GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

www.scienceandnature.org

IDENTIFICATION OF POTENTIAL THERMO TOLERANT BIVOLTINE SILKWORM BREEDS THROUGH PHENOTYPIC AND MOLECULAR APPROACH

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ABSTRACT
The silkworm, *Bombyx mori* is a well domesticated insect whose quantitative traits decline sharply at high temperature. Especially, bivoltines are more prone to high temperature than multivoltines. High temperature is the primary constraint in restricting the bivoltine rearing in tropical countries like India. Identification of potential parents is an essential prerequisite for the development of thermo tolerant breeds. This study aims to identify the potential bivoltine silkworm breeds tolerant to high temperature through phenotypic as well as molecular approach. The 3rd day of 5th instar larvae of ten bivoltine silkworm breeds were subjected to high temperature of 36 ± 1°C for six hours every day to till spinning. Data on Six rearing traits were collected at 25 ± 1°C (control) and 36 ± 1°C (treated). Based on pupation percentage, two groups of silkworm breeds namely thermo tolerant and thermo susceptible were identified. Five silkworm breeds namely B. Con-1 (63%), SK6 (63%), SK7 (61%), B. Con-4 (59%) and NBO-4 (58%) were found to be tolerant to high temperature. Molecular screening through well-established microsatellite markers namely S0803 and S0816 linked to thermotolerance in silkworm was also carried out. DNA amplified products of B. Con-1, B. Con-4, SK6 and SK7 showed thermo tolerant band with both the markers while D6(M), GEN-3, NBO-1, NBO-2, NBO-3 showed thermo susceptible band. The Spearman's rank correlation coefficient between pupation percentage and S0816 was higher (0.870) than S0803 (0.853). This result reveals a highly significant correlation between the pupation percentage and molecular data. This study identifies B. Con-1, B. Con-4, SK6 and SK7 as potential thermo tolerant silkworm breeds that can be used as parents in developing new silkworm breeds possessing thermotolerance through conventional or molecular breeding techniques.

KEY WORDS: *T*hermotolerance, phenotypic traits, microsatellite markers and molecular breeding

INTRODUCTION

The silkworm, *Bombyx mori* has a long history of domestication as an economically important insect. Apparently, intensive and careful domestication over centuries has deprived this commercial insect of the opportunity to acquire thermotolerance. This defencelessness is more pronounced in bivoltine breeds than in multivoltines. Lack of thermotolerance is a major factor among many factors responsible for poor performance of the bivoltine breeds under tropical conditions (Kumari *et al*., 2011; Krishnaswami, 1978a). The selection of potential parents is the foremost and crucial step in any breeding program. Earlier breeding programs aimed to develop thermo tolerant silkworm breeds through selection of parents were based on the phenotypic trait (pupation percentage) under high temperature condition. However, the phenotype is generally modified by the environment, which may be controlled by epistatic and pleiotropic genes (Van Beuningen and Busch, 1997). This is true in case of thermotolerance as this trait in silkworm is controlled by genetic and environmental factors (Kumar *et al*., 2012; Chandrakanth *et al*., 2015). Therefore, selection of parents based on phenotypic traits along with molecular marker based approach may be a better alternative for a successful breeding program as phenotype expression is highly variable due to their interactions with the environment. It is also believed that selection based on the traits linked to the DNA marker could provide a solution to this problem, as they are environmentally neutral (Knapp, 1998). Furthermore, if marker-trait associations are robust and environment × genotype interactions have been characterized, marker assisted breeding program can be employed successfully which markedly shortens the breeding period (Collard *et al*., 2005).

