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IDENTIFICATION OF MICROSATELLITE MARKERS LINKED TO THERMOTOLERANCE IN SILKWORM BY BULK SEGREGANT ANALYSIS AND *IN SILICO* MAPPING

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Silkworm, being a poikilothermic insect, its growth and development is affected by environmental factors especially, temperature. In tropical countries like India, it has considerable effect on silk production due to the prevailing of hot climatic conditions. Previous attempts to evolve silkworm breeds and hybrids tolerant to high temperature by traditional breeding methods have not yielded the desired results. Hence application of new strategies like marker assisted selection (MAS) could be the most effective strategy for developing a thermo-tolerant bivoltine silkworm for sustainable silk production in India. As a prelude, in this study it is aimed to identify simple sequence repeat (SSR) markers closely linked with thermotolerance in silkworm. To do so, 20 silkworm breeds were evaluated at high temperature (36°C) and based on pupation percentage, two multivoltines (Nistari and Cambodge) and two bivoltines (SK4C and BHR3) were identified as thermo-tolerant and one bivoltine (CSR2) was identified as the susceptible breed. These breeds were screened with 85 SSR markers drawn from different linkage groups and out of those, only 11 markers (12.9%) showed distinct polymorphism between thermo-tolerant and susceptible breeds. Further, bulked segregant analysis (BSA) was performed using 11 polymorphic SSR primers, by comparing the SSR profiles of the tolerant (Nistari) and susceptible (CSR2) parents, their F_1 and F_2 bulks. Nevertheless, only 5 markers generated clear differences in the amplified DNAs between the bulks corresponding to that of the parents suggesting that the DNA regions amplified by these

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SSR markers are closely linked to thermotolerance in *B. mori*. The results obtained through bulk segregant analysis was further confirmed by genotyping 5 linked SSR markers using 140 individual F_2 progenies. Of these 5 markers, highest Spearman's rho correlation coefficient was shown by S0816 indicating a high degree of closeness between the genotypic and phenotype variations in F_2 population. Furthermore, we have also attempted to locate the genes near to S0816 by *in silico* approach and upshot revealed 3 genes nearer to its sequence on the *B. mori* genome. The BGIBMGA005249 gene was found to be located nearest to S0816 at a distance of 14.8 Kb. But, further studies are required in this regard to derive a relationship between the thermotolerance and the functional role of identified genes nearer to the closest marker, so that the identified markers can be used to develop a thermo-tolerant silkworm breed through MAS.

Key words: Bulk segregant analysis, *In silico mapping*, Marker assisted selection, Silkworm, Thermotolerance

INTRODUCTION

The silkworm (*Bombyx mori* L.) is an important source of livelihood for subsistence farmers engaged in silk production in many countries and is believed to be a central model for Lepidopteran genomics and genetics. It is a domesticated insect, which is completely dependent on humans for its survival (XIA *et al*., 2009). Being poikilothermic, silkworms are vulnerable to high temperatures (BENCHAMIN and JOLLY, 1986) and based on voltinism, silkworm breeds are classified as uni-, bi- and multi-voltine. Among them, multivoltines being tropical in origin can tolerate high temperature conditions but produce poor quality silk; whereas, bivoltines of temperate origin are prone to high temperature conditions but can produce silk of good quality.

Sericulture in India is predominantly practised in hot tropical regions; rearing of silkworms under such conditions will adversely affect pre- and post-cocoon parameters. Therefore, F_1 hybrids developed by crossing females of native multivoltine and males of exotic bivoltine breeds as parents are popular, because these cross breeds are more tolerant to high temperature, but produce non-gradable silk (LAKSHMI *et al*., 2011). Hence, in order to increase the production of gradable silk with superior quality in India, there is a necessity for the development of thermo-tolerant bivoltine silkworm breed/hybrid, which can be reared throughout year in the tropics of India. However, it is a difficult task because the thermotolerance trait in *B. mori* is influenced by genetic and environmental factors, and their interactions. The earlier attempts made by many silkworm breeders have led to the development of new bivoltine breeds and hybrids. Though, they performed well under controlled laboratory conditions but failed to sustain at the farmers' level (KUMAR *et al*., 2011). One of the possible reasons for the failure of popularization of these breeds was the selection of parents solely based on their phenotypic performance without considering the genetic factors. However, parent selection based on per se performance, and genetic marker may be better option to obtain a wide genetic base, which can yield superior segregants enabling effective selection during the course of evolution of lines suitable for tropical regions (MOORTHY *et al*., 2007). Thus, the molecular dissection of thermotolerance by identification of DNA markers associated with thermotolerance trait would greatly facilitate breeding of thermo-tolerant bivoltine silkworm breed by marker assisted selection (MAS). Hence, the primary objective of the present work was to identify the markers tightly linked to the thermotolerance trait in *B. mori*.

The bulk segregant analysis (BSA) is one of the best methods used to reduce the large number of markers to a few, which is specific and tightly linked to the trait. This technique involves comparing two pooled DNA samples of individuals from a segregating F_2 population originating from a single mating. Initially, DNA markers, which show distinct polymorphism between the contrasting parents (tolerant and susceptible), are identified. Using these polymorphic markers, the DNA profiles of the parents, F_1 progeny and F_2 bulks segregating for the target trait (tolerant and susceptible) are then compared. Thus, the DNA markers that are polymorphic between the bulks are likely to be linked to the target gene. This technique relies on the temporary populations like F_2 and backcrosses (BC), which are simple to produce and saves time and cost (MICHELMORE *et al*., 1991).

