than, the 'native' state of a given protein. The molecular chaperonins, a ubiquitous class of proteins, are believed to bind to and stabilize the partially folded intermediates in the folding pathways and thus promote folding by decreasing the rate of off-pathway folding reactions[19,20]. Alternatively, the rate of folding reactions of a protein can be affected by altering the rate of a limiting on-pathway reaction. In principle, pro-sequences may influence the overall rate of protein folding by either of these two mechanisms. The latest findings of Baker *et al.*[6] indicate that the pro-sequence in alpha-lytic protease increases the rate of folding by over seven orders of magnitude by directly stabilizing the rate-limiting on-pathway intermediate state[16]. Whether this is a universal mechanism for the action of pro-sequences is not clear at the moment and this should be an interesting area of research in coming years.

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Genetic differentiation at *Adh* locus in Indian natural populations of *Drosophila melanogaster*

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Ten Indian geographical populations of *D. melanogaster* collected along 20°N latitudinal range revealed significant clinal variation (3% for 1° latitude) at the *Adh* locus, and Adh^F allelic frequency correlated significantly with increase in latitude. The data on interpopulational genotypic and allelic frequency heterogeneity as well as F_{ST} value of 0.25 revealed significant genic divergence at the *Adh* locus. Patterns of ethanol utilization and ethanol tolerance in larval and adult individuals revealed significant genetic divergence. LC_{50} values revealed clinal variation in the range of 9.25 per cent to 15.8 per cent, i.e. southern populations of *D. melanogaster* depicted significant lower ethanol tolerance compared with north Indian populations. The parallel occurrence of latitudinal genetic divergence at the *Adh* locus and for ethanol tolerance in colonizing populations could be maintained by balancing natural selection varying spatially along the north–south axis of the Indian subcontinent. The present data further support and validate the hypothesis that occurrence of parallel or complementary latitudinal clines across different continental populations provide strong evidence of natural selection maintaining such clinal variation.

COLONIZING species populations offer excellent material for micro-evolutionary studies[1,2]. Studies on biogeo-

graphy, ecology and adaptive physiological traits in global populations of *D. melanogaster* revealed that Afrotropical populations constitute the ancestral populations which later colonized Eurasia and more recently to America and Australia[3]. Most studies on allozymic polymorphism had been made on US and Australian populations of *D. melanogaster* while Asian populations remain unexplored[4-6]. The Indian geographical populations of *D. melanogaster* have not been investigated so far for populational, ecological, quantitative and behavioural genetic studies. Recently, inversion clines in Indian populations of *D. melanogaster* have been reported[7,8]. But the data on allozymic polymorphism are still lacking.

Natural populations of *D. melanogaster* have been found to be polymorphic at the *Adh* locus and generally contained both the common electrophoretic alleles[9]. The Adh-S and Adh-F allozymes revealed different biochemical properties, i.e. the Adh-F allozyme was more active than the Adh-S allozyme, but Adh-S possessed higher thermostability than Adh-F[10,11]. A geographic trend in the frequencies of Adh^S and Adh^F alleles at the *Adh* locus was observed in the form of latitudinal clines in continental populations[12]. However, such data are lacking for Indian geographical populations. Since the gel electrophoretic analysis has helped in elucidating the genetic structure of geographical populations of diverse taxa, therefore, it was considered to characterize the extent of genic divergence at *Adh* locus in latitudinally varying Indian natural populations.

The natural food resources of most *Drosophila* species consist of fermenting fruits. Since the larvae are physically immersed in such media, they are required to cope with short chain alcohols at various concentrations[13]. Thus alcohol dehydrogenase (ADH) is known to be involved in both the utilization and detoxification of exogenous alcohols. Adaptation to ethanol had been found to be a complex process and the ADH induction occurred in the juvenile life stages[14]. The Indian subcontinent represents a diverse array of climatically variable habitats and there is little information on ethanol tolerance analysis in *D. melanogaster* populations. Thus, the present study reports ethanol tolerance in ten Indian natural populations of *D. melanogaster*.