Molecular markers are broadly classified as PCR and non-PCR based markers. Among PCR based markers, microsatellites are noteworthy for their reproducibility, multiallelic nature, codominant inheritance and good genome coverage (Powell *et al*., 1996). They are extensively used in diversity studies, marker-trait associations, linkage and mapping analysis, marker assisted breeding and genotype selections in many animal and plant systems (Collard *et al*., 2005; Moorthy *et al*., 2017). Recently, five microsatellite markers linked to thermotolerance trait in Indian silkworm breeds have been reported, out of which two microsatellite markers *viz*., S0803 and S0816 were found to be tightly associated to the thermotolerance trait (Chandrakanth *et al*., 2015). In addition to the pupation percentage of bivoltine silkworm breeds at high temperature, microsatellite markers associated with thermotolerance (S0803 and S0816) trait can also be utilized to screen bivoltine silkworm breeds for thermotolerance. Selection based on phenotypic and genetic data would be more appropriate and will yield more information about thermotolerance of bivoltine

breeds than the selection based solely on either of them. Therefore in this study, ten bivoltine silkworm breeds were screened to identify the promising thermo tolerant bivoltine silkworm breeds through a combined molecular and phenotypic approach.

MATERIALS & METHODS

Silkworm rearing and high temperature treatment

Ten silkworm breeds namely B.Con-1, B.Con-4, D6(M), GEN-3, NBO-1, NBO-2, NBO-3, NBO-4, SK6 and SK7 were used in this study. Rearing of silkworms was conducted by adopting standard technique as suggested in Krishnaswami (1978b). Feeding was provided three times in a day with mulberry leaves of S1635 variety. Rearing was conducted under recommended temperature and humidity till 2nd day of the 5th instar. From 3rd day of the 5th instar to till spinning, 100 larvae of each breed were exposed to high temperature of 36 ± 1°C for a duration of six hours a day in a chamber called 'SERICATRON' (Environment chamber meant for controlling temperature and humidity). The larvae reared in the recommended temperature were considered as control. The mature larvae on the 7th/8th day of the 5th instar were picked and mounted on to the plastic collapsible mountages. The cocoons were harvested on 6th day after spinning and the floss of the cocoons was removed. During rearing, data on the economically important traits like larval weight (g), pupation percentage (%), yield/10000 larvae by weight (kg), single cocoon weight (g), single shell weight (g) and shell percent (%) was collected. Pupation percentage change over control was also calculated. In addition, morphological characters like larval marking, cocoon colour and cocoon shape were also recorded.

DNA extraction

Three moths of each silkworm breed were grounded with liquid nitrogen in a prechilled pestle and mortar. The homogenized tissue was transferred to a fresh Oakridge tube containing 5 ml of DNA extraction buffer (50 mmol Tris HCI, pH 8.0, 100 mmol NaCl, 20 mmol EDTA) having 100 µg/mL proteinase K and incubated for 1 hour at 55°C. The phenol/chloroform extraction was carried out with Phenol followed by Phenol:chloroform (25:24) and Phenol:chloroform:isoamylalcohal (25:24:1). The DNA was precipitated by adding sodium acetate (pH 5.2) and ethanol. Purified DNA was dissolved in 1X Tris– EDTA buffer (pH 8.0) and quantified using spectrophotometer.

PCR amplification employing microsatellite primers

The PCR reaction mixture was prepared in a 20 µL volume containing 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). PCR reactions were performed on a PTC 200 Thermocycler Engine (Bio-Rad Laboratories, Hercules, CA, USA) with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec; annealing at 56°C for 40 seconds and extension at 72°C for 1 min followed by a 10 min 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. Two microsatellite primer pairs *viz*., S0803 and S0816 which were reported to be linked to thermotolerance in silkworm were used in this study.

Statistical analysis

Data on six rearing traits of ten silkworm breeds were subjected to Independent sample T test with control and treated as two groups. The DNAs of ten breeds were amplified at two locus *viz*., S0803 and S0816 linked to thermotolerance in silkworm. These amplified bands were scored as '1' for thermo susceptible homozygous condition and '2' for thermo tolerant homozygous condition and '0' for heterozygous condition. In order to see the marker-trait relationship, the scored molecular data was correlated with the pupation percentage of the silkworm breeds at 36 ± 1ºC by using Spearman's rho correlation coefficient.

