JEB®

Journal of Environmental Biology

p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Triveni Enterprises (Educational Services Set-up)

Original Research

DOI : http://doi.org/10.22438/jeb/44/3(SI)/JEB-24

Molecular cloning and development of RAPD-SCAR markers for the selection of thermo-tolerant line of tropical tasar silkworm

I.G. Prabhu[1]*, Manjappa[2], M.M. Baig[1], N. Kumar[1], A.K. Sinha[3] and S.Kutala[1]

[1]Central Tasar Research & Training Institute, Central Silk Board, Ranchi – 835 303, India

[2]Central Sericultural Research & Training Institute, Central Silk Board, Mysore – 570 008, India

[3]Ranchi University, Ranchi - 834 008, India

***Corresponding Author Email :** immanual.gilwish@gmail.com ***ORCiD:** https://orcid.org/0009-0003-7069-1150

Received: 07.12.2022 *Revised*: 10.05.2023 *Accepted*: 12.05.2023

Online Copy

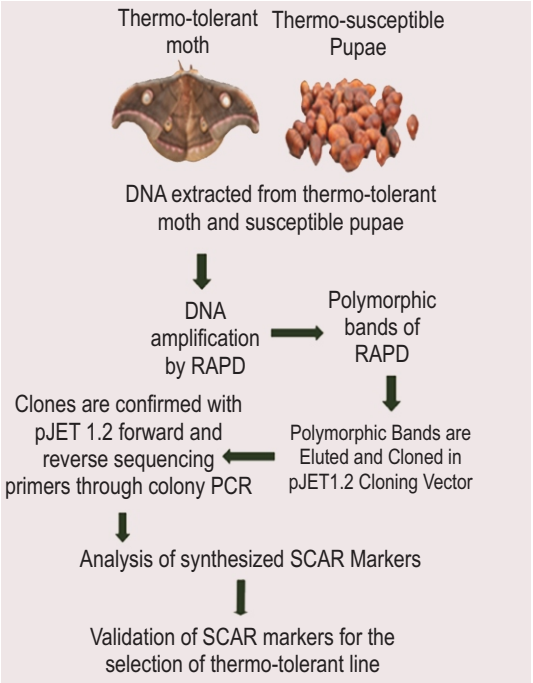
Abstract

Aim: To develop random amplified polymorphic DNA (RAPD) -sequence-characterized amplified region (SCAR) markers for selecting thermo-tolerant line of tropical tasar silkworm.

Methodology: In the environmental chamber, Daba BV cocoons of *A. mylitta* were exposed to high temperature at 46°C/4 hr for 3 days. After the emergence, genomic DNA was extracted from thermo-tolerant moths and thermo-susceptible pupae. The genomic DNA was amplified using 30 RAPD random decamer primers. The improved RAPD fragments that can differentiate thermo-tolerant and susceptible line of *A. mylitta* were eluted, cloned in pJET1.2 vector and sequenced. SCAR markers were developed based on the sequence and validated in the subsequent generations.

Results: Among 30 RAPD primers, OPK04, OPAJ15 and OPA17 generated polymorphic bands to differentiate thermo-tolerant and susceptible line of *A. mylitta*. These polymorphic bands were eluted, cloned and sequenced. Sequencing of three cloned fragments revealed that clone PB1 comprised of 1412 bp, clone PB2 comprised of 704 bp and clone PB3 comprised of 931 bp. Sequence specific stable multiplex SCAR markers TT-PB1, TT-PB2 and TT-PB3 were designed and synthesized. PCR amplification was performed using DNA templates of 10 thermo-tolerant and 10 thermo-susceptible samples. SCAR marker TT-PB1 was observed to be more specific to thermo-tolerant line of tropical tasar silkworm and validation with 25 samples each in next generation also supported the specificity of TT-PB1.

Interpretation: Among three SCAR markers, TT-PB1 showed more specificity for selecting the thermo-tolerant line of *A. mylitta*. Therefore, the study provides an effective and precise PCR-based molecular marker system for selecting thermo-tolerant lines in tropical tasar silkworm to overcome seed crop loss due to high temperature stress in tasar rearing hotter zones.



Key words: *Antheraea mylitta*, cloning, RAPD, SCAR marker, Thermo-tolerance

How to cite : Prabhu, I.G., Manjappa, M.M. Baig, N. Kumar, A.K. Sinha and S.Kutala: Molecular cloning and development of RAPD-SCAR markers for the selection of thermo-tolerant line of tropical tasar silkworm. *J. Environ. Biol.*, **44**, 464-471 (2023).

Introduction

The survival and reproduction of an organism depends on its capacity to deal with extreme temperatures. These extreme temperatures influence the behavioral and physiological mechanisms linked with reproduction, growth and development (Angilletta *et al.,* 2002). Extreme temperatures can lead to irreversible fitness loss and death of insects (Dillon *et al.,* 2009). Insects have complex life cycles, and at different life stages (ages/instars) they may experience different seasonal environments, inhabit different habitats, and experience different environmental stresses. Additionally, they may respond differently depending on their physiological sensitivity (Kingsolver *et al.,* 2011). Therefore, extreme temperatures have different negative effects on different life stages of insects (Chiu *et al.,* 2015). The tropical tasar silkworm, *Antheraea mylitta* Drury is an economically important wild sericigenous insect. During pupal diapause, pupae have to survive in extreme climatic conditions and approximately 30% of seed cocoon loss has been encountered due to high temperatures (42°C), especially during peak summer from May to June (Narain *et al.,* 2001).

