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Phylogenetic analysis of tasar silkworm, *Antheraea mylitta* using SSR and ISSR primers

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Abstract
The diverse strains of the tasar silkworm, *Antheraea mylitta* were analysed using the simple sequence repeat anchored polymerase chain reaction (SSR and ISSR -anchored PCR). The SSR and ISSR amplification of 7 silkworm strains/eco-races (16 individuals in each, with seven primers, which generated polymorphism) yielded a total of 887 bands, out which 420 were (47.3 %) polymorphic (SSR). A total of 85 bands were produced out of which 46 were polymorphic (54%) for ISSR. Most of the bands were observed within the range 130 to 1250 bp, which is in accordance with the allelic size of the primers taken for studies. A dendrogram was constructed using POPGENE 1.32 version and WINBOOT (*XP* Windows) for assessing phylogenetic relations. These results suggest that these markers could be effectively utilised for identifying the genetic variability among tasar ecoraces.

Keywords: tasar silkworm, *Antheraea mylitta* drury, SSR primers, phylogeny

Introduction

The tropical tasar silkworm, *Antheraea mylitta* D, a lepidopteran insect producing commercial tasar silk, is wild in nature, distributed in various geographical regions as ecotypes. They show variation in phenotypic traits like fecundity, voltinism, cocoon weight, silk ratio and host plant preference (Sinha*, et al.,* 1994) [13]. There are nearly 34 ecoraces reported (Jolly *et al*., 1974) [5], of which seven commercial varieties are used in the present study (Sengupta *et al*., 1993), to identify the genetic similarity.

A century back, *Antheraea* sp were widely distributed throughout the tropical and subtropical belts of India (Siddique *et al*., 1992). In spite of the massive deforestation they are still found in well-demarcated ecozones (Jolly, 1974) [5] and mostly differentiated based on phenotypic traits (cocoon weight, colour, *etc*.). Hence proper genetic characterization is an important step for appropriate conservation and utilization of wild genetic materials in breeding program (Chatterjee, *et al*., 2004) [1]. Several molecular markers like RAPD, RFLP, SSR, ISSR and SNP have been developed in case of *Bombyx mori* (Yasukochi, 1998, Tan *et al*., 2001, Reddy *et al*., 1999, Nagaraju, *et al.,* 2002, Cheng *et al*., 2004) [15, 14, 10, 8, 2]. In 2006, Mahendran *et al.,* [6] have identified and characterized an *MboI*-digested genomic DNA fragments, which were used as RFLP markers to distinguish the closely related ecoraces of tropical tasar silkworm *A. mylitta* D.

Simple sequences repeats (SSR), also called microsatellites, are tandem repeats of di-, tri-, tetra- and penta- nucleotides, which are abundant and occur at multiple sites in all prokaryotic and eukaryotic genomes (Field and Wills 1996, Gur-Arie *et al*. 2000) [3, 4]. The important feature of this class of repetitive DNA is its hypervariability, mainly expressed as a variation in the copy number of tandem repeats at a particular locus; it has been proven to be the most powerful genetic marker. Due to their short repeat length and the limited interaction of individual loci, they can be readily studied through PCR amplifcations (Moore *et al*. 1991) [7].

ISSRs (Inter simple sequence repeats / Repeat-anchored primers that amplify regions between SSR) are also proved useful for detecting genetic polymorphisms in microsatellite and intermicrosatellite loci and demonstrated as a novel technique for fingerprinting and differentiating closely related individuals (Zietkiewicz *et al*. 1994) [17]. A study based on the studies of phylogenetic relationships of different ecoraces and genetic variation revealed that RFLP markers were used to distinguish the closely related ecoraces of tropical tasar silk producing insect *A. mylitta* Drury (Mahendran *et al.* 2006) [6].

Genetic diversity and differentiation among different populations of the wild silkworm *A. mylitta* is essential for efficient management and conservation of any animal genetic resources in gene banks. Since, SSRs are co dominant markers and can reveal multiple alleles at a single locus and also been extensively used in the diversity analysis of animal and plant system and ISSR primers also showed the suitability in genetic differentiation among different plant and animal genomes and also found useful in various silkworm races, both these markers were selected for the present study and phylogenetic analysis of different ecoraces and genetic variation was carried out.

