Molecular phylogeny of the *nasuta* subgroup of *Drosophila* based on 12S rRNA, 16S rRNA and CoI mitochondrial genes, RAPD and ISSR polymorphisms

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The *nasuta* subgroup is a cluster of morphologically almost similar forms with a wide range of geographic distribution. During the last three decades nature of inter-relationship among the members has been investigated at different levels of organization. The phylogenetic relationships of the members of the *nasuta* subgroup of the *immigrans* species group of *Drosophila* was made by employing Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats-PCR (ISSR-PCR) polymorphisms, mitochondrial 12S rRNA, 16S rRNA and Cytochrome C Oxidase subunit I (CoI) gene sequences. The phylogenetic tree generated by RAPD analysis is in nearly complete congruence with the classification based on morphophenotypic characters. The 12S and 16S rRNA genes were highly conserved across the *nasuta* subgroup and revealed only 3 and 4 variable sites respectively, of which only one site was informative. The CoI gene, on the other hand, revealed 57 variable sites of which 25 sites were informative. All the three species of orbital sheen complex were included in a major cluster in the phylogenetic trees derived from mitochondrial gene sequence data consistent with the morphophenotypic classification. The CoI analysis placed two species of frontal sheen complex, *D. n. nasuta* and *D. n. albomicans* in two different clades and this is inconsistent with morphological classification. The molecular clock suggested that divergence between the *kohkoa* complex and the *albomicans* complex occurred ~2.2 MYA, indicating recent evolution of the *nasuta* subgroup. The higher transition bias in the mitochondrial genes reported in the present study also suggested recent evolution of the *nasuta* subgroup.

Key words: CoI ISSR, *Drosophila*, evolution, RAPD, rRNA

INTRODUCTION

The *nasuta* subgroup of the *immigrans* species group of *Drosophila* is an assemblage of morphologically almost identical taxa with wide geographic distribution in Indo-Pacific regions extending from Hawaii to eastern tropical Africa and from tropical Australia to South Japan (Wilson et al., 1969; Kitagawa, et al., 1982). Based on the patterns of the frons in males, the members of the *nasuta* subgroup have been placed under three categories. The males of the frontal sheen complex species *D. n. nasuta*,

D. n. albomicans, *D. n. kepulauana* and *D. kohkoa* have a continuous silvery patch on the frons, the males of the orbital sheen complex species *D. s. sulfurigaster*, *D. s. albostrigata*, *D. s. neonasuta, D. s. bilimbata,* and *D. pulaua* have prominent whitish orbits along the edges of the compound eyes and the third category consists of *D. pallidifrons, Taxon F, Taxon I and Taxon J* in which the males are devoid of any marking on the frons (Wilson et al., 1969; Nirmala and Krishnamurthy 1972). The *nasuta* subgroup is one of the extensively investigated assemblages for its evolutionary biology (Ranganath, 2002). From the beginning of the pioneering work of Wilson et al., (1969), phylogeny of the *nasuta* subgroup has been extensively studied at various levels of organi-

zations namely, morphophenotypes (Nirmala and Krishnamurthy, 1972), hybridization and hybrid sterility (Ramachandra and Ranganath, 1988), karyotypes (Ranganath and Hägele, 1981; Wakahama et al., 1983; Ranganath and Ushakumari, 1987; Rao and Ranganath, 1991), hybrid salivary chromosomes (Lambert, 1978; Rajasekarasetty et al., 1980), heterochromatin and satellite DNA (Ranganath et al., 1982; Ranganath and Ushakumari, 1987), isozymes and allozymes (Kanapi and Wheeler, 1970; Ramesh and Rajasekarasetty, 1980), glue proteins (Ramesh and Kalisch, 1989), courtship patterns (Tanuja et al., 2001), courtship song (Shao et al., 1997), mitochondrial DNA (Chang et al., 1989; Yu et al., 1999), and male accessory gland proteins (Ram and Ramesh, 2001).

In spite of such in depth analysis of evolutionary phylogenetics of the *nasuta* subgroup, a consensus pattern among its members is yet to emerge. Molecular markers such as Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR-PCR) polymorphism studies developed in past decade have provided new insights to resolve such intricacies among closely related taxa (Zietkiewicz et al., 1994; Ballard, 2000). Further the sequence data analysis is considered to be the most efficient mode to look on to these aspects of evolutionary entities. Thus in the present investigation RAPD, ISSR-PCR polymorphisms, mitochondrial rRNA and CoI gene sequences, have been taken up with the aim to shed light on both the molecular divergence and the evolutionary interrelationships among the members of *nasuta* subgroup of *immigrans* species group of *Drosophila*. Perusal of the literature has revealed that these molecular markers were sensitive enough to resolve the systematic, evolutionary and phylogenetic intricacies in different groups of plant and animal systems (Nagaraju et al., 2001; Zietkiewicz et al., 1994; Ballard, 2000). In the current study, the phylogenetic tree generated by RAPD markers is in nearly complete congruence with the classification based on morphophenotypic characters. The ISSR-PCR markers and mitochondrial gene sequences, on the other hand, revealed phylogenetic relationships that do not correspond with morphological classification.

