Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268
e-ISSN: 2395-3454

Vol. 17 | No. 4 | pp. 1315-1324 | 2018

Original Research Paper | Open Access

Development of EST-Derived SSR Markers for Tasar Ecoraces and Their Application in Genetic Diversity Analysis

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Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 21-03-2018
Accepted: 11-06-2018

Key Words:
Antheraea mylitta ecoraces
Tasar silkworm
Biodiversity
EST-SSR marker
Single nucleotide polymorphism

ABSTRACT

The wild tropical tasar silkworm, *Antheraea mylitta* polyphagous sericigenous lepidopteran insect, producing tasar silk of commercial importance is distributed in various parts of India as ecoraces, with variations in phenotypic traits like fecundity, voltinism, cocoon weight, silk ratio, etc. In spite of their superior quality silk, they encounter problems like their gradual decrease in number and identification. These populations are very difficult to separate based on morphological traits. The assessment of genetic structure of each population is considered as prerequisite for understanding and preserving natural biodiversity. Hence in the present investigation, genomic DNA of distinct populations of *A. mylitta* was extracted and screened for polymorphism by using EST-derived SSR markers. The DNA profiles based on these markers suggest that they could be effectively utilised for identifying the genetic variability among tasar ecoraces. The alignment of sequences obtained from genomic PCR products has identified potential EST-SSR marker to recognise single nucleotide polymorphism by comparing various tasar ecoraces.

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). There are nearly 34 ecoraces reported (Jolly et al. 1974), of which seven commercial varieties are used in the present study (Sengupta et al. 1993), to identify the genetic similarity. 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). In 2006, Mahendran (2006) has 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.

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) 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). The RFLP molecular markers have been extensively used to study the genetic diversity of population dynamics in many species of plants and animals (Zeitkiewicz et al. 1994, Tsumura et al. 1996, Dayanandhan et al. 1997, Gabierslsen et al. 1998, Wolfe et al. 1998b, Knox et al. 1999).

Expressed sequence tags (ESTs) are the sequences of complementary DNA (cDNA) clones that represent transcribed genes (Hatey et al. 1998). These are short sequences, a few hundred base pairs in length, which are derived by partial, single pass sequencing of the inserts of randomly selected cDNA clones (Adams et al. 1991).

In recent times, microsatellite markers were developed from six populations of *Antheraea assamensis* for analysis of population structure and genetic variation (Arunkumar et al. 2012). The present work is being envisaged to involve larger populations and representatives of *A. mylitta,* 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.

Andhra local ecorace of tasar silkworm, *Antheraea mylitta* Drury, which is an exclusive race of the State of Andhra Pradesh and Telangana, is well known for its superior commercial qualities. It is on the verge of extinction

due to certain weaknesses like poor egg-laying behaviour, voltinism, erratic emergence, non-uniform silk deposition in cocoons and pupal mortality. In the present investigation, an attempt is made to study genetic compatibility of Andhra Local in relation to the other ecoraces. It aims to develop informative molecular markers that could be used to analyse genetic structure and phylogenetic status of the tasar silkworm *A. mylitta*. Hence, EST Wildsilkbase source has been tapped to synthesize the primers and test for the presence of single nucleotide polymorphisms (SNPs).

MATERIALS AND METHODS

Collection of tasar ecoraces: The male and female moths from populations representing seven selected areas of Andhra local ecorace from Warangal district (Telangana) and Daba TV and Daba BV ecoraces from Mahadevpur, Karimnagar districts of Telangana, Bhandara ecorace from Maharasthra (Bhandara), Sukinda from Orissa (Sukindergarh), Raily from Chhattisgarh (Bastar) and Modal from Orissa (Keonjhar) were randomly collected for the present study.

Genomic DNA extraction and purification: Genomic DNA was extracted from randomly selected individual moth from each generation of each line using phenol-chloroform method (Suzuki et al. 1972), which was modified by Nagaraju & Nagaraju (1995). The DNA was dissolved in TE buffer (Tris-EDTA, pH 8.0) and quantified on 0.8% agarose gel or using a Nanodrop, and an uniform concentration of 100 ng/μL was obtained by diluting with the TE buffer (pH 8.0). Gel extraction is the process of extracting the desired DNA fragment intact following the agarose gel electrophoresis by dissolving the gel. Gel purification kits (Qiagen) were used for extraction of desired DNA fragments from the agarose gel by following the manufacturer's instructions. Genetic characterization of seven genotypes with EST primers was done.

Primer designing: Primers were designed for sequences retrieved from Wildsilkbase-EST database. The available cSNP sequences were retrieved from wildsilkbase. The forward and reverse primers were designed from selected contig sequences in the *A. mylitta* using the software Justbio and Oligocalc Bioinformatic online tool by checking the GC content (50-60 above) and length (15-30) of the nucleotide sequence. A total of 24 primers were designed and synthesized at 25 nmole scale (Tables 1-2).

