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Exploring the genetic variability in *Bombyx mori* L. with molecular marker

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Abstract: Investigation was carried out to analyze the diversity in different silkworm races using Inter Simple Sequence Repeat (ISSR) molecular marker for the selection of diverse parents. Among the primers used for ISSR analyses, the primer ISSR2 and ISSR3 generated highest number of fragments. All the primers exhibited 100 per cent polymorphism across 30 silkworm races analyzed. The similarity coefficients ranged from 0.33 to 1.00. Of the pairwise combinations, NB7 and Kollegal Jawan showed the lowest similarity index (0.33), whereas the highest similarity index was recorded between C. Nichi and P_4D_3 (1.00), followed by CSR3 and CSR2xCSR4 (0.98). The mean similarity index was 0.68. There was distinct grouping between the multivoltine and bivoltine races when grouped with marker generated by ISSR PCR. The 2D diagram of the PCA analysis of the markers generated by the different ISSR primers helped to visualize the two major clusters which included the multivoltines and bivoltines separately. The grouping of bivoltines in the PCA analysis clearly showed higher similarity among bivoltines as compared to the multivoltines. Since the combining ability of Pure Mysore was excellent with bivoltine races for hybridization and also because of its high amylase activity; it was selected as donor parent for the F_1 cross. CSR2 was selected as recurrent parent because of its better quantity and quality parameters. Superiority with regard to biological parameters was found with F_1 (Pure Mysore x CSR-2) over the parents.

Keywords: Molecular diversity, *Bombyx mori*, ISSR marker, multivoltines, bivoltines

Introduction

The silkworm, *Bombyx mori* L. is the well-studied domesticated lepidopteran insect. After the discovery of restriction endonucleases and the polymerase chain reaction (PCR), molecular markers were developed and used as powerful tool in analyzing the phylogeny, evolution, ecology and population dynamics of insects (Symondson and Liddell, 1996). Recent developments in silkworm genome analysis provide tools and techniques which, coupled with conventional breeding will help silkworm geneticists and breeders to improve strains. A wide array of DNA marker techniques is available for genetic studies. All DNA markers reflect differences in DNA sequences. Major applications of these DNA markers in silkworms include diversity analysis, mapping genes and marker assisted selection (Nagaraju and Goldsmith, 2002). Selection of parental strains for cross-breeding programme based on genetic distance determined by DNA marker evaluation facilitates the development of new silkworm races.

Material and methods

The multivoltine and bivoltine silkworm races selected for the study are given in the table 1. DNA was extracted from adult moths and amplified using thermocycler. The PCR products were electrophoresed in 4 per cent denaturing polyacrylamide gel electrophoresis and resolved by silver staining procedure (Panaud *et al*., 1996). The bands were scored with the presence of bands as (1) and absence of bands (0) and missing band as (3). The data obtained by scoring the ISSR profiles of different primers were subjected to cluster analysis. Similarity matrix was constructed using Jaccard's (Jaccard, 1908) coefficient. Sequential agglomerative hierarchical non-overlapping (SAHN) clustering was done using unweighted pair group method with arithmetic averages (UPGMA) method. Data analysis was done using NTSYS pc version 2.02 (Rohlf, 1998). A total of five ISSR primers were used for amplifying the genomic DNA (Table 2).

Based on molecular diversity, one parent from multivoltine and another from bivoltine were selected and the crosses were made. The observations on various biological parameters and yield related parameters were recorded and F_1 genetic profile was verified along with parents using ISSR marker. Analysis of variance (ANOVA) of different observations made on the treatments was performed and means were compared by the least significant difference (LSD) (Gomez and Gomez, 1984).

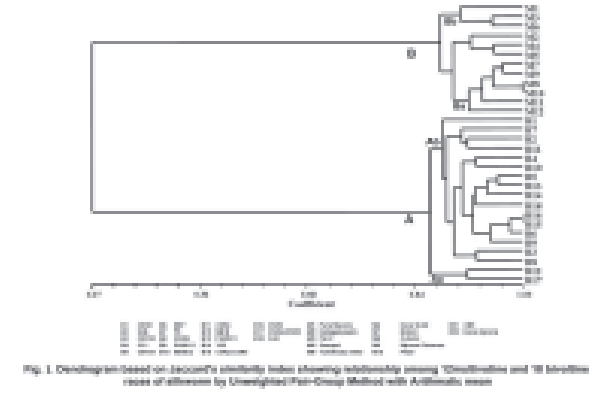
Results and discussion

All the five ISSR primers used in the study produced unambiguous markers. Among the primers used for ISSR analysis the primer ISSR2 and ISSR 3 generated highest number of fragments. All the primers exhibited 100 per cent polymorphism across 30 silkworm races analyzed. The similarity index values obtained for each pairwise comparison among the 30 races is given in the table 3. The similarity coefficients ranged from 0.33 to 1.00. Of the pairwise combinations, NB7 and Kollegal Jawan showed the lowest similarity index (0.33), where as the highest similarity index was recorded between C. Nichi and P4D3 (1.00), followed by CSR3 and CSR2xCSR4 (0.98). The mean similarity index was 0.68.

