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Genetic diversity and phylogenetic relationship as revealed by inter simple sequence repeat (ISSR) polymorphism in the different Ecoraces of Indian tropical tasar silkworm *Antheraea mylitta* drury

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Abstract

The tasar silkworm, Antheraea mylitta D., is a semi-domesticated (Lepidoptera: Saturniidae), wild sericigenous insect. The tropical tasar silkworm is cultivated in the dense, humid, tropical forests of eastern, central and southern India. It is feeds on eight primary food plants. The primary food plants are Terminalia Arjuna, Terminalia tomentosa, Shorea robusta, Lagerstroemia parviflora, L. speciosa, L. indica, Zizyphus and Hardwickia binata. 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.

Keywords: *Antheraea mylitta,* Tropical, Ecoraces, Co-dominant, Microsatellites, Phylogeny, Genetic diversity.

Introduction

The Indian tropical tasar silk insect is found in wild semi-domesticated conditions *Antheraea mylitta Drury* is a semi-domesticated species and natural fauna of tropical India, This species is polyphagous; primarily feeds on *Shorea robusta, Terminalia arjuna* and *Terminali tomentosa* and secondarily lives on at least a dozen other host plants[1]. Tasar silkworm has a wide range of distribution in varied geo-climatic condition[2-5], distributed in different geographical locations and habitats in this country.

The distribution of tasar silkworm *Antheraea mylitta* in the Indian sub- continent is wide ranging between 10° to 32° N latitude and 76° to 93° E longitude with varied environmental conditions covering 17 states *viz.,* Himachal Pradesh, Nagaland, Assam, Meghalaya, West Bengal, Odisha, Jharkhand, Madhya Pradesh, Chhattisgarh, Andhra Pradesh, Maharashtra, Uttar Pradesh, Manipur, Jammu & Kashmir, Rajasthan, Karnataka, Kerala and one Union Territory Dadar Nagar and Haveli reveals that these ecoraces are morpho variants of *Antheraea mylitta* of India[6,7]. The geographic range of population distribution is limited mainly in five types of soli *viz.,* red loamy, sandy red, black clayey, lateritic and forest hill under varied ecological conditions.

Molecular markers are known to have many advantages over morphological and biochemical markers, as they are more stable and independent of environmental influences[8,9]. Since RAPD and ISSR[10,11], were identified as potential molecular marker systems, their use in the analysis of phylogeny and in population genetics has been documented in a wide variety of organisms[12,13]. ISSR markers are useful in detecting genetic polymorphisms among closely related plants or animals by generating a large number of markers that target multiple microsatellite loci distributed across the genome. Potential molecular marker systems and their use in phylogenetic studies as well as genetic diversity in populations have been documented in a wide variety of organisms[14-16]. Molecular markers have also been used extensively to assess genetic diversity among *Antheraea mylitta* ecoraces[17,18].

The present investigation ISSR primers to study the genetic characterization, phylogenetic analysis and genetic diversity present among different ecoraces of *Antheraea mylitta*. Hence, in this study ISSR markers were used to unravel the genetic relationship of seven distinct ecoraces of *Antheraea mylitta*. Tasar ecoraces which were morphologically and geographically distinct was studied using ISSR primers generated distinct and robust bands showing polymorphism, the genetic material collected from diverse of India displayed variable inherent polymorphism was found, to be highest in Daba TV of Telangana (81.82%), followed by Bhandara and Daba BV (72.73%) of Maharashtra and Telangana; Raily and Modal (63,64%) of Chhatisgarh and Orissa; sukinda (54.55%) of Orissa and Andhra local (45.45%) of Telangana (Table-4 and Figure-4).

The present investigation on genetic diversity of seven tasar populations as unraveled by ISSR primers proved effective for understanding the evolutionary processes such as gene flow, natural selection, and genetic drift taking place in a population. As there is more number of tasar ecoraces.

Materials 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 (Figure-1, Table-1). From each population, 20 to 30 cocoons were collected and kept until emergence of the adult moth. Genomic DNA from 13 individual moths of each population was extracted separately following.

