RESEARCH ARTICLE

The *Drosophila bipectinata* species complex: phylogenetic relationship among different members based on chromosomal variations

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Abstract

Making interspecific hybridizations, where possible remains an unparalleled option for studying the intricacies of speciation. In the *Drosophila bipectinata* species complex comprising of four species, namely *D. bipectinata*, *D. parabipectinata*, *D. malerkotliana* and *D. pseudoananassae*, interspecific hybrids can be obtained in the laboratory, thus bequeathing an ideal opportunity for studying speciation and phylogeny. With the view of investigating the degree of divergence between each species pair, we planned to study the polytene chromosomes of the F_1 hybrids, as it would mirror the level of compatibility between the genomes of the parental species. Two sets of crosses were made, one involving homozygous strains of all four species from India and the other including homozygous strains from different places across the globe. Polytene chromosomes of F_1 larvae from both sets of crosses had similar configurations. In F_1 larvae from crosses involving *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana*, complex configurations (depicting overlapping inversions) could be detected in different arms. However, they were fairly synapsed, indicating that the differences are only at the level of gene arrangements. The polytene chromosomes of larvae obtained by crossing *D. pseudoananassae* with the other three species were very thin with gross asynapsis in all the arms, demonstrating that the genome of *D. pseudoananassae* is widely diverged from rest of the species. The overlapping inversions (reflected in complex configuration), are inferred in the light of earlier chromosomal studies performed in this complex.

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Introduction

A species complex constitutes a group of closely related species which have diverged enough to be reproductively isolated from one another but not enough to prohibit hybridization when confined together in a no choice situation (Kopp and Barmina 2005). One example is the *Drosophila bipectinata* complex which comprises of four closely related species, namely *D. bipectinata*, *D. parabipectinata*, *D. malerkotliana* and *D. pseudoananassae*. Taxonomically, this complex belongs to the subgroup *ananassae* of the considerably spread *melanogaster* species group. They have a wide distribution and spread across the Oriental–Australian biogeographic zone. All the four species occur sympatrically over the parts of this range of distribution (Kaneshiro and Wheeler 1970; Bock 1971a; Bock and Wheeler 1972; Okada 1981).

The members of this complex being very suitable for doing evolutionary studies have been extensively utilized by a number of scientists working in the field of population genetics, behaviour and evolution, and has served in understanding the intricacies of speciation (Yang *et al.* 1972; Bock 1978; Hegde and Krishnamurthy 1979; Crossely 1986; Gupta *et al.* 1993; Kopp and Barmina 2005; Kopp *et al.* 2006; Mishra and Singh 2006, 2007; Banerjee and Singh 2012, 2014; Signor *et al.* 2013; Singh and Singh 2013, 2014a, b, 2015; Banerjee and Singh 2015a, b, c; Singh and Banerjee 2015).

While, all studies have pointed one thing clearly that *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana* are closely related to each other and *D. pseudoananassae* is distantly related to these three, the other details of their phylogeny hardly mach each other.

The phylogenetic relationship drawn, especially, using intraspecific and interspecific chromosomal inversions have given contrasting results. Bock (1971b) studied intraspecific and interspecific chromosomal inversions in all the four species of the complex. A total of 20 inversions could be detected as extant polymorphisms in these species. Further in the interspecific hybrids too (F_1 larvae obtained by crossing

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the species) 20 autosomal inversions were detected. From his consummate work on inversions in this complex, Bock constructed a phylogeny whereby *D. malerkotliana*, *D. pseudoananassae* and a population ancestral to *D. bipectinata* and *D. parabipectinata* were said to be derived from a common ancestral population. Jha and Rahman (1972, 1973), made a reference map (camera lucida drawing) of the salivary gland chromosomes of *D. malerkotliana*. They studied the polytene chromosomes of the F_1 hybrids of *D. bipectinata* and *D. malerkotliana* and by interpreting the complex configurations of polytene loops, they depicted that the two species differ by seven paracentric inversions, some of which may have been polymorphic in the common ancestral species and the two arrangements (inverted and standard) have become fixed respectively in the two species. Most recently, Tomimura *et al*. (2005) prepared polytene map of a structurally homozygous stock of *D. parabipectinata* collected from Brunei Borneo in 1971 and used it as a reference standard sequence for all the four species of the complex. In their extensive study involving various geographical populations of all the species and subspecies of the complex, they could detect as many as 87 inversions in the complex and also described their breakpoints. They also studied polytene of the interspecific hybrids and found that only two arrangements were shared interspecifically. On the basis of the characteristic differences in gene arrangements among the four species, they proposed a phylogeny, different from that of Bock's (1971b). According to their phylogeny, *D. pseudoananassae* derives directly from *D. malerkotliana*, from which *D. bipectinata* and *D. parabipectinata* have also been derived.

Molecular phylogeny has also been studied in detail in this complex. Kopp and Barmina (2005) combined phylogenetic and population genetic approaches to reconstruct the evolutionary history of the complex, which included reconstruction of the order and timing of speciation events, the extent of genetic differentiation among species and variation within species and the levels of gene flow across species boundaries. For accomplishing this, they used sequences of one mitochondrial and six nuclear loci. Further, Kopp *et al.* (2006) studied divergence at the Y-chromosome in this complex and confirmed the utility of Y-chromosomal loci in construction of phylogeny. From these studies it was concluded that nucleotide divergence among *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana* is extremely low and they diverged not very long back in the evolutionary time scale about 283,000–385,000 years ago. These three species were found to have more shared polymorphisms than fixed differences. On the other hand, *D. pseudoananassae* was found to be a distant outgroup. According to the phylogeny reconstructed by Kopp and Barmina (2005), the common ancestor of *D. bipectinata* and *D. parabipectinata* diverged from *D. malerkotliana* prior to the separation of the former two species. They also said that *D. pseudoananassae* diverged very long back in the evolutionary time.