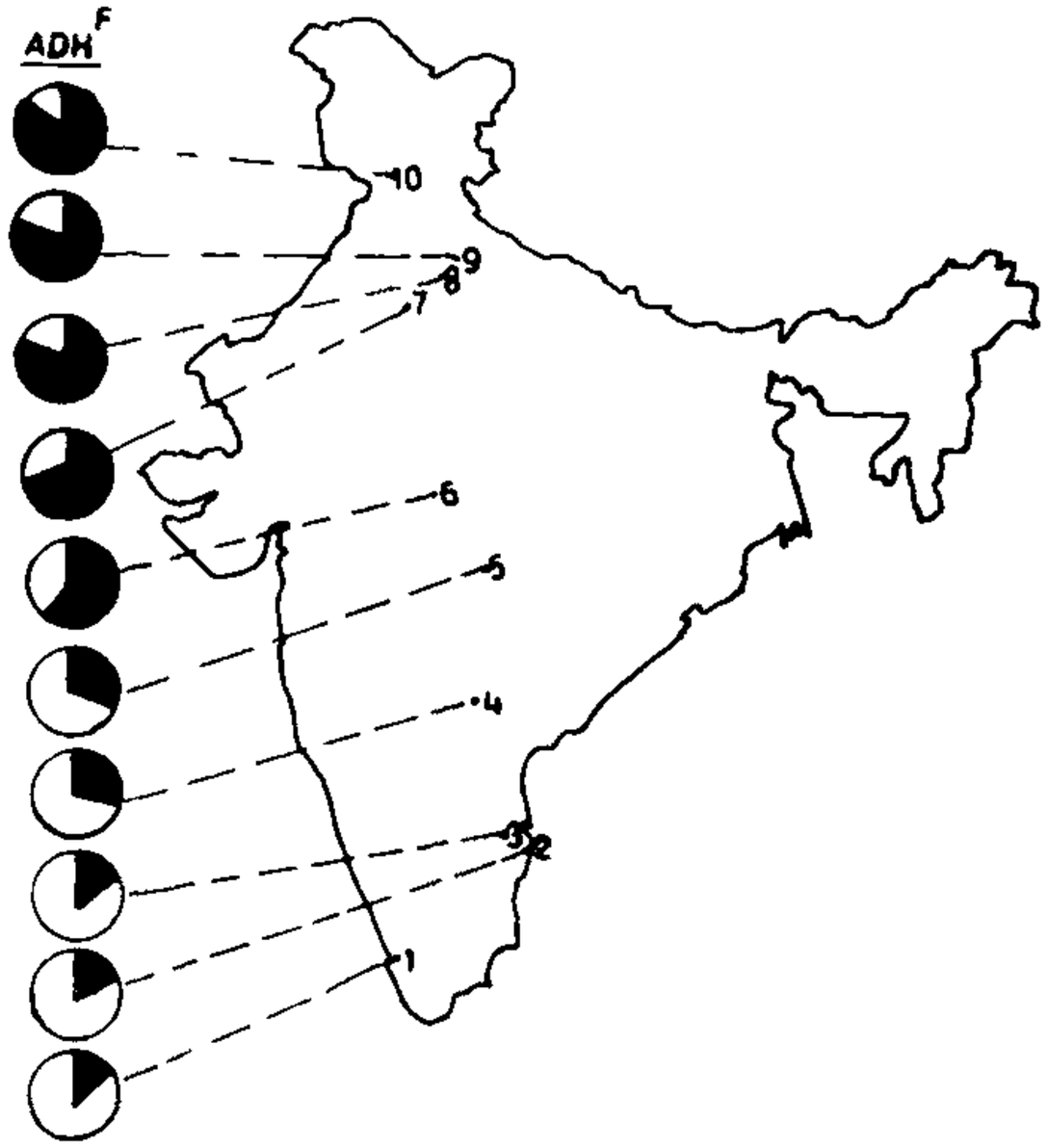

Figure 1. Map depicting collection sites as well as geographical distribution of Adh^F allelic frequency in ten Indian populations of *Drosophila melanogaster*. The frequency of Adh^F allele is shown by black area in each pie diagram. The places of collections and their respective latitudes are given in Table 1.

