NGS STUDIES AND QUANTITATIVE ASSESSMENT OF COCOON TRAITS IN   F1 AND F2 HYBRID POPULATIONS OF TASAR SILKWORM, *A. MYLITTA*

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ABSTRACT

The tasar silkworm, *Antheraea mylitta* Drury, lepidopteran insect, producing tasar silk of commercial importance is distributed in various parts of India as ecoraces, with variations in phenotypic and quantitative 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. Molecular markers have been widely used in studies of the genetics of wild populations to understand genotypic diversity within and between populations and evolutionary processes. Hence in the present investigation, Next-generation sequencing (NGS) technologies and *Insilco* analysis provide reliable large-scale DNA sequencing. They are used extensively for *de novo* sequencing for quantifying expression levels through DNA sequencing and in population genetic studies.

Keywords: *Antheraea mylitta* Drury, Tasar silkworm, quantitative traits, genotypic diversity, Next-Generation Sequencing

INTRODUCTION

The tasar silkworm *A. mylitta* D is commercially exploited in India for production of vanya silk and a large number of poor families depend on tasar culture for their livelihood. It has a wide range of distribution in varied geo-climatic condition [1, 7, 12, 13, 14]. This forest based activity is providing employment and economic support to several tribal families in the pre- cocoon sector, in addition to several others of the country in the post- cocoon activities.

Andhra local ecorace of *A. mylitta* D possesses superior **commercial cocoon traits**, but its commercialization could not be under taken due to weak voltinism, asynchronised moth emergence (35-40%), poor fecundity (165-205), less amenable to human handling and heavy crop loss during late age silkworm rearing stages resulting in low productivity (10-12 cocoons/dfl). However, this genetic resource material is bestowed with superior commercial characters like low denier (7%), high reelability (66%) and higher silk ratio (16.8%).

Daba is an adapted ecorace of *A. mylitta* D. with commercial exploitation under tasar seed sector. This ecorace shows sustenance in cultivating breeding pocket with moderate fecundity (200-250), S.R. %

(13.50-14.50%) and good survival (50-60 cocoons/dfl). Hence, the breeding programme has been formulated involving two ecoraces *viz*., Daba TV and Andhra local ecoraces. This work aims at the improvement of Andhra local ecorace with bestowed with superior characters *i.e.,* survivability and fecundity of Daba TV.

Genetic variants in the natural population and diverse gene in the individual of the population are the prime need for evolving improved breeds and cross breeds with better tolerance and quantitative traits. Since, Tasar silkworm *A. mylitta* is under commercial exploitation in the country, improved breeds/races are need of the hour [5].

Back-crossing is a well known and long established breeding plan where a character is introgressed from a domesticated or wild relative donor parent into the genomic background of a recurrent parent, which progress better with selection of genetically diverged parental breeds. The backcross breeding of silkworm using parents with preferred traits and selection in subsequent generations offer superior varieties [2].

Earlier studies on the **molecular characterization** of genome extracted from

silk glands of Daba TV and Andhra local ecorace of *A. mylitta* D has revealed individual and strain specificity of the ecoraces with some genetic proximity between the two ecoraces. The fact that at the molecular level there is little or no difference between Daba TV and Andhra local ecoraces, it is a significant observation showing compatibility between the ecoraces which may, in future lead to evolving a totally new strain of *A. mylitta* [9].

In general, the aim of silkworm **breeding** is not only to enhancement of fecundity, continuance of quality cocoon production in variable climatic and geographic conditions leading to genetic improvement of traits either incorporating desirable genes and subsequently profitability of sericulture industry. In an attempt to overcome the problems faced by Andhra local ecorace, a comprehensive breeding program was suggested by earlier works [8, 10]. The tasar silkworm, *Antheraea mylitta* D has wide genetic as well as phenotypic variability in its population; also a good material for the exploitation of heterosis, besides it has many constraints in silkworm hybridizations [11].

The ecorace conservation is essential to utilize their valuable genes in enhancing productivity and to build variation in new population through hybridization. Modern sequencing methods like Next-Generation Sequencing (NGS) technologies and Insilco analysis are used in population genetic studies to investigate the evolutionary forces affecting genetic variation.

