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Studies on isozyme variations in a few members of *Drosophila nasuta* subgroup

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Abstract. Isozyme variation at six enzyme loci has been studied involving nine members of *Drosophila nasuta* subgroup, by employing polyacrylamide gel electrophoretic technique. Alleles at three loci namely Acph, Aph and α-Est are found to be highly polymorphic; whereas at β-Est locus the alleles are less variable while at α-Gpdh and To loci, the alleles are found to be least variable. Null alleles are encountered in low frequencies at α-Est, β-Est and c-Gpdh enzyme loci. The allelic frequencies obtained at the six enzyme loci have been utilised as a yardstick to measure the extent of genetic relationship between the species studied. The genetic identity and genetic distance between these closely-related species have been estimated by adopting the Nei's formula. These findings have been discussed with reference to earlier cytogenetic and hybridisation studies made on this subgroup.

Keywords. *nasuta* subgroup ; morphophenotypic complex ; isozymes ; genetic identity ; genetic distance ; *Drosophila nasuta.*

1. Introduction

In recent years, analysis of isozymes through gel electrophoresis has been extensively employed in the study of population genetics. Hunter and Markert (1957) developed the " zymogram " technique to demonstrate the occurrence of isozymes. This method involved the electrophoretic separation, usually of crude extract, followed by demonstration of zones of enzymatic activity with the help of specific histochemical staining procedures applied directly to the electrophoretic medium.

Hubby and Throckmorton (1965) who studied differences in the soluble proteins in the species of *virilis* group were the first to present the possibility of interpreting phylogenetic relationships by the comparison of the " biochemical traits ". Many investigators (Johnson *et al* 1966; Hubby and Throckmorton 1968; Nair *et al* 1971; Yang *et al* 1972; Ayala *et al* 1974a; Zouros 1974; Lakovaara *et al* 1976) have exploited this potent technique to detect enzyme variation in different species of *Drosophila.* This has culminated in establishing the genetic basis of their species relationships. Thus, the availability of techniques to detect isozyme variations by electrophoretic assay has made it possible to analyse a previously inaccessible part of the genome and thus provide an estimate of the genetic similarity or dissimi-

larity between various populations of a species or between different species of a group or subgroup at a number of loci.

The *nasuta* subgroup of the *immigrans* group of *Drosophila* represents a typical cluster of closely related species which are morphologically almost similar. Females of this subgroup are morphologically similar and cannot be told apart; while the males have silvery markings on the frons. The extent of such marking varies. Based on this character, Nirmala and Krishnamurthy (1972) have divided this subgroup into three morphophenotypic complexes namely,

(i) Frontal sheen complex, which includes species with silvery sheen over the entire frons—*D. nasuta nasuta, D.n. albomicana, D.n. kepulauana* and *D. kohkoa.*

(ii) Orbital sheen complex, which includes species with silvery markings confined to the sides of the frontal orbits—*D. nixifrons, D. sulfurigaster sulfurigaster, D.s. albostrigata, D.s. bilimbata, D.s. neonasuta and D. pulaua.*

(iii) Species without any such silvery markings on the frons—*D. pallidifrons.*

Kanapi and Wheeler (1970) made inconclusive preliminary studies on the isozyme variation in only three members of the *nasuta* subgroup. However work on the genetic differences based on the isozyme assay is wanting for most of the members of this subgroup. Hence the present project was undertaken to study the isozyme variation in nine species of the subgroup, to estimate the genetic identity and hence the genetic distance among these members and to compare the genetic data obtained by these biochemical analyses with those of cytogenetic findings.

2. Materials and methods

The members of the *nasuta* subgroup which constituted the material for the present study are given in table 1. The geographic origin of these members are also given. Equal number of males and females of each species were used for the present studies. Acid phosphatase (Acph), alkaline phosphatase (Aph), α-esterase (α-Est), β-esterase

Table 1. Members of *D. nasuta* subgroup involved in the present studies.

Species	Geographic origin
D.n. nasuta	Coorg (Karnataka, India)
D.n. albomicana	Okinawa
D.n. kepulauana	Sarawak
D. kohkoa	Gulf of Thailand
D. pulaua	Sarawak
D.s. neonasuta	Coorg (Karnataka, India)
D.s. sulfurigaster	Wau (New Guinea)
D.s. bilimbata	Fiji
D.s. albostrigata	Mt. Makelins (Laguna)

(*β*-Est), *α*-glycerophosphate dehydrogenase (*α*-Gpdh) and tetrazolium oxidase (To) are the enzymes studied in the present investigation. Three-day old males and females formed the sample. Each fly was ground with 0·2 ml of double-distilled water (glass-distilled) in small tissue grinders and kept separately. Assays were made by disc electrophoresis as described by Davis (1964) with slight modifications (Rajasekarasetty *et al* 1976) where polyacrylamide gel was used as supporting medium. The electrophoresis was carried out at 4° C with appropriate current for desired length of time. The gels were then incubated in an appropriate staining solution. After the enzyme bands appeared, the gels were washed in distilled water and then fixed in 7% acetic acid.

2.1. *Assay and staining procedures*

The following tray buffers were used:

(A) 0·3 M sodium hydroxide boric acid buffer—pH 8·65.

(B) Tris borate EDTA buffer of 87 mM tris (hydroxymethyl) methylamine, 8·7 mM boric acid, 1 mM EDTA and 1 mM NAD—pH 9·0.

The activity of acid phosphatase and alkaline phosphatases was detected by their capacities to hydrolyse *α*-naphthyl phosphate to liberate *α*-naphthol, at pH 5·0 and 8·5 respectively. The released *α*-naphthol is coupled with a diazonium salt (fast blue salt RR) which form a stain at the sites of enzyme activity.

