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STUDIES ON GENETIC VARIABILITY OF TASAR ECORACES AS REVEALED THROUGH MICROSATELLITE MARKERS.

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ABSTRACT
The tropical tasar silkworm *Antheraea mylitta* D., a wild sericigenous and polyphagous insect. is cultivated in the dense, humid, tropical forests of eastern, central and southern India. Its primary food plants are *Terminalia Arjuna, Terminalia tomentosa and Shorea robusta.* The present studies of genetic relations is based on phylogeny of tasar ecoraces using SSR markers, which further provides molecular evidence of the fact that climatic factors, the changes at DNA level and its wide range of distribution in varied geographic conditions would lead to genetic divergence ultimately leading to the formation of new ecorace.

KEYWORDS: *Antheraea mylitta*, ecorace, microsatellites, phylogeny, genetic diversity.

INTRODUCTION

Simple sequence repeats (SSR) otherwise known, as microsatellites are tandemly repeated DNA sequence motifs present in eukaryotic genomes. SSR markers became very popular because they are highly reproducible, multi-allelic, co-dominant, following Mendelian inheritance and are amenable to high throughput automation. Each SSR locus can easily be amplified by using a polymerase chain reaction (PCR) knowing the DNA sequence flanking the repeat region specifically. SSR markers, DNA markers are highly variable, can be amplified from a variety of organisms, are haploid and have high evolutionary rate which permits recovery of historical events without the need of sequencing (Vijayan *et al.*, 2010).

Formerly, thirteen diverse strains of the silkworm *Bombyx mori* were analyzed using the simple sequence repeat anchored polymerase chain reaction (SSR – anchored PCR) or Inter –SSR-PCR (ISSR-PCR, Reddy *et al*., 1999).

A recent study, 12 contigs were selected at random from *Antheraea mylitta* cSNP data freely downloaded from http://www.cdfd.org.in/ wildsilkbase. The 12 forward and reverse EST-SSR primers were designed for PCR amplification with genomic DNA of a seven tasar ecoraces. The sequences using forward and reverse primer were obtained in the five ecoraces (Andhra local, Daba TV, Daba BV, Modal and Sukinda) in graphical representation and also in the form of aligned sequences. From this data a precise observation of alignment of nucleotide sequences with reference primer (primer 1) has revealed that at 60 regions, there was polymorphism at nucleotide level. This is the most valuable observation excerpted from the recent investigation (Renuka, 2014).

The present work is being envisaged to involve larger populations and representatives encompassing various regions of our country for phylogenetic studies and evolve future breeding programme of Andhra local ecorace, as a strategy to conserve the dwindling population, which is on the brink of extinction.

MATERIAL AND METHODS

Isolation of Genomic DNA

Genomic DNA was extracted from 13 randomly selected individual moths from each generation of each line and the control group by the use of the phenol-chloroform method (modified by Nagaraja and Nagaraju, 1995).

SSR based molecular analysis of polymorphic study of different ecoraces of *Antheraea mylitta* D.

Simple sequence repeats (SSR) otherwise known, as microsatellites are randomly repeated DNA sequence motifs present in eukaryotic genomes. SSR markers became very popular because they are highly reproducible, multi-allelic and Co dominant markers.

The reaction mixture is given a momentary spin for thorough mixing of the cocktail components. Then 0.2ml PCR tubes are loaded in a thermal cycler {(Research Master Cycler PTC 200, Eppendorf) (Table. 2)}.

The thermal cycler is programmed as follows
Profile 1: 94°C for 10 min - Initial denaturation
Profile 2: 94°C for 30 sec - Denaturation
Profile 3: 48-60°C for 35sec - Annealing
Profile 4: 72°C for 45 sec - Extension
Profile 5: 72°C for 10 min - Final extension
Profile 6: 4°C for infinity to hold the sample.
Profile 2, 3 and 4 are programmed to run for 35 cycles.

Phylogenetic Study of different ecoraces of Tasar Silkworm, *Antheraea mylitta* D.
The Phylogenetic relationship among tasar ecoraces was 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 *Antheraea 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).

