

## Preliminary Study on Isozyme Variation in Silkworm Germplasm of *Bombyx mori* (L.) and its Implication for Conservation

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### ABSTRAK

Diversiti genetik di dalam dan di kalangan dua belas ulat sutera ras *Bombyx mori* telah dikaji menggunakan enzim metabolisme iaitu, *a*-esterase (*a*-EST), *b*-esterase (*b*-EST), glukosa 6-fosfat dehidrogenase (G6PD) dan asid fosfatase (ACP). Satu penelitian diversiti genetik di dalam dan di kalangan ras menunjukkan bahawa 28% variasi yang diperhatikan berlaku di kalangan ras dan variasi selebihnya (72%) di dalam ras. Kadar purata aliran gen antara bandingan pasangan demi pasangan ras dua belas didapati sangat tinggi (0.62). Perkaitan genetik ras dua belas diperlihatkan oleh dendrogram UPGMA, menunjukkan pengelompokan genetik ras dalam 6 kelompok. NB4D2 dan NB18 adalah secara genetiknya ras serupa manakala BL-24 dan Nistari pula secara genetiknya jauh. Populasi ras ulat sutera J-112 dan NB4D2 masing-masing menunjukkan diversiti genetik tertinggi, % polimorfisme dan lebih alel. Diversiti genetik mereka yang kaya perlu dieksploitasi dalam program pemuliharaan dan pembiakbakaan.

### ABSTRACT

Genetic diversity within and among twelve silkworm *Bombyx mori* races was investigated using metabolic enzymes viz., *a*-esterase (*a*-EST), *b*-esterase (*b*-EST), glucose 6-phosphate dehydrogenase (G6PD) and acid phosphatase (ACP). A perusal of genetic diversity within and among races indicated that 28% of the observed variation occurred among races and the rest of the variation (72%) within races. The average rate of gene flow between pair wise comparisons of the twelve races was found to be very high (0.62). Genetic relatedness of the twelve races revealed by the UPGMA dendrogram, showed genetic grouping of races in six clusters. NB4D2 and NB18 are genetically similar while BL-24 and Nistari are genetically distant races. Populations of silkworm races J-112 and NB4D2 showed the highest genetic diversity, % polymorphism and more alleles, respectively. Their rich genetic diversity needs to be exploited in conservation and breeding programme.

### INTRODUCTION

Central Sericultural Germplasm Resources Centre (CSGRC), Hosur, India, maintains a large number of silkworm (*Bombyx mori* L.) genetic resources totaling 398 (65 multivoltine, 313 bivoltine and 20 mutant) silkworm accessions. Genetic evaluation of such resources is useful in the context of conservation and utilization in the silkworm breeding programme. Many of the accessions have been characterized for morphological characters as well as for important economic traits such cocoon weight, shell weight,

silk ratio, cocoon filament length, etc (Thangavelu *et al.* 1997; Thangavelu *et al.* 2001).

Biochemical characterization through protein profiling of isozyme is needed to know the genetic constitution of an individual and thereby the genetic diversity among and within the races. The isozyme technique is one of the molecular tools in biochemical methods to ascertain the extent of variability available among the species, subspecies and races (Pushpalatha and Vijayan 1999).

Understanding the genetic constitution of an individual in the population of races and

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allelic variations through isozyme studies is known to reflect the differential catalytic ability of allelic genes and their significant role in the adaptive strategy of the genotypes (Parkash *et al.* 1992). Among the various known isozymes, esterases, amylase, acid phosphatase and alkaline phosphatase have been studied extensively since they are the groups of enzymes involved in the metabolic rate of fat body of silkworm involved in gonadal maturation, maintenance of cell viability, metabolic activities of silk gland and defense functions (Yoshitake and Eguchi 1965; Yoshitake and Akiyama 1965; Eguchi *et al.* 1965; Eguchi and Yoshitake 1967; Battacharya *et al.* 1990).