Methods

Isofemale lines were established from population samples of *D. melanogaster* from ten Indian geographical sites (Cochin to Dalhousie; 9° 58′ N to 33° 0′ N, Figure 1). Homogenates of single individuals were subjected to electrophoresis at 250 V and 25 mA at 4° C for 4 h. Three slices of each gel were stained for the related and overlapping enzyme systems, i.e. ADH, octanol dehydrogenase (ODH) and aldehyde oxidase (AO) by standard staining procedures[15]. On the basis of comparison of three gel slices stained for ADH, AO and ODH, it was found that ODH and AO included the two anodal zones while the single cathodal zone was found to be true ADH. Isoelectrophoretic variants (having similar electrophoretic mobility but differing in thermostability) were screened by following the technique of Trippa *et al.*[16]. Temperature and time for the heat treatment were selected on the basis of several preliminary experiments. The isoelectrophoretic thermoresistant (tr) and thermosusceptible (ts) variants were examined in species individuals by heat treating the enzyme *in situ* in the starch gel slices for 12 min at 42° C. The electromorph patterns were compared in the control and treated gel slices. Genetic control of ADH banding patterns was interpreted from the segregation patterns of ADH electromorphs of parents, F_1 and F_2 progeny of several single-pair matings. The genetic indices were calculated by following standard statistical formulae[17]. The log-likelihood χ^2 test (*G-test*) was used to assess whether the observed genotypes were in agreement with those expected on the basis of Hardy–Weinberg equilibrium[18].

The adult ethanol tolerance was assessed following standard procedures[19,20]. Adult survivorship was expressed as the number of adults alive after various time intervals. The LD_{50} values were calculated as the number of hours at which 50% of flies had died and

were estimated by linear interpolation. The ethanol resource utilization values were represented as LT_{50} maximum/LT_{50} control, i.e. if this ratio was > 1, the ethanol vapours were utilized as resource and if this value was < 1, it represented stress. The ethanol threshold concentration was obtained at LT_{50} maximum/LT_{50} control = 1. The larval ethanol tolerance behaviour of geographical populations of *D. melanogaster* was analysed by following standard method[21]. The relative numbers of the larvae out of a total of ten on the two sectors of Agar petri plates (with and without a particular ethanol concentration) were noted after 20 min for each ethanol concentration. Five replicates were tested at each ethanol concentration at 20° C for each of the populations. The threshold between attraction and avoidance after 20 min was calculated for different ethanol concentrations.

Results

Genetic basis of ADH variation

The ADH electrophoretic phenotypes included segregating two-banded patterns (of either faster or slower mobilities) and three-banded patterns at a single polymorphic zone of ADH activity in *D. melanogaster*. Species-specific genetic crosses between individuals having triple-banded ADH patterns produced 1:2:1 proportions of off-springs with alternating two-banded variants and triple-banded patterns in accordance with monogenic control of ADH electrophoretic phenotypes. Thus, the observed ADH electromorphs were represented by post-translational or conformational isozymes, i.e. homozygous genotypes depicted two-banded patterns. The present observations on ADH electrophoretic phenotypes agreed with earlier reports in *D. melanogaster* that for some of NAD requiring dehydrogenases occurrence of more than one electromorph in homozygotes was due to post-translational differential binding of NAD molecules.