The staining reaction of *α* and *β*-esterases on gels, after electrophoresis, is achieved by trapping enzymatically-released naphthol (or a naphthol-derivative) from 1-naphthyl acetate and 2-naphthyl acetate respectively, with the diazonium salt (fast blue salt RR). This reaction produces an insoluble stain at the sites of enzymatic activity.

The localisation of *α*-glycerophosphate dehydrogenase on electrophoretic media is made by incubating the electrophoresed gels in a solution containing *α*-glycerophosphate as the substrate and the oxidised form of the coenzyme (NAD). The reduced NAD formed during the reaction passes electrons through an intermediate electron carrier phenazonium methosulphate, to a tetrazolium compound (nitro blue tetrazolium) resulting in the formation of an insoluble purple diformazan dye at the sites of enzyme activity.

The enzyme tetrazolium oxidase on gels is localised by its capacity to oxidise the tetrazolium salt (nitro blue tetrazolium) which is used as a substrate in the presence of phenazonium methosulphate.

The assay and staining procedures for different enzymes are as follows:

(i) *Acid phosphatase* (*Acph*): Tray buffer A, electrophoresis for 2 hr with 80 V. Stain: 500 mg polyvinylpyrrolidone, 100 mg sodium 1-naphthyl phosphate, 100 mg fast blue salt RR, 60 mg magnesium chloride, 60 mg manganese chloride and 2 g of sodium chloride, dissolved in 100 ml of 0·125 M acetate buffer (acetic acid sodium acetate buffer) of pH 5·0.

(ii) *Alkaline phosphatase* (*Aph*): Tray buffer A, electrophoresis for 2 hr with 80 V. Stain: The constituents and the quantity are same as above, but dissolved in 100 ml of 0·05 M tris-HCl buffer (pH 8·5).

(iii) *α-Esterase* (*α-Est*): Tray buffer A, electrophoresis for 2 hr with 80 V. Stain: 100 mg 1-naphthyl acetate dissolved in 2 ml acetone, to which 2 ml of distilled water was added and mixed with 50 ml of 0·1 M phosphate

buffer (pH 5·9) to which 100 mg of fast blue salt RR was added. The whole solution was mixed with 50 ml of 0·1 M phosphate buffer (pH 6·5).

(iv) *β-Esterase (β-Est)* : Tray buffer A, electrophoresis for 2 hr with 80 V. Stain: The constituents and the quantity and the preparation of stain was same as above except for the substrate where 2-naphthyl acetate was used instead of 1-naphthyl acetate.

(v) *α-Glycerophosphate dehydrogenase (α-Gpdh)* : Tray buffer B, electrophoresis for 2½ hr with 100 V. Stain: 25 mg β–NAD, 20 mg nitro blue tetrazolium, 180 mg EDTA, 800 mg sodium α-glycerophosphate, 5 mg N-methyl-phenazonium methosulphate dissolved in 100 ml of 0·05 M tris-HCl buffer (pH 8·5). The gels were incubated in dark at 37° C.

(vi) *Tetrazolium oxidase (To)* : Tray buffer B, electrophoresis for 2½ hr with 100 V. Stain: 25 mg β-NAD, 20 mg nitro blue tetrazolium, 5 mg N-methyl phenazonium methosulphate dissolved in 100 ml 0·05 M tris-HCl buffer (pH 8·5). The gels in the incubating solution were exposed to light, till white bands appeared on blue background.

Since the mobility of a protein in an electric field depends on the number of charges on the protein, the sign of the net charges, the degree of dissociation which is a function of pH and the magnitude of the electrical field potential, care was taken to maintain the pH of buffers and the current, always constant for particular assays.

Different alleles at each enzyme locus are named following the genetic nomenclature used by Ayala *et al* (1971). At each locus, one allele which is the most common (when all the species analysed are compared) is given the value of 1·00. Other alleles are named with reference to that standard. An allele designated 0·95 codes for an enzyme which migrates 5 mm less than the standard, towards the anode and an allele coding for an enzyme whose migration is 5 mm more than the standard, is designated 1·05. The abbreviations used to designate each enzyme is already given at the beginning of this section. The same abbreviations are used to represent genes coding for the enzymes and the alleles are written as super-scripts over the symbol representing the locus. Thus $To^{1.00}$, $To^{1.08}$, $To^{1.10}$ and $To^{1.15}$ are the four alleles at To enzyme locus.

3. Results

Totally six isozyme systems have been studied in nine members of the *nasuta* sub-group. The number of genomes sampled from each species, different alleles at each enzyme locus and their frequencies are included in tables 2 to 7. Perusal of these tables indicates that, of the six isozyme systems studied, three namely, Acph, Aph and α-Est are highly polymorphic. The frequencies of alleles are highly variable at these three isozyme loci where no two species have similar patterns of allelic frequencies. At β-Est locus, the variation is less, while α-Gpdh and To loci have least variation both in number of allelic variants and their frequencies.

Null alleles, the products of which do not display *in vitro* catalytic activity, are detected in three namely α-Est, β-Est and α-Gpdh enzyme systems. These null alleles whenever found, were always very low in frequency.

Table 2. Allelic frequencies at Acph locus in members of *Drosophila nasuta* subgroup.

