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## GENETIC DIVERSITY AND DIFFERENTIATION AMONG POPULATIONS OF THE TASAR SILKWORM, *ANTHRAEA MYLITTA* DRURY, REVEALED BY ISSR MARKERS (LEPIDOPTERA: SATURNIIDAE)

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G. Renuka and G. Shamitha\*

Department of Zoology, Kakatiya University, Warangal-506009, Telangana.

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\*Corresponding Author Email: [vodithalashamitha@gmail.com](mailto:vodithalashamitha@gmail.com)

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### ABSTRACT

*Antheraea mylitta* Drury, the semi-wild silk-producing lepidopteran insect commonly known as tasar silkworm is unique to India and is distributed over a wide tropical forest range covering the states of Telangana, Andhra Pradesh, Bihar, Chhattisgarh, Madhya Pradesh, Maharashtra and Orissa. The populations found in different areas are known by their specific local names and are considered as different ecotypes, but it is difficult to separate the populations on the basis of morphological and life-cycle traits and thus molecular characterization was attempted. The present communication relates to the results obtained from the analysis of polymorphism unraveled by twelve ISSR primers for 16 populations of *A. mylitta* belonging to seven ecotypes and this analysis also identified 12 markers. In the present study genetic differentiation by using a polymorphic profile generated by ISSR marker system.

### KEY WORDS

*Antheraea mylitta*, tasar silkworm, ecotypes, morphological, polymorphism, ISSR primers.

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#### Introduction

Andhra local ecorace of tasar silkworm, *Antheraea mylitta*, which is an exclusive race of the state of Andhra Pradesh and Telangana, is well known for its superior commercial qualities. It is one of the verges of extinction due to certain weaknesses like poor egg-laying behaviour, voltinism, erratic emergence, non-uniform silk deposition in cocoons and pupal mortality. The present investigation is an attempt to study the genetic proximity of the other ecoraces of *Antheraea mylitta*, bring about an idea of breeding of Andhra local ecorace, without losing its beneficial commercial characters and suggest methods to overcome its weaknesses.

ISSR, are presumably non-coding loci and are dispersed throughout the genome. It is used to study genetic diversity analysis in diverse organisms - animal and plant. The ISSR primers contain

microsatellite motifs and three anchoring nucleotides at the 5' terminal end and were used in genetic analysis (Santana et al., 2009) and marker-association studies (Srivastava et al., 2007).

Earlier, genetic diversity and differentiation was revealed among different populations of the wild silkworm *Antheraea mylitta* was examined using ISSR markers (Chatterjee et al., 2004, Kar P.K., et al., 2005). Since ISSR primers (Inter Simple Sequence Repeat) primers also showed the suitability in genetic differentiation among different plant and animal genomes and also found useful in various silkworm races, these primers are selected for the present study.

The knowledge of genetic diversity in crop improvement is essential for high yield and gain hybrid vigour (Razdan et al., 1994), the present study is taken up to analyse the genetic closeness and

differentiation Andhra local ecorace with that of other ecoraces of Tasar silkworm, *Antheraea mylitta*. In the present study, we present the PCR-ISSR based phylogenetic analysis of seven tasar silkworm populations. For these studies several Microsoft software programs like POPGENE 1.32 (ISSR) was used.

### Materials and methods

Randomly selected male and female moths from populations representing seven selected areas of our country - Andhra local ecorace from Warangal district (Telangana) and Daba TV and Daba BV ecoraces from Mahadevpur, Karimnagar districts of Telangana, Bhandara ecorace from Maharashtra (Bhandara), Sukinda from Orissa (Sukindergarh), Raily from Chhatisgarh (Bastar) and Modal from Orissa (Keonjhar) were collected for the present study. The present investigation is an analysis of polymorphism unraveled by 14 ISSR primers for 16 populations of *A. mylitta* belonging to seven ecotypes.

#### Genomic DNA isolation

Genomic DNA was extracted from 16 randomly selected individual moths from each generation of each line by the use of the phenol-chloroform method (Suzuki et al., 1972) later it was modified by Nagaraju and Nagaraju (1995). DNA was dissolved in TE buffer (Tris-EDTA, pH 8.0) and Quantification was done on 0.8% agarose gel and a uniform concentration of 100 ng/ $\mu$ l was obtained after serial dilution with the TE buffer (pH 8.0) or using nanodrop.

