Genetic basis of hybrid male sterility among three closely related species of *Drosophila*

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The genetic basis of hybrid male sterility among three closely related species, *Drosophila bipecticuata, D. parabipecticuata* and *D. malerkotliana* has been investigated by using backcross analysis methods. The role of Y chromosome, major hybrid sterility (MHS) genes (genetic factors) and cytoplasm (non-genetic factor) have been studied in the hybrids of these three species. In the species pair, *bipecticuata - parabipecticuata*, Y chromosome introgression of *parabipecticuata* in the genomic background of *bipecticuata* and the reciprocal Y chromosome introgression were unsuccessful as all males in second backcross generation were sterile. Neither MHS genes nor cytoplasm was found important for sterility. This suggests the involvement of X-Y, X-autosomes or polygenic interactions in hybrid male sterility. In *bipecticuata - malerkotliana* and *parabipecticuata - malerkotliana* species pairs, Y chromosome substitution in reciprocal crosses did not affect male fertility. Backcross analyses also show no involvement of MHS genes or cytoplasm in hybrid male sterility in these two species pairs. Therefore, X-autosome interaction or polygenic interaction is supposed to be involved in hybrid male sterility in these two species pairs. These findings also provide evidence that even in closely related species, genetic interactions underlying hybrid male sterility may vary.

**Keywords:** Hybrid male sterility, Genetic analysis, *Drosophila*

Hybrid male sterility is the most common postzygotic reproductive barrier encountered during the study of speciation. It follows Haldane's rule\(^5\), which states, “When in the F\(_1\) offspring of two different animal races (or species) one sex is absent, rare or sterile, that sex is the heterozygous (heterogametic) one”. Investigations on the genetic basis of sterility in male hybrids were initiated through the pioneering works of Sturtevant\(^2\) and Dobzhansky\(^3\). The genetic analyses of sterility in hybrids between two sibling species of *Drosophila* (*D. pseudoobscura* and *D. persimilis*) revealed that sterility is due to X-autosome interactions\(^5\). There are two explanations for this interaction which are based on the assumptions that (1) there is a unique and species specific balance of genes in the X chromosome and the autosomes peculiar to themselves and different from other species\(^4\), and (2) there are complementary interactions of genes present on autosomes and X chromosome\(^1,5\). Male hybrids contain a complete homospecific set of autosomes and X chromosome from the maternal species but lack X chromosome in the corresponding homospecific set of paternal species. This perturbs genic balance between X chromosome and autosomes resulting into sterility. In hybrid females, the complete homospecific set of autosomes and X chromosome from each parental species permits X-autosome balance resulting into fertility. Analysis of hybrid sterility among *D. sechellia, D. mauritiana* and *D. simulans* has suggested that X-autosome interactions are mainly involved in hybrid male sterility\(^6\).

Recently, studies on two subspecies of *D. pseudoobscura* (USA and Bogota) have also revealed that X-autosome interactions are playing an important role in hybrid male sterility\(^7\). A large number of investigations on hybrid male sterility in different species of *Drosophila* following the backcross analysis of Dobzhansky\(^8\) largely point to the role of X chromosome in hybrid male sterility\(^8,13\).

The major difference between heterogametic and homogametic sexes of most species is the presence of Y chromosome in the former placing an obvious contribution of X - Y or Y-autosome incompatibilities towards hybrid male sterility\(^14\). The X-Y interaction hypothesis was first proposed by Haldane\(^12\), which implicates genic interactions between X and Y chromosomes resulting into
preferential hybrid sterility in heterogametic sex. The effects of Y chromosome on hybrid male sterility ranges from no detectable effect between *D. viridis* and *D. novamexicana* to significant Y effect between geographic races of *D. pseudobscura* to significant Y effect between geographic races of *D. micromelana*, *D. texana* and *D. viridis*, *D. mojavensis* and *D. arizonensis* and *D. hydei* and *D. neohydei* including the subtle effect between *D. sceliphilis* and *D. simulans*. In the sibling species pair *D. simulans* and *D. mauritiana*, this is the most likely explanation for hybrid male sterility. Cytoplasmic incompatibilities were also suspected to play important role in hybrid sterility.

