RESEARCH ARTICLE

Introgressive hybridization and evolution of a novel protein phenotype: glue protein profiles in the *nasuta–albomicans* complex of *Drosophila*

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Abstract

Glue proteins are tissue-specific proteins synthesized by larval salivary gland cells of *Drosophila*. In *Drosophila nasuta nasuta* and *D. n. albomicans* of the *nasuta* subgroup, the genes that encode the major glue protein fractions are X-linked. In the present study, these X-linked markers have been employed to trace the pattern of introgression of *D. n. nasuta* and *D. n. albomicans* genomes with respect to the major glue protein fractions in their interracial hybrids, called cytoraces. These cytoraces have inherited the chromosomes of both parents and have been maintained in the laboratory for over 400–550 generations. The analysis has revealed that cytoraces with *D. n. albomicans* X chromosome show either *D. n. nasuta* pattern or a completely novel pattern of glue protein fractions. Further, quantitative analysis also shows lack of correlation between the chromosomal pattern of inheritance and overall quantity of the major glue protein fractions in the cytoraces. Thus, in cytoraces the parental chromosomes are not just differentially represented but there is evidence for introgression even at the gene level.

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Introduction

Diagnostic genetic markers provide a powerful means for identifying hybrid organisms and for characterizing patterns of introgression (Avise 1994). They also help in resolving questions about the ancestry of putative hybrid populations (Barton and Gale 1993). In cases where ancestry of the hybrids is known, such markers can help in providing insights into the pattern of introgression of parental genomes. *Drosophila nasuta nasuta* ($2n = 8$) and *D. n. albomicans* ($2n = 6$), belonging to the frontal sheen complex of *nasuta* subgroup of *Drosophila immigrans* species group, are the parents for many hybrid lineages experimentally created under laboratory conditions. *D. n. nasuta* and *D. n. albomicans* are morphologically almost identical, allopatric sibling species. Despite their striking karyotypic divergence they are cross-fertile and hence considered as chromosomal races (reviewed in Ranganath 2002). Interracial hybridization involving these two races followed by karyotypic screening of hybrid lineages over generations revealed fixation of different combinations of parental chromosomes among different hybrid lines. These true-breeding chromosomal introgressants were called cytoraces. The cytoraces have been maintained in the laboratory for over 400–550 generations, and these cytoraces along with the parents are referred to as the *nasuta–albomicans* complex (reviewed in Ranganath and Aruna 2003).

The karyotypic introgression of parental genomes in the cytoraces is well characterized at a gross level, where an entire chromosome is considered as a unit. Various studies in the *nasuta–albomicans* complex, such as cytogenetic changes (Tanuja *et al*. 1999a,b), morphometric trait analysis (Harini and Ramachandra 1999a,b, 2000), a few fitness parameters (Ramachandra and Ranganath 1988), mating preferences (Tanuja *et al*. 2001a,b) and isozymes (Aruna and Ranganath 2004), have revealed a complex pattern of interracial divergence among parents and cytoraces. But these

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studies could not provide markers that would help us to get an insight into the path of introgression. In the present study we tried to analyse the level of introgression among cytoraces using glue proteins as markers.