RESULTS

The rearing performances of ten bivoltine silkworm breeds at 25 ± 1ºC and 36 ± 1ºC were studied. In the studied traits, the performance of the silkworm breeds were reduced at 36 ± 1ºC when compared to 25 ± 1ºC. The morphological characters like larval marking, cocoon colour and cocoon shape of the 10 bivoltine silkworm breeds are presented in Table 1. There was no much variation in the morphological characters of silkworm breeds under study. With respect to larval marking, expect D6(M), all the silkworm breeds were plain larvae. All the silkworm breeds spun white coloured cocoon. With respect to cocoon shape, five silkworm breeds (GEN-3, NBO-1, NBO-2, NBO-3 and NBO-4) spun oval shaped cocoons and other five (B.Con-1, B.Con-4, D6(M), SK6 and SK7) spun dumbbell shaped cocoons.

TABLE 1: Morphological characters of the silkworm breeds under the study

#	Breeds	Larval marking	Cocoon colour	Cocoon shape
1	B.Con-1	Plain	White	Dumbbell
2	B.Con-4	Plain	White	Dumbbell
3	D6(M)	Marked	White	Dumbbell
4	GEN-3	Plain	White	Oval
5	NBO-1	Plain	White	Oval
6	NBO-2	Plain	White	Oval
7	NBO-3	Plain	White	Oval
8	NBO-4	Plain	White	Oval
9	SK6	Plain	White	Dumbbell
10	SK7	Plain	White	Dumbbell

Rearing performance of silkworm breeds at 25 ± 1°C and 36 ± 1°C

Six basic rearing traits *viz*., larval weight, pupation percentage, cocoon yield / 10,000 larvae by weight, single cocoon weight, single shell weight and shell percent that are considered to be important parameters for a successful silkworm rearing were recorded and analyzed in 10 bivoltine silkworm breeds at 25 ± 1°C (control) and 36 ± 1°C (treated). At 25 ± 1°C, all the breeds performed better with higher value for rearing traits compared to their performance at 36 ± 1°C. Independent sample T test revealed significant differences between control and treated groups in all the rearing traits (Table 2). The 5th instar age and total larval duration was one day lesser in treated batches when compared to control.

All the silkworm breeds performed better at 25 ± 1 ºC than at 36 ± 1°C considering all the rearing traits. Larval weight was highest in B.Con-4 (38.40 g) and lowest in NBO-2 (30.32 g). Similarly, highest and lowest pupation percentage was recorded with NBO-4 (94%) and NBO-2 (85%), respectively. Cocoon yield /10,000 larvae and single cocoon weight were highest in B. Con-1 (13.59 kg, 1.510 g) and lowest in NBO-1 (9.85 kg, 1.107 g). Single shell weight and shell percent were highest in GEN-3 (0.254 g, 20.51%) and lowest in B. Con-1 (0.219 g, 17.11%) (Table 2).

All the rearing traits were found to be declined at 36 ± 1°C when compared to 25 ± 1°C. NBO-4 and NBO-2 showed highest (26.88 g) and lowest (20.12 g) larval weight, respectively. Similarly, highest and lowest pupation percentage was recorded with B. Con-1 (63%) and NBO-2 (32%), respectively. Cocoon yield /10,000 larvae and single cocoon weight were highest in B. Con-1 (7.02 kg, 1.066 g) and lowest in NBO-2 (3.03 kg, 0.823 g). Single shell weight and shell percent were highest in GEN-3 (0.185 g, 18.32%) and lowest in NBO-2 (0.145 g, 15.42%) (Table 2).

Percentage reduction / change in pupation percentage

Percentage reduction / change in pupation percentage was calculated in the treated batches over their control. NBO-2 was severely affected by high temperature than other breeds with 62.35% reduction in pupation percentage while B. Con-1 was found to be least affected with pupation percentage reduction of 30%. Percent change in pupation percentage of silkworm breeds at high temperature over control is presented in Figure 1.