With the advancement in the genomics and availability of new molecular tools, silkworm genome has been greatly explored by successively constructing molecular linkage maps using markers like random amplification of polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs), microsatellites and single nucleotide polymorphisms (SNPs) (SHI *et al*., 1995; PROMBOON, *et al*., 1995; YASUKOCHI, 1998; TAN *et al*., 2001; MIAO *et al*., 2005; YAMAMOTO *et al*., 2006), laying a strong foundation for mapping *B. mori* genes associated with the traits of commercial importance. Among these markers, microsatellites, also known as simple sequence repeats (SSRs) have gained considerable interest because of their reproducibility, multiallelic nature, codominant inheritance and good genome coverage (POWELL, 1996). SSRs are tandem repeats of 1-6 bp of DNA sequence originated primarily due to slipped strand mispairing (LEVINSON and GUTMAN, 1987) and subsequent errors during DNA replication/ repair/ recombination (KATTI *et al*., 2001) or unequal crossing over between sister chromatids (INNAN *et al*., 1997).

Phenotype variations in a particular trait are likely to be linked to the microsatellite polymorphism present in the *B. mori* genome. For instance, LI *et al*. (2006) mapped the densonucleosis non-susceptible gene (*nsd-Z*) on 15^{th} chromosome of *B. mori* by using SSR markers. Further, ZHAO *et al.* (2006) mapped BmNPV-resistance gene of *B. mori* and also 3 SSR markers linked to I (yellow blood inhibitor) gene was identified on 9^{th} chromosome of *B. mori* (LI *et al.*, 2008). In addition, the genome assembly of *B. mori* has been greatly improved with the generation of 6× and 3× draft genome sequences created by Chinese group and Japanese group in 2004 (MITA *et al*., 2004; XIA *et al*., 2004), respectively. With the redevelopment of SilkDB and integration of highly efficient and convenient genome browser tools like SilkMap provides a useful resource to map the gene of interest.

In this direction, earlier ZHAO *et al*. (2010) attempted and identified 5 SSR markers linked to thermotolerance in silkworm and mapped them on 8^{th} chromosome using backcross population of thermo-tolerant variety - Dong 34 and thermo-sensitive variety - Ou17. However the question arises, how these markers can be utilized universally in MAS in silkworm due to the fact that Indian and Chinese breeds may not share a common genetic background and further, these markers cannot be applied directly to Indian breeds without prior testing and validation. Keeping this in view, in this study, it is aimed to validate the already identified markers through BSA in Indian silkworm breeds and to identify additional SSR markers that are tightly linked to the thermotolerance trait and confirmation through correlation analysis between phenotypic changes and genotypic variations in F_2 population. Further, to locate the SSR marker strongly associated with thermotolerance in accordance with the silkworm database by *in silico* approach.

MATERIALS AND METHODS

Screening and identification of tolerant and susceptible genotypes

In order to identify tolerant and susceptible genotypes, 20 silkworm breeds (Table 1) were selected based on earlier reports and three days old 5th instar larvae of these breeds were subjected to high temperature (36°C) for 6 hours in a SERICATRON (chamber meant to control constant temperature and humidity during silkworm rearing) as suggested by KUMAR *et al*. (2011). The procedure was continued till spinning. The matured larvae were mounted on plastic mountages and allowed to spin cocoons. By estimating the pupation percentage, the silkworm breeds were considered as thermo-tolerant or susceptible. The experiment was performed twice and in triplicate with 100 larvae in each replication.

Preparation and rearing of F_2 combinations

The most tolerant multivoltine (Nistari and Cambodge) and susceptible (CSR2) parents identified in this study were used to raise two F_1 combinations viz., Nistari ♀ x CSR2 ♂ and Cambodge ♀ x CSR2 ♂. These F_1s were reared and selfed to prepare F_2 layings. The larvae from single disease free laying of each of the two F_2 combinations were exposed to high temperature (36ºC). The moribund larvae, which were characterized by puffing of body, low feeding and low gripping ability were considered as susceptible. The larvae, which survived and spun good cocoons and subsequently, metamorphosed into moths, were considered as tolerant.

DNA extraction

Genomic DNAs were extracted from moths of tolerant and susceptible breeds, F_1s and tolerant F_2 progenies using DNAzol reagent (Invitrogen, USA) as per manufacturer's protocol. Posterior silk gland of F_2 susceptible progeny was used as the source of DNA because the identification of susceptible progenies (dying) was possible only during treatment period at larval stage. Therefore, the posterior silk gland of susceptible larvae were dissected and stored at -80ºC until use. The DNAs were quantified using an Ultraspec-1100 pro UV-spectrophotometer (Amersham BioSciences, Hongkong, China).

