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Niranjan Kumar
Silkworm Breeding and Genetics, Central Tasar Research and Training Institute, Ranchi, Jharkhand, India

Immanual Gilwax Prabhu
Silkworm Breeding and Genetics, Central Tasar Research and Training Institute, Ranchi, Jharkhand, India

Manjappa
Silkworm Breeding and Genetics, Central Tasar Research and Training Institute, Ranchi, Jharkhand, India

Ajit Kumar Sinha
Silkworm Breeding and Genetics, Central Tasar Research and Training Institute, Ranchi, Jharkhand, India

Alok Sahay
Silkworm Breeding and Genetics, Central Tasar Research and Training Institute, Ranchi, Jharkhand, India

Correspondence
Niranjan Kumar
Silkworm Breeding and Genetics, Central Tasar Research and Training Institute, Ranchi, Jharkhand, India

Development of RAPD-SCAR markers for the identification of *Antheraea mylitta* with high shell weight

Niranjan Kumar, Immanual Gilwax Prabhu, Manjappa, Ajit Kumar Sinha and Alok Sahay

Abstract
Antheraea mylitta is a tropical tasar-silk producing insect distributed across India. Even though, economically important traits of tasar silkworm are highly influenced by ecosystem, its genotype also plays a vital role in the expression of high yield traits. Development of DNA based molecular marker for the selection of highly productive lines are imperative to improve the production of tasar silk. The present study was intended to develop Sequence-Characterized Amplified Region (SCAR) markers from the improved Random Amplified Polymorphic DNA (RAPD) fragments for the selection of high shell weight line. High shell weight (> 2g) and low shell weight (< 1g) lines were selected. Genomic DNA from both the groups were analysed for polymorphism using 40 decamer RAPD primers. Primers OPW9, OPA11 and OPJ18 generated the polymorphic fragment at the size of 630, 620 and 220 bp respectively to distinguish high and low shell weight groups. The polymorphic bands were cloned, sequenced and converted into stable multiplex SCAR markers HSWP1, HSWP2 and HSWP3. Among three, HSWP1 and HSWP2 were highly specific to high shell weight line. This study developed stable multiplex SCAR markers for the precise selection of high shell weight bearing tropical tasar silkworm to enhance the productivity of tasar silk.

Keywords: *Antheraea mylitta*, sequence-characterized amplified region, random amplified polymorphic DNA, multiplex, polymorphism

1. Introduction
Indian tasar silkmoth, *Antheraea mylitta* is an economically important wild species distributed in different geographical locations and habitats in the country. Because of the various ecological conditions prevailing in different regions, several morphovariants called ecoraces, have been identified in *A. mylitta* [1]. Totally forty four ecoraces are reported in this species, which feed primarily on *Terminalia tomentosa*, *T. arjuna* and *Shorea robusta* and also on a number of secondary food crops [1]. Tasar cocoons are reported to be the largest among all the silk-producing insects in the world [2]. The ecoraces are uni, bi or trivoltine depending upon the geo-ecological conditions and differ from each other in several qualitative and quantitative traits [3], such as cocoon weight and colour, larval colour, and so on. Silk fibre produced by *A. mylitta* has its own distinctive colour and is thicker than *Bombyx mori* silk. However, the tasar silk has higher tensile strength, elongation, and stress-relaxation values than the silk secreted by the domesticated silkworm *B. mori* [4-5]. Since this silkworm is heterogeneous, with a high level of heterozygosity [6], the development of stable heterotic breeds or lines are difficult. On the other hand, there is a need for the improvement of tasar silk production and provide employment for poor farmers requires the use of highly productive breeds of this silkworm. To select the homogeneous population with high yield trait, the identification of molecular markers associated with high shell weight is imperative.
Molecular markers are categorized into non-PCR-based and PCR-based. Non-PCR-based markers are informative but practical involvement of radioactive isotopes, high cost, absolute requirement of sequence information and sophisticated laboratory set up limits their usage [7-8]. PCR-based markers have been widely used because of their technical simplicity, ease of screening a large number of samples in a short period of time and can be carried out in a moderately equipped laboratory. Specific, semi-specific and arbitrary primers can be used in various combinations to target DNA motifs/genes of interest [7]. In recent years PCR based molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR) and other DNA markers are the most preferred marker systems as

they provide more detailed genetic information due to either the increased variability of loci or the greater numbers of available loci [6, 9-13].

