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MOLECULAR DYNAMICS OF GENOMIC DNA OF SILKWORM BREEDS FOR SCREENING UNDER HIGHER TEMPERATURE REGIMES UTILISING ISSR-PRIMERS

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Abstract: An experimental design was programmed to understand the level of dynamics of gDNA of silkmoth and the screening the parameters of the silkworm is relation to higher temperature regimes. The Bivoltine silkworm breeds namely, CSR_2, CSR_4, KA, NB_4D_2 and JROP were chosen for the investigations and exposed to the higher temperature of $35+1^0C$, $40+1^0C$ understood that these breeds are highly sensitive towards extreme temperature under natural condition, however the development of robust hardy bivoltine silkworm breeds at the age of 5^{th} instar imposed to $35+1^0C$, $40+1^0C$ in the BOD incubator for about 4hrs daily with the interval of 1hr gap till the day of commencement of spinning for about 7 different generations in order to built up a increased range of tolerance in the system and investigated the gDNA pattern and banding profile in the selected breeds by utilizing 5 different UBC-primers as a marker in parental breeds and also different combinations of hybrids of silkworm and constructed the dendograms by using UPGMA method indicating with boot strap values. The gDNA is unique for the polymorphism of the banding pattern in the amplification of ISSR-PCR products and the phylogenetic inference was drawn on the basis of an evidence of dissimilarity index (I-F). Based on the result obtained in the present study the highest level of temperature tolerance observed in KA and CSR_2 breeds with low level of genetic distance between the breeds on the basis of gene frequency evidenced by the boot strap values in the constructed dendogram with the help of molecular markers.
Key Words: gDNA, ISSR- primers, Silkworm Breeds and Higher temperature.

INTRODUCTION

The overall production of raw silk is a reflection of the genetic endowment of the silkworm breeds concerned to its racial trait. Any given genetic endowment of an organism has its own range of reaction in a given environment. Although several characters in *Bombyx mori* L. exhibit mendelian inheritance, the quantitative traits of economic importance are under control constituents of polygenic inheritance. It is obvious that the most of the

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quantitative characters are affected by both genetic and environmental factors (Bulmer, 1980). There is a vast array of polygenic variability controlling the quantitative traits in silkworm, *Bombyx mori* L. It was estimated that for one economic trait, in two silkworm genotypes reared under two environments, as many as 24 different G × E interactions are possible (Allard and Bradshaw, 1964). However, our present understanding of G × E interactions is that, by rearing the breeds in laboratory environment precise predictions cannot be made about their phenotypic expression in different environments but for measuring their phenotypic and genetic mean as well as the environmental component of variance. Nevertheless, the results of breed × location interaction studies have shown that in general, the performance of the breed itself will be the best indicator of the suitability of its genotype to the prevailing environment. In view of the above, it is opined that selection of the genotype based on their performance in the native environment would help to integrate various factors leading to isolation of region / season specific tropical breeds for commercial exploitation. The environment can have direct effect on the phenotype, for example through nutrition, disease incidence and management, but can have no effect on the genotype. The environment can indirectly affect the genotype and the alteration of gene frequency so that, only certain experimental animals are selected as parents for the next generation and others are ignored.

Materials and Methods: Two disease free layings of the five bivoltine breeds namely, NB_4D_2, KA, JROP, CSR_2 and CSR_4 each about 450-500 eggs were obtained from Germplasm Bank, Department of Studies in Sericulture Science, Mysore. The eggs were incubated at 25±1^{o}C temperature and 80-85% relative humidity for about 10-12 days till their hatching. Wooden trays of 90 × 70 × 10 cm in size were washed with water, dried and disinfected with 2-4% formalin solution and covered with paraffin paper of the same size. The layings were black boxed at blue egg stage in order to obtain uniform hatching and the hatched larvae were brushed on clean and labeled wooden trays. The larvae of all the selected bivoltine breeds were reared under standard rearing conditions on four feeding schedules (Krishnaswami, 1978). Mulberry leaf of M_5 variety (*Morus alba*) harvested from the garden of Sericulture department and were chopped into suitable size according to stage of larvae fed to the silkworm. The silkworm bed was cleaned after II moult and thereafter every day. Healthy silkworms were maintained till the day spinning. The ripened healthy worms were allowed to spin the cocoons on bamboo mountages. The cocoons were harvested on 5th day of spinning and selection of cocoons were made on following day and preserved in wooden