The stressful temperature induces detrimental effects infertility, fecundity, immunity, and embryonic development in *A. mylitta* (Malik and Malik, 2009; Pandey *et al.,* 2010; Kumar *et al.,* 2012). Similarly in *B. mori*, temperature stress adversely affects the testicular size, spermatogenesis, fertility, fecundity, growth rate and efficiency of food utilization (Ye *et al.,* 2000; Kumar *et al.,* 2000; Muniraju *et al.,* 2004; Hussain *et al.,* 2011). The selection of thermo-tolerant line of tropical tasar silkworm is essential to overcome the loss due to high temperature stress. Conventionally, selective breeding mainly focus on improving the thermo-tolerance in silkworm. Traditional breeding technique in tropical tasar silkworm is time consuming. Recent developments in biotechnology utilize DNA markers to unravel genetic polymorphisms and evaluate the genetic basis for the observed phenotypic variability. Over the past thirty years, various molecular marker techniques have been employed in Marker Assisted Selection (MAS) to enhance the efficiency and effectiveness of selecting desired traits in breeding programs. RFLP, AFLP, RAPD, ISSR, SSR, CAPS, dCAPS,STS and SNP have been used for the Marker assisted selection (Fu *et al.,* 2013; Khadivi-Khub and Soorni, 2014; Samathmika *et al.,* 2021).

There are limited studies on Marker assisted selection in tropical tasar silkworm breeding program. RAPD derived sequence-characterized amplified region (SCAR) markers were first developed in 1993 by Paran and Michelmore in lettuce for downy mildew resistance genes. SCAR markers are stable molecular markers that convert the dominant markers (RAPD) into co-dominant markers through molecular cloning (Rajesh *et al.,* 2013; Kantipudi *et al.,* 2021). SCAR markers have a high level of polymorphism and longer primers with high sequence specificity (Kumla *et al.,* 2012). The procedure becomes simple when RAPD is combined with SCAR markers using primers designed from the polymorphic sequence of RAPD amplicons, as reported Niranjan *et al.* (2018). In the present study, polymorphic RAPD bands between thermo-tolerant and susceptible line were eluted from agarose gel, followed by cloning and sequencing. Based on the sequence, SCAR markers were developed to select the thermo-tolerant line in tropical tasar silkworm.

Materials and Methods

DNA extraction from *A. mylitta*: Cocoons of *A. mylitta* were exposed to high temperature (46±1°C/4hr) in an environmental chamber and the procedure was continued for three days. Based on the emergence of healthy moths from the cocoons, fecundity and hatchability after treatment, the silkworm lines were considered as thermo-tolerant and susceptible. The experiment was replicated as triplicate. Genomic DNA was extracted from thermo-tolerant moths and thermo-susceptible pupae using conventional phenol– chloroform based DNA extraction method (Nagaraja and Nagaraju, 1995) and purified using PureLink™ Genomic DNA (Invitrogen, USA) as per manufactures protocol with slight modification. Purified genomic DNA samples were diluted with 1XTE buffer to final concentration of 10 ng μl^{-1} and stored at -20°C.

Amplification of DNA by RAPD: Genomic DNA of thermo-tolerant and susceptible lines of *A. mylitta* was amplified using 30 decamer RAPD primers which were synthesized from Eurofins MWG Operon, Bangalore, India (Table 1). A total of 25 µl PCR reaction mixture consisted 2.5 µl buffer (10X Dream Taq DNA polymerase buffer containing 15 mM $MgCl_2$, Fermentas); 2.5 µM dNTPs (from 10-mM stock, Fermentas), 20 pM primer, 1u of Dream Taq DNA polymerase (Fermentas) and 30 ng of genomic DNA. Amplification was executed through a thermal cycler (Biorad, USA) with 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 a final extension of 72 °C for 10 min. After amplification, the products were electrophoresed on 1.5% agarose gel, employing 1 kb ladder as a molecular weight standard, stained with ethidium bromide and documented.

Cloning, identification and sequencing of DNA fragments: Three polymorphic bands which differentiates thermo-tolerant and susceptible lines of *A. mylitta* were excised from the agarose gel and purified using Gene JET purification column (Thermo Scientific, USA). Purified DNA fragments were ligated into blunt end cloning vector pJET1.2 (Thermo Scientific, USA) and transformed into *Escherichia coli*. Luria-Bertani agar plates with 100 µg μl^{-1} of ampicillin were used for selecting the recombinant clones. Screening of recombinant colonies was preformed through positive selection and insertion was verified by PCR using pJET 1.2 forward and reverse sequencing primers (pJET1.2-F: 5´-CGACTCACTATAGGGAGA GCGGC-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 following the Sanger method (AgriGenome Labs Pvt. Ltd., Kerala).

Bioinformatics analysis by online program BLAST: The homology of sequenced DNA products with other species was analysed through Basic Local Alignment Search Tool (BLAST) in GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

Designing of SCAR primers: For designing Multiplex SCAR primers, the nucleotide sequence of three cloned RAPD fragments was utilized with the help of Oligo Perfect Primer Designer available online at https://www.thermofisher.com/in/en/oligo-design-tools/oligoperfect. html. The sequences of primers and amplificon lengths are listed in Table 2.

Analysis of multiplex SCAR markers: PCR amplification was performed using DNA template from 10 thermo-tolerant moths and 10 thermo-susceptible pupae *A. mylitta* (20 samples). For 20 µl PCR reaction, 2 µl of genomic DNA was added to a mixture containing 10 µl of 2X Dream Taq PCR MasterMix, 20 pmoles of three SCAR primers, and the remaining was adjusted with double distil water. PCR was performed in T100 Thermal Cycler (Biorad, USA) with an initial pre-denaturation at 95 °C for 120 sec, followed by 35 cycles at 94 °C for 40 sec, 60 °C for 30 sec, 72 °C for 60 sec, and final extension at 72°C for 300 sec. After amplification, the products were electrophoresed on 2% agarose gel. Gels were visualized by staining ethidium bromide and the images were documented using Image Quant (GE Healthcare Life Sciences, UK). Similar procedure was followed for validation while selecting the thermo-tolerant line.