#### PCR amplification of the DNA with ISSR primers

ISSR primers synthesized by Eurofins Genomics India Pvt. Ltd, Bangalore, were tested for their efficacy in amplification of DNA. Fourteen primers were tested, out of which one primer (UBC 809) has produced reproducible robust bands and selected for studies. PCR amplification was carried out (Chatterjee et al., 2004) on PCR Research Eppendorf Master Cycler gradient, PTC 200, using 20 $\mu$ l reaction mixture, as explained below.

#### PCR amplification of DNA with ISSR primers

A total of 14 inter simple sequences repeat (ISSR) primers from Eurofins Genomics India Pvt. Ltd, Bangalore, were used for the study, of which one primer showed high percentage of polymorphism. PCR amplification was done in 20 $\mu$ l reaction mixtures containing 2 $\mu$ l of 30-50ng DNA, 2.0 $\mu$ l 10X PCR buffer (MBI Fermentas), 0.3 $\mu$ l of 25mM dNTPs, 1.5 $\mu$ l of 25mM MgCl<sub>2</sub>, 1.0 $\mu$ l primer, 0.2 $\mu$ l of Taq DNA polymerase and 13 $\mu$ l of MQ. The PCR schedule was 95 $^{\circ}$  C for 5 min followed by 35 cycles of 94 $^{\circ}$  C for 30s, 40-45 $^{\circ}$  C for 45s, 72 $^{\circ}$  C for 45s, and a final extension of 10 min at 72 $^{\circ}$  C.

#### Separation and detection of PCR products

The PCR products were resolved on 1.2% agarose gel in Tris-Boricacid/EDTA buffer (pH 8.0) and electrophoresis was carried out with a constant voltage of 50V in parallel with DNA standard markers. Gel was stained with ethidium bromide (0.5 $\mu$ g/ml), it runs for 2-3 hrs on 50 volts and photographed with a Gel Documentation Unit (model no12 200069, 230V capacity) and store the images digitally on the attached computer (with specific software installed) in JPJ format. In the present work, 1 Kb and MassRuler<sup>TM</sup> DNA ladders of MBI Fermentas Inc. were used as markers to estimate the size of PCR amplified products. Binary scoring of the profiles was done visually.

#### Genetic data analysis

The Phylogenetic relationship among tasar ecoraces were analyzed by generating the Phylogenetic tree by Nei (1972) genetic distance using UPGMA analysis through POPGENE software [1.32 version] (Yeh et al., 1999). The PCR amplified bands were scored visually by different ecoraces of *A. mylitta* on the basis of their presence (1) or absence (0). The scores obtained were then pooled for constructing a single data matrix, which was used for estimating the proportion of polymorphic loci, Nei (1973) gene diversity (h), gene flow (Nm), coefficient of gene differentiation (GST), Nei (1978) unbiased genetic distance (D). Significant test and construction of a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations were carried out by using POPGENE [version 1.32] (Yeh et al., 1999) computer program. Band sharing based intra- population similarity indices (S1) were

calculated for all possible comparisons according to the following formula: Similarity index (S1) =  $2NAB/(NA+NB)$ .

#### 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 sent to Zero, whereas if the population 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 software (Yeh et al., 1999) using Nei (1972) standard genetic distance equation.

#### Scoring for Dominant markers (ISSR)

For dominant markers such as RAPDs, AFLPs and ISSRs, it is generally assumed that each band represents a different locus and that the alternative to a band at the gel position characteristic of that locus is the absence of a band anywhere in the gel (Pradeep et al., 2011). In the present studies, it is expressed in the form of '1' for presence and '0' for absence of the band using software Popgene 1.32 version for the construction of phylogenetic tree based on genetic distance.

In the profile of dendrograms for ISSR using Popgene 1.32version, the level of polymorphism is expressed as the percentage of all loci that are polymorphic. It

also gives details about bootstrap values, no. of alleles, gene flow, genetic distance, gene diversity, etc.