Material and Methods

DNA Extraction

Seven morphologically distinct populations of *Antheraea mylitta* collected from different regions of Central and eastern region India, were used for the study: wild cocoons of Andhra local ecorace from warangal, Daba TV and Daba BV ecoraces from Adilabad, Khammam, districts of Telangana, Bhandara ecorace from Bhandara, district of Maharasthra, Sukinda and Modal from Sukindergarh, Baripada districts of Orissa, Raily from Bastar district of Chhattisgarh ecoraces from distant ecopockets of country were collected by exploring the natural

habitats (Fig.1, Table-1). From each population, 20 to 30 cocoons were collected and kept until emergence of the adult moth. Genomic DNA from 16 individual moths of each population was extracted separately following.

Genomic DNA was extracted from 16 randomly selected individual moths from each generation of each line and the control group by the use of the phenol-chloroform method. The DNA was incubated with RNAse A and re extracted before diluting to the desired level in the TE buffer (1mM Tris-HCl, 1 mM EDTA, pH 8.0). Quantification was done on 0.8% agarose gel and a uniform concentration of 100ng /µl was obtained after serial dilution with the TE buffer (pH 8.0) against standard uncut lambda DNA. Genomic DNA of the ecotypes from each generation will be mixed at equal volumes to make a bulk sample of that generation.

Table 1: Origin and characteristics of Tasar Silkworm, *Antheraea mylitta* Drury.

Sl. No	Ecorace	Site of collection	Food plant	Cocoon availability	Level of adaptability
1.	Andhra local	Mahadevpur Karimnagar (Telangana)	*Terminalia arjuna* and *T. tomentosa*	Forest collection	Wild
2.	Daba TV	Karimnagar (Telangana)	*Terminalia arjuna* and *T. tomentosa*	Silkworm rearing	Wider adaptability
3.	Daba BV	Warangal (Telangana)	*Terminalia arjuna* and *T. tomentosa*	Silkworm rearing	Wider adaptability
4.	Modal	Keonjhar (Orissa)	*Shorea robusta*	Forest collection	Wild
5.	Sukinda	Bhandara, Nagpur (Maharashtra)	*Terminalia arjuna* and *T. tomentosa*	Silkworm rearing	Wider adaptability
6.	Raily	Bastar(Chhatisgarh)	*Shorea robusta*	Forest collection	Wider adaptability
7.	Bhandara	Bhandara (Maharashtra)	*Terminalia arjuna* and *T. tomentosa*	Forest collection	Wild