The different races were broadly grouped into two clusters namely cluster A (18- bivoltines) and cluster B (12-multivoltines). There was distinct grouping between the multivoltine and bivoltine races when grouped with marker

generated by ISSR PCR. Cluster A is further divided into two broad clusters Aa (NB18 and CSR4x CSR2) and Ab with other 16 bivoltines. Among the subcluster Ab, B1 stood alone from the rest and CSR2xCSR4 and CSR3 had the maximum similarity values.

Multivoltines in cluster B were broadly grouped into two clusters Bb (Kolar gold, PA12 and Pure Mysore), and all the other multivoltines grouped in the other cluster Ba. Cluster Ba was further divided into two subclusters and C. Nichi and P4D3 showed the maximum similarity index values (Fig. 1). The 2D diagram of the PCA analysis of the markers generated by the different ISSR primers also indicated similar results and helped to visualize the two major clusters which included the multi and bivoltines separately. The grouping of bivoltines in the PCA analysis clearly showed higher similarity among bivoltines as compared to the multivoltines (Fig. 2).



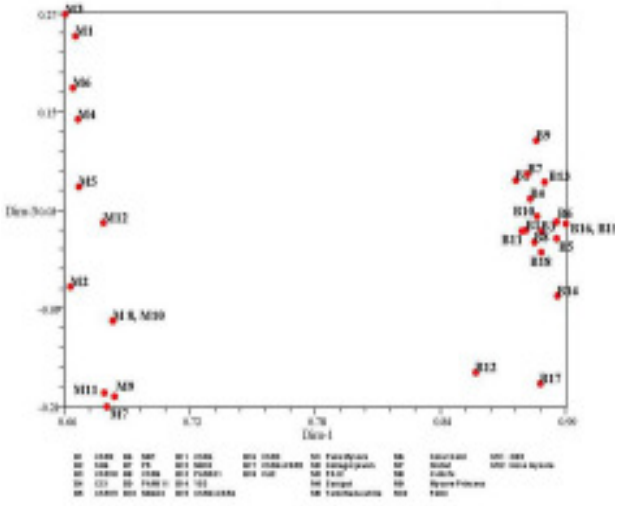


Fig.2 Pattern of relationship among 12 multivoltine 15 bivoltine races of silkworm by PCA analysis of ISSR data

Table 2. List of ISSR primers used for genetic diversity analysis

Primer	Sequence
ISSR01	$(GA)_9$-A
ISSR02	$(GA)_9$-C
ISSR03	$(GA)_9$-T
ISSR04	CCCGGATCC$(GA)_9$
ISSR05	CCCGGATCC$(CT)_9$

Table 1. Multivoltine and Bivoltine silkworm races

Multivoltine races	
Races	Parentage / Source
Kolar Gold	(PM x NN6D) X S.H
Kollegal Jawan	(PM x NN6D) X S.H
C.Nichi	J1 X C4
Sarupat	CSGRC,Hosur
P4D3	CSGRC,Hosur
PA12	CSGRC,Hosur
Tamil Nadu White	PM X C.Nichi
Hosa Mysore	Ae_4 X Pure Mysore
Mysore Princess	(PM x NN6D) X S.H
CB5	CSGRC,Hosur
Nistari	CSGRC,Hosur
Pure Mysore	CSGRC,Hosur
Bivoltine races	
NB4D2	(Kokko x Seihaku) x (N124 x C 124)
SH6	CSGRC,Hosur
YS3	CSGRC,Hosur
CSR2	Shunrei x Shogetsu
CSR3	BN18 XBCS25 (out crossed with CC1)
CSR4	BN18 x BCS25 (out crossed with NB4D2)
CSR6	Shunrei x Shogetsu
CSR18	B201 x BCS12
CSR19	B201 x BCS12
NB7	Kinshu x Showa
NB18	(Kokko x Seihaku) x (N124 x C 124)
CC1	(KA x NB1) x (NB7 x SPC1)
CA2	(NB7 xSPC2)
PAM101	CSGRC,Hosur
PAM111	CSGRC,Hosur
P5	CSGRC,Hosur
CSR2X4	CSR2X CSR4
CSR4X2	CSR 4X CSR2