Scoring for Co-dominant markers (SSR)
With co-dominant markers, such as allozymes, RFLP and SSR, each recognizable allele at a given locus is ordinarily associated with a single band at a unique position in the gel. Thus, in the case of diploid organisms for a given locus a homozygote will have one band and a heterozygote will have two. Null alleles (no band) are rarely seen. Also, if there are multiple alleles per locus, as expected for SSRs, which are highly variable, the total number of bands expressed by all the individuals in a sample will likely be much greater than the number of loci involved.

In the present studies genetic relations of various ecoraces by scoring the PCR-SSR profiles was done. Here it is expressed as '1' for absence, '2' , '3', '4', *etc*. for the presence of one, two, three, *etc*., for the construction of genetic distance based phylogenetic tree using software popgene 1.32.

In the profile of dendrograms for SSR using popgene 1.32., the level of polymorphism is expressed as the percentage of all loci that are polymorphic. It also gives details about no. 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 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.

Table.1: Molecular Characterization of the Tasar ecoraces using SSR primers

S.No	Locus Name	5'-3'primer sequence	Annealing temperature (°C)	$MgCl_2$ (mM)	Allele size(bp)	Comment
1	Amysat 002	F5'ATTGTAATCATAATCCTATCG 3' R-5'CCGAAACAACCTACATTCC3'	52	2.0	221	Not worked
2	Amysat 005	F-5'CTGCCCCCTTCTGTCAG3' R-5'CTCACTACACGGGTTCCC3'	65	1.5	200	Worked polymorphic
3	Amysat 006	F-5'GATCTCGTCGGACGAGG3' R-5'GATCGCGCTTGCGGTT3'	52	2.0	163	Not worked
4	Amysat 007	F-5'CACAACATGTAAATCGGAAG3' R-5'TCGCAAGATGTGGGTATCA3'	52	2.0	168	Worked polymorphic
5	Amysat 009	F-5'TAACGGAAAAGCCCAGAGTG3' R-5'CCATGGCTTATGGTTTTTGC3'	55	1.5	219	Not worked
6	Amysat 010	F-5'CAGACAGACAGACGGAGTGC3' R-5'AACCGAGCAGCAAACCCTAT3'	57	2.0	187	Not worked
7	Amysat 014	F-5'CGACAGCACTTACAGCGACT3' R-5'GGACGAACTCAAGGCTGTTC3'	60	1.5	194	Not worked
8	Amysat 024	F-5'TGGAAGATCCAACAGCATACA3' R-5'GATGAGGCACGGACACCTAC3'	54	3.0	239	Worked polymorphic
9	Amysat 026	F-5'CGCCACTTGCTCAGGAAT3' R-5'CACGAGATGAGGTTGTGGTG3'	48	1.5	142	Worked polymorphic
10	Amysat 029	F-5'TGTGAAGTGCCATATACAGAAGG3' R-5'TCAAAAACGACGTCAAGGTG3'	56	2.5	155	Not worked
11	Amysat 030	F-5'ACGTGCTAGTGCA3' R-5'CATTCCACTACATCA3'	57	2.5	250	Not worked
12	Amysat 033	F-5'GAGCCCTTGAGTATGCTGCT3' F-5'ACACGCCTCAACAACTCCTC3'	48	1.5	159	Worked monomorphic
13	Amysat 034	F-5'ATAGGTATGTGGGGTGAATC3' R-5'CACAACACTCGCAGTGCG3'	48	2.5	202	Worked polymorphic
14	Amysat 036	F-5'TGAACGATTAAAATTGCATTAGG3' R-5'CGTGGGGTTCTACCACCTAC 3'	57	1.0	227	Worked Polymorphic
15	Amysat 037	F-5'CGCACGCGCACACT 3' R-5'CCTATACACAAGCGAGTAGT 3'	54	1.5	122	Not worked

Table.2: PCR amplification of DNA with SSR primers.