Sequence-characterized amplified region (SCAR) markers are stable molecular markers derived from RAPD. The basic principle is to convert the dominant markers into co-dominant markers to reduce the tediousness of RAPD by molecular cloning [14-16]. RAPD and SCAR markers have been used to identify ecoraces and estimate the genetic variability in tasar silkworms [11-12]. The SCAR marker has been developed for the identification of right parental stock of tasar silkworms with high yield trait in breeding programs designed to enhance the productivity of tasar silk [17]. In the present study, DNA fragments were amplified with the DNA template of high and low shell weight tasar silkworm, using an improved RAPD followed by DNA ligation, cloning, and sequencing. Then the multiplex SCAR markers were developed for the specific selection of high shell weight lines of *A. mylitta*.

2. Materials and methods

2.1 Genomic DNA extraction from *A. mylitta*

Genomic DNA was extracted from the individual pupal fat body of *A. mylitta* selection line with high shell weight (> 2g) and low shell weight (< 1g) using the previously described method with few modification [18]. Fat bodies were ground in liquid nitrogen using a mortar and pestle. Extraction buffer (100mM Tris-HC1, pH 8.0, 50 mM NaCl, 50 mM EDTA and 1% SDS) and proteinase K (100 µg/mL) was added to the ground tissue and incubated at 37 °C for 2 h with occasional swirling. The DNA was extracted twice with phenol-chloroform-isoamylalcohol (24:24:1) and once with chloroform. The supernatant DNA was ethanol-precipitated, resuspended in TE (10 mM Tris-HCI, 1 mM EDTA, pH 8.0) buffer and incubated at 37 °C for 1 h after addition of RNase A (100 µg/mL). DNA was reextracted with phenol-chloroform and ethanol precipitated as described earlier. Extracted genomic DNA was quantified on 0.8% agarose gel electrophoresis and diluted to a uniform concentration of 10 ng/µL for the RAPD.

2.2 Amplification of DNA by RAPD

A set of 40 decamer RAPD primers were synthesized from Eurofins MWG Operon, Bangalore, India (sets OPA, OPAJ, OPBC, OPC, OPJ, OPK and OPW). The PCR was performed in a 25 µl reaction mixture containing 2.5 µl buffer (10 X DreamTaq DNA polymerase buffer containing 15 mM MgCl2, Fermentas); 2.5 µM dNTPs (from 10-mM stock, Fermentas), 20 pM primer (random decamer primer), 1 unit of DreamTaq DNA polymerase (Fermentas) and 30 ng of genomic DNA. Amplification was performed in a thermal cycler (Techne, UK) using the following program: 1 cycle of 94 °C for 4 min, 40 cycles of 94 °C for 1 min, 37 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 10 min. PCR products were resolved on 1.5% agarose gel, employing 1 kb ladder as a molecular weight standard, stained with ethidium bromide (0.5 µg ml^{-1}) and run in 1X TBE (100 mM Tris-HCl, pH 8.0, 83 mM Boric acid, 1 mM EDTA, pH 8.0) at 80 voltage. Gels were visualized with UV transilluminator (UV teck, Bangalore Genei) and photographed.