MATERIALS AND METHODS

***Drosophila* species** Seven taxa belonging to two complexes within the *nasuta* subgroup were studied. They are:
1) Frontal sheen complex: (i) *D. n. nasuta* (Mysore), (ii) *D. n. albomicans* (Okinawa), (iii) *D. n. kepulauana* (Brunei, Borneo), and (iv) *D. kohkoa* (Thailand),
2) Orbital sheen complex: (i) *D. s. sulfurigaster* (New Ireland), (ii) *D. s. neonasuta* (Mysore) and (iii) *D. s. albostrigata* (Cambodia).

These fly stocks were obtained from the Drosophila Stock Center, Department of Zoology, University of Mysore, Mysore, India.

DNA preparation Genomic DNA was prepared from about 50–100 flies following the standard method of Sambrook et al., (1989).

RAPD-PCR Fourteen random primers (Operon Technologies, Alameda, USA) were used: OPA-08, OPA-13, OPC-02, OPD-08, OPE-02, OPF-05, OPF-06, OPF-09, OPG-16, OPG-17, OPL-03, OPN-18, OPP-01 and OPP-02. PCR amplification of DNA was performed according to Williams et al., (1990). Southern hybridization was carried out to confirm the homology of co-migrating fragments of different species by following the standard procedure of Sambrook et al., (1989).

ISSR-PCR Totally 10 primers (4 designed in-house: $T_3(ATT)_4$, $CGA(ATT)_4$, $(YR)_8RYRY$ and $T(GT)_9$ and 6 obtained from University of British Columbia: UBC-807, UBC-810, UBC-811, UBC-812, UBC-842 and UBC-881 were used in the present investigation. PCR amplification was performed according to Zietkiewicz et al., (1994).

Mitochondrial DNA sequences The primer sets that were used to amplify the fragments of mitochondrial genes: 12S rRNA gene- SR-J-14199: TAC TAT GTT ACG ACT TAT (Location- 14182-14199), SR-N-14594: AAA CTA GGA TTA GAT ACC C (Location- 14594-14612), 16S rRNA gene- LR-J-13017: TTA CGC TGT TAT CCT AA (Location- 13001-13017), LR-N-13398: CAC CTG TTT AAC AAA AAC AT (Location- 13398-13417) and CoI gene- CI-J-1632: TGA TCA AAT TTA TAA T (Location- 1617-1632), CI-N-2191: GGT AAA ATT AAA ATA TAA ACT TC (Location- 2191-2213). The location of the primers refers to *D. yakuba* mitochondrial DNA sequences (Kambhampati and Smith, 1995). The *D. yakuba* sequence from NCBI database (Accession No. NC_001322) was used as outgroup.

Sequencing and analysis of the DNA sequences The PCR amplified products were sequenced using ABI Prism 377 automated sequencer (PE Biosystems). The sequences obtained from all the species were analyzed using GCG software (University of Wisconsine, Genetics Computer Group, Version 5.0) and CLUSTALV.

Phylogenetic analyses The gel images of RAPD and ISSR-PCR were analyzed for the presence or absence of a given amplification product of each species. Polymorphism between species was calculated (an index of genetic distance 1-F values) using the formula of Nei and Li, (1979). Phylogenetic trees were constructed using UPGMA (WINBOOT) computational programs. Phyloge-

netic analysis of the mitochondrial sequences was carried out by Neighbour Joining (NJ) method and Maximum Parsimony (MP) method with Kimura 2 parameter model as implemented in MEGA version 2.1 (Kumar et al., 2001). Bootstrap analysis (1000 bootstrap replications) was employed to test the reliability of the topologies of phylogentic trees in the present study. The AT percent and nucleotide substitution was calculated using DNASP 4.00 (Rozas et al., 2003).

The average substitution rate of CoI gene is calculated based on the divergence of CoI gene sequences between *D. melanogaster* and *D. yakuba* (8.7%), which took 6.1 million years (Russo et al., 1995). The divergence time was estimated using MEGA 2.1.