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[19]. 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 1ng _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.

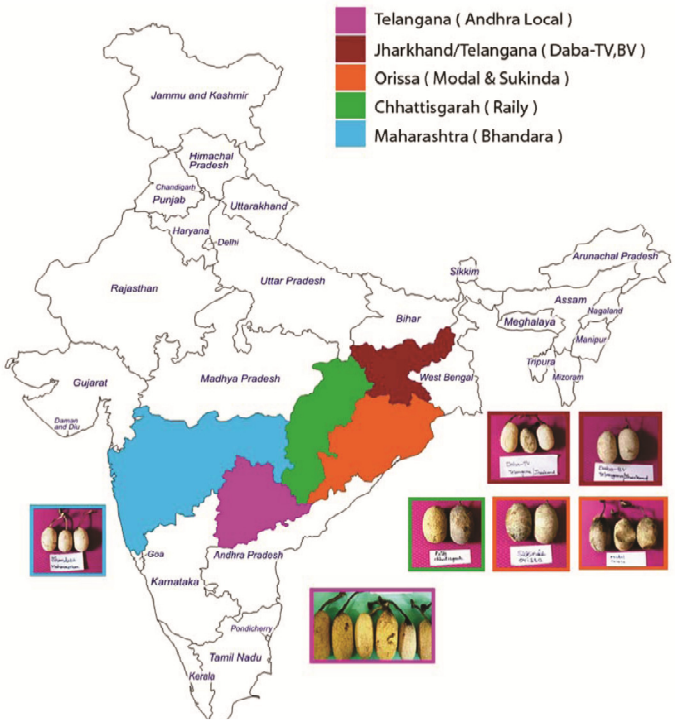


Figure-1: Collection of Tasar *Antheraea mylitta* samples from selected parts of the country. The map template was downloaded from google-maps.com and it using Adobe Photoshop.

Table-1: Collection of Tasar *Antheraea mylitta* samples from selected parts of the country.

S.No	Ecorace	Site of collection
1	Andhra local	Warangal Telangana
2	Daba TV	Khammam, Adilabad Telangana
3	Daba BV	Warangal, Karimnagar Telangana
4	Modal	Baripada Orissa
5	Sukinda	Sukindagarh Orissa
6	Raily	Bastar Chhatisgarh
7	Bhandara	Bhandara Maharashtra

PCR amplification of the Genomic DNA with ISSR primer: ISSR primers synthesized by Eurofins Genomics India Pvt. Ltd, Bangalore, were tested for their efficacy in amplification of DNA.

Fourteen (14) Oligos were tested, out of which two primers (UBC 861, UBC 864) has produced reproducible robust bands and showed high percentage of polymorphism, selected for studies. PCR amplification was carried out[20] on PCR Research Eppendorf Master Cycler (Table-2).

PCR amplification was done in 20µl reaction mixtures containing 2µl of 30-50ng DNA, 2.0µl 10X PCR buffer (MBI Fermentas), 0.3µl of 25mM dNTPs, 1.5µl of 25mM $MgCl_2$, 1.0µl primer, 0.2µl of Taq DNA polymerase and 13µl of MQ (Table-3).

The PCR thermal cycler is programmed as follows; 95º C for 5 min - Initial denaturation, 94°C for 30 sec –denaturation, 40-45º C for 45sec – Annealing, 72°C for 45 sec – Extension 72°C for 10 min - Final extension: 4°C for infinity to hold the sample, are programmed to run for 35cycles.

The PCR products were resolved on 1.2% agarose gel in Tris-Boric acid/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 (0.5µg/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 Mass Ruler TM 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.

Table-2: Molecular characterization of the ecoraces *Antheraea mylitta* using ISSR primers.