Knowledge on genetic variability is important for breeding (Frankel and Brown 1983; Frey *et al.* 1983) and useful to improve the specific set of characters in low yielding silkworm stocks. Maintenance of variability within population is one of the most important aspects in the conservation of genetic resources (Zeng *et al.* 2003). This in mind, four isozymes viz., a-esterase (a-EST), b-esterase (b-EST), glucose 6 phosphate dehydrogenase (G6PD) and acid phosphatase (ACP), were utilized to characterize the individuals in the population of 12 silkworm races for studying the genetic variation and their polymorphic features for maintenance and genetic conservation strategies in the silkworm germplasm stocks.

## MATERIALS AND METHODS

Twelve silkworm races (6 Multivoltines and 6 Bivoltines) from the germplasm stocks of CSGRC, Hosur, India were selected for isozyme characterization (Table I). Ten individual moths

from each of 12 silkworm races were homogenized in extraction buffer (200 mM Tris-Cl, pH 7.5; 5mM MgCl<sub>2</sub>; and 250 mM NaCl) and centrifuged at 10,000 rpm for 10 min (Joy and Gopinathan, 1995). The supernatant was subjected to electrophoresis as described by Laemmli (1970) on 7.5 % non-denaturing polyacrylamide gel.

### Enzyme Visualization

The gels were incubated in respective stains for a-EST (E.C.Number 3.1.1.1), b - EST (E.C.Number. 3.1.1.1), G6PD (E.C.Number. 1.1.1.49) and ACP (E.C.Number.3.1.3.1) for 30 minutes at 37°C (Richardson *et al.* 1986). The stained gels were visualized under a bright illuminator and photographed using Kodak DC4800 gel documentation system (Biovis, India). The isozymes were numbered in the order of decreasing mobility from the cathode. The locus that specifies the isozyme with the least cathodal migration was labeled as A, the next as B, C, D, E and so on (Fig. 1).

### Statistical Analysis

#### Genetic Variation within Races

Within each race, the observed number of alleles per locus ( $N_a$ ), the effective number of alleles per locus ( $N_e$ ), observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_e$ ) (Nei, 1973) and the number and per cent of polymorphic loci were determined. In addition, for each locus the observed and the expected heterozygosity of an individual over the total analyzed races were estimated (Nei, 1973). Wright's F-statistics (Wright, 1951) viz.,  $F_{IS}$ -inbreeding coefficient

TABLE 1  
Details of the silkworm races, their origin and class for isozyme analysis

Sl.No	Accessions	Races	Origin	Class
1	BMI-0058	BL-23	India	Evolved breed
2	BMI-0059	BL-24	India	Evolved breed
3	BMI-0017	Nistari	WestBengal	Original
4	BMI-0027	O	WestBengal	Evolved breed
5	BMI-0001	Pure Mysore	Karnataka	Original
6	BMI-0009	Kollegal Jawan	Karnataka	Evolved breed
7	BBI-0083	CC-1	Karnataka	Evolved breed
8	BBI-0082	NB7	Karnataka	Evolved breed
9	BBE-0010	J-112	Japan	Evolved breed
10	BBE-0009	B-40	Japan	Evolved breed
11	BBI-0081	NB18	India	Evolved breed
12	BBI-0044	NB4D2	India	Evolved breed



