

RESEARCH NOTE

Pattern of mating preference of interspecific hybrid females and phylogeny in the *Drosophila bipectinata* species complex

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Introduction

If one has to understand speciation through hybridization, few models can prove to be better than *Drosophila*. This is not only because it can be cultured in the laboratory and crosses made conveniently but also due to the fact that speciation seems to occur very fast in *Drosophila*, with the genus having a large number of species. All species of *Drosophila*, however, do not share identical relationships and there are some which are more closely related than others, having achieved distinct species status only in the recent past. This turns out to be a boon for speciation biologists who enjoy the rare privilege of catching a glimpse of species still in the act of acquiring a distinct status. In *Drosophila* taxonomy, groups of such very closely related species are called species complexes: recently separated, having achieved just enough divergence to cause reproductive isolation, yet not sufficient to forego hybridization (Kopp and Barmina 2005). Indeed, the possibility of getting hybrids from absolute species opens new prospects for an evolutionary biologist, providing insights into how genomes of two independently diverging lines would function or interact when brought together in an individual.

As it turns out, the *Drosophila bipectinata* species complex, which includes four very closely related species: *D. bipectinata*, *D. parabipectinata*, *D. malerkotliana* and *D. pseudoananassae* is very suitable for carrying out evolutionary studies, involving interspecific hybrids. All the four species occur sympatrically over some parts of their range of distribution: the Oriental–Australian biogeographic zone (Kopp and Barmina 2005). Thus, it is believed that they must have diverged in sympatry and the case being so natural selection played the most prominent role during their divergence.

Being such a favourable model for doing evolutionary studies, this complex has not escaped the interest of evolutionary biologists and much research has been carried out towards working out the phylogenetic relationship among them (Bock 1971a, b, 1978; Jha and Rahman 1972; Tomimura *et al.* 2005; Kopp and Barmina 2005; Mishra and Singh 2006a; Kopp *et al.* 2006; Banerjee and Singh 2012). The fact that accumulation of differences does not stop with the completion of speciation is well proven in this complex as in three of its members, *D. bipectinata*, *D. pseudoananassae* and *D. malerkotliana* subspecies have been discovered (Matsuda *et al.* 2005; Kopp and Barmina 2005), and *D. parabipectinata* stands as the only species in this complex without any subspecies. This is because it is perhaps the youngest among the four and has originated from *D. bipectinata* only in the recent past (Banerjee and Singh 2012).

Speciation is a multidimensional event and certainly when it is the question of four species diverging from each other, the paths taken may not be common. Each species pair may have a different story to tell. For example, in this complex, the cause of hybrid male sterility has been worked out extensively among different species pairs and incompatibility between the X and Y chromosomes, between the X chromosome and an autosome and Y chromosome have been implicated in sterility (Mishra and Singh 2006b, 2007). However, while hybrid studies involving only hybrid males, to learn the details of male sterility, have been done extensively in this species complex, hybrid females though being fertile have not been studied in much detail. There is only one preliminary report where hybrid females were backcrossed to parental males to check whether there was a significant difference in the number of matings with each parental male (Bock 1978). However, the conclusions drawn from this study were not very clear.

The male traits (courtship signals) and recognition of these traits constitute the rituals before the act of mating. They are determined by distinct sets of genes that form their own

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network and the expressions of the genes in a network are sex-limited (Fierst 2013). Mate recognition system is very elaborate in *Drosophila* and is more of a female territory (Laturney and Moehring 2012). It shows a polygenic mode of inheritance and plays an important role in sexual isolation and thereby speciation (Ritchie 1996). Therefore, we were interested in determining how this system is affected in hybrid females. Receptivity and preference are two important attributes of the recognition system and show both intra-specific and interspecific variations. Preference and receptivity functions of hybrids are intermediate between those of the parental species, if they additively inherited (Tomaru *et al.* 2004), while, when the recognition system of one of the parental species is dominant over the other, it shows a more prominent expression in the hybrids. Receptivity may also be a measure of the degree of compatibility of the genomes of parental species, the less the compatibility, the less receptive the females due to distortion in the mate recognition system. The present study aims at testing the mating preference of hybrid daughters from reciprocal crosses involving the four species of this complex.

