Incipient sexual isolation in the *nasuta-albomicans* complex of *Drosophila*: No-choice experiments

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Drosophila nasuta nasuta and *Drosophila nasuta albomicans* are cross-fertile races of *Drosophila*. Hybridization between these races in the laboratory has given rise to new races (Cytoraces), among which karyotypic composition differs from one another and also from those of the parental races. In this study, we search for the evidence of incipient reproductive isolation among the parental races and four Cytoraces by assessing the fraction of no-matings, mating latency and copulation duration in all possible types of homo- and heterogamic crosses (N = 4184). In no-choice conditions, the latency time (time to initiation of copulation) is lower in homogamic crosses than in heterogamic crosses for both parental races and Cytoraces. Latency time and copulation duration are negatively correlated, whereas fraction of no matings is positively correlated with latency time. Thus these six closely related races of the *nasuta-albomicans* complex show the initiation of the earliest stages of pre-zygotic isolation, manifested as a tendency for matings to be initiated earlier and more often, and for a longer duration, among homogamic rather than heterogamic individuals.

1. Introduction

Drosophila nasuta nasuta ($2n$ = 8) and *Drosophila nasuta albomicans* ($2n$ = 6) are a pair of allopatric sibling chromosomal races of the *nasuta* subgroup of *Drosophila* (Nirmala and Krishnamurthy 1972; Ranganath and Hägele 1981). Interracial hybridization of these races followed by the maintenance of hybrids for many generations has resulted in the evolution of populations whose karyotypic composition is different from those of parents and these hybrid populations have been termed Cytoraces (Ramachandra and Ranganath 1986, 1990). The assemblage consisting of the parental races, namely *D. n. nasuta* and *D. n. albomicans* and the newly evolved Cytoraces are treated as '*nasuta-albomicans* complex' of *Drosophila* (Ramachandra and Ranganath 1996). Cytogenetic and morphophenotypic divergence has been recorded among a few members of this new complex (Tanuja *et al* 1998, 1999a, b). Now we report the divergence for three important components of prezygotic reproductive isolation, namely the incidence of no-mating, mating latency and duration of copulation among six 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: (i) *D. n. nasuta* ($2n$ = 8; Coorg strain, India); (ii) *D. n. albomicans* ($2n$ = 6; Okinawa strain, University of Texas collections, 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 and $2n$ = 8 in females; Ramachandra and Ranganath 1990).

2.2 *Mating experiments*

Fifty eggs were collected in fresh half-pint-milk bottles with wheat cream agar medium to avoid larval competition during the development, and incubated at 22°C. Once the flies started emerging from these bottles, virgin

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females and males from each of these bottles were isolated and transferred to fresh media vials within 6 h of their eclosion and maintained separately at 22°C. After aging these virgin flies for 5 days, they were used for no-choice mating experiments in which one male was allowed to mate with a female of either its own race (homogamic) or of a different race (heterogamic).

The matings were carried out in the empty vials plugged with cotton. Flies were aspirated into the vials, to avoid etherization before the experiment. The mating activities of these flies were recorded for about 5 h starting at 07·00 h. Mating latency was measured as time (in minutes) taken from the introduction of a male and a female together into an empty vial until the initiation of copulation. Copulation duration was measured as time (in minutes) taken from the initiation to the termination of copulation. For each of the cross, the fraction of vials in which no matings occurred during the 5 h of observation was also recorded.

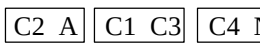
In the present study, with six different races, 36 crosses were made. The total number of matings attempted was 4184. The following statistical comparisons were made for the three traits studied: (i) Among homogamic matings of six races. (ii) Among males of six races, with trait values averaged over all matings with females of other races. (iii) Among females of six races, with trait values averaged over all matings with males of other races. (iv) Among homo-parental (homogamic matings of parental races); homo-cytorace (homogamic matings for each of the four Cytoraces); hetero-parental (cross between two parental races); hetero-cytorace (cross among any two Cytoraces); hetero-mixed (heterogamic matings between a parental race and a Cytorace) types of matings. (v) Correlation coefficients between three measures of reproductive isolation namely mating latency, copulation duration and the fraction of no-matings have been computed.