## Results

### The genetic variability revealed through ISSR markers

A summary of genetic variation statistics for all loci is depicted in Table 1. Average number of alleles observed was for  $1.5000 \pm 0.5189$ ,  $1.6429 \pm 0.4972$ ,  $1.5714 \pm 0.5136$ ,  $1.5000 \pm 0.5189$ ,  $1.7143 \pm 0.4688$ ,  $1.5714 \pm 0.5136$  and  $1.6429 \pm 0.4972$  for AL, D TV, D BV, MD, SUK, RL and BH, respectively, while it was  $2.000 \pm 0.000$  when all populations were taken together. Similarly, in AL, D TV, D BV, MD, SUK, RL and BH the average number of effective alleles were  $1.2130 \pm 0.2927$ ,  $1.2385 \pm 0.2571$ ,  $1.2730 \pm 0.3072$ ,  $1.1381 \pm 0.1632$ ,  $1.3006 \pm 0.2969$ ,  $1.2219 \pm 0.2650$  and  $1.1497 \pm 0.1158$  respectively, while average for the total populations was  $1.2244 \pm 0.2159$  when all populations were taken together. The observed number of alleles ranged from 1-2 (UBC 809-1,2,3,7,8,11,14 and UBC 809-4,5,6,9,12,13), while effective number of alleles ranged from 1.000 - 1.9780 (UBC 809-1,2,3,7,8,14 and UBC 809-9) (Tables 2).

**Table 1: Origin and characteristics of Andhra local, DabaTV, Daba BV, Modal, Sukinda, Raily and Bhandara ecoraces.**

Ecorace	Place of origin	Place of use / practice	Primary food plant	Cocoon availability	Level of adaptability	Pre dominant cocoon colour	Diapause period (months)	Voltinism	Average larval weight (grams)	Average Larval span (days)
Andhra Local	Adilabad (Andhra Pradesh)	Andhra Pradesh	T. arjuna, T. tomentosa	Forest collection	Wild	Cremish /yellow	4	Trivoltine	25	42
Daba	Singhbhum (Jharkhand)	Entire tasar produci	T. arjuna,	Silkworm rearing	Wider adaptability	Grey	6-7	Bi/ Trivoltine	42	31

		ng areas	T. tomentosa							
Modal	Simlipal (Orissa)	Simlipal	Shorea robusta	Forest collection	Wild	Blackish Grey	4-10	Uni /Bi/ Trivoltine	45	32
Sukinda	Sukindagarh (Orissa)	Entire tasar producing areas	T. arjuna, T. tomentosa	Silkworm rearing	Wider adaptability	Yellow	4	Trivoltine	35	36
Raily	Bastar (Chhattisgarh)	Bastar	Shorea robusta	Forest collection	Wider adaptability	Blackish Grey	4-10	Uni /Bi/ Trivoltine	45	32
Bhandara	Bhandara (Maharashtra)	Bhandara	T. arjuna, T. tomentosa	Forest collection	Wild	Blackish grey/ yellow/ creamish	5	Trivoltine	30	35

**Table 2: Nei's Analysis of gene diversity in subdivided populations.**

Population genetics parameters for the 16 populations of seven ecoraces of *Antheraea mylitta*.

Ecorace-wise data on the number of alleles (Na), number of effective alleles (Ne), h= genetic diversity; I=Shannon Index; Ht= Total Heterozygosity; Hs = Sample Heterozygosity

