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[Short Communication]

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Genetic Variability in the Natural Populations of Daba Ecorace of Tasar Silkworm (*Antheraea mylitta* Drury), as Revealed by ISSR Markers

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Genetic diversity within the natural populations of Daba ecorace of *Antheraea mylitta* Drury was studied using individual silkworms collected from the South Singhbhum district of Jharkhand state of India with 21 inter simple sequence repeat (ISSR) primers. A total of 148 bands were produced, of which 79% was polymorphic. The pair wise genetic distance among the individuals varied from 0.186 to 0.329. The dendrogram grouped the individuals into 3 major clusters. Nei's heterozygosity analysis revealed 0.265 ± 0.18 variability within the population. The high genetic variability present within the natural population of Daba ecorace of *A. mylitta* is indicative of their adaptational strategy in nature and have much importance for *in situ* conservation as well as utilization in breeding programs.

Key words: *Antheraea mylitta,* Daba ecorace, Genetic diversity, ISSR, Tasar silkworm

Introduction

Antheraea mylitta Drury, the Indian tropical tasar silkworm is a polyphagous, wild to semi-wild sericigenous species feeding primarily on *Terminalia tomentosa, T. arjuna* and *Shorea robusta.* The tasar silkworm has a wide distribution throughout the humid tropical region of India between 80 – 88°E longitude and 16 – 24°N latitude (Thangavelu and Sinha, 1993), covering the states of Maharashtra, Karnataka, Andhra Pradesh, Madhya Pradesh, Chhattisgarh, Orissa, Jharkhand, Bihar and West Bengal.

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The different populations of this silkworm present in various agro-climatic conditions exhibit high degree of polymorphism in their phenotypic expressions. Environmental conditions such as temperature, relative humidity and rain fall in different seasons are reported to cause temporal variations in characters like fecundity, hatchability, cocoon weight, shell weight and filament lengths (Srivastava *et al.*, 2000). Photoperiod, which has a latitudinal basis, is also seen to influence the pattern of voltinism of these populations (Kar *et al.*, 2000). Altitude is another factor, which changes voltinism in wild silk moths as *A. mylitta* behaves as trivoltine, bivoltine and univoltine in low, medium and high altitudes, respectively (Nayak and Dash, 1991). Thus, these different populations of natural variants are called ecoraces or ecotypes.

Some of the important ecotypes being exploited in India for silk production are Sukinda, Bhandara, Raily, Sarihan, Andhra local, Daba, Modal, Laria and Tira. Among these ecoraces, Daba and Sukinda are presently under commercial exploitation through planned breeding programs. The natural populations of Daba are present in the forest of south Singhbhum district of the state of Jharkhand. Considering the economic importance and amenability to systematic rearing of Daba ecorace on *T. arjuna* and *T. tomentosa* plantations, attempts are being made to evolve high yielding pure lines to exploit the hybrid vigour. Breeding experiments revealed that Daba ecorace is a good combiner for absolute silk yield (Siddiqui *et al.*, 1998). Information on the genetic variability present within a population is a prerequisite to plan and execute appropriate breeding and conservation strategies. Since no report on the genetic variability in the Daba ecorace is available at molecular level, the present study was undertaken with an aim to determine the extent of genetic variability present in the natural population of Daba in the south Sighbhum district of Jharkhand, India.

Molecular markers are known to have many advantages

over morpho-biochemical markers, as they are more stable and environmentally independent (Bernatzky and Tanksley, 1989; Gepts, 1993). Hence molecular markers like inter-simple sequence repeats (ISSR) (Zietkiewicz *et al.*, 1994; Prevost and Wilkinson, 1999) have been used for investigating genetic relatedness and diversity in plant and animal populations (Tsumura *et al.*, 1996; Ghislain *et al.*, 1999; Reddy *et al.*, 1999; Abbot, 2001; Bornet *et al.*, 2002). Recently, Chatterjee *et al.* (2004) have used ISSR primers to study the genetic diversity present among different populations of Raily and between a few other ecoraces of *A. mylitta*. These studies prompted us to select ISSR primers for the present investigation.

Material and Methods

Genetic materials used

The nature grown Daba cocoons were collected randomly from a forest region of south Singhbhum district of Jharkhand between 22.60°N latitude, 85.82°E longitude, and at an average altitude of 209 meters above mean sea level, covering an area of approximately 120 square kilometer. The predominant food plant was *Terminalia tomentosa*. The quantitative traits like cocoon weight, shell weight and shell ratio etc. were recorded. Cocoons were randomly mixed and thirteen of them comprising seven males ($D_1 - D_7$) and six females ($D_8 - D_{13}$) were used for DNA extraction and ISSR-PCR study.