The cocktail for PCR amplification for respective SSR fragments is prepared as follows. The reaction mixture (10µl) contains:

PCR products	Amysat005	Amysat007	Amysat024	Amysat026	Amysat033	Amysat034	Amysat036
Genomic DNA(10ng/µl)	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
10xPCR buffer	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
25mM $MgCl_2$	0.6 µl	0.6 µl	0.6 µl	0.6 µl	0.6 µl	0.6 µl	0.6 µl
1Mm dNTPs	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
Forward primer	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
Reverse primer	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
TaqDNA polymerase	0.1µl	0.1µl	0.1µl	0.1µl	0.1µl	0.1µl	0.1µl
MQ	4.3 µl	4.3 µl	4.3 µl	4.3 µl	4.3 µl	4.3 µl	4.3 µl

RESULTS AND DISCUSSION

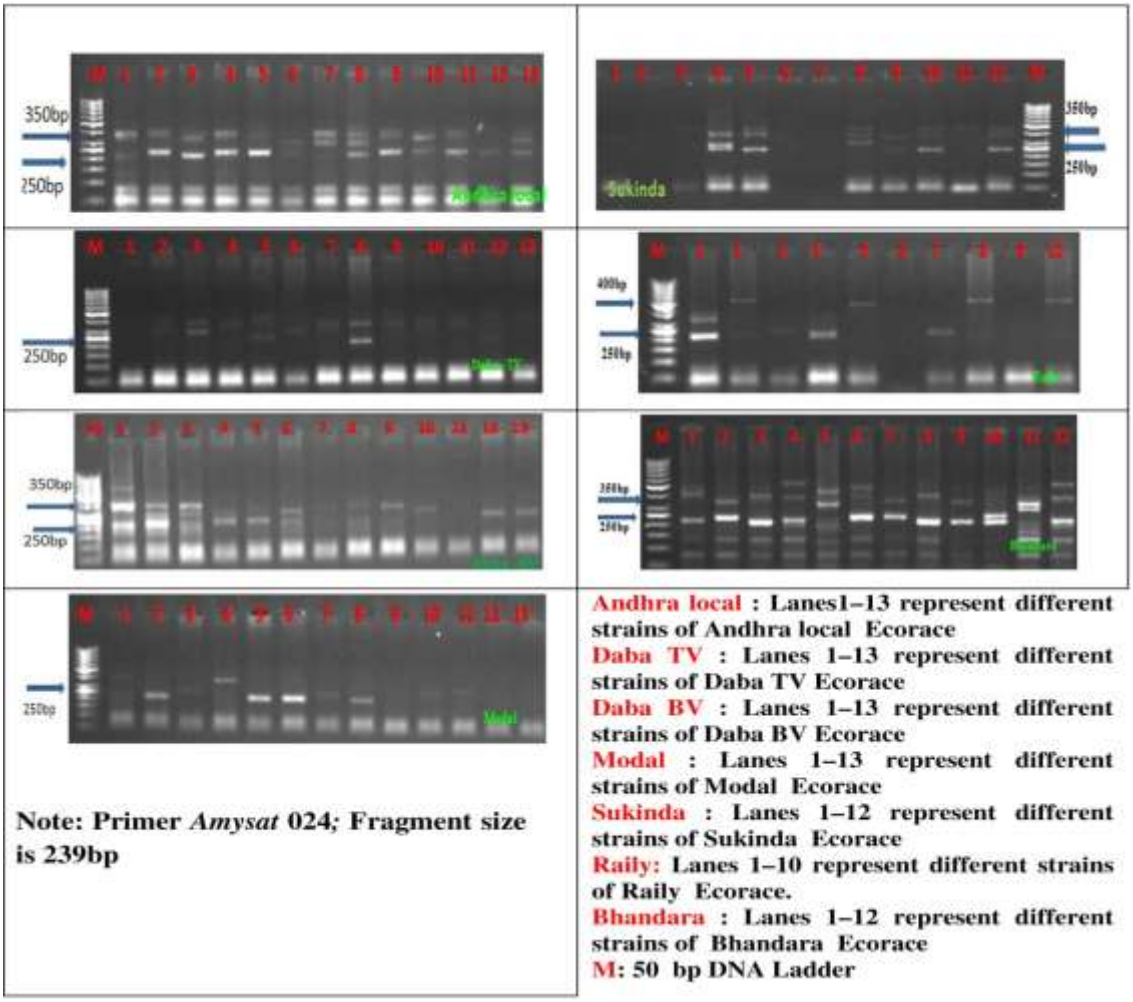

Fig. 1: SSR profiles generated from genomic DNA of 13 strains from different individuals of (Andhra local, Daba-TV, Daba-BV, Modal, Sukinda, Raily, Bhandara) ecoraces of Tasar Silkworm, *Antheraea mylitta* D using the primer *Amysat024*.