Populational genetic structure

The data on observed and expected genotypes, sample size, allelic frequencies, heterozygosity values and application of *G*-test for fit to Hardy–Weinberg expectations at polymorphic *Adh* locus in *D. melanogaster* populations are given in Table 1. The allelic frequency patterns at *Adh* locus revealed significant clinal variation (along South–North axis) among Indian populations. The extent of clinal variation at *Adh* locus was found to be significantly higher (3% with 1° latitude; $r = 0.96$; $b = 0.036$) and revealed significant deviations from Hardy–Weinberg equilibrium at *Adh* locus in Indian populations. (Tables 1 and 2; Figure 2). The genotypic as well as allelic frequency patterns at *Adh* locus revealed significant interpopulation heterogeneity (75.82) and allelic frequency heterogeneity (378.46) on the basis of contingency chi-square test among the Indian populations. The data on Wright's fixation index ($F_{ST} = 0.25$) revealed significant genic divergence at *Adh* locus in Indian populations (Table 2).

The statistical comparison of *Adh* allelic frequency data in Indian populations of *D. melanogaster* with those of other allopatric populations (Afrotropical, Chinese, Japanese and European) revealed (a) consistency of the direction of latitudinal clines on the different continents; (b) the extent of latitudinally related range of allelic frequencies differed significantly at *Adh* locus among Indian versus Afrotropical populations as well as Indian versus European populations (Table 3). Thus, the direction of latitudinal cline was found to be similar among different allopatric populations but the allelic frequencies differed significantly on the basis of Student's *t* test (Table 3).

Cryptic variation

Indian populations of *D. melanogaster* revealed occurrence of wide-spread heat stability polymorphism in addition to electrophoretic variation at polymorphic *Adh*

Table 1. Data on alcohol dehydrogenase (ADH), observed and expected genotypes, allelic frequencies, heterozygosities (obs./exp.), Wright's coefficients (f), effective number of alleles (n_e) and *G*-values for log-likelihood χ^2 test for fit to Hardy–Weinberg equilibrium in ten Indian geographical populations of *D. melanogaster*

		Obs. and exp. genotypes				Allelic freq.		Het.			
Population	Latitude	FF	SS	FS	Sample size	F	S	Obs./exp.	f	n_e	G-values
Cochin	9° 58′ N	5/1 90	129/124.36	23/30.74	157	0 11	0 89	0.15/0.20	0.25	1.25	5.78*
Madras	13° 04′ N	8/2 73	128/122 61	26/36.64	162	0.13	0 87	0.16/0.23	0.30	1.43	10 40*
Tirumala	13° 40′ N	12/3.79	113/104 43	23/39.78	148	0.16	0 84	0.15/0 26	0 42	1.36	20.30*
Hyderabad	17° 20′ N	10/3.83	60/54.29	17/28 87	87	0.21	0.79	0.20/0.33	0.39	1.19	13.20*
Nagpur	21° 09′ N	16/7.38	48/40.18	18/34 44	82	0.30	0.70	0 21/0.42	0 47	1 72	18 51*
Bhopal	23° 16′ N	21/16.93	15/10 45	18/26.61	54	0 56	0 44	0.33/0.49	0 32	1 97	5 83*
Rohtak	28° 94′ N	62/56 40	13/6.96	28/39 63	103	0.74	0.26	0 27/0.38	0 29	1 62	8.54*
Saharanpur	29° 58′ N	78/70.57	12/5.61	26/39 81	116	0.78	0.22	0 22/0 34	0 34	1 52	11.72*
Dehradun	30° 19′ N	80/74.24	10/4 64	26/37.12	116	0.80	0 20	0 22/0 32	0.31	1.47	8.75*
Dalhousie	33° N	90/84 05	10/4 05	25/36.90	125	0.82	0 18	0 20/0 29	0 32	1 42	10 92*

*Significant at 5% level, F and S represent fast and slow electromorphs respectively.