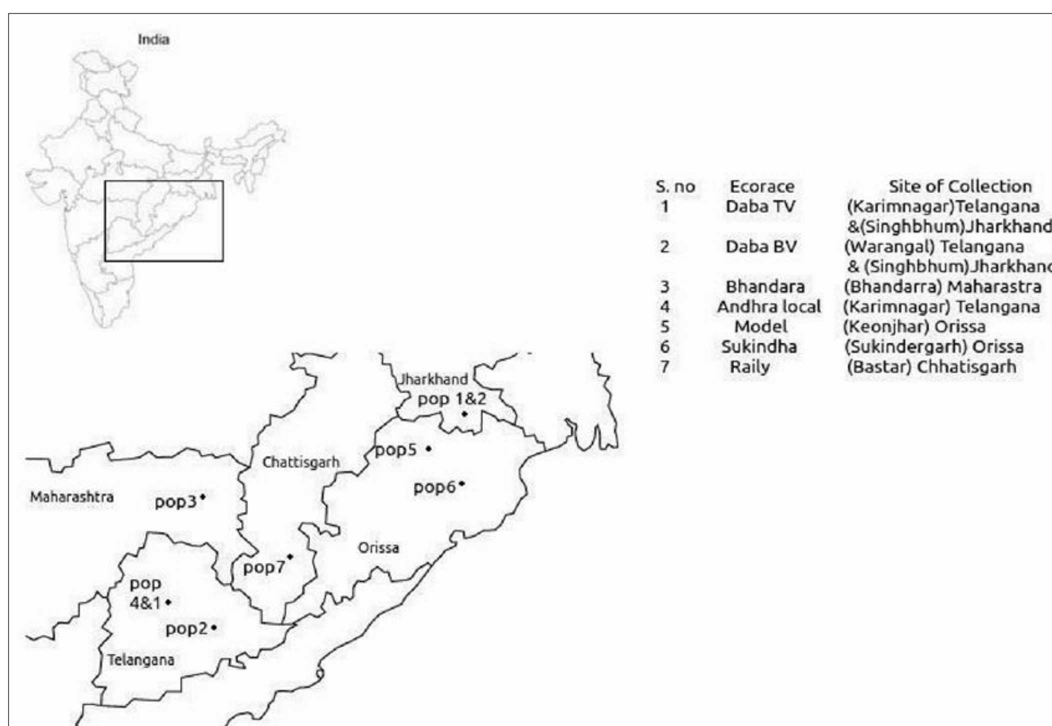


Fig 1: The rearing sites of ecoraces of Tasar Silkworm, *Antheraea mylitta* Drury.

PCR Amplification of Genomic DNA with SSR and ISSR primers

The amplification of genomic DNA was carried out in a PTC-200 Thermal-cycler using two oligonucleotide primers that hybridize to opposite strands. The location of the primers in the template determines the length of the amplicon. following the standard procedure 10 SSR and one ISSR primers will be procured from PCR cycling conditions were standardized for each fragment to be amplified based on the melting temperature (*T*m) of primers by using Research Master Cycler PTC 200, Eppendorf will be tested for their efficacy in amplification of Silkworm DNA employed in this study. PCR amplification of the DNA was carried in the Thermal-Cycler, PTC 200. The amplified products were analysed by gel electrophoresis.

Scoring for SSR and ISSR markers

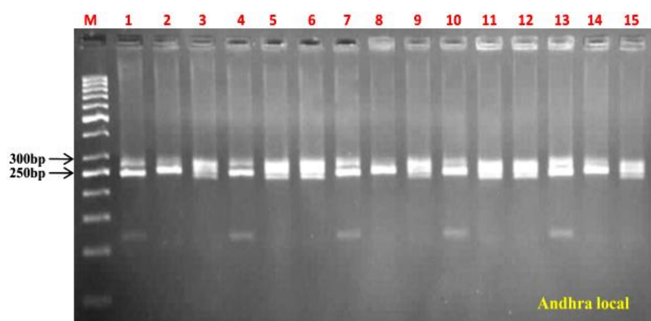
Binary coding was used to score gel and each band of primer was scored of 7 different ecoraces of 16 individuals and 10 SSR and 01ISSR primers with 100 to 1250 base pairs species was calculated and utilizing these distances, species were clustered following UPGMA method (Fig 2& 3).