Table. 3: Nei's Original Measures of Genetic Identity and Genetic distance of *Antherea mylitta* D Genotypes, by PCR-SSR maker data.

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*******************************************************************************
**                                                                           **
**        Nei's Original Measures of Genetic Identity and Genetic distance   **
**                    [See Nei (1972) Am. Nat. 106:283-292)]                 **
**                                                                           **
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pop ID	1	2	3	4	5	6	7
1	****	0.8631	0.8523	0.8609	0.8919	0.9098	0.8170
2	0.1472	****	0.9060	0.9320	0.9518	0.9616	0.8987
3	0.1598	0.0987	****	0.9244	0.8793	0.9010	0.7997
4	0.1498	0.0704	0.0786	****	0.9385	0.9486	0.8309
5	0.1144	0.0493	0.1286	0.0635	****	0.9545	0.8858
6	0.0946	0.0391	0.1042	0.0528	0.0466	****	0.9018
7	0.2022	0.1068	0.2235	0.1852	0.1212	0.1034	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Andhra local, Daba-TV, Daba-BV, Modal, Sukinda, Raily were found to have a genetic distance of 0.2022, 0.1068, 0.2235, 0.1852, 0.1212 and 0.1034 with that of Bhandara. The dendrogram produced by UPGMA of Nei's genetic distance for all populations (7×13 = 91) is presented in fig.2 {(Phylogenetic tree of SSR) (Table. 3)}.

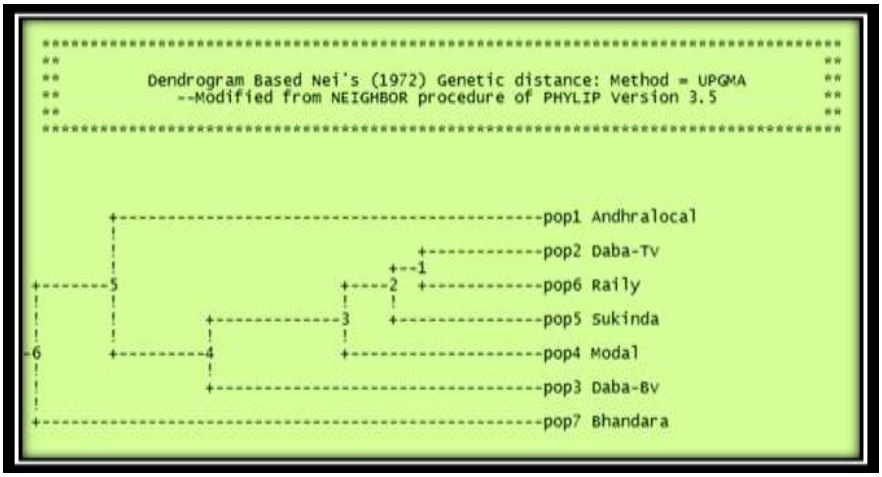

Fig. 2: UPGMA dendrogram depicting genetic diversity of *Antheraea mylitta* D genotypes, obtained by PCR-SSR primers.

Table. 4: Calculation of mean genetic distance of *Antheraea mylitta* genotypes using SSR primers (as calculated from Table 3).

Sl. No.	Ecoraces	Mean values
1.	Andhra local	0.08525
2.	DabaTV	0.1322
3.	DabaBV	0.1000
4.	Modal	0.0872
5.	Sukinda	0.0734
6.	Raily	0.1570

Table. 5: Genetic variation statistics in the ecoraces of *Antheraea mylitta, a*s revealed by Phylogenetic Analysis based on SSR primers.