trays. The moths emerged on 10^{th} to 12^{th} day were allowed for pairing for about 4 hours. The depaired females were retained in the cellules on craft brown paper for oviposition. The egg sheets were disinfected by dipping in 2% formalin solution and dried after washing in running tap water at room temperature. The female moths, after oviposition were subjected to pebrine detection by single mother moth examination. The layings were treated to hydrochloric acid with a specific gravity of 1.04 within 24 hours of oviposition to prevent embryonic diapause development and to continue the embryonic development in the following generation The ambient temperature (28±1°C) was the control treatment and was based on tropical countries where the air temperature in summer is 35-40°C in the daytime. Two imposed temperatures of 35±1°C and 40±1°C were tested using a BOD (Biological Oxygen Demand) incubator. The temperature stressed larvae were exposed to 2 × 2 hour periods at a high temperature (35±1°C or 40±1°C) separated by a 4 hour "rest" period at 28±1°C. Control batches were maintained at a constant 28±1°C. The exposing duration to thermal stress was started from the first to fifth day of fifth instar. Appropriate plastic boxes (25 × 18 × 7 cm in size) with a net lid were made and used to transfer larvae from the rearing house to the BOD. After the heat treatment, the tested larvae were transferred to the standard rearing condition at 28±1°C. All experimental silkworms in control and treated batches were not fed during incubation and however fresh mulberry leaves were fed 15 minutes after the temperature treatment. The humidity in the BOD was maintained as in rearing house about 75 ±2 % by using wet foam rubber pads. Sheets were disinfected by dipping in 2% formalin solution and dried after washing in running tap water at room temperature. The female moths, after oviposition were subjected to pebrine detection by single mother moth examination. The layings were treated to hydrochloric acid with a specific gravity of 1.04 within 24 hours of oviposition to prevent embryonic diapause development and to continue the embryonic development in the following generation.

RESULTS

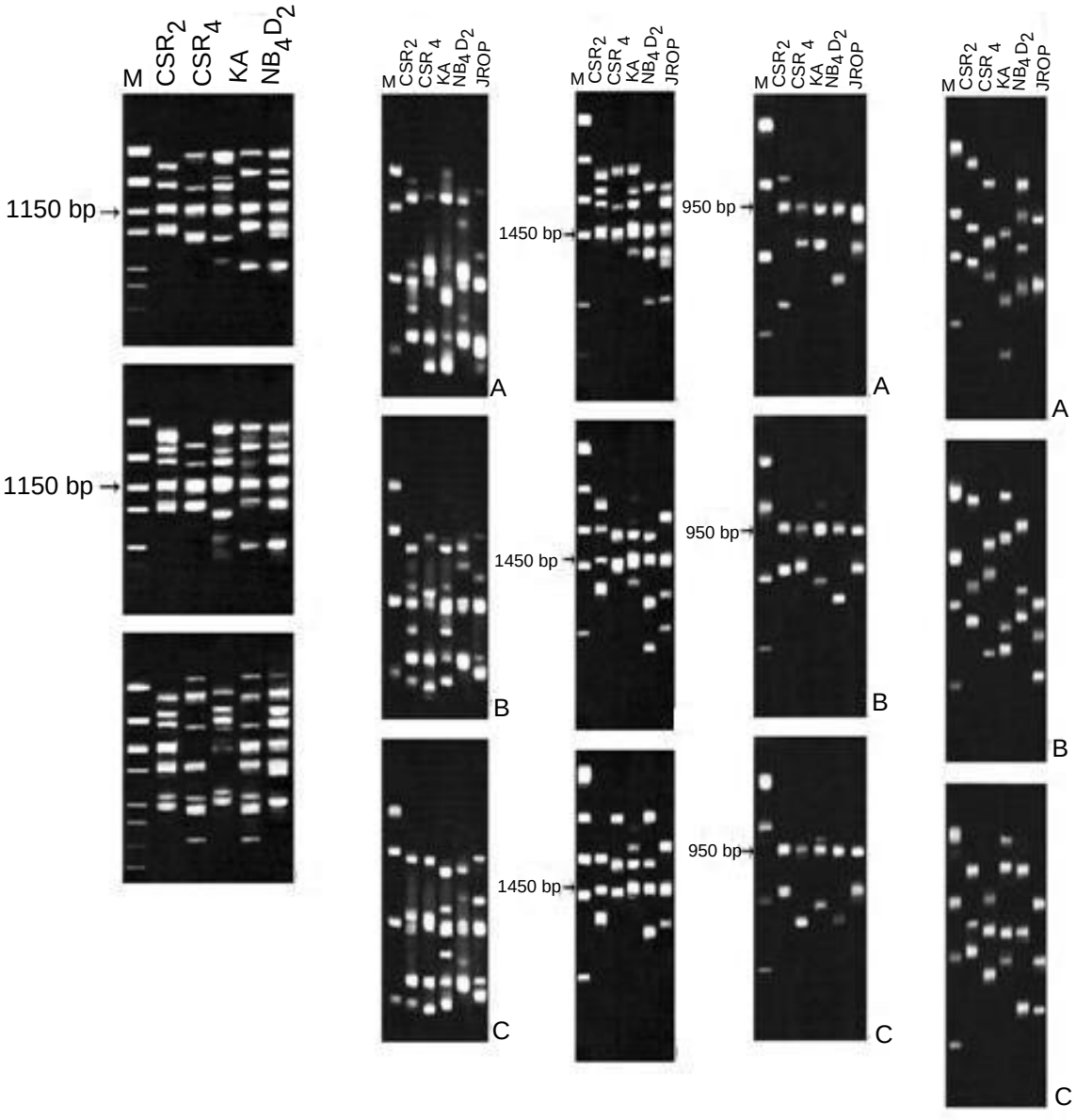
To analyze and study the ISSR banding patterns, among the twelve different UBC primers were used initially for screening with gDNA in one of the breeds namely, JROP. Finally, five UBC primers were selected used based on quality of ISSR-PCR products obtained to carryout ISSR on gDNA samples of all the selected bivoltine breeds throughout the investigations The ISSR-PCR was performed three times with the selected primers and an excellent reproducible and informative profile was obtained for all the breeds .The bands were scored with care by