Materials and methods

Drosophila stocks

The following stocks of the four species were used: *D. bipectinata*-PN 99, *D. parabipectinata*-Mys, *D. malerkotliana*-RC91 and *D. pseudoananassae*-KB284. The strains of *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana* have been collected from various places of India. The strain of *D. pseudoananassae* has been collected from Brunei Island, Brunei. All stocks are being maintained in the laboratory in simple yeast-agar culture medium at ~ 24°C with 12 h cycle of light and darkness.

Experimental design

To obtain hybrids, reciprocal crosses were set up, using 7-day-aged flies, in food vials. Virgin hybrid daughters of reciprocal crosses were collected and aged for 7 days. Meanwhile parental males were also collected and aged for 7 days. Mating preference of hybrid daughters was tested with parental males by following the male choice technique, in which a male of one of the parental species was confined with two hybrid daughters, one each from a reciprocal cross in a food vial. Hybrid daughters of one of the crosses were marked by wing clipping because they are morphologically indistinguishable. Getting hybrids turned out to be very difficult where *D. pseudoananassae* was involved, so in crosses involving *D. pseudoananassae*, a minimum of 24 sets of each male choice cross were set up. In male choice crosses involving other pairs, not less than 50 sets of each male choice cross were set up. After 5 days the males were discarded and the females were dissected to check for

sperms in their ventral receptacle and spermathecae under the microscope. Thus, they were scored as being mated or unmated.

Statistical analysis

Chi square values were calculated from a contingency table, under the assumption of random mating, to check whether there is a significant difference in the number of matings of hybrid daughters derived from reciprocal crosses. Since, in all except two crosses, daughters from reciprocal crosses exhibited no difference in their preference pattern, data across two female types were pooled and chi square values were calculated to see whether the number of matings was significantly different in male choice crosses involving different parental males.

Results

Results of the male choice crosses involving hybrid daughters and parental males are provided in table 1. In crosses involving *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana*, the pattern of mating preference of daughters derived from reciprocal crosses was found to be similar, as the difference in the number of matings with parental males was insignificant ($P > 0.90$). However, the daughters were partial towards one of the parental males as the differences between the total numbers of mated hybrid daughters with each parental male in male choice crosses were found to be highly significant ($P < 0.001$). In the crosses involving *D. pseudoananassae* and the other three species, it was very difficult to get hybrids, especially in those involving *D. pseudoananassae* males (data not shown). Thus, the degree of crossability of *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana* is poor with *D. pseudoananassae*. Daughters derived from reciprocal crosses involving *D. bipectinata* and *D. pseudoananassae* exhibited nonrandom mating with *D. bipectinata* males ($P < 0.001$), with daughters of *D. bipectinata* females and *D. pseudoananassae* males mating more with *D. bipectinata* males, compared to hybrid females from the other reciprocal cross. Similarly, daughters derived from reciprocal crosses involving *D. parabipectinata* and *D. pseudoananassae* exhibited nonrandom mating with *D. pseudoananassae* males ($P < 0.001$), with daughters of *D. parabipectinata* females and *D. pseudoananassae* males preferring *D. pseudoananassae* males, compared to hybrids from the reciprocal cross. This also accounted for a significant difference ($P < 0.05$) between the total number of matings with each parental male, in the species pairs *D. bipectinata*-*D. pseudoananassae* and *D. parabipectinata*-*D. pseudoananassae*. Daughters derived from reciprocal crosses involving *D. malerkotliana* and *D. pseudoananassae*, exhibited random mating with each parental male. However, the number of matings was significantly more ($P < 0.05$) with *D. malerkotliana* males.

Table 1. Number of matings of interspecific hybrids in male choice experiments.