Therefore, the phylogenies drawn from these studies are not consistent. Taking in view of all the studies that have been done so far in this complex, we thought of ruminating the polytene chromosomes of the interspecific hybrids (after Bock 1971b; Jha and Rahman 1972, 1973 and Tomimura *et al.* 2005), to relook into the chromosomal configurations again, since the conclusions drawn especially by Bock (1971b) and Tomimura *et al.* (2005) are contrasting. In polytene chromosomes of the interspecific hybrids in *Drosophila*, since the homologues chromosomes are from different species they can give a direct peek into the nucleotide sequence divergence and arrangement differences by way of looking at the chromosomal asynapsis and inversions loops respectively. Polytene chromosomes of interspecific hybrids can offer invaluable glimpses into the possible mechanism of speciation (whether nucleotide divergence or divergent fixing of polymorphic gene arrangements played the larger role during speciation). Going through earlier literatures involving this complex certainly points out that rearrangements must have played a greater role. This study is also an attempt to validate those done by Bock (1971a) and Tomimura *et al.* (2005) and establish a phylogeny among the members of this complex.

Materials and methods

Fly stocks

Initially, the chromosomal analysis of all the available laboratory stocks of the four species was carried out to pick strains for interspecific hybridization. This was done to screen the stocks and basically seek homozygous stocks. Table 1a–e gives the details of all the stocks of the four species available in our laboratory. From each stock at least 10 larvae were used for analysis. For isofemale lines, absence of inversion heterozygosity in 10 larvae is enough to claim that the line is homozygous. However, in case of mass cultures more number of larvae needs to be checked to confirm homozygosity.

Stocks having different gene arrangements in the polytene arms (reflected in heterozygous inversion loops) preferably should not be used for interspecific hybridization to study polytene chromosomes of the hybrids as, in such a case, the set of chromosomes got from one or each of the parents may be one of the two arrangements commonly found in them (and not a fixed standard arrangement). Thus, the mode of pairing of polytene chromosomes in the interspecific hybrids may not truly reflect the interspecific difference per se.

Crosses

Since we are not proficient at comprehending the precise banding pattern of each chromosomal arm and are only acquainted with the gross landmarks, it was not possible for us to claim securely that a homozygous stock for a certain arm was homozygous for the standard gene arrangement and not an inversion which may have come to be fixed in the laboratory stock. The laboratory stocks are maintained by transferring small number of flies in each generation. Thus,

Table 1. Details of the laboratory stocks of *D. bipectinata* species complex.

Name of stock	Place of the collection	Year of collection
(a) Mass culture stocks of *D. bipectinata*, collected from various places in India		
Mysore (Mys-88)	Mysore (Karnataka)	1988
Alipurduar (AD-93)	Alipurdur (West Bengal)	1993
Siliguri (SL-93)	Siliguri (West Bengal)	1993
Trivandrum (TD-93)	Thiruvananthapuram (Kerala)	1993
Arumanai (AR-96)	Arumanai (Tamil Nadu)	1996
Pune (PN-99)	Pune (Maharashtra)	1999
Bodhgaya (B.Gaya-02)	Bodh Gaya (Bihar)	2002
Raebareli (RB-03)	Raebareli (Uttar Pradesh)	2003
Navsori (NV-03)	Navsori (Gujarat)	2003
Nilgiri (NG-03)	Nilgiri (Tamil Nadu)	2003
(b) Isofemale lines of *D. bipectinata*		
Siliguri (SL-01)	Siliguri (West Bengal)	2001
Akola (Akl-12)	Akola (Maharashtra)	2012
IROT 8[#]	Iriomote (Japan)	–
K-aaj 072 146[#]	Nairobi (Africa)	–
K-aaj 078 174[#]	Kota Kinabalu (Malaysia)	–
(c) Details of the laboratory stocks of *D. malerkotliana*		
BHU 87	Banaras (Uttar Pradesh)	1987
Baripada 87	Baripada (Orissa)	1987
RC 91	Raichur (Karnataka)	1991
B. Gaya 02	Bodh Gaya (Bihar)	2002
RB 03	Raebareli (Uttar Pradesh)	2003
Mys	Mysore (Karnataka)	1999
GL 10	Gwalior (Madhya Pradesh)	2010
PU 14	Puri (Orissa)	2014
K-aan 007[#]	Rio Claro (Brazil)	–
K-aan 038[#]	Mombasa (Kenya)	–
K-aan 046[#]	Cebu (Philippines)	–
(d) Details of the laboratory stocks of *D. parabipectinata*		
Mys	Mysore	1988
Indo	Celebes (Indonesia)	1978
Kaan 065 B15[#]	Thailand	–
(e) Details of the laboratory stocks of *D. pseudoananassae*		
KB 1062	Brunei Island (Brunei)	2003
KB 466	Brunei Island (Brunei)	2003
KB 1000	Brunei Island (Brunei)	2003
KB 284	Brunei Island (Brunei)	2003
Bang 04	Bengaluru (Karnataka, India)	2004
K-aap 001 CMG242[#]	Chiang Mai (Thailand)	–

[#]Obtained from Prof. M. Matsuda, Kyorin University, Mitaka, Japan. All KB lines of *D. pseudoananassae* were kindly provided by Prof. Artyom Kopp, University of California, Davis, USA. All are isofemale lines.