	Ecoraces	Na	Ne	h	I	Ht	Hs
1	Andhra local	1.5000± 0.5189	1.2130 ±0.2927	0.1392± 0.1664	0.2217±0.2 495	0.1392± 0.0277	0.1392± 0.0277
2	Daba TV	1.6429±0.4 972	1.2385 ±0.2571	0.1640 ±0.1512	0.2677±0.2 288	0.1783±0. 0234	0.1698±0.0209
3	Daba BV	1.5714 ±0.5136	1.2730 ±0.3072	0.1756 ±0.1771	0.2738±0.2 636	0.1783±0. 0234	0.1698±0.0209
4	Modal	1.5000 ±0.5189	1.1381 ±0.1632	0.1059 ±0.1173	0.1826±0.1 961	0.1609±0. 0156	0.1516±0.0134
5	Sukinda	1.7143 ±0.4688	1.3006± 0.2969	0.1972 ±0.1630	0.3147±0.2 370	0.1609±0. 0156	0.1516±0.0134
6	Raily	1.5714 ±0.5136	1.2219 ±0.2650	0.1505 ±0.1572	0.2436±0.2 385	0.1505±0. 0247	0.1505±0.0247
7	Bhandara	1.6429±0.4 972	1.1497 ±0.1158	0.1214± 0.0939	0.2167±0.1 677	0.1214±0. 0088	0.1214±0.0088

**Table 3: List of primers used for ISSR analysis and the resulting polymorphic DNA bands.**

Primer	Sequence (5'-3')	Annealing temperature (°C)	MgCl <sub>2</sub> (mM)	Total number of bands amplified	Number of polymorphic bands	Polymorphism (%)
UBC809	AGA GAG AGA GAG AGA GG(17)	40- 45 °C	25	84	78	92.85

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

Ecorace	Number of polymorphic loci	Percentage of polymorphic loci
Andhra local	7	50.00
Daba TV	9	64.29
Daba BV	8	57.14
Modal	7	50.00
Sukinda	10	71.43
Raily	8	57.14
Bhandara	9	64.29

**Table 5: Calculation of mean genetic distance**

Ecoraces	Mean values
1. Andhra local	0.0127
2. DabaTV	0.0206
3. DabaBV	0.01446
4. Modal	0.0187
5. Sukinda	0.01366
6. Raily	0.0159

#### Gene diversity in the populations

The genetic diversity in the seven populations is presented in Table 2. The total genetic diversity (Ht) varied between 0.0297 and 0.4073 with a mean of 0.1625± 0.0164. Within-population genetic diversity (Hs) ranged from 0.0270 to 0.3657 with a mean of 0.1556± 0.0145. Gene differentiation (Gst) ranged from 0.0098 (UBC809-5) to 0.1106 (UBC 809-9) with a mean of 0.0423. Nei's diversity (h) ranged from 0.0000 (UBC 809- 1,2,3,7,8,11,14) to 0.4944 (UBC 809-9). When individual populations were considered, total genetic diversity in AL, D TV, D BV, MD, SUK, RL and BH were

0.1392± 0.1664, 0.1640 ±0.1512, 0.1756 ±0.1771, 0.1059 ±0.1173, 0.1972 ±0.1630, 0.1505 ±0.1572 and 0.1214± 0.0939, respectively. The sample genetic diversity was calculated to be the same as the total genetic diversity when the populations were taken separately in Andhra local, Raily and Bhandara.

An alternative approach for calculating the within population variation is Shanon's diversity index which does not assume Hardy-Weinberg equilibrium. Average Shanon's diversity index were 0.2217±0.2495, 0.2677±0.2288, 0.2738±0.2636, 0.1826±0.1961,

0.3147±0.2370, 0.2436±0.2385 and 0.2167±0.1677 AL, D TV, D BV, MD, SUK, RL and BH respectively (Table 2).

## Discussion

### **Genetic characterization of ecotypes with ISSR primers**

In the present investigation, the genetic diversity of 7 tasar ecoraces which were morphologically and geographically distinct was studied using ISSR primers. Out of the 12 ISSR primers tested, only one ISSR primer (UBC 809) 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 (54%). Multiple bands varying in size from 200 to 900bp are seen in almost all the strains (Fig. 1). Such a large range of variability might have been caused by small insertions/deletions in the genomic region amplified between the microsatellite anchored regions (Reddy et al., 1999). 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 investigation, screening of genomic DNA from 16 individuals of seven populations using one ISSR primer (UBC809) yielded several reproducible amplicons. The average no of amplicons produced per DNA sample was 2-6 per primers, with sizes ranging from 200-1250bp. The percentage of polymorphism was 92.85 (Table 3).

