Genetic interactions underlying hybrid male sterility in the *Drosophila bipectinata* species complex

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Understanding genetic mechanisms underlying hybrid male sterility is one of the most challenging problems in evolutionary biology especially speciation. By using the interspecific hybridization method roles of Y chromosome, Major Hybrid Sterility (MHS) genes and cytoplasm in sterility of hybrid males have been investigated in a promising group, the *Drosophila bipectinata* species complex that consists of four closely related species: *D. pseudoananassae*, *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana*. The interspecific introgression analyses show that neither cytoplasm nor MHS genes are involved but X-Y interactions may be playing major role in hybrid male sterility between *D. pseudoananassae* and the other three species. The results of interspecific introgression analyses also show considerable decrease in the number of males in the backcross offspring and all males have atrophied testes. There is a significant positive correlation between sex - ratio distortion and severity of sterility in backcross males. These findings provide evidence that *D. pseudoananassae* is remotely related with other three species of the *D. bipectinata* species complex.

**Key words:** *D. bipectinata* species complex, genetic interactions, hybrid male sterility

**INTRODUCTION**

Recent resurgence of interest on speciation has unfolded several important mysteries, particularly of reproductive isolation in *Drosophila* (Singh, 1994; Laurie, 1997; Singh and Kulathinal, 2000; Sawamura and Tomaru, 2002; Coyne and Orr, 2004; Orr, 2005; Mishra and Singh, 2005a). However, several milestone achievements are still awaiting and one of them is the genetic interactions underlying hybrid male sterility. Each species exists with its own history of accumulated genetic modifications in course of its evolution. Therefore, in interspecific hybrids, the coexistence of genomes from two different species may results into genetic incompatibilities i.e., any kind of deviation from perfect development of individuals with mixed genomes (Sawamura, 1999) that may lead to hybrid sterility or inviability. To understand the genetic mechanisms underlying hybrid male sterility or inviability, interspecific introgression has been the most favoured approach in different model systems like *Drosophila*, mice, rice etc. (Matsubara et al., 2003; Oka et al., 2004; Mishra and Singh, 2005a). In *Drosophila*, although X-autosome, X-Y, Y-autosome and other interactions such as polygenic interactions and role of Major Hybrid Sterility (MHS) genes in hybrid male sterility are documented, it is difficult to assign any particular interaction for a particular species cross because different species behave differently for such interactions (Mishra and Singh, 2005a). Even among closely related species the genetic interactions underlying hybrid male sterility may vary (Mishra and Singh, 2005b). Therefore, investigations on genetic interactions involved in hybrid male sterility in closely related species is continued as a favoured experimental approach. Sex chromosomes have predominant role in hybrid male sterility (Mishra and Singh, 2005a). Further, interactions between X and Y chromosomes and their individual interactions with autosomes are important in understanding the genetic basis of hybrid male sterility and inviability (Sawamura et al., 2004; Mishra and Singh, 2005a). Recent views on the genetics of hybrid male sterility are broadly categorized into two schools: one believes in polygenic interactions, which include two variants viz strong (identity of genes have little importance - Naveira and Maside, 1998; Carvajal et al., 1996) and weak (identity of genes are important because some genes cause hybrid problems but others do not - Wu et al., 1996), and the other believes in genes of major effect (Orr, 1992). Therefore, role of Major Hybrid
Sterility (MHS) genes in hybrid male sterility becomes important for either confirming or ruling out the involvement of genes of major effect. The role of cytoplasm has also been reported in hybrid male sterility in Drosophila (Ehrman, 1963; Orr, 1989). Sterility in hybrid males has been correlated with segregation distortion that turned out into sex - ratio distortion (Tao et al., 2001; Orr and Irving, 2005). Further, spermatogenesis is one of the most sensitive processes of development in hybrids (Presgraves and Orr, 1998; Laurie, 1997; Sawamura, 1999), and the degree of perturbation during spermatogenesis is correlated with sterility in hybrid males that is reflected through the morphology of testis (Dobzhansky, 1951). If the degree of perturbation is higher during spermatogenesis, no sperm will be formed and testes will be atrophied (Mishra and Singh, 2005b). However, Y chromosome in hybrid male sterility has been correlated with segregation distortion that turned out into sex - ratio distortion (Tao et al., 2001; Orr and Irving, 2005). Further, spermatogenesis is one of the most sensitive processes of development in hybrids (Presgraves and Orr, 1998; Laurie, 1997; Sawamura, 1999), and the degree of perturbation during spermatogenesis is correlated with sterility in hybrid males that is reflected through the morphology of testis (Dobzhansky, 1951). If the degree of perturbation is higher during spermatogenesis, no sperm will be formed and testes will be atrophied (Mishra and Singh, 2005b).