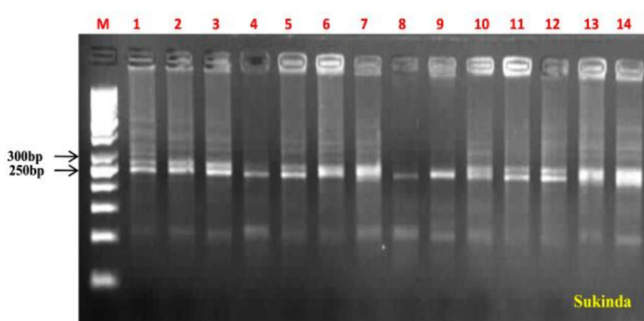
Statistical Analysis

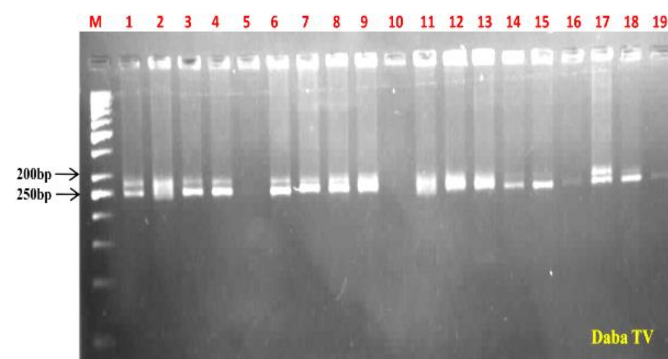
In the profile of dendrograms for SSR using POPGENE 1.32. version and ISSR using WINBOOT (*XP* Windows) for the construction of phylogenetic tree based on genetic distance. The level of polymorphism was expressed as the percentage of all loci that are polymorphic. It also gives detail about number of alleles, gene flow, genetic distance, gene diversity etc.

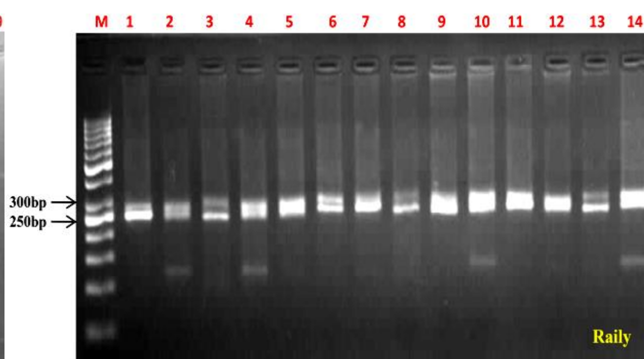
Genetic distance (D)
Genetic distances are designed to express the genetic differences between two populations as a single number. If there are no differences, the distance could be set to zero, whereas if the populations have no allele in common at any locus the distance may be set equal to its maximum value, 1. The genetic distance (*D*) was calculated by POPGENE and WINBOOT (XP windows) softwares (Yeh *et al.* 1999) [16] using Nei (1972) [9] standard genetic distance equation.