Results and Discussion

Genomic DNA of thermo-tolerant and susceptible lines were extracted and purified from the fat body tissue of tasar silkworm pupae. Genetic polymorphism among thermo-tolerant and susceptible lines of tropical tasar silkworm were screened through 30 RAPD random decamer primers of OPA, OPAJ, OPBC, OPC, OPJ, OPK and OPW sets. On 1.5% agarose gel, PCR products of RAPD resolved showed considerable variations. The amplifications were obtained with 27 primers, of which only 11 produced polymorphic bands between the samples. The polymorphic bands were observed at different length, ranging from 200 – 3000 bp and the number of bands produced by each primer varied from 5 to 14 with polymorphism varying between 56–94%. RAPD primers could unravel significant DNA polymorphism among thermo-tolerant and susceptible lines of *A. mylitta*. High degree of polymorphisms can be revealed by RAPD analysis without any prior DNA sequence information of the species (Noormohammadi *et al.,* 2013; Zhang *et al.,* 2013). RAPD patterns are usually more complex, sometime clear discrimination of band of interest is not possible. This complex pattern may also lead to misidentification of desired bands, if the mobility of DNA fragments is somewhat uneven, resulting in band shifts (Hardrys *et al.,* 1992; Sales *et al.,* 2001). The stability and specificity can be significantly improved for testing different alleles by converting dominant RAPD marker into codominant SCAR markers (Rajesh *et al.,* 2013; Liu *et al.,* 2020; Baite *et al.,* 2020; Mei *et al.,* 2020).

The use of SCAR markers is preferred over other methods such as RAPD, RFLP, AFLP, IRAP, ISSR, and SSR as SCAR markers provide a more accurate and specific identification of single or few bands rather than complex pattern. The use of SCAR markers is preferred over other methods such

Table 1: Sequence of oligonucleotide used in RAPD

Name	Sequence
OPW5	GGCGGATAAG
OPW6	AGGCCCGATG
OPW9	GTGACCGAGT
OPW12	TGGGCAGAAG
OPW16	CAGCCTACCA
OPW18	TTCAGGGCAC
OPW20	TGTGGCAGCA
OPK04	CCGCCCAAAC
OPK10	GTGCAACGTG
OPJ18	TGGTCGCAGA
OPA02	TGCCGAGCTG
OPA11	CAATCGCCGT
OPA14	TCTGTGCTGG
OPA16	AGCCAGCGAA
OPA17	GACCGCTTGT
OPAJ03	AGCACCTCGT
OPAJ04	GAATGCGACC
OPAJ07	CCCTCCCTAA
OPAJ08	GTGCTCCCTC
OPAJ15	GAATCCGGCA
OPAJ17	ACCCCCTATG
OPAJ19	CAAACGTCGG
OPBC12	CCTCCACCAG
OPBC20	AGCACTGGGG
OPC02	GTGAGGCGTC
OPC09	CTCACCGTCC
OPC15	GACGGATCAG
OPC16	CACACTCCAG
OPC17	TTCCCCCCAG
OPW4	CAGAAGCGGA

Table 2: Sequences of SCAR primers, PCR condition and product size

SCAR	5'-Primer Sequence (5´-3´)	5'-Primer Sequence (5´-3´)	Amplicon Size (bp)	Tm(°C)
TT-PB1	TCTCTGAGACCCTCACCAGG	CAAGCAACACAATGGCGTCA	300	60.0°C
TT-PB2	TGGGGATGTGCATCACTTGA	CCAGCTCCATTGTTCCCCAA	202	60.0°C
TT-PB3	TTACCACCATCGCATGTCGG	ACGCTTATGATGCCAGCCAT	100	60.5°C

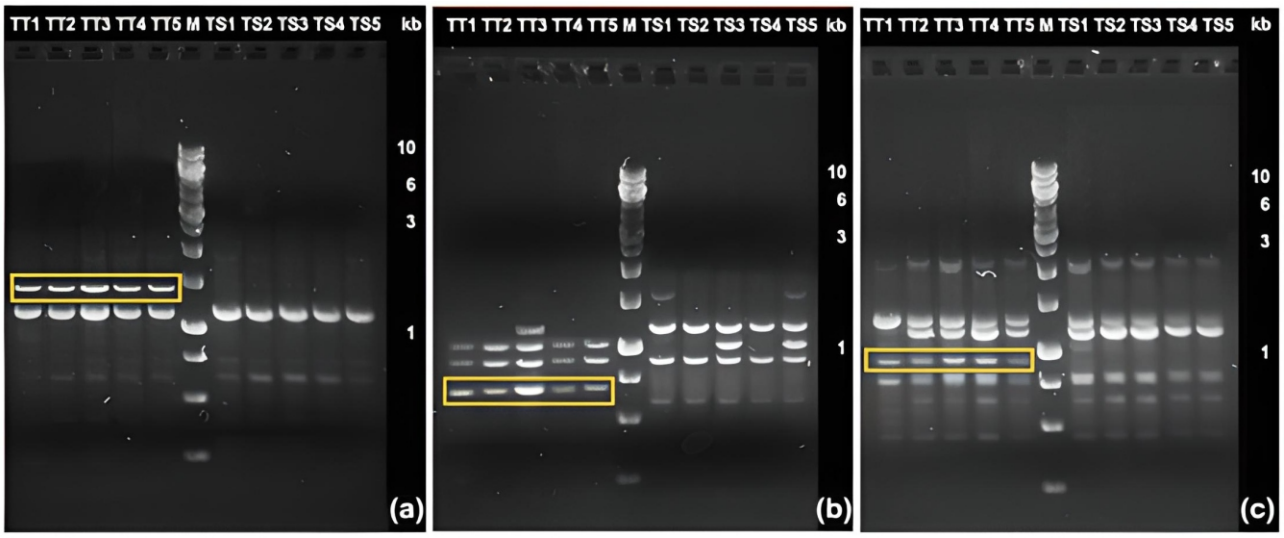


Fig. 1: RAPD polymorphism between thermo-tolerant and susceptible lines of A. mylitta. TT1–TT5, thermo-tolerant moth DNA; TS1-TS5, thermo-susceptible pupal DNA; M, marker. (a) RAPD DNA fragment from primer OPK04 and the yellow box indicates the band cut from an improved RAPD fragment – PB1; (b) RAPD DNA fragment from primer OPAJ15 and the yellow box indicates the band cut from an improved RAPD fragment – PB2; (c) RAPD DNA fragment from primer OPA17 and the yellow box indicates the band cut from an improved RAPD fragment – PB3.