Glue proteins are developmental-stage-specific and tissue-specific secretory proteins synthesized by larval salivary gland cells in *Drosophila* (Lane *et al*. 1972). Just prior to pupation, they are expectorated to the exterior and are thought to have a role in attaching the puparium to a solid surface (Korge 1977; Riddiford 1993; Lehmann 1996). Glue protein electrophoresis patterns in *D. nasuta* subgroup members are simple and major glue fractions follow X-linked pattern of inheritance in a codominant fashion. The *D. n. nasuta* and *D. n. albomicans* glue protein patterns differ with respect to all the X-linked fractions (Ramesh and Kalisch 1988, 1989a). Thus, the X-linked glue protein fractions can serve as good markers for introgression studies as their inheritance pattern can be traced among cytoraces, wherein distribution of *D. n. nasuta* and *D. n. albomicans* chromosomes is well known. In cytorace 1 and cytorace 2, *D. n. albomicans* X chromosomes were recorded (Ramachandra and Ranganath 1986), whereas in cytorace 3 and cytorace 4, *D. n. nasuta* X chromosomes were reported (Ramachandra and Ranganath 1990). Therefore, employing these X-linked glue protein markers, an attempt has been made to study the level of introgression of *D. n. nasuta* and *D. n. albomicans* genomes in these four cytoraces. The study provides an insight into the pattern of introgression of parental genes in cytoraces as far as glue proteins are concerned, and also evidence for the emergence of a novel glue protein pattern in one of the cytoraces.

Material and methods

Fly stocks

For the present study, the parents, namely (i) *Drosophila nasuta nasuta* ($2n = 8$; Coorg strain, India, 201.001), and (ii) *D. n. albomicans* ($2n = 6$; Okinawa, University of Texas collections, 3045.11), as well as four cytoraces, namely (iii) cytorace 1 (Cyt-1; $2n = 7$ in males, $2n = 6$ in females; Ramachandra and Ranganath 1986), (iv) cytorace 2 (Cyt-2; $2n = 6$; Ramachandra and Ranganath 1986), (v) cytorace 3 (Cyt-3; $2n = 8$; Ramachandra and Ranganath 1990), and (vi) cytorace 4 (Cyt-4; $2n = 7$ in males, $2n = 8$ in females; Ramachandra and Ranganath 1990), were employed. Diagrammatic representation of the karyotypes of *D. n. nasuta*, *D. n. albomicans* and the four cytoraces is given in figure 1. The age of these cytoraces is around 400–550 generations. Synchronized cultures were developed following a procedure modified from Delcour (Ramachandra and Ranganath 1988). These cultures were maintained under uniform conditions of relative humidity and 22 ± 1°C temperature in wheat cream agar medium seeded with yeast.

Preparation of samples, electrophoresis and pattern analysis

Late third-instar larvae were sexed, and salivary glands from 50 male and 50 female larvae of each race were dissected out separately in physiological saline. Each pair of glands was immediately fixed in 95% ethanol, and glue protein samples were prepared by dissolving in 20 μl sample buffer (Ramesh and Kalisch 1988). The samples were left at room temperature overnight and, prior to loading for electrophoresis, were boiled at 100°C for 10 min and centrifuged at 300 *g* for 10 min. Twenty μl of sample was loaded in each well. Electrophoretic separation was by SDS-PAGE (T = 13.7%, C = 3.5%), following the procedure of Ramesh and Kalisch (1988), at 70 V at room temperature. Protein bands were visualized by staining the gel with Coomassie Brilliant Blue (CBB) R-250 (Laemmli 1970) for 3–4 h. Subsequently, the gels were destained, documented and scored for protein patterns. The molecular masses of the variable protein fractions were determined relative to protein molecular mass marker (PWM-M, Bangalore Genei, India) using molecular analyst software (Bio-Rad, USA). On the basis of the number of differences, a dendrogram was constructed using MEGA 2.0 software (Kumar *et al*. 2001)

Volume analysis of major glue protein fractions

Volume analysis of the major glue protein fractions localized with CBB R-250 was done following the method of Ravi and Ramesh (2002). Briefly: The gel documentation system (Gel Doc 1000, Bio-Rad, USA) used in the present investigations for image analysis has light detectors. Using its 'volume analysis' mode, one can determine the relative differences in the intensities of individual band areas (optical density per sq mm). In terms of absolute quantitation, this reflects the concentration of the protein in specific bands. These OD values were determined for 25 females and 25 males. The level of significance for the quantitative differences of the major glue protein fractions among different strains/races was determined by means of ANOVA using SPSS 10.0 software, followed by multiple comparisons using Duncan's multiple range test (DMRT).