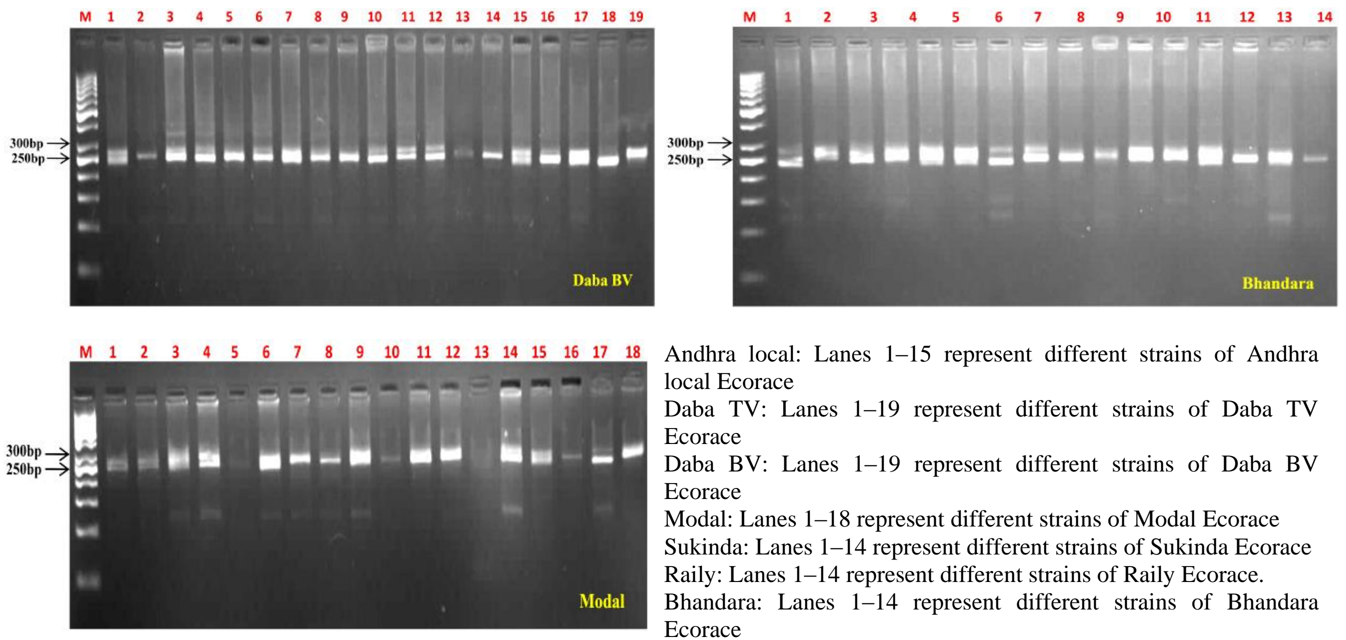
Results and Discussion
In the present studies, genetic relations of various genotypes by scoring the PCR-SSR and ISSR profiles were done. The polymorphic loci generated by SSR marker systems were scored using popgene 1.32 software. The level of polymorphism was expressed as the percentage of all loci that are polymorphic in the profile of dendrograms for SSR. the germplasm collected from various zones of India displayed variable genetic polymorphism and was found to be highest in Bhandara, (72.41%), followed by the samples Daba TV, Daba BV and Modal from Karimnagar, Warangal of Telangana and Keonjhar have displayed (68.98%) and Raily from Bastar of Maharashtra (65.52%). The samples Sukinda and Andhra local from Karimnagar and Sukindergarh were least diverse and displayed only 55.17% polymorphism (Table 4).
The screening of genomic DNA from individuals of seven populations using ISSR primer (UBC809) yielded several reproducible amplicons. The average no of amplicons produced per DNA sample was 2-6 per primer, with sizes ranging from 200-1250 bp. The primer generated distinct and robust bands showing polymorphism across 5 different amplifications (using 5 different sample populations of each ecorace). A total of 85 bands were produced out of which 46 were polymorphic. Multiple bands varying in size from 200 to 900bp are seen in almost all the strains (Fig. 3). Among the 14 loci, at five loci there was polymorphism showing a minimum of 2 and a maximum of 4 bands. As ISSR markers are reliable, reproducible and have been used to estimate genetic diversity among closely related populations (Vogel *et al*., 1997), the study can become more helpful if it is done with greater number of primers and individuals. In the present studies, the germplasm collected from various zones of India displayed variable genetic polymorphism and was found to be highest in Sukinda, (71.43%), followed by the samples Daba TV and Bhandara (64.29%); Daba BV and Raily ((57.14%). The samples of Andhra local and Modal were least diverse and displayed only 50% polymorphism. (Table 4).
AL, DT, DB, Modal, Sukinda, Raily were found to have a genetic distance of 0.1967, 0.1932, 0.2554, 0.2217, 0.1495 and 0.2644 with that of Bhandara [Table 2(a)]. The dendrogram produced by UPGMA of Nei's genetic distance for all populations (7×16 = 112) is presented in Fig.4 (Phylogenetic tree of SSR).
The genetic distance values based on ISSR analysis are presented in (Table 3). The highest value of 0.645 was between Sukinda and Daba BV and the lowest value was 0.11, between *Bhandara* and *Modal.* The distance matrix based on ISSR data sets was used to construct a dendrogram, which is shown in (Fig. 5).











Andhra local: Lanes 1–15 represent different strains of Andhra local Ecorace
Daba TV: Lanes 1–19 represent different strains of Daba TV Ecorace
Daba BV: Lanes 1–19 represent different strains of Daba BV Ecorace
Modal: Lanes 1–18 represent different strains of Modal Ecorace
Sukinda: Lanes 1–14 represent different strains of Sukinda Ecorace
Raily: Lanes 1–14 represent different strains of Raily Ecorace.
Bhandara: Lanes 1–14 represent different strains of Bhandara Ecorace

Note: Primer *Amysat* 025*;* Fragment size is 247bp

Fig 2: SSR profiles generated from genomic DNA of 16 strains from different individuals of (A.L, D. TV, D. BV, Modal, Sukinda, Raily, Bhandara) ecoraces of tasar silk worm, *Antheraea mylitta using the primer Amysat025.*