Ecorace	Place of collection	Number of polymorphic loci	Percentage of polymorphic loci%
Andhra local	Warangal, Telangana	12	57.12
Daba TV	Adilabad, Telangana	9	42.86
Daba BV	Karimnagar, Telangana	12	57.14
Modal	Baripada, Orissa	11	52.38
Sukinda	Sukindergarh, Orissa	12	57.14
Raily	Bastar, Chhatisgarh	10	47.62
Bhandara	Maharashtra	10	47.62

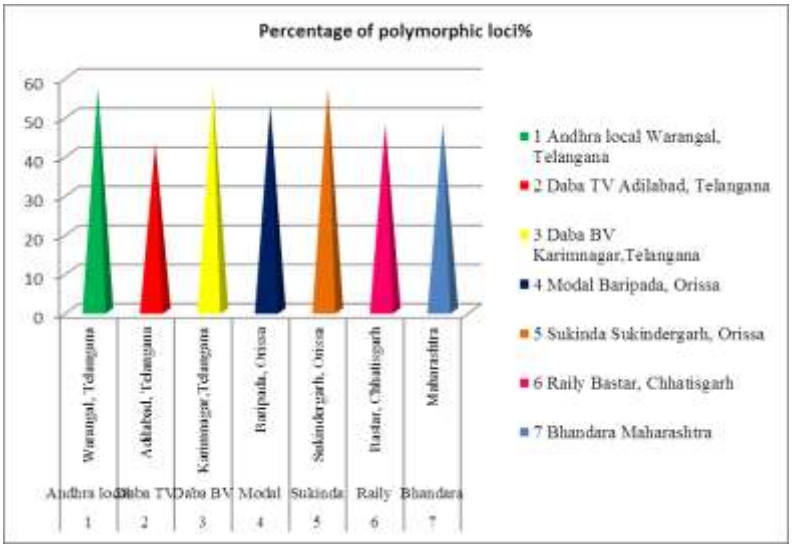

Fig. 3: Polymorphic loci percentage (%) of the ecoraces of *Antheraea mylitta,* as revealed by phylogenetic analysis based on SSR primers.

Table. 6: Polymorphism in Tasar ecoraces using SSR Primers.

S.No	Primer	Annealing temperature (°C)	MgCl2 (mM)	Total number of bands amplified	Number of polymorphic bands	Polymorphism (%)
1	Amysat005	65	25mM	100	71	71%
2	Amysat007	52	25mM	106	50	47.16%
3	Amysat024	54	25mM	61	23	37.70%
4	Amysat026	48	25mM	93	46	49.46%
5	Amysat033	48	25mM	79	-	-
6	Amysat034	48	25mM	77	36	46.75%
7	Amysat036	57	25mM	79	19	24.05%

Table. 7: Nei's Analysis of gene diversity in subdivided populations. Population genetics parameters for the 13 populations of seven ecoraces of *Antheraea mylitta* D.

S. No	Ecoraces	Na	Ne	h	I	Ht	Hs
1	Andhra local	1.5714±0.5071	1.3100±0.3774	0.1837±0.1962	0.2802±0.2795	0.2149 ± 0.0387	0.1570 ± 0.0209
2	Daba TV	1.4286± 0.507	1.2104±0.3160	0.1304±0.1773	0.2019±0.2608	0.2149 ±0.0387	0.1570 ± 0.0209
3	Daba BV	1.5714±0.5071	1.3215±0.3740	0.1905±0.1988	0.2885±0.2843	0.2216 ± 0.0400	0.1910±0.0306
4	Modal	1.5238±0.5118	1.3464±0.4284	0.1916±0.2186	0.2823±0.3062	0.2216 ± 0.0400	0.1910±0.0306
5	Sukinda	1.5714±0.5071	1.3822±0.4009	0.2187±0.2122	0.3224±0.3027	0.2145± 0.0392	0.1961± 0.0344
6	Raily	1.4762±0.5118	1.2997±0.3818	0.1735±0.2060	0.2581±0.2958	0.2145± 0.0392	0.1961± 0.0344
7	Bhandara	1.4762±0.5118	1.2923±0.3676	0.1724±0.2011	0.2578±0.2915	0.1724±0.0405	0.1724±0.0405

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

The genetic analysis using Simple sequence repeat anchored polymerase reaction (SSR- anchored PCR) has gained attention as a means of characterizing complex genomes. SSRs are widely used in population genetics and linkage map construction because of their high levels of polymorphism and reproducibility (M.W. Li, *et al.*, 2015) and their genome-wide distribution. Molecular markers are known to have many advantages over morphological and biochemical markers, as they are more stable and independent of environmental influences (Bernatzky & Tanksley, 1989; Gepts, 1993).

