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Deciphering genetic differences among the natural populations of *Drosophila malerkotliana*on the basis ofquantitative data of common paracentric inversions

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Abstract:*Drosophila malerkotliana* is a widely distributed species of Oriental-Australian zoogeographical region. It has its evolutionary significance, due to its genetic connection with other three species (*D. bipectinata, D. parabipectinata* and *D. pseudoananassae*) of *bipectinata* species complex, which have collectively been analyzed to study phylogenetic affinities among them. The genetic differentiation among the natural populations of this species has sparsely been studied by earlier workers. We analyzed chromosomal polymorphism in six natural populations, aligned in north to south vertical axis and found four paracentric inversions to be ubiquitously established/distributed in all such populations of this species. The data procured regarding the frequency of different karyotypes of these four commonly occurring inversions in its six populations were used to determine the genetic differentiation in them. Since flies were sampled from two distant zones of India, one north and other south, a perusal of the role of distance in genetic differentiation was also considered. The results provided distinct genetic differentiation between north and south populations with evidence that the frequency of four commonly occurring inversions exceed considerably among the four south Indian populations than the two north Indian populations. Further, the dendrogram constructed on the basis of genetic identity among the fifteen paired groups reveal two discrete genetic groups, one for north and the other for south populations. Thus, this paper discusses the effect of latitudinal variation on genetic differentiation in *D. malerkotliana* and the probable evolutionary forces that might have caused substantial variation among its natural populations

Index Terms:*Drosophila malerkotliana,* Paracentricinversions, chromosomal polymorphism, latitudinal variation, genetic differentiation.

I. Introduction

Population genetical studies depend upon the analysis of genetic variation among the individuals of populations which in turn help to understand the evolutionary trend of the species. Environmental factors as well as evolutionary forces like mutation, natural selection, migration and genetic drift are the prime factors which cause genetic variations among the different natural populations of a species. Earlier population geneticists preferred *Drosophila* species to investigate genetic variation among their natural populations by considering commonly occurring inversions as genetic markers (Dobzhansky and Sturtevant 1938; Dobzhansky et al., 1950;Da Cunha, 1960; Dobzhansky, 1970; Bock, 1971a; Naseerulla and Hegde 1993; Banerjee and Singh 1996; Singh 1998, 2001; Ananina *et al.,* 2004; Singh and Singh 2021). Inversions are the segments of chromosomes that lie 180^{o} inverted in its position and are recognized as paracentric (that does not include centromere) or pericentric (that includes centromere) inversions. Paracentric inversions are very common in several *Drosophila* species due to their adaptive superiority (Kapun and Flatt 2019). Chromosomal polymorphism due to paracentric inversions in *Drosophila* is an adaptive trait (Dobzhansky 1970; Sperlich and Pfriem 1886).The Oriental-Australian zoogeographical region is rich in Drosophilid species due to its varied ecological conditions (Bock 1971b, 1978; Bock and Wheeler 1972; Banerjee and Singh 2012,2017; Singh 2019) and one of the species complexes, i.e., *bipectinata* species complex is well documented to be inhabitant of this region (Bock 1971a, b; Singh and Singh 2021). Bock initiated his studies on the genetic polymorphism of *D. bipectinata* species complex that comprises four morphologically very similar species; *D. bipectinata,D. parabipectinata,D. malerkotliana* and *D. pseudoananassae* (Bock 1971a, b). Considering the significance of this species complex, a number of researchers worked on various genetical aspects of its members to establish phylogenetic connections among them (Bock, 1971b, 1978; Bock and Wheeler, 1972; Singh and

Rhomberg, 1987;Tomimura *et al.*, 2005; Singh and Sisodia, 2008; Singh and Banerjee 2016, Singh and Singh 2018, 2020, 2021).