Molecular screening

The DNAs of ten bivoltine silkworm breeds were tested with two microsatellite markers *viz*. S0803 and S0816 that are reported to be linked to thermotolerance in silkworm. The amplified DNAs at both the loci S0803 and S0816 showed homozygous thermo tolerant banding pattern with B.Con-1, B.Con-4, SK6 and SK7 while D6(M), GEN-3, NBO-1, NBO-2 and NBO-3 showed homozygous thermo susceptible banding pattern (Fig. 2 and 3). But, amplified DNA of NBO-4 showed homozygous thermo tolerant banding pattern at locus S0816 and homozygous thermo susceptible banding pattern at locus S0803 (Fig. 2 and 3).

Spearman's Rho correlation coefficient

Molecular data was correlated with the pupation percentage (indicator of thermotolerance) using Spearman's Rho correlation coefficient. The results revealed that there was significant correlation between the microsatellite markers used and pupation percentage. The correlation coefficient between pupation percentage and S0816 was higher (0.870) than S0803 (0.853). The results of Spearman's Rho correlation coefficient are presented in Table 3.

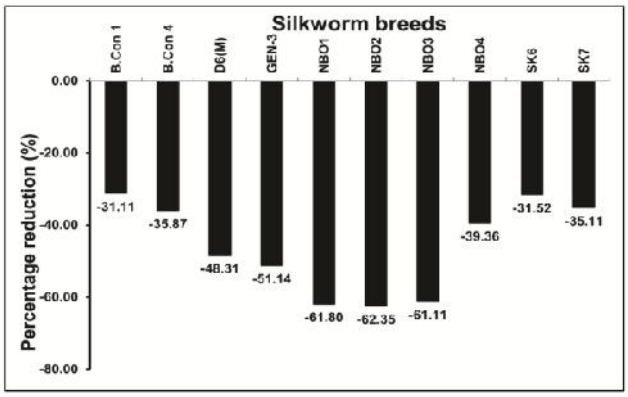
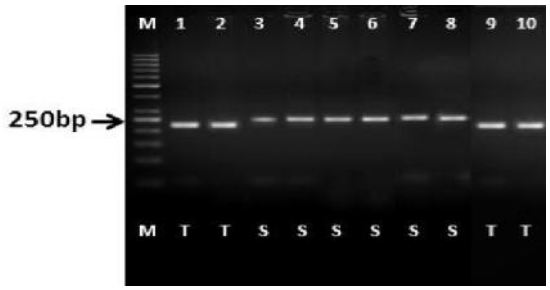


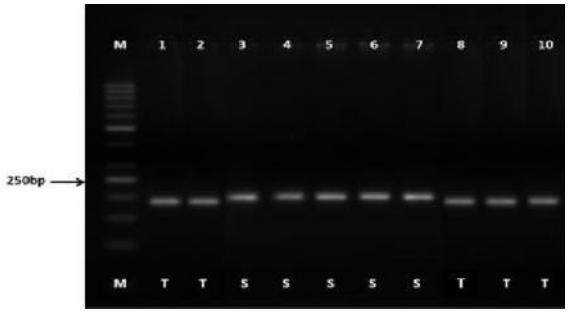
FIGURE 1: Percent changes in pupation percentage of silkworm breeds at 36 ± 1°C over control.



S- Thermo susceptible , T-Thermo tolerant

M- 50 bp ladder; 1-B.Con-1; 2-B.Con-4; 3-D6(M); 4-GEN-3; 5-NBO-1; 6- NBO-2; 7- NBO-3; 8- NBO-4; 9- SK6 and 10- SK7

FIGURE 2: S0803 amplification banding pattern of ten bivoltine silkworm breeds.