Statistical analyses for (i) through (iv) above was by means of analysis of variance (ANOVA) followed by Tukey's HSD test for unequal sizes (Spjotvoll-Stoline test) for multiple comparisons. All statistical analysis were done on Statistica for Windows release 5·0B.

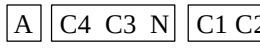
3. Results

In the present study with six races of *nasuta-albomicans* complex, a total of 4184 crosses were attempted. Of this, 2715 crosses resulted in matings. Table 1 provides the mean values of mating latency, copulation duration and per cent of no-matings in each of the 36 crosses of the present experiment. Of the six races, Cytorace 2 and *D. n. albomicans* have the highest (113·72 min) and the lowest (68·06 min) mean mating latency respectively. Similarly, the mean maximum duration of copulation was in *D. n. albomicans* (20·4 min) while the lowest was in Cytorace 2 (15 min). The differences among six races for mating latency ($F_{5, 536} = 4{\cdot}85$; $P = 0{\cdot}00024$) and for copulation duration ($F_{5, 536} = 24{\cdot}01$; $P = 0{\cdot}0001$) are found to be significant. The table 2 has the means of the three parameters for a male of a particular race across the females of six races as well as of female of a race across the males of the other races. These means would help us to compare the performance of males and females of six races under study on the parameters analysed. The two-way ANOVA computed to these means shows the significant difference among males and among females for the parameters mating latency and copulation duration (table 2). Further, pair-wise comparisons were made with the help of Tukey's HSD test and based on the level of differences, the six races under study can be organized in a hierarchical pattern, wherein the races within a cluster do not show significant differences. The hierarchy of the males of six races for mating latency could be represented as

Similarly the males of six races copulate for different duration and it is hierarchically represented as



The sequence of females for mating latency is



while the relative position of females of six races for duration of copulation is

The results are subjected to another type of analysis. Based on the mating type, the 36 crosses can be grouped into homogamic and heterogamic categories. Similarly, based on the identity of the races involved in each cross, the 36 crosses can be organized into five groups, such as homo-parental, homo-cytorace, hetero-parental, hetero-cytorace and hetero-mixed. The mean values of the three parameters for each of these groups are presented in table 3. The overall mating latency of homogamic matings is significantly less than that of heterogamic matings ($F_{1, 2711} = 16{\cdot}48$; $P = 0{\cdot}00005$) while insignificant difference is seen for the duration of copulation ($F_{1, 2711} = 0{\cdot}52$; $P = 0{\cdot}4730$). Similarly, the two-way ANOVA has shown significant impact of race identity (parental vs Cytoraces vs mixed of both homogamic and heterogamic matings) on mating latency ($F_{2, 2711} = 15{\cdot}14$; $P = 0{\cdot}0000003$) and copulation duration ($F_{2, 2711} = 23{\cdot}75$; $P = 0{\cdot}0000003$). Further, among five groups, pair-wise comparisons

showed homo-parentals have the least mating latency while hetero-cytoraces have the longest duration of mating latency. Homo-parental copulate for longer duration than homo-cytorace, hetero-cytorace and hetero-mixed categories. Homo-cytorace has the shorter duration of copulation than hetero-parental and hetero-mixed categories. With regard to the incidence of no-mating, two-way ANOVA has shown insignificant differences both for mating type (homogamic vs heterogamic) and of race identity (parental vs Cytorace vs mixed) (table 3).

By considering the values of each of the 36 crosses from table 1, the incidence of no-matings ranges from 15·34% (*D. n. albomicans*) to 39% (Cytorace 2) in homogamic situation, while it ranges from 10% (C1♂ X C4♀) to 70% (C2♂ X C3♀) in heterogamic matings. Analysis of correlation among three parameters analysed showed positive correlation between no-matings and mating latency ($r = 0{\cdot}73$; $P < 0{\cdot}001$); while negative correlation is recorded between no-matings and copulation duration ($r = -0{\cdot}51$; $P = 0{\cdot}001$); as well as between mating latency and copulation duration ($r = -0{\cdot}45$; $P = 0{\cdot}006$).