The Drosophila bipectinata complex consists of four closely related species – D. bipectinata Duda 1923, D. parabipectinata Bock 1971, D. malerkotliana Parshad and Paika 1964, and D. pseudoananassae Bock 1971, where females are indistinguishable and males can be differentiated by their sex comb pattern and abdominal tip pigmentation (Bock, 1971,1978; Bock and Wheeler, 1972; Singh and Singh, 2001; Mishra and Singh, 2005b; Kopp and Barmina, 2005; Tomimura et al., 2005). Based on this colouration, two subspecies of D. malerkotliana (D. m. malerkotliana – black abdominal tip and D. m. pallens – yellow abdominal tip), and D. pseudoananassae (D. p. pseudoananassae – yellow abdominal tip and D. p. nigrens – black abdominal tip) have been reported (Bock, 1971; Okada, 1981; Singh and Singh, 2001). Recently, two subspecies (Asian and Pacific) of D. bipectinata have been reported, which show partial reproductive isolation (Kopp and Frank, 2005). However, Matsuda et al. (2005) have described three subspecies of D. bipectinata viz. D. bipectinata bipectinata (population from Southeast Asia), D. b. szenitancii (population from Papua New Guinea) and D. b. pacificiae (population from South Pacific Ocean). The D. bipectinata species complex is unique in having phylogenetically two very closely related species (D. bipectinata and D. parabipectinata), third species (D. malerkotliana) being closer to first two species, and fourth species (D. pseudoananassae) being distantly related with the other three species (Bock, 1971; Yang et al., 1972; Mishra and Singh, 2006). These features make the D. bipectinata species complex an interesting model system for evolutionary studies with particular reference to speciation. In the interspecific hybrids, F1 females are fertile while males are sterile (Bock, 1978; Mishra and Singh, 2006). Investigation on genetic basis of hybrid male sterility among D. bipectinata, D. parabipectinata and D. malerkotliana revealed that there is no role of MHS genes and cytoplasmic factors in sterility of hybrid males (Mishra and Singh, 2005b). However, Y chromosome is involved in hybrid male sterility in the cross between D. bipectinata and D. parabipectinata but not between D. bipectinata and D. malerkotliana, and D. parabipectinata and D. malerkotliana (Mishra and Singh, 2005b). The genetic interactions underlying hybrid male sterility of D. pseudoananassae with the other three species are unknown. Therefore, the involvement of genetic interactions in hybrid male sterility between D. pseudoananassae and the other three species has been undertaken during the present study. The roles of Y chromosome, MHS genes and cytoplasmic factors in hybrid male sterility of crosses between D. pseudoananassae and the other three members of this complex are investigated.

MATERIALS AND METHODS

The strains of the four species employed in the present study are D. bipectinata Pune, D. parabipectinata Mysore, D. malerkotliana malerkotliana Raichur (all three from India) and D. pseudoananassae nigrens KB284 (collected from Brunei in 2003). They are maintained in the laboratory on simple yeast – agar culture medium at approximately 24°C. For hybridization, 5–7 virgin females (7 days old) of one species were kept with 5–7 bachelor males of another species in a food vial of 3" (height) x 1" (diameter). The schemes and methods used in the present investigations are exactly the same as performed in the previous study (Mishra and Singh, 2005b). Additionally, sex ratio was scored in the offspring of first- and second - generation backcrosses.

RESULTS

Role of Y chromosome in hybrid male sterility

The essence of this hybridization scheme is to put the Y chromosome of one species into the genetic background of another species through interspecific introgression.