PCR amplification of DNA with EST-SSR primers: PCR was performed to amplify the target genomic region in 20 μL reaction volume. In 20 μL, 1 μL of template (50-100 ng/μL) was mixed with 2.5 μL of 10X PCR buffer, 1 μL of 5 mM dNTP's (individual nucleotide sets from fermentas), 1 μL of 25 mM $MgCl_2$, 0.5 U of polymerase enzyme (Taq, fermentas), 1 μL of 10 picomole forward and reverse primers. PCR reaction was performed in a Thermal cycler (Eppendorf) and the cycling conditions vary for each primer pair. The amplified product was analysed by agarose gel electrophoresis. The PCR schedule was 95°C for 5 min followed by 32 cycles of 95°C for 50s, 50-58°C for 50s, 72°C for 40s and a final extension of 10 min at 72°C. The PCR cycling conditions were standardized for the each fragment to be amplified based on the melting temperature (Tm) of primers. The PCR products were resolved on 1.0% agarose gel. Gel was stained with ethidium bromide (0.5 mg/mL) and photographed with gel documentation system (Fig. 1).

Cloning, sequencing and analysis: The EST markers generated amplified products of the five ecoraces at 473 bp. The amplicons were ligated separately into pTZ57R/T using T/A PCR product Insta clone PCR cloning kit. After transformation of *E. coli* DH5α cells, recombinant colonies were selected and plasmid DNA was extracted by the alkaline lysis method (Sambrook et al. 2001). The presence of the insert was confirmed by restriction digestion of plasmid DNA using *Hind*III and *Eco*RI. The plasmid DNA was purified and sequenced (Fig. 2).

Molecular data analysis- uploading of EST-SSR data: Out of the seven ecoraces taken up, the DNA from Raily and Bhandara ecoraces did not amplify. Hence, the sequence alignment of EST-SSR data of five tasar ecoraces was carried out using software Bioedit and ClustalW. The sequence was analysed by carrying out BLAST against NCBI nucleotide database (BLASTx) to obtain a non-redundant protein sequences (nr).

The phylogenetic tree was constructed for the five different ecoraces of *A. mylitta* by using Mega 4.0 software (Tamura et al. 2007). In the profile of dendrograms for EST-SSR using Mega 4, two phylogenetic trees based on bootstrap values and genetic distance were obtained (Table 3).

RESULTS AND DISCUSSION

Genetic characterization of ecotypes with EST- SSR primers: EST analysis is an effective approach for novel gene identification, homologous gene comparison and transcription profiling (Li et al. 2002). In recent times, EST analysis was used to characterize the cDNA library from Chinese oak silkworm, *Antheraea pernyi* (Yu-Ping et al. 2009). The current molecular systematics depends on PCR amplification of a few universal genes to provide phylogenetic data. The EST sequences can provide rich data for phylogenetic and genomic studies. The ESTs provide a more immediately available source of genomic data for genome sequencing used for phylogenetic studies (Rudd 2003). ESTs have been generated for gene discovery and genome sequencing and

Table 1: List of EST Primers (Forward and Reverse 5'-3' synthesized at 25 n mole scale).

Contig	Primer Name	Forward Primer	Reverse Primer	Comment
Contig205	1	GCT GGC ATC AGA TGC TGC	GGT CTC CGA CGC TAT CGC TGC	473bp fragment was amplified
Contig209	2	GCG TAC ATA GTA GCT GTC GTG	GGT GCC GAA GGA ATT GCG AC	
Contig211	3	GCC ATC TTG GCA ATG GC	GAT GGC TCC CAG GGC ATG	
Contig215	4	CG CAC TGT GGC TCT CGA AG	GGC CAG CCG TAT GGA CTG G	Not worked
Contig221	5	GCA CCA GTG GCC ACA GC	CTA CTG TGG CTT TGG CCG	
Contig222	6	CGG GTA AGA CCA TCA CCC	GCG AGT CTT AAC GGC TAG GGC	
Contig236	7	GGA AGT CGC CTT CTG CGC CAG	GGC GAG AGC TTC CAC CTC	
Contig243	8	GGG TCC GTA GGG ACG CCC	CTG TGT GAG CGG CAG TCG C	
Contig247	9	GCG TAC ATA GTA GCT GTC GTG	CCA TGA ATA CGG ACG TCG CC	
Contig247	10	GCT GTC GTG TCA GCG TTG G	GCC ATG AAT ACG GAC GTC GC	
Contig241	11	GG GCT AGA AGC CGT AGT TTC	CAT GGC GAC CAT GCC TCG C	
Contig244	12	GCC GGT GTA GCC AGC TC	GCG ATC GCC GGG CTT CTC	

Note: Primers synthesized by Integrated DNA technologies, in collaboration with Department of Biotechnology, University of Hyderabad, Hyd., 2011.

