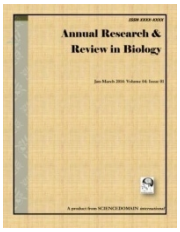

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Inter Simple Sequence Repeat (ISSR) Markers for Assessment of Genetic Polymorphism and Phylogenetic Relationships of the Silkworm *Bombyx mori* L.

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Authors' contributions

This work was carried out by the author SB. The authors designed the study, wrote the protocol, and wrote the author SB of the manuscript. All authors read and approved the final manuscript for publication.

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ABSTRACT

An attempt was made to understand the genetic polymorphism and phylogenetic relationships among the eighteen silkworm races/breeds comprising six each of bivoltines (BVs), multivoltines (MVs) and autosomal mutants of the silkworm *Bombyx mori* belonging to both indigenous and exotic origins, which were drawn from the germplasm of the Department. The DNA was extracted from the whole moth and amplification was carried out with inter simple sequence repeat (ISSR) marker technique. Four ISSR primers namely UBC-812, UBC-813, UBC-826 and UBC-827 were utilized of which two primers UBC-812 and UBC-826 generated a total of 88 clear, distinct and reproducible bands ranging from 500bp to 2000bp corresponding to an average of 44 bands/primer. Among 88 bands 18 bands were monomorphic and remaining 70 were polymorphic. Thus, the percentage of polymorphism revealed for UBC-812 primer was 100%, whereas UBC-826 revealed 60.86% indicating the occurrence of genomic variation from the inbred population.

The unweight pair group method with arithmetic means (UPGMA) was constructed from the ISSR marker profiles have clearly discriminated the BVs, whereas MVs and an autosomal mutant namely *pre* and a BV race namely C_{108} were genetically inter-linked together in cluster-I and II respectively. The significance of genetic polymorphism and phylogenetic relationships through the ISSR study are herein discussed.

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Keywords: Bombyx mori; genetic polymorphism; phylogenetic relationship; ISSR markers.

1. INTRODUCTION

The conservation of genetic diversity both within and among natural populations is a fundamental goal for conservation of biological science. Henceforth, the knowledge utilizing different biotechnological methods are prioritized and also an essential component of plant and animals (as well as insects) resource management and they are becoming increasingly important for the conservation strategies utilizing different markers. However, a DNA molecular marker in essence detects nucleotides sequence variation at a particular location in the genome. The genetic variation/diversity must be found between the parents of the chosen cross for the marker to be informative among their offspring and to allow its pattern of inheritance to be analyzed. DNA markers can generate fingerprints, which are distinctive patterns of DNA fragments resolved by PCR- agarose-electrophoresis and detected by staining or labeling. The advent of the PCR was a breakthrough for molecular marker techniques and made possible many fingerprinting methods.

Hence, it may clear that, the sericulture scientists efficiently have been attempting different PCR marker techniques at molecular level are RAPD, AFLP, RFLP, SSR *etc*. These markers were extensively used to describe the genetic constitution of an individual and also be used to determine the genetic polymorphism, diversity and relationship, *etc* existing in a population. Among the all marker techniques, the ISSR are most widely applied and probably because they do not require the knowledge of genome sequences, protocol is relatively simple, rapid and cost effective and also high reproducible in nature, which has being proved by various sericulture scientists *viz*., [1,2,3,4,5,6,7] in the silkworm *Bombyx mori.* Apart, in several plants too, ISSR markers have been successfully used for the assessment of genetic diversity in Corn [8] and for linkage to a specific gene [9], for cultivar identification in Oil seed rape and potatoes [10,11], for mapping of plant chromosomes [12]. Keeping the above concept an ISSR-PCR based advanced molecular technique is utilized to enrich the knowledge of molecular genetics of the silkworm *Bombyx mori*.