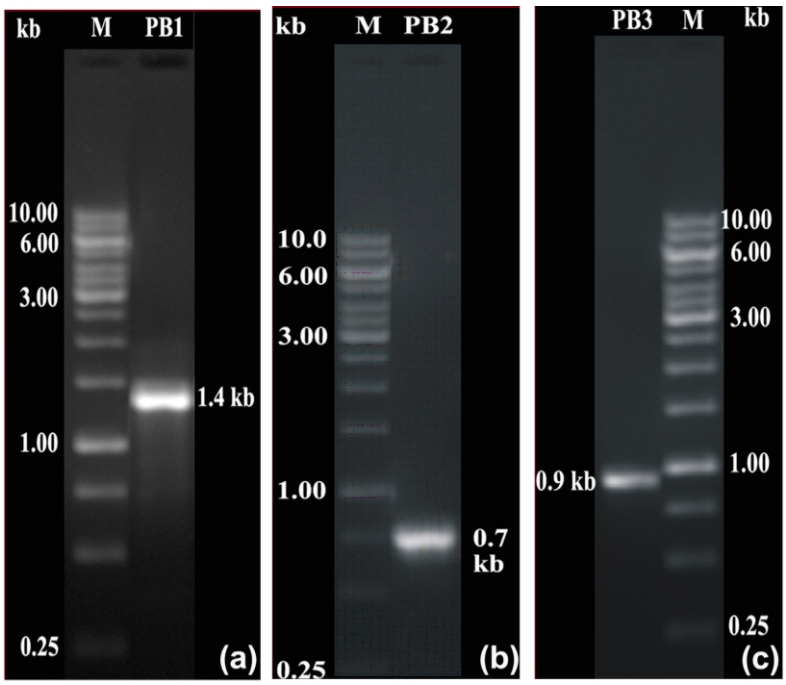


Fig. 2: Identification of positive clones of RAPD fragments by colony PCR amplification with pJET1.2 forward and reverse sequencing primers (a) Identification of the clone of PB1; (b) Identification of the clone of Pb2; (c) Identification of the clone of PB3. ¬Lane M indicates 1kb DNA ladder from the size of 250 to 10000 bp.

as RAPD, RFLP, AFLP, IRAP, ISSR, and SSR as SCAR markers provide a more accurate and specific identification of single or few bands rather than a complex pattern. Combining RAPD analysis with SCAR markers can enhance the authenticity of identifying specific yield traits. RAPD bands unique to differentiate thermo-tolerant and susceptible lines were produced by primers OPK04, OPAJ15 and OPA17. In Fig. 1, the yellow box indicates the bands labeled with PB1 by primer OPK04 (Fig. 1a), PB2 by primer

(a)
```
   1 CCGCCCAAAC CCCATGTTTC CGTCATGACC GCCCAGGGCG ATAAGATCTT CGCCTGAAGA
  61 AATCTCTGCT ATGGTTCTCA CAGATGAAGG AAACTGCTGA AGCCTACCTT GGAAAGAAGG
 121 TCACCCACGC CGTCGTTACT GTACCTGCTT ACTTCAACGA TGCCCAGCGT CAGGCCACCA
 181 AGACGCCGGT GTTATTGCTG TTAACGTCAT GAGGATCATC AACGAGCCTA CTGCCGCCGC
 241 CATCGCGTAC GGTCTCGACA AGAAGGAAGG AGAGAAGAAT GTGCTCGTAT TTGACTTGGG
 301 TGCGGTACCT TCGACGTGTC TCTGCTTACC ATCGACAATG GCGTATTTGA GGTGGTCGCC
 361 TAACGGTGAC ACTCACCTTG GTGGTGAAGA TTTCGACCAA AGAGTTATGG AGCACTTCAT
 421 CAAACTGTAC AAGAGAAGAA GGGCAAGGAT ATCAGAAAAG ACAACCGTGC CGTCCAGAAA
 481 TTGCGTCGTG AAGTCAGAAA GCCAAGAGAG CGTTGTCTTC CGGTCACCAG GTCAAGATTG
 541 AGATCGAATC GTTCTTTGAG GGCGAAGACT TCTCTGAAAC TCTCACCAGG GCGAATTCGA
 601 GGAGTTGAAC ATGGATCTGT TCAGATCTAC TCTTAAACCT GTCAGAAAGT ATTGGAGGAC
 661 GCCGACATGA ACAAGAAGGA CGTCGATGAA ATTGTGCTGG TCGGTGGTTC TACCCGTATC
 721 CCTAAGGTGC AGCAGCTCGA AGGAGTTCTT CAACGGCAAG GGCCATCCCG CGGTATCAAC
 781 CCTGATGAGG CCGCGCCTAC GGTGCCGCCG TCCAGGCCGG CGTGCTCAGC GGTGAACAGA
 841 CACTGACGCC ATCGTGCTGC TCGACGTCAA CCCTCTTACC ATGGGTATCG AGACTGTCGG
 901 AGGCGTCATG ACCAAGCTGA TCCCACGTAC ACCGTTATCC CCACCAAGAA GTCACAGACT
 961 TCTCTACCGC CAGCGACACC AACACACTGT CACATCCAGG TTTACGAGGG AGAGCGCCCC
1021 ATGACCAAGG ACAACCATTT GCTTGGTAAA TTCGATCTTA TGGCATTCCT CCGGCACCGA
1081 GAGGCATTCC TCAGATCGAG GCACCTTCGA AATTGACGCC AACGGTATTC TGCAGTATCT
1141 GCCAGGATAA GGGAACCGGA AATCGCGAGA AGATTGTCAT CACCAACGAC CAAAACAGAC
1201 CACCAGAAGA CATCGAGAGG ATGATTAAGG ATGCTGAGAA GTTGCAGATG ATGACAAGAA
1261 ACTAAAGGAG CGCGTCGAGT CCAGAAATGA ACTAGAGCTA CGCTTACTCG ATCAAGAACC
1321 AGCTCCAAGA TAAGGAGAAA CTTGGTGCTA AGGTCAGCGA TGATGACAAA GCGAAGATGG
1381 AGGAAGCTAT CGACGCCGCG ATCAAACCCG CC
```