### **Phylogenetic analysis on the basis of ISSR markers**

Recent advances in molecular biology have introduced a new generation of markers which are versatile tools having a number of advantages over the conventional phenotypic markers. Among the PCR based marker techniques, ISSRs are stable, reproducible and one of the simplest and widely used, involving amplification of DNA segment present at amplifiable distance in between two identical microsatellites repeat regions oriented in opposite directions. Hence, they are extensively used in phylogenetic analysis and populations studies. ISSR technique has been employed in Indian Mulberry sp to understand genetic inter-relationships among parents which are a pre-requisite for any breeding program (Vijayan, 2005).

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. The distance matrix based on ISSR data sets was used to construct a dendrogram, which is shown in (Fig. 2).

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 (Chhattisgarh) 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 (Fig. 3, 3a).

Genetic relations of various genotypes by scoring the PCR-ISSR profiles were done. The polymorphic loci generated by ISSR marker system were scored by 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. 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).

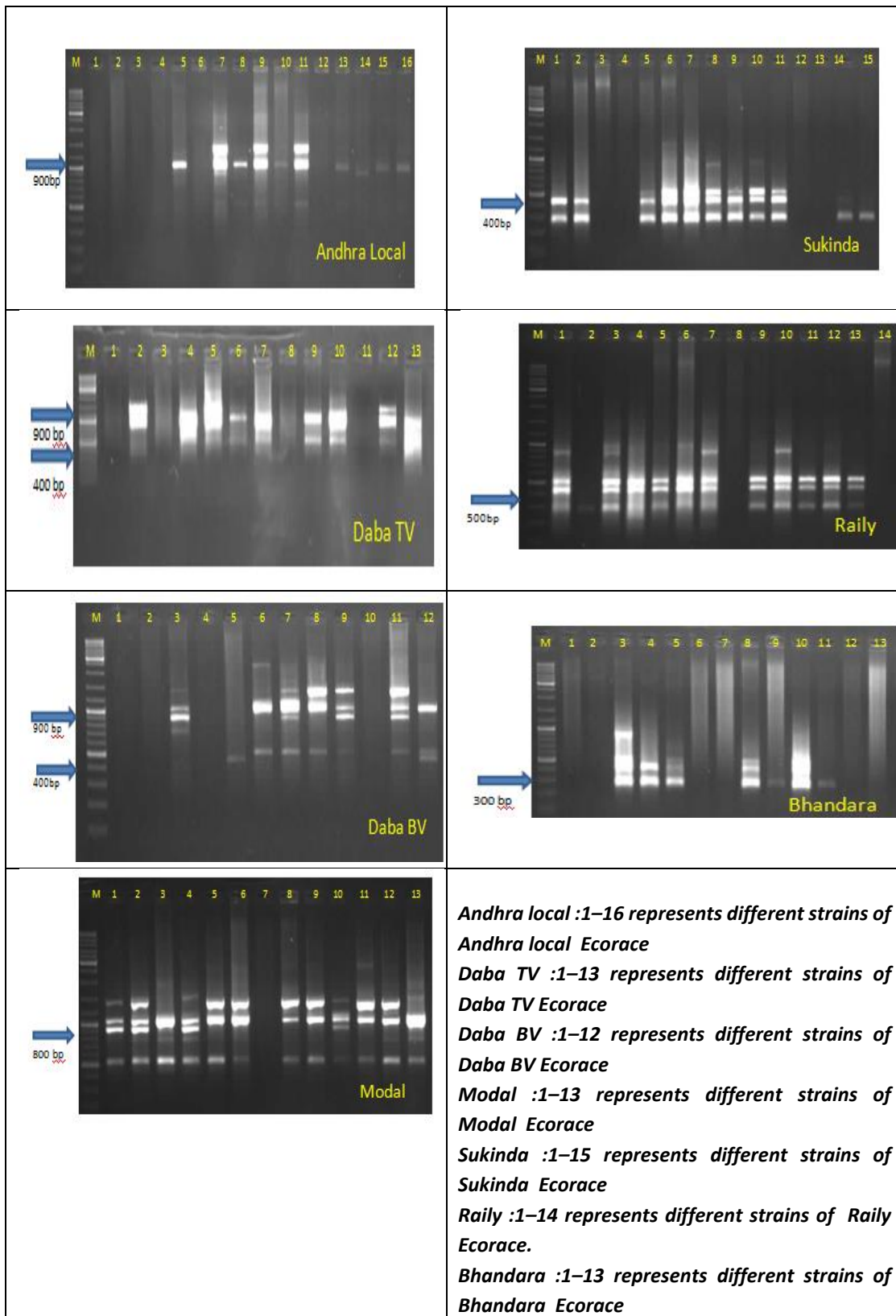
It can be inferred from the dendrograms (Fig. 2) obtained using ISSR primers, Andhra local and Daba BV, Daba TV and Raily are found to be genetically close, while Modal- Bhandara cluster and Sukinda have shown some genetic distance within the population.