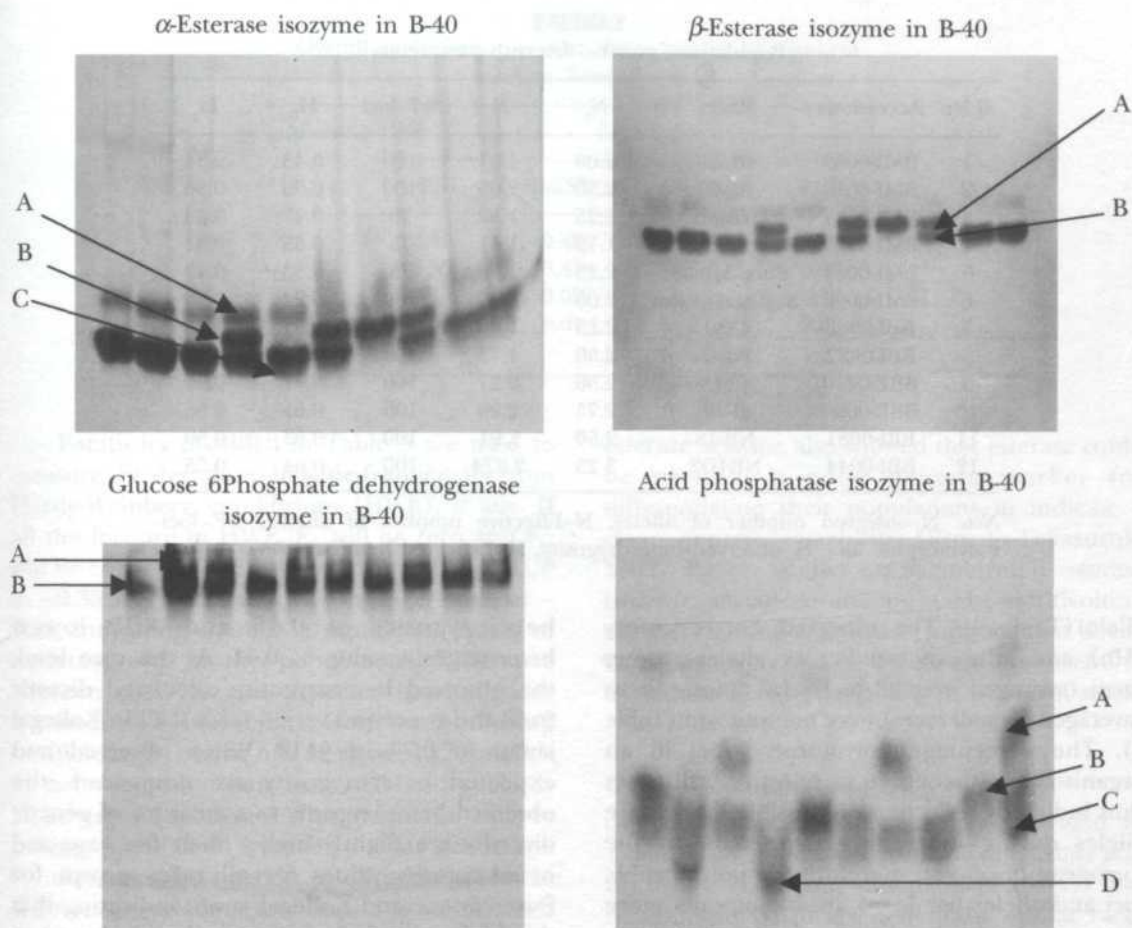


Fig. 1: Isozyme polymorphism in four loci in the B-40 race of *Bombyx mori*. A,B,C and D are alleles

within races;  $F_{IT}$  - inbreeding coefficient over the total analyzed races;  $F_{ST}$  - reduction in inbreeding coefficients due to differences among races or genetic differentiation of races at the level of all the loci, were also analyzed. All these statistical measures were determined using the POPGENE version 1.31 (Yeh *et al.* 1999).

*Genetic Variation between Races*

Estimates of Nei's genetic distance unbiased for sample size (Nei 1978) for each pair wise combination of races were calculated using POPGENE version 1.31 and an unweighted pair group method analysis (UPGMA) of dendrogram was constructed (Yeh *et al.* 1999).

**RESULTS AND DISCUSSION**

Twelve silkworm races were selected for studies on genetic variability. Four isozymes, *viz.*,  $\alpha$ -EST,  $\beta$ -EST, G6PD and ACP were analyzed in

the larval hemolymph samples of 10 individuals from each race. Results are presented in Table 2. The value of  $N_a$  (number of allele/locus) ranged from 1.75 (O race) to 3.25 (NB4D2 race). The value of observed heterozygosity ranged from 0.22 (Kollegal jawan) to 0.77 (J-112).