Crosses		n	No. of mating	Chi-square	Total no. of mating	Chi-square
Females (daughters of)	Males					
<i>D. bipectinata</i> (♀) × <i>D. parabipectinata</i> (♂)	<i>D. bipectinata</i>	54	15	03.08	39	13.69**
<i>D. parabipectinata</i> (♀) × <i>D. bipectinata</i> (♂)		54	24			
<i>D. bipectinata</i> (♀) × <i>D. parabipectinata</i> (♂)	<i>D. parabipectinata</i>	59	36	00.28	75	
<i>D. parabipectinata</i> (♀) × <i>D. bipectinata</i> (♂)		59	39			
<i>D. bipectinata</i> (♀) × <i>D. malerkotliana</i> (♂)	<i>D. bipectinata</i>	55	35	00.14	72	11.82**
<i>D. malerkotliana</i> (♀) × <i>D. bipectinata</i> (♂)		55	37			
<i>D. bipectinata</i> (♀) × <i>D. malerkotliana</i> (♂)	<i>D. malerkotliana</i>	70	26	02.34	61	
<i>D. malerkotliana</i> (♀) × <i>D. bipectinata</i> (♂)		70	35			
<i>D. parabipectinata</i> (♀) × <i>D. malerkotliana</i> (♂)	<i>D. parabipectinata</i>	61	17	0.03	35	30.59**
<i>D. malerkotliana</i> (♀) × <i>D. parabipectinata</i> (♂)		61	18			
<i>D. parabipectinata</i> (♀) × <i>D. malerkotliana</i> (♂)	<i>D. malerkotliana</i>	54	01	00	02	
<i>D. malerkotliana</i> (♀) × <i>D. parabipectinata</i> (♂)		54	01			
<i>D. bipectinata</i> (♀) × <i>D. pseudoananassae</i> (♂)	<i>D. bipectinata</i>	31	25	11.32**	32	6.16*
<i>D. pseudoananassae</i> (♀) × <i>D. bipectinata</i> (♂)		31	07			
<i>D. bipectinata</i> (♀) × <i>D. pseudoananassae</i> (♂)	<i>D. pseudoananassae</i>	29	16	02.14	17	
<i>D. pseudoananassae</i> (♀) × <i>D. bipectinata</i> (♂)		29	11			
<i>D. parabipectinata</i> (♀) × <i>D. pseudoananassae</i> (♂)	<i>D. parabipectinata</i>	28	03	00.58	08	4.31*
<i>D. pseudoananassae</i> (♀) × <i>D. parabipectinata</i> (♂)		28	05			
<i>D. parabipectinata</i> (♀) × <i>D. pseudoananassae</i> (♂)	<i>D. pseudoananassae</i>	24	14	16.20**	15	
<i>D. pseudoananassae</i> (♀) × <i>D. parabipectinata</i> (♂)		24	01			
<i>D. malerkotliana</i> (♀) × <i>D. pseudoananassae</i> (♂)	<i>D. malerkotliana</i>	30	15	02.50	24	4.00*
<i>D. pseudoananassae</i> (♀) × <i>D. malerkotliana</i> (♂)		30	09			
<i>D. malerkotliana</i> (♀) × <i>D. pseudoananassae</i> (♂)	<i>D. pseudoananassae</i>	34	09	0.30	16	
<i>D. pseudoananassae</i> (♀) × <i>D. malerkotliana</i> (♂)		34	07			

n, total number of females tested; *, $P < 0.05$; **, $P < 0.001$.

Discussion

Pattern of mating preference of hybrid daughters derived from reciprocal interspecific crosses involving *D. bipectinata*, *D. parabiepectinata* and *D. malerkotliana* was found to be similar which provides evidence that mate recognition in these species has no influence of the sex of parental species. In other words, the genes responsible for mate recognition do not show any maternal effect. The fact that the daughters of the reciprocal crosses among these species prefer one of the parental males better over the other proves that the genome of one of the parental species is dominant over the other. In our previous study, it was postulated that between *D. bipectinata* and *D. parabiepectinata*, *D. bipectinata* is the ancestral species and *D. parabiepectinata* is the derived one (Banerjee and Singh 2012). Our hypothesis was based on the model proposed by Watanabe and Kawanishi (1979), which says that it is the derived females which discriminate against ancestral males, for maintaining the integrity of the gene pool in the face of invasion from the ancestral population. Thus, while the ancestral females have the capacity to recognize derived males (*D. bipectinata* females mate randomly with males of *D. parabiepectinata* and those of its own species), the derived females are more strict when it comes to mate recognition (*D. parabiepectinata* females prefer males of its own species than *D. bipectinata* males). In the present study, it was found that hybrid daughters from reciprocal crosses prefer *D. parabiepectinata* over *D. bipectinata*. Hybrid daughters have both the relaxed recognition system of *D. bipectinata* (in terms of recognizing *D. parabiepectinata*) and the rigid recognition system of *D. parabiepectinata* (in terms of recognizing *D. bipectinata*). We believe that in the hybrid females, the relaxed recognition system being more ancient is dominant over the strict system. Thus, in terms of the ability to recognize or the strictness in recognizing a mate the daughters behave more like the ancestral parent, *D. bipectinata*, ironically in doing so they do not find the ancestral male suitable as a mate. There is a possibility that the recognition system of *D. parabiepectinata* is dominant but we believe that since the same of *D. bipectinata* is more ancient, it is playing a more prominent role in a hybrid daughter, as far as recognition is concerned.