From the Table 5, showing genetic distance, it can be inferred that among the 6 (A.L, DBV, Modal, Sukinda, Raily) populations Daba TV (mean value=0.0206) shows higher genetic distance from the other populations and that it is genetically distant from other ecoraces, while the ecoraces Andhra Local, DabaBV and Sukinda shown more genetic closeness.

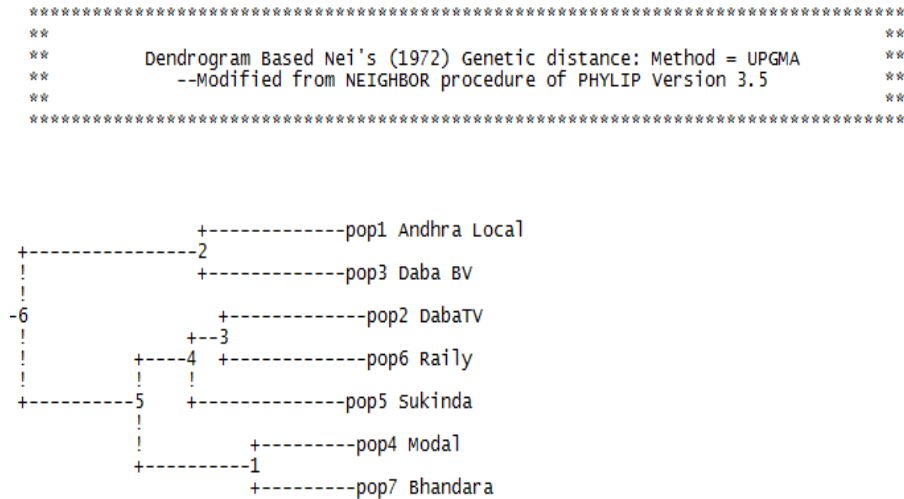
### **The order of genetic closeness may be summarized as follows**

DabaTV < Modal < Raily < DabaBV < Sukinda < Andhra local

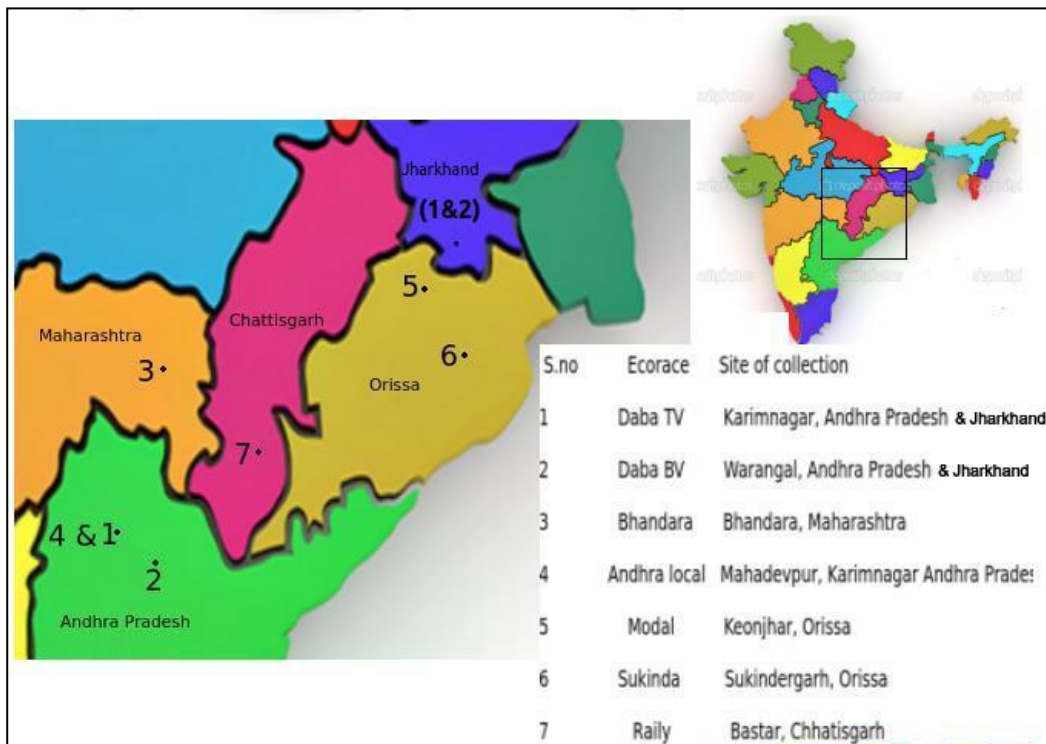
Population A.L and Daba BV are found to be genetically close according to phylogenetic tree.