Table 2. Statistical analysis of ADH variability in natural populations of *D. melanogaster*

Genetic indices	Values
Inter-population *Adh* allelic frequency heterogeneity*	378.46**
Inter-population genotypic heterogeneity	75.82**
Wright's F_{ST} analysis	0.254
Regression coefficient of Adh^F allelic frequency with latitude	0.036**

*On the basis of contingency χ^2 analysis,
**Significant at 5% level.

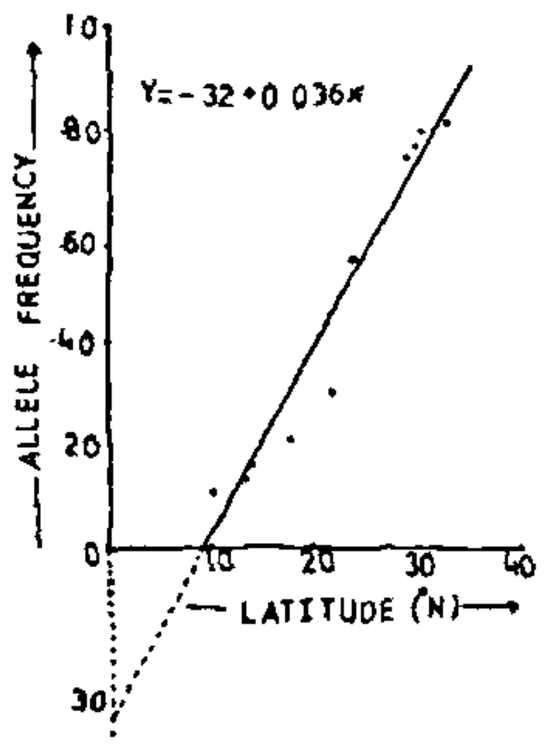

Figure 2. Relationship of Adh^F allelic frequency with latitude in ten Indian natural populations of *D. melanogaster*.

locus (Table 4). In *D. melanogaster* populations, the Adh^S (tr) allelic frequency was negatively correlated with increasing latitude. The statistical *t* test comparison of electrophoretic versus cryptic variation revealed significant increase in effective number of alleles as well as heterozygosity at *Adh* locus in *D. melanogaster* populations.

Ethanol tolerance in D. melanogaster

The intraspecific variation for ethanol tolerance among ten geographical populations of *D. melanogaster* was found to be significantly different along the north–south axis of the Indian subcontinent. The adult individuals were analysed for their potential to utilize the ethanol vapours in a closed system and the data on ethanol utilization as well as ethanol tolerance of ten geographical populations are given in Table 5.

The data on larval ethanol preference behaviour towards a range of ethanol concentrations (1 to 15 per cent) are given in Table 5. The larval ethanol threshold values varied from 6 per cent in Cochin populations to 12 per cent in Dalhousie populations. The larval individuals of ten populations have revealed slightly lesser ethanol tolerance than those of adults but the pattern of clinal variation was found to be similar for both adult as well as larval stages (Table 5).

The data on LT_{50} max/LT_{50} control (which are the measures of resource versus stress) for ten *D.*

Table 3. Statistical comparison of *Adh* allelic frequencies of Indian versus other continental populations of *D. melanogaster*

	India	Japan	China	Europe	Afrotropical
N	6	6	6	6	6
F (Range)	0.13–0.82	0.50–0.93	0.57–0.80	0.92–0.94	0.01–0.38
(Mean)	0.49	0.73	0.75	0.93	0.09
S (Range)	0.18–0.87	0.07–0.50	0.20–0.43	0.06–0.08	0.62–0.99
(Mean)	0.51	0.27	0.25	0.07	0.91
t	—	1.43	1.62	3.33*	3.73*
Reference	Present study	24	25	22	22, 23

N–Number of populations analysed; allelic frequencies range include minimum and maximum values; F and S denote electromorphs; *t*–Student's *t* test;
*Significant at 5% level.