Species \ Alleles	0·72	0·74	0·75	0·78	0·80	0·82	0·85	0·88	0·90	0·91	0·93	0·95	0·98	1·00	1·02	1·03	1·06	1·10
D.n. nasuta (*n*=80)	1·00	..	1·00	..	0·33	..	0·66	0·15	0·03	..	0·07	..	0·07	10·0	0·40	..	..	..
D.n. albomicana (*n*=96)	0·57	..	0·70	0·43	0·43	..	0·33	..	..	..	0·37	0·30	0·40	0·57	..	..	..	..
D.n. kepulauana (*n*=60)	1·00	1·00	0·20	..	0·30	..	..	0·10	..	0·90	0·80	0·10	0·20	0·40	..	..	0·40	..
D. kohkoa (*n*=48)	..	..	..	1·00	..	..	0·33	..	0·60	0·33	..	..	0·53	1·00	..	..	..	..
D. pulaua (*n*=84)	1·00	..	..	..	1·00	0·07	..	..	1·00	..	..	..	1·00	1.00	..	0·07	..	..
D.s. neonasuta (*n*=88)	0·63	..	0·30	0·20	0·80	0·20	..	0·50	..	..	..	..	0·50	0·50	..	..	..	0·50
D.s. sulfurigaster (*n*=68)	0·30	.	0·97	..	0·50	0·50	..	0·50	0·50	..	..	0·30	1·00	0·53	..	..	..	..
D.s. bilimbata (*n*=66)	0·76	..	0·56	0·36	0·88	0·08	..	..	0·52	..	..	..	0·24	0·72	..	0·04	..	..
D.s. albostrigata (*n*=62)	1·00	..	1·00	0·50	0·43	0·50	..	0·87	0·50	..	..	..	0·50	1·00	..	..	..	..

Note: *n* = Number of genomes analysed.

Table 3. Allelic frequencies at Aph locus in members of *Drosophila nasuta* subgroup.

Species \ Alleles	0·88	0·89	0·92	0·93	0·94	0·95	0·97	1·00	1·02	1·05	1·07	1·09	1·12	1·14	1·15	1·17	1·19	1·23	1·27
D.n. nasuta (*n*=66)	..	0·17	0·20	..	..	0·17	0·40	0·33	0·77	0·20	0·20	..	0·20	..	0·63	0·33	..	0·17	..
D.n. albomicana (*n*=48)	..	..	0·80	..	..	..	0·80	0·80	0·53	..	..	0·53	0·80	0·53	..	..	..	..	0·73
D.n. kepulauana (*n*=82)	..	..	..	..	..	1·00	1·00	..	0·50	..	..	..	..	..	..	1·00	0·80	0·30	..
D. kohkoa (*n*=80)	..	..	0·80	1·00	..	..	..	1·00	..	0·90	..	..	..	..	..	0·80	1·00	..	..
D. pulaua (*n*=60)	..	..	..	..	1·00	0·77	..	0·97	..	0·50	0·50	..	..	..	..	..	1·00	0·50	0·27
D.s. neonasuta (*n*=68)	0·73	0·13	..	..	1·00	0·06	0·17	0·50	0·43	..	0·50	..	0·50	..	..	0·50	..	..	..
D.s. sulfurigaster (*n*=68)	..	0·80	0·80	..	..	0·87	0·13	0·67	..	..	..	..	0·80	0·67	..	0·47	0·13	..	..
D.s. bilimbata (*n*=56)	..	0·44	1·00	..	0·72	..	0·20	0·28	..	..	0·60	..	..	0·40	..	0·68	0·08	..	..
D.s. albostrigata (*n*=78)	..	0·17	1·00	..	1·00	..	0·37	0·78	0·15	..	..	0·33	0·07	..	..	..	0·05	0·05	..

Note: *n* = Number of genomes analysed.

Table 4. Allelic frequencies at α-Est locus in the members of *Drosophila nasuta* subgroup.

Species \ Alleles	0·87	0·88	0·90	0·92	0·95	0·97	1·00	1·03	1·05	1·08	1·10	1·13	1·15	1·18	1·23	1·25	1·28	1·30	1·35	Null alleles
D.n. nasuta (*n*=78)	..	..	..	0·72	0·72	0·24	0·32	0·08	0·04	0·16	0·20	0·04	0·44	0·04	0·20	..	..	..	..	0·04
D.n. albomicana (*n*=78)	..	..	..	1·00	1·00	..	0·73	..	..	..	..	..	..	0·17	..	0·63	..	..	..	..
D.n. kepulauana (*n*=68)	..	..	1·00	..	..	..	1·00	..	..	..	..	..	..	..	..	1·00	..	..	..	..
D. kohkoa (*n*=72)	..	..	..	1·00	1·00	..	..	..	..	..	1·00	..	..	..	..	..	..	..	..	..
D. pulaua (*n*=72)	0·16	..	..	1·00	..	..	1·00	..	..	..	1·00	..	..	..	..	0·64	..	..	0·36	..
D.s. neonasuta (*n*=58)	..	..	..	0·10	0·95	0·70	0·35	1·00	..	..	..	..	..	0·10	..	..	0·65	..	..	..
D.s. sulfurigaster (*n*=62)	..	..	..	1·00	..	..	1·00	1·00	..	..	..	..	0·27	0·73	..	..	..	..	..	..
D.s. albostrigata (*n*=68)	0·40	0·40	0·33	0·60	0·33	..	0·60	..	..	..	0·33	..	..	0·60	0·33	..	..	0·20	..	..

n = Number of genomes analysed,

Table 5. Allelic frequencies at β-Est locus in the members of *Drosophila nasuta* subgroup.

