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Sexual isolation between two sibling species of *Drosophila: D. ananassae* and *D. pallidosa*

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Sexual isolation, a form of premating reproductive barriers, is one of the elementary causes of speciation. In the present study, investigations on sexual isolation between two sympatric sibling species, namely *Drosophila ananassae* and *D. pallidosa* have been undertaken by employing four different choice conditions (multiple-, female-, male- and no-choice). Mating success was scored by direct observation in Elens–Wattiaux mating chamber. In all the four choice conditions, homogamic matings were more frequent than heterogamic ones, which indicates preferential mating between males and females of the same species. The values of isolation estimate in all the four techniques are close to zero, suggesting the presence of strong sexual isolation between these two sibling species. In female choice method, where the sex ratio was 1 female : 2 males, males of the two species differ in their mating success. Similarly, in male-choice method with sex ratio 1 male : 2 females, females of the two species differ in their mating success. However, in multiple- and no-choice methods where the sex ratio was 1 : 1 (equal number of males and females), no difference was found in mating success of the two types of males and females. From these results, it is concluded that there is strong ethological isolation between *D. ananassae* and *D. pallidosa*, which is not affected by different experimental conditions. However, mating propensity is influenced by sex ratio in these two sibling species.

Keywords: *Drosophila*, experimental conditions, mating propensity, sexual isolation, sibling species.

UNDERSTANDING the mechanisms of speciation is one of the prime targets for evolutionary biologists. Reproductive isolation can be broadly categorized into two forms: pre-mating and post-mating. Pre-mating isolation is the interaction between individuals of pure species while post-mating isolation is a phenomenon of developmental defects[1]. In sympatric species, prezygotic isolation has evolved faster than postzygotic isolation, whereas in allopatric species, prezygotic and postzygotic isolation evolved at the same rate[2]. Sexual selection and isolation are stronger in sympatric populations than in allopatric ones in the semi-species group of *Drosophila paulistorum*[3]. Sexual isolation is a form of premating barriers to gene exchange, where opposite sexes of different populations fail to mate due to behavioural incompatibility. Genes controlling sexual behaviour are likely to control species-specific differences in courtship and are involved in reproductive isolation of closely related species of *Drosophila*[4]. Different patterns of sexual isolation exist in interspecific and intraspecific populations of *Drosophila*[5]. Investigations on sexual isolation between those sympatric species where post-mating isolation is absent, have a great potential to unravel the mechanisms of speciation.

D. ananassae and *D. pallidosa* are an excellent species pair for such studies. They belong to the *D. ananassae* complex of the *ananassae* species subgroup of the *melanogaster* species group[6]. *D. ananassae* is a cosmopolitan and circumtropical species, but *D. pallidosa* is endemic to the islands of the South Pacific Ocean[7]. In spite of their sympatric distribution, post-mating reproductive barriers, such as hybrid sterility or hybrid inviability do not exist between them[6,8]. In addition, clear morphological differences are observed only in body colouration and number of rows in the sex-comb[9]. It suggests that the phylogenetic separation in *D. ananassae* and *D. pallidosa* must have been a recent event in speciation of the melanogaster group[6]. Sexual isolation has been considered to be crucial in maintaining the integrity of the gene pool of the two species, despite their sympatric habitat and absence of post-mating isolation[9]. In the laboratory, strong sexual isolation between the two species has been confirmed[8,10,11] by employing male- and no-choice techniques. These previous studies were insufficient to clarify mating success difference under different choice techniques and sex ratio. Different choice conditions are multiple-, male-, female- and no-choice. In the experiment employing multiple-choice technique, males and females of the two types closely correspond to natural conditions. In no-choice experiments, one type of male is placed with one type of female and it does not represent a choice situation. In male-choice experiments,

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one type of male is placed with both types of females. Thus, males can choose one type of the two types of females. In female-choice experiments, one type of females are placed with both types of males and thus, females can choose one of two males[12]. These experimental techniques have been used to score mating success in various *Drosophila* species. Variation in the results due to different choice situations and sex ratio has been found in some cases with respect to the patterns of mating and mating propensity[13–17]. During the present study, sexual isolation has been measured between both species, *D. ananassae* and *D. pallidosa* using all the four techniques (multiple-, female-, male- and no-choice) and the results are represented in this communication.