Among the four species of this complex, *D. bipectinata* and *D. malerkotliana* are comparatively more in their density of distribution. Chromosomal polymorphism of *D. bipectinata* has substantially been studied by different researchers to know the extent of genetic variation among its natural and laboratory populations (Bock 1971b; Banerjee and Singh 1996; Banerjee and Singh 2017; Singh and Singh 2018,2021). A study conducted recently on the genetic differentiation of seven Indian natural populations of *D. bipectinata* based on the distribution of three common inversions of this species has clearly shown marked genetic variation among its north and south populations (Singh and Singh 2021). Some evolutionary biologists working on the members of this species complex selected *D. malerkotliana* for the study of its genetic link with its allies to establish their phylogenetic closeness (Bock, 1971a; Bock and Wheeler, 1972; Matsuda *et al.*, 2005, 2009; Tomimura *et al.*, 2005; Mishra and Singh, 2006; Banerjee and Singh, 2012, 2017; Singh, 2019). Besides this, the genetic polymorphisms have also been studied by considering chromosomal and protein polymorphisms in this species (Bock, 1971b; Jha and Rahman, 1972; Gupta and Panigrahy, 1990; Das and Singh, 1992; Singh and Banerjee, 1996; Kumar and Singh, 2017; Singh, 2013; Kumar, Singh and Singh 2019; Singh and Singh, 2020, 2022).

In the present work, *D. malerkotliana* fliessampled from six different places of India were cultured and their third instar larvae wereobserved for the presence of inversions and any other structural chromosomal aberrations. Since four paracentric inversions were found to be commonly occurring in all the six populations, genetic variations among these populations could be ascertained based on the differential distribution of these four inversions. Data available for the different karyotypes of these inversions of the natural populations were employed to compute some essential population genetical parameters to see the level of genetic differentiation among the natural populations of *D. malerkotliana* and the probable causes for such variation.

II. Materials and Methods

Flies of*D. malerkotliana* were collected from six different geographical localities of India from fruit and vegetable markets. Two populations, Lucknow (LKO) and Varanasi (VNS) separated by a distance of nearly 300km came from north, whereas, remaining four populations Madurai (MDR), Rameshwaram (RMM), Trivandrum (TVM) and Nagercoil (NGC) were collected from southern part of India. The south Indian populations were separated by a minimum distance of 100km (between TVM and NGC). Female flies brought to the laboratory were individually cultured in food vials to raise isofemale lines. Third instar larvae randomly selected from individual vials were used for the preparation of their salivary gland chromosomes (polytene chromosomes).

For the preparation of the polytene chromosomes, the lacto-aceto-orcein staining method was used. In this endeavour, a third instar larva randomly selected from an isofemale line was dissected in insect saline (0.67%) to separate out its salivary glands. The salivary glands were then treated with fixative (acetic acid and methanol in the ratio of 1:1) for about thirty seconds and then subjected to LWA (lactic acid-1:water-2: acetic acid-3) for about ten seconds. The glands were then stained in 2% lacto-aceto-orcein for half an hour. Finally, the stained glands were washed (in 45% acetic acid) and squashed in mountant (lactic acid and 60% acetic acid in 1:1 ratio) by pressing the glands covered with glass cover. To identify different chromosome arms and inversion breakpoints, the polytene chromosomes map provided by Jha and Rahman (1972) and Panigrahy (1984) was considered.

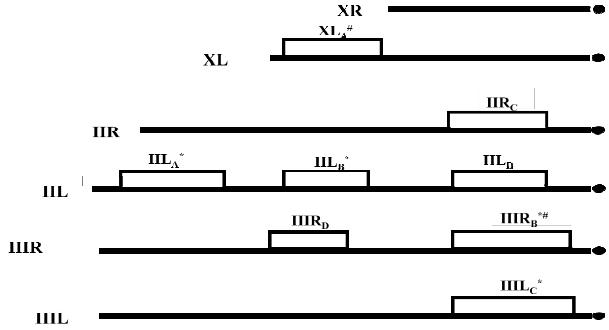
III. Inversion karyotypes and their analysis

The normal banding pattern at the inversion locus is referred as ST/ST whereas, the same locus having inverted gene order is indicated as IN/IN. Individuals with heterozygosity are referred ST/IN. This implies with all the four common inversions where they have been karyotypically identified with their specific nomenclature. By observing the different chromosome arms in the polytene chromosomes, the type of karyotype at different inversion loci was recorded for the individual larva. Once the number of three karyotypes of a polymorphic locus was available, the frequency of ST (normal gene arrangement) and IN (inversion arrangement) could be calculated by following the Hardy-Weinberg formula.