**Fig. 1. ISSR profile generated from genomic DNA of 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.**

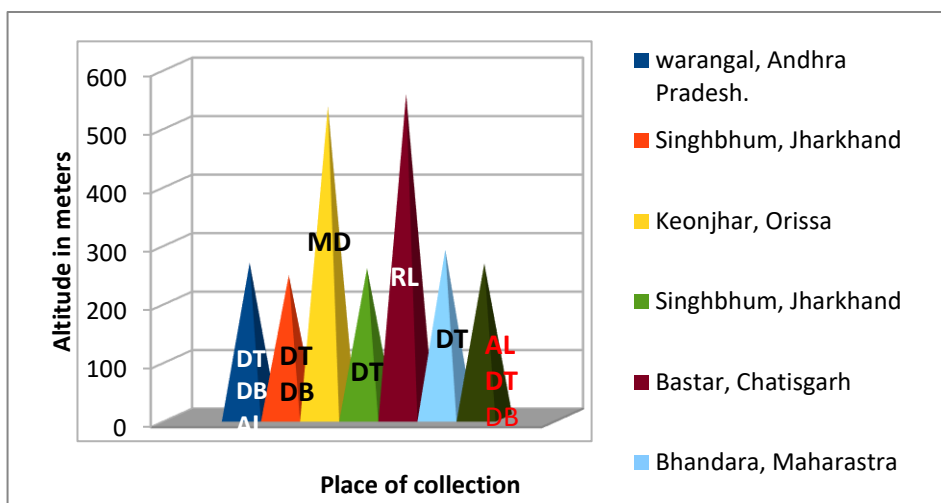


**Fig. 2. Phylogenetic analysis using ISSR primers UPGMA dendrogram depicting ecorace genetic diversity of *Antheraea mylitta* genotypes, obtained by PCR-ISSR marker data. (POPGENE version 1.32).**



**Fig 3. The rearing sites of ecoraces of Tasar Silkworm, *Antheraea mylitta*.**





**Fig. 3a. Graph depicting the comparison of altitude and site of availability of tasar ecoraces.**

AL (Andhra Local) – in Karimnagar and Warangal of Telangana; MD (Modal), SK (Sukinda), RL (Raily) – in higher altitudes; DT (Daba TV), DB (Daba BV) – in lower altitudes; BN (Bhandara) – in Maharashtra; DB(Daba BV), DT(Daba TV) in Telangana, Jharkhand, Maharashtra.

### Conclusion:

The molecular characterisation using ISSR markers suggest that these markers could be effectively utilised for identifying the genetic variability among tasar ecoraces. Among the ecoraces studied, Andhra local and Daba BV are found to be genetically close according to ISSR phylogenetic tree.

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**\*Corresponding Author:**

**G. Shamitha\***

Email: [vodithalashamitha@gmail.com](mailto:vodithalashamitha@gmail.com)