As high-throughput DNA sequencing technologies are generating vast amounts of data, and fast, flexible and memory-efficient implementations are needed to facilitate analyses of thousands of samples simultaneously, the **Next-Generation Sequencing (NGS)** can now be considered as an established analysis technology for research applications across the Life Sciences, however, the analysis workflow still requires substantial bioinformatics expertise. NGS has emerged as a standard technology for molecular profiling assays *viz*., transcriptome sequencing (RNA-Seq), whole-genome and whole-exome sequencing (WGS/WXS), genome-wide association studies (GWAS), *etc*. [4]. The study has been designed to develop genotype mapping of parental tasar populations having contrasting cocoon characteristics as well as hybrid populations.

MATERIAL AND METHODS

The present study comprises of comparative evaluation of certain cocoon and post cocoon

characters of parental and hybrid populations of tasar silkworm, *A. mylitta.* D., based on their economic viability.

Cocoon and Post-cocoon Parameters of Tasar silkworm, *A. mylitta* (Andhra local and Daba-TV)

Hybrid Population

The weights of the cocoon, pupa and shell of Tasar silkworm, *A. mylitta,* Daba-TV, Andhra local Ecoraces, were measured using standard Electronic Balance of Citizen-make in terms of grams. The length of cocoon and peduncle; width of the cocoon were measured by using *Vernier Calipers* and the Shell Thickness was measured by using Screw Gauge. The post cocoon parameters *viz.*, Shell Ratio was measured according to standard procedures and recorded.

Shell Ratio (%)

The shell ratio is calculated by the following formula

$$\text{Shell ratio (\%)} = \frac{\text{Shell Weight}}{\text{Cocoon Weight}} \times 100$$

Reelability (%)

The reelability of cocoons for economic reeling is the ease with which the cocoons yield the bave in reeling, which is called the reelability of cocoons. This is calculated by the following formula:

$$\text{Reelability (\%)} = \frac{\text{Weight of the silk reeled}}{\text{Weight of the cocoons}} \times 100$$

Denier

The difference in thickness of the size of the bave from beginning to the end is so gradual and minute in tasar cocoon that it does not interfere with the quality of the size of ultimate raw silk reeled. Denier is obtained by the following formula:

$$\text{Denier} = \frac{\text{Weight of the silk reeled}}{\text{Length of the silk reeled}} \times 9000$$

Genomic DNA isolation

The Genomic DNA isolation and the quantification of the 1-8 samples of Tasar Silkworm, *A. mylitta* D Parental ecoraces (Andhra local, Daba TV, F_1 and F_2) of each line by using HiPurATM Insect DNA Purification Kit. DNA was dissolved in TE buffer (Tris-EDTA, pH 8.0), revealed an ideal concentration of DNA ng / µl of the genomic sample *i.e.,* between 1.8 – 1.9 in 260 / 280 ratio) checked against 1 kb standard DNA ladder was obtained. It been observed that DNA has been isolated without any protein or any other contamination and was used for further studies in PCR and NGS analysis. Genetic characterization of 4 populations *viz.,* parental ecoraces (Andhra local, Daba TV), F_1 and F_2 were done [15 modified by 3].

Database & Tools used for analysis of sequence

The genomic DNA of *A. mylitta* was sequenced independently using the Illumina

NextSeq500; 75 paired-end and data analysis were done by FastQC[1], bowtie[2], and Tassel-3[3] respectively. Total no of sample sequences are 8 and PstI-HF – MluC1 enzymes were used. Later, the sequence was Converted using tag Count, This plugin derives a tag Count list for each sample. It keeps only good reads having a barcode and a cut site. Trims off the barcodes and truncates sequences that have a second cut site, or read into the common adapter.

In the current studies, Tassel3 UNEAK pipeline, *a denovo* GBS analysis approach was opted due to the lack of reference genome. The entire protocol **(Figure 1)** is explained step by step in the following sections.