Several studies were performed on genetic analysis of hybrid male sterility in different species pairs of *Drosophila* and the results show that there are varied reasons for hybrid male sterility. The pursuit of genetic analysis of hybrid sterility in closely related species continues to be a favored experimental approach. The *D. bипектина* species complex offers an ideal opportunity for hybrid sterility studies as it consists of four closely related species among which the genetic basis of hybrid male sterility is unknown. The four species are *D. bипектина* Duda 1923, *D. parabipectiniana* Bock 1971, *D. malerkolliana* Parshad and Paika 1964 and *D. pseudoananasae* Bock 1971. There are two subspecies of *D. malerkolliana*, *D. m. malerkolliana* and *D. m. pallida*. *D. pseudoananasae* also has two subspecies namely *D. p. pseudoananasae* and *D. p. nigrens*. Females of all the six species and subspecies are indistinguishable. *D. parabipectiniana*, *D. m. malerkolliana* and *D. p. nigrens* show a distinct sexual dimorphic pattern where the last three abdominal segments are completely melanized in males, but not in females. *D. bипектина*, and *D. parabipectiniana* are freely crossable species in one direction and both of them also cross easily with *D. malerkolliana*. The degree of crossability between the three species and *D. pseudoananasae* is low (Mishra and Singh, unpublished data). In all crosses, hybrid males are completely sterile while females are fertile. The fertility of F1 females permits backcrossing to study genetic interactions underlying hybrid male sterility, which is the basis of reproductive isolation among these species. The size of testis and seminal vesicle is similar in *D. bипектина*, *D. parabipectiniana* and *D. malerkolliana* but differs in *D. pseudoananasae* (Mishra and Singh, unpublished data). Further, studies on size of testis and seminal vesicle as well as spermatid individualization and sperm motility in the hybrids of four species reinforces that *D. bипектина*, *D. parabipectiniana* and *D. malerkolliana* are similar while *D. pseudoananasae* differs from these three species (Mishra and Singh, unpublished data). In this communication, we report the results of studies on the genetic basis of hybrid male sterility among the three closely related species: *D. bипектина*, *D. parabipectiniana* and *D. malerkolliana*.

Materials and Methods

*Drosophila* stocks — The three species employed during present study are *D. bипектина*, *D. parabipectiniana* and *D. malerkolliana* from Pune (Maharashtra), Mysore and Raichur (Karnataka), respectively and are indigenous to India. All the stocks were maintained in the laboratory on simple yeast-agar medium at approximately 24°C.

Test of male sterility — Sperm motility in four day old male was used for assessing fertility. Males with motile sperm were scored as fertile while those having immotile sperm were scored as sterile.

Scheme for testing role of Major Hybrid Sterility (MHS) genes in hybrid sterility — The method of Zeng and Singh was followed to detect any major X-linked or dominant autosomal sterility genes. In this method, an F1 female, which produced 50% sterile sons, is chosen for a BC1 cross. If it carries one or more major sterility gene(s), in the second backcross, about half or more of her BC1 F2 daughters would carry the gene(s) and hence will produce 50% or more sons that are sterile. If none of her daughters produces approximately 50% sterile sons then there is no role of major hybrid sterility gene in sterility. To perform the experiment, for *bипектина* — *parabipectiniana* species pair, females of *bипектина* were crossed with males of *parabipectiniana* and F1 females obtained were backcrossed to *bипектина* males (BC1). From the BC1 F2 offspring, virgin females were collected and crossed individually with 3:4 *bипектина* males. The progeny obtained from this backcross were assessed for male fertility. Those females, which have produced at least 30-40% sterile sons, were selected for next generation backcross. From these selected females, virgin daughters were collected and were backcrossed to *bипектина* males individually by confining one female with 3:4 males. The backcross progeny obtained were assessed for male fertility. If major X-linked or dominant
autosomal genes are involved in hybrid male sterility, the number of sterile sons should be around 50% otherwise it would decrease. Similar crosses were made in bipectinata-malerkotliana and para-bipectinata-malerkotliana species pairs.

Scheme for testing the role of Y chromosome in hybrid male sterility — In this scheme, backcrosses were made to generate Y chromosome of one species in the genetic background of the other species. F1 Hybrid females were backcrossed to males of the paternal species and BC1 F2 males were then crossed to females of the maternal species in subsequent generations. In this way, the Y chromosome of paternal species male gradually replaces the Y chromosome of maternal species in the background of X chromosome, autosomes and cytoplasm of the maternal species. This experiment was performed for bipectinata-parabipectinata, bipectinata-malerkotliana and parabipectinata-malerkotliana species pairs.

Scheme for testing the role of cytoplasm in hybrid male sterility — The hybrid females were recurrently backcrossed to the paternal species males so that entire nuclear genome of maternal species was replaced by that of the paternal species. This permitted the test for compatibilities between the nuclear genome of one species with the cytoplasm of another species in male fertility. Cytoplasmic incompatibility is expected to increase the number of sterile males progressively through backcross generations. This experiment was performed for bipectinata-parabipectinata, bipectinata-malerkotliana and parabipectinata-malerkotliana species pairs.