D. pseudoananassae and D. bipectinata species pair

To introgress the Y chromosome of D. pseudoananassae into the genetic background of D. bipectinata, females of D. bipectinata (bi) were crossed with males of D. pseudoananassae (ps). The F1 females (bi ps) were then backcrossed to the D. pseudoananassae males. The BC1 males obtained from this backcross were assessed for fertility, where 28% males were found fertile (Table 1). The BC1 males (bi ps ps) contained Y chromosome from D. pseudoananassae while cytoplasm from D. bipectinata and a combination of autosomes from both D. bipectinata and D. pseudoananassae but X chromosome recombiant of D. bipectinata or D. pseudoananassae. These males were again backcrossed to the D. bipectinata females and the second - generation backcross (BC2) males (bi – bi ps ps) were assessed for fertility. They carry Y chromosome from D. pseudoananassae, and X chromosome and cyto-
plasm from *D. bipectinata*. All of them were sterile and had atrophied testes (Table 1) blocking further attempts to pursue the next generation backcrosses. In introgressing the Y chromosome of *D. bipectinata* into the genetic background of *D. pseudoananassae*, the BC2 males (ps-psbibi) were also sterile and had atrophied testes indicating the involvement of Y chromosome in hybrid male sterility (Table 1).

**D. pseudoananassae and D. parabipectinata species pair** The introgression of Y chromosome of *D. pseudoananassae* into the genetic background of *D. parabipectinata* was performed by hybridizing the females of *D. parabipectinata* (pa) to the males of *D. pseudoananassae* and then backcrossing the F1 females (pa ps) to *D. pseudoananassae* males. The number of fertile BC1 males (pa ps ps) was 26% (Table 1). These males were again backcrossed to *D. parabipectinata* females to obtain the second - generation backcross (BC2) males (pa – pa ps ps). All BC2 males were sterile with atrophied testes (Table 1). Therefore, further backcross was not carried out. When Y chromosome of *D. parabipectinata* is introgressed into the genetic background of *D. pseudoananassae*, the BC2 males (ps-pspapa) also had atrophied testes and were sterile suggesting the role of Y chromosome in hybrid male sterility.

**D. pseudoananassae and D. malerkotliana species pair** *D. malerkotliana* (ma) females were crossed with males of *D. pseudoananassae* to introgress the Y chromosome of *D. pseudoananassae* into the genetic background of *D. malerkotliana*. The F1 females (ma ps) were backcrossed to the males of *D. pseudoananassae* in the same way as previously performed and backcross males (ma ps ps) were assessed for male fertility (Table 1). The percentage number of fertile BC1 males was 34 (Table 1). These males were again backcrossed to females of *D. pseudoananassae* in the same way as previously performed and backcross males (ma ma ps) were assessed for male fertility (Table 1). The percentage number of fertile BC1 males was 26% (Table 1). These males were again backcrossed to females of *D. malerkotliana* to obtain BC2 offspring. All the BC2 males (ma-ma ps ps) were sterile and had atrophied testes, which did not permit further backcrosses (Table 1). When Y chromosome of *D. malerkotliana* is introgressed into the genetic background of *D. pseudoananassae*, the BC2 males (ps-psmama) were also sterile having atrophied testes (Table 1). Therefore, Y chromosome has role in sterility of hybrid males between *D. pseudoananassae* and *D. malerkotliana*.

**Role of cytoplasm in hybrid male sterility** The esse-
nce of the scheme of cytoplasmic introgression is to mod-
ulate the hybridization in such a way that entire nuclear
genome of maternal species can be replaced with that of
paternal species so that the cytoplasmic compatibility of
maternal species can be assessed with the nuclear
genome of paternal species.