Results

Pattern analysis

Pattern of introgression of X-linked glue protein fraction in cytoraces: *D. n. nasuta* (Coorg) and *D. n. albomicans* (Okinawa) are parents of the four cytoraces employed in the present analysis. This analysis was taken up to understand the level of introgression of parental glue protein X-linked fractions among cytoraces. The analysis revealed the presence of 11 major glue protein fractions with three variant phenotypes. Each race (the parents as well as cytorace) had five major fractions (figure 2). There were no sex-specific differences nor intrapopulation variations in the glue protein patterns. Following Ramesh and Kalisch (1989b), these fractions were grouped into four domains (table 1).

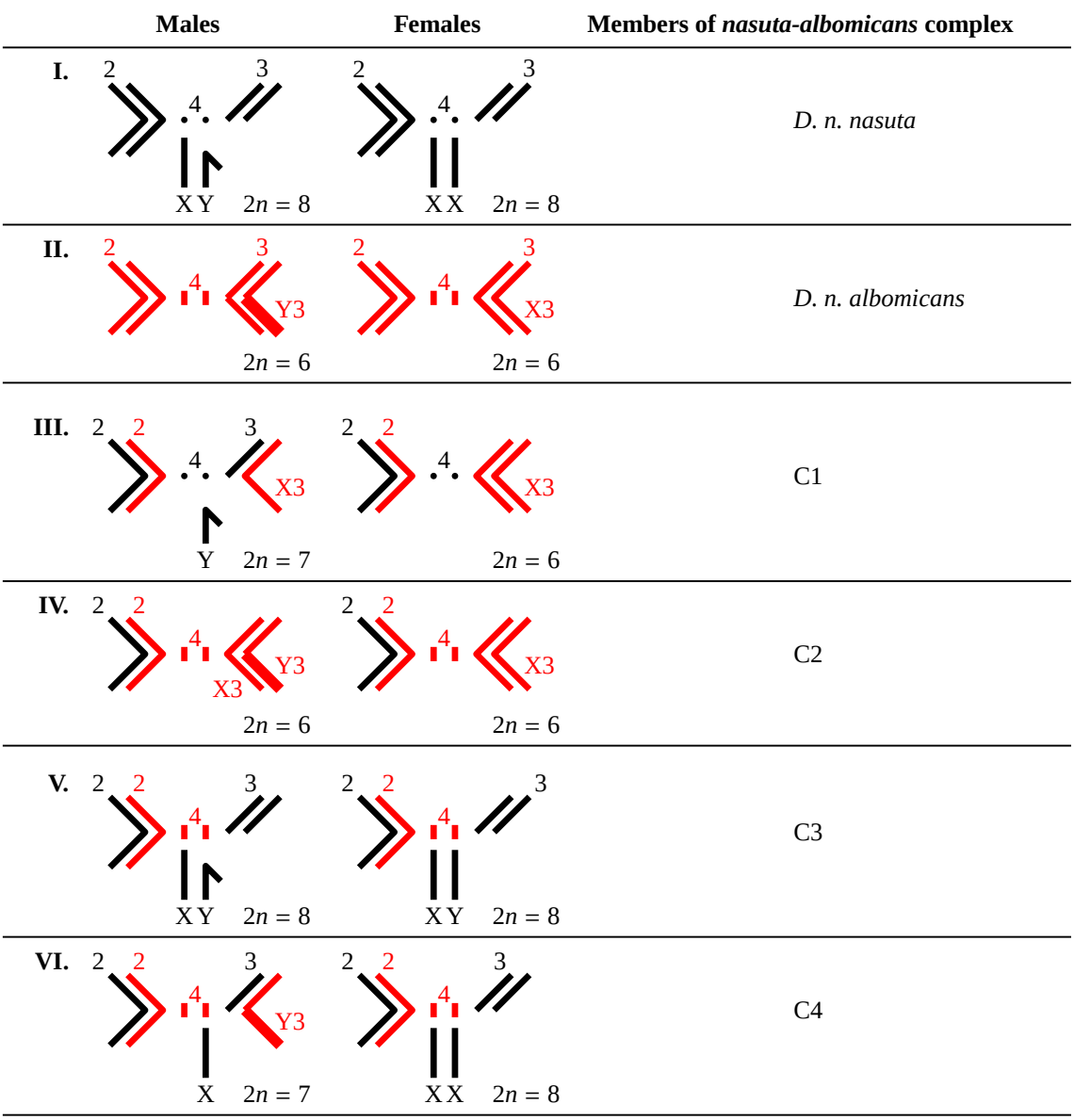

Figure 1. Illustration of the karyotypic composition of *D. n. nasuta*, *D. n. albomicans* and the four cytoraces. The patterns of representation of the parental chromosomes in the cytoraces are denoted by two colours.

Table 1. Glue protein fractions and quantitation by optical density (OD) values of overall major fractions in parents and four cytoraces.

		Molecular mass of glue protein				
Parent or race	Total no. of major fractions	I <100 kDa	II* <60 kDa to >30 kDa	III* <30 kDa to >20 kDa	IV 14 kDa	Combined OD values of major fractions*
D. n. nasuta	5	<100	43	30, 28	14	33.93 ± 0.37^{a}
D. n. albomicans	5	<100	35	25.5, 23	14	44.81 ± 0.5^{b}
Cytorace-1	5	<100	43	30, 28	14	39.23 ± 0.5^{c}