S.No.	Oligo Name	5'-3'primer sequence	No. of bands amplified	No. of polymorphic bands	Polymorphism (%)
1	UBC807	AGA GAG AGA GAG AGA GT(17)	-		
2	UBC808	AGA GAG AGA GAG AGA GC(17)	-		
3	UBC809	AGA GAG AGA GAG AGA GG(17)	-		
4	UBC811	GAG AGA GAG AGA GAG AC(17)	-		
5	UBC812	GAG AGA GAG AGA GAG AA(17)	-		
6	UBC813	CTC TCT CTC TCT CTC IT(17)	-		
7	UBC822	TCT CTC TCT CTC TCT CA(17)	-		
8	UBC827	ACA CAC ACA CAC ACA CG(17)	-		
9	UBC841	GAG AGA GAG AGA GAG AYC(18)	-		
10	UBC844	CTC TCT CTC TCT CTC TCRC(19)	-		
11	UBC855	ACA CAC ACA CAC ACA CYT(18)	-		
12	UBC861	ACC ACC ACC ACC ACC ACC (18)	172	165	95.93
13	UBC864	ATG ATG ATG ATG ATG ATG (18)	81	62	76.54
14	UBC873	GAC AGA CAG ACA GACA(16)	-		

Table-3: PCR amplification of DNA with ISSR primers. The cocktail for PCR amplification for respective ISSR fragments is prepared as follows. The reaction mixture (20µl) contains.

PCR products	UBC861	UBC864
Genomic DNA(100ng/µl)	2.0 µl	2.0 µl
10xPCR buffer	2.0µl	2.0µl
25mM $MgCl_2$	1.5 µl	1.5 µl
dNTPs	0.3µl	0.3µl
Primer	1.0 µl	1.0 µl
Taq DNA polymerase	0.2µl	0.2µl
MQ	13.0 µl	13.0µl

Phylogenetic study of different ecoraces of Tasar Silkworm, *Antheraea mylitta.:* The Phylogenetic relationship among tasar ecoraces were analyzed by UPGMA analysis through POPGENE software [1.32 version][21] .

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[22]. In the present studies, it is expressed in the form of '1' for presence and '0' for absence of the band The scores obtained were then pooled for constructing a single data matrix, which was used for estimating the proportion of polymorphic loci[23] gene diversity (h), gene flow (Nm), coefficient of gene differentiation (GST)[24] and genetic distance (D) were calculated.

Results and discussion

DNA Polymorphsim as revealed by ISSR primers: A high amount of polymorphism was observed at the DNA level primer system (Figure-2). The 2 ISSR primers 13 individuals are studied in each of the 7 ecoraces of Tasar Silkworm*, Antheraea mylitta.* Generated a total of 253 bands, out of which 227 bands produced by primer UBC 861 and UBC 864 were polymorphism showing 95.93% and 76.54% (Table-2) The bands between 300bp to 900bp region (Figure-2, Figure-3).