Cocooning and pupal duration in F1 hybrids was 7-8 days time while it has prolonged in F2 hybrids to 8-10 days. Cocoons were harvested after full pupation. The pupal formation in F1 Hybrids was 12-14 days while it prolonged in F2 Hybrids to 14-17 days, which was higher when compared to that of parental ecoraces {(6-7 days), **(Table 1)**}.
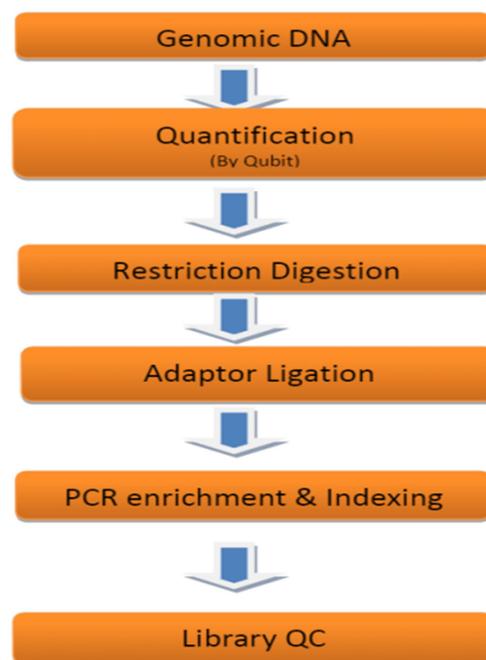

Figure 1: Experimental Work flow for Genotyping by Sequencing Library Preparation

Table 1: Cocoons harvested and Pupal duration of F1 and F2 hybrids of Tasar silkworm, *A.mylitta*

Cocoon Formation in days		Pupal Formation in days	
F1	F2	F1	F2
7-8	8-10	12-14	14-17

Table 2: Cocoon Characteristics of F1 and F2 Hybrids of Tasar Silkworm, *A.mylitta*.

S. No.	Silkworm Type	Cocoon Weight (grams)	Pupal Weight (grams)	Shell Weight (grams)	Shell ratio (%)	Weight of the filament (grams)	Length of the filament in (meters)	Reelability (%)	Denier
1	AL	6.43±2.16	5.30±2.06	0.99±0.30	15.48%	0.59	580	9.17	9.1
2	DTV	7.17±0.43	5.60±0.52	1.47±0.19	20.57%	0.88	872	12.27	9.0
3	F1 (AL ♂ + DTV ♀)	8.75±1.36	6.90±1.29	1.14±0.22	13.05%	0.68	674	7.77	9.08
4	F2 (F1♂ + AL ♀)	9.19±0.78	7.98±0.74	1.21±0.20	13.16%	0.72	714	7.83	9.07

The average cocoon weight, Pupal weight, Shell weight (gm), of Tasar silkworm *A.mylitta* (Andhra local Parental, Daba-TV Parental, F1and F2 Hybrid) standard deviation of Cocoon weight were 6.43±2.16 (S.D), 7.17±0.43 (S.D), 8.75±1.36 (S.D), and 9.19±0.78 (S.D)) and Pupal weight 5.30±2.06 (S.D), 5.60±0.52 (S.D), 6.90±1.29 (S.D), and 7.98±0.74 (S.D), while Shell weight (gm), 0.99±0.30 (S.D), 1.47±0.19 (S.D), 1.14±0.22 (S.D), and 1.21±0.20 (S.D). The average Shell ratio (%), Reelability (%), Denier, weight of the filament (gms) and Filament length (cm) of Tasar silkworm *A. mylitta* (Andhra local Parental, Daba-TV Parental, F1 and F2 Hybrid) were 15.48, 20.57, 13.05, and 13.16 respectively and reelability (%) of (Andhra local Parental, Daba-TV Parental, F1and F2 Hybrid) 9.17, 12.27, 7.77, 7.83 while denier 9.1, 9.0, 9.08, 9.07 and weight of the filament(gms) 0.59, 0.88, 0.68, 0.72 Filament length (m) 580, 872, 674 and 714 respectively **(Table 2)**.

 ➢ The cocoon and pupal weights were greater in F1 when compared to F2 populations, while it was more than the parental Andhra Local.
 ➢ The shell weight has decreased gradually from F1 to F2 and also when compared to parental Daba TV.