DNA extraction and PCR

Individual pupa was crushed in liquid nitrogen, added lysis buffer (pH 7.5) containing 200 mM Tris-HCl, 25 mM EDTA, 300 mM NaCl and 2% of SDS and incubated at 37°C overnight in the presence of proteinase K. DNA was extracted through phenol-chloroform extraction and precipitation with ethanol in the presence of 3 M sodium acetate. The precipitated DNA was washed with 70 % alcohol and dissolved in Tris-EDTA buffer (pH 8.0). RNA contamination was removed through treatment with RNAse and the DNA was re-preciptated after another round of phenol: chloroform extraction steps. The extracted DNA was dried, dissolved in TE buffer and diluted to 10 ng/µl through serial dilution and quantification on 0.8% agarose gel against uncut λ-DNA (10 ng/µl) as standard.

PCR amplification of the DNA with ISSR Primers

Twenty-one ISSR primers from University of British Columbia (ISSR Kit # 9) were used for the study (Table 1). PCR amplification was done in an MJ Research Thermal-Cycler, PTC200 (MJ Research Inc. Watertown, Massachusetts, USA), using 20 µl reaction mixture containing 30 ng DNA, 2.0 µl of 10X PCR Buffer of Genei, Bangalore, India, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.15 µM individual ISSR Primer and 1 unit of *Taq* DNA polymerase. The PCR schedule was 94°C for 2 min followed by 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min and a final extension of 10 min at 72°C.

Table 1. List of ISSR primers used and polymorphism generated among the silkworms

Sl. no.	5-3 Primer sequence	No. of fragments	% Polymorphism
807	AGAGAGAGAGAGAGAGT	9	100
808	AGAGAGAGAGAGAGAGC	7	85.7
809	AGAGAGAGAGAGAGAGG	9	77.8
810	GAGAGAGAGAGAGAGAT	10	100
811	GAGAGAGAGAGAGAGAC	9	100
812	GAGAGAGAGAGAGAGAA	8	75
825	ACACACACACACACACT	6	100
826	ACACACACACACACACC	5	80
830	TGTGTGTGTGTGTGTGG	1	0
834	AGAGAGAGAGAGAGAGYT	8	62.5
835	AGAGAGAGAGAGAGAGYC	4	75
840	GAGAGAGAGAGAGAGAYT	8	75
841	GAGAGAGAGAGAGAGAYC	4	75
842	GAGAGAGAGAGAGAGAYG	4	75
855	ACACACACACACACACYT	9	66.6
861	ACCACCACCACCACCACC	7	85.7
864	ATGATGATGATGATGATG	9	88.9
881	GGGTGGGGTGGGGTG	11	81.8
884	HBHAGAGAGAGAGAGAG	11	63.6
885	BHBGAGAGAGAGAGAGA	6	66.6
886	VDVCTCTCTCTCTCTCT	3	100

Note, Y = (A,G,C,T); B = (C,G,T); H = (A,C,T); V = (A,C,G); D = (A,G,T).

The PCR products were resolved on 1.5% agarose (GIBCO, Bethesda Research Laboratory, Paisley, Scotland) gel in Tris Boric Acid/EDTA buffer (1 × TBE) using 0.6 cm thick gel of 20 cm (w) × 25 cm (L) and electrophoresis was done with a constant voltage of 60V from Amersham Pharmacia power supply units, EPS 400/200 for three hrs. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed with the gel documentation system (Syngene corporation, Frederick, MD, USA) Binary scoring of the profiles was done on the basis of presence (1) or absence (0) of bands.

Statistical analyses

Genetic heterozygosity within the population was worked out using the software POPGENE (Yeh, 1998). Similarly,

the genetic diversity within the population was also estimated by pairwise comparison of the silkworm using Dice's coefficient $(D) = 1 - [(2N_{ab})/(2N_{ab} + N_a + N_b)]$, where N_{ab} is the number of bands that are shared by the genotypes 'a' and 'b' and N_a is the number of bands present in 'a' and N_b is the number of bands present in 'b' (Sneath and Sokal, 1973). A dendrogram was generated from the above matrix using unweighted pair group method with arithmetical averages (UPGMA) (Sneath and Sokal, 1973) on PHYLIP 3.5c software program (Felsenstein, 1993). The robustness of the dendorgram was tested by estimating cophenetic correlation for the dendrogram and comparing it with the original genetic dissimilarity matrix, using Mantels matrix correspondence test (Mantel, 1967). Bootstrap value for each node was also calculated using the free software WINBOOT (Yap and Nelson, 1996).