The DNA isolation and the quantification of the 91 individuals of the7 ecoraces (Andhra local, Daba TV, Daba BV, Bhandara, Modal, Sukinda and Raily) in the Tasar Silkworm, *Antheraea mylitta* D, revealed an ideal concentration of DNA ng/µl of the genomic sample *i.e.*, between 1.6 – 1.8 in 260/280 ratio) checked against 1 kb standard DNA ladder was obtained. It has been observed that DNA has been isolated without any protein or any other contamination and was used for further studies in PCR and Phylogenic analysis.

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 and Gur-Arie *et al.*, 2000) SSRs are a small array of tandemly arranged bases (1–6) dispersed throughout the genomes. SSRs as DNA markers have advantages over many other markers as they are highly abundant and polymorphic, co-dominantly inherited, analytically simple and readily transferable (Weber, 1990).

SSRs are indeed excellent for studies of population genetics and mapping (Jarne and Lagoda, 1996; Goldstein and Schlotterer, 1999) and now the marker of choice in most areas of molecular genetics as they are highly polymorphic even between closely related lines, require low amount of DNA, can be easily automated for high throughput screening, can be exchanged between laboratories, and are highly transferable between populations (Dandin S.B. and Gupta *et al.*, 1999).

Simple sequence repeats and single nucleotide polymorphism are the most preferred marker systems due to their higher reproducibility, co-dominant inheritance and easy amenability to high-throughput automation. Genetic characterization of 7 ecotypes with SSR primers was done. Out of the 15 primer combinations tried (Generous gift from Dr. K. P. Arun Kumar CDFD, 2014), 7 primer combinations have shown to have desired size fragments and six primers Amysat005, Amysat007, Amysat024, Amysat026, Amysat034 and Amysat036 (of allelic size 200, 168, 239, 142, 202 and 227 respectively) have shown polymorphism and one primer Amysat033 have shown monomorphism (of allelic size 159).

As is evident from figures 1, the SSR amplification of 7 silkworm strains/ecoraces (13 individuals in each, with seven primers, which generated polymorphism) using 7 SSR primers yielded a total of **595** bands, out of which

245 bands were polymorphic (41.17 %). During scoring, all the bands present in both polymorphic and monomorphic profiles were selected. The average no. of amplicons produced per DNA sample was 2-6 per primers, most of the bands were observed within the range of 150-500 bp, which is in accordance with the allelic size of the primers taken for studies.

Among the 21 alleles, at 13 alleles there was polymorphism showing a minimum of 2 and a maximum of 4 bands in each lane. The six markers were polymorphic within the strain. The amplification products were found around the following base pair regions:

- 250- 400 bp region in the Amysat024 and Amysat26 primers
- 200-400 bp region in the Amysat005 and Amysat034
- 200-250 bp region in the Amysat036
- 200 bp region in the Amysat033
- 150-168 bp region in the Amysat007.

In the present studies, among 07 polymorphic microsatellites used on 7 populations, 13 loci showed within population polymorphism in all populations. The most diverse locus (Amysat 026) has 5 alleles in Andhra local; (Amysat034) have shown 4 alleles in Daba-BV and (Amysat 005) has shown 3 alleles in Bhandara (Amysat 007) and (Amysat 036) have shown 2 alleles in ecoraces Daba-TV, Daba-BV and Sukinda and (Amysat033) has shown one alleles in all the 7 ecoraces studied.

From the findings it is abundantly clear that SSR Amysat026 primer generated polymorphic bands among the ecoraces, followed by Amysat024 and Amysat 005. These primers can be effectively used in genetic and phylogenetic studies. However, SSR Amysat033 primer generated monomorphic bands among the ecoraces.

The phylogenetic analysis using molecular markers like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), fluorescent dye labelled ISSR PCR reaction (FISSR-PCR) analysis have been developed to distinguish the genetic diversity among silkworm species (Yasukochi, 1998; Reddy *et al.*, 1999; Tan *et al.*, 2001; Nagaraju *et al.*, 2002; Cheng *et al.*, 2004; Mahendran *et al.*, 2005; Mahendran *et al.*, 2006). A recent studies on the seven ecoraces of *A. mylitta* D on molecular characterisation has revealed that SSR primers could be effectively utilised for genetic analysis of tasar silkworm (Renuka, 2014).