**D. pseudoananassae and D. bipectinata species pair**

To introgress the nuclear genome of *D. bipectinata*
into the cytoplasmic background of *D. pseudoananassae*,
the females of *D. pseudoananassae* were crossed to the
males of *D. bipectinata* and the F₁ females (ps bi) obtained
were backcrossed to the *D. bipectinata* males. In this way, the backcross males and females (BC₁) have
cytoplasm from *D. pseudoananassae* and nuclear genome
from both *D. pseudoananassae* and *D. bipectinata*. The
BC₁ females were then recurrently backcrossed to the *D.
bipectinata* (paternal species) males. Since the females
always remained from the maternal species (*D. pseudoa-
nanassae*), the cytoplasmic part always comes from *D.
pseudoananassae* but the nuclear genome from *D. bipec-
тинata* gradually replaces the nuclear genome of *D.
pseudoananassae* in the subsequent backcross genera-
tions. The male fertility is assessed in the backcross gen-
erations (psbibi2, psbibi3, and psbibi4), where fertility of
males was found to increase in the subsequent genera-
tions and in fourth - generation backcross all males were
fertile (Fig. 1). There was no loss of fertility if the flies
(bipsps4) were sib mated for next three generations. In
the reciprocal cross also the fourth backcross generation
flies (psbibi4) were completely fertile and there was no
effect of cytoplasmic introgression (Fig. 1). Therefore,
cytoplasmic factors are compatible between *D. pseudoa-
nanassae* and *D. bipectinata*.

**D. pseudoananassae and D. parabipectinata**
The nuclear genome of *D. parabipectinata* can be introgressed
into the cytoplasmic background of *D. pseudoananassae*
by crossing the females of *D. pseudoananassae* to the
males of *D. parabipectinata*. The F₁ females (ps pa) were
backcrossed to the *D. parabipectinata* males to introgress
the nuclear genome of *D. parabipectinata* into the cyto-
plasmic background of *D. pseudoananassae*. The off-
spring obtained from the backcross were BC₁ (ps pa pa)
. The BC₁ females were again backcrossed to the males of *D. parabipectinata* and fertility of BC₂ males
(pspapa2) was assessed. The recurrent hybridization of
the backcross females to the *D. parabipectinata* males
was carried out. In fourth - generation backcross, all
males were completely fertile (Fig. 1). The fourth gener-
ation offspring (ps pa pa 4) were sib mated for the next
three generations and no loss of fertility was found. The

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**Fig. 1.** Fertility of backcross males having heterospecific cytoplasm. Crosses between A. female *D. bipectinata* and male *D.
pseudoananassae*, B. female *D. pseudoananassae* and male *D. bipectinata*, C. female *D. parabipectinata* and male *D.
pseudoananassae*, D. female *D. pseudoananassae* and male *D. parabipectinata*, E. female *D. malerkotliana* and male *D.
pseudoananassae*, F. female *D. pseudoananassae* and male *D. malerkotliana*. 
Hybrid male sterility in the *D. bipectinata* species complex

The reciprocal cross also showed the similar results (almost all the papsp4 males were fertile) suggesting that cytoplasmic factors are compatible between *D. pseudoananassae* and *D. parabipectinata*. **D. pseudoananassae** and **D. malerkotliana** As performed in the previous crosses, females of the maternal species i.e., *D. pseudoananassae* were crossed with the males of the paternal species i.e., *D. malerkotliana* and the F1 females were recurrently backcrossed to the paternal species males. In this cross also the fourth - generation backcross males (pamama4) were fertile and there was no loss of fertility after sib mating for the next three generations (Fig. 1). The reciprocal cross also showed compatibility in cytoplasmic factors between *D. pseudoananassae* and *D. malerkotliana* (Fig. 1). Therefore, cytoplasmic factors are not involved in hybrid male sterility in the cross between *D. pseudoananassae* and *D. malerkotliana*.

**Role of MHS genes in hybrid male sterility** Zeng and Singh (1995) have introduced a scheme to determine the role of any X–linked or dominant autosomal MHS genes in hybrid male sterility, which is shown in Fig. 2.

According to this scheme, females of one species were crossed with males of another species. The F1 females were then backcrossed to the males of maternal species. In the first -generation backcross (BC1) offspring, individual females were crossed with multiple males to obtain BC2 generation flies. Each BC1 female will produce a matriarchal family and thus a number of matriarchal families were established. Fertility of sons (BC2) from each family has been scored, which indicates whether or not the mother (BC1) of the family carried any MHS gene. If mother carried MHS genes, half of her sons would receive the gene and become sterile. Similarly, half of her daughters will also receive the gene and will carry it to the next generation. The daughters (BC2) of one of the sterility –gene –carrying mothers were then individually backcrossed to multiple maternal species males and their sons (BC3) were assessed for fertility. If 50% or more BC3 males were sterile then daughters from the BC3 families were again individually crossed to the multiple males of maternal species. If MHS genes are playing any role in hybrid male sterility then in the subsequent backcross generations, 50% of the males will be sterile, as daughters will carry 50% of the genome to the next generation.