In the present study, an effort is being made to assess the genetic polymorphism and phylogenetic relationships through ISSR analysis based on their gene pool of origin more accurately among the selected eighteen races/breeds of the silkworm *Bombyx mori.*

2. MATERIALS AND METHODS

2.1 Silkworm Races/Breeds/Mutants and DNA Extraction

Two voltine groups, six each of bivoltines *viz,* C_{108}, Kalimpong-A (KA), NB_4D_2, CSR_2, P_{31} and NB_{18}, multivoltines, Pure Mysore (PM), Nistari, C.nichi, MU_1, *npnd* and Hosa Mysore (HM) and six autosomal mutants, zebra, ursa, knobbed, pere, lemon and pre of mulberry silkworm (*Bombyx mori*) were drawn from the germplasm of Department of Studies in Sericulture, University of Mysore. After incubation of eggs at 25±10ºC and relative humidity of 80±5%, black box was carried out on the 8^{th} day to achieve uniformity in hatching. Single laying of each of the pure races was selected. The larvae hatched from each laying were reared separately under uniform laboratory conditions [13] and their qualitative and quantitative traits are enlisted in Table 1. For DNA extraction, whole moth body tissue from ten individuals belonging to two sexes selected at random was collected from all the genotypes according to the procedure followed for different plant and animal tissues [14]. DNA was quantified utilizing 0.8% agarose gel electrophoresis and diluted to a uniform concentration (10ng/µl) for ISSR finger printing.

Table 1. Distinguished characters of 18 races/breeds/mutants selected for the present study

Races/ Breeds	Voltinism	Larval characters	Cocoon colour	Cocoon shape	Larval duration (h)	Single cocoon weight (g)	Single shell weight (g)	Shell ratio (%)	Origin
C_{108}	BV	Plain	White	Oval	552±2.05	2.05±0.016	0.398±0.007	19.41± 0.015	China
KA	BV	Plain	White	Oval	557±2.06	1.88±0.030	0.390±0.006	20.74±0.017	India
NB_4D_2	BV	Plain	White	Dumb-bell	557±2.04	2.00±0.031	0.388±0.007	19.40±0.016	India
CSR_2	BV	Plain	White	Oval	562±2.81	2.20±0.02	0.424±0.003	21.20±0.013	India
P_{31}	BV	Marked	White	Dumb-bell	547±1.10	2.00±0.01	0.389±0.002	19.45±0.011	Exotic
NB_{18}	BV	Plain	White	Dumb-bell	552±3.00	2.20±0.032	0.399±0.009	19.00±0.016	India
PM	MV	Plain	Light green	Spindle	629±4.51	1.00±0.02	0.140±0.002	14.00±0.015	Indo-China
Nistari	MV	Marked	Golden yellow	Spindle	504±2.00	1.06±0.01	0.138±0.002	13.01±0.013	India
C.nichi	MV	Plain	White	Dumb-bell	486±1.51	0.92±0.01	0.108±0.001	11.73±0.010	India
MU_1	MV	Plain	Green	Oval	525±2.01	1.30±0.02	0.211±0.002	16.23±0.016	India
$npnd_2$	MV	Marked	White	Oval	480±1.10	0.91±0.01	0.106±0.001	11.64±0.011	Japan
HM	MV	Plain	Dark green	Oval	525±1.28	1.16±0.02	0.184±0.002	16.14±0.014	India
zebra	BV	Marked	White	Dumb-bell	542±1.85	1.86±0.028	0.312±0.002	16.77±0.010	Exotic
ursa	BV	Marked	White	Dumb-bell	582±2.70	1.80±0.029	0.280±0.008	15.55±0.012	Exotic
knobbed	BV	Plain	White	Dumb-bell	528±1.85	1.90±0.02	0.330±0.002	17.36±0.012	Exotic
pere	BV	Marked♀♀ Plain♂♂	White	Dumb-bell	504±1.28	1.92±0.01	0.327±0.002	17.03±0.015	Exotic
lemon	BV	Plain	White	Dumb-bell	509±1.20	1.60±0.01	0.262±0.002	16.37±0.010	Exotic
pre	MV	Marked	White	Oval	510±1.25	1.36±0.01	0.227±0.002	16.69±0.010	Exotic

Index: BV= Bivoltine, MV= Multivoltine,

Table 2. List of ISSR primers and their sequences/status of polymorphism

Sl. No.	Primers	Primer sequences	Monomorphic bands	Polymorphic bands	% of polymorphism
1	UBC-812	GAGAGAGAGAGAGAGAA	0	42	100
2	UBC-826	ACACACACACACACACC	18	28	60.86
3	UBC-813	CTCTCTCTCTCTCTCTT	-	-	-
4	UBC-827	ACACACACACACACACG	-	-	-
	Grand total		18	70	160.86
Average percentage of polymorphism from the UBC-812 & UBC-826 primers					80.43