Table 2: Oligonucleotide specifications of the primer.

Primer code	1F -1R Amfb0566 Amfb0843 Amfb1203 Amfb0737
Forward sequence	5'GCTGGCATCAGATGCTGC3'
Reverse sequence	5'GGTCTCCGACGCTATCGCTGC3'
Fragment size (range in bp)	473
Annealing temp°C	55°C
GC%	1F-61.1% 1R- 66.6%
DNA bases (25 nmoles)	18 (Forward) 21 (Reverse)

Phylogenetic analysis using EST-SSR primers

Table 3: Pair-wise genetic distance of the tasar ecoraces, *Antheraea mylitta*, based on EST-SSR phylogeny.

	1	2	3	4	5
1. DabaTV R	-	-	-	-	-
2. Daba BV F	7.122	-	-	-	-
3. Modal F	7.604	6.995	-	-	-
4. Sukinda R	9.413	6.658	37.381	-	-
5. Andhra local R	10.812	42.330	12.252	45.893	-

Table 4: Types of purine and pyrimidine exchanges occurred in the sequences of tasar ecoraces.

Mutation type	Number	Number of Exchanges occurred				
Transitions	19	purine->purine	G to A 9	A to G 10	-	-
Transitions	8	pyrimidine->pyrimidine	T to C 3	C to T 5	-	-
Transversions	7	purine->pyrimidine	A to T 1	A to C 2	G to C 4	G to T nil
Transversions	8	pyrimidine->purine	T to A 1	T to G 1	C to A 1	C to G 5

are available in public databases.

The sequences using forward and reverse primer (Amfb0566 Amfb0843 Amfb1203 Amfb0737) were obtained for five ecoraces (Andhra local, Daba TV, Daba BV, Modal and Sukinda). From the electropherogram, only those sequences, which have shown nucleotide variations have been selected (Fig. 3). From this data, a precise observation of alignment of nucleotide sequences with reference primer

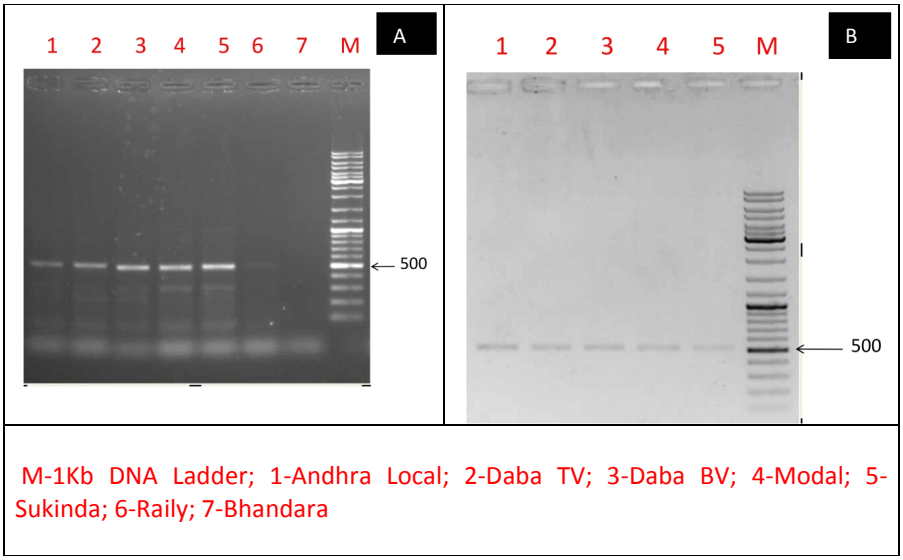


Fig. 1: A. EST-PCR profile with Amfb0566 Amfb0843 Amfb1203 Amfb0737 primer; B. Gel elution profile with Amfb0566 Amfb0843 Amfb1203 Amfb0737 primer.
{PCR amplified 500bp fragment with IST set of EST primer. 6 and 7 samples are not amplified. (our fragment size is 473bp)}.