We followed the same scheme to test the involvement of MHS genes in sterility of hybrid males in the crosses of *D. pseudoananassae* with other three species of the *D. bipectinata* complex. Females of the maternal species (*D. pseudoananassae*) were crossed to the males of paternal species (either *D. bipectinata*, *D. parabipectinata* or *D. malerkotliana*). The F1 females were then backcrossed to the males of *D. pseudoananassae*. The BC1 females obtained were then individually backcrossed to multiple males of *D. pseudoananassae* as performed among *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana* (Mishra and Singh, 2005b). Interestingly, in crosses between *D. pseudoananassae* females and *D. bipectinata* males, 21 out of 50 BC1 females produced more than 50% BC2 sterile sons (see Appendix). However, when the BC2 females from those vials where 50% or more males were sterile have been individually backcrossed to *D. pseudoananassae* males, the BC3 offspring had less than 50% sterile males. Only 4 vials out of 20 showed about 40% sterile sons. BC3 females from these three vials were again backcrossed to the *D. pseudoananassae* males and fertility of BC4 males were assessed. Almost all the BC4 males were fertile (see Appendix). Therefore, involvement of MHS genes in hybrid male sterility between *D. pseudoananassae* and *D. bipectinata* has been ruled out. However, no backcross progeny (BC2) were obtained (50 vials tested) in the cross between *D. parabipectinata* and *D. pseudoananassae* (pa –papsps) and *D. malerkotliana* and *D. pseudoananassae* (ma – mapsps), which blocks further backcrosses. Therefore, role of MHS genes among hybrids of *D. pseudoananassae*, *D. parabipectinata* and *D. malerkotliana* could not be tested.

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Fig. 2. Scheme for testing the role of MHS genes in hybrid male sterility.

* 1 denotes one species while 2 denotes another species
Sex-ratio distortion In each of the four species, the sex ratio is 1:1. In the hybrids of *D. pseudoananassae* with the other three species of the *D. bipectinata* complex, sex ratio (female: male) was scored in the first- and second-generation backcross offspring and fertility of males in both backcross generations were also scored. When *D. pseudoananassae* was female parent and *D. parabipectinata* was male parent, the BC2 offspring (ps-ps pa pa) contained almost all females (Table 1). The reciprocal cross also showed the similar results (pa – pa ps ps) (Table 1). The number of BC2 males was four to ten fold lesser than the number of females in the crosses between *D. bipectinata* and *D. pseudoananassae*, and *D. malerkotliana* and *D. pseudoananassae* (Table 1). However, the sex ratio was almost 1:1 if the BC2 females were either ‘ps pa pa 2’, ‘pa ps ps 2’, ’ma ps ps 2’, ‘ps ma ma 2’, ‘bi ps ps 2’ or ‘ ps bi bi 2’. It shows that the incompatibilities were much higher when the Y chromosome was introgressed than when cytoplasm was introgressed into alien species. Further, the number of fertile males was also higher in case of cytoplasmic introgression than in case of Y chromosome introgression (e.g., ps pa pa 2 vs. ps –ps pa pa) (Table 1). The presence of atrophied testes in BC2 males when Y chromosome was introgressed also reflects the higher perturbation during spermatogenesis. Thus, the severity of sterility and sex-ratio distortion were higher in backcross generations when Y chromosome was introgressed. It is also suggested that there is higher incompatibilities between *D. pseudoananassae* and the other three species of the *D. bipectinata* complex, which is reflected through morphology of testes and sex-ratio distortion.