Polymerase chain reaction (PCR) and SSR analysis

A total of 85 microsatellite markers were selected based on earlier reports of PRASAD *et al*. (2005), LI *et al*. (2005) and MIAO *et al*. (2005) and further information of these markers on different linkage groups of silkworm. The PCR reaction mixture consisted of 20 ng genomic DNA, 1X PCR buffer, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 0.4 µM primers and 1 unit of *Taq* polymerase (Thermo Fisher Scientific, Waltham, MA, USA) in a 20 µL reaction volume. PCR were performed on a PTC 200 Thermocycler Engine (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycle of an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s; annealing at 38-56°C for 40 s and extension at 72°C for 1 minute followed by 10 minutes of final extension at 72°C. The amplified products were resolved on 3% of Metaphor agarose gels prepared by adding two parts of metaphor agarose and one part of agarose.

Bulked segregant analysis (BSA)

Tolerant and sensitive bulks were prepared from F_2 population of Nistari ♀ x CSR2 ♂ by pooling aliquots, containing equivalent amounts of total DNA from each of 10 sensitive and 10

tolerant individuals. The bulks were tested with the informative markers that are polymorphic between the tolerant and susceptible breeds.

Confirmation of polymorphic markers and marker-thermotolerance trait correlation

To determine the association of phenotypic variation in thermotolerance with microsatellite polymorphism, we carried out genotyping of 5 linked markers by using 140 (67 were thermo-tolerant and 73 were thermo-susceptible) F_2 individuals from Nistari ♀ x CSR2 ♂. The PCR amplified products of F_2 individuals were scored as 1, 2, 3 and 0 (null) by comparing with PCR amplification products of parental DNA. The DNA bands inherited from the tolerant parent (Nistari) were designated as "1" (homozygous). Similarly, the DNA bands inherited from the susceptible parent (CSR2) were designated as "3" (homozygous). The DNA bands derived from both the parents (tolerant and susceptible) were designated as "2" (heterozygous). Using Spearman's rho correlation, informative markers identified from BSA were tested for confirmation. The Spearman's rho correlation coefficient (SCC) would reveal the degree of closeness between the each genotyped marker and the phenotype variation. The PCR protocol and methodology for amplified product detection were adopted as aforesaid.

In silico mapping of SSR markers based on sequence information

BLASTn was performed using the sequences of forward and reverse primers as query sequences with default parameters. The hit sequences with high identity were retrieved from GenBank. The genomic sequences thus obtained were mapped on silkworm genome by using SilkMap tool available at SilkDB database. SilkMap is a tool developed to map any protein or nucleotide sequences on silkworm chromosomes. The SilkDB database includes the latest annotated genome sequences, and hence SilkMap is a convenient tool for mapping genes of interest. The markers closely associated with thermotolerance were mapped. Further, to detect the gene nearer to the closely associated marker, the up and down stream sequences of the closest marker were ascertained for the gene with a browser window size of 10Kb.

RESULTS

Identification of tolerant and susceptible genotypes

Twenty silkworm breeds consisting of two multivoltines and eighteen bivoltines were evaluated at 36°C during 5^{th} instar to identify genotypes having contrasting response to thermotolerance. Based on the pupation percentage, Nistari and Cambodge were identified as highly tolerant, SK4C and BHR3 were categorised as moderately tolerant and CSR2 as the susceptible breed (Table 1).

Phenotyping of F_2 population and chi-square analysis

To understand the phenotypic variation and segregation pattern at high temperature, two F_2 populations developed by mating two most tolerant breeds i.e., Nistari and Cambodge and susceptible breed CSR2 were exposed to 36°C. Sixty-seven out of 140 progeny of Nistari ♀ x CSR2 ♂ and 116 of 224 progeny of Cambodge ♀ x CSR2 ♂ of F_2 segregants were found to be tolerant to high temperature treatment (Table 2). Surprisingly, in F_1 population, all were not tolerant (Data not shown). The χ^2 test for independence indicated that the ratio deviates from the expected ratio of 3:1 ($P > 0.05$) (Table 2).

Table 1. Morphological characters and pupation percentage of twenty silkworm breeds

Sl. No.	Breed	Larval marking	Cocoon shape	Cocoon colour	Percentage pupation (%)	Locality
1	ATR16	Plain	Oval	White	19.22	Dehradun
2	ATR29	Marked	Dumbbell	White	40.44	Dehradun
3	BHR2	Marked	Dumbbell	White	43.89	Berhampore
4	BHR3	Marked	Dumbbell	White	59.67	Berhampore
5	B37	Plain	Oval	White	12.56	Hosur
6	CSR2	Plain	Oval	White	12.22	Mysore
7	CSR17	Plain	Oval	White	27.89	Mysore
8	CSR46	Plain	Oval	White	40.67	Mysore
9	CSR47	Marked	Dumbbell	White	28.33	Mysore
10	CSR50	Plain	Oval	White	36.33	Mysore
11	CSR51	Marked	Dumbbell	White	18.56	Mysore
12	D6(P)	Marked	Dumbbell	White	28.22	Berhampore
13	D6(P)N	Marked	Dumbbell	White	44.40	Berhampore
14	NN6D	Plain	Oval	White	44.22	Hosur
15	S38	Plain	Oval	White	22.67	Hosur
16	SK3	Plain	Oval	White	32.44	Berhampore
17	SK4	Marked	Dumbbell	White	38.56	Berhampore
18	SK4C	Marked	Dumbbell	White	60.89	Berhampore
19	Nistari*	Marked	Spindle	Yellow	85.11	Berhampore
20	Cambodge*	Plain	Spindle	Yellow	84.00	Berhampore