Expected frequency of three karyotypes (ST/ST, ST/IN and IN/IN) was calculated by considering the frequency of ST and IN of a specific inversion locus. To test significant difference between observed and expected values, chi squire (χ^2) test was performed. This analysis helped to know that a particular locus is in H-W equilibrium or not. Inbreeding coefficient (F) was also calculated by using the equation $F = H_e - H_o / H_e$,where H_e and H_o are expected and observed heterozygosity respectively. This parameter was calculated by considering the commonly occurring inversion loci and taking average value for each population. Genetic identities (**I**) were also calculated by using the formula given by Nei (1972) and by this formula we computed the value of **'I'** for fifteen comparisons in *D. malerkotliana*.

IV. Results

Polytene chromosomes examination of hundreds of third instar larvae of all the six populations of *D. malerkotliana* enabled us to identify total eight different types of paracentric inversions and an autosomal translocation in left arm of the second chromosome. The banding pattern of the translocated part clearly revealed that a part of terminal end of 3R was attached with 2L resulting into the forked appearance of approximately 20% part of terminal end of 2L (Singh and Singh 2015). Out of the eight inversions, two new inversions were observed for the first time in this species. In these two new inversions, one was located on right arm of third chromosome and the other in the left arm of the X-chromosome (being reported for the first time). The breakpoints of all the eight inversions are depicted through line diagram in Figure1.



***Commonly occurring inversions, #newly reported inversions**

Fig. 1. Line diagram showing location of eight paracentric inversions observed in different chromosome arms of *D. malerkotliana*

Frequencies of ST and inversion arrangements in different chromosome arms of six natural populations of *D. malerkotliana* are presented in Table 1 and the data have been depicted through bar diagram in Figure 6. The upper segment depicts the frequency of inversion arrangement and the lower segment (distinguished by color) shows the frequency of ST arrangement for each inversion locus. The frequency of ST gene order ranged from 0.84 to 0.93 considering all the gene loci and therefore, the inversion gene order remained to range from 0.07 to 0.16. However, the perusal of gross data clearly indicate higher frequency of inversion gene order in the natural population of south Indian places compared to two populations, i.e., LKO and VNS.

Table 1. Frequencies of ST and inversion arrangements in different chromosome arms of six natural populations of *D. malerkotliana* (Chi squire (χ^2) values have been computed based on Hardy-Weinberg equilibrium analysis)

Inversions	Gene Arrangements	POPULATIONS					
		LKO	VNS	MDR	RMM	TVM	NGC
		Frequencies					
IIL$_A$	ST	0.93	0.92	0.91	0.89	0.85	0.84
	IIL$_A$	0.07	0.08	0.09	0.11	0.15	0.16
χ2		2.77	2.37	1.89	0.611	1.21	2.54
IIL$_B$	ST	0.92	0.91	0.9	0.88	0.87	0.87
	IIL$_B$	0.08	0.09	0.10	0.12	0.13	0.13
χ2		1.77	1.47	1.11	3.40	2.61	2.18
IIR$_C$	ST	0.93	0.90	0.89	0.92	0.9	0.88
	IIR$_C$	0.07	0.10	0.11	0.08	0.10	0.12
χ2		2.77	**6.59***	**5.79***	2.28	0.92	3.05

*** P<0.05**

Table 2. Estimates of average observed and expected heterozygosity and inbreeding coefficient (F) in six natural populations of *D. malerkotliana*

POPULATIONS	Ho ± SE	He ± SE	F ± SE	χ^2
LKO	0.117±0.013	0.147±0.011	0.20±0.020	0.612
VNS	0.127±0.010	0.176±0.013	0.27±0.018	1.364
MDR	0.138±0.007	0.188±0.010	0.26±0.019	1.323
RMM	0.160±0.008	0.195±0.017	0.20±0.023	0.628
TVM	0.180±0.013	0.225±0.016	0.20±0.024	0.90
NGC	0.186±0.013	0.240±0.013	0.22±0.025	1.215
Mean	0.151±0.011	0.20±0.014	0.23±0.021	1.01±0.08

Table 2 shows average observed and expected chromosomal heterozygosity and inbreeding coefficient (F) in the natural populations of *D. malerkotliana* and the data have been depicted through bar diagram in Figure 7. The average observed heterozygosity ranged from 0.117 to 0.186 being lowest in LKO and highest in NGC. An increasing trend in the level of heterozygosity was observed from the extreme north population (LKO) to the southern edge of India (NGC) (figure 8).