Table 4. Patterns of cryptic allelic frequencies at *Adh* locus on the basis of post-electrophoretic heat denaturation technique in six Indian natural populations of *D. melanogaster*

	Tirumala		Nagpur		Bhopal		Rohtak		Saharanpur		Dalhousie	
	tr	ts	tr	ts	tr	ts	tr	ts	tr	ts	tr	ts
Adh^F	—	0.16	0.05	0.25	0.26	0.30	0.34	0.40	0.37	0.41	0.49	0.33
Adh^S	0.55	0.29	0.37	0.33	0.17	0.27	0.09	0.17	0.07	0.15	0.07	0.11
H & H′	0.27	0.59	0.42	0.69	0.49	0.74	0.38	0.69	0.35	0.67	0.30	0.63
H′-H	0.32		0.27		0.25		0.31		0.32		0.33	
n_e & n'_e	1.37	2.42	1.72	3.24	1.97	3.86	1.62	3.20	1.52	3.01	1.42	2.72
n'_e/n_e	1.77		1.88		1.96		1.97		1.98		1.92	

H & n_e are heterozygosity and effective number of alleles on the basis of electrophoresis while H′ & n'_e are such indices on the basis of post-electrophoretic heat denaturation technique; n'_e/n_e = increase in effective number of alleles; H′-H = increase in heterozygosity.

Table 5. *Adh* allelic frequencies, per cent ethanol tolerance and ethanol utilization (LT_{50} max/LT_{50} control), adult LC_{50} ethanol concentration and larval ethanol threshold values of ten latitudinally varying Indian populations of *D. melanogaster*

	Allelic frequency		Larval ethanol	Adult ethanol		Adult LC_{50}
Population	Adh^F	Adh^S	(threshold values)	Tolerance (threshold values)	Utilization (LT_{50} max/ LT_{50} control)	(ethanol conc.)
Cochin (9° 58′ N)	0.11	0.89	6.0	9.0	1 15	9.25
Madras (13° 04′ N)	0.13	0.87	6.0	10.0	1.17	9.8
Tirumala (13° 40′ N)	0.16	0.84	7.5	10.25	1.20	10.6
Hyderabad (17° 20′ N)	0.21	0.79	8.0	10.4	2.00	10.8
Nagpur (21° 09′ N)	0.30	0.70	9.0	12.75	1.97	12.0
Bhopal (23° 16′ N)	0.56	0.44	9.5	11.4	2.6	12 0
Rohtak (28° 54′ N)	0.74	0.26	10.0	13.25	2.81	12.8
Saharanpur* (29° 58′ N)	0.78	0.22	13.4	14.75	4.00	13.5
Dehradun (30° 18′ N)	0.80	0.20	12.0	13.2	3.10	14.0
Dalhousie (33° N)	0.82	0.18	12.0	15.0	3 48	15.8

*Population sample from a winery.

melanogaster populations have shown latitudinal variation (Table 5). The adult ethanol threshold values were found to vary clinally in the range of 9 per cent to 15 per cent among ten Indian populations from south to north localities (Table 5). The ethanol concentrations up to 13 per cent served as a resource for north Indian populations while a maximum of 9 per cent ethanol concentration could be utilized by south-Indian populations.

The LC_{50} ethanol concentrations were calculated from mortality data of adults after four days of ethanol treatment and LC_{50} values revealed clinal variation in the range of 9.25 per cent to 15.8 per cent, i.e. southern populations of *D. melanogaster* depicted significant lower ethanol tolerance compared with north Indian populations (Table 5). Thus, the ethanol utilization indices as well as ethanol tolerance threshold values in larval and adult individuals were found to vary latitudinally (Table 5).

In order to test whether *Adh* allelic frequency changes and ethanol tolerance potential are correlated with latitude, statistical analysis of correlation was carried out for all the ten geographical populations of *D. melanogaster*. The statistical correlations were found to be significantly high among latitudinal variation versus larval and adult ethanol tolerance versus Adh^F allelic frequency (Table 6). Thus, both the traits of ethanol utilization and ethanol tolerance have revealed adaptive significance and are being maintained by natural selection mechanisms.