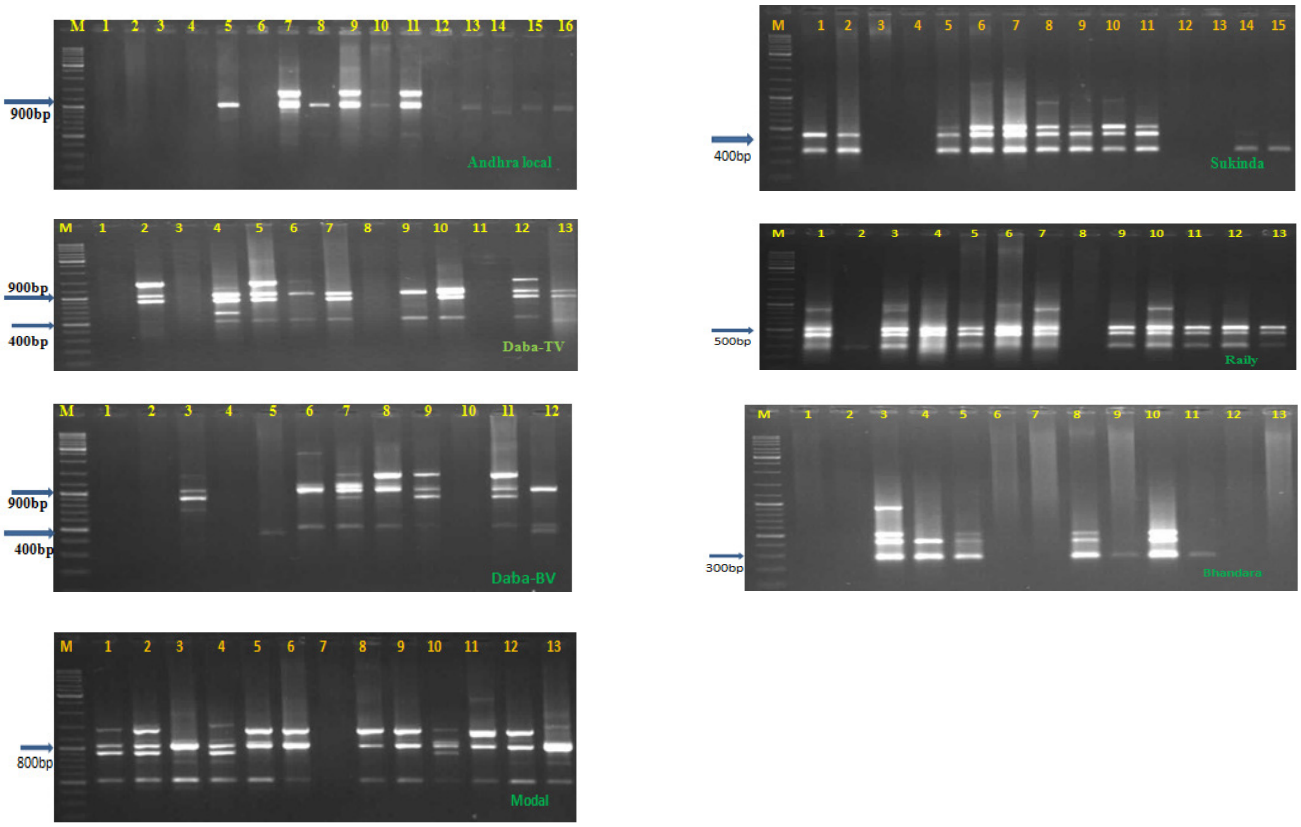


Figure-2: ISSR markers generated from 13, different individuals of seven ecoraces Tasar Silkworm, *Antheraea mylitta* using the primer *UBC 861.*