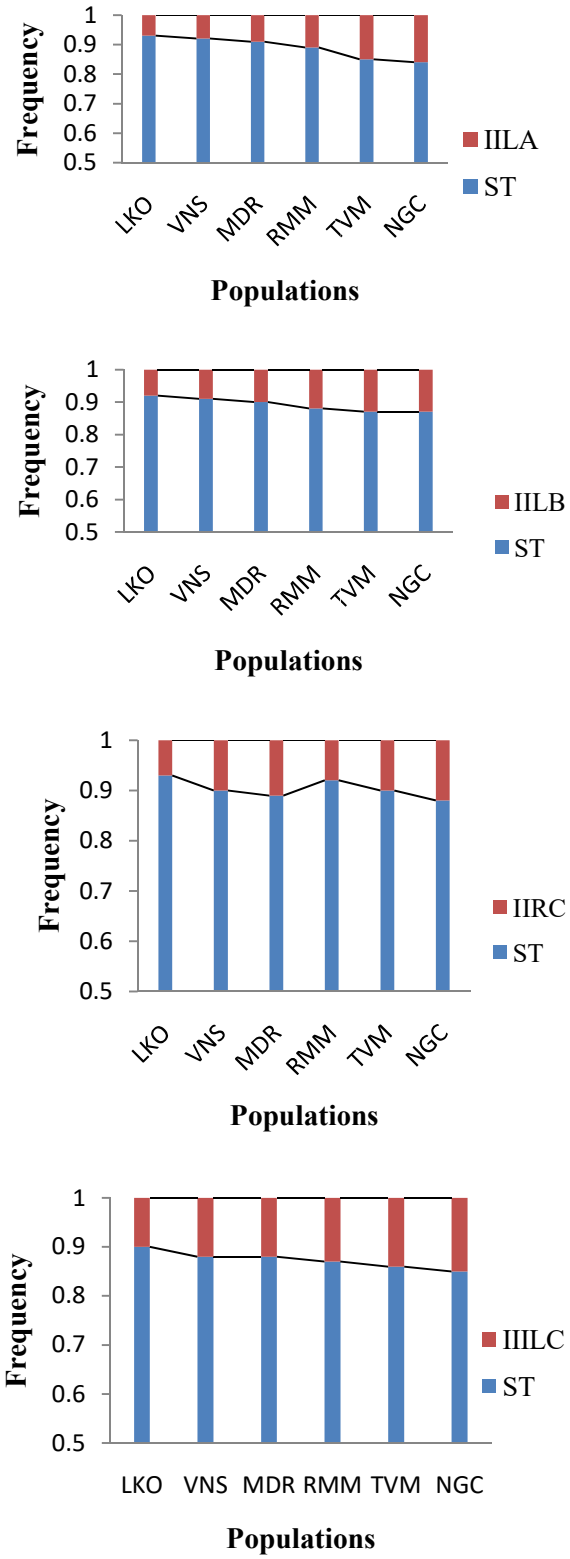


Figure 6. Frequency of ST and inversion gene arrangements for four commonly occurring inversions loci in six natural populations of *D. malerkotliana*

The level of inbreeding computed through inbreeding coefficient indicate that maximum inbreeding occurred in VNS population (0.270) followed by MDR (0.260) giving an indication that these two populations might have been derived from individuals having common descend. Although the value of F does not reflect inbreeding at higher level and the observed values ranging between 0.200 to 0.270 deems to be quite genuine for natural populations of *Drosophila.*

The data regarding the presence of number of heterozygous inversions and individuals bearing them is shown in the table 3. To test random distribution of inversions in the population, χ^2 analysis was performed applying Poisson distribution. To test difference between observation and expectation, χ^2 analysis was performed. The results indicate that there is significant difference between observation and expectation ($p<0.001$). The mean number of heterozygous inversions per individual was found to be 0.62 (MDR) to 0.76 (LKO and NGC). The results of this analysis revealed that except two populations, i.e., LKO and VNS, the other four showed non-random distribution of heterozygous inversions indicating that in these four natural populations of *D. malerkotliana* certain combinations of genotypes were favored due to selection.