RESULTS

RAPD analysis The number and size of the amplified products varied depending upon the sequences of random primers and DNA samples used. The size of the amplified products ranged 300–2000 bp. A considerable amount of polymorphism was detected for all the 14 primers. A total of 124 fragments was scored and of these, about 88% (109 bands) were polymorphic and shared by at least two species, while 11 fragments were monomorphic (shared by all the species including members of both frontal and orbital sheen complexes). There are also fragments, which are species specific that are not shared by members of the *nasuta* subgroup. The occurrence of common and unique bands between species is valuable and can be used to gain phylogenetic information. However, it is important to demonstrate the homology of co-migrating bands before venturing into such comparisons, particularly when RAPD analysis is used to resolve species level phylogeny. Southern hybridization of RAPD gels was carried out using a purified single band reamplified in the presence of radiolabeled α ^{32}P nucleotide to demonstrate the homology between co-migrating bands. Autoradiographs of the two southern blots of RAPD profiles generated by OPA-08 and OPF-06 revealed that all fragments of the same size (the co-migrating bands) hybridized to the probe without cross hybridizing to any other bands (data not shown). The dissimilarity index (1-F) was calculated based on Nei and Li (1979). Among the frontal sheen complex, the least genetic dis-

tance (1-F) of 0.429 was recorded between *D. kohkoa* and *D. n. albomicans*, while the highest of 0.553 was between *D. kohkoa* and *D. n. nasuta*. Among the orbital sheen complex, the least 1-F value is 0.317 and it was between *D. s. albostrigata* and *D. s. neonasuta*, while the highest of 0.411 was between *D. s. neonasuta* and *D. s. sulfurigaster*.

The UPGMA (WINBOOT) analysis of the RAPD data (Fig. 1) revealed the relative positions of the members of the *nasuta* subgroup. The members of the orbital sheen complex namely, *D. s. sulfurigaster*, *D. s. albostrigata* and *D. s. neonasuta* along with *D. n. nasuta* from the frontal sheen complex formed one lineage, and other frontal sheen complex members namely *D. n. kepulauana, D. n. albomicans* and *D. kohkoa* formed the other lineage. Among three subspecies of orbital sheen complex, *D. s. albostrigata* and *D. s. neonasuta* are very close in their evolutionary relationship compared to *D. s. sulfurigaster*. Among frontal sheen complex, *D. kohkoa* and *D. n. albomicans* are more closely related and formed a distinct sub-
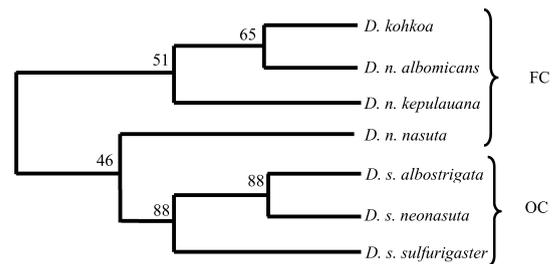
Fig. 1. UPGMA (WINBOOT) phylogenetic tree of the *nasuta* subgroup based on RAPD analysis. Numbers on nodes indicate the bootstrap values. (FC: frontal sheen complex; OC: orbital sheen complex)
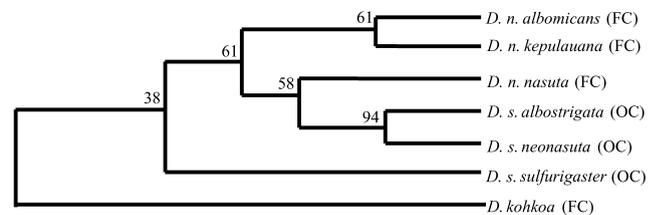
Fig. 2. UPGMA (WINBOOT) phylogenetic tree based on ISSR profile of the *nasuta* subgroup. Numbers on the nodes indicate bootstrap values. (FC: frontal sheen complex; OC: orbital sheen complex)

Table 1. Summary of sequence variation features of 12S, 16S rRNA and CoI mitochondrial genes of *D. nasuta* subgroup

	Total Length (bp)	Variable sites	Informative sites	Percentage of Informative Sites (%)	Average A+T Content (%)
12S rRNA	433	3 (17)	1 (1)	0.23 (0.23)	75.2 (75.1)
16S rRNA	418	4 (27)	1 (2)	0.24 (0.49)	77.9 (78.0)
CoI	539	57 (111)	25 (30)	4.64 (5.57)	65.3 (65.7)

The values within parentheses are obtained when the outgroup (*D. yakuba*) is included.

clade, while *D. n. kepulauana* shared a close relationship with this subclade.