Cytorace-2	5	<100	39	22.5, 21	14	42.07 ± 0.36^{d}
Cytorace-3	5	<100	43	30, 28	14	38.57 ± 0.5^{c}
Cytorace-4	5	<100	43	30, 28	14	36.37 ± 0.4^{e}

*Values with the same superscript letter are not significantly different at 5% level according to DMRT (ANOVA on combined OD: $F_{5,294} = 87.405$; $P < 0.05$).

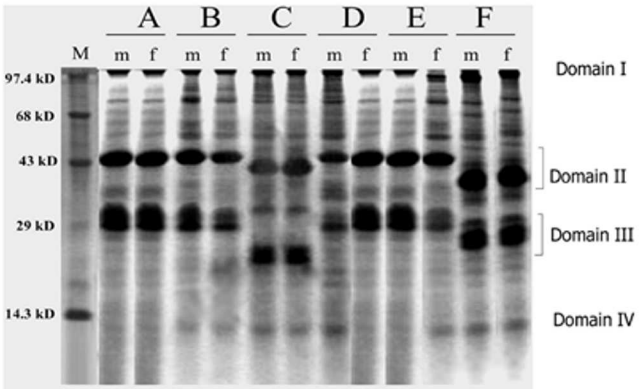

Figure 2. Glue protein electrophoretic patterns in (A) parent *D. n. nasuta* (Coorg), (B) cytorace-1, (C) cytorace-2, (D) cytorace-3, (E) cytorace-4, (F) parent *D. n. albomicans* (Okinawa). M, Marker; f, female; m, male.

Domains I and IV were monomorphic, with no variation in molecular mass of the fractions across the six members (parents and cytoraces), whereas the fractions representing domains II and III were polymorphic. Domain I was represented by a single fraction of molecular mass <100 kDa and domain IV was represented by a 14-kDa fraction. Domain II, which also comprised a single fraction in all the races, was represented by a 43-kDa fraction in four of the six races, namely *D. n. nasuta*, Cyt-1, Cyt-3 and Cyt-4, and by a 35-kDa fraction in *D. n. albomicans* and a 39-kDa fraction in Cyt-2. Domain III was represented by a doublet in each race, with 30-kDa and 28-kDa fractions in four of the six members analysed, namely *D. n. nasuta*, Cyt-1, Cyt-3 and Cyt-4, and 25.5-kDa and 23-kDa fractions in *D. n. albomicans* and 22.5-kDa and 21-kDa fractions in Cyt-2.