an inverted arrangement found initially in high frequency may eventually become fixed due to founder effect. Therefore, to confirm that the interspecific differences inferred through the analysis of polytene chromosome morphology of the F_1 larvae are true fixed differences between the species, among the homozygous stocks of the four species, two sets were settled upon for making interspecific crosses. One set involved homozygous strains of all the four species from India and the other included homozygous strains from different places across the globe. Since only one laboratory stock of *D. malerkotliana* was found to be free of inversion heterozygosity, it was common in both sets of crosses. The following stocks were used in first set of crosses: *D. bipectinata*, Mys from Mysore; *D. parabipectinata*, Mys from Mysore; *D. malerkotliana*, GL-10 from Gwalior; *D. pseudoananassae*, Bang 04 from Bengaluru. The stocks used in second set of crosses: *D. bipectinata*, IROT from Iriamote, Japan; *D. parabipectinata*, Indo from Indonesia; *D. malerkotliana*, GL-10 (Gwalior) from India; *D. pseudoananassae*, KB284 from Brunei Island, Brunei.

Given that an F_1 larva gets one set of chromosomes from each parent, the polytene chromosomes of F_1 larvae from a pair of reciprocal crosses would not be different. Therefore, crosses were made only in one direction, making it as six crosses per set. For getting interspecific F_1 larvae, virgin females and males of the requisite strains of each species were collected and aged for seven days after which crosses were set up in vials containing mixture food (which has a

higher proportion of yeast and lesser proportion of agar–agar, so that the larvae were healthy). Twenty pairs of flies were put in each vial.

Method for polytene squash preparation

When the F_1 hybrid larvae arrived, polytene squash preparations were made by using the lacto-aceto orcein method. The salivary glands of the third instar larvae were dissected out in insect's saline (0.67%). The fat bodies were removed and the glands were fixed in aceto methanol for 30 s. They were then treated with LWA (a solution containing lactic acid, water and acetic acid in the ratio 1:2:3) for 10 s. They were next stained in 2% lacto aceto orcein for an hour. After an hour, the glands were washed in 45% acetic acid and mounted and squashed in a solution containing lactic acid and 60% acetic acid in the ratio 1:1. At least 10 well spread preparations from each cross were observed and good preparations were used for microphotography.

Results

Inversions found in the laboratory stocks of the complex

The polytene chromosomes of all the four species are morphologically similar. We followed the nomenclature of Jha and Rahman (1972), Gupta and Panigrahy (1990) and Tomimura *et al.* (2005) which is slightly different from Bock's (1971b). His 3L has been designated 3R and 3R has been designated 3L by us as 3L and 3R of *D. malerkotliana* (Jha and Rahman 1972) closely resembles 3L and 3R of *D. ananassae*, named by Ray-Chaudhuri and Jha (1965).

Altogether, 11 inversions were found to persist in heterozygous condition in the laboratory stocks of the four species. In *D. malerkotliana*, only one stock, i.e. GL 10, was found to be free of any inversion heterozygosity.

Chromosomal configurations in the interspecific hybrids

It was very difficult to cross *D. pseudoananassae* with the other three species of the complex, and the larvae obtained were very weak and thin. In spite of several trials, good polytene squash preparations could not be made from the second set of crosses *D. pseudoananassae* × *D. bipectinata* and *D. pseudoananassae* × *D. malerkotliana*. For all the other crosses, corresponding polytene chromosomes of F_1 larvae from both sets had identical configurations. Therefore, the configurations were the true reflection of the fixed interspecific differences. The following observations could be made:

- In polytene chromosomes of F_1 larvae of *D. bipectinata* × *D. parabipectinata* complex configurations were found in all the autosomal arms (2L, 2R, 3L and 3R) and also the X chromosomal arms (figures 1a; 2a; 3a; 4a; 5). At the tip of 3L, a simple heterozygous loop was also detected (figure 3a). The most complex configuration was found in 2L (figure 1a) and it spanned the entire length of the arm. Pairing was nevertheless very good, i.e. there was perfect synapsis.
- In polytene chromosomes of F_1 larvae of *D. bipectinata* × *malerkotliana*, 2R was found to pair perfectly with no complex loops. The other autosomal arms were found