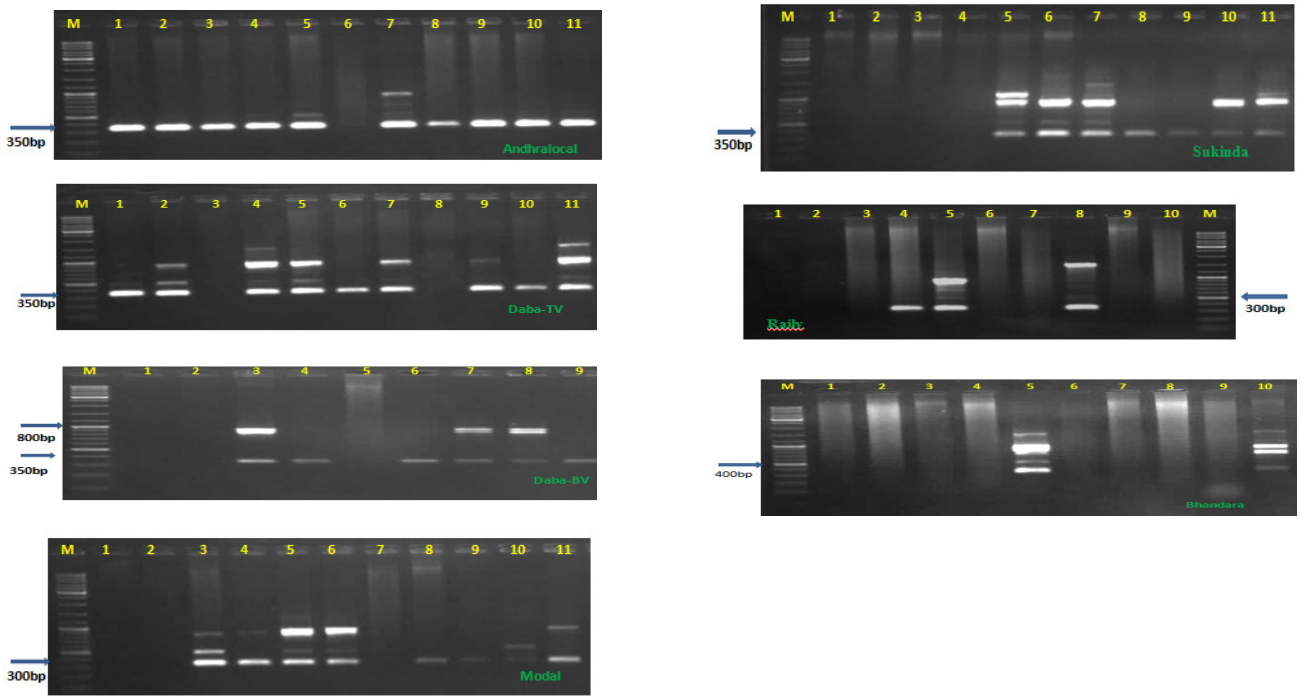


Figure-3: ISSR markers generated from 11 individuals of seven ecoraces Tasar Silkworm, *Antheraea mylitta* using the primer *UBC864.*

Table-4: Genetic variation statistics in the ecoraces of *Antheraea mylitta.* As revealed by phylogenetic analysis based on ISSR primers.

Ecorace	Place of collection	Number of polymorphic loci	Percentage of polymorphic loci%
Pop1	Warangal, Telangana	5	45.45
Pop 2	Adilabad, Telangana	9	81.82
Pop 3	Karimnagar,Telangana	8	72.73
Pop 4	Baripada, Orissa	7	63.64
Pop 5	Sukindergarh, Orissa	6	54.55
Pop 6	Bastar, Chhatisgarh	7	63.64
Pop 7	Maharashtra	8	72.73

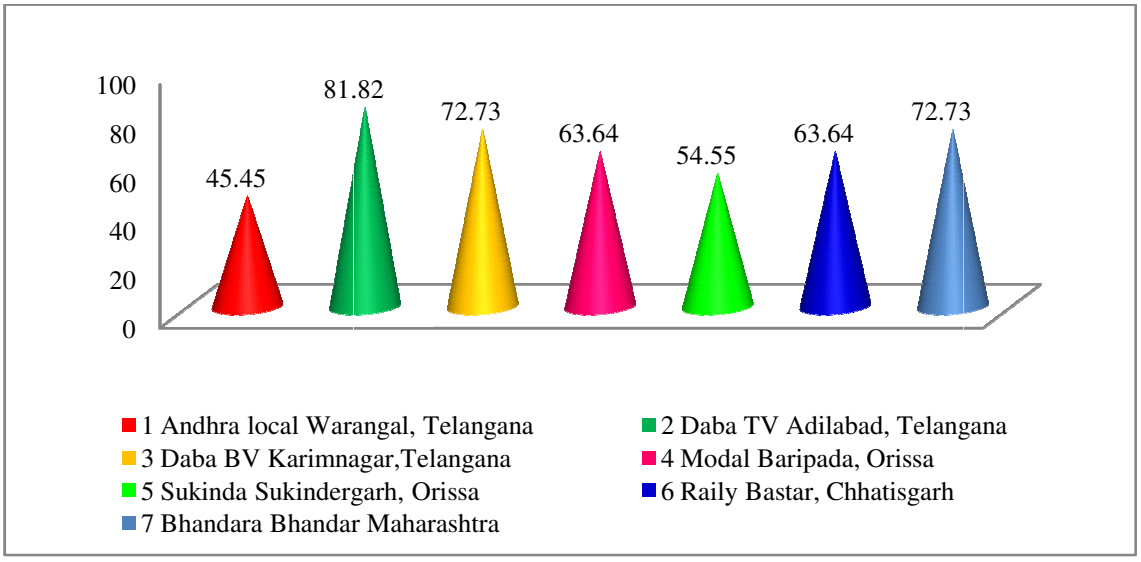
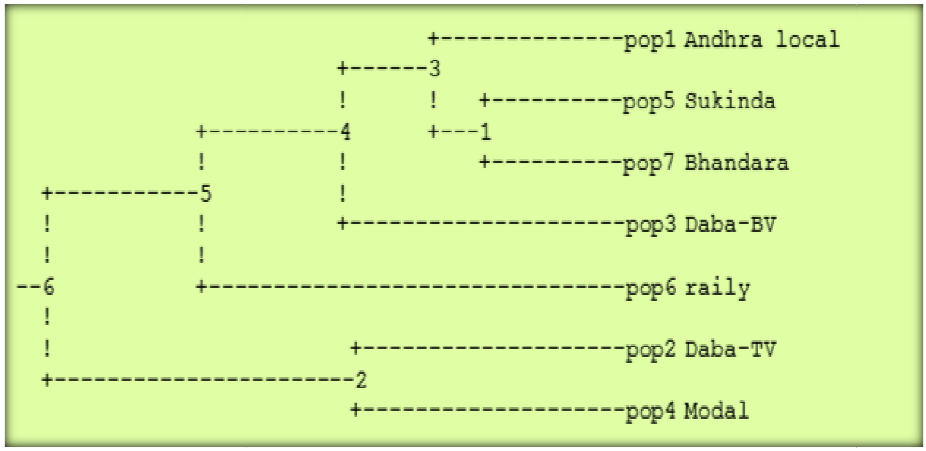