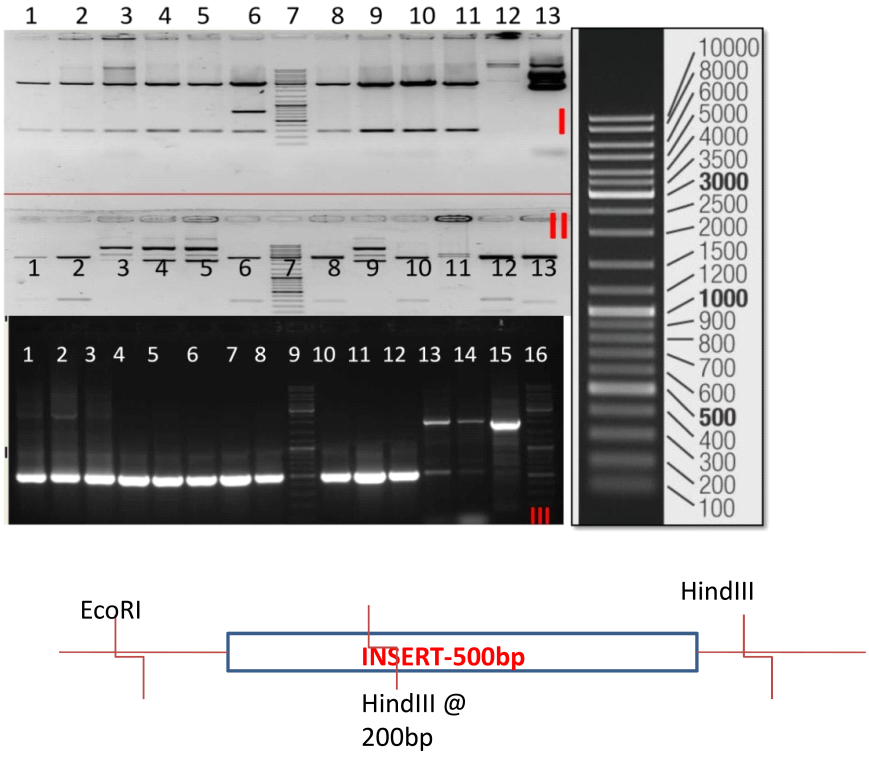


Fig. 2: Double digestion of different ecoraces clones with Amfb0566 Amfb0843 Amfb1203 Amfb Primer.
I- Double digestion: 1-6: 6 different colonies of Andhra local; 7: ladder; 8-13: 6 different colonies of Daba TV.
II- Double digestion: 1-6: 6 different colonies of Daba BV; 7: ladder; 8-12:4 different colonies of Modal; 13-15: 4 different colonies of Sukinda.
III-PCR confirmation: 1-2: Andhra local clones; 3-5: Daba TV clones; 6-8:Daba BV clones; 10-12: Modal clones; 13-15: Sukinda clones.