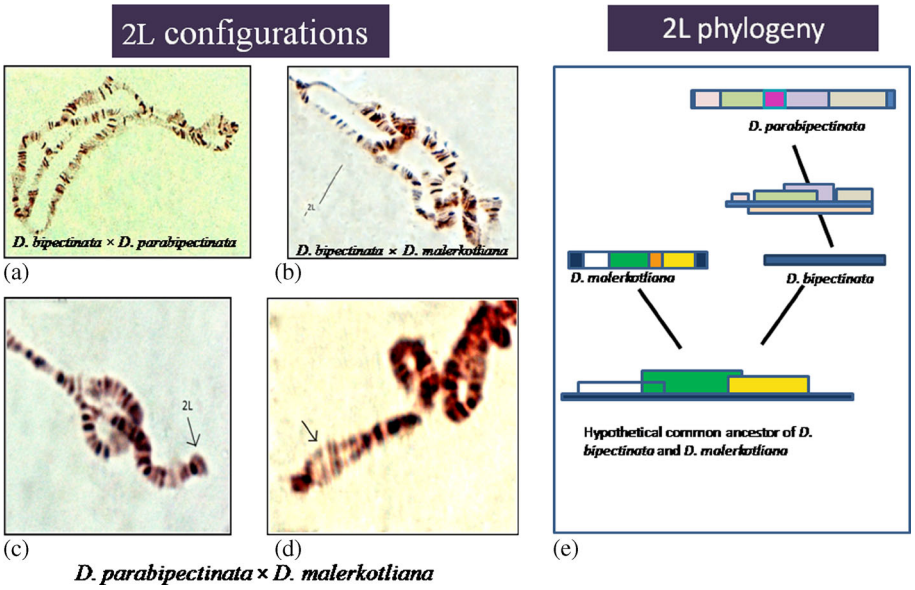

Figure 1. 2L chromosomal configurations in interspecific hybrids in the *D. bipectinata* species complex. (a) Complex configuration in 2L of hybrids of *D. bipectinata* × *D. parabipectinata.* (b) Complex configuration in interspecific hybrids of *D. bipectinata* × *D. malerkotliana.* (c) Paracentric loop in the interspecific hybrids of *D. parabipectinata* × *D. malerkotliana.* (d) Asynapsis at the tip in the interspecific hybrids of *D. parabipectinata* × *D. malerkotliana.* (e) Diagrammatic depiction of the 2L phylogeny.

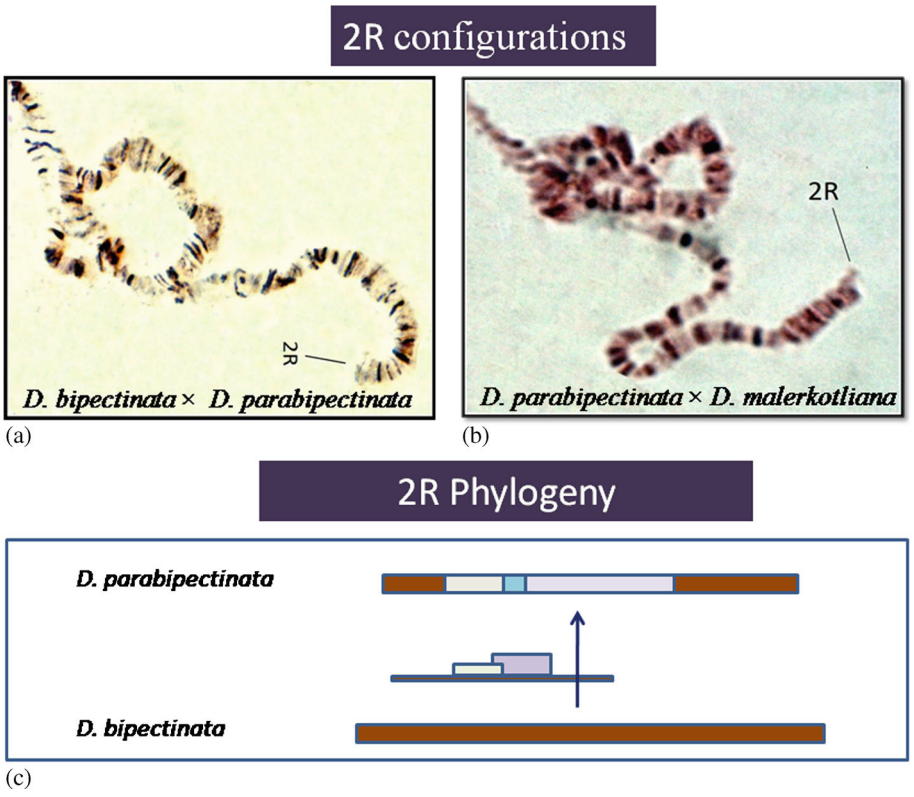

Figure 2. 2R chromosomal configurations in interspecific hybrids in the *D. bipectinata* species complex. (a) Complex configuration in interspecific hybrids of *D. bipectinata* × *D. parabipectinata.* (b) Complex configuration in interspecific hybrids of *D. parabipectinata* × *D. malerkotliana.* (c) Diagrammatic depiction of 2R phylogeny.

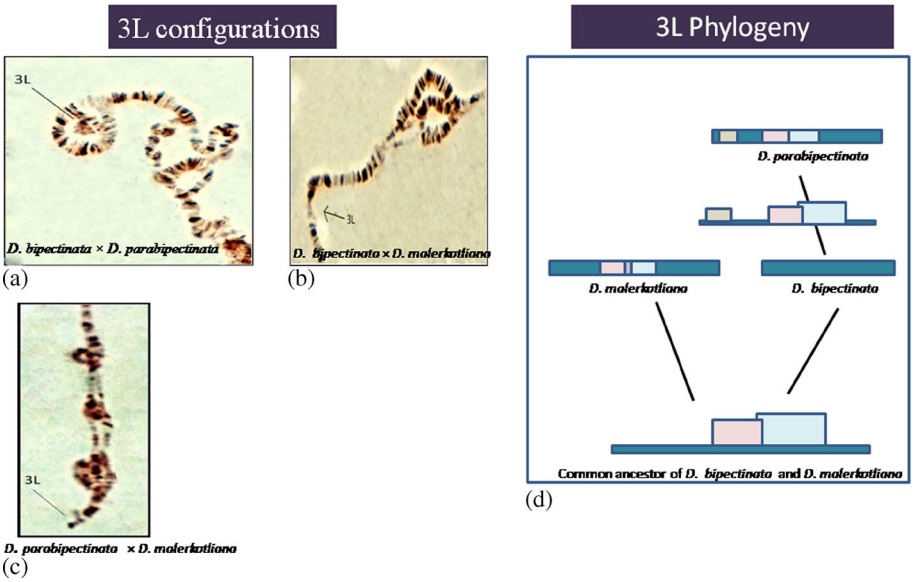

Figure 3. 3L chromosomal configurations in interspecific hybrids in the *D. bipectinata* species complex. (a) Complex configuration in interspecific hybrids of *D. bipectinata* × *D. parabipectinata.* (b) Complex configuration in interspecific hybrids of *D. bipectinata* × *D. malerkotliana.* (c) Complex configuration in interspecific hybrids of *D. parabipectinata* × *D. malerkotliana.* (d) Diagrammatic depiction of 3L phylogeny.