Figure-4: Polymorphic loci percentage (%) of the ecoraces of *Antheraea mylitta*. As revealed by phylogenetic analysis based on ISSR primers.



Source: the Phylogenetic tree by genetic distance using UPGMA analysis through POPGENE software 1.32 version.
Figure-5: Dendrogram showing grouping of the seven populations of *Antheraea mylitta* using UPGMA analysis.

Genetic distance of *Antheraea mylitta* genotyps using ISSR Primers

Table-5: Calculation of mean genetic distance of *Antheraea mylitta* Genotyps using ISSR Primers.

Sl. No.	Ecoraces	Mean values
1.	Andhra local	0.1394
2.	DabaTV	0.1182
3.	DabaBV	0.1339
4.	Modal	0.1279
5.	Sukinda	0.1245
6.	Raily	0.1042

Gene diversity in the populations of *Antheraea mylitta*

Table-6: Nei's Analysis of Gene Diversity in Subdivided Populations[25]. *Molecular Evolutionary Genetics* (p. 187-192)].

Locus	Sample Size	H_t	H_s	G_{st}	N_m
UBC861-1	91	0.4898	0.3888	0.2061	1.9258
UBC861-2	91	0.4898	0.3719	0.2406	1.5779
UBC861-3	91	0.4985	0.3111	0.3760	0.8299
UBC861-4	91	0.4342	0.2434	0.4394	0.6380
UBC861-5	91	0.1039	0.0879	0.1535	2.7576
UBC861-6	91	0.0840	0.0609	0.2759	1.3125
UBC861-1	91	0.4995	0.3956	0.2079	1.9047
UBC861-2	91	0.2753	0.2536	0.0789	5.8333
UBC861-3	91	0.2898	0.2198	0.2417	1.5690
UBC861-4	91	0.0840	0.0744	0.1149	3.8500
UBC861-5	91	0.0430	0.0372	0.1348	3.2083
Mean	91	**0.2993**	**0.2222**	**0.2574**	**1.4427**
St. Dev	-	**0.0368**	**0.0190**	-	-

*Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst; See McDermott and McDonald, Ann. Rev. Phytopathol. 31:353-373 (1993). The number of polymorphic loci is: 11, The percentage of polymorphic loci is: 100.00.

The genetic diversity in the seven populations is presented in the total genetic diversity (Ht) with a mean of 0.2993 ± 0.0368. Within - population genetic diversity (Hs) with a mean of 0.2222 ± 0.0190. Gene differentiation (Gst) with a mean of 0.2574 and Gene flow (Nm) with a mean 1.4427 was calculated (Table-6).