Set -I
M 1 2 3 4 5 6 7
1200bp
1100bp
500bp

Set - III
M 1 2 3 4 5 6 7
3000bp
1000bp
500bp

Set -II
M 1 2 3 4 5 6 7
3000bp
1000bp
500bp

Set - IV
M 1 2 3 4 5 6 7
3000bp
1000bp
500bp

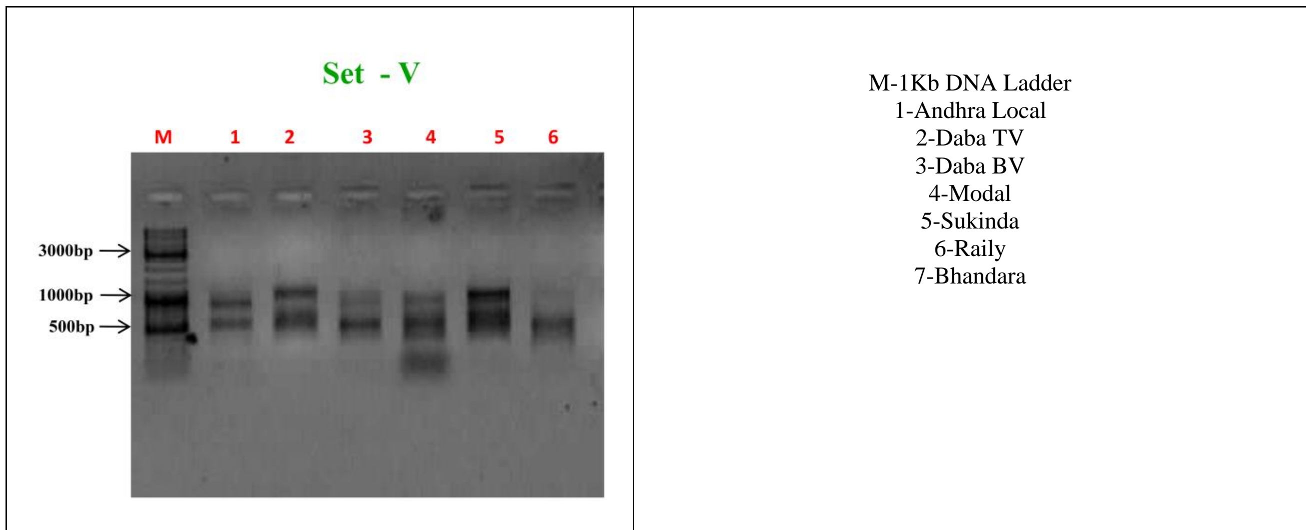


Fig 3: ISSR profile generated from genomic DNA of V different individual sets of (A.L, D. TV, D. BV, Modal, Sukinda, Raily, Bhandara) ecorace of tasar silk worm, *Antheraea mylitta.*using the primer UBC 809.

Table 2(a): Nei's Original Measures of Genetic Identity and Genetic distance.

Pop ID	1	2	3	4	5	6	7
1		0.7581	0.6808	0.7519	0.8317	0.6939	0.8214
2	0.2770		0.8506	0.8860	0.8648	0.8228	0.8243
3	0.3845	0.1618		0.7791	0.7610	0.7683	0.7746
4	0.2851	0.1211	0.2496		0.8488	0.9267	0.8012
5	0.1842	0.1452	0.2731	0.1639		0.8196	0.8611
6	0.3654	0.1951	0.2636	0.0761	0.1990		0.7677
7	0.1967	0.1932	0.2554	0.2217	0.1495	0.2644	

AL, DT, DB, Modal, Sukinda, Raily were found to have a genetic distance of 0.1967, 0.1932, 0.2554, 0.2217, 0.1495 and 0.2644 with that of Bhandara. The dendrogram produced by UPGMA of Nei's genetic distance for all populations (7×16 = 112) is presented in fig 4(Phylogenetic tree of SSR),{ Table 2(a)}.