Table 3. The observed and expected numbers of larvae carrying heterozygous inversions and the mean number of heterozygous inversions per larva in six natural populations of *D. malerkotliana*

Populations	No. of heterozygous inversions present in the larvae				χ^2	Mean no. of heterozygous inversions per individual
	0	1	2	3		
LKO	22 (23.87)	21 (18.14)	6 (6.89)	2 (1.75)	0.75	0.76
VNS	21 (23.55)	20 (15.78)	4 (5.28)	1 (1.18)	2.08	0.67
MDR	16 (21.52)	23 (13.34)	1 (4.17)	--	10.82*	0.62
RMM	17 (23.50)	26 (15.51)	2 (5.12)	--	10.78*	0.66
TVM	26 (22.63)	28 (16.07)	1 (5.71)	1 (1.35)	14.77*	0.71

d.f. 2; *P<0.05

Since four commonly occurring inversions have been observed to be polymorphic in *D. malerkotliana*, the different karyotypic combinations for each inversion locus was analyzed in detail. The three karyotypes of inversion IIL_A located in 2L for six natural populations are presented through bar diagram in Figure 2. In all the six populations, the karyotype ST/ST was observed to be exceedingly high than other two karyotypes i.e., ST/IIL_A and IIL_A/IIL_A. However, there is substantially distinct increase in the heterozygosity in south Indian populations (MDR, RMM, TVM and NGC) as compared to two populations of north India (LKO and VNS). Figure 3 depicts the karyotypic combinations formed due to IIL_B in six populations of *D. malerkotliana*. In this case also, ST/ST karyotype was found maximally in all the populations followed by ST/IIL_B and IIL_B/IIL_B. The third commonly occurring inversion observed in 2R, IIR_C was found to be present in all the populations analyzed with a similar trend of highest occurrence of ST/ST combinations followed by its inversion heterozygotes and inversion homozygotes (figure 4). In figure 5, the three inversion karyotypes of 3L formed due to $IIIL_C$ have been displayed. In this case also, almost similar trend is followed for the karyotypes ST/ST, ST/$IIIL_C$ and $IIIL_C$/$IIIL_C$ as have been seen with other 3 inversion loci.

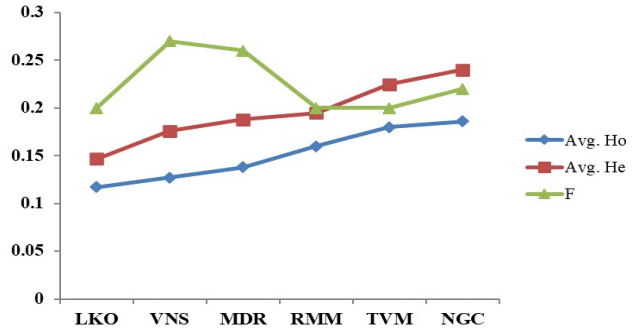


Figure 7. Graphical presentation of average observed and expected heterozygosity (Ho and He) and inbreeding coefficient (F) in six natural populations of *D. malerkotliana*

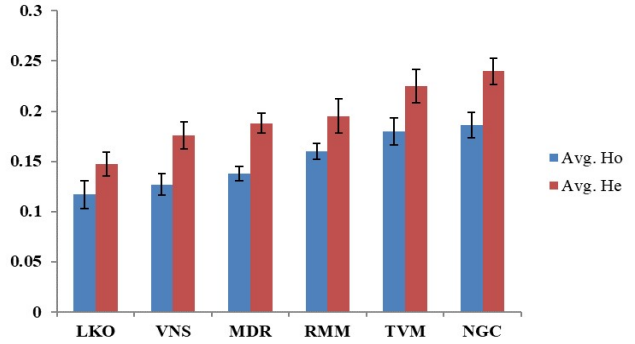


Figure 8. Bar diagram showing average frequencies of observed and expected heterozygotes (Ho and He) in natural populations of *D. malerkotliana.*