The mass culture stock (MYS) of *D. ananassae* was established in the laboratory from the naturally impregnated females collected from Mysore in 2000. The stock of *D. pallidosa* (NAN 57), an isofemale line collected at Lautoka, Fiji in 1981 was provided by M. Matsuda, Japan. A preliminary test of mating propensity was carried out in different geographical strains of *D. ananassae* and finally the stock (MYS) of *D. ananassae* having the same mating propensity as that of the *D. pallidosa* stock (NAN 57) was chosen. This precaution was taken because differences in mating propensity strongly affect the choice tests, which can lead to misinterpretation of discrimination[18].

Stocks of the two species were maintained on simple yeast-agar culture medium at approximately 24°C temperature in the laboratory. Virgin females and bachelor males of both strains were separated under anesthesia within 2–4 h of eclosion to avoid previous exposure of females to male courtship, which may otherwise affect the results[19]. Virgin females and males were kept in separate food vials in batches of ten. This number was fixed to avoid bias due to density effects[20].

Flies were aged for seven days. One day before the experiment, virgins were taken and examined under anesthesia for any obvious morphological distortion that might have taken place during the period of ageing. Only normal flies were used. Depending upon the experimental methods, flies of *D. ananassae* (both males and females) were marked in order to distinguish them from those of *D. pallidosa*. Marking was done by putting a drop of nail polish on the thorax. It has been previously shown that this type of marking has no effect on the mating performance of *D. ananassae* flies[21,22].

In order to study sexual isolation between *D. ananassae* and *D. pallidosa*, different experimental techniques were used.

(i) Multiple-choice: Females and males of both species of *D. ananassae* and *D. pallidosa* were used in equal ratio, i.e. 15 flies of each species and of each sex. The total number of flies in each replicate was 60 and sex ratio was 1 female : 1 male.

(ii) Female-choice: Fifteen females of one species were placed with 15 males of each of two species. The total number of flies in each replicate was 45 and sex ratio was 1 female : 2 males.

(iii) Male-choice: Fifteen males of one species were placed with 15 females of each of two species. The total number of flies in each replicate was 45 and sex ratio was 1 male : 2 females.

(iv) No-choice: The flies were not given a choice and male of one species was confined with females of one species. Thus, four different combinations were carried out in no-choice experiments. The total number of flies in each replicate was 30 and sex ratio was 1 female : 1 male.

Flies were introduced into an Elens–Wattiaux mating chamber[23] without etherization, females were introduced first. Mating was observed for 60 min. When a pair commences mating, it was taken out with an aspirator and kept in separate empty vials[24]. Later, mated types were identified using stereomicroscope. The tests were performed between 7.00 and 11.00 AM in a temperature-controlled room (approximately 24°C) under normal laboratory light conditions.

The number of different mating combinations between *D. ananassae* and *D. pallidosa* is presented in Table 1. There were more homogamic matings than heterogamic matings providing evidence for preferential mating between females and males of the same species. Isolation estimates were calculated for all the four choice sets (Table 1) using the formula of Merrell[25]. Values of isolation estimate are close to zero in all the experimental sets. This implies that sexual isolation between *D. ananassae* and *D. pallidos*a is strong. The χ^2 values calculated for 1 : 1 ratio on marginal totals to assess the relative mating propensity of the two sexes of both species are presented in Table 1. There are significant differences between females and males of the two species in male-choice and female-choice respectively. On the other hand, differences are not significant in multiple- and no-choice experiments. However, *D. ananassae* flies are more successful in mating than *D. pallidosa*. Thus, there is effect of sex ratio on mating propensity of the species studied.

The results revealed the presence of preferential mating between *D. ananassae* and *D. pallidosa* in both directions. Isolation estimate values are close to zero, indicating strong sexual isolation between the two sibling species, which reinforces the previous findings[9,11,26]. In *Drosophila*, sexual isolation is influenced by two major signals, e.g. cuticular hydrocarbons (pheromones) and wing vibration[11,27]. Virgin females and young males produce species-specific pheromones that elicit male courtship. The antennae contain receptors for both auditory as well as chemical stimuli. Males and females receive auditory courtship signals through their antennae whose aristae serve as receptors[5]. Removal of the antennae of females reduces their recep-

Table 1. Number of matings in 60 min in a mating chamber and χ^2 for 1 : 1 ratio on marginal totals to assess relative sexual activity of both sexes of *D. ananassae* (A) and *D. pallidosa* (P) flies under different choice conditions (data based on five replicates)