* multivoltine breeds

Table 2. Phenotypes of F_2 progenies and results of chi square test

F_2 crosses	**Tolerant**	**Susceptible**	**Ratio**	**χ^2**
Nistari ♀ x CSR2 ♂	67	73	0.48: 0.52	55.01
Cambodge ♀ x CSR2 ♂	116	108	0.52: 0.48	64.38

SSR polymorphism and bulked segregant analysis (BSA)

Using eighty-five SSR primers derived from all linkage groups, the DNAs of 4 thermo-tolerant breeds (Nistari, Cambodge, SK4C and BHR3) and 1 susceptible breed (CSR2) were screened for polymorphism. Eleven (12.9%) markers showed distinct polymorphism between thermo-tolerant and susceptible breeds. Though, few other markers were polymorphic between the selected breeds, they did not reveal distinct polymorphism between tolerant and susceptible breeds and such markers were discarded. The primer details are listed in Table 3.

Table 3. Details of microsatellite markers polymorphic between the parents

Sl. No.	Locus symbol	Primer sequence (5'→3')
1	Fl0516*	CGACTCACTCCTTTTATTTTATTGACTCT CGGATCGAGTACTGCAATGCG
2	Fl0648*	AAGTAACAACAATACCGAGACTGACG TTATTACCTACCTTACATTGTGCATTTG
3	Fl0656*	CCAGACAATCATTAGATGGACAAGA TCATTTACCCGTTGAACGCTTAT
4	Sat2604*	GCTCGCCATATGCAATCCTC CGTCATTGCCTTCATTTCAGTTC
5	S0801**	GCGGTAATGCCGTGCCC GGTTTTAATAATAATACTGGATTTCTGGTT
6	S0803**	AAGTTCTTTACCAGTTCACAGACAGC CGCCATGCAACTGTCGTCAC
7	S0809**	AACATTTGCTTAGGACTGAATTTACAC AATAATAACTTTTACACGCACCTACACTT
8	S0813**	CCAGGAAATTCCAACCAGTAGCC ACTTACCACTACACCAGACGGAC
9	S0816**	GAAATCCGTTTGAAGAATCCACA CATCCGTTGAATGAGTATCGTTTG
10	S0819*	GGCATAATCGCATCGCTCG TCACGAAATGCCAATCATAACTG
11	S0820*	GAGGTACCAGTGATTGCAGACGT CCCAGGTGTACTCGGAGTCATTTA

*polymorphic between parents

**polymorphic between parents and, thermo–tolerant and –susceptible bulks

BSA was applied to identify markers tightly linked to the thermotolerance trait. Two bulks containing each of 10 tolerant and 10 susceptible larvae were selected from F_2 population of Nistari ♀ x CSR2 ♂ and they were screened with 11 polymorphic markers. SSR markers were presumed to be linked if they generated same banding pattern (homozygous) as Nistari for tolerant bulk and banding pattern (homozygous) as CSR2 for susceptible bulk. SSRs, which generated banding pattern as F_1 (heterozygous) for either of the bulks or if they evert banding pattern compared to their phenotypic nature, such primers were discarded. Out of 11 polymorphic markers, only five SSR markers namely S0801, S0803, S0809, S0813 and S0816 showed banding pattern similar to tolerant and susceptible parents. Hence these markers are most likely to be tightly linked to the thermotolerance trait in *B. mori*.

Genotyping of F_2 population and marker-thermotolerance trait correlation

In this study, pupating capacity of the larvae at high temperature condition was used as a phenotype marker. Hence, the correlation between the variation in the phenotype and the microsatellite markers reveals degree of closeness of the markers with the phenotype. All the 5

linked microsatellite markers were significantly correlated ($P > 0.01$) with the phenotype. Spearman's rho correlation coefficient revealed a significantly high correlation between S0816 and phenotype (0.722), followed by S0803 (0.656) and S0809 (0.598) (Table 4). All the markers segregated in the expected ratio of 1:2:1 ($P > 0.05$) (Table 5).

Table 4. Results of Spearman's rho correlation

Microsatellite marker	Spearman's rho correlation coefficient
S0801	0.375
S0803	0.656
S0809	0.598
S0813	0.487
S0816	0.722

Table 5. Results of chi square test for segregation of markers

	F_2 progenies				
Marker	**AA**	**AB**	**BB**	**Ratio**	χ^2
S0801	41	64	35	0.29: 0.45: 0.25	1.54
S0803	35	68	37	0.25: 0.49: 0.26	0.17
S0809	39	65	36	0.28: 0.46: 0.26	0.84
S0813	38	67	35	0.27: 0.48: 0.25	0.39
S0816	35	67	38	0.25: 0.48: 0.27	0.39

In silico mapping of SSR markers based on sequence information

According to the correlation results, we located all the 5 markers linked to thermotolerance, to locate the genes controlling the trait, the genomic sequences associated with the markers were retrieved by BLAST search which were located on *B. mori* chromosomes. Using a SilkMap tool, we constructed a sequence based map of 8th chromosome, which includes 3 SSR markers (S0803, S0809 and S0816) and 3 gene sequences (BGIBMGA005249, BGIBMGA005250 and BGIBMGA005272) located on up and downstream sequences of S0816. The S0816, BGIBMGA005249, BGIBMGA005250 and BGIBMGA005272 were located on scaffold nscaf2827 of 8th chromosome of *B. mori* genome. The 16.2 Kb upstream and 149.6 Kb downstream sequences of S0816 of *B. mori* genome were examined to locate the nearest gene. The search spans a total distance of 165.7 Kb. The results showed that BGIBMGA005249, BGIBMGA005250 and BGIBMGA005272 are closer to S0816, and the BGIBMGA005249 is nearest to S0816, which is located at 14.8 Kb physical distance on upstream of S0816 (Table 6) (Figure 1).