2.3 Cloning, selection and sequencing of DNA fragments

Three various RAPD bands specific to high shell weight lines were excised from the agarose gel and purified using GeneJET purification column. Gel eluted polymorphic DNA bands were cloned in blunt end cloning vector pJET1.2 for sequencing. The PCR amplified gel eluted product with 3'-dA overhangs was blunted with DNA blunting enzyme. Blunting reaction mixture consists of 10 µl of 2X reaction buffer, 1 µl of eluted DNA fragment, 6 µl of nuclease free water and 1 µl of DNA blunting enzyme were mixed together and incubated at 70ºC for 5 min. Following incubation, 1 µl of pJET1.2/blunt Cloning Vector and 1 µl of T4 DNA ligase were added with the above suspension. Ligation mixture was incubated at room temperature (22ºC) for 5 min. After incubation, ligation mixture was transformed into *Escherichia coli* DH5α through calcium chloride transformation. The colonies grown on plates were confirmed as clones with insert. The insertion was verified by colony PCR using pJET1.2 forward and reverse sequencing primers (pJET1.2-F: 5´-CGACTCACTATAGGGAGAGCGGC-3´, pJET1.2-R: 5´-AAGAACATCGATTTTCCATGGCAG -3´). After amplification the polymorphic fragment length was confirmed in 1.5% agarose gel electrophoresis. The cloned DNA fragments were then sequenced using the Sanger method.

2.4 Bioinformatics analysis by online program BLAST

The homology of the sequenced DNA with already available sequence data of other species were analysed by online program BLAST from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

2.5 Designing and analysis of SCAR markers

The nucleotide sequence of each of the cloned RAPD fragments were used to design Multiplex SCAR primers using online OligoPerfect Primer Designer (https:// www. thermofisher.com/in/en/oligo-design-tools/oligoperfect.html) (Table 1). PCR amplification was performed using the DNA template from 10 high shell weight pupae and 10 low shell weight pupae of *A. mylitta* (20 samples in total). The 20 µL PCR reaction system was prepared as follows: 10 µL of 2X Taq PCR MasterMix, 20 pmoles of three SCAR primers, and 2 µL of genomic DNA (10 ng), with the remaining volumes filled by ddH2O. PCR was performed in an Thermal Cycler with an initial pre-denaturation for 120 s at 95 °C followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s. The final extension step was performed at 72 °C for 5 min. The amplified PCR products were separated by electrophoresis on a 2.0% agarose gel in 1 × TAE buffer. Gels were visualized by 0.5 µg/mL ethidium bromide staining and the images documented using ImageQuant (GE Healthcare Life Sciences, UK).

3. Results and discussion

3.1 RAPD Analysis

The DNA was extracted and purified from five high shell weight and five low shell weight pupae. The purified DNA of all the samples was used for RAPD to analyse the polymorphic sequence present between the high shell weight and low shell weight Daba with 40 decamer RAPD primers of OPA, OPAJ, OPBC, OPC, OPJ, OPK and OPW sets. The PCR products of the RAPD resolved on 1.5% agarose gel and stained with ethidium bromide shows considerable variations. The number of bands produced by each primer varied from 5 to 12 and polymorphism varied from 55 – 92%. The polymorphic bands were observed at different length, from 200 – 3000bp. Thus the RAPD primers could unravel significant DNA polymorphism among high and low shell weight lines of Daba.

High degrees of polymorphisms can be revealed by RAPD analysis without any prior DNA sequence information of the species, and is easy to manipulate [19-26]. Less DNA template is needed for RAPD and relatively easy to handle but due to poor reproducibility and stability, leads to the restriction in practical application. The specificity and stability can be greatly improved for the testing of different alleles by the conversion of dominant RAPD marker into codominant SCAR markers [14-16]. SCAR markers are also advantageous as they identify single or few bands instead of a complex pattern and they are more precise than RAPD, RFLP, AFLP, IRAP, ISSR and SSR. So the identification of specific yield traits becomes more authentic and well-verified if RAPD analysis is combined with SCAR markers.