Species \ Alleles	0·92	0·95	0·97	0·98	1·00	1·02	1·03	1·05	1·10	1·15	1·20	1·25	1·28	1·30		1·35	Null alleles
D.n. nasuta (*n*=86)	..	0·96	..	..	0·96	..	..	0·46	..	..	0·06	..	..	0·43	0·46	..	0·03
D.n. albomicana (*n*=80)	..	..	..	..	0·50	..	..	1·00	1·00	..	0·50	..	0·50	1·00	..	..	..
D.n. kepulauana (*n*=56)	0·33	0·63	..	0·20	0·20	..	..	0·21	0·06	0·21	0·33	0·06	..	..	..	..	..
D. kohkoa (*n*=52)	..	0·40	..	0·60	0·40	..	0·60	..	..	..	..	..	..	0·40	..	..	..
D. pulaua (*n*=66)	..	..	..	..	1·00	..	..	1·00	..	..	..	0·96	..	1·00	..	..	..
D.s. neonasuta (*n*=78)	..	0·06	0·62	..	0·25	0·06	..	..	..	..	..	..	..	..	..	0·38	..
D.s. sulfurigaster (*n*=64)	..	..	..	..	1·00	..	..	..	..	..	1·00	..	..	..	..	..	..
D.s. bilimbata (*n*=90)	..	0·86	..	..	0·06	..	..	0·80	..	..	0·06	..	0·36	0·56	..	..	..
D.s. albostrigata (*n*=88)	..	..	..	..	1·00	..	..	..	..	..	0·43	0·33	..	1·00	..	..	..

Note: *n* = Number of genomes analysed.

Table 6. Allelic frequencies at α-Gpdh locus in the members of *Drosophila nasuta* subgroup.

Species	Alleles 0·85	0·90	0·95	0·98	1·00	1·02	1·05	Null alleles
D.n. nasuta (*n*=48)	0·20	1·00	..	..	..	..	..	..
D.n. albomicana (*n*=68)	..	0·82	..	..	0·18	..	..	..
D. kohkoa (*n*=64)	..	..	..	..	1·00	..	..	..
D. pulaua (*n*=66)	..	..	..	0·20	1·00	..	..	..
D.s. neonasuta (*n*=76)	..	..	..	0·07	0·46	0·43	0·03	..
D.s. sulfurigaster (*n*=88)	..	..	..	0·29	0·82	..	..	0·06
D.s. bilimbata (*n*=84)	..	..	..	0·28	0·41	0·31	..	..
D.s. albostrigata (*n*=60)	..	..	0·60	..	0·40	..	..	..

Table 7. Allelic frequencies at To locus in various members of *Drosophila nasuta* subgroup.

Species	Alleles 1·00	1·08	1·10	1·15
D.n. nasuta (n=60)	1·00	..	..	1·00
D.n. albomicana (*n*=96)	1·00	..	..	1·00
D.n. kepulauana (*n*=46)	1·00	..	..	1·00
D. kohkoa (*n*=64)	..	0·67	0·33	1·00
D. pulaua (*n*=48)	1·00	..	..	0·86
D.s. neonasuta (*n*=98)	1·00	..	..	0·89
D.s. sulfurigaster (*n*=86)	1·00	..	..	0·79
D.s. bilimbata (*n*=90)	1·00	..	..	..
D.s. albostrigata (*n*=84)	1·00	..	..	0·89

Two types of alleles are recognised. They are, " shared alleles " and " unique alleles ". The shared alleles are those which are common in two or more species while the unique alleles are restricted to single species. Further, the unique alleles may be high or low in frequency.

To evaluate the degree of genetic differentiation among the species of the *nasuta* subgroup involved in the present analysis, coefficients of genetic identity (*Ixy*) and genetic distance (*Dxy*) between different species have been calculated by applying Nei's formula (Nei 1971, 1972). These measures are given by the following formula:

$$Ixy = \frac{\Sigma x_i y_i}{[\Sigma x_i^2 \Sigma y_i^2]^{1/2}}, \text{ and } Dxy = -\log_e Ixy$$

where x_i and y_i are the frequencies of the ith allele in the species x and y, and the summation is overall alleles of the isozyme systems studied. The coefficients

or the values of genetic identity and genetic distances thus calculated on the basis of allelic frequencies at different enzyme loci among different species of the *nasuta* subgroup analysed, are given in table 8. The highest value of genetic identity is evident between *D.n. nasuta* and *D.n. albomicana* ($Ixy = 0{\cdot}687$) and the least identity is between *D.n. kepulauana* and *D. kohkoa* ($Ixy = 0{\cdot}281$). Highest value of genetic distance is between *D. kohkoa* and *D.n. kepulauana* ($Dxy = 1{\cdot}462$) and the least value of genetic distance is between *D.n. nasuta* and *D.n. albomicana* ($Dxy = 0{\cdot}375$).

4. Discussion

Gel electrophoresis allows an investigator to analyse the biochemical products of the genetic loci. Electrophoresis with polyacrylamide as the supporting medium is a sensitive method for detecting minor differences in overtly identical molecules. The amino acid substitutions which results in a charge difference in a protein molecule is readily detectable by this method. Henning and Yanofsky (1963) based on their studies with *Escherichia coli* showed that 7 out of 9 mutationally altered forms of tryptophan synthetase had differences in migration rate in polyacrylamide. They opined that similar large proportion of single amino acid substitutions can be detected by this means.

Table 8. Values of genetic identity, Ixy (above diagonal separation) and distance, Dxy (below diagonal separation) estimated among different members of *Drosophila nasuta* subgroup (following Nei's formula, 1972).

Ixy / *Dxy*	*D.n. nasuta*	*D.n. albomicana*	*D.n. kepulauana*	*D. kohkoa*	*D. pulaua*	*D.s. neonasuta*	*D.s. sulfurigaster*	*D.s. bilimbata*	*D.s. albostrigata*
D.n. nasuta		0·687	0·509	0·429	0·521	0·527	0·527	0·546	0·632
D.n. albomicana	0·375		0·453	0·435	0·553	0·472	0·583	0·584	0·648
D.n. kepulauana	0·675	0·792		0·281	0·474	0·382	0·428	0·440	0·406
D. kohkoa	0·846	0·833	1·270		0·542	0·349	0·414	0·428	0·485
D. pulaua	0·652	0·593	0·747	0·613		0·494	0·571	0·639	0·678
D.s. neonasuta	0·641	0·751	0·962	1·053	0·705		0·549	0·612	0·573
D.s. sulfurigaster	0·641	0·540	0·849	0·882	0·561	0·600		0·574	0·685
D.s. bilimbata	0·605	0·538	0·821	0·849	0·448	0·491	0·555		0·680
D.s. albostrigata	0·459	0·434	0·902	0·724	0·389	0·557	0·378	0·386	

" Protein studies allow information to be obtained only from those elements of the genome, that specify protein structure and these may not represent all genetic elements. They comprise, however, a sufficiently large category so that their analysis may provide useful information concerning evolutionary changes in genes and gene pools " (Hubby and Throckmorton 1965).