ISSR analysis A total of 245 amplified products as scored and the size of the bands ranged 200–3000 bp. Out of 245 fragments, 238 (97%) were polymorphic between species. Three bands were unique to orbital sheen complex and one band was unique to the frontal sheen complex. The number of amplified products generated by a primer varied from 12 to 40 and most of the primers yielded more than 20 bands per primer. The primer T(GT)$_9$ and UBC-810 amplified 40 and 32 scorable products respectively, while the primer UBC-881 amplified a minimum of 12 products. Among the pairwise comparisons of ISSR scored products the least 1-F value among all species was 0.504 between *D. s. albostrigata* and *D. s. neonasuta*, while the highest is 0.789 between *D. kohkoa* and *D. s. albostrigata*. The genetic distance also varied between the members of each exophenotypic complex. In the frontal sheen complex, the least is 0.603 between *D. n. albomicans* and *D. n. kepulauana* and the highest was 0.759 between *D. kohkoa* and *D. n. kepulauana*. Among orbital sheen complex, the lowest value of 0.504 was noticed between *D. s. albostrigata* and *D. s. neonasuta* and the highest of 0.696 was recorded between *D. s. albostrigata* and *D. s. sulfurigaster*.

The UPGMA based phylogenetic tree (Fig. 2) showed *D. n. albomicans* and *D. n. kepulauana* of frontal sheen complex in one clade, while *D. s. albostrigata* and *D. s. neonasuta* of orbital sheen complex in another clade. *D. n. nasuta* was closer to the members of the orbital sheen complex namely *D. s. albostrigata* and *D. s. neonasuta* than to its sister members of the frontal sheen complex. On the other hand *D. kohkoa* of the frontal sheen complex and *D. s. sulfurigaster* of the orbital sheen complex occupied independent distinct positions.

Mitochondrial DNA sequence analysis In the present study, we sequenced a part of mitochondrial 12S rRNA, 16S rRNA and CoI genes. The average sizes of the fragments analyzed were 433 bp, 418 bp and 539 bp for 12S rRNA, 16S rRNA and CoI genes, respectively. The overall frequencies of A, T, G, C for 12S rRNA were 39.1, 36.2, 14.4 and 10.3%, for 16S rRNA gene it was 38.5, 39.5, 12.4 and 9.6%, and for CoI gene 37.9, 27.5, 17.8 and 16.8%, respectively. The A+T content for the fragments of 12S rRNA, 16S rRNA and CoI genes were 75.2%, 77.9% and 65.3% respectively within the *nasuta* subgroup (Table 1). We also determined the transition bias ratio for these three gene sequences. Substitutions in 12S rRNA are exclusively transitions, while the rates of transitions are higher for 16S rRNA and CoI sequences (Fig. 6).

The number of informative sites of the three genes varied greatly. The highest number of polymorphic sites

was found in CoI (57), followed by 16S rRNA (4) and 12S rRNA (3) (Table 1). Whereas, when the outgroup (*D. yakuba*) was included in the analysis, the three genes showed 111, 27 and 17 polymorphic sites, respectively. Kimura 2-parameter model of MEGA 2.1 revealed a range of genetic distance from 0 to 0.005 in case of 12S rRNA gene, 0 to 0.007 for 16S rRNA gene and 0 to 0.094 with CoI gene among the members of *nasuta* subgroup when the outgroup was not included. The sequences of these mitochondrial gene fragments were submitted to the GenBank (accession numbers: 12S rRNA AF332701-07, 16S AF387332-38 and CoI AY633549-55).

Phylogenetic trees were constructed for the mitochondrial sequences and the topologies of the trees were tested for confidence levels by bootstrap analysis. Always a 50% bootstrap consensus tree topology was considered. The NJ and MP trees showed similar topologies (data not shown). The NJ tree of 12S rRNA (Fig. 3) showed two major clades, one with *D. n. kepulauana* and all the remaining subspecies came out as a separate clade in

Fig. 3. 50% bootstrap consensus Neighbour Joining (MEGA) phylogenetic tree of the *nasuta* subgroup based on mitochondrial 12S rRNA gene sequences. (FC: frontal sheen complex; OC: orbital sheen complex)

Fig. 4. 50% bootstrap consensus Neighbour Joining (MEGA) phylogenetic tree of the *nasuta* subgroup based on mitochondrial 16S rRNA gene sequences. (FC: frontal sheen complex; OC: orbital sheen complex)
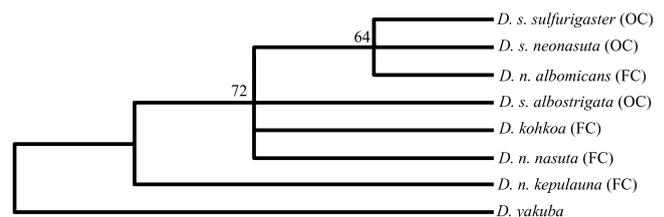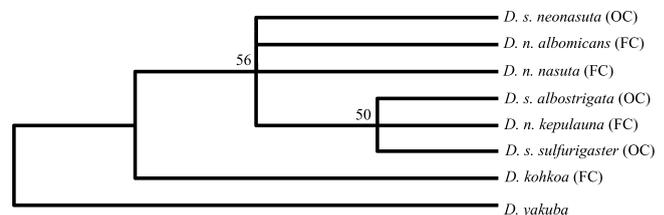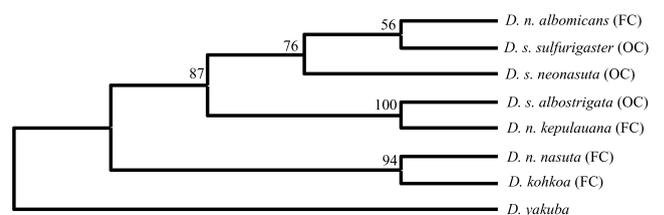