Table 6. The result of sequence based in silico mapping of the linked markers

Sl. No.	Marker	Hit scaffold	Scaffold position	Chromosome
1	S0801	nscaf2855	3677520	10
2	S0803	nscaf2970	2741964	8
3	S0809	nscaf2970	244957	8
4	S0813	nscaf2993	6878182	12
5	S0816	nscaf2827	1211210	8
6	BGIBMGA005249	nscaf2827	1196335..1198818	8
7	BGIBMGA005250	nscaf2827	1194864..1195656	8
8	BGIBMGA005272	nscaf2827	1360873..1363798	8

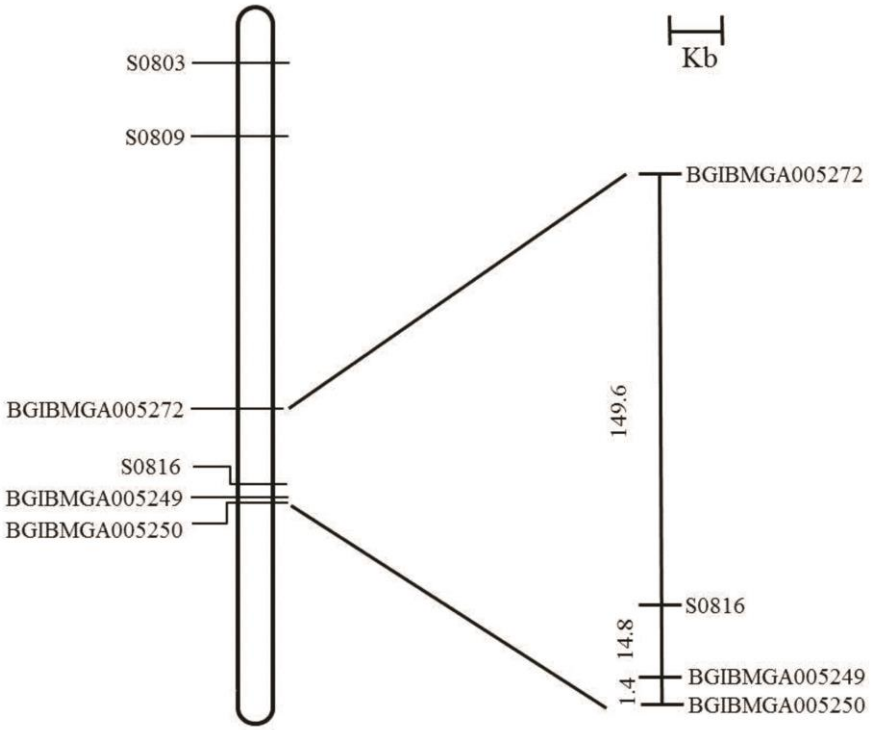

Figure 1: *In silico* map of the markers on 8th chromosome

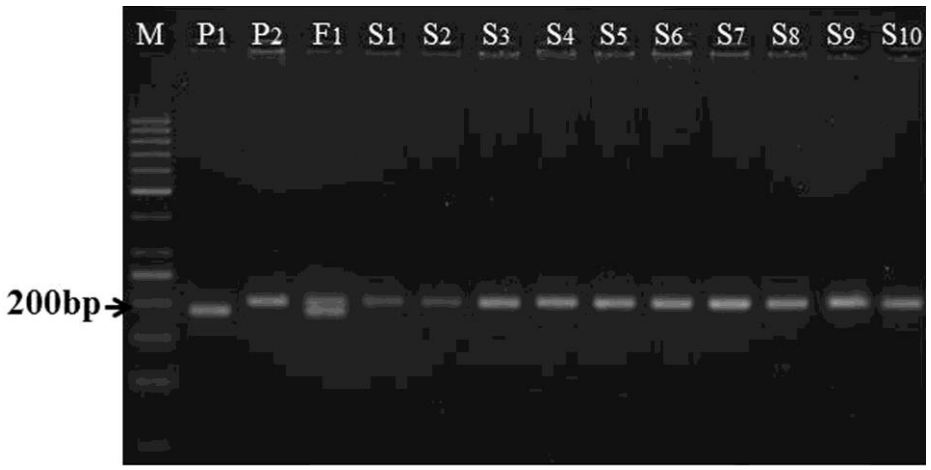

Figure 2(a): S0816 amplification banding pattern of P_1-Nistari, P_2-CSR2, F_1 and S_1-S_{10}-F_2 thermo-susceptible progeny