Discussion: Molecular characterisation of Tasar ecoraces, *Antheraea mylitta* using ISSR primers: ISSR markers are usually located in non-coding regions and are selectively neutral. Because ISSR primers generate multi locus fingerprinting profile, ISSR analysis has been applied in studies involving genetic identity, parentage, clone and strain identification as well as gene mapping studies[26]. ISSR markers, evolve faster as they are genomic regions with microsatellites that exhibit variable mutation rates and high level of polymorphism[27].

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 14 ISSR primers tested, two ISSR primers *viz.,* UBC 861 and UBC 864 generated distinct and robust bands showing polymorphism. ISSR primers yielded a total of 253 bands, out of which **227** bands, varying in size from 300 to 900 bp are seen in almost all the strains (Figure2, Figure-3).

The ISSR primer UBC861 has generated 6 alleles in Daba BV; 5 in Daba TV; 4 in Modal and Raily; 3 in Sukinda and Bhandara and 2 bands in Andhralocal ecoraces. Whereas, the ISSR primer UBC864 has generated 5 alleles in Bhandara; 4 in Daba TV; 3 in Andhralocal, Sukinda, Modal and Raily; 2 bands in Daba BV ecoraces.

In the present study, screening of 13 individuals of 7 populations using two ISSR primers**,** UBC 861 and UBC864 yielded several reproducible amplicons. The average no. of amplicons produced per DNA sample was 2-6 per primers, with sizes ranging from 300-900 bp. The percentage of polymorphism was (89.7 %). ISSRs exhibit the specificity of microsatellite markers, but need no sequence information for primer synthesis enjoying the advantage of random markers[28].

Phylogenetic analysis of tasar ecoraces as revealed through ISSR markers: ISSR data of ecoraces genetic diversity analysis of *Antheraea mylitta* does not show region – wise clustering. Several individual clusters have been obtained (Figure-5).

Cluster 1: Sukinda- Bhandara formed an individual cluster 1, found to be genetically close.
Cluster 2: Daba-TV- Modal formed an individual cluster 2 found to be genetically close.
Cluster 3: Andhra local formed an individual cluster 3 and found to be closer to cluster 1 (Sukinda and Bhandara populations).
Cluster 4: Daba-BV formed an individual cluster 4 and found to be closer to cluster 3(Andhra local).
Cluster 5: Raily formed an individual cluster 5 found to be closer to cluster 4.

The dendrogram (Figure-5) using ISSR primers, Sukinda and Bhandara, Daba TV and Modal are found to be genetically close, Andhra Local, Daba BV and Raily shown some genetic distance within the population.

The maintenance of genetic polymorphism in natural populations can also reflect the process of adaptation to environmental heterogeneity[29,30]. On the other hand, molecular characterization with ISSR primers helped in bringing out more genetic information, which can be directly correlated with the difference in the distribution[20].

All the 22 microsporidians identified from the tasar silkworm differed in their ISSR profiles indicating genetic variability. ISSR-PCR is one of the simplest and quickest marker systems with high reproducibility, including the virtue of its unique efficiency in distinguishing even closely related organisms, it may be important for proper identification of different species of microsporidian[31]. It is evident that ISSR primers could detect more polymorphism than RAPD, RFLP and isozyme analysis in closely related organisms[14,15,17,32]. The high level of polymorphism realized from this study further proves the efficacy of ISSR technique.

Genetic distance, it can be seen that among the 6 populations (*i.e*., Daba TV, Daba BV, Modal, Sukinda and Raily), Andhra local (mean value=0.1394) shows higher genetic distance from other populations and that it is genetically distant from other ecoraces. Daba BV (0.1339) is closer to AL. It can also be observed that the lowest genetic distance was found in Raily (0.1042). Modal and Sukinda have shown some genetic closeness {(0.1279 and 0.1245) (Table-5)}.