Of the four cytoraces, three had glue protein patterns similar to that of *D. n. nasuta*, and clustered along with *D. n. nasuta* in the dendrogram (figure 3). *D. n. albomicans* formed an independent lineage, as did Cyt-2, which exhibited neither the *D. n. nasuta* nor the *D. n. albomicans* pattern. Domain II of Cyt-2 was a 39-kDa fraction, which represents an average of the domain II fraction molecular masses of *D. n. nasuta* and *D. n. albomicans*, while domain III was represented by 22.5-kDa and 21-kDa fractions, instead of the 30-kDa/25.5-kDa or 28-kDa/23-kDa fractions in the parents.

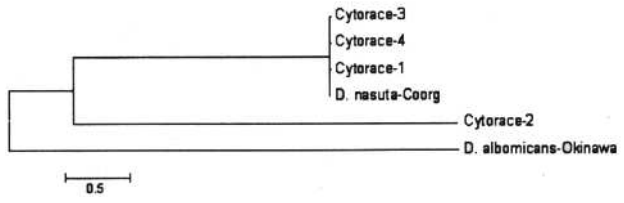

Figure 3. Dendrogram displaying the relationship among the parents and cytoraces.

Cross-check for the fidelity of Cyt-2 glue protein pattern: To confirm the consistency and linkage of domain II and III glue protein fractions in Cyt-2, we crossed Cyt-2 with *D. n. nasuta* and analysed the F_1 hybrid pattern. These domains are inherited in a codominant fashion in F_1 females, while F_1 males show only the maternal glue protein pattern, thus clearly indicating X linkage (figure 4).

To sum up, Cyt-3 and Cyt-4 which have *D. n. nasuta* (Coorg) X chromosome show *nasuta* glue protein pattern, Cyt-1 which has *D. n. albomicans* X chromosome shows *nasuta* pattern, and Cyt-2 shows a novel protein pattern.

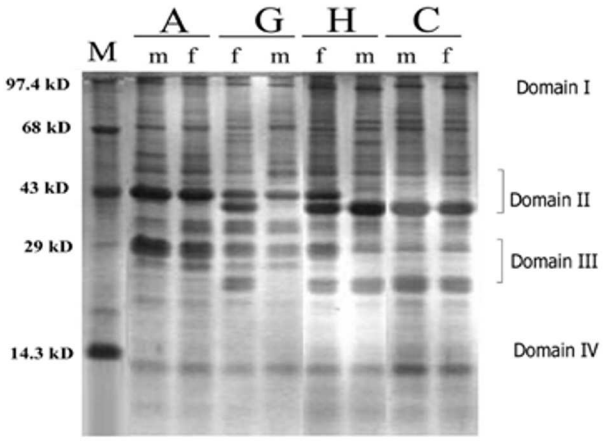

Figure 4. Glue protein patterns in *D. n. nasuta* (Coorg), cytorace-2 and their hybrids. (A) *D. n. nasuta* (Coorg), (G) hybrid between *D. n. nasuta* ♀ and cytorace-2 ♂, (H) hybrid between *D. n. nasuta* ♂ and cytorace-2 ♀, (C) cytorace-2. M, Marker; f, female; m, male.