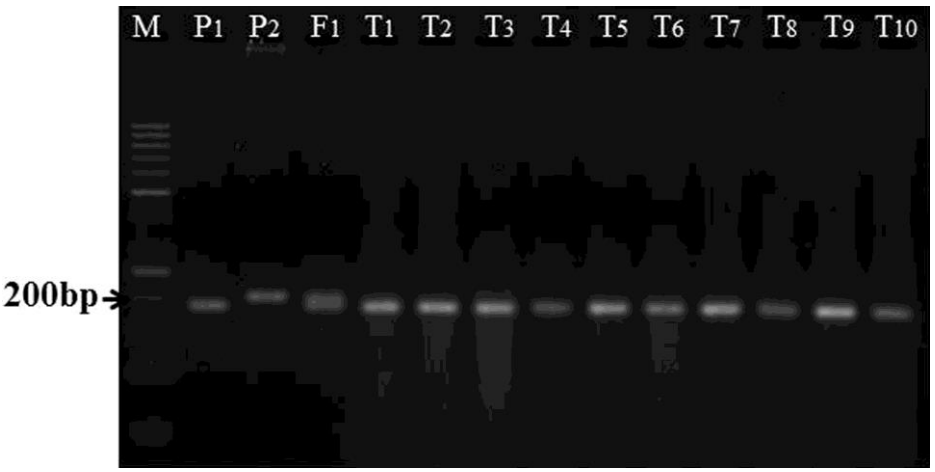

Figure 2(b): S0816 amplification banding pattern of P_1-Nistari, P_2-CSR2, F_1 and T_1-T_{10}-F_2 thermo-tolerant progeny

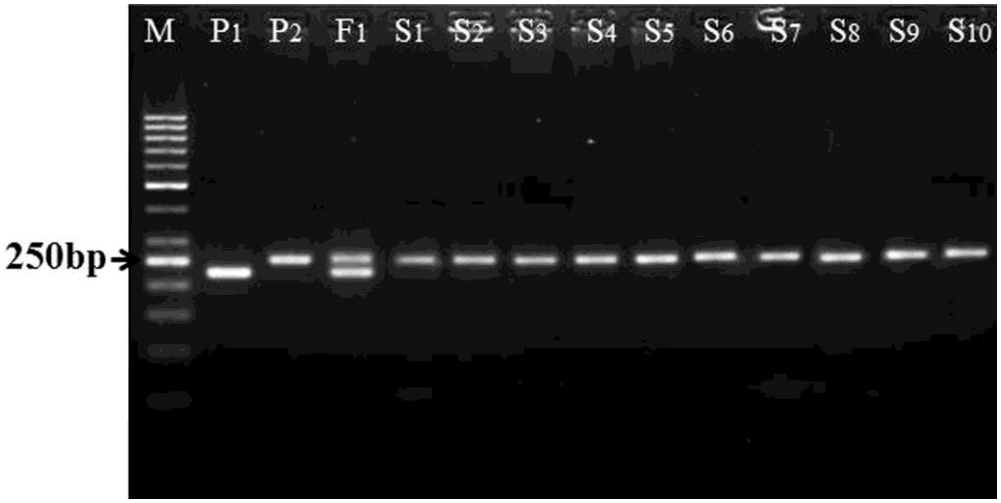

Figure 3(a): S0803 amplification banding pattern of P_1-Nistari, P_2-CSR2, F_1 and S_1-S_{10}-F_2 thermo-susceptible progeny

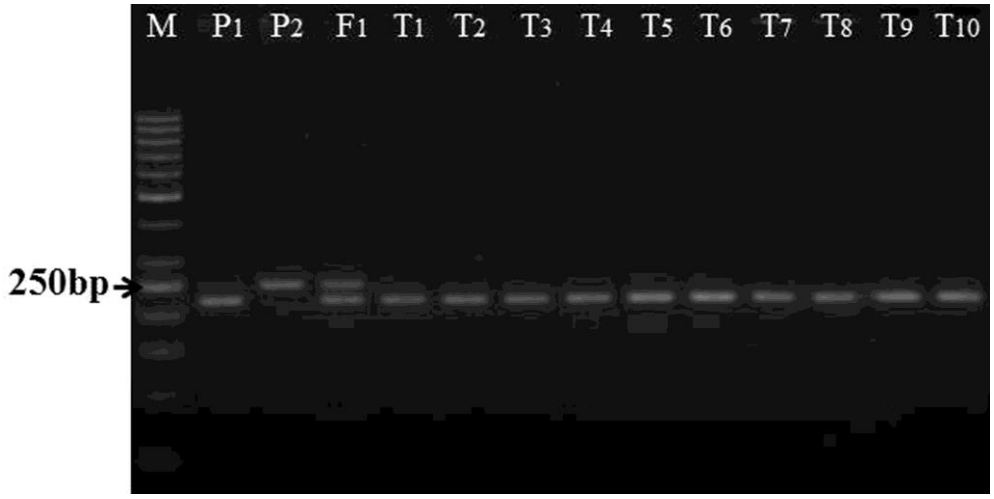

Figure 3(b): S0803 amplification banding pattern of P_1-Nistari, P_2-CSR2, F_1 and T_1-T_{10}-F_2 thermo-tolerant progeny