To continue the story further, daughters of *D. bipectinata* and *D. malerkotliana* are partial towards *D. bipectinata*. In our previous study, it was found that the pair exhibited symmetrical sexual isolation, and thus it was postulated that *D. bipectinata* and *D. malerkotliana* have come from a common ancestor. The fact that the hybrid daughters recognize *D. bipectinata* males better over *D. malerkotliana*, indicates that the genes responsible for mate recognition are more dominating in *D. bipectinata*.

The daughters of *D. parabiepectinata* and *D. malerkotliana* have poor receptivity, as they mate infrequently with both the parental males, worse with *D. malerkotliana* males. This indicates that though fertile, their mate recognition system is affected severely due to hybridization. Therefore, the

genomes of *D. parabiepectinata* and *D. malerkotliana* are incompatible. Behaviourally, the pair exhibit asymmetrical isolation (Banerjee and Singh 2012), so they ought to be close. However, the effect on mate recognition system indicates otherwise. In this study, the hybrid daughters were found to mate significantly more with *D. parabiepectinata* males than *D. malerkotliana*. Thus, the mate recognition system of the hybrid daughters is similar to *D. parabiepectinata*. Here, we have not explained our results as done for the species pair *D. bipectinata*–*D. parabiepectinata* because being an ancestral-derived species pair, their mate recognition systems must have coevolved under such pressure. However, in case of *D. parabiepectinata* and *D. malerkotliana*, the mate recognition systems must have evolved independently. Moreover, a smaller effective population size in *D. parabiepectinata* may also be responsible for greater genetic divergence between *D. parabiepectinata* and *D. malerkotliana* than *D. malerkotliana* and *D. bipectinata*, as proven in the study by Kopp and Barmina (2005). The present study has led us to give a different explanation for the asymmetrical sexual isolation observed between this species pair. While sexual isolation is the first to arise in the process of speciation (Nanda and Singh 2012), it need not be strengthened once other mechanisms take over to bring about complete reproductive isolation. Thus, asymmetrical isolation arose earlier during the process of speciation and was not strengthened further (leading to symmetry), while divergence at other loci continued, with the result that though the pair has accumulated enough incompatibilities, it still shows asymmetrical reproductive isolation.

It was very difficult to hybridize *D. pseudoananassae* with the other three species of the complex. It has been proven earlier in our laboratory that the degree of crossability is poor when *D. pseudoananassae* is one of the parent (Mishra and Singh 2006a). When *D. pseudoananassae* was the male parent, obtaining progeny turned out to be all the more difficult. Thus, the courtship rituals of *D. pseudoananassae* has diverged to such an extent that the females of the other three species recognize it with difficulty. It may also be possible that the females of *D. bipectinata*, *D. parabiepectinata* and *D. pseudoananassae* have evolved a hostile reproductive tract environment, killing sperm, from the males of a distantly related species such as *D. pseudoananassae*.

Daughters of *D. bipectinata* females \times *D. pseudoananassae* males mate more with *D. bipectinata* males than the daughters of the other reciprocal cross. The possible explanation for this can be that there is some maternal effect associated with *D. pseudoananassae* females which does not allow daughters of *D. pseudoananassae* females \times *D. bipectinata* males to recognize *D. bipectinata* males. Indeed, maternal effect for female preference has been reported by Butlin and Ritchie (1989). Similar is the case with the species pair *D. pseudoananassae*–*D. malerkotliana*, though here the difference between the numbers of matings in reciprocal crosses with *D. malerkotliana* males is not statistically significant.

Daughters of *D. parabiepectinata* females × *D. pseudoananassae* males mate more with *D. pseudoananassae* males than the daughters of the other reciprocal cross. Thus, the maternal effect associated with *D. pseudoananassae* females does not play any role here and this preference pattern cannot be explained with any possible hypothesis and needs further investigations.

This study is one of its kind, as the rich literature concerning *Drosophila* speciation and hybridization studies has no mention of this type of study on mating pattern of hybrid females except for a study by Tomaru *et al.* (2004) in the *melanogaster* species complex involving the species *D. sechellia*, *D. melanogaster* and *D. simulans*. Though this study is not sufficient for drawing a solid conclusion regarding the phylogeny, it does give an idea about the relationship shared by each species pair in the *biepectinata* complex.

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