2.2 ISSR Finger Printing

PCR amplification of genomic DNA with primers were done according to the standardized procedure [15] on a Multi Gene-II Research Thermal Cycle (Labnet International Inc, Central Ave, Union city, California, USA) using 20µl of reaction mixture. The PCR schedule followed was 94ºC for 2 minutes of 1 cycle (Pre-denaturation step), followed by 35 cycles of 94ºC for 30 seconds (Denaturation step), followed by 50ºC of 35 cycles for 30 seconds (Annealing step), then 35 cycle's extension step at 72ºC for 2 minutes followed by elongation extension step at 72ºC for 10 minutes of 1 cycle. The PCR product was separated on 1.5% agarose gel using a constant voltage of 50v from Bio-Tech 500 power package. PCR product was loaded with 3µl of tracking dye and run until the dye was 7-8 cm from the wells. The profiles were stained with ethidium bromide and documented by the Alpha Innotech Gel Documentation System (US) and the bands were numbered sequentially from higher to lower based on molecular weight for each profile. Four ISSR primers namely UBC-812, UBC-813, UBC-826 and UBC-827 (obtained from the University of British Colombia, Canada) were selected (Table 2) for present study. The data obtained were analyzed and bands observed were scored as present (1) or absent (0) directly from the profiles. The experiment was repeated twice and bands with prominent and reproducible were selected for the analysis. The statistical measures on percentage of polymorphism were determined using the computer software package pop gene version 1.31 [16].

3. RESULTS

All amplification reactions were carried out at least thrice in order to make sure consistency and repeatability of fingerprints generated using selected ISSR primers. All the DNA bands were found to be spread over the DNA molecular weight ranging from 500 bp to 2000 bp depending on the amplified DNA fragments as described below.

3.1 Inter Population Genetic Polymorphism through ISSR Markers

The profiles of the selected eighteen silkworm races were generated using a total 4 primers of which only 2 primers have output resolute profiles (Figs. 1 & 2), which produced clear, distinct and reproducible amplified products. Indeed, these primers were found to generate identical banding pattern. A total of 88 clear, distinct and reproducible bands were observed corresponding to an average of 44 bands per primer (Table 2). Further, this table elaborates the details of genetic polymorphism. It is observed that 18 bands were monomorphic in nature from the total 88 bands and a total of 70 bands were polymorphic in nature and percentages of polymorphism obtained were 100 and 60.86 from UBC-812 and UBC-826 primers respectively indicating an average of 80.43% genetic polymorphism per primer, thereby indicating the occurrence of high level genomic variation in selected population. ISSR profiles for UBC-812 marker (Fig. 1) clearly demonstrated differential expression of DNA banding pattern. It is evident from results that, an highest of 4 bands were observed in P_{31} race, whereas, a lowest of 1 band was observed in KA and zebra genotypes, followed by 3 bands each in C_{108}, NB_4D_2, C.nichi, MU_1 and *npnd* races. On the other hand, the ISSR UBC-826 profile (Fig. 2) also produced an highest of 4 bands in Nistari, *C. nichi*, *pere* genotypes followed by 3 bands each in C_{108}, KA, NB_{18}, MU_1 and *pre* genotypes and a lowest of 1 band was recorded in KA and zebra genotypes.