DISSCUSSION

Identification of genotypes with contrasting characters to develop segregating population is an important step in identification of genetic markers. The selected genotypes should be highly divergent to the trait of interest. Hence, we examined phenotypic variability for thermotolerance in

20 silkworm breeds by evaluating them at high temperature (36°C). Based on the phenotype information, Nistari and Cambodge were identified as most thermo-tolerant, SK4C and BHR3 as moderately thermo-tolerant and CSR2 as susceptible breeds. Nistari and Cambodge are multivoltine breeds and it is a known fact that multivoltines are tolerant to high temperature conditions. Of bivoltines, SK4C and BHR3 were found to be tolerant. SK4C was bred through heat esterase selection (MOORTHY *et al*., 2008) and BHR3 was bred through exposing them at high temperature over the generations (RAO *et al*, 2008) justifying our findings. CSR2 is a bivoltine breed, which is widely reared in Southern India. It was bred from highly productive Japanese hybrids at favourable temperature (SREEKUMAR *et al*., 2011).

For identifying informative markers, we have used all the four tolerant and one susceptible breeds and screened their DNA with 85 SSR markers. Of them, only 11 markers were able to discriminate tolerant and susceptible breeds. All the 11 markers showed the same banding pattern among tolerant bi- and multi-voltines. After this confirmative assay, Nistari which showed highest pupation percentage at high temperature with 2.4 times more survival rate than CSR2 was selected to develop F_2 segregating population. Furthermore the distinct polymorphism showed by 11 markers between the thermo-tolerant and susceptible breeds was confirmed by the heterozygous form of both the polymorphic products in the F_1s. Genetically, the bivoltines and multivoltines are highly divergent and thus, it is difficult to generate an allele common to tolerant bi- and multi-voltine silkworm breeds.

Bulked segregant analysis was initially proposed for screening qualitative traits known to express variation at a single locus of large effect (GIOVANNONI, 1991; MICHELMORE *et al.*, 1991). However, the simplicity and low cost of BSA have led to its use for more complex traits, including traits whose genetic control is unknown. Further, the advantage of BSA method is that it detects only tightly linked markers because crossing over generates a heterozygous pool in the F_2 and the weakness of this method is lack of statistical power as it is not be possible to know how many individuals contribute to each of the co-dominant markers in a pooled F_2 sample scored as heterozygous using PCR. KUMAR *et al*., (2002) used BSA method in silkworm to identify ten RFLP markers closely linked to the cocoon shell characters by screening low and high bulks for cocoon weight and shell character in F_2. Recently, one SNP marker has been identified to be linked to the cocoon traits on linkage group 4 of the *B. mori* through BSA using F_2 population of Pure Mysore and CSR2 (SREEKUMAR *et al*., 2011). These findings suggest the possibility of applying BSA in silkworm for identification of markers tightly linked to the thermotolerance trait. Accordingly, in this study, BSA was performed with 11 informative microsatellite markers, which revealed 5 markers tightly linked to thermotolerance in silkworm, *B. mori*.

One hundred and forty F_2 individuals of Nistari ♀ x CSR2 ♂ were genotyped by using 5 linked markers in order to determine marker-trait association through correlation analysis and to know the independent segregation of each marker by chi square test. The results revealed that all the 5 markers segregated independently. The amplification pattern of S0816 and S0803 is shown in Figure 2 (a and b) and Figure 3 (a and b), respectively. The phenotypic variation of thermotolerance in F_2 population was strongly correlated to genotypic variation of marker S0816 than others. It indicates that among the 5 linked markers, S0816 is strongly associated with thermotolerance trait in *B. mori*. These results are concurrent with the findings of ZHAO *et al*. (2010), in which they have mapped 5 markers linked to thermo-tolerant gene (*KN*) on chromosome 8 using backcross population of Chinese silkworm breeds as parents. These SSRs which often

have flanking regions highly conserved in related species, allows the use of the same primer pairs in related genomes.

In the present study, 5 (S0803, S0809, S0816, S0819 and S0820) markers linked to thermo-tolerant gene (*KN)* reported by ZHAO *et al*. (2010) were also used, but among them two (S0819 and S0820) did not generate distinct polymorphism between thermo-tolerant and susceptible breeds. However, in our study we were able to find additionally, 2 SSR markers (S0801 and S0813), which were polymorphic between parents and also associated with thermotolerance in Indian silkworms, but these markers are not reported by ZHAO *et al*. (2010) as they were not polymorphic between Chinese silkworm breeds. The possible reason may be that the marker-thermotolerance relationship was studied by ZHAO *et al*. (2010) by constructing maps based on recombination frequency that varies from one genetic background to another. We used four thermo-tolerant and one susceptible breed for SSR polymorphism which had different genetic backgrounds and hence, there is a possibility of losing or gaining some markers when compared to the markers identified in other populations. Moreover, results of phenotypic assessment of thermotolerance in F_2 populations obtained from Nistari ♀ x CSR2 ♂ and Cambodge ♀ x CSR2 ♂ indicated a significant deviation from the expected ratio of monogenic inheritance (3:1) suggesting that the trait is controlled by polygenes. The thermotolerance ability of *B. mori* tends to reduce with increase in temperature. Other than high temperature, the thermotolerance of silkworm also varies greatly depending upon the developmental stage, leaf quality and humidity.