Fig. 5. 50% bootstrap consensus Neighbour Joining (MEGA) phylogenetic tree of the *nasuta* subgroup based on mitochondrial CoI gene sequences. (FC: frontal sheen complex; OC: orbital sheen complex)

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(A)        7 34 38 41 44 46 49 58 155 206 222 223 226 259 285 286 297 305 334 366 382
Dsa        A  A  T  T  A  A  T  G  G   A   A   T   T   T   A   A   A   A   T   T   A
Dnk        -  .  .  .  .  .  .  .  .   .   .   .   .   .   .   .   .   .   .   .   .
Dsn        -  .  .  .  .  .  .  .  .   .   .   .   .   .   .   .   .   C   .   -   .
Dna        -  .  .  .  .  .  .  .  .   .   .   .   .   .   .   .   .   C   .   .   -
Dss        -  .  .  .  .  .  .  .  .   .   .   .   .   .   .   .   .   C   .   -   .
Dnn        .  .  .  .  .  .  .  .  .   .   .   .   .   .   .   G   .   .   .   .   .
Dk         -  .  .  .  .  .  .  .  .   .   .   .   C   .   .   .   .   .   .   .   .
Dy         -  G  A  -  T  G  A  A  A   T   T   A   A   .   T   T   G   T   .   .   .
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(B)        2 32 35 38 42 47 63 84 86 169 175 176 177 212 213 214 215 252 262 280 285 294 342 361 362 373 379 390 406
Dsa        A  A  A  T  A  G  T  C  A  T   T   T   T   A   A   A   A   A   T   T   A   T   T   G   T   G   T   T   -
Dnk        .  .  .  -  .  .  .  .  .  .   .   .   .   .   .   .   T   .   .   .   .   .   .   A   C   .   .   .
Dsn        .  .  .  .  .  .  .  .  .  .   .   .   G   T   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Dna        .  .  .  .  .  .  .  .  .  .   .   .   .   .   .   .   T   .   .   .   .   .   .   .   .   .   .   .
Dss        .  .  .  .  .  .  .  .  .  .   .   .   .   .   .   .   T   .   .   .   .   A   .   .   .   .   .   -
Dnn        .  .  .  .  .  .  .  .  .  .   .   .   .   .   .   .   T   .   .   .   .   .   .   .   .   .   .   .
Dk         .  .  .  .  .  .  .  .  .  .   .   .   .   .   .   .   T   .   .   .   .   A   .   .   .   .   .   .
Dy         G  T  T  A  .  A  A  T  G  A   A   A   A   T   .   .   T   T   A   A   T   A   A   A   .   T   A   A   G
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(C)        1 13 15 16 17 18 22 25 31 37 43 49 52 55 64 70 73 78 91 94 97 100 102 106 111 117 118 120 121 123 133 139 142 148 151 169 175 184 186 193 208 226 247 256 258 262 268 273 274 277 278 289 295 298 301
Dsa        G  A  A  C  A  G  T  G  T  T  G  G  T  G  A  A  A  A  T  C  G  T   A   T   A   A   A   G   T   A   T   T   A   T   T   A   A   T   A   T   T   T   G   T   A   G   T   A   G   A   A   T   A   T   A   T
Dnk        .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Dsn        .  G  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  T   A   .   .   G   .   G   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   C   .   .   .   .
Dna        .  .  .  .  C  .  .  .  C  C  .  .  .  .  .  .  .  .  .  .  .  T   A   .   .   G   .   G   .   .   .   .   .   C   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   C   .   .   .   .   .   .
Dss        .  .  T  .  .  .  .  C  C  A  .  .  .  .  .  .  .  .  .  .  .  T   .   .   .   G   G   .   C   .   .   C   C   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   C   .   .   .   .   .   .