Volume analysis

Quantity of major glue protein fractions among members of nasuta–albomicans complex: Volume analysis of the five overall major glue protein fractions among parents and cytoraces revealed highest OD values for *D. n. albomicans* and least for *D. n. nasuta* (table 1). The overall quantity of major glue fractions in the cytoraces was in between those in the two parents. Among the cytoraces, the OD value was highest for Cyt-2 and least for Cyt-4, and these two were found to be significantly different from each other. On the other hand, the difference in overall quantity of major glue protein fractions between Cyt-1 and Cyt-3 was not significant. Differences in OD values were significant for Cyt1/Cyt3 to Cyt-4 comparisons, even though the patterns of major glue fractions were identical. The sum intensities of glue protein fractions of all the four cytoraces were significantly different from those of both parents, and this is indicated in the DMRT clustering, wherein Cyt-1 and Cyt-3 cluster together whereas *D. n. nasuta*, Cyt-2, Cyt-4 and *D. n. albomicans* form independent clusters (table 1). The results of this semiquantitative analysis are consistent with the glue protein pattern analysis and also indicate a trend that does not fit into the expected chromosomal pattern of inheritance.

Further, even though Cyt-3 and Cyt-4 have *D. n. nasuta* X chromosome, their OD values were significantly different.

Therefore, only the X-linked fractions (domain II and domain III) were separately analysed. The statistical analysis revealed that the difference in OD values for the X-linked fractions of Cyt-3 and Cyt-4 was not significant. However, for Cyt-1 and Cyt-2, which have *D. n. albomicans* X chromosome, the OD values for the X-linked fractions were significantly different. On the whole, it appears that Cyt-2 has diverged the most from both the parents as well as the other cytoraces, as far as glue proteins are concerned.

Discussion

We have examined genetic variation of developmental-stage-specific and tissue-specific glue protein fractions among six members of the *nasuta–albomicans* complex employing SDS-PAGE to understand the pattern and extent of introgression among cytoraces using X-linked glue protein genes as markers. Apart from providing insight into the pattern/level of introgression among the hybrid lineages of the *nasuta–albomicans* complex, our study also provides a comprehensive picture of glue protein variation among these parental and hybrid lineages. The glue protein fractions were classified into four domains by Ramesh and Kalisch (1989b) according to homologies of their mobility and molecular mass. The present study revealed that cytorace-2 has a novel glue protein pattern.

The major glue protein fractions representing domains II and III of *D. n. nasuta* as well as *D. n. albomicans* show differences in mobility. F_1 hybrid analysis has confirmed their X-linkage with a codominant inheritance pattern (Ramesh and Kalisch 1988, 1989a). As no recombinant patterns were localized among F_1 hybrids, these fractions were considered to be inherited as a single unit (Ramesh and Kalisch 1989a). The karyotypic representation of the parental chromosomes (*D. n. nasuta* (Coorg) and *D. n. albomicans* (Okinawa)) in the chromosomes of the cytoraces is known (figure 1). The cytoraces have homologous X chromosome of one of the parents (Ramachandra and Ranganath 1986, 1990) and, as expected, heterozygosity for glue protein pattern was not noticed in any of the four cytoraces. On the basis of knowledge of the karyotypes of the cytoraces, we expected that cytoraces with *D. n. nasuta* or *D. n. albomicans* X chromosome would have *D. n. nasuta* or *D. n. albomicans* glue protein pattern, respectively, in the absence of introgression of glue protein genes from the chromosome of one parent into the chromosome of the other. Cyt-3 and Cyt-4 possess the *D. n. nasuta* X chromosome, and as expected they show *D. n. nasuta* parent glue protein pattern, thus confirming lack of recombination. However, Cyt-1, which possesses the *D. n. albomicans* X chromosome, has *D. n. nasuta* glue protein pattern. This could be due to one of two causes: (i) a recombination event during the early stages of establishment of this cytorace when there was coassociation of the X chromosomes of the parents, or (ii) presence of a *D. n. nasuta* modifying enzyme even if the glue protein gene was of *D. n. albomicans*.