gel documentation system, Image acquisition and analysis software, Version 3000, Alpha Innotech Corporation, Copyrights 1993-2009 and large amount of polymorphism was recognized both at interspecific as well as intraspecific level.

Amplified products of the five selected bivoltine breeds after seven continuous generations at ambient temperature (28±1°C). 94 products (bands) were scored and the size of the bands ranging from 340-1820 bp Out of 94 fragments, 82 (87.23%) were polymorphic Thus, the resultant ISSR assay with 94 bands from five primers showed both quantitative and qualitative profile of the five selected bivoltine breeds after seven continuous generations at ambient temperature condition (28±1°C). In relation to quantity of scorable products of the each separate primer, most of the primers yielded more than 15 bands in all the breeds, as well as the primers UBC-809 and UBC-842 revealed the maximum of 26 and minimum of 11 of bands respectively.

Amplified products of the five selected bivoltine breeds after seven continuous generations at 35 ± 1°C, 90 products (bands) were scored and the size of the bands ranging from 340-1800 bp Out of 90 fragments, 69 (76.67%) were polymorphic Thus, the resultant ISSR assay with 90 bands from five primers showed both quantitative and qualitative profile of five selected bivoltine breeds after seven continuous generations at 35±1°C temperature. In relation to quantity of scorable products of the each separate primer, most of the primers yielded more than 15 bands in all the breeds, as well as the primers UBC-809 and UBC-842 revealed the maximum of 25 and minimum of 10 of bands respectively. Amplified products of the five selected bivoltine breeds after seven continuous generations at 40 ± 1°C A total of 95 products (bands) were scored and the size of the bands ranging from 340-1840 bp. Out of 95 fragments, 87 (91.58%) were polymorphic Thus, the resultant ISSR assay with 95 bands from five primers showed both quantitative and qualitative profile of five selected bivoltine breeds after seven continuous generations at 40±1°C temperature. In relation to quantity of scorable products for the each separate primer, most of the primers yielded more than 15 bands in all the breeds, as well as the primers UBC-809 and UBC-842 revealed the maximum and minimum number of bands at 40±1°C, respectively by 30 and 10.

ISSR and Phylogenetic inference and data analysis based on the formula of Nei and Li (1979). The dissimilarity index (1-F) value of each pair wise comparison of ISSR scored products were obtained from the five selected bivoltine breeds at three different temperature and ten selected bivoltine hybrids from respective control and temperature based parents. The relationship of the breeds was resolved on dendrograms (parsimony tree of UPGMA).