Dnn        A  .  .  .  C  .  .  C  .  .  .  .  A  .  .  .  .  .  .  .  .  T   A   .   A   .   .   .   .   .   .   .   .   .   .   G   .   .   .   .   C   A   .   .   .   .   A   .   C   .   .   .   .   .   .   .
Dk         A  .  T  C  .  .  C  .  .  .  A  G  .  .  G  .  T  A  .  .  A  G   .   .   .   .   .   .   .   T   .   .   G   .   .   C   .   .   A   .   .   G   A   .   .   G   A   .   .   C   G   .   .   .   .   .
Dy         A  G  .  .  A  A  A  .  A  A  A  A  A  .  G  T  .  A  T  .  A  G   .   .   G   T   A   A   G   A   A   T   .   .   G   T   A   T   .   C   .   A   A   G   A   A   .   A   T   .   .   .   A   G   A
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           307 309 312 313 319 322 327 330 331 336 337 340 343 346 352 358 361 364 367 369 370 372 373 375 376 379 381 385 388 391 394 396 403 421 424 427 430 433 436 439 445 457 465 472 475 478 481 487 493 496 526 529 535 536 538 539
Dsa         T   A   G   G   A   A   T   A   C   T   T   G   C   A   C   A   A   G   T   G   A   G   A   G   G   T   G   T   G   A   A   G   A   A   G   G   C   A   T   G   C   A   A   A   T   T   A   A   T   A   A   T   A   -   C   T
Dnk         .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   A   .   .   .
Dsn         C   .   .   A   G   .   .   .   .   .   .   .   .   .   .   .   A   .   .   .   .   .   .   .   .   .   A   .   A   .   .   .   .   A   .   .   .   .   T   .   .   .   .   .   .   .   .   .   .   .   .   .   A   .   .   .
Dna         .   .   .   A   G   .   .   .   .   .   T   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   A   .   A   .   .   .   .   A   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   A   .   .   .
Dss         C   .   .   .   G   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   A   .   A   .   .   .   .   A   .   .   .   .   .   .   .   .   .   .   .   .   .   .   -   .   .   .   .   .   .   .
Dnn         .   .   .   .   G   .   .   .   .   .   T   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   A   .   A   .   G   .   .   A   .   .   C   .   T   .   C   .   .   .   .   T   .   .   .   .   .   .   .   .   .   .
Dk          .   G   .   .   .   G   G   C   .   G   .   .   T   .   .   .   .   .   .   .   .   .   .   .   .   .   G   .   .   A   .   .   .   .   .   .   T   G   C   C   C   .   C   T   G   .   .   .   C   A   .   .   .   .   .   .
Dy          A   .   A   A   G   .   .   .   A   .   A   A   T   G   A   T   T   T   .   A   T   A   T   A   A   A   .   A   A   T   T   A   T   T   .   T   T   G   A   A   T   C   .   .   C   C   .   .   .   T   T   A   .   A   T   A
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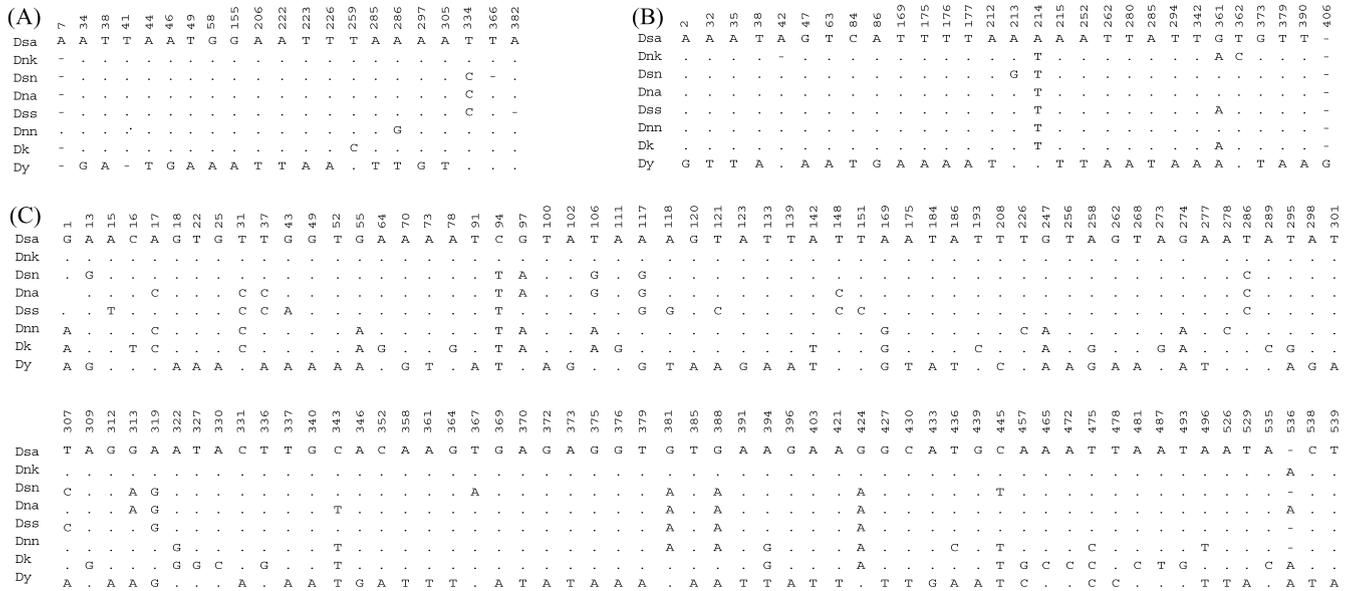
Fig. 6. Polymorphic sites of (A) 12S rRNA, (B) 16S rRNA and (C) CoI genes of mitochondrion with *D. s. albostrigata* as master sequence. Only those nucleotides that differ from the master sequence at these positions are shown. Dots denote identity with master sequence. '–' indicates absence of any nucleotide. Number above the figure is the relative position of the nucleotide. **Dsa**: *D. s. albostrigata*, **Dnk**: *D. n. kepulauana*, **Dsn**: *D. s. neonasuta*, **Dna**: *D. n. albomicans*, **Dss**: *D. s. sulfurigaster*, **Dnn**: *D. n. nasuta* and **Dk**: *D. kohkoa*. The sequence of *D. yakuba* (**Dy**) is included as an outgroup