3.2 Cloning of RAPD amplification fragment

Among 40 primers used for RAPD amplification, OPK10, OPA16, OPBC20 and OPC02 were not shown any complementarity with the template DNA. Even though other 36 primers generated more polymorphic bands between the samples, they have not shown specific polymorphism between the high and low shell weight lines except OPW9, OPA11 and OPJ18. OPW9, OPA11 and OPJ18 generated the polymorphic fragment at the size of 630, 620 and 220 bp respectively and the bands are marked with a white arrow (Fig. 1). The indicated polymorphic bands were eluted from the agarose gel and 3'-dA overhangs was blunted with DNA blunting enzyme. Blunted DNA was ligated with blunt end cloning vector pJET1.2. The recombinant vector was transformed to competent *E. coli* DH5α cells and recombinant clones were selected through the insertional inactivation of lethal gene *eco47IR* in the multiple cloning site of cloning vector. Further, clones were confirmed through the colony PCR using pJET1.2 forward and reverse sequencing primers. The amplified products were confirmed with agarose gel electrophoresis and bands were observed at 630, 620 and 220 bp for clone containing polymorphic DNA fragment of OPW9, OPA11 and OPJ18 respectively. Finally, positive clones were selected for Sanger Sequencing.

3.3 Sequencing and characterization of polymorphic RAPD fragments

Sequencing of the above mentioned three cloned polymorphic RAPD fragments revealed that clone of OPW9 consisted of 627 nucleotides, OPA11 consisted of 608 nucleotides and OPJ18 consisted of 217 nucleotides. BLAST searches of the nucleotide sequences in GenBank database showed homology of OPW9, OPA11 and OPJ18 fragments to *Manduca sexta* ecdysone receptor homologue (MsEcR) gene, *A. pernyi* trehalase 1A (TRE-1A) mRNA and *A. yamamai* mariner transposon gene for transposase respectively (Fig. 2).

3.4 Development of high and low shell weight lines specific SCAR markers and analysis of PCR amplicons

To generate stable SCAR markers from polymorphic fragments of RAPD markers, three pairs of primers HSWP1, HSWP2 and HSWP3 were designed and synthesized based on cloned sequences. The expected length of the bands was at 301, 209 and 101 bp for HSWP1, HSWP2 and HSWP3 respectively. The designed SCAR primer pairs were then used to amplify the genomic DNA from 10 high shell weight and 10 low shell weight DNA samples to test the lines-specificity in amplification. The amplification results are shown in Fig. 3. The PCR results by SCAR marker HSWP1 indicated that the PCR products with expected size were observed only in high shell weight samples and it shows high specificity towards selection of high shell weight lines. HSWP2 primers generated bands only in high shell weight DNA samples but not in all the same samples. HSWP3 SCAR primers showed loss of the original RAPD polymorphisms and same were also reported by several research groups [11, 27-31]. SCAR marker HSWP1, which is highly specific to high shell weight line amplified the region coding for ecdysone receptor homologue gene. It has been reported that phytoecdysteroid structurally similar to ecdysteroid used commercially to increase productivity in sericulture by increasing cocoon weight, shell weight and shell percentage [32-33]. Accordingly, the amplified region might have crucial role in the shell weight of tropical tasar cocoon.

4. Conclusion

The phenotypes of tropical tasar silkworm is greatly influenced by environmental factors. Main objective of the present study was to develop reliable DNA-based molecular marker to identify breeds/lines with high shell weight. We developed robust and simple multiplex PCR based SCAR markers to distinguish between high and low shell weight groups of *A. mylitta* Daba. SCAR markers HSWP1 and HSWP2, which are specific for high shell weight were found to be greatly reproducible and used to analyse the inheritance pattern of this yield trait. Further research is required to identify the complete sequence of marker genes and their pattern of expression.