**DISCUSSION**

*D. pseudoananassae* is phylogenetically distantly related with the other three species (*D. bipectinata*, *D. parabipectinata* and *D. malerkotliana*) of the *D. bipectinata* complex (Mishra and Singh, 2006) and therefore the degree of genetic incompatibility between *D. pseudoananassae* and the other three species is supposed to be higher. Genetic incompatibility between species may result into hybrid sterility and inviability (Sawamura, 1999). To understand the mechanisms of hybrid male sterility, interspecific introgressions between *D. pseudoananassae* and the other three species were performed and the results obtained are used to discuss the putative role of Y chromosome, MHS genes and cytoplasmic factors in sterility of hybrid males. Interestingly, the interspecific introgression of Y chromosome between *D. pseudoananassae* and the other three species causes considerable decrease in the number of males in backcross offspring (Table 1). This sex-ratio distortion is correlated with genetic incompatibility between species and also perturbation in spermatogenesis leading to sterility in backcross males (Table 1).

The results of all reciprocal crosses between *D. pseudoananassae* and the other three species showed that Y chromosome is involved in hybrid male sterility (Table 1). There may be three possible interactions of Y chromosome: X-Y, Y-autosome and Y-cytoplasm. Since F1 males carry a haplo–set of conspecific autosomes to the Y chromosome, they should be fertile if there would be Y-dominant autosomal interactions in hybrid male sterility. It is evident from Fig. 1 that cytoplasmic factors are compatible among the species. Therefore, Y-cytoplasm interactions are also ruled out. Hence, the plausible interactions causing hybrid male sterility are X–Y interactions or Y-recessive autosomal interactions. It is also supported by the description of Zeng and Singh (1993) who suggested the scheme (used for introgression of Y chromosome) as a general method for assessing the role of X-Y interactions in hybrid male sterility. In the crosses between *D. pseudoananassae* and *D. bipectinata*, *D. pseudoananassae* and *D. parabipectinata*, and *D. pseudoananassae* and *D. malerkotliana*, there was no effect of cytoplasm in hybrid male sterility (Fig. 1). If cytoplasmic incompatibilities had role in hybrid male sterility then percentage of fertile males would have decreased in the subsequent backcross generations. However, we found that fertility of males increased in backcross generations (Fig. 1). Further, no MHS genes were detected in hybrid male sterility between *D. pseudoananassae* and *D. bipectinata*, although we could not test MHS genes effect in the other species crosses. The other possible genetic interactions that may underlie in hybrid male sterility are X-autosome and autosome-autosome interactions and the involvement of polygenes. The autosome-autosome interactions are not a very common cause of hybrid male sterility but it is reported in *D. hydei* and *D. neohydei* (Schäfer, 1978). Therefore, its role needs to be tested before it is ruled out in the hybrids of *D. pseudoananassae* with other three species of the *D. bipectinata* complex. Polygenes may cause sterility in hybrid males either through their additive effects (Naveira and Maside, 1986) or disruption of synergistic interactions (Pantazidis et al., 1993; Palopoli and Wu, 1994; Sawamura et al., 2004).

Sterile males of the *D. bipectinata* complex hybrids contain either testes of normal size with immotile sperm or atrophied testes without any sperm and the severity of perturbation of spermatogenesis is reflected through the testis size i.e., atrophied testes have higher perturbation than testes of normal size with immotile sperm (Mishra and Singh, 2006). Among the four species, *D. pseudoananassae* is phylogenetically remotely related with the other three species and F1 hybrid males obtained by
crossing D. pseudoananassae with the other three species contained atrophied testes. On the other hand, F1 hybrid males of the crosses among D. bipectinata, D. parabipectinata and D. malerkotliana contained testes of normal size but with immotile sperm (Mishra and Singh, 2006). From these results, it is inferred that species hybrids with higher genetic compatibilities have lesser perturbance in spermatogenesis and species hybrids with lesser genetic compatibilities have higher disturbance in spermatogenesis. Similarly, it is also inferred that the Y chromosome of D. pseudoananassae is genetically more incompatible to other three species than the incompatibilities of Y chromosome of other three species among themselves as in the former case testes were atrophied in second generation backcross males (Table 1) while in the latter case the second generation backcross males contained testis of normal size with immotile sperm (Mishra and Singh, 2006).