*Genetic Variation within Races*

Four-enzyme system representing four loci was resolved with sufficient consistency and clarity (Fig. 1). All the four loci were found to be polymorphic. The per cent of polymorphic loci was found to vary from 75% in the races of Nistari, O and Pure Mysore and 100% in BL-23, BL-24, Kollegal jawan, CC-1, NB-7, J-112, B-40, NB18 and NB4D2 (Table 2). The genetic "richness" as measured by the observed number of alleles indicated that all loci were equally rich with 4 to 5 alleles except for G6PD (3

TABLE 2  
Population genetic diversity parameters

Sl.No	Accessions	Races	N <sub>a</sub>	N <sub>e</sub>	%P loci	H <sub>o</sub>	H <sub>e</sub>
1	BMI-0058	BL-23	2.00	1.51	100	0.43	0.34
2	BMI-0059	BL-24	2.50	2.05	100	0.59	0.56
3	BMI-0017	Nistari	2.25	1.82	75	0.47	0.34
4	BMI-0027	O	1.75	1.51	75	0.35	0.31
5	BMI-0001	Pure Mysore	2.25	1.98	75	0.33	0.42
6	BMI-0009	Kollegal jawan	2.00	1.40	100	0.22	0.30
7	BBI-0083	CC-1	2.25	1.85	100	0.57	0.45
8	BBI-0082	NB-7	2.50	1.73	100	0.55	0.40
9	BBE-0010	J-112	2.50	2.27	100	0.77	0.56
10	BBE-0009	B-40	2.75	2.29	100	0.65	0.56
11	BBI-0081	NB-18	2.50	1.91	100	0.53	0.50
12	BBI-0044	NB4D2	3.25	2.274	100	0.64	0.55

Note: N<sub>a</sub>-observed number of alleles, N<sub>e</sub>-Effective number of alleles, %P loci-Polymorphic loci, H<sub>o</sub>-observed heterozygosity, H<sub>e</sub>-Expected heterozygosity.

allele)(Table 4). The observed heterozygosity (H<sub>o</sub>) and effective number of alleles among races (averaged over all loci) and among locus (averaged over all races) were not uniform (Table 3). The percentage polymorphic loci in an organism are susceptible to selective influences thus indirectly reflecting on possible loss of rare alleles during inbreeding depression in the European bison and therefore the polymorphic loci and alleles per locus are biologically more important to measure the genetic diversity among any species (Hartl and Pucek 1994). All the four loci were polymorphic in all twelve-silkworm races.

Observed average heterozygosity, expected average heterozygosity, and F-statistics (F<sub>IS</sub>, F<sub>IT</sub> and F<sub>ST</sub>) each are listed in Table 3. Four loci viz.,  $\alpha$ -EST,  $\beta$ -EST, G6PD and ACP showed heterozygosity.  $\alpha$ -EST showed highest

heterozygosity of 0.66 and ACP lowest heterozygosity value of 0.41. At the race level, the observed heterozygosity calculated directly from the genotypes varied from 0.22 in Kollegal jawan to 0.77 in J-112. When observed and expected heterozygosity are compared, the observed heterozygosity as a measure of genetic diversity are slightly higher than the expected heterozygosity values for all races except for Pure Mysore and Kollegal jawan indicating that this higher heterozygosity may be attributed to different geographic origin of the twelve silkworm races involved in this study (Table 1). It is reported that such genetic diversity expressed either as average number of alleles or heterozygosity is usually higher in any species with wider geographic ranges, higher fecundity, greater longevity and larger population sizes (Nevo *et al.* 1984).