T-Thermo tolerant, S- Thermo susceptible, M- 50 bp ladder; 1-B.Con-1; 2-B.Con-4; 3-D6(M); 4-GEN-3; 5-NBO-1; 6-NBO-2; 7- NBO-3; 8- NBO-4; 9- SK6 and 10- SK7

FIGURE 3: S0816 amplification banding pattern of ten bivoltine silkworm breeds.

TABLE 2: Rearing performance of bivoltine silkworm breeds under normal and high temperature conditions

#	Breed	Larval wt. (g)		Pupation percent (%)		Cocoon yield /10,000 larvae (kg)		Single Cocoon Wt. (g)		Single Shell Wt. (g)		Shell %	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
1	B.Con-1	38.10	21.38	90	63	13.59	7.02	1.510	1.066	0.219	0.178	17.11	16.69
2	B.Con-4	38.40	26.66	92	59	13.01	6.01	1.414	0.989	0.223	0.166	17.29	16.79
3	D6(M)	32.54	21.91	89	46	11.68	4.38	1.312	0.866	0.242	0.152	19.36	17.55
4	GEN-3	34.38	24.48	88	43	10.38	4.26	1.180	0.898	0.254	0.185	20.51	18.38
5	NBO-1	35.87	24.56	89	34	9.85	3.25	1.107	0.837	0.225	0.165	19.06	17.61
6	NBO-2	30.32	20.12	85	32	10.00	3.03	1.176	0.823	0.221	0.145	18.45	15.42
7	NBO-3	30.36	20.36	90	35	11.15	3.74	1.239	0.953	0.227	0.151	18.32	15.84
8	NBO-4	34.91	26.88	94	58	11.87	6.24	1.263	1.007	0.229	0.157	18.13	15.60
9	SK6	36.11	25.00	92	63	11.39	6.88	1.238	1.028	0.225	0.165	18.17	16.06
10	SK7	34.12	23.95	94	61	12.20	6.90	1.298	1.066	0.232	0.178	17.87	16.70
	Mean	34.51**	23.53**	90.30**	49.40**	11.51*	5.17*	1.274**	0.953**	0.230**	0.164**	18.43**	16.66**
	SD ±	2.67	2.33	2.65	12.13	1.17	1.52	0.11	0.09	0.01	0.01	0.95	0.92
	Min	30.32	20.12	85.00	32.00	9.85	3.03	1.107	0.823	0.219	0.145	17.11	15.42
	Max	38.40	26.88	94.00	63.00	13.59	7.02	1.510	1.066	0.254	0.185	20.51	18.38

* Significant at P < 0.05; ** Significant at P < 0.01

TABLE 3: The results of Spearman's Rho correlation coefficient

#	Microsatellite marker	Primer sequence (5'→3')	Spearman's rho correlation coefficient
1	S0803	AAGTTCTTTACCAGTTCACAGACAGC CGCCATGCAACTGTCGTCAC	0.853
2	S0816	GAAATCCGTTTGAAGAATCCACA CATCCGTTGAATGAGTATCGTTTG	0.870

DISCUSSION

All the silkworm breeds showed a decline in the rearing parameters at 36 ± 1°C rearing temperature in comparison to 25 ± 1°C. Water is a vital component required for proper metabolic activity and maintaining optimum growth in silkworm. During higher temperatures, evapo-transpiration of water at body surfaces and respiratory epithelium of tracheal system significantly increases (Rahmathulla, 2012). This problem in association with loss of water from mulberry leaves through evaporation resulting in poor moisture content leading to the faster drying of the same makes it difficult for the silkworm to ingest it. This results in non-feeding or poor feeding of leaves by silkworm, which negatively affects the growth and in turn the productivity of silkworm (Kumar *et al*., 2002). These processes jointly increase the vulnerability of other biological processes to heat stress resulting in decline in the performance of the silkworm (Kumari *et al*., 2011). In this study, the rearing traits were gradually decreased at higher rearing temperature. Similar observation of decline in the rearing traits due to high temperature in silkworm was also reported by Kumar *et al*. (2002) and, Singh and Kumar (2010).