Posttranslational modification, mutation and intragenic recombination have been suggested as causes for the origin of unexpected electromorphs in hybrids (Woodruff 1989). The present study showed that the glue protein profile of Cyt-2 (with *D. n. albomicans* X chromosome) is like neither of the parental profiles but rather an altogether novel pattern. The novel pattern in Cyt-2 could be attributed to one of the processes listed by Woodruff (1989). However, Zajonz *et al*. (1996) have shown that mobility differences observed in the X-chromosomal glue protein fractions in wild-type 'Sa' strain of *D. n. nasuta* and *D. n. albomicans* were not due to their association with carbohydrates. The differences seen in the electrophoretic mobility of X-chromosomal glue proteins of Cyt-2 in the present investigation may also not be due to glycosylation. It must be emphasized that posttranslational modification, mutation and intragenic recombination are neither mutually exclusive nor the only possible mechanisms that might have led to the variant profile observed in Cyt-2. Further studies may throw light on this.

In the present study volume analysis gave higher intensities for *D. n. albomicans* than *D. n. nasuta* but among the cytoraces the quantities for Cyt-1 and Cyt-3 lie around the midpoint between these parents. This possibly indicates stabilizing selection, wherein the quantities are moving away from both extremities represented by the parents (Strickberger 1985). On the other hand, Cyt-4 has significantly lower and Cyt-2 significantly higher value compared to the middle value, and this is in accordance with the karyotypic data (figure 1). It is possible that quantity of glue protein, an essential material for pupal attachment, is buffered in the cytoraces.

The dendrogram based on glue protein pattern (figure 3) indicates clustering of *D. n. nasuta* (Coorg), Cyt-1, Cyt-3 and Cyt-4. The clustering of Cyt-3, Cyt-4 and *D. n. nasuta* is in line with the karyotype data (Ramachandra and Ranganath 1986, 1990), while clustering of Cyt-3 with Cyt-4 and *D. n. nasuta* with Cyt-1 agree with isozyme data (Aruna and Ranganath 2004). Presence of Cyt-1, Cyt-2 and *D. n. albomicans* in different clusters is neither in accordance with karyotype data (Ramachandra and Ranganath 1986, 1990) nor in agreement with sternopleural bristle data (Harini and Ramachandra 1999b). The independence of Cyt-2 from both the parents is supported by mating preference experiments (Tanuja *et al*. 2001b), differences in body weight (Harini and Ramachandra 2000), copulation duration (Tanuja *et al*. 2001a), and isozyme data (Aruna and Ranganath 2004). Thus, Cyt-2 has diverged from both parents for most of the traits studied so far. Further, the confirmatory cross between Cyt-2 and *D. n. nasuta* for consistency and linkage of X-linked fractions has clearly shown that the glue protein pattern on SDS-PAGE can be employed as a marker for Cyt-2 among the six members analysed. On the other hand, volume analysis has shown clustering of Cyt-1 and Cyt-3, whereas *D. n. nasuta*, Cyt-2, Cyt-4 and *D. n. albomicans* form independent clusters (table 1). This is not

in accordance with karyotype data (Ramachandra and Ranganath 1986, 1990), morphometric traits (Harini and Ramachandra 1999a,b, 2000), mating preferences (Tanuja *et al*. 2001b), or isozyme data (Aruna and Ranganath 2004). Thus, the pattern of introgression for each trait shows lack of correlation for unrelated characters. Even the qualitative (pattern analyses) and quantitative (volume analysis) analyses of glue proteins do not correlate. Thus, introgression of genomes seems to have led to a mosaic pattern of genetic reorganization among the cytoraces.

The present study of glue protein pattern analysis in the *nasuta–albomicans* complex of *Drosophila* has thrown light for the first time on introgression of *D. n. nasuta* glue protein genes into the *albomicans* X chromosome in one of the cytoraces. Further, the appearance of a novel glue protein pattern among the cytoraces is evidence for the role of introgressive hybridization followed by recombination in the emergence of new characteristics during raciation. Thus, the members of the *nasuta–albomicans* complex appear to be a unique assemblage of closely related yet diversified forms which can be employed to understand regulation as well as expression of glue protein genes in different genetic backgrounds.

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