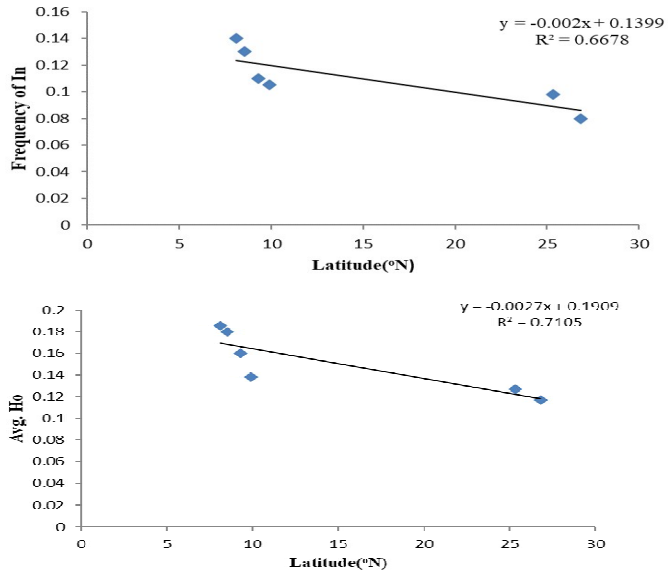


Figure 9. Scattered dots showing observed heterozygosity and frequencies of inversions with respect to latitude in natural populations of *D. malerkotliana*.

A correlation study was also done to see whether average heterozygosity show interrelationship with latitudinal position and frequency of inversions vs. latitudinal position. This analysis revealed distinct significant negative correlation (measured through Pearson's correlation coefficient) between average H_o vs. latitudinal position and same trend for frequency of inversion vs. latitudinal position (figure 9).

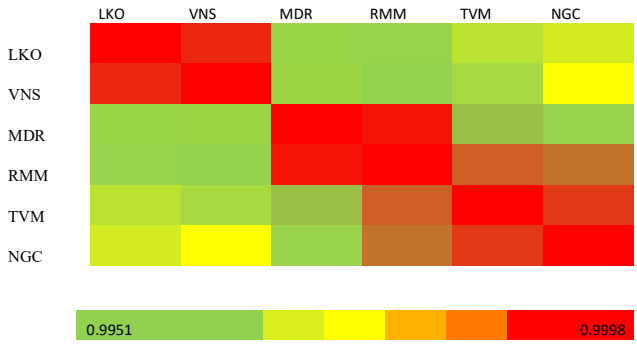


Figure 10. Pairwise comparison of genetic identity (I) in Indian natural populations of *D. malerkotliana.*

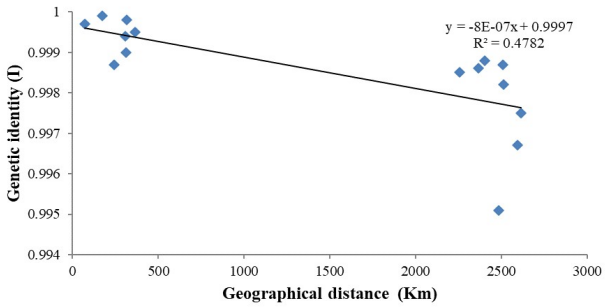


Figure 11. Correlation between Geographical distance (Km) and genetic identity (I) in Indian natural populations of *D. malerkotliana.*

A pairwise comparison of genetic identity (I) of these populations of *D. malerkotliana* is shown in figure 10. Since six populations have been analyzed, therefore, 15 comparisons have been possible to see the level of genetic identity based on their genetic makeup. LKO and VNS showed maximum identity followed by populations coming from south India like, TVM vs. NGC; MDR vs. NGC; RMM vs. TVM and RMM vs. NGC. Populations like VNS vs. NGC; LKO vs. NGC and LKO vs. TVM were genetically distantly related as they showed I value substantially less than other.