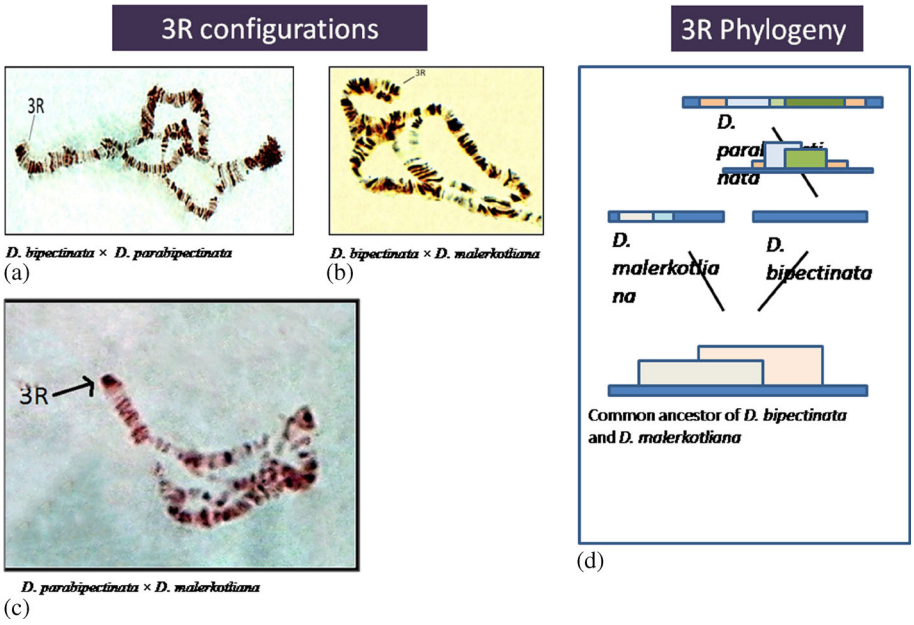

Figure 4. 3R chromosomal configurations in interspecific hybrids in the *D. bipectinata* species complex. (a) Complex configuration in interspecific hybrids of *D. bipectinata* × *D. parabipectinata.* (b) Complex configuration in interspecific hybrids of *D. bipectinata* × *D. malerkotliana.* (c) Complex configuration in interspecific hybrids of *D. parabipectinata* × *D. malerkotliana.* (d) Diagrammatic depiction of 3R phylogeny.

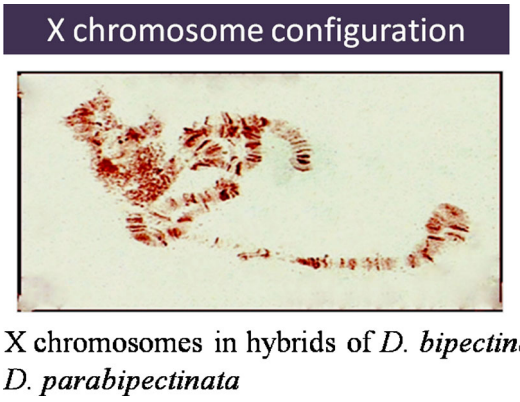

Figure 5. X chromosomal configuration in interspecific hybrids of *D. bipectinata* × *D. parabipectinata.*

to have complex configurations (figures 1b; 3b; 4b). X chromosomes were found to be completely paired.

- In polytene chromosomes of F_1 larvae of *D. parabipectinata* × *malerkotliana*, asynapsis was found near the tip of 2L (figure 1d) and along the entire length of 3L (figure 3c). 2L also exhibited a paracentric loop at the tip (figure 1c). 2R and 3R exhibited complex configurations (figures 2c; 4c). X chromosomes were found to be completely paired without any complex loops or asynapsis.
- The arms of polytene chromosomes of F_1 larvae from the three crosses involving *D. pseudoananassae* were asynapsed along their entire lengths, interspersed with extremely knotted loops.

Discussion

Over the past 50 years many models of chromosomal speciation have been proposed (Rieseberg 2001). It has been argued by several authors that separation or splitting of a population or species into two species is the common route taken when polymorphic arrangements become a threat to the fitness of a population. It is then that a population becomes fixed for one or more chromosomal rearrangements (White 1978). Our study also reveals that chromosomal rearrangement is the key, if not the lone player in speciation in this complex. Studies on nucleotide divergence by Kopp and Barmina (2005), divulged the meagre nucleotide divergence especially among *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana*. Given such small nucleotide divergence, morphological differences (among the males of three species) are far from being just barely evident, they are in fact very prominent). In addition to this, there are behavioural differences and strong reproductive isolation among these species (see review by Singh and Banerjee 2016). Therefore, more than divergence at the level of single nucleotides, rearrangement of gross chromosomal blocks must have played a greater role and have influenced regulation of gene expression in a big way causing speciation. This fact in place, the arrangement differences between the different species pairs evident in the interspecific hybrids was utilized by us for predicting a phylogeny. We believe this to be an unparalled way of predicting the direction of evolution and phylogeny.