Genetic relations of the seven ecoraces by scoring PCR-SSR 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.

Andhra local, Daba-TV, Daba-BV, Modal, Sukinda, Raily were found to have a genetic distance of 0.2022, 0.1068, 0.2235, 0.1852, 0.1212 and 0.1034 with that of Bhandara (Table 3). The dendrogram produced by UPGMA of Nei's genetic distance of *Antheraea mylitta* D genotypes, based on PCR-SSR for all populations (7×13 = 91) is presented in (Fig. 2).

Phenogram based on UPGMA algorithm from SSR data of ecoraces genetic diversity analysis of *Antheraea mylitta* does not show region–wise clustering. Several individual clusters have been obtained (Fig. 2).

Cluster 1: Daba-TV- Raily formed an individual cluster 1, which is found to be genetically close.
Cluster 2: Sukinda formed an individual cluster 2 and found to be closer to cluster 1
Cluster 3: Modal formed an individual cluster 3 and found to be closer to cluster 2 (Sukinda populations), than cluster 1(Daba-TV and Raily populations).
Cluster 4: Daba-BV formed an individual cluster 4 and found to be closer to cluster 3 (Modal populations).
Cluster 5: Andhra local formed an individual cluster 5 was not included in any cluster, and found to be closer to cluster 4 than cluster 3.
Cluster 6: Bhandara formed an individual cluster 6, Bhandara seem to have diverse genotype and a rare one which was not included in any cluster, and found to be closer to cluster 5 than cluster 4.

It can be inferred from the dendrogram obtained using SSR primers, Daba TV and Raily are found to be genetically close, Sukinda, Modal, Daba BV, Bhandara and Andhra Local have shown some genetic distance within the population.

From Tables 3 and 4 on genetic distance, it can be seen that among the 6 populations (*i.e.*, Andhra local, Daba TV, Daba BV, Modal, Sukinda), Raily (mean value=0.1570) shows higher genetic distance from other populations and that it is genetically distant from other ecoraces. It can also be observed that the lowest genetic distance was found in ecoraces Sukinda, (0.0734) Andhra local, (mean value=0.08525) Modal (mean value=0.0872) have shown some genetic closeness.
The order of genetic closeness may be summarized as follows:
Raily* < *Daba TV* < *Daba BV* < *Modal* < *Andhra local* < *Sukinda

- Daba TV is genetically less close than Raily.
- Daba BV is genetically less close than Daba TV.
- Modal is genetically less close than Daba BV.
- A.L is genetically less close than Modal.
- Sukinda is genetically less close than A.L.

Populations of Raily and Daba TV are found to be close within the populations according to genetic distance and phylogenetic tree.

In the present studies, the germplasm collected from various zones of India displayed variable genetic polymorphism and was found to be highest in Sukinda and Daba BV (57.14) of Orissa and Karimnagar; followed by Andhra local (57.12%) of Telangana, Modal (52.38%) of Orissa; the samples Raily & Bhandara of Chhatisgarh and Maharashtra and Daba TV of Telangana were least diverse and displayed only 47.62% and 42.86% polymorphism (Table 5 & Fig 3).

In the present investigation, screening of genomic DNA from 13 individuals of 7 populations using 7 SSR primers yielded several reproducible amplicons. The average no. of amplicons produced per DNA sample were 2-6 per primers, with sizes ranging from 150-500 bp. The percent polymorphism was 71% in Amysat 005, whi1e it was 47.16%, 37.70%, 49.46%, 46.75% and 24.05% in primers Amysat007, Amysat024, Amysat026 and Amysat034 and Amysa036t respectively, while it was monomorphic in Amysat033 (Table.6). It is clear from the studies, that, SSR markers generated from 07 random primers revealed sufficient polymorphism to investigate and compare the genetic diversity of seven silkworm species. Thirteen individual samples were studied from each strain. According to Nei, (1978), the number of molecular marker loci is more important than sample size. In this study, 19-71 SSR reproducible loci were analyzed for each of the thirteen strains of seven ecoraces.