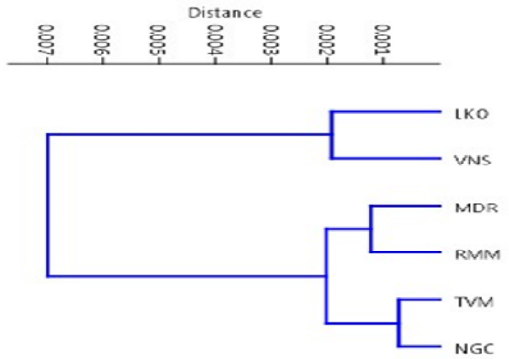
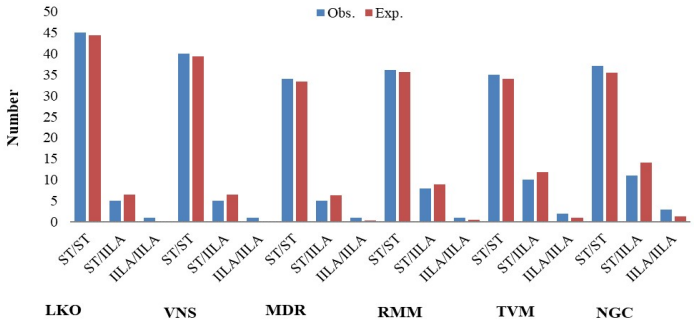


Figure 12. Phylogenetic tree constructed on the basis of UPGMA considering the values of genetic identities among the paired populations of *D. malerkotliana*

Figure 11 shows correlation between geographical distance (Km) and genetic identity measured for the fifteen pairs and scattered dots indicates two clusters one showing higher identity among the populations separated by less distance and a separated cluster for distantly located populations. A dendrogram showing genetic relationships among the six populations of *D. malerkotliana* (figure 12)shows that the south Indian populations make a compact cluster with MDR vs. RMM and TVM vs. NGC. The North Indian populations i.e., LKO vs. VNS make an independent cluster and have substantially differentiated from South Indian populations.



Figur2. Bar diagram showing observed and expected numbers of different karyotypes in 2L due to ST/IIL$_A$ inversion in six natural populations of *D. malerkotliana*

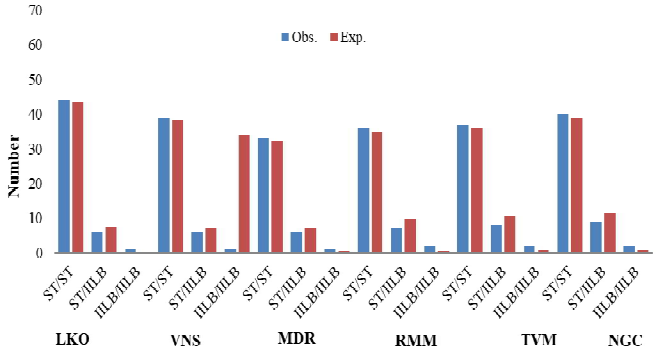


Figure 3. Bar diagram showing observed and expected numbers of different karyotypes in 2L due to ST/IIL$_B$ inversion in six natural populations of *D. malerkotliana*

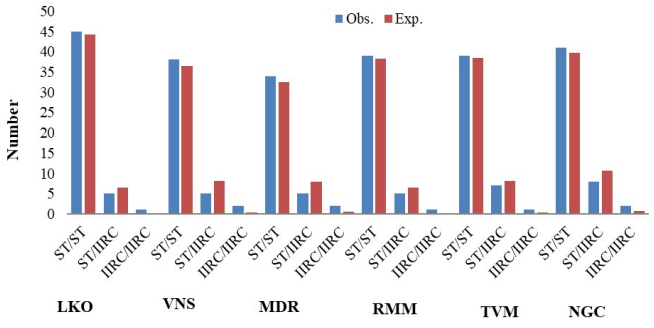


Figure 4. Bar diagram showing observed and expected numbers of different karyotypes in 2R due to ST/IIR$_C$ inversion in six natural populations of D. malerkotliana

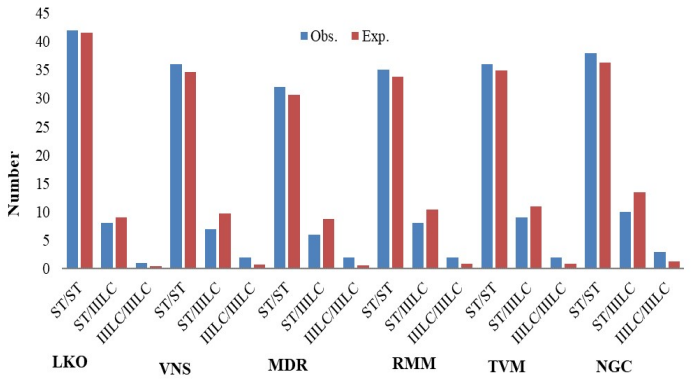


Figure 5. Bar diagram showing observed and expected numbers of different karyotypes in 3L due to ST/IIIL$_C$ inversion in six natural populations of *D. malerkotliana*