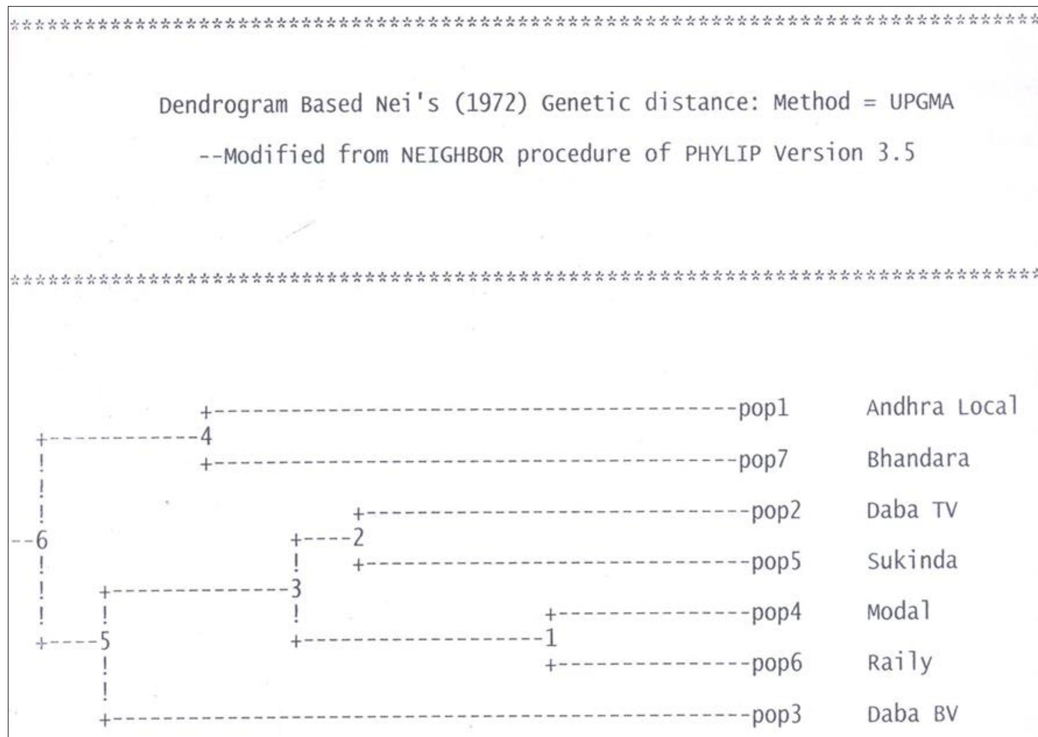


Fig 4: UPGMA dendrogram depicting ecorace genetic diversity of *Antheraea mylitta* D genotypes, obtained by PCR-SSR marker data.

Phylogenetic analysis using ISSR primers

Table 3: Dice genetic similarity distance matrix values based on ISSR data between seven *Antheaea mylitta* strains.

Pop ID	AL	DTV	DBV	MODAL	SUKINDA	RAILY	BHANDARA
AL	1.000000						
DTV	0.640000	1.000000					
DBV	0.666667	0.571429	1.000000				
MODAL	0.600000	0.571429	0.434783	1.000000			
SUKINDA	0.642857	0.620690	0.645161	0.416667	1.000000		
RAILY	0.583333	0.640000	0.592593	0.300000	0.642857	1.000000	
BHANDARA	0.476190	0.363636	0.416667	0.117647	0.560000	0.476190	1.000000