(b)
```
   1 GAATCCGGCA GTCAGATGAC GGGTGGAGAG CGAGTTCCCT TACTCGATAT CGAAACGAAC
  61 GGTTCGCAAG AAGTCTCGTA TGATGAGCAG AGTCTGTCTG TTCCGACGGT ATATCCAACC
 121 GCGTTATTAA CTTCTACCCG TCCAACTCAT CGTGATATTG CATCTATTTC AATGCCGCTA
 181 TGCTAACTGT ATCTTTCCCG CGGCGTGGAA AGAAGCGGAC GTTATCGGCA TACACAAACC
 241 CGGTAACCAA AAAATAATCC GACGAGTTAC CGCCCGATTA GCCTCCTCAT GTCTCTGGCA
 301 ACTGTATGAG CGTCTGCTTA CAAACGCCTT CAGAGCTTCG TCTCATCCAA GGGCATTCTC
 361 ATCGATGAAC AATTCGGATT CGTGCAAATC ACTCATGCGT ACAACAGGTG CACCGCCTCA
 421 CGGAGCACAT TCTCGTAGGA CTAACCGACA AAACCGATCT ACACGGGAGC CCTCTTCTTC
 481 GACGTCGCAA AAGCGTCGAC AAGTCTGGCA CAATGGTTTG ATTTTCAAAC TTTTCAACAT
 541 GGGCGTACCG GCAGTCTCGT GCTCATCATA CGGGACTTCT TGTCAACCGC TCTTTTCGAT
 601 ATCGAGAGAG GGAACCCTCC TCCCCGCGAC CACTCAGGCT GGAATCCGCA AGGCTCCGTT
 661 CTCTCTCCGC TCCTCTTTAG TTTATTTATT AACGACGGCC TAAG
```

(c)
```
   1 CTGGCGAACA TTGCCTGCAC TTTTTTTCCG TATCGGTTGC AACACATTCT CTTGCGACTT
  61 TTGAAGCATC GTGACGTCCG ACAAGTATTT GTAATATATG TTATTACATT AATTTTATAT
 121 TAATATTTAT GTGTGGGTTT TGAAGCGTCT TGACGCTCCA CACATACATA TATTAATATT
 181 TGAATTGTTT ATTGTTTGTA TCATTTTTTA TCAGTGTCTG TGCTTCCATT CTACATTAAT
 241 TACATACAAA TACACTAGAC ACACATTACT ATACATAGAC TAGTCCAGTG GGAGCCTAAA
 301 AGGAACACCG AACCTGTAAG GTAAGTGGTT TTACAATACT TCCGTTTGAA TTATTAGAGT
 361 TGCTGTTTAA ATAAAAGTTT GACTCAGAAT GGTCATGAAG CCGAATTGAA ATATTGATAG
 421 GCCCGGTACA ATTCGGTTTT GCTGTGGTGA CTATCTCGTA CATACAAATT GCTTGCATCT
 481 CGTTCTAAAT GAATAAAAAA AATAGGTATT TAAAACTAGT AATTTTGAAA AACTAAGCAT
 541 GTCACATGTA CGGTTAAAAT TAGATGTCGC TCATTTTGTG TAACGTGTAA AGGCACAATT
 601 GTAAACCAAT TTAAATGATT TTTTTTTTTA ATTTGATTAT ATTCGGGGAT GGTCTCACAT
 661 GTATTTAAAG AACATATGAA ACATCGTAAG GTAATTAAAT ACCGGTATTT TTATTTACTT
 721 CTCTTTACAT TTTGAAAGTT ATAATTCTAG TACTTAATGA TAGTAAATAA AAAAAAAACG
 781 ATCAATATAA CACACTGTGG GATTTAGTTG ATAAATCATT TTTTTTTTCT AAATATTATA
 841 TAAACAATTT CTTTTACATA CAAAAATTAT TCCATAAAAA AAAAAAAATA CAGTCGAATT
 901 GATAACCTCC TCCTTTTTTT GACAAGCGGT C
```

Fig. 3: Cloned sequence detail by Sanger sequencing. The sequence of clone PB1 (A) with 1412 bp, PB2 (B) with 704 bp and PB3 (C) with 931 bp.

OPAJ15 (Fig. 1b) and PB3 by primer OPA17 (Fig. 1c). The indicated polymorphic bands were eluted from agarose gel and purified, and ligated to pJET1.2 by blunt end ligation.