4. Discussion

Reproductive isolation is hypothesized to develop gradually between genetically differentiated sub populations and ultimately lead to speciation (Rice and Hostert 1993). Reproductive isolation is an important asset of a species. It enshrines the uniqueness and the integrity of a species. Divergence of characters pertaining to reproductive isolation is of considerable interest in evolutionary biology. There has been considerable progress during the last decade in our understanding of the evolution of reproductive isolation (Hollocher and Wu 1996; True *et al* 1996; Capy *et al* 2000). Any analysis of speciation should include a systematic study of traits involved in pre-zygotic and post-zygotic isolation. Most of the studies related to pre-zygotic isolation have concentrated on mating preference through different choice experiments. On the other hand, significant information is not available on mating latency and duration of copulation among diversifying populations, although these traits could be the earliest steps in the acquisition of pre-zygotic reproductive isolation.

Table 1. Mean values along with standard errors (SE) of mating latency (ML) and copulation duration (CD) as well as per cent of no-matings (NM%) recorded in 36 crosses among six races of the *nasuta-albomicans* complex.

	Races					
Males / Females	N	A	C1	C2	C3	C4
N	NM%: 29·55 ML: 88·61 ± 5·64 CD: 20·4 ± 0·34 (220)	NM%: 39·53 ML: 98·21 ± 5·91 CD: 14·99 ± 0·51 (253)	NM%: 22·08 ML: 96·19 ± 9·68 CD: 18·08 ± 0·59 (77)	NM%: 40·00 ML: 133·32 ± 7·89 CD: 14·39 ± 0·47 (150)	NM%: 28·00 ML: 101·42 ± 7·42 CD: 17·42 ± 0·45 (100)	NM%: 33·00 ML: 90·01 ± 9·41 CD: 17·13 ± 0·49 (100)
A	NM%: 17·65 ML: 70·54 ± 4·09 CD: 20·58 ± 0·32 (225)	NM%: 15·34 ML: 68·06 ± 4·38 CD: 18·18 ± 0·30 (150)	NM%: 10·53 ML: 74·09 ± 5·64 CD: 19·16 ± 0·47 (114)	NM%: 16·67 ML: 100·56 ± 7·43 CD: 19·98 ± 0·34 (126)	NM%: 13·00 ML: 66·89 ± 6·26 CD: 19·36 ± 0·40 (100)	NM%: 16·00 ML: 77·25 ± 7·36 CD: 18·97 ± 0·57 (100)
C1	NM%: 30·16 ML: 99·23 ± 11·90 CD: 18·52 ± 0·76 (63)	NM%: 44·16 ML: 136·77 ± 11·89 CD: 15·07 ± 0·69 (77)	NM%: 33·33 ML: 69·89 ± 5·52 CD: 16·03 ± 0·53 (93)	NM%: 42·50 ML: 129·52 ± 8·11 CD: 13·03 ± 0·43 (120)	NM%: 56·00 ML: 167·09 ± 12·86 CD: 19·20 ± 0·69 (100)	NM%: 25·00 ML: 110·89 ± 8·72 CD: 19·17 ± 0·53 (100)
C2	NM%: 56·13 ML: 136·53 ± 9·71 CD: 18·81 ± 0·72 (155)	NM%: 64·67 ML: 121·26 ± 10·36 CD: 14·13 ± 0·77 (150)	NM%: 49·38 ML: 138·54 ± 12·79 CD: 14·32 ± 1·17 (81)	NM%: 39·00 ML: 113·72 ± 11·19 CD: 15·00 ± 0·45 (100)	NM%: 68·00 ML: 144·91 ± 13·38 CD: 15·59 ± 0·96 (100)	NM%: 39·00 ML: 132·11 ± 10·69 CD: 18·33 ± 0·73 (100)
C3	NM%: 41·00 ML: 68·86 ± 7·62 CD: 18·91 ± 0·35 (100)	NM%: 36·00 ML: 100·76 ± 10·56 CD: 17·81 ± 0·62 (100)	NM%: 65·00 ML: 137·26 ± 16·17 CD: 17·17 ± 0·91 (100)	NM%: 70·00 ML: 144·97 ± 14·72 CD: 16·63 ± 0·95 (100)	NM%: 28·00 ML: 86·25 ± 8·18 CD: 17·00 ± 0·27 (100)	NM%: 21·00 ML: 68·25 ± 7·67 CD: 17·58 ± 0·48 (100)
C4	NM%: 24·00 ML: 77·35 ± 6·90 CD: 18·92 ± 0·29 (100)	NM%: 44·00 ML: 126·12 ± 12·41 CD: 17·25 ± 0·57 (100)	NM%: 10·00 ML: 81·14 ± 6·76 CD: 19·46 ± 0·54 (100)	NM%: 36·00 ML: 96·41 ± 9·13 CD: 16·73 ± 0·55 (100)	NM%: 39·00 ML: 103·39 ± 10·91 CD: 18·41 ± 0·41 (100)	NM%: 35·00 ML: 80·11 ± 8·37 CD: 18·29 ± 0·58 (100)

N, *D. n. nasuta*; A, *D. n. albomicans*; C1-C4, Cytoraces. Number in parentheses indicate number of matings attempted.