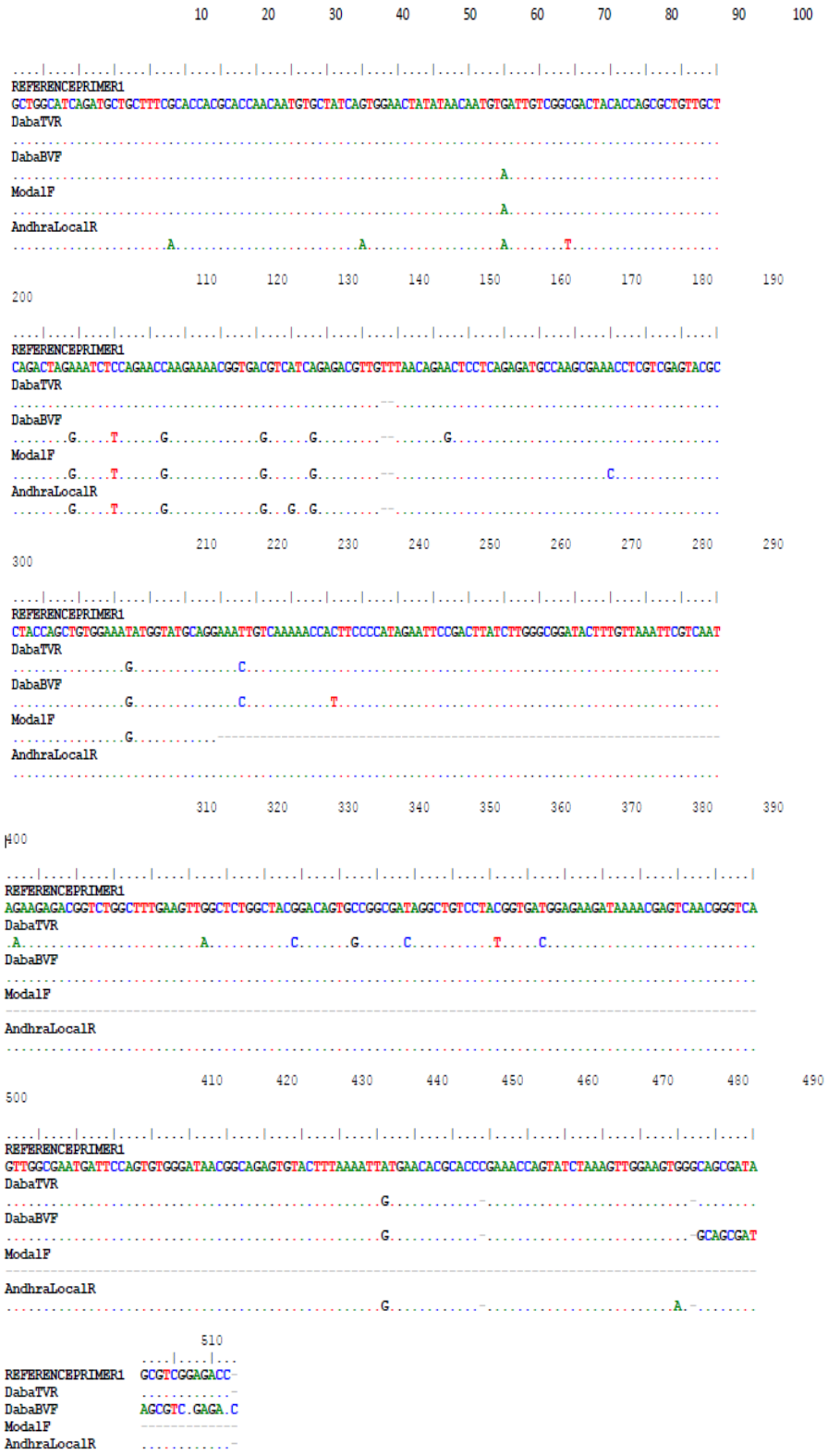
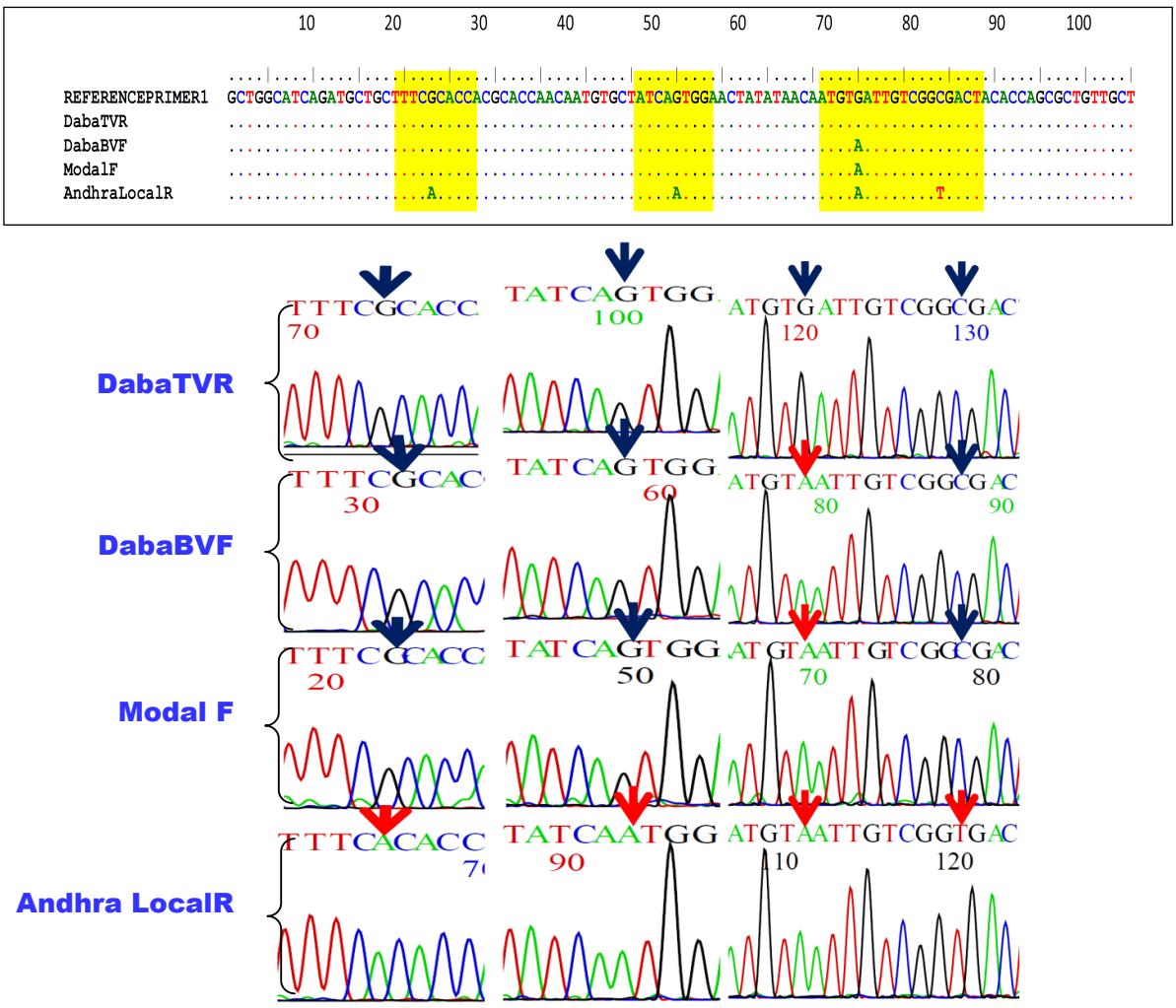


Fig.3: DNA sequences of five tasar ecoraces obtained from sequencing of genomic PCR products.