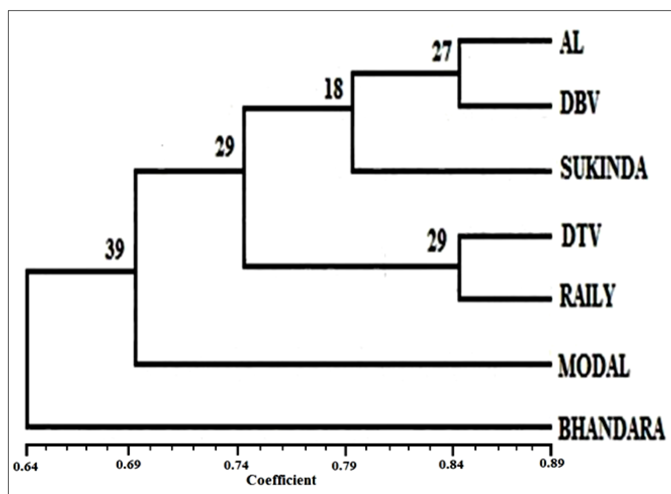


Fig 5: UPGMA dendrogram depicting ecorace genetic diversity of *Antheraea mylitta* D. genotypes, obtained by PCR-ISSR UBC 809 marker data. Genetic distances are indicated below the tree. Values at nodes refer to the number of times the node occurred in 100 bootstrap replicates [WINBOOT(XP windows)].

Table 2(b): Calculation of mean genetic distance from the table 2(a).

Sl. No.	Ecoraces	Mean values SSR
1.	Andhra local	0.1822
2.	DabaTV	0.2646
3.	DabaBV	0.18625
4.	Modal	0.1858
5.	Sukinda	0.2081
6.	Raily	0.21348

Table 4: Genetic diversity analysis in the ecoraces of *A. mylitta.*

Ecorace	Number of polymorphic loci		Percentage of polymorphic loci	
	SSR	ISSR	SSR	ISSR
Andhra local	16	7	55.17	50.00
Daba TV	20	9	68.97	64.29
Daba BV	20	8	68.97	57.14
Modal	20	7	68.97	50.00
Sukinda	16	10	55.17	71.43
Raily	19	8	65.52	57.14
Bhandara	21	9	72.41	64.29

It can be inferred from the dendrograms (Fig.4) obtained using SSR primers Andhra local - Bhandara; Daba TV - Sukinda, Modal- Raily are found to be genetically close, while Daba BV seem to have diverse genotype.

The From Table 2 (a) and (b) on genetic distance, it can be seen that Daba TV (mean value=0.2646) shows higher genetic distance among the other 6 populations (*i.e*., Andhra local, Daba BV, Modal, Sukinda, Raily), which implies that Daba TV genetically distant from other ecoraces. It can also be observed that the lowest genetic distance was found in ecoraces Andhra local, Daba BV, Modal (same genetic distance of 0.18) have shown more genetic closeness. Lower the genetic distance, genetically closer are the ecoraces.

In the present studies, phylogenetic relation between ecoraces was determined based on ISSR by constructing dendrogram with bootstrap values of 1000 replicates using Winboot (XP Windows). The fragments that appeared on dendrogram were scored as diallelic for each assigned locus (1=band present, 0=band absent). Phylogeny analysis indicates that ecoraces clustered mostly according to geographic distribution.

Andhra local and DabaBV were found to have clustered according to geographical distribution (both are found in Telangana). Sukinda seems to have formed a separate line (Sukindergarh, Orissa). Daba TV (Telangana) and Raily (Chhatisgarh) are clustered into one, geographically closer than other ecoraces, but distant to the above. Modal (Keonjhar, Orissa) and Bhandara (Maharashtra) have formed two separate and lines, indicating a higher geographic distance from the other populations. The morphological characters of these ecoraces also reveal that modal and bhandara have the highest and lowest filament length, indicating diverse genotype among the populations studied. These observations are in accordance with earlier studies (Mahendran *et al*., 2006) [6], in which the ecoraces were found to have clustered according to geographical distribution and morphological characters. Since geographically closely situated populations tend to be genetically more similar, the study needs further probing with regard to random mating.

Conclusion

The molecular characterisation using SSR and ISSR markers suggest that these markers could be effectively utilised for identifying the genetic variability among tasar ecoraces. Among the ecoraces studied, Andhra local ecorace was found to be genetically close to Bhandara and Daba BV within the populations in relation to genetic distance based on SSR and ISSR phylogenetic trees respectively and found to have clustered according to geographical distribution.

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