace 2 are given in table 5. In these experiments *D. n. nasuta* had more homogamic matings with the individuals of *D. n. nasuta* and Cytorace 2, as opposed to Cytorace 2 which had more heterogamic matings. Similarly, with *D. n. albomicans* and Cytorace 2, *D. n. albomicans* had more of conspecific matings while Cytorace 2 had more of non-conspecific matings. In Cytorace 2 and Cytorace 1 combination too, Cytorace 1 had more of homogamic matings while Cytorace 2 had almost random matings. A similar situation is seen in Cytorace 2 and Cytorace 4 combination. On the other hand, in Cytorace 2 and Cytorace 3

combination, Cytorace 2 had more of homogamic while Cytorace 3 had more of heterogamic matings.

4. Discussion

In *Drosophila*, the most efficient and usual mode of reproductive isolation is due to ethological and/or mating differences (Marin 1991). Two closely related species are effectively isolated if enough differences in courtship behaviour exist between them. Because of the importance of such a mechanism, efforts have been made to measure the degree of sexual isolation between different pairs of species, races and strains of *Drosophila* (Marin 1991). Even though, there are several reports of complete sexual isolation between pairs of species a number of examples of incomplete inter-specific isolation have been reported (Patterson 1947). Such incomplete reproductive isolation is very crucial since they throw light on the acquisition and the evolution of sexual isolation.

It has been shown that "choice-experiments" are useful to measure sexual isolation between *Drosophila* strains or

Table 5. Multiple-choice experiments: Comparisons between homogamic and heterogamic matings of the males and the females of Cytorace 2 with the males and females of other races.

Crosses	No. of crosses made	No. of mating recorded <i>n</i> (%)	Homogamic matings			Heterogamic matings			Σc^2	Isolation index
			Crosses			Crosses				
Males and females			♂	♀	<i>n</i> (%)	♂	♀	<i>n</i> (%)		
<i>D. n. nasuta</i> / Cytorace 2	150	134 (89.33)	N	N	44 (32.84)	N	C2	10 (7.46)	N: 20.16*	N: 0.63
			C2	C2	22 (16.42)	C2	N	58 (43.28)	C2: 3.78*	C2: 0.37
<i>D. n. albomicans</i> / Cytorace 2	150	143 (95.33)	A	A	71 (49.65)	A	C2	06 (4.20)	A: 53.18*	A: 0.78
			C2	C2	14 (9.79)	C2	A	52 (36.36)	C2: 2.44	C2: 0.39
Cytorace 1/ Cytorace 2	150	141 (94.00)	C1	C1	54 (38.29)	C1	C2	29 (20.58)	A: 2.64	A: 0.15
			C2	C2	28 (19.86)	C2	C1	30 (21.27)	C2: 20.74*	C2: -0.57
Cytorace 2/ Cytorace 3	100	89 (89.00)	C2	C2	46 (51.69)	C2	C3	14 (15.73)	C1: 6.94*	C1: 0.30
			C3	C3	11 (12.36)	C3	C2	18 (20.22)	C2: 0.00	C2: -0.02
Cytorace 2/ Cytorace 4	100	96 (96.00)	C2	C2	24 (25.00)	C2	C4	25 (26.04)	C1: 6.30*	C1: 0.28
			C4	C4	31 (32.29)	C4	C2	16 (16.67)	C2: 0.02	C2: -0.03
									C2: 16.02*	C2: 0.53
									C3: 0.16	C3: -0.12
									C2: 11.38*	C2: 0.44
									C3: 1.24	C3: -0.24
									C2: 0.00	C2: -0.04
									C4: 0.44	C4: 0.12
									C2: 1.22	C2: 0.19
									C4: 4.18*	C4: 0.32

* $P < 0.05$.