which a distinct subclade was formed comprising of *D. s. sulfurigaster, D. s. neonasuta and D. n. albomicans*. The NJ tree of 16S rRNA gene sequences (Fig. 4) also showed two distinct clades, one with *D. kohkoa* and the other with *D. s. sulfurigaster, D. s. albostrigata and D. n. kepulauna* which formed a subclade. The NJ tree of CoI (Fig. 5) formed two major clades. One clade included the frontal sheen complex species, *D. n. nasuta* and *D. kohkoa* and the other clade comprised of *D. n. albomicans, D. s. sulfurigaster, D. s. neonasuta, D. s. albostrigata* and *D. n. kepulauna*. This clade, however, further resolved in to two branches: one with *D. s. albostrigata* and *D. n. kepulauna* and the other with *D. n. albomicans, D. s. sulfurigaster* and *D. s. neonasuta* which again formed two clades, one with *D. n. albomicans* and *D. s. sulfurigaster* and the other with *D. s. neonasuta*. Since the bootstrap values were robust the groupings based on CoI could be considered reliable.

We determined the time of divergence of 'albomicans' and 'kohkoa' complexes based on the divergence of CoI gene sequences between *D. melanogaster* and *D. yakuba*. Considering the substitution rate of CoI gene in *Drosophila* as 1.4% per million years (Russo et al., 1995), the 'albomicans' and 'kohkoa' complexes diverged approximately 2.2 MYA.

DISCUSSION

Yu et al., (1999) identified two major clusters i.e. *kohkoa* and *albomicans* complexes in the *nasuta* subgroup based on their geographic distribution and mitochondrial ND4

and ND4L sequences. The 'kohkoa' complex included *D. s. sulfurigaster, D. pulaua, D. kohkoa* and *Taxon F*, while the 'albomicans' complex comprised *D. albomicans, D. nasuta, D. s. neonasuta, D. s. albostrigata, D. pallidifrons, Taxon I* and *Taxon J*. In the present study the two complexes differed in morphophenotypic complexes *i.e.* frontal sheen and orbital sheen complexes. Of the five phylogenetic trees generated in the present study using five different marker systems, the one generated out of RAPD markers is in nearly complete congruence with the classification based on morphophenotypic characters. It showed two distinct clusters, one including three subspecies of frontal sheen complex (*D. kohkoa, D. n. albomicans D. n. kepulauana*) and the other with all the subspecies of orbital sheen complex (*D. s. albostrigata, D. s. neonasuta* and *D. s. sulfurigaster*) and *D. n. nasuta* of the frontal sheen complex. Though *D. n. nasuta* belongs to frontal sheen complex, it has been clustered with orbital sheen complex, which is inconsistent with the morphological classification (Fig. 1). The three subspecies of orbital sheen complex are considered closely related since they are hybridizable among themselves to a certain degree. The clustering together of the subspecies of orbital sheen complex based on RAPD data is consistent with the earlier reports based on interspecific hybridization (Ramachandra and Ranganath, 1988), isozymes (Kanapi and Wheeler, 1970; Ramesh and Rajasekarsetty, 1980), courtship song (Shao et al., 1997) and male accessory gland proteins (Ram and Ramesh, 2001). All the three subspecies of orbital sheen complex were included in a major cluster in the phylogenetic trees derived from

12S rRNA, 16S rRNA and CoI sequence data consistent with the morphophenotypic classification. Out of these, the bootstrap values were quite high for the tree generated based on CoI gene and hence the results could be considered reliable.