The order of genetic closeness as follows:
Andhra local < Daba BV< Modal < Sukinda < Daba TV < Raily
Populations of Sukinda-Bhandara and Daba-TV - Modal are found to be close within the populations according to 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 Daba TV of Telangana (81.82%), followed by Bhandara and Daba BV (72.73%) of Maharashtra and Telangana; Raily and Modal (63, 64%) of Chhattisgarh and Orissa; sukinda (54.55) of Orissa and Andhra local (45.45) of Telangana (Table-4 and Figure-4).

The present study, 13 individuals of 7 populations using 2 ISSR primers yielded several reproducible amplicons. The average no. of amplicons produced per DNA sample were 2-6 per primers, with sizes ranging from 300-900 bp. The percent polymorphism was 95.93 in UBC861, whi1e it was 76.54% in UBC864 primers (Table-2).

Genetic variability and genetic structure revealed of Tasar Ecoraces through ISSR markers: The Table-4 clearly demonstrate that higher polymorphism and genetic diversity are present in (81.82% and 0.3055 respectively) in Daba- TV populations, while they are lowest in Andhra local ecorace (45.45% and 0.1356 respectively), compared to other populations. The higher polymorphism can be further evident from the average number of observed alleles (1.8182) and effective alleles (1.5336) in the Daba TV and the lowest in Andhra local population in comparison to the other five populations.

A higher polymorphism in Daba TV was also evident in earlier reports, which could be due to the greater chance of inbreeding and random mating[32]. The present study substantiates the view, as among the seven ecoraces it was only Daba TV, which has compatibility to mate with Andhra local ecorace and produce

hybrids. The present investigation of hybridisation based on backcross method between two contrasting genetically variable ecoraces *viz.,* of Andhra local and Daba TV was successful for two successive crops. This is attributed to the fact that both these populations are inhabited in geographically almost same altitude. It could be one of the reasons of their compatibility of mating and production of F1 and F2 generations with quality cocoons. This is also corroborated by the study on genetic differentiation as revealed by ISSR markers which indicated negligible possibility of genetic mixing seems in high altitude[32].

In the present studies, the genetic differentiation (G_{ST} = 0.2574) observed among seven populations indicate that populations are in the threshold of genetic differentiation (Table. 6). Similar observations were made earlier[32]. The study also indicates that a marginal increase in gene flow (N_m= 1.4427) possibly indicates the initiation of genetic drift, which may lead to the formation of variants of the existing ecoraces.

Nm value greater than 1.0 is considered necessary to prevent divergence resulting from genetic drift[33]. The value of gene flow (N_m) < 1.0 (fever than one migrant per generation into a population) or equivalently, a value of gene differentiation (G_{ST}) > 0.25 is generally regarded as the threshold quantities beyond which significant population differentiation occurs[34].

Hence the present study on genetic diversity of seven tasar ecoraces as unraveled by ISSR primers proved effective for understanding the evolutionary processes such as gene flow, natural selection, and genetic drift taking place in a population. As there is more number of tasar ecoraces, it paves the way for further analysis of genetic structure of tasar populations, to decipher the phenotypic variations amongst the ecoraces.

Summary: PCR-ISSR based phylogenetic analysis using popgene 1.32 in the 7 tasar ecotypes revealed that Sukinda & Bhandara, Daba TV & Modal are found to be genetically close, Andhra Local, Daba BV and Raily shown some genetic distance within the population. Andhra local shows higher genetic distance from other populations. It can also be observed that Daba BV is closer to AL and the lowest genetic distance was found in Raily. Modal and Sukinda have shown some genetic closeness. Populations of Sukinda-Bhandar and Daba-TV - Modal are found to be close. The percent polymorphism was 95.93 in UBC861, whi1e it was 76.54% in UBC864 primers. As there are more number of tasar ecoraces, it paves the way for further analysis of genetic structure of tasar populations, to decipher the phenotypic variations amongst the ecoraces.

Conclusion

The present work, based on genetic characterization of 7 tasar ecotypes using ISSR generated polymorphism, emphasized not only on the genetic closeness of Andhra local ecorace in relation to other ecoraces, but it went further probing its compatibility to mate with them.

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