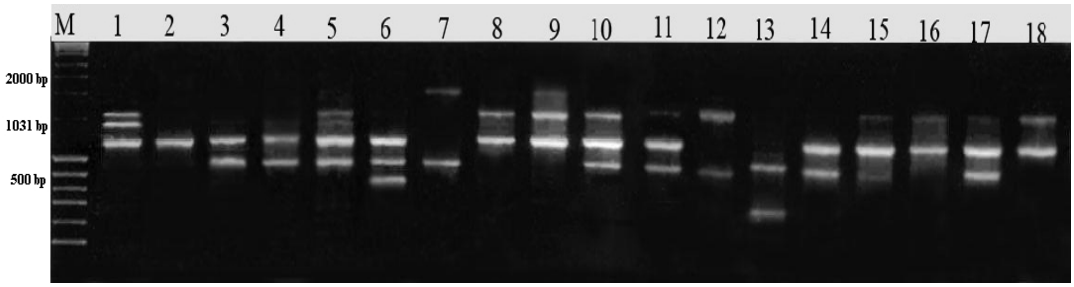

Fig. 1. ISSR amplification pattern using the primer UBC-812

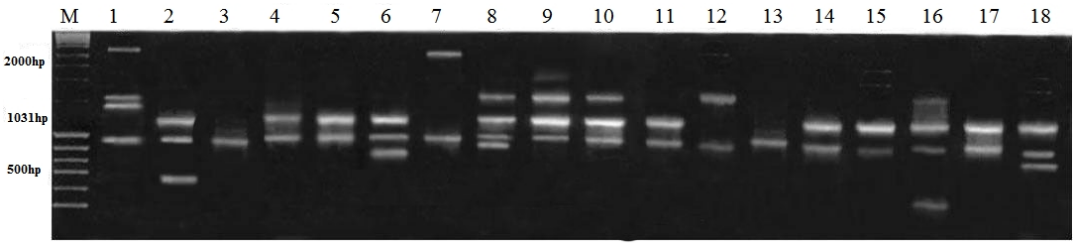

Fig. 2. ISSR amplification pattern using the primer UBC-826

3.2 Inter Population Genetic Relationship Revealed Through ISSR Markers

The cluster analysis based on the ISSR profiles through the UPGMA method has generated the dendrogram (Fig. 3), which has clearly separated into two major clusters. Cluster-I includes bivoltine races and cluster-II includes multivoltine races. Further, the mutants were clustered into BV group (cluster-I). The genetic relationship was measured for all the races/breeds and they scored a maximum and minimum genetic distance of 0.25 and 0.1 respectively. Further, this figure elaborated the individual races cluster relationship. The genetic distance measurement of the cluster-I showed an highest value of 0.20 and least of 0.1. Further, this cluster is divided into three sub-clusters, wherein the KA and CSR_2 breeds were clustered into lowest genetic distance sub-cluster-a (0.1) and highest 0.3 genetic distance was sub-cluster-b by NB_4D_2 and pere. Interestingly the zebra and ursa mutants were clustered into separate sub-cluster-c, wherein, their genetic distance was 0.3. Further, from the analysis it is evident that NB_{18}, lemon and *pre* autosomal mutants were inter-related for the sub-cluster-a and b with genetic distance from 0.4 to 0.20 respectively.

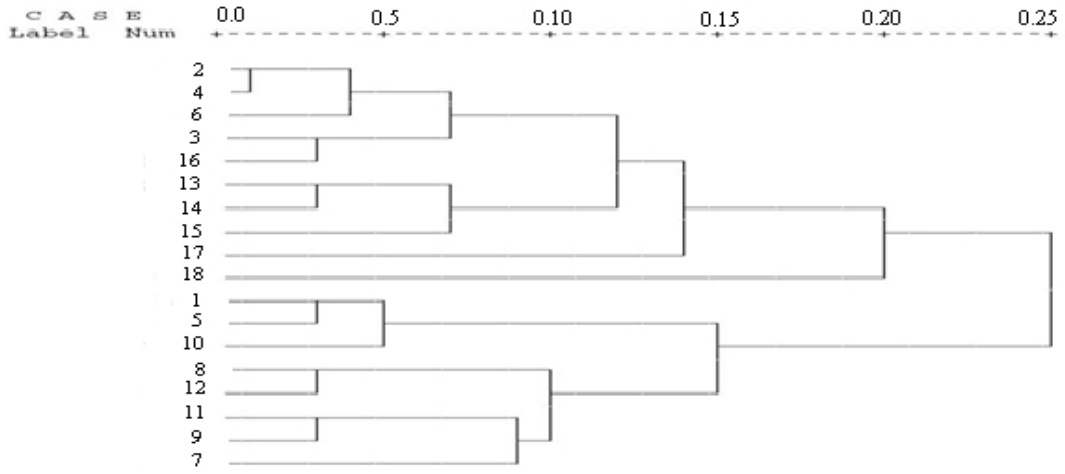

Fig. 3. Dendrogram showing the genetic relationships among eighteen races/breeds/mutants of the silkworm *Bombyx mori.*

Index: M=Marker, 1=C_{108}, 2=KA, 3=NB_4D_2, 4=CSR_2, 5=P_{31}, 6=NB_{18}, 7=PM, 8=Nistari, 9=C.nichi, 10=npnd, 11=MU_1, 12=HM, 13=zebra, 14=ursa, 15=knobbed, 16=pere, 17=lemon and 18=pre.