V. Discussion

During the present study, in the course of screening of the isofemale lines, we were able to find out seven autosomal and single X chromosome inversions in natural populations of *D. malerkotliana.* The X chromosomal inversion was found only in a single larva out of the eighty larvae analyzed in the Varanasi population of *D. malerkotliana.* The X chromosomal polymorphism has never been reported by any other work in *D. malerkotliana,* and in our case too, its frequency was found to be very low, being detected in a single larva. Close analysis of the configurations and the breakpoints of other inversions reveal that rest of the inversions have been reported by earlier researchers too (Bock 1971b; Jha and Rahman 1972; Tomimura et al., 2005; Banerjee and Singh 2017).

Genetic differentiation of natural populations may be influenced by ecological variation, geographical distance and evolutionary forces operating in such populations. We found distinct genetic structuring among the natural populations of *D. malerkotliana*. The north populations showed ample genetic differences from the south populations. Between the two north populations, the possibility of gene flow would be only possible if it is being assisted by human activity, i.e., through fruit and vegetable transportation. The two cities of north are nearly 300km apart, however, the exchange of fruits and vegetables may be the sources of intermixing of the two populations which has consequently resulted more genetic closeness between them. Although, we expected that the north populations may be genetically much different from each other owing to bottleneck effect which they experience during summer and winter seasons. Among the south populations, the genetic differentiation is least observed between MDR and RMM giving an indication that the gene flow between these two populations is larger than the remaining two populations (TVM and NGC). Although, these four populations of south India did not markedly differ amongst them when pair wise compared with the two populations of north. Thus, there is role of genetic differentiation due to longer distance as larger distance, at least refrain gene flow among the populations.In total, fifteen genetic identity values helped us to scrutinize the pairs of populations that maximally resemble genetically from each other and therefore, the pairwise genetic identity (I) values help to support 'isolation by distance' effect (Wright 1943).

The distribution of four inversions all along the stretch of more than 2600km distance is evidence that these inversions have been established as commonly occurring and might be conferring adaptive significance to this species. The dendrogram constructed on the basis of genetic identity values reveal distinct genetic variation between the north and south populations. Lucknow and Varanasi, the two north populations make one cluster that get distinctly differentiated from the four south populations. Among the south populations, maximum genetic similarity was recorded between Trivandrum and Nagercoil populations.

During the early 1970s, the genetical investigations involving the four species of *bipectinata* species complex was initiated and the two major features which formed such analysis were morphological and cytogenetical traits particularly, polytene chromosome analysis (Bock 1971b; Bock and Wheeler 1972). By now, there are sufficient reports pertaining to the evolutionary history, phylogenetic relationship and population genetics of the *bipectinata* species complex (Narda 1968; Bock 1971b; Bock and Wheeler 1972; Banerjee and Singh 1996, 1997; Tomimura et al 2005;Mishra and Singh 2006; Singh and Sisodia 2008; Banerjee and Singh 2017, Singh and Singh 2021). However, out of the four species of this complex, two species, i.e., *D. bipectinata* and *D. malerkotliana* found more attention owing to their wider distribution and prevalence all across their distribution zone. According to Marcow and O'Grady (2005), *D. malerkotliana* is a sub cosmopolitan species. Prakash et al. (2014) found high stress resistance level in *D. malerkotliana* and suggested it as being responsible for its invasion and ecological success on different continents, as compared to *D. bipectinata*.

Earlier researchers who worked on these two prime members; *D bipectinata* and *D. malerkotliana* of this species complex reported the adaptive importance of inversion polymorphism and found that *D. bipectinata* possesses comparatively higher polymorphism than other members of this complex (Bock 1971b; Gupta and Panigrahy 1990; Tomimura et al., 2005; Merot 2020). Through our previous study (Singh and Singh 2021), it also seems that *D. bipectinata* is more chromosomally polymorphic than the remaining three because nine paracentric inversions have been observed in *D. bipectinata* in which four have been reported for the first time whereas, in *D. malerkotliana*, eight paracentric inversions have been observed in which two are the new ones.

VI. Acknowledgement

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