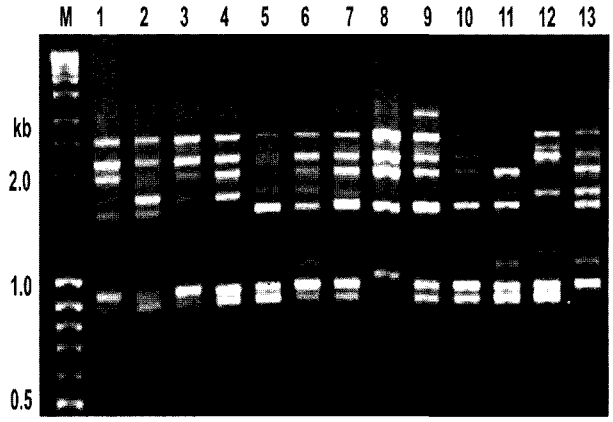

Fig. 1. PCR profiles generated by primer UBC864 with genomic DNA of thirteen individuals of Daba ecorace of *A.mylitta*. Lanes 1 – 7 are males ($D_1 - D_7$) and 3 – 13 are females ($D_8 - D_{13}$). M is the molecular weight marker.

Results and Discussion

Phenotypic variability

The cocoons showed variations on single cocoon weight, which varied from 8.3 g to 10.4 g with an average of 9.3 ± 0.68 g. The single shell weight also varied from 1.5 g to 2.1 g with an average of 1.75 ± 0.18 g. It is well documented that the tasar silkworm *Antheraea mylitta* shows considerable variation within and between populations in traits like voltinism and cocoon characters (Thangavelu and Sinha, 1993; Srivastava *et al.*, 2002). The variability in the cocoon characters observed in the present study thus agrees with these findings.

Genetic variability

A high amount of polymorphism was observed at the DNA level (Fig. 1). The 21 ISSR primers generated a total of 148 bands, of which 117 were polymorphic thus showing 79% polymorphism among the silkworms. The bands were between ~450 bp to ~3,000 bp. The number of bands generated by individual primers varied from 3 (UBC 886) to 11 (UBC 881 and UBC 884) (Table 1) and the band distribution among the individuals showed considerable variability (Table 2). Nei's genetic heterozygosity was 0.231 ± 0.19, 0.247 ± 0.20 and 0.265 ± 0.18, respectively for males, females and for the total population. The average genetic distance based on Dice's coefficient was 0.262 with a range of 0.186 (between D_{10} and D_{11}) to 0.329 (between D_9 and D_{13}). Thus, it could be seen that there is considerable amount of genetic variability present in the ecorace of Daba. This in turn points to the fact that the phenotypic variability exhibited by this ecorace in characters like fecundity, hatchability, cocoon weight, shell weight, absolute silk yield, filament length etc. (Srivastava *et al.*, 2000) is not purely due to the environmental factors but, has a certain amount of genetic base as evident from the above data. This is further clear from the dendrogram realized through the UPGMA analysis. The dendrogram grouped the 13 silkworms into three main clusters (Fig. 2). Though the bootstrap vales are not highly significant in all nodes, the high co-phenetic correlation coefficient of these clusters ($r = 0.95$; $p = 0.001$) obtained through the Mantel test reveals that the clustering truly depicts the genetic variability present among the individual silkworms. Since, the natural populations of out breeding species are expected to hold high genetic diversity, the genetic polymorphism unraveled by the ISSR markers with these 13 silkworms of Daba ecorace is quite expected.

The earlier studies on biological, physio-genetic and behavioural characters of different ecoraces of *A. mylitta* reflect that the genetic variability is the result of adaptation to different eco-geographical conditions and the food plants on which it feeds as there exists tremendous inter as well as intra-population variability in different ecoraces (Singh and Srivastava, 1997). Similarly, wide variability has also been recorded for different biochemical parameters among the ecoraces (Srivastava *et al.*, 2001). Although studies have been carried out systematically on morphological, quantitative, biochemical and behavioural traits, the characterization of ecoraces of this species is still inconclusive probably due to phenotypic and behavioral plasticity. Under this circumstance, the findings of the present study is of much significance in formulating strategies aimed at proper conservation and utilization of this economically important insect. Recently Chatterjee *et al.* (2004) using molecular tools, for the first time, reported presence of high genetic variability within and between populations of Raily ecorace of *A. mylitta*. Simi-

Table 2. Presence and absence of bands generated by ISSR primers in individuals of Daba ecorace of *A. mylitta*