Phylogenetic inference of the five selected bivoltine breeds after exposure of seven continuous generations at ambient temperature condition (28±1°C). The perusal of data shows that the least 1-F value among all the breeds at ambient temperature condition (28±1°C) was 0.305 between NB_4D_2 and CSR_2 and the highest was revealed 0.659 between NB_4D_2 and KA breeds. The relationship between the five selected bivoltine breeds after seven continuous generations at ambient temperature (28±1°C) condition is represented in the form of dendrogram. In the dendrogram, the information is clear and supported by Bootstrap values. CSR_2 is closer to KA breed as well as JROP, NB_4D_2 and CSR_4 were placed in

separate positions based on distance of Bootstrap values.

Phylogenetic inference of the five selected bivoltine breeds after exposure of seven continuous generations at 35 ± 1°C temperature regime. The study of data shows that the least 1-F value among all the breeds at 35±1°C temperature was 0.300 between JROP and CSR_2, while the highest was 0.590 between NB_4D_2 and CSR_2 breeds. The relationship between the five selected bivoltine breeds after seven continuous generations at 35±1°C temperature was shown in the form of dendrogram. In the dendrogram, the information is clear and supported by Bootstrap values. KA is closer to CSR_2 breed as well as JROP, NB_4D_2 and CSR_4 are placed in separate positions with an evidence of distance of Bootstrap values.

Phylogenetic inference of the five selected bivoltine breeds after exposure of seven continuous generations at 40 ± 1°C temperature regime. The perusal of results shows that the least 1-F value among all the breeds at 40±1°C temperature was 0.218 between KA and CSR_2, while the highest was 0.571 recorded between KA and CSR_4 breeds. The relationship of all the five selected bivoltine breeds after seven continuous generations at 40±1°C temperature has shown an evidence in the dendrogram. The information is clear and supported by Bootstrap values. KA is closer to CSR_2 breed as well as NB_4D_2 and CSR_4 are placed in the same position. Further, JROP breed is placed in separate positions.

DISCUSSION

The ISSR-PCR technique shown to be a reliable and reproducible in molecular biology for comparative and evolutionary genetical distance studies in the silkworm, L. (Sethuraman *et al*., 2002). ISSR and SSRs, due to their abundance and dispersal in the genome, have been preferred to study the relationship between closely related populations (Deshpande *et al.,* 2001). The present ISSR profile is highly reproducible, consistent and informative of all the primers used. The reproducibility of ISSR assay lies in the principle of ISSR-PCR. The ISSR assay is based on the use of primers which are not arbitrary, but designed a prior to anchor to anonymous simple sequence repeat (SSR) loci (Zietkiewicz *et al*., 1994; Wolfe *et al*., 1998) and are long and repetitive in nature. The primers require a stringent annealing temperature and due to low primer-template mismatch the ISSR-PCR yields highly reproducible patterns. The present assay has given more emphasis and an excellent consistent and reproducible profile.

The ISSR assay has yielded multiple products with differential molecular weight that ranges from 200-3000 bp. The present study has proven the efficiency of ISSR-PCR in

generating high level of polymorphism in the bivoltine silkworm breeds. Genetic diversity and differentiation among populations of the Indian eri silkworm, *Samia Cynthia ricini*, revealed by ISSR markers (Vijayan *et al.,* 2006).Genetic differentiation induced by artificial selection through four continuous generations in an inbred population of the silkworm, revealed by RAPD and ISSR marker systems (Appukuttan *et al*., 2005). The high variability in the number of the band products amplified by all the primers in the present investigation and suggests the hyper variable nature and type of simple sequence repeats in the genomes of different breeds and hybrids. The great number of distinct products amplified by 5 selected primers were recorded and high amount of polymorphic bands revealed that the ubiquitous and hypervariable nature of ISSR markers and it suggests that the applicability of ISSR-PCR in genome fingerprinting at the interspecies level (Zietkiewicz *et al*., 1994).