Indeed phenotype screening of F_2 population revealed that the thermotolerance trait in silkworm might be controlled by polygenes thereby increasing the possibility of having additional markers linked to thermotolerance and involvement of many genes in providing thermotolerance to silkworm. When attempted to validate markers identified by ZHAO *et al*. (2010), we have also identified 2 more markers closely associated with thermotolerance in Indian silkworm. Hence, our study differs from other studies in emphasizing the QTL (Quantitative Trait Loci) nature of thermotolerance. Concurrent to this, in *Drosophila melanogaster*, MORGAN and MACKAY (2006) mapped seven quantitative trait loci (QTL) (3 QTLs influence cold-stress resistance and 4 affect heat-stress resistance) affecting thermotolerance on second and third chromosomes by backcrossing each recombinant inbred (RI) line to both parental lines (Oregon-R and 2b). In maize also, FROVA and GORLA (1994) identified 5 QTLs linked to injury caused by high temperature on pollen germinability and six QTLs for pollen tube growth as a measure of pollen thermotolerance. Furthermore, BARAKAT *et al.* (2011) reported that three SSR markers were linked to grain filling rate (GFR) by quantitative trait loci (QTL) analysis of the F_2 population of bread wheat. These studies confirmed that the thermotolerance trait is controlled by a QTL.

Further, we have also attempted to map the gene near to the closely associated marker to thermotolerance, which may represent thermo-tolerant gene (*KN*) per se we found 3 (BGIBMGA005249, BGIBMGA005250 and BGIBMGA005272) genes near to the tightly associated marker for thermotolerance trait of which, the BGIBMGA005249 gene was found to be located nearest to S0816 at a distance of 14.8 Kb.

CONCLUSION

This study was able to identify two more SSR markers and validated the known markers in Indian silkworm breeds. The tightly linked microsatellite marker, S0816 can be used as the target marker for screening during the breeding process. Accordingly, the marker S0816 has been used to select the thermo-tolerant bivoltine parent (donor with target gene) along with productive

parent (recurrent) and breeding program is initiated to develop bivoltine breed suitable to tropical climatic conditions of India.

Further, the biochemical mechanisms known to protect *B. mori* during high temperature conditions are heat shock proteins (Hsps) and catalase (VELU *et al*., 2008; NABIZADEH and KUMARA, 2011). Hence, as a futuristic approach the analysis of functional roles of BGIBMGA005249, BGIBMGA005250 and BGIBMGA005272 genes in the developed near isogenic line during thermal stress will be undertaken and that would provide new insights on molecular mechanisms involved in thermotolerance in silkworm.

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IDENTIFIKACIJA MIKROSATELITSKIH MARKERA POVEZANIH SA TERMOTOLERANTNOSŠĆU U SVILENE BUBE ANALIZOM BULK SEGREGANT *IU SILICO* MAPPING

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Izvod

Rast i razvoj svilene bube, kao poikilotermnog insekta, je pod uticajem faktora sredine, posebno, temperature. U tropskim zemljama kao što su India, ima značajan uticaj na proizvodnju svili zbog preovlađujućih toplih klimatskih uslova. Prethodni pokušaji da se razviju hibridi svilene bube tolerantni na visoke temperature tradicionalnim metodama uzgoja nisu dale željene rezultate. Otuda primena novih strategija, kao što su selekcija na osnovu markera (MAS), mogu biti najefikasniji za razvoj termo-tolerantne bivoltine svilene bube za održivu proizvodnju svile u Indiji. Cilj ove studije je da se identifikuju SSR markeri blisko povezani sa termotolerancijom svilene bube. 20 kukuljica su procenjeni na visokoj temperaturi (36°C), a na osnovu procenta ppljenja dva (Nistari i Kambodža) i dva bivoltines (SK4C, BHR3) identifikovani su kao termo-tolerantni i jedan bivoltine (CSR2) je identifikovan kao osetljiva rasa. Od 85 SSR markera samo 11 markera(12.9%) je pokazala jasan polimorfizm između termo-tolerantni i osetljivim rasa. Dalje, segregant analiza (BSA) je izvedena korišćenjem 11 polimorfnih SSR prajmera, upoređivanjem SSR profila od tolerantnog (Nistari) i osetljivih (CSR2) roditelja, njihove F_1 i F_2. Samo pet markera generišu jasne razlike Rezultati dobijeni analizom BSA su dodatno potvrđeni genotipizacijom sa 5 SSR markera koristeći 140 individualno F_2 potomstvo. Od 5 markera, najviši koeficijent je pokazao S0816 ukazuje na visok stepen bliskosti između genotipova i fenotipa varijacija u F_2 populaciji. Osim toga mi smo takođe pokušali da lociramo gene blizu S0816 *In siliko* pristupom i ishod je da 3 gena su bliže sekvenci na *B. mori* genomu. BGIBMGA005249 gen je utvrđeno da se nalaze najbliže S0816 na udaljenosti od 14.8 KB. Međutim, dalja istraživanja su potrebna da s eodredi odnos između termotolerantnosti i funkcionalne uloge identifikovanih gena tako da identifikovani markeri mogu da se koristiti za razvoj termo-tolerantne svilene bube kroz MAS.

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