PCR-SSR based phylogenetic analysis using popgene 1.32 in the 7 tasar ecotypes revealed that Daba TV and Raily are found to be genetically close, Sukinda, Modal, Daba BV, Bhandara and Andhra Local have shown some genetic distance within the population. The ecoraces Raily showed a higher genetic distance from other populations It can also be observed that the Sukinda, Andhra local, and Modal have shown some genetic closeness. Populations of Raily and Daba TV are found to be close within the populations according to genetic distance and phylogenetic tree. The percent polymorphism was 71% in Amysat 005, whi1e it was 37.70% and 49.46% in primers Amysat024 and Amysat026 respectively.

Polymorphic profile generated by SSR primers from 7 different strains of *Antheraea mylitta* (Table. 6) was further analyzed by generating the Phylogenetic tree by Nei (1972) genetic distance using UPGMA analysis through POPGENE software [1.32 version] (Yeh *et al*., 1999).

Genetic analysis is essential to understand the evolutionary processes such as gene flow, natural selection, and genetic drift taking place in a population. The moderate mean coefficient of genetic differentiation (G_{st} = 0.2896) observed among the populations shown in the present studies indicates that the populations are in the verge of genetic differentiation as they have accumulated about 25% genetic variability among themselves. This is in corroboration with earlier studies. As the studies on genetic diversity is used in conservation studies, which is one of the major objectives of the present investigation, a moderately high percentage of polymorphic loci *i.e.*, 42.86%-57.14%, as revealed by phylogenetic analysis based on SSR primers, implies that populations have been differentiated into separate gene pools (Vijayan *et al*, 2006 and Renuka, 2016).

The population genetic parameters calculated through the SSR markers of seven populations are presented in Table 7. The observed (Na) number of alleles was recorded highest for the Andhra local, Daba BV and Sukinda with the value of 1.5714±0.5071. The minimum value for Na (1.4286±0.507) was recorded Daba TV. It can also clearly demonstrate from dendrogram obtained by PCR-SSR marker data (Fig. 2), as individual clusters were formed in these ecoraces.

The highest number of effective (N_e) alleles, Nei's gene diversity (h) and Shanon's information index (I) was recorded highest in Sukinda ecorace *i.e.*, 1.3822±0.4009, 0.2187 ± 0.2122 & 0.3224±0.3027 respectively, while lowest of these values were seen in Daba TV *i.e*, 1.2104±0.3160, 0.1304 ±0.1773 & 0.2019±0.2608 respectively. As can be seen from the dendrogram, the cluster 3 has diverged to produce four populations, wherein Sukinda formed an individual cluster 2 and found to be closer to cluster 1 (Daba TV and Raily). It is also evident when we observe the total genetic diversity H_t and Sample genetic diversity H_s, which were highest in both Sukinda and Raily (0.2145± 0.0392 and 0.1961± 0.0344), while lowest in Bhandara (0.1724±0.0405 and 0.1724±0.0405), which formed a separate cluster.

These observations clearly indicate that these ecoraces are genetically variable and diverged into separate strains based on geographic conditions and adaptability, as displayed by their distinct and unique phenotypic characteristics. It is also signified by the value of the gene flow (N_m) estimate of 1.2268, indicating the exchange of genes between populations. As, N_m value greater than 1.0 is considered necessary to prevent divergence resulting from genetic drift (Wright, 1951) and that high gene flow between populations precludes local adaptation and will also impede the process of speciation (Barton *et al.*, 1985).

The factors like latitude, longitude *etc.*, of distinct areas leads to marked differences expressing wide variations in phenotypic, physiological, behavioural, commercial and technological traits. Various populations thus, isolated geographically over centuries have adapted to a particular ecological niche and referred to as ecological races (Suryanarayana *et al.*, 2005).

The present studies of genetic relations based on phylogeny of tasar ecoraces using co-dominant microsatellites, further provides molecular evidence of

the fact that climatic factors, the changes at DNA level and its wide range of distribution in varied geographic conditions would lead to genetic divergence ultimately leading to the formation of new ecoraces.

CONCLUSION

From the findings it is abundantly clear that SSR Amysat026 primer generated polymorphic bands among the ecoraces, followed by Amysat024 and Amysat 005. These primers can be effectively used in genetic and phylogenetic studies. However, SSR Amysat033 primer generated monomorphic bands among the ecoraces.

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