The above two schemes were followed from Zeng and Singh.6

Results

D. bipectinata and D. parabipectinata species pair

Role of Y chromosome in hybrid male sterility — The introgression of D. parabipectinata Y chromosome into the genetic background of D. bipectinata was carried out by crossing bipectinata females with parabipectinata males and then backcrossing the hybrid females to parabipectinata males. Thus, the backcross males (bipapa) contain Y chromosome from parabipectinata and X chromosome and cytoplasm from bipectinata. The backcross progeny contained 58% fertile males. Fertile males were again backcrossed to the bipectinata females. All male offspring were blocking further attempts to pursue next generation backcross. Similarly, in the reciprocal crosses also the introgression of D. bipectinata Y chromosome into D. parabipectinata was unsuccessful. Therefore, it is suggested that Y chromosome is involved in hybrid male sterility.

Role of cytoplasm in hybrid male sterility — The backcross males (bipapa) have cytoplasm from D. bipectinata and nuclear genome from both D. parabipectinata and D. bipectinata and are 58% fertile. The backcross females were recurrently crossed with D. parabipectinata males, which cause replacement of the nuclear genome of D. bipectinata with that of D. parabipectinata. In subsequent backcross generations (Fig. 1A), male fertility increased steadily (bipapa2 = 97%, bipapa3 = 98% and bipappa4 = 100% fertile males). Sib mating between fourth generation flies showed no further loss of fertility for the next three generations. In reciprocal cross also, there was no effect of cytoplasmic introgression on fertility of males (Fig. 1D), which suggests no role of cytoplasm in hybrid male sterility.

D. bipectinata and D. malerkotliana species pair

Role of Y chromosome in hybrid male sterility — To introgress the Y chromosome of D. malerkotliana into D. bipectinata, F1 females of the crosses D. bipectinata females and D. malerkotliana males were crossed to the males of D. malerkotliana. The BC1 F2 males, which contain Y chromosome from D. malerkotliana, were repeatedly crossed with the D. bipectinata females. In every subsequent backcross generations, autosomes from malerkotliana were replaced with that of bipectinata while Y chromosome remained from malerkotliana. Fertility of backcross males increased with the increasing of autosomal contribution from bipectinata (Fig. 2B) in successive backcross generations (bi-bimama = 80.09%, bi2-bimama = 94.59% and bi3-bimama = 98.73%). The males and females from fourth generation were sib mated for next three generations without further loss of male fertility. The reciprocal introgression of Y chromosome gave the same results (Fig. 2E). Therefore, Y chromosome is not involved in hybrid male sterility.

Role of cytoplasm in hybrid male sterility — To introgress the nuclear genome of malerkotliana into the cytoplasm of bipectinata, F1 females obtained by crossing bipectinata females to the malerkotliana
males were recurrently backcrossed to the *malerkotliana* males. In the first backcross generation, nearly 60% males were fertile. In subsequent generations (Fig. 1B), the male fertility increased steadily (bimama2 = 87%, bimama3 = 95% and bimama4 = 98%). Thus, the nuclear genome of *malerkotliana* is compatible with the cytoplasm of *bipeclillata*. In the reciprocal crosses also, there was no effect of cytoplasm on backcross male fertility, which suggests that cytoplasm has no role in hybrid male sterility.

**D. parabipecinata** and **D. malerkotliana** species pair

**Role of Y chromosome in hybrid male sterility**

To introgress *D. malerkotliana* Y chromosome into *D. parabipecinata*, females of *D. parabipecinata* were crossed with males of *D. malerkotliana* and the F1 females obtained were backcrossed to the males of *D. malerkotliana*. In the offsprings, the male progeny carried Y chromosome from *D. malerkotliana* and autosomes and X chromosome from *D. parabipecinata*. The number of fertile backcross males (pamama) is 86%. These males were again backcrossed to the *D. parabipecinata* females and the offspring produced (pa-pamama) contained 73% fertile males. In subsequent generations, the number of fertile males increased (Fig. 2C) with replacement of autosomes of *D. malerkotliana* with that of *D. parabipecinata* (pa2-pamama = 92%, pa3-pamama = 99%). There was no loss of fertility in fourth generation males (pa3-pamama) when kept for sib mating for next three generations. In the reciprocal crosses, the same pattern of fertility was observed (Fig. 2F), which suggests no role of Y chromosome in the hybrid male sterility.