The six cytogenetically closely related members of the *nasuta-albomicans* complex of *Drosophila* have been used in the present investigation. Of these, *D. n. nasuta* and *D. n. albomicans* are the parental races. The Cytorace 1, Cytorace 2, Cytorace 3 and Cytorace 4 are the products of interracial hybridization between two parental races (Ramachandra and Ranganath 1986, 1990). Since their origin, these races have been maintained in the laboratory.

We have examined these very initial stages of pre-zygotic isolation events among six closely related races of the *nasuta-albomicans* complex of *Drosophila* in all possible types of homo- and heterogamic crosses ($N = 4184$). None of the heterogamic crosses showed complete pre-zygotic isolation (total absence of mating), but there are detectable differences for the parameters under study. The performance of six races during homogamic matings can be summed up as follows: for mating latency: Cytorace 2 > *D. n. nasuta* > Cytorace 3 > Cytorace 4 > Cytorace 1 > *D. n. albomicans*; for duration of copulation: *D. n. nasuta* > Cytorace 4 > *D. n. albomicans* > Cytorace 3 > Cytorace 1 > Cytorace 2 and for incidence of no-matings Cytorace 2 > Cytorace 4 > Cytorace 1 > *D. n. nasuta* > Cytorace 3 > *D. n. albomicans*. Thus there is a clear indication of interracial divergence for the said parameters.

Since the heterogamic crosses among six races are possible for each race, the performance of males of a race with the females of other five races and similarly of females with the males of other five races have revealed very interesting picture. The males of *D. n. nasuta* have the least mating latency and the longest duration of copulation, while the males of Cytorace 2 showed exactly the opposite trend. On the other hand, the females of *D. n. albomicans* showed the minimum mating latency with a prolonged period of copulation, while the females of Cytorace 2, like its males, copulate quickly and also has relatively longer duration of copulation. Thus, this comparison also is suggestive of gender-specific differences among races for pre-zygotic parameters under investigation. The divergence among races under study for mating latency and copulation duration is also reflected in comparisons of the pooled data of homo- vs heterogamic matings as well as pooled data of parental vs Cytoraces vs mixed matings. It is also interesting to note that even though the two parental races are inter fertile, the hybrid Cytoraces show a degree of incipient reproductive isolation among themselves.

In a few studies on the pattern of isolation between two races/species, differences have been documented between reciprocal crosses and this has been referred to as asym-

Table 2. Mean values along with standard errors (SE) of mating latency (ML), copulation duration (CD) and per cent of no-matings recorded for males and females of six races when each one of them was crossed with the individuals of other races along with the summary of two-way ANOVA.

	Races					
	N	A	C1	C2	C3	C4
Mean of males	NM%: 33·47 ML: 90·19 ± 2·79 CD: 19·36 ± 0·17	NM%: 40·24 ML: 108·53 ± 3·40 CD: 16·24 ± 0·22	NM%: 30·97 ML: 99·52 ± 3·59 CD: 17·37 ± 0·27	NM%: 40·80 ML: 119·75 ± 3·78 CD: 15·96 ± 0·20	NM%: 38·67 ML: 111·66 ± 4·05 CD: 17·83 ± 0·20	NM%: 28·17 ML: 93·11 ± 3·64 CD: 18·25 ± 0·23
Mean of females	NM%: 33·67 ML: 101·29 ± 3·00 CD: 17·07 ± 0·19	NM%: 15·38 ML: 76·23 ± 2·31 CD: 19·37 ± 0·16	NM%: 39·06 ML: 118·89 ± 4·17 CD: 16·84 ± 0·24	NM%: 53·94 ML: 131·18 ± 4·57 CD: 16·03 ± 0·32	NM%: 43·50 ML: 101·06 ± 4·27 CD: 17·52 ± 0·22	NM%: 31·33 ML: 94·09 ± 3·67 CD: 18·18 ± 0·19