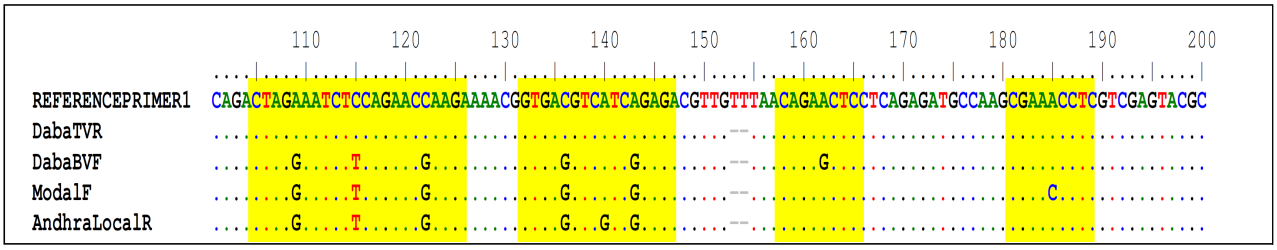
Note: Correlate results with reference primer

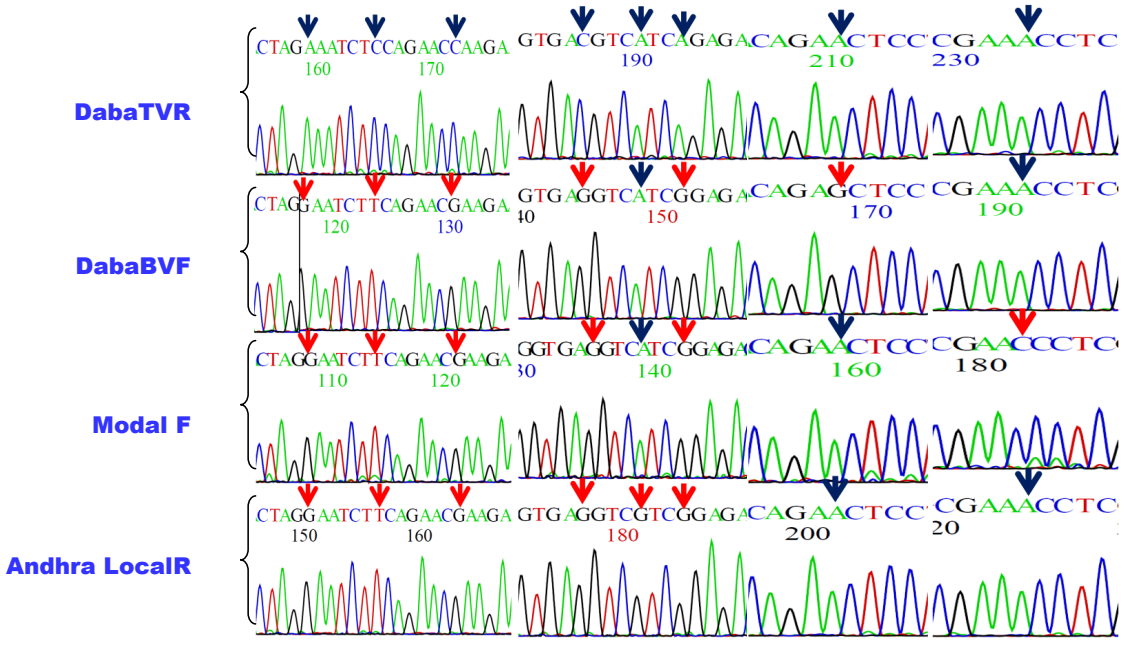
Fig. 4a: SNP discovery by alignment of sequence traces obtained from direct sequencing of genomic PCR products.

(primer 1) has revealed that at 60 regions, there were polymorphism at nucleotide level (Fig. 4a-b). This is the most valuable observation excerpted from the present investigation. Out of the sixty, the nucleotide replacements were 12 (Andhra local), 10 (Daba TV), 30 (Daba BV) and 8 (Modal), while in Sukinda, sequences have shown much variability, which can be observed from the phylogenetic tree based on genetic distance (Fig. 5). It can be inferred from the present studies that Daba BV has shown replacements in maximum number of nucleotides owing to its bivoltine nature and the rest of the ecoraces being trivoltine. In Andhra local and Daba TV there were at least five and six specific nucleotides respectively which were not seen in the remaining three ecoraces, which suggests of their uniqueness. From the whole sequence data, various transitions and transversions were noted (Table 4).