**Role of cytoplasm in hybrid male sterility**

The role of cytoplasm in the hybrid male sterility was tested by introgressing the nuclear genome of *D. malerkotliana* into the cytoplasmic background of *D. parabipecinata*. For this, *D. parabipecinata* females were crossed to the *D. malerkotliana* males and the F1 females obtained were repeatedly backcrossed to *D. malerkotliana* males. The first backcross (pamama)
offsprings contain cytoplasm from D. parabipectinata but nuclear genome from both D. parabipectinata and D. malerkotliana and contained 86% fertile males. In the subsequent backcross generations (Fig. 1C), the nuclear genome of D. parabipectinata was gradually replaced with D. malerkotliana and the fertility of males increased accordingly (pamama2 = 91.92%, pamama3 = 92.81% and pamama4 = 100%). In the reciprocal cross also, the fertility of backcross males increased in subsequent generations (Fig. 1F). Therefore, it is suggested that cytoplasm has no role in hybrid male sterility.

Role of major hybrid sterility gene — Genetic studies on Drosophila have accumulated evidence for few major hybrid sterility (MHS) genes responsible for causing sterility in hybrid males. The role of MHS genes in the above three species pairs was also investigated by using the scheme described by Zeng and Singh. In each cross, the progeny obtained from backcross females were assessed for male fertility. In each cross, around 70 females were individually tested for their sons’ fertility but no female produced 50% or more sterile sons. Those females, which produced around 30-40% sterile sons, were again backcrossed for second generation progeny. If MHS genes were involved in sterility then these genes should be transmitted to the next generations causing sterility in at least 30-40% sons. However, next generation males were fertile. These findings ruled out the role of any major X-linked or dominant autosomal MHS genes in hybrid male sterility in these three species pairs.

Discussion
The main objective of present study was to test the role of X and Y chromosome and their interactions with autosomes in hybrid male sterility among D. bипектината, parabipectinata and malerkotliana. Several factors have been reported to cause hybrid male sterility in Drosophila. Among them, the X chromosome, Y chromosome and their interactions with autosomes play a predominant role. Apart from this, involvement of few MHS genes in hybrid male sterility has also been documented. In bипектината – parabipectinata species pair, which are phylogenetically much close, Y chromosome in the reciprocal crosses has been attributed in hybrid male sterility. The Y chromosome can interact with the X chromosome, autosomes or the cytoplasm. The cytoplasmic introgression experiment showed that cytoplasm of both species is compatible with their genome (Fig. 1A, D), which ruled out the Y – cytoplasm interactions in hybrid male sterility. Further, if Y-autosomes interactions would be the cause of hybrid male sterility then some of the F1 males should be fertile as in the hybrids of D. virilis and D. texana. Complete sterility of hybrid males rules out the second possibility of Y– autosomes interaction. From the present results (role of major hybrid sterility genes), it is evident that there was no involvement of major X-linked or dominant autosomal hybrid sterility genes in this species pair. The possibilities that remains are X–Y interactions, X - autosomes interaction, autosomes - autosomes interactions and involvement of polygenes. In
bipectinata-malerkotliana and parabipectinata-malerkotliana species pairs, there was no effect of Y chromosome and cytoplasm in hybrid male sterility (Fig. 1 B, C, E, F; Fig. 2, A, B, C, D). Further, it is evident from the results that MHS genes do not play any role in sterility in these species pairs. These findings ruled out the involvement of Y chromosome, few major X-linked and dominant autosomal hybrid sterility genes and cytoplasm in determining hybrid sterility in these species pairs. Therefore, it is suggested that either X- autosome interactions or polygenes are involved in the hybrid male sterility in bipectinata-malerkotliana and parabipectinata-malerkotliana species pairs. As demonstrated in other species of Drosophila, polygenes may affect hybrid male sterility either by epistatic interactions 26, through disruption of synergistic interactions 27 or through additive effects 28. Although autosome-autosome interactions is not very common cause of hybrid male sterility and has been reported only in D. hydei and D. neoeder 29 but its role in sterility of hybrids of these three species cannot be ruled out.

These findings provide evidence that even in very closely related species, the genetic interactions involved in hybrid male sterility may be varied. In D. bipectinata, D. parabipectinata and D. malerkotliana, it is the first report on genetic interactions underlying hybrid male sterility. Apart from this, it indicates the involvement of Y chromosome during early phase of speciation in the bipectinata complex as it plays a major role in sterility of hybrids of D. bipectinata and D. parabipectinata, which are phylogenetically very close species. In the hybrids of D. parabipectinata and D. malerkotliana and also D. bipectinata and D. malerkotliana, the absence of role of Y chromosome and expected involvement of interactions of X chromosome and autosomes or polygenes suggests that the role of Y chromosome in hybrid sterility might be taken up by either X chromosome and autosomes or polygenes in later stage of speciation.

The present study provides several potential avenues for future research in D. bipectinata species complex for investigating the role of genetic factors involved in hybrid male sterility both at cytogenetics and molecular level. Further scope exists for studies on the role of X - Y interaction and interactions of X chromosome with different autosomes in hybrid male sterility in these three species by using phenotypic markers on X chromosome and autosomes.

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