Primers		Individuals												
		D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9	D_{10}	D_{11}	D_{12}	D_{13}
807	P	2	4	3	4	3	1	1	4	3	3	2	4	3
	A	7	5	6	5	6	8	8	5	6	6	7	5	6
808	p	3	3	3	3	4	4	3	4	5	3	4	3	3
	A	4	4	4	4	3	3	4	3	2	4	3	4	4
809	P	3	5	4	5	8	6	7	6	6	7	7	4	6
	A	6	4	5	4	1	3	2	3	3	2	2	5	3
810	P	3	1	3	5	3	3	2	4	4	5	5	5	2
	A	7	9	7	5	7	7	8	6	6	5	5	5	8
811	P	4	3	3	4	5	4	4	4	2	4	3	3	5
	A	5	6	6	5	4	5	5	5	7	5	6	6	4
812	P	3	4	3	3	3	5	5	5	5	5	5	5	4
	A	5	4	5	5	5	3	3	3	3	3	3	3	4
825	P	3	4	5	5	3	3	3	4	2	3	2	2	2
	A	3	2	1	1	3	3	3	2	4	3	4	4	4
826	P	3	3	4	2	3	4	3	2	3	2	3	2	3
	A	2	2	1	3	2	1	2	3	2	3	2	3	2
830	P	1	1	1	1	1	1	1	1	1	1	1	1	1
	A	0	0	0	0	0	0	0	0	0	0	0	0	0
834	P	6	7	5	7	5	3	5	6	6	5	5	5	4
	A	2	1	3	1	3	5	3	2	2	3	3	3	4
835	P	3	4	3	3	4	3	3	4	4	2	4	4	3
	A	1	0	1	1	0	1	1	0	0	2	0	0	1
840	P	4	4	4	4	4	5	5	5	3	3	5	5	3
	A	5	5	5	5	5	4	4	4	6	6	4	4	6
841	P	1	2	3	1	2	3	2	3	2	2	1	2	3
	A	4	3	2	4	3	2	3	2	3	3	4	3	2
842	P	3	2	4	3	2	2	4	2	3	2	2	2	2
	A	2	3	1	2	3	3	1	3	2	3	3	3	3
855	P	7	7	6	6	6	7	8	7	7	6	6	5	7
	A	2	2	3	3	3	2	1	2	2	3	3	4	2
861	P	5	3	7	4	6	5	4	4	2	3	5	5	5
	A	2	4	0	3	1	2	3	3	5	4	2	2	2
864	P	9	7	7	7	6	8	7	5	7	6	7	6	7
	A	0	2	2	2	3	1	2	4	2	3	2	3	2
881	P	6	9	10	8	7	7	4	9	7	6	9	8	8
	A	5	2	1	3	4	4	7	2	4	5	2	3	3
884	P	7	7	9	9	8	8	7	9	8	9	7	7	8
	A	4	4	2	2	3	3	4	2	3	2	4	4	3
885	P	5	3	4	5	5	4	3	4	4	4	4	5	5
	A	1	3	2	1	1	2	3	2	2	2	2	1	1
886	P	3	2	2	2	1	2	2	2	2	2	1	2	1
	A	1	2	2	2	3	2	2	2	2	2	3	2	3

P, presence in the silkworm and A, absence in the silkworm

larly, in another lepidopteran insect *Bombyx mori,* genetic diversity among different breeding stocks has been estimated using molecular markers like ISSR (Reddy *et al.*, 1999). These studies together with the present result clearly indicate that ISSR primers can be effectively used for estimating the genetic diversity in the populations of *A. myliita.*

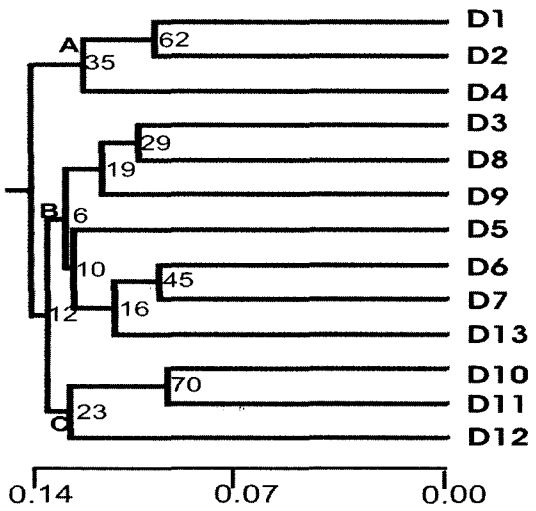

Fig. 2. Dendrogram realized from Dices coefficient using UPGMA analysis. D_1 to D_7 are male and $D_8 - D_{13}$ are female individuals. A, B and C are different clusters. The bootstrap values are given at the nodes.

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