The positive selection was adopted to screen the positive clones on LB agar plate with ampicillin. DNA insert ligated into the cloning site disrupts the lethal restriction enzyme gene within the vector and finally, the bacterial cells carrying recombinant plasmids form the colonies. The selected positive clones PB1, PB2 and PB3 were finally confirmed through colony PCR using pJET1.2 forward and reverse sequencing primers (Fig. 2). The amplified products were confirmed through agarose gel electrophoresis and the bands were observed at 1.4 kb, 0.7 kb and 0.9 kb for clones PB1(Fig. 2a), PB2 (Fig. 2b) and PB3 (Fig. 2c),

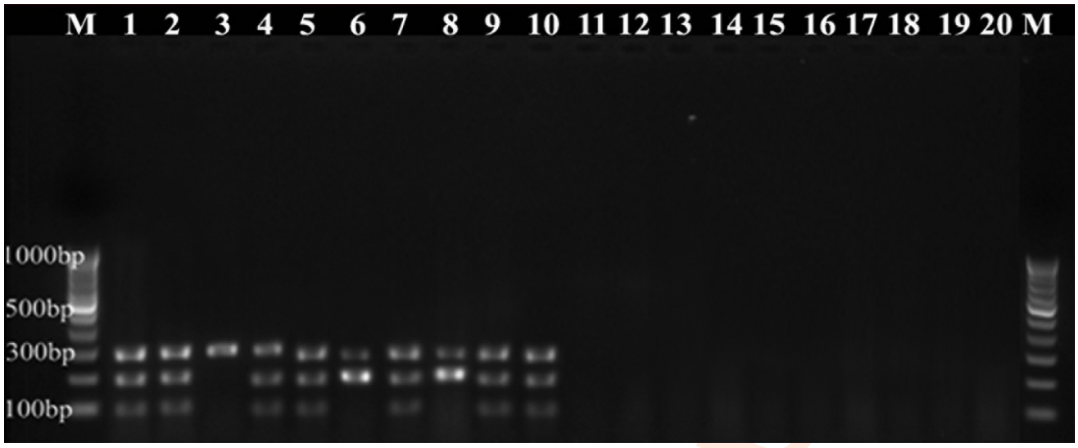


Fig. 4: Development of multiplex SCAR markers and analysis of PCR amplicons of thermo-tolerant and susceptible lines of *A. mylitta*. Multiplex SCAR markers atL1–L10 of thermo-tolerant line and L11-20 of thermo-susceptible lines of *A. mylitta*; Lane M indicates DNA molecular weight of 100bp DNA ladder from the size of 100 to 1000 bp. TT-PB1 SCAR marker amplicon size was 300 bp, TT-PB2 SCAR marker amplicon size was 202 bp, and TT-PB3 SCAR marker amplicon size was 100 bp.

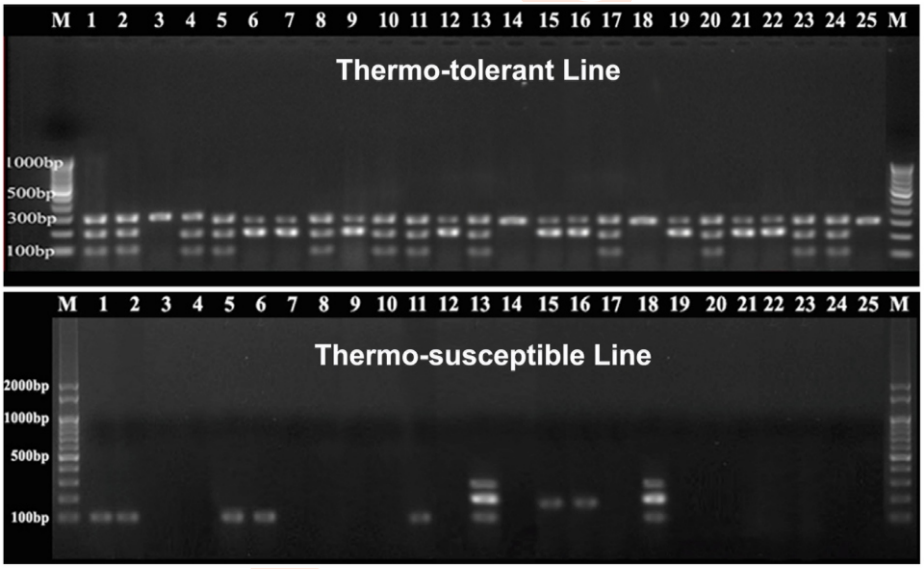


Fig. 5: Validation of developed multiplex SCAR markers and analysis of PCR amplicons of thermo-tolerant and susceptible lines of *A. mylitta* in next generation of the selection of thermo-tolerant line. Multiplex SCAR markers of L1–L25 each for thermo-tolerant and susceptible lines of *A. mylitta*; Lane M indicates the DNA molecular weight of 100bp DNA ladder and 200bp DNA ladder for thermo-tolerant and susceptible gels respectively.

respectively. Sanger sequencing was performed for positive clones. Sequence of three clones comprising polymorphic RAPD fragments revealed that clone of PB1 consisted 1412 bp, PB2 consisted 704 bp and PB3 consisted 931 bp (Fig. 3).

The sequence information was used to design oligonucleotide primers for SCARs. Three pairs of primers were synthesized, and the details are presented in Table 2. With BLAST analysis, one of the three fragments, PB3 did not show homology with any of the sequences in the GenBank, which may due to RAPD amplified product from non-coding regions of tasar silkworm genome. PB2 showed homology to Non-LTR retrotransposon DNA sequences in *Bombyx mori*. PB1 showed significant similarity with Heat Shock Protein Cognate 3 of *Papilio xuthus* (Asian swallow tail). In total three pairs of SCAR markers, TT-PB1, TT-PB2 and TT-PB3, were designed and synthesized based on the cloned sequences of polymorphic fragments of RAPD markers. The expected length of the bands was 300, 202 and 100 bp for TT-PB1, TT-PB2 and TT-PB3, respectively. The designed SCAR primer pairs were then used to amplify the

genomic DNA from 10 thermo-tolerant line and 10 thermo-susceptible line to test the selection specificity in amplification. The amplification results are shown in Fig. 4. The PCR results of SCAR markers TT-PB1, TT-PB2 and TT-PB3 indicated that the PCR products with expected size were not observed in thermo-susceptible samples. TT-PB1 amplification was observed in all the thermo-tolerant lines but TT-PB2 and TT-PB3 shows the specificity of about 80–90%. Developed SCAR markers were validated in the subsequent selection process with 25 samples each of thermo-tolerant and susceptible lines (Fig. 5).