CSR4 x CSR2 was distinctly different from CSR2 among the bivoltines even though CSR 2 was one of the parents in the crossbreed. This indicates the genetic distinctness of crossbreeds. Among multivoltines, Pure Mysore and Hosa Mysore had lower genetic similarity. It might be because of Hosa Mysore derived from Pure Mysore. The grouping of bivoltines in the PCA analysis clearly showed higher similarity among bivoltines as compared to the multivoltines. These results showed that ISSR markers are useful for fingerprinting of silkworm races. Although most ISSR loci are dominant rather than co-dominant, ISSR markers offer several advantages for genotyping, the major one being the rapid production of a large number of markers in a cost-effective manner (Nagaraju and Goldsmith, 2002). Because of these advantages, the ISSR technique has potential use in silkworm breeding, germplasm evaluation and genetic mapping. Exploitation of heterosis played a vital role in increasing silk production to a great extent (Thangavelu, 1997). In view of this, it is essential to gather a thorough knowledge on the pedigree of the pure races in order to determine their genetic background and to utilize them appropriately in cross breeding programmes to maximize

Table 4. Comparison of F_1 (PM x CSR2) along with its parents, PM and CSR2 for biological and yield related parameters

Treatments	Larval weight	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Hatching (%) (g)	ERR (%)	Single filament length (m)	Denier	Amylase content (µg /100µl/h)
PM	0.941 ± 0.03	0.526 ±0.023	0.406 ±0. 008	0.083 ± 0.003	87.27±1.7	94.99±0.98	306.58 ±4.3	1.04±0.2	1507.07±0.986
CSR2	3.088±0.09	2.491 ±0.004	1.860±0.007	0.556±0.05	91.90±0.94	72.43±0.58	1165.02 ±6.5	3.13±0.03	135.43±1.1091
PMXCSR2	3.055± 0.039	2.5041±0.0039	1.879±0.0036	0.563±0.0032	91.90±0.093	93.60±0.58	1246.05±4.37	3.032±0.0032	1496.09±3.779
S.Em.±	0.0803	0.0196	0.0091	0.0051	1.7712	1.0475	13.7699	0.0710	8.235
C.D. (0.05)	0.1648	0.0403	0.0186	0.0105	3.8591	2.5633	30.0023	0.1547	20.151

Fig. 3. . Confirmation of F1(PMxCSR2)using ISSR markers

heterosis (Mano *et al.*, 1992).

Performance of F_1 hybrid depends upon proper selection of suitable parents and the nature of genetic divergence between them. The races Pure Mysore (PM) and CSR2 were selected as parents and crossed to obtain the F_1 progeny. Among multivoltines, Pure Mysore, has long history of adaptability to various climatic conditions in India. Similarly among bivoltines, CSR2 showed distinct difference from other bivoltine races as per ISSR analysis was also selected. The biological and yield related parameters in F_1 and its parents are presented in table 4. The larval weight of PM x CSR2 was 3.055 g, whereas PM and CSR2 recorded 0.941 g and 3.088 g, respectively (Table 4). Cocoon weight of F_1 was higher (2.504 g) than the better parent CSR2 (2.491 g). Similar pattern was exhibited in shell weight as in cocoon weight. Hatching per cent of eggs were similar in parents and F_1. Effective rate of rearing in F_1 (93.60%) was closer to Pure Mysore (94.99%). Heterosis was observed in single filament length in F_1. The single filament length of F_1 was 1246.05 m, as compared to its parents PM (306.58 m) and CSR2 (1165 m). PM x CSR2 hybrid recorded intermediary denier value of 3.032 as compared to the parents CSR2 (3.13) and Pure Mysore (1.04). The genetic pattern of F_1 was verified using ISSR3 and ISSR4 (Fig. 3). Hybrid vigour was noticed in the present study as reported by Ravindra Singh *et al*. (2001).

Table 3. Similarity index values among 30 silkworm races using 5 inter-simple sequence repeats