Like earlier studies on interspecific chromosomal inversions, we too found interesting results from our studies on

the chromosomal analysis of the F_1 interspecific hybrids in this complex. If one has to start looking from the degree of synapsis, then the polytene chromosomes of the hybrids of *D. bipectinata* × *D. parabipectinata* and *D. bipectinata* × *D. malerkotliana* showed excellent synapsis (figures 1, a&b; 2, a&b; 3, a&b; 4, a&b) indicating that there is very good homology between the chromosomes of *D. bipectinata* and *D. parabipectinata*, *D. bipectinata* and *D. malerkotliana*. The degree of synapsis deteriorated in the F_1 hybrids of *D. parabipectinta* and *D. malerkotliana* (figures 1d; 3c) and was worst in those from all the crosses involving *D. pseudoananassae*. The synapsis was so poor and the loops so knotted that it was not possible to infer arrangement differences between the parental species involved. The level of synapsis in interspecific hybrids is reflective of the degree of compatibility between the parental species involved. In fact, Naveira *et al.* (1986) devised a new method for mapping gene differences between species through comparison of pairing patterns in two *Drosophila* species and their hybrids. Poor synapsis is reflective of remote relatedness between the parental species. Therefore, consistent with earlier findings in this complex, *D. psedoananassae* is widely diverged from the rest of the three species of this complex.

The complex configurations in most of the autosomal arms indicate that there is difference in gross gene arrangements between *D. bipectinata* and *D. parabipectinata*; *D. bipectinata* and *D. malerkotliana; D. parabipectinata* and *D. malerkotliana*. Especially, the configurations of 2L and 3R were found to be very complex. We could affirm the following from our results, discussed also in the light of earlier studies.

Configuration of 2L in hybrids of D. bipectinata and D. parabipectinata

2L exhibited a configuration, involving almost the whole arm (figure 1a), which was not recorded either by Bock (1971b) in his figure 6a or Tomimura *et al.* (2005) in their study (their figure 4b). Just behind the goblet, found near the tip, a simple loop could be distinguished, depicting a single gene arrangement difference (inversion), between *D. bipectinata* and *D. parabipectinata*. Precisely at the end of this, originated a large loop spanning the rest of the arm and this large inversion loop included within itself, three inversion loops (figure 1a). Therefore, the 2L of the two species differ by a small, simple inversion, a large inversion and three included inversions within the large inversion. Of these three, two looks overlapping and the third looks independent.

Configuration of 2L in hybrids of D. bipectinata and D. malerkotliana

2L revealed three overlapping inversions (figure 1b). Bock (1971b) in his figure 6c has shown two small overlapping inversions. Jha and Rahman (1972) found three overlapping inversions and suggested that a series of three paracentric inversions occurred to change the standard gene arrangement of *D. malerkotliana* to that of *D. bipectinata* or vice versa. A close look at the sketch of the inversion complex that they made and superimposing it with the microphotograph, we reveal that the configurations could be identical. The only doubt being that, in the sketch of Jha and Rahman (1972), one of the breakpoints lie just near the tip and in the microphotograph, it lies a little away from the point described by Jha and Rahman (1972, in their figure 6a–c). Tomimura *et al.*'s (2005) finding on the other hand does not match with ours. They found two independent paracentric inversions to separate *D. bipectinata* and *D. malerkotliana* (their figure 4a). Thus, we go with Jha and Rahman (1972) and conclude that 2L of *D. bipectinata* and *D. malerkotliana* differ by three overlapping paracentric inversions.

Configuration of 2L in hybrids of D. parabipectinata and D. malerkotliana

In some preparations, we found the homologues to be asynapsed near the tip of 2L (figure 1d), pointing towards some incompatibility. A simple paracentric loop was also found near the proximal end (figure 1c). Bock (1971b) and Tomimura *et al.* (2005) too found this loop. However, they also found other configuration differences, which we could not detect. Hence, through our study we conclude that *D. parabipectinata* and *D. malerkotliana* differ by a single arrangement difference in 2L.

2R configurations in hybrids of D. bipectinata and D. parabipectinata

2R exhibited two overlapping inversions lying towards the chromocenter (figure 2a), Bock (1971b, figure 8a) detected a large inversion and a small inversion near the tip. Tomimura *et al.* (2005) also detected two heterozygous loops (figures 4, e&f), one towards the tip and the other towards the chromocenter. One of the two inversions found in our study may be the one found by Tomimura *et al.* (2005). The other does not look like the one found by Bock as that inversion was at the terminus, and the inversion in this study lies near the distal end.

2R configuration in hybrids of D. bipectinata and D. malerkotliana

2R was found to be perfectly synapsed and no inversion loops could be detected. Bock (1971b, figure 8c), however, detected two overlapping inversions one of them being common with the one found near the chromocenter in hybrid larvae of *D. bipectinata* and *D. parabipectinata*. However, Jha and Rahman (1972) and Tomimura *et al.* (2005) also found 2R to be completely synapsed. Thus, we conclude that there is no structural difference between 2R of *D. bipectinata* and *D. malerkotliana*.

2R configuration in hybrids of D. parabipectinata and D. malerkotliana

A pair of overlapping inversions was found near the chromocenter (figure 2b). Both of these appeared to be the

ones found in hybrids of *D. bipectinata* and *D. parabipectinata*. Bock (1971b, figure 8b) also found two overlapping inversions but they were near the tip. Therefore, the differences between the pair found by him do not coincide with the differences found by us. Figures by Tomimura *et al.* (2005) did not have any depiction of 2R of hybrids of *D. parabipectinata* and *D. malerkotliana*. We conclude that *D. malerkotliana* and *D. bipectinta* have common gene arrangements in 2R that differ identically from the arrangements found in *D. parabipectinata*.