Of the four taxa of the frontal sheen complex analyzed in the present study, *D. n. nasuta* and *D. n. albomicans* are morphologically identical and cross-fertile. Hybrid populations of these can be maintained for any number of generations. Earlier studies have shown that *D. n. albomicans* with 2n=6 is derived from *D. n. nasuta* with 2n=8, through a centric fusion between autosomes and sex chromosomes and hence are treated as chromosomal races. A number of studies based on different marker systems (Chang et al., 1989; Shao et al., 1997; Yu et al., 1999; Ram and Ramesh, 2001) have shown unambiguous evolutionary affinities between the two races. In all these studies *D. kohkoa* and *D. n. kepulauana* are distantly placed with reference to these chromosomal races namely *D. n. nasuta* and *D. n. albomicans*. In the present study, ISSR and 16S rRNA based analyses placed *D. kohkoa* as a distinct species from the rest of the species, CoI revealed close affinity among *D. n. nasuta* and *D. kohkoa*, whereas only 12S rRNA analysis included *D. n. kepulauana* in a separate clade.

The NJ tree of CoI gene revealed two distinct clades, one consisting of *D. n. albomicans, D. s. sulfurigaster, D. s. neonasuta, D. s. albostrigata* and *D. n. kepuluana*- the *albomicans* complex and the other included *D. n. nasuta* and *D. kohkoa*- the *kohkoa* complex. Contrary to Yu et al.',s (1999) observation based on ND4 gene, in our study the *D. s. sulfurigaster* was included in *albomicans* complex and *D. n. nasuta* was in *kohkoa* complex suggesting different mutation rates in various mitochondrial genes in the *nasuta* subgroup. In contrast to the previous observations CoI analysis placed *D. n. nasuta* and *D. n. albomicans* in two different clades.

The high A+T content of all the three mitochondrial genes in the species studied agrees with the earlier reports on other insects including *Drosophila* (Clary and Wolstenholme, 1985; Yu et al., 1999; Crozier and Crozier, 1993; Mitchell et al., 1993). However, our results show that the A+T bias is relatively low in CoI gene as compared to 12S rRNA, 16S rRNA reported in the present study and ND4 and ND4L mitochondrial genes (Yu et al., 1999). The transition bias observed in 12S rRNA, 16S rRNA and CoI sequences is consistent with the mitochondrial DNA of other *Drosophila* species (Tamura, 1992; Moriyama and Powell, 1997). The transition bias is accompanied by A+T richness in mitochondrial DNA of *Drosophila*, which causes an apparently lower transition/transversion ratio in less closely related species as contended by Tamura, (1992) and Yu et al., (1999). De Salle et al., (1987) observed a much higher rate of transitions especially in closely related Hawaiian *Drosophila*. In

the present study, the exclusive transitions in the 12S rRNA sequence and higher transitions in the 16S rRNA and CoI sequences (Fig. 6) are suggestive of the lower nucleotide divergence and closer affinity of the species of the *nasuta* subgroup. This is also consistent with the estimated genetic distances and low rate of nucleotide substitutions in the three sequences. DeSalle et al., (1987) have proposed that the number of sites that accept substitutions is very small because of a functional constraint (that is peculiar to a particular taxonomic group), which impedes the mitochondrial DNA sequence divergence. Therefore, transition bias in the mitochondrial genes reported in the present study suggests recent divergence of the members of the *nasuta* subgroup.

At an average substitution rate of 1.4% per million year, 8.7% substitution in the CoI gene between *D. melanogaster* and *D. yakuba*, accounts for divergence time of ~6.1 million years (Russo et al., 1995). Using this information, we calibrated the molecular clock of the *nasuta* subgroup and estimated that the *kohkoa* complex and *albomicans* complex have diverged ~2.2 MYA. Our results suggest that the divergence within the *nasuta* subgroup occurred much later than that within *melanogaster* subgroup (Russo et al., 1995).

Morphological, cytological, hybridization and molecular data are not in complete agreement in uncovering the phylogenetic affinities of the *nasuta* subgroup. Even the informative CoI gene sequence data does not correspond with the morphological classification of the members of this group indicating that morphology could be a result of congruent evolution rather than indicative of homologous traits across the species group. Mitochondrial introgression through natural hybridization process could be an alternate possibility but we have not come across natural hybrids in the *nasuta* subgroup.

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