TABLE 3  
Genetic diversity measures based on each locus over all population

LOCUS	N <sub>a</sub>	N <sub>e</sub>	H <sub>o</sub>	H <sub>e</sub>	F <sub>IS</sub>	F <sub>IT</sub>	F <sub>ST</sub>	Nm
$\alpha$ -EST	5.00	2.98	0.66	0.66	-0.33	0.01	0.26	0.70
$\beta$ -EST	4.00	2.37	0.54	0.58	-0.27	0.01	0.22	0.84
G6PD	3.00	1.97	0.48	0.49	-0.29	0.03	0.25	0.73
ACP	4.00	2.73	0.41	0.63	0.02	0.39	0.38	0.40
Mean	4.00	2.51	0.52	0.59	-0.22	0.12	0.28	0.62

Note: N<sub>a</sub>-observed number of alleles, N<sub>e</sub>-Effective number of alleles, H<sub>o</sub>-observed heterozygosity, H<sub>e</sub>-Expected heterozygosity, F<sub>IS</sub>-inbreeding coefficient within population, F<sub>IT</sub>-inbreeding coefficient over the population, F<sub>ST</sub>-genetic differentiation and Nm -Gene flow.



TABLE 4  
Overall allele frequencies at four loci in 12 silkworm  
races of *Bombyx mori*

Alleles	Locus			
	a-EST	b-EST	G6PD	ACP
A	0.45	0.48	0.38	0.41
B	0.32	0.41	0.60	0.40
C	0.11	0.08	0.01	0.15
D	0.04	0.01	-	0.02
E	0.05	-	-	-

F-statistics provided in Table 3 are used to measure the deviations in gene frequencies from Hardy-Weinberg equilibrium (HWE), if any. If all the loci are in HWE,  $F_{IS}$  will be zero and  $F_{IT}$  will be equal to  $F_{ST}$ .  $F_{IS}$  ranged from 0.02 for ACP to -0.33 for  $\alpha$ -EST and the average value as -0.22, indicating there is a significant excess of heterozygosity in the populations. Difference between observed and expected heterozygosity is a measure of inbreeding. Here the inbreeding coefficient ( $F_{IT}$ ) value of 0.12 showed 12% deficit in heterozygosity than expected under Hardy Weinberg Equilibrium. This might be due to mating (gene flow) between similar genotypes.  $F_{ST}$  measures the fixation of different alleles in different populations and is used as an indicator of divergence among populations. Genetic differentiation among races was low with mean  $F_{ST} = 0.2842$  implying that 28.42 % of total variation was between races and 71.58% of total genetic variation was within races. The rate of gene flow ( $Nm=0.6297$ ) was found to be very high among races. The high rate of gene flow might be the reason for the low level of genetic differentiation among the populations. Thus it is found that the silkworm races in Germplasm stocks of Hosur, India exhibit a pattern of genetic diversity characterized by a moderate degree of inter-population genetic diversity and a rather high intra-population genetic diversity. Further a high level of heterozygosity was found only in a-esterase. The important aspect of the present work is that the observation of high level of heterozygosity (0.66) at a- EST locus indicates that this esterase may be considered as a good genetic marker for studies of genetic variability in silkworm genetic resources. Similar studies on the population genetic variability among the sugarcane borer *Diatraea saccharalis* through

esterase isozyme also showed that esterase could be used as a good genetic marker for differentiating their populations to indicate a clear genetic variability (Ruvolo-Takasusuki 2002). Earlier studies on haemolymph esterase isozyme variations among eight multivoltine silkworm germplasm stocks indicate that allelic variations expressed in the isozyme are useful to determine genetic variability in silkworm stocks (Das *et al.* 1992).

#### Genetic Variation between Races

Genetic distance coefficients are provided in Table 5. Of the 12 silkworm races, Nistari and BL-24 are genetically distant races (0.949) and NB4D2 and NB18 are genetically similar races (0.029). This is in agreement with earlier studies on microsatellites in thirteen diverse silkworm races that the above breeds viz., NB4D2 and NB18 were also found in one cluster group because they are derived from a common Japanese double hybrid (Damodar Reddy *et al.* 1999). The genetic relatedness of the twelve silkworm races as revealed by the UPGMA dendrogram (Fig. 2) showed relatedness among the evolved breeds (NB4D2 and NB18) while this was not found among local races (Pure Mysore and Nistari). The branches in the dendrogram reflect the genetic differentiation among populations of different races. The populations of twelve silkworm races covered a broad geographical range and thus enzymatic differentiation is expected to exist among them. An understanding of the genetic diversity and population genetic structure is not only important for the conservation of species but is also essential for maintenance of genetic diversity within the populations and improvement of races through breeding (Millar and Westfall 1992).