3L configuration in hybrids of D. bipectinata and D. parabipectinata

A simple paracentric loop was found near the tip and a pair of inversions was found covering a large portion of the arm (figure 3a). These three inversions are identical to the ones described by Bock (1971b, figure 13a) in 3R which has been named as 3L by us and Tomimura *et al.* (2005, figure 5b). Our microphotograph superimposes perfectly with theirs.

3L configuration in hybrids of D. bipectinata and D. malerkotliana

A pair of inversions was detected by us (figure 3b). This looked identical to the overlapping inversions found in hybrids of *D. bipectinata* and *D. parabipectinata*. Bock (1971b, figure 13c) described these inversions to be overlapping. Jha and Rahman (1972) found the same configuration but their honed observation and carefully made sketches led them to describe the two inversions not as overlapping but the whole of one inversion included within the other with one of the breakpoints being common for both the inversions (see their figure 7, b&c). Figures in Tomimura *et al.* (2005) have no depiction of 3L in *D. bipectinata* × *D. malerkotliana* hybrids. Since, Jha and Rahman (1972) even described the breakpoints and the configuration looks identical to what Bock (1971b) and we found, we would go with Jha and Rahman in concluding that *D. bipectinata* and *D. malerkotliana* differ by two inversions, one included within the other.

3L configuration in hybrids of D. parabipectinata and D. malerkotliana

We found the whole of 3L to be asynapsed (figure 3c). However, Bock (1971b) and Tomimura *et al.* (2005) also found a simple paracentric loop at the tip of the arm. Asynapsis in the arm is reflective of poor compatibility between the homologues.

3R configuration in hybrids of D. bipectinata and D. parabipectinata

3R configuration (figure 4a) could exactly be superimposed on Bock's figure 10a of his 3L. He described the configuration as having three regions of homologous pairing within limits of a complex inversion. This configuration was said to be a product of three inversions, the first and third having the same breakpoints that is one inversion included within the other and the third overlapping with some regions of both the inversions. The configuration of 3R in figure 5d from Tomimura *et al.* (2005) also looked similar. Therefore, it is concluded that 3R of *D. bipectinata* and *D. parabipectinata* differ by three inversions.

3R configuration in hybrids of D. bipectinata and D. malerkotliana

The complex configuration of 3R shows two overlapping inversions as four breakpoints are evident (figure 4b). The configurations of 3R as depicted in Bock (1971b, figure 10c, his 3L) and sketches of Jha and Rahman (1972, figure 8, a&b) perfectly fits with our microphotograph. However, Bock said that the two species differ by three inversions in 3R and Jha and Rahman said that they differ by only two overlapping inversions. Close inspection of the configuration suggests that there are only two overlapping inversions and there is a small region of asynapsis without any looping. There is no mention of 3R configuration in hybrids of *D. bipectinata* and *D. malerkotliana* in Tomimura *et al.* (2005).

3R configuration in hybrids of D. parabipectinata and D. malerkotliana

The complex configuration of 3R depicts three overlapping inversions (figure 8c) Bock (1971b) said that two overlapping inversions are present. However, his figure 10b could be perfectly superimposed over our microphotograph. Close inspection indeed reveals three pairs of breakpoints in the configuration indicating that 3R of *D. parabipectinata* and *D. bipectinata* differ by three inversions. Tomimura *et al.* (2005, figure 5e) does not match with ours.

X chromosomal configurations

Bock (1971a), found the X chromosomal arms of all the crosses to be homosequential. Jha and Rahman (1972) too found the X chromosomal arms of *D. bipectinata* × *D. malerkotliana* to be homosequential. However, in our study, X chromosomal arms of hybrids of *D. bipectinata* and *D. parabipectinata* were found to have a large pericentric inversion complex (figure 5). This matched with the figure 3, a&d of Tomimura *et al.* (2005). Since, in their figure both the loops touch the chromocenter and in our microphotograph (depicting both the arms) too, the involvement of chromocenter is evident, we conclude that the inversions are overlapping, spanning both the arms of the X chromosome. The X chromosomal arms of hybrids of *D. bipectinata* × *D. malerkotliana* and *D. parabipectinata* × *D. malerkotliana* were found to be homosequential. In hybrids of crosses involving *D. pseudoananassae* as one of the parents, the preparations were so poor that we could not identify the X chromosomes. Tomimura *et al.* (2005), found arrangement differences in hybrids of other crosses too.

Phylogeny based on chromosomal arrangements

Figures 1e, 2c, 3d and 4d depicts the phylogenetic relationship shared between the three more closely related species, *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana* based on differences in chromosomal arrangements. Interpreting differences in the chromosomal arrangements, we propose that *D. bipectinata* and *D. malerkotliana* must have come from a common ancestral population and *D. bipectinata* has given rise to *D. parabipectinata*.

Since hybrids involving *D. pseudoananassae* as one of the parents exhibited asynapsis in the chromosomal arms, we propose that it must have diverged long back in the evolutionary history.

Evolutionary significance of the arrangement differences

Differences in chromosomal arrangements that we found in this study neither matches entirely with what Bock found nor align with the findings of Tomimura *et al.* (2005). If *D. bipectinata*, *D. parabipectinata* and *D. malerkotliana* arose from a common ancestral population, there must have been common configurations and shared arrangements in the interspecific hybrids. However, apart from a few, we did not find common arrangements. Therefore, these three species do not seems to have come from a common ancestor.