In general, if mutations take place at random, the transitions over transversions ratio should be 0.5. However, the ratio traced from the above is equal to 1.8, which indicates a clear bias towards transitions. It can be inferred that each type of transitional change is produced 1.8 times as often as each type of transversional change. A comparative study of transitions and transversions in rodents and human beings was reported earlier (Collins et al. 1994) and results obtained from a study of human SNPs from EST sequence trace databases gave a transition to transversion ratio of 1.7 (Picoult et al. 1999).

It is observed from the above data that guanine has mostly found to replace adenine and cytosine in purine -> purine transitions and pyrimidine -> purine transversions. The sequences of Daba BV and Sukinda were rich in AT and





Note: Correlate results with reference primer

Fig. 4b: Heading for graphs: SNP discovery by alignment of sequence traces obtained from direct sequencing of genomic PCR products.

GC repeats (Table 5). It is also seen that Daba BV has the highest TT and AA repeats in their sequences. This may be due to the distinctness of being bivoltine and geographical adaptation of the ecoraces. A similar observation was made in recent times (Pradeep et al. 2005) on the involvement of A/T rich regions in larval duration under tropical conditions.

In the present investigation, the SNP discovery by alignment of sequences obtained from direct sequencing of genomic PCR products by comparing sequences retrieved from Wildsilkbase has identified potential EST marker to recognize SNP by comparing various tasar ecoraces, which was hitherto not reported so far.

Phylogenetic analysis of EST-SSR markers: The field of molecular systematics, which depends on PCR amplification to provide phylogenetic data, is slowly but surely maturing, as researchers are now making rapid arrangements of phylogeny on the basis of small and sparsely sampled data sets (Murphy et al. 2001, Nardi et al. 2003). One of the goals of phylogenomics is to generate data sets that result in topologies that are robust to the addition of new data and stable to changes in assumptions of analyses. While the growing number of genome sequences may eventually be used for phylogenetic inferences across a broad sample of taxa, ESTs provide a more immediately available source of genomic data (Rudd 2003). Hence universal primers were designed, which can be used with standard genomic extracts from dried specimens that facilitate lepidopteran phylogenomics (Niklas et al. 2008). In *B. mori*, an EST database was constructed from genes of tissues and developmental stages, with a view to analyse the complete analysis of *Bombyx* genome (Mita et al. 2004).

The sequences of ecoraces so obtained were subjected to further analysis. The sequence alignment was carried out using Clustal W in Bioedit available at MEGA 4.0 software (Tamura et al. 2007). After alignment we con-

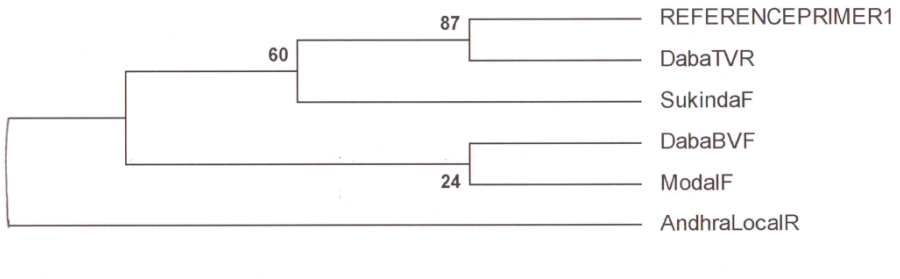


Fig. 5: Phylogenetic tree generated by EST-SSRs, based on UPGMA.