Following the first electrophoretic studies of Wright (1961, 1963) in *D. melanogaster*, various workers have made use of this technique to analyse the genomes in different members of the *virilis* group (Hubby and Throckmorton 1965; Lakovaara *et al* 1976), the *mesophragmatica* species group (Nair *et al* 1971), the *bipectinata* species complex (Yang *et al* 1972), the *willistoni* group (Ayala and Powell 1972; Ayala and Tracey 1974), the *mulleri* subgroup (Zouros 1974), the *ananassae* complex (Hegde 1979) to detect isozyme variations and thus estimate the genetic similarity and dissimilarity between the species of the group/subgroup studied. In the light of the above findings, the authors have analysed isozyme variations in nine members of the *nasuta* subgroup namely, *D.n. nasuta, D. n. albomicana, D. n. kepulauana, D. kohkoa, D. pulaua, D. s. neonasuta, D. s. sulfurigaster, D. s. bilimbata* and *D. s. albostrigata*, at six isozyme loci, to estimate the genetic identity and genetic distance among them.

Perusal of tables 2–7 reveals that in the nine members which are involved in the present study, the hydrolytic enzyme loci are highly polymorphic. Totally 18 allelic variants for Acph, 19 for Aph, 19 allelic forms for α-Est; and for β-Est 16 allelic variants are encountered. On the other hand, the glycolytic enzyme locus namely α-Gpdh has only 7 allelic variants and the locus coding for isozymes of To, has only 4 allelic variants. Further, none of the members possesses all the allelic variants at any polymorphic enzyme locus. Similar pattern of polymorphism of allelic variants at the hydrolytic enzyme loci and enzymes of glycolytic pathway are reported in two populations of *D. ananassae* (Gillespie and Kojima 1968), the members of Hawaiian *Drosophila* (Rockwood *et al* 1971), two subspecies of *D. equinoxialis* (Ayala *et al* 1974b), the natural populations of *D. persimilis* (Prakash 1977) and in the members of *ananassae* complex (Hegde 1979). King and Ohta (1975) and Ohta and Kimura (1975) opined that the observed patterns of variation at various loci can be produced by very small but differing levels of natural selection relative to mutation rates at different loci. Kojima *et al* (1970) opined that greater polymorphism at hydrolytic enzyme loci is maintained because these enzymes act on heterogeneous substrates which originate from external environment while Prakash (1977) is of the opinion that higher levels of polymorphism are maintained at the loci coding for hydrolytic enzymes due to weak natural selection or selective neutrality, but the natural selection is more stringent against variation at the loci coding for enzymes of the glycolytic pathway. The nine different species of *nasuta* subgroup involved in the present studies are maintained in the laboratory, which is fairly homogeneous environment, when compared to situations in natural populations. Present studies on the enzyme analysis in these laboratory stocks have revealed the occurrence of a high degree of polymorphism at the hydrolytic enzyme loci. These findings of the authors corroborate the explanations of Prakash (1977), offered for the maintenance of genic polymorphism at the hydrolytic enzyme loci in *Drosophila*. The less number of electrophoretic variants encountered at α-Gpdh locus may be attributed to the selective disadvantage of the mutationally altered

new variants. This selective elimination by means of natural selection is obvious since in an adult fly, α-Gpdh is involved in several important functions; one, in the flight muscles, where the soluble NAD-dependent enzyme together with a mitochondrial particle bound and flavine-linked oxidase comprises the α-glycerophosphate cycle (Hansford and Sacktor 1971). This cycle produces energy for flight through electron transfer in mitochondria. The two other important functions of α-Gpdh are the maintenance of the balance between oxidised and reduced NAD in the cytoplasm for glycolysis and supplying glycerophosphate for the synthesis of lipids. Thus, the authors opine that the survival of a new allelic variant is favoured if the physiological properties of the enzyme are unaffected.

From the observations on allelic variants and their frequencies (tables 2–7), two generalisations can be made. First, at any given locus, the frequency of the 'most common allele' (allele 1·00) varies among different species. The observed range in frequency for the common allele is from 0·50 as in *D. s. neonasuta*, to 1·00 as in *D. n. nasuta*, *D. kohkoa*, *D. pulaua* and *D. s. albostrigata*, at Acph locus. The range in the frequency of common allele at Aph locus is from 0·28 as in *D. s. bilimbata*, to 1·00 as in *D. kohkoa*. At α-Est enzyme locus, the frequency range of common allele is from 0·32 (*D. n. nasuta*) to 1·00 (*D. n. kepulauana*, *D. pulaua* and *D. s. sulfurigaster*). The range of this allele is from 0·06 as in *D. s. bilimbata*, to 1·00 as in *D. pulaua*, *D. s. sulfurigaster* and *D. s. albostrigata*, at β-Est locus. The frequency range of common allele at α-Gpdh locus is from 0·18 as in *D. n. albomicana*, to 1·00 as in *D. kohkoa* and *D. pulaua*. At To locus, the frequency of the common allele is 1·00 in all the members in which the allele is encountered. The second generalisation is that the number of allelic variants and their frequencies varies from species to species except at To locus.