In the hybrids of *D. bipectinata* and *D. parabipectinata*, all arms (autosomal and sex chromosomal) were having complex configurations. Therefore, there are gross arrangement differences between these two species. The pairing between the homologues was nevertheless found to be good. Bock did not find as many arrangement differences as we found between the two species and said that a common ancestral population gave rise to *D. bipectinata* and *D. parabipectinata*. This does not seems possible, as a common ancestral population could not have bore the burden of so many polymorphisms. Inversion polymorphisms associated with heterosis, come at their own cost. It is known that inversion heterozygotes have a higher selective value than the corresponding homozygotes (Wright and Dobzhansky 1946). Therefore, reduced fitness of the corresponding homozygotes lower the optimum reproductive capacity of the population which has too many polymorphisms. We thus propose that it was not a common ancestral population that gave rise to *D. bipectinata* and *D. parabipectinata,* but one gave rise to the other through transient polymorphisms. From earlier studies in the past dealing with sexual isolation, we predicted that *D. bipectinata* is the ancestral population and *D. parabipectinata* is derived from *D. bipectinata* (Banerjee and Singh 2012). Also, *D. bipectinata* is more widespread and genetically more variable. Hence, we postulate that it is the ancestral species which gave rise to *D. parabipectinata*.

D. bipectinata and *D. malerkotliana* were found to differ by a number of arrangement differences but not as many as found between *D. bipectinata* and *D. parabipectinata*. The X chromosomes and 2R were found not to have any arrangement difference. We go with Jha and Rahman in saying that a common ancestral population gave rise to two species. The evolutionary advancement of a population, having a number of polymorphisms is only possible through the act of budging from the *status quo*. Thus, escaping the impasse of Haldane's effect, the common ancestral population must have got divided into two distinct populations (through decrease in the heterotic properties of inversion polymorphism). Within each of the two populations now, different gene arrangements were fixed and speciation progressed (Haldane 1957; Carson 1959). Reproductive isolation and hybrid male sterility might have arisen in due course of time because of the inferiority of the interpopulation hybrids strengthening reproductive isolation.

We found a little difficult to come up with a 3L phylogeny. *D. parabipectinata* differs from *D. bipectinata* by a pair of inversions in the middle of the arm and a simple paracentric inversion at the tip. Surprisingly, the same pair of inversions also separates *D. bipectinata* and *D. malerkotliana*, their hybrids exhibiting the same configuration in the middle of the arm. *D. bipectinata* and *D. malerkotliana* are derived from a common ancestral population which must have been polymorphic for both the inversions and one of the arrangements got fixed in each species (figure 3d). It is perhaps coincidental that the same inversions arose in *D. parabipectinta* anew while it was separating from *D. bipectinata*. These may be inversion susceptible spots in the arm. Tomimura *et al.* (2005) suggested that transposable elements might mediate the occurrence of inversions in natural populations of *D. bipectinata*. Like its parent species, *D. bipectinata* must also have had these inversions from *D. malerkotliana*, owing to a rare hybridization and after a brief phase of polymorphism, one of the arrangements got fixed in *D. parabipectinata* (figure 3d). We do not go with the seemingly more probable hypothesis that *D. malerkotliana* gave rise to *D. parabipectinata* or vice versa because 3L of hybrids of *D. malerkotliana* and *D. parabipectinata* exhibited asynapsis indicating that they cannot be as close as an ancestral-derived pair. Moreover, earlier studies, and configurations at other arms also suggests that *D. malerkotliana* is more distantly related to *D. parabipectinata* than it is to *D. bipectinata*.

Hybrids involving *D. pseudoananassae* as one of the parents, were in general very thin and their glands also did not look well developed. Also, the polytene preparations were poor and homologues were asynapsed. Asynapsis interspersed with extremely knotted configurations only indicates that *D. pseudoananassae* is very distantly related to the other three species.

We do not buy the phylogeny proposed by Tomimura *et al.* (2005), because even in their study poor synapsis in chromosomal arms of hybrids involving *D. pseudoananassae* as one of their parents is evident. Their work is very extensive and interesting involving large number of populations (including even the subspecies) and meticulous mapping of the breakpoints. In fact, some of their observations in hybrid polytene do match with our observations. Yet, phylogenies should not just rely on arrangement differences, the compatibility of

chromosomes reflected in the degree of synapsis should also be taken into account. Apart from this, studies of all nature should be considered before drawing any conclusion. Bock's phylogeny and our phylogeny differ only in one detail: while he said that *D. bipectinata* and *D. parabipectinata* have come from a common ancestral population, we propose that *D. bipectinata* has given rise to *D. parabipectinata*. Some of the arrangement differences between *D. bipectinata* and *D. parabipectinata* observed by Bock do not match with ours. The inconsistency in results is surprising because the differences between the species ought to be fixed (Patterson and Stone 1952). However, since we had made two sets of each cross and observed at least 10 well-prepared slides, we are quite confident about our findings.

In conclusion, the ancestral-derived relationship between *D. bipectinata* and *D. parabipectinata* is a saga of acute gene arrangement differences without much divergence at the level of nucleotides. On the other hand, the relationship between *D. pseudoananassae* and the other three species is a saga of strong nucleotide divergence accompanied by some arrangement differences. It must have diverged long back in the evolutionary history from the same common ancestor that gave rise to *D. bipectinata* and *D. malerkotliana*. *D. bipectinata* and *D. malerkotliana* diverged from this ancestor and goes on accumulating differences, contesting to spread across the globe.

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