TABLE 5  
Genetic relationships among 12 races of silkworm based on Nei's genetic distance

Sl.No	Races	BMI-0058	BMI-0059	BMI-0017	BMI-0027	BMI-0001	BMI-0009	BBI-0083	BBI-0082	BBE-0010	BBE-0009	BBI-0081	BBI-0044
1	BMI-0058	****											
2	BMI-0059	0.265	*****										
3	BMI-0017	0.445	0.949	****									
4	BMI-0027	0.089	0.449	0.386	****								
5	BMI-0001	0.396	0.484	0.6152	0.751	****							
6	BMI-0009	0.148	0.532	0.672	0.152	0.401	****						
7	BBI-0083	0.139	0.038	0.945	0.317	0.353	0.267	****					
8	BBI-0082	0.662	0.442	0.939	0.565	0.360	0.366	0.328	****				
9	BBE-0010	0.281	0.087	0.568	0.267	0.403	0.320	0.106	0.188	****			
10	BBE-0009	0.446	0.240	0.763	0.363	0.535	0.538	0.248	0.240	0.122	****		
11	BBI-0081	0.139	0.148	0.680	0.356	0.179	0.247	0.036	0.234	0.184	0.281	****	
12	BBI-0044	0.074	0.250	0.584	0.240	0.216	0.206	0.090	0.406	0.290	0.299	0.029	****



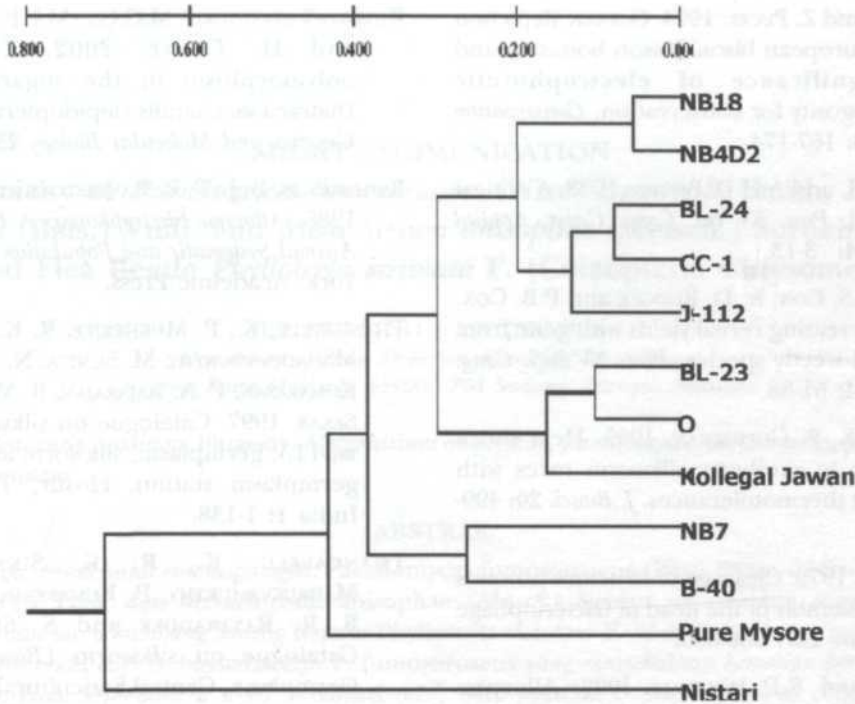


Fig. 2. Dendrogram showing clustering of populations of twelve silkworm races based on Nei's (1978) genetic distance: method =UPGMA

**CONCLUSION**

The genetic diversity and population genetic structure of twelve silkworm races revealed in this study would be utilized for an effective conservation plan and breeding strategies. Based on the results observed in this study, it is inferred that populations of silkworm races J-112 and NB4D2 would be very useful in a breeding programme, because they harbour higher levels of genetic diversity and more alleles.

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