Present investigations have enabled the authors to distinguish three patterns of allelic variations. They are,

A. Shared alleles—alleles common to two or more species. These may represent the alleles from ancestral gene pool.

B. Unique alleles—restricted to particular species which occur in high frequencies (considered to be high if the frequency of the allele is 0·50 and above). These include;

(i) $Acph^{0.74}$ limited to *D.n. kepulauana* with a frequency of 1·00.
(ii) $Acph^{1.10}$ which is restricted to *D.s. neonasuta* has a frequency of 0·50.
(iii) $Aph^{0.88}$ is limited to *D.s. neonasuta* with a frequency of 0·73.
(iv) $Aph^{0.93}$ in *D. kohkoa* has a frequency of 1·00.
(v) $Aph^{1.15}$ found in *D.n. nasuta* has a frequency of 0·63.
(vi) α-$Est^{1.28}$ is restricted to *D.s. neonasuta* with a frequency of 0·65.
(vii) β-$Est^{0.97}$ in *D.s. neonasuta* has a frequency of 0·62.
(viii) β-$Est^{1.03}$ is limited to *D. kohkoa* with a frequency of 0·60.
(ix) α-$Gpdh^{0.95}$ with a frequency of 0·60 in *D.s. albostrigata*.
(x) $To^{1.08}$ is limited to *D. kohkoa* with a frequency of 0·67.

C. Minor alleles—which are in low frequency (considered to be low if the frequency of the allele is 0·20 and below) are restricted to single member of the sub-

group. The following are the minor alleles encountered in the present investigations;,

(i) α-Est$^{1.05}$ with a frequency of 0·04, α-Est$^{1.08}$ with a frequency of 0·16 and α-Est$^{1.13}$ with a frequency of 0·04 are restricted to *D.n. nasuta.*

(ii) α-Est$^{1.30}$ with a frequency of 0·20 is limited to *D.s. albostrigata.*

(iii) β-Est$^{1.02}$ which is present only in *D.s. neonasuta* has a frequency of 0·06.

(iv) α-Gpdh$^{0.85}$ is limited to *D.n. nasuta* and has a frequency of 0·20.

(v) α-Gpdh$^{1.05}$ is restricted to *D.s. neonasuta* with a frequency of 0·03.

A similar pattern of allelic frequencies has been reported in different populations of *Drosophila* (Prakash *et al* 1969; Johnson 1971), sibling species of *willistoni* group (Ayala *et al* 1970), different species of *bipectinata* species complex of *Drosophila* (Yang *et al* 1972). On the basis of the above findings in the members of *nasuta* subgroup, the authors are of the opinion that the maintenance of unique alleles with high frequency in different members of the subgroup, may be the result of point mutations which are favoured by selection and the occurrence of minor alleles in low frequencies may be due to selective disadvantage suffered by the point mutations or these minor alleles might have recently arisen through point mutations.

Null alleles are detected in three of the six enzyme loci studied. α-Est, β-Est and α-Gpdh are the three loci in which null alleles are encountered. The null alleles with a frequency of 0·04 and 0·03 at α-Est and β-Est enzyme loci respectively are evident in *D.n. nasuta.* Further, the null alleles with a frequency of 0·06 is encountered in *D.s. sulfurigaster* at α-Gpdh locus. The possession of null alleles does not necessarily mean a change in the structural gene. The gene may be present in the organism but inactive or it may be present and active but may not be sufficiently active, to be detected on gels in single fly assays.

Based on the allelic frequencies at various enzyme loci studied, the coefficients of genetic identity and genetic distance have been calculated by applying Nei's formula (Nei 1971, 1972). According to the results thus obtained (table 8), the relationship sequence of *D.n. nasuta* to other members of the subgroup is that, it is closely related to *D.n. albomicana* ($Ixy = 0·687$, $Dxy = 0·375$), than to *D.s. albostrigata* ($Ixy = 0·632$, $Dxy = 0·459$), than to *D.s. bilimbata* ($Ixy = 0·546$, $Dxy = 0·605$), than to *D.s. sulfurigaster* ($Ixy = 0·527$, $Dxy = 0·641$), than to *D.s. neonasuta* ($Ixy = 0·527$, $Dxy = 0·641$), than to *D. pulaua* ($Ixy = 0·521$, $Dxy = 0·652$), than to *D.n. kepulauana* ($Ixy = 0·509$, $Dxy = 0·675$), than to *D. kohkoa* ($Ixy = 0·429$, $Dxy = 0·846$). Similarly *D.n. albomicana* is closely related to *D.n. nasuta* ($Ixy = 0·687$, $Dxy = 0·375$), than to *D.s. albostrigata* ($Ixy = 0·648$, $Dxy = 0·434$), than to *D.s. bilimbata* ($Ixy = 0·584$, $Dxy = 0·538$), than to *D.s. sulfurigaster* ($Ixy = 0·583$, $Dxy = 0·540$), than to *D. pulaua* ($Ixy = 0·553$, $Dxy = 0·593$), than to *D.s. neonasuta* ($Ixy = 0·472$, $Dxy = 0·751$), than to *D.n. kepulauana* ($Ixy = 0·453$, $Dxy = 0·792$), than to *D. kohkoa* ($Ixy = 0·435$, $Dxy = 0·833$). The relationship of *D.n. kepulauana* with other members of the subgroup is that, *D.n. kepulauana* is closely related to *D.n. nasuta* ($Ixy = 0·509$, $Dxy = 0·675$), than to *D. pulaua* ($Ixy = 0·474$, $Dxy = 0·747$), than to *D.n. albomicana* ($Ixy = 0·453$, $Dxy = 0·792$), than to *D.s. bilimbata* ($Ixy = 0·440$, $Dxy = 0·821$), than to *D.s. sulfurigaster* ($Ixy = 0·428$, $Dxy = 0·849$), than to *D.s. albostrigata* ($Ixy = 0·406$, $Dxy = 0·902$), than to *D.s. neonasuta* ($Ixy = 0·382$, $Dxy =$