Table 1: Sequences of SCAR primers, PCR condition and product size

SCAR	5´-primer Sequence (5´-3´)	5´-primer Sequence (5´-3´)	Size (bp)	Tm(ºC)
HSWP1	CAGCAGCAGCAGCAACAG	CACTCCGCCTGAAGAACCC	301	60
HSWP2	TCGACAGTTCCCGAAAGATCTC	TCGCTGTTGTTGTCATACCAC	209	59
HSWP3	TGCCTTGCACTGCTGAACA	GCTGAGCGCTTGCGATTATC	101	60

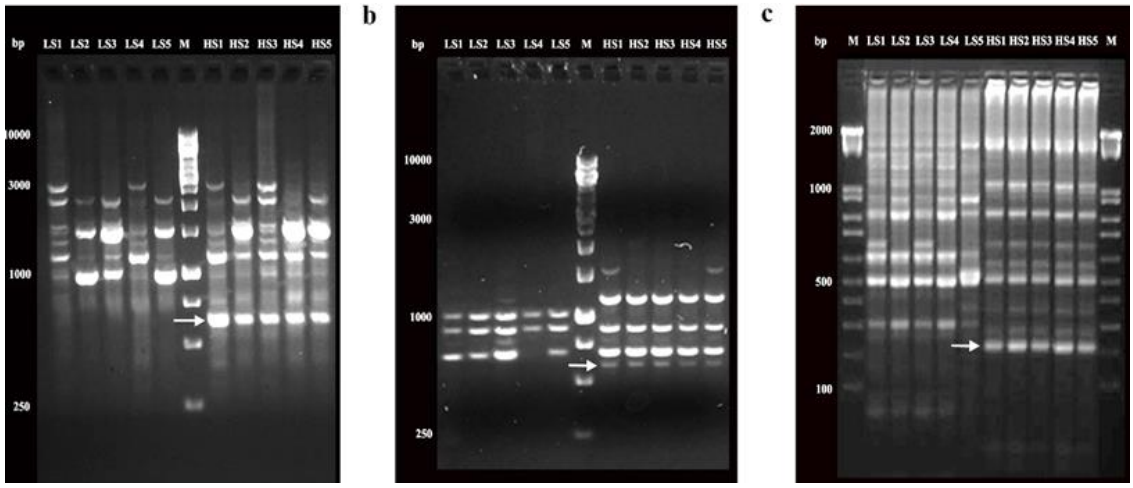

Fig 1: RAPD polymorphism between high and low shell weight lines of *A. mylitta*. LS1–LS5, low shell weight pupal DNA; HS1-HS5, high shell weight pupal DNA; M, marker. (a) RAPD DNA fragment from primer OPW9. The white arrow indicates the band cut from an improved RAPD fragment. (b) RAPD DNA fragment from primer OPA11. The white arrow indicates the band cut from an improved RAPD fragment. (c) RAPD DNA fragment from primer OPJ18. The white arrow indicates the band cut from an improved RAPD fragment.

Fig 2: BLAST searches of the cloned nucleotide sequences. (A) Cloned fragment of OPW9 primer showed homology with *Manduca sexta* ecdysone receptor homologue (MsEcR) gene. (B) Cloned fragment of OPA11 primer showed homology with *A. pernyi* trehalase 1A (TRE-1A) mRNA. (C) Cloned fragment of OPJ18 primer showed homology with *A. yamamai* mariner transposon gene for transposase.

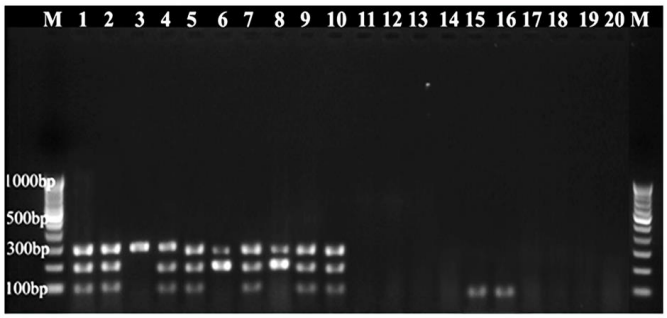

Fig 3: Validation of multiplex SCAR markers through analysis of the PCR amplicons of high and low shell weight DNA. 1–10, high shell weight pupal DNA; 11-20, low shell weight pupal DNA; M, marker. HSWP1 SCAR marker amplicon size is 301bp, HSWP2 SCAR marker amplicon size is 209bp, and HSWP3 SCAR marker amplicon size is 101bp.

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