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18
M1	1.00																													
M2	0.88	1.00																												
M3	0.93	0.86	1.00																											
M4	0.92	0.93	0.93	1.00																										
M5	0.88	0.92	0.89	0.96	1.00																									
M6	0.89	0.87	0.96	0.91	0.93	1.00																								
M7	0.83	0.94	0.83	0.90	0.92	0.88	1.00																							
M8	0.87	0.90	0.87	0.92	0.93	0.91	0.96	1.00																						
M9	0.85	0.91	0.86	0.88	0.89	0.87	0.97	0.96	1.00																					
M10	0.87	0.90	0.87	0.92	0.92	0.91	0.96	1.00	0.96	1.00																				
M11	0.82	0.89	0.86	0.85	0.86	0.87	0.94	0.93	0.97	0.93	1.00																			
M12	0.86	0.85	0.90	0.89	0.90	0.94	0.90	0.94	0.90	0.94	0.92	1.00																		
B1	0.40	0.39	0.40	0.38	0.36	0.39	0.39	0.39	0.40	0.39	0.41	0.41	1.00																	
B2	0.42	0.40	0.39	0.39	0.36	0.36	0.39	0.39	0.40	0.39	0.39	0.37	0.87	1.00																
B3	0.39	0.40	0.37	0.38	0.37	0.37	0.39	0.38	0.38	0.38	0.38	0.39	0.89	0.92	1.00															
B4	0.38	0.34	0.36	0.35	0.37	0.36	0.37	0.38	0.38	0.38	0.37	0.38	0.85	0.89	0.90	1.00														
B5	0.38	0.35	0.35	0.35	0.36	0.36	0.36	0.36	0.37	0.36	0.38	0.37	0.88	0.88	0.89	0.92	1.00													
B6	0.34	0.33	0.34	0.33	0.34	0.35	0.35	0.35	0.36	0.35	0.37	0.36	0.91	0.85	0.90	0.89	0.93	1.00												
B7	0.38	0.35	0.37	0.38	0.38	0.36	0.38	0.39	0.39	0.39	0.38	0.38	0.83	0.90	0.89	0.88	0.88	0.91	1.00											
B8	0.35	0.37	0.34	0.37	0.38	0.36	0.40	0.38	0.38	0.38	0.37	0.38	0.85	0.86	0.93	0.90	0.90	0.93	0.92	1.00										
B9	0.37	0.33	0.36	0.34	0.34	0.35	0.36	0.35	0.36	0.35	0.36	0.35	0.87	0.90	0.88	0.91	0.91	0.95	0.93	0.91	1.00									
B10	0.38	0.34	0.36	0.35	0.37	0.36	0.37	0.38	0.38	0.38	0.38	0.40	0.85	0.86	0.87	0.93	0.93	0.93	0.88	0.90	0.91	1.00								
B11	0.37	0.36	0.36	0.37	0.35	0.33	0.37	0.36	0.38	0.36	0.38	0.36	0.88	0.92	0.90	0.90	0.90	0.89	0.92	0.87	0.88	0.87	1.00							
B12	0.39	0.41	0.36	0.39	0.38	0.35	0.40	0.38	0.39	0.38	0.39	0.37	0.84	0.82	0.86	0.83	0.88	0.85	0.83	0.85	0.81	0.83	0.85	1.00						
B13	0.36	0.34	0.35	0.33	0.34	0.36	0.36	0.36	0.37	0.36	0.38	0.37	0.91	0.89	0.90	0.93	0.96	0.96	0.88	0.90	0.95	0.93	0.90	0.85	1.00					
B14	0.38	0.37	0.36	0.35	0.35	0.34	0.38	0.37	0.39	0.37	0.40	0.38	0.90	0.90	0.92	0.92	0.95	0.91	0.87	0.88	0.90	0.92	0.92	0.90	0.95	1.00				
B15	0.34	0.33	0.34	0.33	0.34	0.35	0.35	0.35	0.36	0.35	0.37	0.36	0.91	0.85	0.90	0.89	0.93	1.00	0.91	0.93	0.95	0.93	0.89	0.85	0.96	0.91	1.00			
B16	0.35	0.33	0.36	0.34	0.33	0.34	0.35	0.34	0.36	0.34	0.36	0.36	0.90	0.87	0.88	0.88	0.91	0.98	0.93	0.91	0.96	0.91	0.91	0.84	0.95	0.90	0.98	1.00		
B17	0.39	0.40	0.37	0.38	0.39	0.37	0.42	0.42	0.43	0.42	0.42	0.40	0.85	0.83	0.88	0.87	0.90	0.90	0.85	0.87	0.85	0.90	0.87	0.92	0.90	0.92	0.90	0.88	1.00	
B18	0.37	0.36	0.37	0.35	0.36	0.38	0.36	0.36	0.37	0.36	0.38	0.38	0.38	0.90	0.83	0.88	0.88	0.95	0.94	0.86	0.88	0.89	0.88	0.88	0.80	0.80	0.81	0.88	0.92	1.00

B1	CSR2		B6		NB7	B11	CSR4	B16		CSR3		M1	Pure Mysore		M6		Kolar Gold	M11	CB5											
B2	SH6		B7		P5	B12	NB18	B17		CSR4xCSR2M2		Kollegal jawan			M7		Nistari	M1	2Hosa mysore											
B3	CSR18		B8		CSR6	B13	PAM101	B18		CA2		M3	PA12		M8		C-Nichi													
B4	CC1		B9		PAM111	B14	YS3					M4	Sarupat		M9		Mysore Princess													
B5	CSR19		B10		NB4D2	B15	CSR2xCSR4					M5	TamilNadu white		M10		P4D3													

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