0·962), than to *D. kohkoa* (*Ixy* = 0·281, *Dxy* = 1·270). The relationship of *D. kohkoa* with other species of the subgroup shows the following sequence: *D. kohkoa* is closely related to *D. pulaua* (*Ixy* = 0·542, *Dxy* = 0·613), than to *D.s. albostrigata* (*Ixy* = 0·485, *Dxy* = 0·724), than to *D.n. albomicana* (*Ixy* = 0·435, *Dxy* = 0·833), than to *D.n. nasuta* (*Ixy* = 0·429, *Dxy* = 0·846), than to *D.s. bilimbata* (*Ixy* = 0·428, *Dxy* = 0·849), than to *D.s. sulfurigaster* (*Ixy* = 0·414, *Dxy* = 0·882), than to *D.s. neonasuta* (*Ixy* = 0·349, *Dxy* = 1·053), than to *D.n. kepulauana* (*Ixy* = 0·281, *Dxy* = 1·270). The relationship of *D. pulaua* with the remaining members is that, it is closely related to *D.s. albostrigata* (*Ixy* = 0·678, *Dxy* = 0·389), than to *D.s. bilimbata* (*Ixy* = 0·639, *Dxy* = 0·448), than to *D.s. sulfurigaster* (*Ixy* = 0·571, *Dxy* = 0·561), than to *D.n. albomicana* (*Ixy* = 0·553, *Dxy* = 0·593), than to *D. kohkoa* (*Ixy* = 0·542, *Dxy* = 0·613), than to *D.n. nasuta* (*Ixy* = 0·521, *Dxy* = 0·652), than to *D.s. neonasuta* (*Ixy* = 0·494, *Dxy* = 0·705), than to *D.n. kepulauana* (*Ixy* = 0·474, *Dxy* = 0·747). Further, *D.s. neonasuta* is closely related to *D.s bilimbata* (*Ixy* = 0·612, *Dxy* = 0·491), than to *D.s. albostrigata* (*Ixy* = 0·573, *Dxy* = 0·557), than to *D.s. sulfurigaster* (*Ixy* = 0·549, *Dxy* = 0·600), than to *D.n. nasuta* (*Ixy* = 0·527, *Dxy* = 0·641), than to *D. pulaua* (*Ixy* = 0·494, *Dxy* = 0·705), than to *D.n. albomicana* (*Ixy* = 0·472, *Dxy* = 0·751), than to *D.n. kepulauana* (*Ixy* = 0·382, *Dxy* = 0·962), than to *D. kohkoa* (*Ixy* = 0·349, *Dxy* = 1·053). The relationship of *D.s. sulfurigaster* with other members of the subgroup is that, it is closely related to *D.s. albostrigata* (*Ixy* = 0·685, *Dxy* = 0·378), than to *D.n. albomicana* (*Ixy* = 0·583, *Dxy* = 0·540), than to *D.s. bilimbata* (*Ixy* = 0·574, *Dxy* = 0·555), than to *D. pulaua* (*Ixy* = 0·571, *Dxy* = 0·561), than to *D.s. neonasuta* (*Ixy* = 0·549, *Dxy* = 0·600), than to *D.n. nasuta* (*Ixy* = 0·527, *Dxy* = 0·641), than to *D.n. kepulauana* (*Ixy* = 0·428, *Dxy* = 0·849), than to *D. kohkoa* (*Ixy* = 0·414, *Dxy* = 0·882). The relationship of *D.s. bilimbata* with other members of the *nasuta* subgroup shows the following sequence; *D.s. bilimbata* is closely related to *D.s. albostrigata* (*Ixy* = 0·680, *Dxy* = 0·386), than to *D. pulaua* (*Ixy* = 0·639, *Dxy* = 0·448), than to *D.s. neonasuta* (*Ixy* = 0·612, *Dxy* = 0·491), than to *D.n. albomicana* (*Ixy* = 0·584, *Dxy* = 0·538), than to *D.s. sulfurigaster* (*Ixy* = 0·574, *Dxy* = 0·555), than to *D.n. nasuta* (*Ixy* = 0·546, *Dxy* = 0·605), than to *D.n. kepulauana* (*Ixy* = 0·440, *Dxy* = 0·821), than to *D. kohkoa* (*Ixy* = 0·428, *Dxy* = 0·849) and *D.s. albostrigata* is closely related to *D.s. sulfurigaster* (*Ixy* = 0·685, *Dxy* = 0·378), than to *D.s. bilimbata* (*Ixy* = 0·680, *Dxy* = 0·386), than to *D. pulaua* (*Ixy* = 0·678, *Dxy* = 0·389), than to *D.n. albomicana* (*Ixy* = 0·648, *Dxy* = 0·434), than to *D.n. nasuta* (*Ixy* = 0·632, *Dxy* = 0·459), than to *D.s. neonasuta* (*Ixy* = 0·573, *Dxy* = 0·557), than to *D. kohkoa* (*Ixy* = 0·485, *Dxy* = 0·724), than to *D.n. kepulauana* (*Ixy* = 0·406, *Dxy* = 0·902). These values of genetic identity and genetic distance can be used as a dependable index for estimating the extent of genetic differentiation between closely related species of the *nasuta* subgroup under study.