The results show that, the performance of ISSR primers varies across the texa which reflects different relative frequencies of microsatellite motifs in the genome of different silkworm breeds and hybrids. In the present investigation, the genetic differentiation induced by artificial selection through seven continuous generations in the silkworm inbred populations could be a consequence of random drift, which causes changes in gene frequencies reflected in changed generation means (Falconer and Mackay 1996). The higher degree of polymorphism generated by ISSR primers in the selection of lines denotes the abundance and variation of SSRs among them (Russel *et al*., 1997). Continuous selection and inbreeding could have induced ahomozygous state of the recessive gene (Strunnikov 1995). That could be accreditedto the selection of the allelic variants that influence the phenotypic characters (Chani *et al.*, 2002). Such a distortion of loci (RFLPs and RAPDs) was observed in barley (Graner *et al.,* 1991, Manninen 2000), potato (Rivard *et al.,* 1996) and in microspore-derived rice (Xu *et al.*, 1997). Stop codons are AT-rich and it can be presumed that mutation of pressure plays a significant role in the occurrence and distribution of stopcodons (Xia *et al*., 2003).Variation in the number of the bands per primer per sample (different groups of breeds and hybrids at different conditions) among all the samples indicated that, the SSRs are relatively abundant and hyper variable and reflects the type of repeats in the genomes of the present study. Present results suggest that inter-SSR-PCR can be used to identify the presence of the repeated elements targeted by the different primers used and to evaluate their distribution within different genomes (Zietkiewicz *et al.*, 1994). Perusal of dendrograms in the present ISSR assay shows that, in the case of selected bivoltine silkworm breeds during seven generations exposure to stressful condition at 40±1°C, the breeds with dumbell shaped

cocoon (NB_4D_2 and CSR_4) clustered in the same ladder, obviously. It can be explained due to magnitude of genetical similarity in a developmental and evolutionary duration through an adaptability procedure by selection at 40±1°C temperature. As well as, all temperature regimes CSR_2 and KA breeds were identified at the same cluster. Study of hybrids in relation to dendrograms shows the different clustering of the same hybrids prepared from two different groups of the parents control groups and improved groups. Further, the change in the gene frequency of the same parents' in relation to be thermo tolerant after seven generations artificial selections under undesirable and stressful temperature condition was confirmed on the basis of results obtained. Based on the clustering of stress based improved hybrids shown lesser variation in clusters it can be suggested that, similarity in the gene frequency becomes more in the breeds and accordingly in their F1 hybrids after artificial selection of parents through several generation under imposed higher temperature regimes.

CONCLUSION

Five selected bivoltine breeds of the silkworm, namely NB_4D_2, KA, JROP, CSR_2 and CSR_4 were reared under ambient and imposed temperature regimes for about seven continuous generations from control and breeds exposed to 40±1°C and silk moths of above said breeds were used throughout the research work. Five different primers namely, UBC-809, UBC-834, UBC-834, UBC-842 and UBC-844 with a specific sequence (AG)8G, (AG)8YT, (AG)8YC, (GA)8YG and (CT)8RC respectively were used to understand the gDNA through analysis of ISSR-PCR profile under imposed acute thermal regimes like 35±1°C and 40±1°C in silk moth adult stage of selected bivoltine breeds. The pattern of analyzed amplified products using five selected primers resulted at 28±1°C (control) in the form of bands profile were scored totally by 94 bands and size of the bands ranging from base pairs of 340-1820. The percentage of polymorphic bands was 87.23 % and recorded. At 35±1°C, the pattern of variation in the amplification of ISSR-PCR products was relatively less and scored by 90 with 76.67% polymorphic in nature in the expression of pattern of bands were observed. At 40±1°C, the polymorphism in gDNA was noticed by 91.58% among the 95bands scored. The genomic DNA on the basis of ISSR analysis in all the five UBC primers in relation to temperature regimes calculated by dissimilarity index (1-F) value of Nei and Li (1979). The phylogenetic inference was drawn on the basis of an evidence of dissimilarity index (1-F) the relationship was represented in the form of dendrogram with Bootstrap values and showed CSR_2 breed is genetically closer to KA breed followed by

JROP, NB_4D_2 and CSR_4 respectively. The values of Bootstrap indicated a distance between NB_4D_2 and CSR_4 was recorded highest, and lowest distance between JROP and CSR2 were observed as evidence in the genetic analysis at 35±1°C. The breeds namely KA and CSR_2 were placed in the same cluster on dendrogram with a lower level of distance under extremely high temperature condition (40±1°C), whereas the highest level of distance was observed between KA and CSR_4 breeds. Mostly, the resulting gene frequency and arrangement of gDNA base pairs in total could be attributed in the manifestation genetic distance between the breeds. Among the ten selected bivoltine hybrids under ambient temperature (28±1°C) the hybrids namely CSR_2 × KA and CSR_4 × KA were closer relationship with an evidence of lesser distance value of Bootstrap in the construction of dendrogram. It was interestingly observed that, the CSR_4 and NB_4D_2 breeds with dumbbell cocoon shape were placed at most close interaction in all the three dendrograms obtained under ambient and imposed temperature regimes. Based on all the results obtained, artificial selection during seven continuous generations generally caused lesser genetic distance between the breeds and hybrids were investigated.

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