Discussion

The present data on clinal variation at *Adh* locus in Indian populations of *D. melanogaster* further support and validate the hypothesis that occurrence of parallel or complementary latitudinal clines across different continental populations provide strong evidence of natural selection maintaining such clinal allozymic variation[4–6]. Latitudinal clines have been reported in Australian populations[5], Afrotropical populations[22,23], Japanese populations[24], and Chinese populations[25]. The occurrence of clinal variation across diverse biogeographical regions cannot be explained on the basis of stochastic processes such as genetic drift and/or gene flow since the continental populations differ significantly in their evolutionary history as well as eco-geographical conditions. The existence of parallel clinal allelic frequency changes at *Adh* locus provides strong evidence for the action of latitudinally related environmental gradients. The biochemical properties of Adh

Table 6. Correlation coefficient (r) values between latitudes and biological variables (Adh^F frequency and ethanol tolerance) in populations of *D. melanogaster*

Parameters	*r*
Latitude versus Adh^F	0.96
Latitude versus ethanol tolerance (Adult)	0.96
Latitude versus ethanol tolerance (Larval)	0.91
Ethanol tolerance (adult) versus Adh^F	0 93
Ethanol tolerance (larval) versus Adh^F	0.91
Ethanol tolerance, adult versus larval	0 96

allozymes have suggested that temperate or cooler places could favour Adh^F while tropical or warm places would select Adh^S allelic variants[26]. The observed clinal pattern at *Adh* locus in Indian populations is in agreement with the known higher thermostability of Adh^S variant. Hence, the observed higher allelic frequency of Adh^S in the South Indian populations could be favoured by tropical environment.

ADH catalyses the oxidation of primary and secondary alcohols to aldehydes and ketones respectively. Secondary alcohols are more toxic than primary alcohols because secondary alcohols are oxidized to ketones rather than less toxic aldehydes[14]. Since strains homozygous for the Adh^F allele show greater *in vitro* ADH activity than do strains homozygous for Adh^S allele with both primary and secondary alcohols, the fast allele may be selected against in the presence of secondary alcohols[10]. The tropical region (southern Indian localities) is characterized by greater plant diversity compared with the northern region[27] and hence result in the production of secondary alcohols through fermentation of diverse sweet plant resources. Thus, it is suggested that the abundance of secondary alcohols in the southern tropical environment of Indian subcontinent might exert selective pressure favouring higher frequency of Adh^S allele. On the contrary the relative absence of secondary alcohols in the fly habitat of the north Indian localities might have favoured Adh^F allele. Thus, the observed clinal variation at the *Adh* locus in Indian populations of *D. melanogaster* seem to be maintained by balancing natural selection varying spatially along the north–south axis of the Indian subcontinent.

The Indian geographical populations of *D. melanogaster* revealed significant genetic divergence in their potential to use ethanol. The adult longevity periods were found to increase significantly at 1 to 9 per cent for south Indian populations and 1 to 12 per cent for north Indian populations. The ethanol threshold values were found to vary clinally in the range of 9 to 15 per cent in the case of adults and 6 to 12 per cent for larvae in geographical populations from south to north localities. The LC_{50} values revealed clinal variation in the range of 9.25 to 15.8 per cent ethanol, i.e. southern populations depicted lower ethanol tolerance compared with the northern populations. The larval individuals of *D. melanogaster* populations revealed lower ethanol tolerance than those of adults but the pattern of clinal variation was found similar for both the adult and larval stages. The ethanol utilization indices as well as ethanol tolerance threshold values in larval and adult individuals were found to vary latitudinally in different Indian populations. The present observations are in agreement with other reports on the evidence of action of natural selection at *Adh* locus as well as for ethanol tolerance in some allopatric populations[28,29]. Thus both these traits have adaptive significance and are being maintained by natural selection mechanisms.

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