D. nasuta subgroup, which includes morphologically almost similar forms, has been divided into three morphophenotypic complexes (Nirmala and Krishnamurthy 1972), based on the pattern of silvery marking on the frons of the males of this subgroup. Cytogenetic studies including hybridisation experiments made on different members of the *nasuta* subgroup (Nirmala and Krishnamurthy 1973–74; Ranganath *et al* 1974) have revealed that *D.n. albomicana* is a chromosomal race

of *D.n. nasuta*. These two species are cross fertile and will breed through for many generations. Further, *D.n. kepulauana* has been established as a semi-species of *D.n. nasuta*. Studies by Nirmala (1973), Ranganath and Krishnamurthy (1976) and Rajasekarasetty *et al* (in press) have shown that the members of the orbital sheen complex, namely, *D.s. neonasuta*, *D.s. sulfurigaster*, *D.s. bilimbata*, *D.s. albostrigata* and *D. pulaua* cross with each other and produce hybrids with different degrees of post-mating isolation (hybrid sterility, back cross sterility). Further, the members of frontal sheen complex are entirely isolated from members of the orbital sheen complex.

In the present investigations, the values of genetic identity, which are obtained based on the allelic frequencies at different enzyme loci, reveal the highest value of genetic identity between *D.n. nasuta* and *D.n. albomicana* ($Ixy = 0 \cdot 687$) than between any two other members of the subgroup analysed. This high value between these two species reflects the amount of similarity of their genome and thus supports the earlier findings of cytogenetic analysis (Ranganath *et al* 1974). The genetic identity values obtained by the authors in the present studies among the members of the orbital sheen complex are also high which conform to their cross-ability with each other (Rajasekarasetty *et al*—in press) but do not agree with the post-mating isolation results (Nirmala 1973; Ranganath and Krishnamurthy 1976). The authors thus opine that the analysis of genomes of the hybrids through the study of isozymes would probably throw some light on the post-mating isolation and genetic differentiation at the level of isozyme loci, in these members of the orbital sheen complex. Similar results of absence of correlations between values of genetic identity and the pattern of post-mating isolation have been reported in the members of *mulleri* subgroup (Zouros 1974). Further, from the genetic identity values obtained in the present investigations of the authors, it is clear that *D.n. kepulauana* is closely related to *D.n. nasuta* ($Ixy = 0 \cdot 509$), than to any other member of the subgroup. This finding of the authors is again in conformity with the earlier cytogenetic results where *D.n. kepulauana* has been established as a semi-species of *D.n. nasuta*, and is cross sterile with other members of the subgroup. From the relationship sequence earlier discussed by the authors, it is evident that *D. kohkoa* is genetically distant from rest of the members of the subgroup analysed, except from *D. pulaua*. This finding supports the results obtained by Rajasekarasetty *et al* (in press) on the pattern of hybridisations, where *D. kohkoa* is completely isolated from other species of the subgroup studied. Similar findings on the correlation between cytogenetic results and isozyme patterns are evident in other species groups of *Drosophila* (Johnson *et al* 1966; Hubby and Throckmorton 1968; Nair *et al* 1971; Yang *et al* 1972; Hegde 1979).

Though *D.n. nasuta*, *D.n. albomicana* and *D. kohkoa* belong to frontal sheen complex, they share high values of genetic identity with *D.s. albostrigata* and *D. pulaua* of the orbital sheen complex, than when compared with the members of their own morphophenotypic complex (table 8). It is interesting to note the high values of genetic identity between *D.n. nasuta* and *D.s. albostrigata* ($Ixy = 0 \cdot 632$), *D.n. albomicana* and *D.s. albostrigata* ($Ixy = 0 \cdot 648$) and between *D. kohkoa* and *D. pulaua* ($Ixy = 0 \cdot 542$). Based on the above findings the authors opine that in these members, the reproductive isolation must have occurred before the accumulation of large amount of genetic divergence. Similar high identity values are reported between cross sterile species namely, *D. gasici* and *D. brncici* of *D. meso-*

phragmatica species group (Nair *et al* 1971), between *D. phaeopleura* and *D. varians* of *D. ananassae* complex (Hegde 1979). Further, Hubby and Throckmorton (1968) reported that *D. paulistorum* has proteins more in common with *D. nebulosa* than with its own sibling *D. willistoni.*

D.n. albomicana which differs from the standard *D.n. nasuta* by two fixed inversions (Rajasekarasetty *et al*—in press), shows highest value of genetic identity (*Ixy* = 0·687). But the value of genetic identity is 0·509 between *D.n. kepulauana* and *D.n. nasuta* even though *D.n. kepulauana* differs from *D.n. nasuta* by two fixed inversions. *D.s. neonasuta* differs from *D.n. nasuta* by two fixed inversions but they have a genetic identity value of 0·527. *D.s. sulfurigaster*, *D.s. bilimbata* and *D. pulaua* which differ from *D.n. nasuta* by similar three fixed inversions exhibit the genetic identity value of 0·527, 0·546 and 0·521 respectively, with *D.n. nasuta.* Further, *D. pulaua* and *D.s. sulfurigaster* show a value of 0·571, *D. pulaua* and *D.s. bilimbata,* a value of 0·639 while *D.s. sulfurigaster* and *D.s. bilimbata* show the value of genetic identity to be 0·574. These three members namely, *D. pulaua, D.s. sulfurigaster* and *D.s. bilimbata* have homosequential chromosomes. Thus the similarity in macrochromosomal banding sequence does not correspond with similarity at the genetic level. Similarly, the large range of genetic divergence between pairs of homosequential species of Hawaiian *Drosophila* indicate that chromosomal similarity does not necessarily correspond with genetic similarity (Rockwood *et al* 1971).

Thus the present investigations on isozyme variation have revealed,

(i) that there exists correlation between the genetic relationship established based on isozyme allelic frequencies and the extent of premating isolation among the nine members of the *nasuta* subgroup;

(ii) the genetic identity values, obtained for the members of the orbital sheen complex of this subgroup, do not lie on par with the pattern of post-mating isolation in these members and

(iii) there is no correspondence between the linear differentiation of the salivary gland chromosomes and the extent of genetic relationships based on isozyme analysis in the members of this subgroup.

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