Experiment		A	P	Total	$\chi^2_{(A, P)}$	$\chi^2_{(A, P)}$	IE
Multiple-choice							
	A	31	1	32			
	P	1	31	32	0	0	0.032
	Total	32	32	–			
Female-choice							
	A	33	0	33	6.60*	0.002	0.081
	P	10	22	32			
	Total	43	22	–			
Male-choice							
	A	48	3	51	2.68	5.44*	0.038
	P	0	30	30			
	Total	48	33	–			
No-choice							
	A	55	0	55	0.07	0.07	0.027
	P	3	55	58			
	Total	58	55	–			

*Significant at $P < 0.05$; df = 1; IE, Isolation Estimate.

tivity to courtship and removes sexual isolation between *D. pseudoobscura* and *D. persimilis*[28,29]. Olfactory signals were one of the sensory bases of mating success associated with genotype frequency in *Drosophila*[30].

In *D. pseudoobscura*, on the basis of chemical cues, females could discriminate males[31,32]. Cuticular hydrocarbon in *D. pallidosa* is (Z,Z)-5,27 tritriacontadiene[33] and in *D. ananassae* is (Z,Z)-5,25-hentricontadiene[34]. The only significant difference in cuticular hydrocarbons contributes to the sexual isolation[35,36]. Female pheromones induce male courtship behaviour in both species. Males of both the species strongly court heterospecific females[11]. Females discriminate courting males by acoustic cues, whether males are conspecific or heterospecific. The result of experiments using wingless males or aristae-less females showed that female sex pheromone was insufficient to isolate these two species sexually, and that the acoustic signals produced by the male wing vibration were critical in achieving sexual isolation between *D. ananassae* and *D. pallidosa*[11]. Courtship songs play an important role in female mate discrimination against courting males. Thus, the mechanism that prevents gene flow between the two species is mate discrimination, and the loci which played a crucial role in the evolution of reproductive isolation, were mapped to distinct position near the *Delta* locus in middle of the left arm of the second chromosome[11].

In the sexual isolation between *D. ananassae* and *D. pallidosa*, we must consider two different situations: choice tests (multiple-, female- and male-choice) where both sexes and species are present, and no-choice test, where there is only one sex of each species. Under conditions of choice, sexual isolation will occur by mate discrimination that is the possibility of comparing and selecting from individuals of both species. Discrimination is mainly based on species-specific chemical, auditory, visual and behavioural signals[37].

In female-choice, interspecific matings are 0.0% (*D. ananassae* females × *D. pallidosa* males) and 13.3% (*D. pallidosa* females × *D. ananassae* males). In male-choice, it is 0.0% (*D. ananassae* females × *D. pallidosa* males) and 4.0% (*D. pallidosa* females × *D. ananassae* males). Thus, this indicates that *D. ananassae* females are less likely to mate with alien males than are *D. pallidosa* females, confirming the previous findings[10,26].

In the present case, *D. ananassae* flies are more active than *D. pallidosa* flies. It has also been reported that being a cosmopolitan species, *D. ananassae* males exhibit high sexual drive and females have a relatively high discriminating capacity[10].

In multiple-choice, where the conditions are like that of nature where both the species are present, their preferential matings are more frequent. Under no-choice test, a female can find a foreign male and sexual isolation will depend fundamentally on the degree to which the sexual behaviour of both species agrees. The factors involved in homospecific mating (male propensity, vigour and female receptivity) must also be involved in heterospecific matings. Thus, isolation between two populations measured by no-choice test would be related to both female receptivity and male mating ability. In male choice, where sex ratio is 1 male : 2 females, females of two species differ in their mating success. Similarly, in female-choice where the sex ratio is 1 female : 2 males, males of the two species differ in their mating success. In contrast, in multiple- and no-choice tests, where the sex ratio is 1 female : 1 male, no

difference was found in mating propensity. Mating propensity is influenced by sex ratio in *D. biarmipes*[14], *D. ananassae*[15] and *D. pseudoobscura*[17], but not in *D. bipectinata*[13]. It has been suggested that the interference between the individuals of the same sex may delay the average time for mating. The pattern of mating is also influenced by different techniques with varying sex ratio. The degree of selective mating between yellow mutant and wild type *D. melanogaster* was measured using the four different techniques. Male- and no-choice tests revealed less isolation than female- and multiple-choices[16].

Thus, sex ratio affects mating propensity and pattern of mating in *Drosophila*, but the results of different studies may vary. Our results provide evidence for strong sexual isolation between *D. ananassae* and *D. pallidosa* and for the effect of sex ratio on mating propensity of the two species.

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