Many traits of silkworm are quantitative in nature. They are largely influenced by environmental factors such as temperature, relative humidity, light and nutrition (Ramesha *et al*., 2010; Zhang *et al*., 2002). Therefore, assessing the degree of phenotypic difference of the quantitative traits is important to understand the genetic steadiness of breeds under varied environmental conditions. In the current study, the phenotypic expression of six traits of economic importance in ten silkworm breeds under 25 ± 1°C and 36 ± 1°C were tested. The variations observed in the phenotypic manifestation for the traits measured can be attributed to the genetic constitution of the breeds and their degree of expression which was influenced by the high temperature during rearing. Five silkworm breeds, B.Con-1, B.Con-4, NBO-4, SK6 and SK7 were found to be tolerant with high pupation percentage of >55 and were also found to be stable with less percentage reduction in pupation percentage at high temperature. Similarly, five silkworm breeds, D6(M), GEN-3, NBO-1, NBO-2 and NBO-3 were found to be thermo susceptible with <55% pupation percentage at high temperature and were severely affected by high temperature than other silkworm breeds. Similar studies were also confirmed by Kumari *et al*. (2011) with different silkworm breeds. APS24 and APS12 silkworm breeds were found to be thermo tolerant by rearing at 32 ± 1°C in the 5^{th} instar (Laksmi *et al*., 2011). Pupation percentages at three different temperatures were recorded by Reddy *et al*. (2002) in cross breed by employing the same procedure of the present study for high temperature treatment. Kumari *et al*. (2011) screened 24 bivoltine breeds of the germplasm by subjecting to high temperature treatment every day from 5^{th} instar 3^{rd} day to till spinning at 36 ± 1°C and identified BD2-S, SOF-BR and BO2 as tolerant breeds, and BO1-S as susceptible.

Current study reveals a negative relationship between the tested temperatures and the rearing parameters of silkworm breeds. Significant differences revealed through Independent sample T test between control and treated groups indicate that high temperature significantly causes reduction in the rearing traits. Five silkworm breeds namely B.Con-1, B.Con-4, NBO-4, SK6 and SK7 were found to be tolerant to high temperature. Among them, B.Con-1 and SK6 showed highest pupation percentage and less percentage reduction in pupation percentage over controls at high temperature. Furthermore, molecular studies conducted also suggests that silkworm breeds namely B.Con-1, B.Con-4, SK6 and SK7 had thermo tolerant gene in their genetic makeup, which was confirmed in both the microsatellite markers but NBO-4 showed association with thermo tolerant gene only with respect to S0816 but not with S0803. Hence, it cannot be used for molecular breeding programs as parents. Similarly, five silkworm breeds, D6(M), GEN-3, NBO-1, NBO-2 and NBO-3 showed thermo susceptible banding pattern with respect to both the microsatellite markers linked to thermo tolerant gene. Similar molecular studies were also conducted by Zhao *et al*. (2010) and Chandrakanth *et al*. (2015). This kind of approach to screen the bivoltine silkworm germplasm both phenotypically and through molecular markers can yield more effective results in selecting the parent for either conventional or molecular breeding programs. Four silkworm breeds identified as thermo tolerant in this study can be effectively utilized for conventional as well as molecular breeding to develop new thermo tolerant silkworm breeds/hybrids.

CONCLUSION

From this study, based on the pupation percentage, percentage reduction in pupation percentage at high temperature over control and screening through molecular marker associated with thermotolerance, it can be confirmed that B.Con-1, B.Con-4, SK6 and SK7 are tolerant to high temperature. Therefore, these silkworm breeds can be effectively utilized for conventional as well as molecular breeding programs to develop new thermo tolerant silkworm breeds/hybrids.

ACKNOWLEDGMENT

We thank Central Silk Board for the financial assistance for carrying out the work.

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