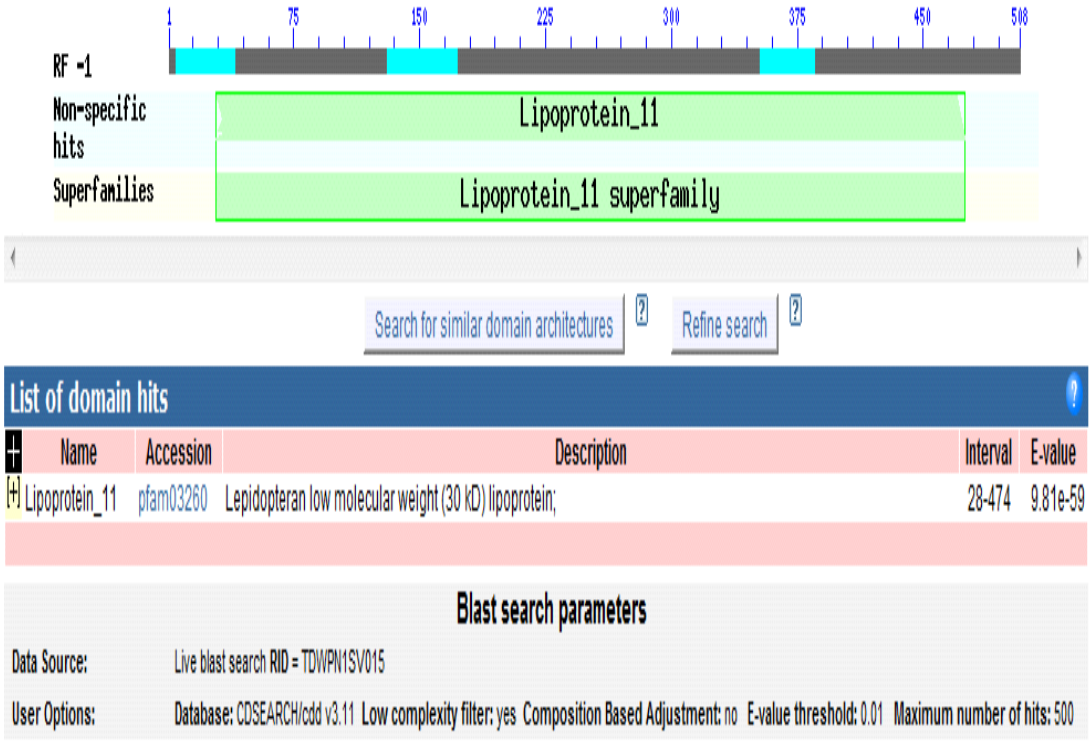


Fig. 6: Lipoprotein-11 superfamily, as revealed by BLAST analysis of the DNA sequences of the five tasar ecoraces.

structed a phylogenetic tree using genetic distance and bootstrap values. The UPGMA (unweighted pair group method analysis) dendrogram based on bootstrap values revealed that, Andhra Local remained as a separate isolate, Daba TV clustered with Sukinda with intra population variability, while, Daba BV clustered with Modal. Daba TV-reference primer clade was supported by bootstrap value of 87%, while its cluster with Sukinda and Daba BV-Modal were supported by 60% and 24% respectively (Fig. 5), revealing that Daba BV and Sukinda of probable origin and Andhra Local as a unique species.

Phylogenetic analysis of tasar ecoraces indicated interrelations amongst tasar ecoraces. The genetic proximity of ecoraces of *Antheraea mylitta*, helps to bring about an idea of breeding of Andhra Local ecorace with other ecoraces, without losing its beneficial commercial characters and suggest methods to overcome its weaknesses.

BLAST analysis of the DNA sequences of tasar ecoraces: The BLAST analysis of the DNA sequences of tasar ecoraces (Andhra Local, Daba TV, Daba BV, Modal and Sukinda (Fig. 6) has shown that the gene belonged to 30 kDa family of lipoproteins which is found in larval, pupal and egg stages of silkworm. These proteins are classified as Lipoprotein_11 superfamily (Accession no: pfam 03260, Fig. 6).

Table 5: The frequency of AT and GC repeats in the forward and reverse sequences of tasar ecoraces.

Sl. No.	Ecorace	AT	GC
1.	Daba TV F	24	24
2.	Daba TV R	34	36
3.	Daba BV F	50	66
4.	Daba BV R	55	52
5.	Modal F	13	14
6.	Modal R	14	16
7.	Andhra local F	38	34
8.	Andhra local R	35	37
9.	Sukinda F	48	67
10.	Sukinda R	48	51
	Total	**359**	**397**

The members of 30-kDa lipoprotein family have also been found in a number of other species of the Lepidoptera order, e.g. *Samia cynthia' ricini*, *Antheraea assama* or *Antheraea mylitta* (Zhang et al. 2012). An analysis of expressed sequence tags (ESTs) derived from several silkworm tissues indicated that the level of expression of the 30-kDa proteins varies. The more ESTs of a particular protein are present in the genome, the higher the expression level of that protein (Sun et al. 2007).

The 30 kDa proteins are multifunctional and permanently required by insects; they therefore exist at various sites throughout the life cycle of the silkworm. During larval development, they are expressed in fat body tissues and haemolymph (Vanishree et al. 2005). It is found that 30-kDa lipoprotein family are the most abundant protein fraction of silkworm fifth instar, which are synthesized in the larval fat body and later secreted into haemolymph (Gamo 1978). From the haemolymph, they are translocated into pupal fat body and utilized during adult development as storage proteins (Kim et al. 2001). They are transported to the yolk granules in adult female moths and act as yolk proteins of the developing silkworm eggs (Chen et al. 1990, Maki et al. 2001). This mechanism which is in corroboration with properties of 30 kDa lipoproteins of *B. mori* (Pietrzyk et al. 2013) can be confirmed by the present studies, as the source of DNA sequences obtained is the genomic DNA of the adult.

ACKNOWLEDGEMENTS

GS is grateful to DBT, New Delhi, for providing funds to carry out the research work (Grant no: BT/PR13300/PBD/19/206/2009).

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