Furthermore, cluster-II was also divided into three sub-clusters, their genetic distance ranging from 0.3 to 0.15. The C_{108} and P_{31} races were clustered into sub-cluster-a and into sub-cluster-b (Nistari and HM) and sub-cluster-c (MU_1 and C.nichi) and their genetic distance was 0.3 respectively and remaining *npnd* and PM races were genetically inter-related to sub-cluster-a and sub-cluster-c and their genetic distance of 0.5 and 0.9 respectively. It is quite interesting to understand the C_{108} and P_{31} races were further away from their parentage instead, they have clustered into cluster-II and their genetic distance was 0.3.

4. DISCUSSION

The silkworm races need to be protected carefully in germplasm with an aim to enhance the conservation in the future. Hence, the ISSR will have an important role in securing silkworm rights by virtue of its unique efficiency in distinguishing even closely related germplasm stocks. The genetic architecture of traits is of key concern to evolutionary geneticists. Many of these issues can be addressed by analysis of a collection of tightly linked markers and the appropriate experimental design [17]. Genetic polymorphic markers have wide potential applications in animal and plant improvement programmes as means for varietal and parentage identifications. These studies explicitly report the importance of repeats and transposable elements in the molecular phylogenetic analysis through DNA profiles (Figs. 1 and 2) of the domesticated silkworm races/breeds and the higher amount of genetic polymorphism realized in this study from closely related populations of silkworm. The highest numbers of 4 DNA bands were observed in P_{31}, Nistari, C.nichi and pere mutant in two primers namely UBC-812 and UBC-826, where all the three of four races were originated from exotic condition. But on the other hand, another few races/mutants of exotic origin have not shown the same trend (C_{108}, *npnd*, zebra, ursa, knobbed, lemon and *pre*). This finding shows that, there is lot of genetic polymorphism existed among the exotic races as well as indigenous races (Table 1). Further, Table 2 reveals the detailed study of genetic polymorphism among the selected races/breeds

from UBC-primers 812 and 826 respectively. The primer UBC-812 showed, number of monomorphic bands were nil and whereas 42 polymorphic bands were observed and obtained the 100 percentage of polymorphism from the said primer. On the other hand from primer UBC-826, 18 monomorphic bands and 28 polymorphic bands are evident and obtained percentage of polymorphism is 60.86. Ultimately, 80.43 percentage of average polymorphism has been calculated from the resulted two primers. In a similar finding on *Trichosanthes dioca* the popular "pointed gourd" an highest genetic polymorphism of 88.7 percentage was clearly evident [18]. Contrary to the above relatively narrow genetic base through polymorphic studies is also evident [19]. It is important that, the low variability might also be due to the existence of genetic incompatibility factors that reflect the combination between germplasm of the two centers of origin [20, 21]. Further, it also indicates that, the UBC-812 primer revealed highest percentage of polymorphism than UBC-826 primer. This kind of higher genetic polymorphism earlier has been reported [22] in two races of domesticated silkworm *Bombyx mori* and also few investigations [4,23] utilizing different primers in *Antheraea mylittae.*

Moreover, ISSR marker technique has been used in phylogenetic investigations based on relative similarity through UPGMA (24). Nevertheless, phylogenetic tree showed the genetic relation among the geographically separated populations but also among individuals within the populations. In this study, the phylogenetic tree (Fig. 3) revealed all the races/breeds/mutants clustered into two major clusters (Cluster-I and II); though tree revealed inter population genetic relationship among the selected population. The data based on the ISSR marker, separated the BV and MV races/breeds into two major separate groups. The five mutants namely, zebra, ursa, knobbed, pere and lemon are clustered in BV group and precocity which is MV race interestingly clustered under BV group. Further, this autosomal mutant and C_{108} were genetically inter-linked together in cluster-I and II respectively. But, if we put a glance towards P_{31} and C_{108} these two BV races were genetically far away from the former group (cluster-I) instead, they were clustered into MV group (cluster-II). It is not sure but may be due to adaptability, effect of environmental factors and genetic mixing between the geographically separated population and in between populations of a territory which leads to genetic mixing [20,18]. However, the author reiterates that through the present study is one of the important venture in the field of ISSR studies yet, a detailed study is required to understand the phylogeny based on intra and inter population (races) genetic diversities utilizing different marker systems added with Nei's statistics which will help in developing species specific or group specific molecular markers for analyzing intra and inter population genetic diversity. Since the selected races/breeds for this investigation have exhibited genetic polymorphism, it is opined by the authors that such polymorphism might be due to their origin from sub-continental and south-east Asia and can be conveniently be used in silkworm molecular genetics and breeding programme.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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