After amplification, it was observed that TT-PB1 showed homology to Heat Shock Protein Cognate 3 which had significant specificity for the selection of thermo-tolerant line compared with TT-PB2 and TT-PB3. In L13 and L18 of thermo-susceptible line, amplicons of all the three SCAR markers were observed which showed that the mortality of temperature treated pupae may be due to factors like diseases, pests, etc. Heat Shock Protein Cognate 3 expression was triggered by the increased concentration misfolded proteins due to protein destabilizing conditions like heat stress (Polina *et al.,* 2019). The results obtained with the SCAR primers TT-PB2 and TT-PB3 showed loss of original RAPD polymorphisms. Such losses were also reported by De Clercq *et al.* (2003), Kengne *et al.* (2001), and Wang *et al.* (2004). SCAR primers were designed based on the internal sequence of polymorphic RAPD fragments which did not include the original RAPD primer sequence. Therefore, RAPD variations may be due to single nucleotide polymorphisms at the priming site and not to indel events (Brisse *et al.,* 2000; Hernandez *et al.,* 2001). In case of TT-PB1, the original RAPD polymorphism was retained. This SCAR markers based characterization provided valuable insights into the genetic and biological diversity within the species (Hiremani and Dubey, 2019; Deng *et al.,* 2022).

Tropical tasar silkworm is highly influenced by environmental factors. Approximately, 30% of seed cocoon loss was encountered in tasar sector due to high temperature stress. In the present study, a reliable DNA-based molecular marker was developed to select the thermo-tolerant line of *A. mylitta*. SCAR marker TT-PB1 was highly specific for the selection of thermo-tolerant line and was found to be highly reproducible, which could be used to analyse the inheritance pattern of this thermo-tolerance trait. Further research is required to identify marker genes' complete sequence and expression pattern.

Acknowledgment

The authors acknowledge the Central Silk Board, Bengaluru for the financial support to carry out this research work (AIT 04002 SI).

Authors' contribution: I.G. Prabhu: Designing of the research plan, cloning and sequence analysis of RAPD polymorphic bands, SCAR markers validation and manuscript preparation; **Manjappa:** Selection and sorting of thermo-tolerant and susceptible line of tropical tasar silkworm; **M.M. Baig:** Performed RAPD for the selection of polymorphic bands; **N. Kumar:** Designing of SCAR markers and optimization; **A.K. Sinha:** Contributed in the preparation of manuscript; **S. Kutala:** Contributed in outlaying of experimental plan and provided molecular laboratory facility for the research work.

Funding: Central Silk Board, Bengaluru - AIT 04002 SI.

Research content: The research content of the manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data availability: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

References

Angilletta, M.J., P.H. Niewiarowskib and C.A. Navas: The evolution of thermal physiology in ectotherms. *J. Therm. Biol.,* **27**, 249–268 (2002).

Baite, M.S., B.K. Upadhyay and S.C. Dubey: Development of a sequence-characterized amplified region marker for detection of *Ascochyta rabiei* causing Ascochyta blight in chickpea. *Folia Microbiol.,* **65**,103–108 (2020).

Brisse, S., J. Dujardin and M. Tibayrenc: Identification of six *Trypanosoma cruzi* lineages by sequence-characterized amplified region markers. *Mol. Biochem. Parasitol.,* **111**, 95–105 (2000).

Chiu, M.C., J.J. Kuo and M.H. Kuo: Life stage-dependent effects of experimental heat waves on an insect herbivore. *Ecol. Entomol.,* **40**, 175–181 (2015).

De Clercq, D., S. Cognet, M. Pujol, P. Lepoivre and M.H. Jijakli: Development of a SCAR marker and a semiselective medium for specific quantification of *Pichia anomala* strain K on apple fruit surfaces. *Posthar. Biol. Technol.,* **29**, 237–247 (2003).

Deng, H., M.A. Khan, X. Liu, J. Fu and Z. Mei: Identification of SCAR markers for genetic authentication of *Dendrobium nobile* Lindl. *Braz. J. Biol.,* **82**, e260394 (2022).

Dillon, M.E., G. Wang, P. A. Garrity and R.B. Huey: Thermal preference in *Drosophila*. *J. Therm. Biol.,* **34**, 109–119 (2009).

Fu, J., L. Yang, M.A. Khan and Z. Mei: Genetic characterization and authentication of *Lonicera japonica* Thunb. by using improved RAPD analysis. *Mol. Biol. Rep*., **40**, 5993-5999 (2013).

Hardrys, H., M. Balick and B. Schierwater: Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.,* **1**, 55–63 (1992).

Hernandez, P., R. de la Rosa, L. Rallo and G. Dorado: Development of SCAR markers in olive (*Olea europaea*) by direct sequencing of RAPD products: Applications in olive germplasm evaluation and mapping. *Theor. Appl. Genet.,* **103**, 788–791 (2001).

Hiremani, N.S. and S.C. Dubey: Phylogenetic relationship among Indian population of *Fusarium oxysporum* f. sp. lentis infecting lentil and development of specific SCAR markers for detection. 3 *Biotech*, **9**,196 (2019).

Hussain, M., S.A. Khan, M. Naeem and M.F. Nasir: Effect of rearing

Online Copy

temperature and humidity on fecundity and fertility of silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). *Pak. J. Zool.,* **43**, 979–985 (2011).

Kantipudi, N.B., T.E. Sheeja, D. Minoo, M.K. Rajesh, K. Samsudeen, E.J. Suraby and I.P.V. Kumar: Random amplified polymorphic DNA (RAPD) and derived techniques. *Meth. Mol. Biol.,* **2222**, 219-247 (2021).

Kengne, P., H.D. Trung, V. Baimai, M. Coosemans and S. Manguin: A multiplex PCR-based method derived from random amplified polymorphic DNA (RAPD) markers for the identification of species of the *Anopheles minimus* group in South-east Asia. *Inse. Mol. Biol.,* **10**, 427–435 (2001).