	Degrees of freedom	Mean square	*F*	*P*-level
Mating latency				
Male	5	7·450696	13·55654	< 0·0001
Female	5	20·63641	37·54794	0
Male * Female	25	2·831858	5·152565	< 0·0001
Copulation duration				
Male	5	666·0601	31·80742	0
Female	5	636·1249	30·37788	< 0·0001
Male * Female	25	120·8216	5·769785	< 0·0001

Note: Male, Strain identity of male; Female, Strain identity of female.

Table 3. Mean values of mating latency, copulation duration and per cent of no-matings for the pooled data of 36 crosses based on different types of homo- and heterogamic matings among six races of the *nasuta-albomicans* complex, along with the summary of Tukey's HSD test for unequal sizes (Spjotvoll-Stoline test) for pair-wise comparisons.

Categories	No· of matings	Mating latency (Mean ± SE)	Copulation duration (Mean ± SE)	No-matings (Mean ± SE)
Homogamic				
Homo-parental	282	$79{\cdot}35 \pm 3{\cdot}72^{a}$	$19{\cdot}40 \pm 0{\cdot}24^{a}$	$22{\cdot}44 \pm 7{\cdot}11^{a}$
Homo-cytorace	260	$87{\cdot}26 \pm 4{\cdot}34^{b}$	$16{\cdot}62 \pm 0{\cdot}24^{b}$	$34{\cdot}14 \pm 2{\cdot}28^{b}$
Heterogamic				
Hetero-parental	362	$82{\cdot}23 \pm 3{\cdot}51^{c}$	$18{\cdot}23 \pm 0{\cdot}32^{c}$	$28{\cdot}83 \pm 11{\cdot}18^{c}$
Hetero-cytorace	681	$113{\cdot}52 \pm 3{\cdot}10^{d}$	$17{\cdot}36 \pm 0{\cdot}20^{d}$	$43{\cdot}41 \pm 5{\cdot}52^{d}$
Hetero-mixed	1130	$98{\cdot}18 \pm 2{\cdot}23^{e}$	$17{\cdot}93 \pm 1{\cdot}40^{e}$	$32{\cdot}46 \pm 4{\cdot}96^{e}$

Following pair-wise comparisons showed significant difference at $P < 0{\cdot}05$.
Mating latency: *a*/*d*; *a*/*e*; *b*/*d*; *c*/*d*; *c*/*e*; *d*/*e*.
Copulation duration: *a*/*b*; *a*/*d*; *a*/*e*; *b*/*c*; *b*/*e*.

metry in isolation. The six races of *Drosophila* under study are extremely closely related races. In the present study of 36 crosses, 18 pairs of reciprocal crosses are available. Of these 18 pairs, 12 pairs constitute heterogamic matings. Comparisons between reciprocal crosses of heterogamic matings involving ancestral and derived races, asymmetry is seen mainly between the reciprocal crosses of *D. n. nasuta* and *D. n. albomicans*, where the ancestral female, that is, *D. n. nasuta* requires more latency to mate and also it copulates for shorter duration with the males of derived race *D. n. albomicans*. Similarly, all the crosses involving the males of the ancestral race *D. n. albomicans* and the females of derived Cytoraces namely Cytorace 1, Cytorace 2, Cytorace 3 and Cytorace 4, it required more time to initiate copulation and copulates for shorter duration than their respective reciprocal crosses. But such a trend is noticed between the reciprocal crosses of *D. n. nasuta*, the other ancestor, and the four Cytoraces.

Experiments using laboratory strains have provided evidence even though the development of reproductive isolation in natural conditions is difficult to observe (Rice and Hostert 1993). The more advanced the stage of speciation of two diverging populations, the more difficult to delineate the genetic/evolutionary events that has set the process into motion. So it will be difficult to understand the process of speciation by looking at the finished products. The evolutionary scenario of the *nasuta-albomicans* complex is extremely interesting in this regard. The present contribution is unique in that it has documented the divergence for one of the very early stages of pre-mating reproductive isolation, which includes mating latency and copulation duration among six races of the *nasuta-albomicans* complex.

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