It is evident from the results presented in Table 1 that in the cross between D. bipectinata females and D. pseudoananassae males, the sex ratio (♀/♂) is 2.55 in BC1 generation and the number of fertile males is 28% (bi ps ps). When there was cytoplasmic introgression, the sex ratio was decreased to nearly 1.1 in the BC2 generation (bi ps ps 2) flies and fertility of males increased to 78%. Contrary to this, when Y chromosome was introgressed in BC2 generation, the sex ratio increased to nearly 7 while the number of fertile males (bi-bi ps ps) decreased to zero. The same relationship is found in the backcrosses with other species (Table 1). Therefore, it is concluded that there is a negative correlation between the fertility of males and sex-ratio distortion in the flies of backcross generations or a positive correlation between severity of sterility of males and sex - ratio distortion in backcross flies. This biased sex ratio may be due either to inviability of males, sex chromosome segregation distortion or sex transformation i.e., males may be transformed into females. Nevertheless, these possibilities are still to be tested. Our findings, however, agree with the results obtained in hybrids of D. mauritiana and D. simulans (Tao et al., 2001), and D. pseudoobscura Bogota and D. pseudoobscura USA (Orr and Irving, 2005).

Interestingly, such sex-ratio distortion was not observed in the backcross offspring in the crosses among D. bipectinata, D. parabipectinata and D. malerkotliana even in the crosses between D. bipectinata and D. parabipectinata where all the backcross males were sterile (data not shown). Therefore, the main reason behind sex-ratio distortion may be the higher genetic incompatibilities between D. pseudoananassae and the other species.

Based on the results reported earlier (Mishra and Singh, 2005b) and obtained during the present study, it can be concluded that in the D. bipectinata species complex, 1) there is no or little role of MHS genes or cytoplasmic factors in hybrid male sterility, 2) Y chromosome may have key role in hybrid male sterility in the crosses between D. bipectinata and D. parabipectinata, and D. pseudoananassae and other three species (however, there is no role of Y chromosome in hybrid male sterility in the crosses between D. bipectinata and D. malerkotliana, and D. parabipectinata and D. malerkotliana), 3) the genetic incompatibility among species is correlated with degree of perturbance of spermatogenesis that is reflected through testes morphology and presence and motility of sperm, and 4) the sex-ratio distortion of backcross males is positively correlated with severity of male sterility.

Therefore, these findings reinforce that D. pseudoananassae is phylogenetically remotely related with other three species and the X-Y interaction may be playing major role in male sterility of its hybrids with other three species. These investigations provide a platform to pursue the finer details of causes of hybrid male sterility and sex-ratio distortion at cytogenetics and molecular level and to understand the evolutionary mechanisms of speciation in the D. bipectinata species complex.

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APPENDIX

For testing the involvement of MHS genes in hybrid male sterility, the raw data is shown below:

The “% sterile males (total number of males)” in different generations of backcrosses in the hybrid of D. pseudoobscura and D. bipectinata are as follows:

**BC2 males:** % sterile males (total number of males)

20.7(53), 22.6(53), 28.3(53), 35.0(60), 36.1(61), 37.3(83), 38.1(76), 38.1 (76), 38.6(132), 39.1(64), 39.5(81), 39.6(64), 40.0(70), 41.0(67), 42.4(92), 42.2(102), 42.9(70), 43.1(65), 43.5(92), 44.9(84), 44.7(85), 44.7(85), 44.7(94), 46.3(105), 47.5(61), 48.1(81), 48.1(81), 48.3(87), 49.3(75), 53.3(90), 53.8(106), 56.4(94), 56.8(118), 57.6(93), 57.9(83), 57.9(83), 57.9(83), 57.9(83), 58.2 (91), 58.5 (83), 58.5 (83), 58.5 (83), 58.5 (83), 60.3(73), 60.6 (109), 61.8(76), 61.8 (76), 64.5(76), 64.9(94), 65.0 (60), 67.1(70), 68.7(83), 79.2(53).

**BC3 males:** % sterile males (total number of males)

0(16), 0(22), 0(24), 0(67), 3.1(32), 3.2(63), 7.4(27), 12.5(48), 15.6(45), 15.9(63), 25.0 (36), 24.5(47), 30.9(42), 31.3(48), 35.9(39), 38.3(60), 43.7(71), 43.7(71), 48.1(54), 49.1(57),

**BC4 males:** % sterile males (total number of males)

0.0(19), 0.0(20), 0.0(21), 0.0(24), 0.0(27), 0.0 (35), 11.6(43),