Khadivi-Khub, A. and A. Soorni: Comprehensive genetic discrimination of *Leonurus cardiaca* populations by AFLP, ISSR, RAPD and IRAP molecular markers. *Mol. Biol. Rep.,* **41**, 4007–4016 (2014).

Kingsolver, J.G., H.A. Woods, L.B. Buckley, K.A. Potter, H.J. MacLean and J.K. Higgins: Complex life cycles and the responses of insects to climate change. *Int. Comp. Biol.,* **51**, 719–732 (2011).

Kumar, C.S., B.M. Sekharappa and S.K. Sarangi: Role of temperature and leaf quality on the larval growth and development during young age of silkworm, *Bombyx mori. J. Exp. Zool.,* **3**, 9–16 (2000).

Kumar, D., J.P. Pandey, Ragini, A.K. Sinha and B.C. Prasad: Temperature discerns fate of *Antheraea mylitta* Drury eggs during embryonic development. *J. Entomol.,* **9**, 220–230 (2012).

Kumla, S., S. Doolgindachbaporn, R. Sudmoon and N. Sattayasai: Genetic variation, population structure and identification of yellow catfish, *Mystus nemurus* (C&V) in Thailand using RAPD, ISSR and SCAR marker. *Mol. Biol. Rep.,* **39**, 5201–5210 (2012).

Liu, X., J. Cheng, Z. Mei, C. Wei, M.A. Khan, J. Peng and J. Fu: SCAR marker for identification and discrimination of specific medicinal *Lycium chinense* Miller from *Lycium* species from ramp-PCR RAPD fragments. 3 *Biotech.,* **10**, 334 (2020).

Malik, M.A. and F.A. Malik: Ontogenic changes in hemolymph biochemical composition in the silkworm, *Bombyx mori* L under thermal stress. *Acad. J. Entomol.,* **2**, 16-21 (2009).

Mei, Z. and M.A. Khan: Genetic authentication of *Eclipta prostrate* (Asteraceae) from *Penthorum chinense* (Penthoraceae) by sequence characterized amplified region (SCAR) markers. *Rev Biol Trop*., **68**,180–188 (2020).

Muniraju, E., B.M. Sekharappa and R. Raghuraman: Food intake and utilization efficiency in silkworm *Bombyx mori* L. (Pure Mysore) reared at different temperature combinations. *Int. J. Trop. Insect Sci.,* **24**, 135–142 (2004).

Nagaraja, G.M. and J. Nagaraju: Genome fingerprinting of the silkworm, *Bombyx mori*, using random arbitrary primers. *Electrophoresis*, **16**, 1633-1638 (1995).

Narain, R., S.S. Rath, B.B.S. Negi, S. Nagendra, V. Kulshrestha, B.C. Prasad, B.R.R.P. Sinha and K. Thangavelu: Integrated package for seed cocoon preservation and seed production in *A. mylitta* D. *Indian Silk*, **10**, 15-17 (2001).

Niranjan, K., I.G. Prabhu, Manjappa, A.K. Sinha and A. Sahay: Development of RAPD-SCAR markers for the identification of *Antheraea mylitta* with high shell weight. *J. Entomol. Zool. Stud.,* **6**, 828-833 (2018).

Noormohammadi, Z., F.Y. Hasheminejad-Ahangarani, M. Sheidai, B.A.S. Ghasemzadeh and O. Alishah: Genetic diversity analysis in Opal cotton hybrids based on SSR, ISSR, and RAPD markers. *Genet. Mol. Res.,* **12**, 256-269 (2013).

Pandey, J.P., P.K. Mishra, D. Kumar, B.M.K. Singh and B.C. Prasad: Effect of temperature on hemocytic immune response of tropical tasar silkworm *Antheraea mylitta* D. *Res. J. Immunol.,* **3**, 169–177 (2010).

Paran, I. and R.W. Michelmore: Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor.Appl. Genet.,* **85**, 985-993 (1993).

Polina, D., D. Bedulina, E. Madyarova, L. Rivarola-Duarte, S. Schreiber, P. F. Stadler, T. Luckenbach and M. Timofeyev: Description of strongly heat-inducible heat shock protein 70 transcripts from Baikal endemic amphipods. *Sci. Rep.,* **9**, 8907 (2019).

Rajesh, M.K., B.A. Jerard, P. Preethi, R.J. Thomas, T.P. Fayas, K.E. Rachana and A. Karun: Development of a RAPD-derived SCAR marker associated with tall-type palm trait in coconut. *Sci. Hortic.,* **150**, 312–316 (2013).

Sales, E., S.G. Nebauer, M. Mus and J. Segura: Population genetic study in the Balearic endemic plant species *Digitalis minora* (*Scrophulareaceae*) using RAPD markers. *Am. J. Bot.,* **88**, 1750–1759 (2001).

Samathmika, R., M. Hassani, B. Heidari, S. Deb, E. Orsini, J. Li, C.M. Richards, L.W. Panella, S. Srinivasan, G. Campagna, G. Concheri, A. Squartini and P. Stevanato: Development of an SNP assay for marker-assisted selection of soil-borne *Rhizoctonia solani* AG-2-2-IIIB resistance in sugar beet. *Biology (Basel),* **11**, 49 pages (2021).

Wang, X., Y. Yang, Y. Cong and D. Duan: DNA fingerprinting of selected *Laminaria* (Phaeophyta) gametophytes by RAPD markers. *Aquaculture,* **238**, 143–153 (2004).

Ye, G., C. Hu and H. Gong: Impact of high temperature on testicular growth and development of valuable silkworm *Antheraea yamamai* (Lepidoptera: Saturiidae). *Ying Yong Sheng Tai Xue Bao.,* **11**, 851–855 (2000).

Zhang, L., H.G. Zhang and X.F. Li: Analysis of genetic diversity in *Larix gmelinii* (Pinaceae) with RAPD and ISSR markers. *Genet. Mol. Res.,* **12**,196-207 (2013).

Online Copy