species (Marin 1997). [It must be pointed out here that we use the word 'choice' in a purely conventional sense. During courtship and mating, males and females of *Drosophila* (and other species) exchange information and stimulate one another. Thus what one might think is a male's 'choice' might in fact be that of the female, and vice-versa.] Perusal of the literature reveals that incipient sexual isolation between different strains/races of *Drosophila* has been recorded in many instances. Patterson (1947) has shown that sexual isolation between *D. mulleri* and *D. aldrichi* is incomplete but the isolation index remains quite high in both directions due to preference for homogamic matings. Documentation of non-random matings has been shown by Kaneshiro (1976) in the different species of *planitibia* group, by Petit *et al* (1976) in French and Japanese stocks of *D. melanogaster*, by Arita and Kaneshiro (1979) in *D. adiantola*, by Ahearn (1980) as well as Kaneshiro and Kurihara (1981) amongst widely distributed strains of *D. silvestris*, by Ehrman and Parsons (1980) in geographic strains of *D. immigrans*, by Singh and Chatterjee (1985) in few of the lines of *D. ananassae*, by Bock (1978) and Singh *et al* (1981) as well as Singh and Chatterjee (1991) in *D. bipectinata*, *D. parabipectinata*, *D. malerkotliana* and *D. pseudoananassae*, by Koepfer and Fenster (1991) in the strains of *D. mercatorum* and by Yoshiyuki and Kimura (1997) between brown and black morphs of *D. elegans*.

In our present investigation, of the six races under study, *D. n. nasuta* and *D. n. albomicans* are the parental races. Interracial hybridization of these two races has resulted in the evolution of four derived Cytoraces. These morphologically identical, cytologically closely related races constitute an important assemblage to study different dimensions of racial divergence. The chromosomes of the parental races are differentially represented in these four Cytoraces (Ramachandra and Ranganath 1986, 1990). In the present study, by conducting male-choice, female-choice and multiple-choice experiments amongst these six races, the pattern of mating preferences, if any, is investigated. *D. n. nasuta* and *D. n. albomicans* have evolved in nature while these four Cytoraces have evolved under laboratory conditions, through hybridization between *D. n. nasuta* and *D. n. albomicans*. The age of these Cytoraces at the time of conducting these experiments was about 300 to 350 generations (10 to 15 years). In the male-choice experiments, the males of *D. n. albomicans* discriminate the females of all other races under study. Among the derived races, the males of Cytorace 1 significantly discriminated the females of *D. n. nasuta*, Cytorace 2 and Cytorace 3 while the males of Cytorace 4 significantly discriminated the females of *D. n. nasuta* and Cytorace 2. In the female-choice experiments, the females of Cytorace 1 significantly discriminate the males of all the five other races under study, whereas the females of

Cytorace 2 discriminate the males of *D. n. nasuta*, *D. n. albomicans* and Cytorace 4. It is interesting to note that the females of parental races (*D. n. nasuta* and *D. n. albomicans*) do not discriminate the males of derived races but the females of derived races namely Cytorace 1 and Cytorace 2 discriminate the males of its parental race. In the multiple-choice experiments, except the males and females of Cytorace 3, individuals of other races namely *D. n. nasuta*, *D. n. albomicans*, Cytorace 1 and Cytorace 4 discriminated against the individuals of Cytorace 2, and have more of homogamic matings, while the males and females of Cytorace 2 due to reduced discrimination, have more of heterogamic matings than homogamic matings. Thus, the mating preference among these six closely related races of the *nasuta-albomicans* complex is far from random. The males of the *D. n. albomicans*, Cytorace 1 and Cytorace 4 in male-choice, the females of Cytorace 1 and Cytorace 2 in female-choice and males and females of *D. n. nasuta*, *D. n. albomicans*, Cytorace 1 and Cytorace 4 against the males and females of Cytorace 2 in multiple-choice experiments have clearly shown non-random matings.

Reproductive isolation is an important characteristic of a species. The acquisition of sexual isolation during anagenesis is a much debated and discussed issue in evolutionary biology. Populations that appear to be evolving reproductive isolation at either pre- or post-copulatory level, provide rare opportunities to follow the event (Krebs and Markow 1989). Interracial hybridization between *D. n. nasuta* and *D. n. albomicans* has acted as an "evolutionary catalyst" and has resulted in the emergence of new races, which are in the process of differentiation. The evolutionary scenario of the *nasuta-albomicans* complex is extremely interesting. These closely related independently evolving members offer a rare and unique opportunity to witness riation under laboratory conditions. The present study is unique in that it has documented the divergence for one of the very early stages of pre-mating reproductive isolation, that is, differential mating preference among six races. Since these races of the *nasuta-albomicans* complex have shown tendencies for isolation, studies are in progress to understand mating behaviour of these strains. Thus this could be a sort of evolutionary experimentation on riation/speciation, almost simulating a natural event in the environs of laboratory.

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