 ➢ Shell ratio was almost the same in F1 and F2
 ➢ Denier of F1 and F2 is similar to that of Daba TV

In F1 generation it was observed that out of 185 (1 dfls) taken, 72 eggs have been hatched, which is an important aspect of the present studies. The significant observation of the present studies is that the **cocoon and pupal weights in F2 hybrid are higher** than that of parental ecorace, Andhra local, which may be due to increased larval period; though it is lower than that in Daba TV, while in F2 it was found to be lower than the parental stock. The *cocoon yield* of F1 and F2 hybrids has been increased when compared to parental Andhra local ecorace. In the two backcross generations, *i.e.,* F1 and F2 denier is almost similar to that of Daba TV parent. In the next season the F1 male and Andhra local female were allowed to mate to obtain the F2 generation. The fecundity obtained in the two crop seasons in F1 and F2 were 185 and 160 respectively, fecundity of F1 and F2 hybrids was found to be more than the parental stock (AL-150 and Daba TV-250). The present study yielded F1 and F2 hybrid cocoons with pupal weight, shell ratio, cocoon width, reelability and shell thickness, which have shown considerable increase when compared to either or both of the

parents. The post -cocoon parameters like cocoon weight Pupal weight increased when compared to the parental types. Shell weight, cocoon length, denier and filament length have decreased when compared to the parental types.

In the two backcross generations, *ie.,* F1 and F2 denier is similar to that of Daba TV parent (though less denier of AL parent is preferable). Based on the recent study, that the commercially important finer denier of tasar silk filament can be attained with minor reduction in silk yield of wild Jata ecorace by adopting repeated backcrossing for four generations with semi domesticated Daba ecoraces [Manohar, 2009], the present breeding method can be further standardised and prolonged to attain superior phenotype quality.

The present investigation, on breeding as one of the strategies for enhancing the productivity and quality of tasar raw silk, is aimed at improvement of Andhra local ecorace in order to bestow it with superior characters like survivability and fecundity of Daba TV in lines with preliminary studies [6], further improved and standardized with backcross method, resulted in yielding better quality cocoons and cocoon yield in the commercial crop seasons.

Tasar culture is a forest based industry best suited to the economy and social structure of developing countries like India. Minimum investment is the most significant feature of Tasar culture. State government should take most priority for raising and maintaining systematic Tasar host plantations, the state government of Telangana as already taken initiatives in this direction as Tasar Food Plants *viz.*, *Terminalia arjuna* have been planted in its scheme of "Harithaharam" (Green Garlands).

In the present studies, the genomic DNA of parental ecoraces - Andhra local and Daba TV of *A. mylitta* and their hybrid populations were sequenced independently using the Illumina NextSeq500 in order to analyze their genetic relationship. The sequencing library revealed that the fragment size ranged between 200bp to 700bp and identified 35877 sites in 8 samples. Further, the phylogenetic tree showed closely and distantly related taxa among the populations.

The sequencing of 8 samples in the present investigation has identified their populations with related to taxons and identified the SNPs at the same location in all the sequences. The cluster analysis of the eight samples has shown that they were of same length and clustered together at i.e., the range is 4 - 9. All such sequences have also

shown unique GC rich regions. *GC content* (guanine-cytosine *content*) is found to be high in **S1** (Andhralocal (male) –F1 Parent), **S5** (Andhralocal (Female) – F2 Parent), and S6 (F1 (male) - – F2 Parent) compared to S2(Daba TV (Female) – F1 Parent), S3 (F1 Population1), S4(F1 Population 2) and S7(F2 Population 1). However, a species with an extremely *low GC-content* is S8.

CONCLUSION

The conservation of Tasar silkworm, *A. mylitta* D, Andhra local ecorace needs further improvement of breeding practices, to enable the viability of F_1 and F_2 generations. The present investigation on PCR based Phylogenetic analysis using Mega Software in 4 tasar populations *viz.,* parental ecoraces [(Andhra local, Daba TV), F_1 and F_2], derived from hybridization between two contrasting genetically variable ecoraces *viz.,* of Andhra local and Daba TV was successful for two successive commercial crops from commercial viewpoint. The purpose of this paper is to stimulate further discussions regarding great challenges we are facing in the quantitative trait studies of high dimensional genomic data produced by next-generation sequencing.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable

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