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RAPD Markers for Understanding of the Genetic Variability among the Four Silkworm Races and Their Hybrids

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Abstract: The development of molecular markers provided a new technological base for selection of parental lines and prediction of hybrid performance and heterosis. In the present investigation, four different silkworm races (*Bombyx mori* L.) were drawn from the germplasm of Department of Studies in Sericulture Science, University of Mysore, Manasagangotri, Mysore and 12 hybrids were prepared. Whole moth body tissue (after egg laying) was collected from all the genotypes for DNA extraction and fifteen random primers (Different types of OPA and UBC) were used. High degree of polymorphism of 100% was detected in case of 4 primers, *viz*., UBC-704, UBC-731, UBC-747 and OPA-05. The lowest percentage of polymorphism (0.0%) was observed when amplified by OPA-01 and OPA-02. The cluster analysis based on UPGMA method based on dissimilarity index values has generated the dendrogram which has clearly separated the bivoltine and multivoltine races into two groups. Pure Mysore is genetically further away from the former groups.

Key words: *Bombyx mori* · Genetic variability · Hybrid · RAPD

INTRODUCTION

Silkworm breeding programs are based on the development and selection of outstanding hybrids from inbred lines [1]. Developing and selecting inbred lines for performance is mainly based on morphological characters although time consuming. The hybrid performance is evaluated from extensive yield trials that are costly and time consuming. In any hybrid program, a large number of crosses are made, while only a few good hybrids are obtained. Silkworm breeding is based around the identification and utilisation of genetic variation. The breeder makes decisions at several key points in the process. First in deciding on the most appropriate parents to use for the initial cross or crosses and then in the selection strategy used in identifying the most desirable individuals amongst the progeny of the cross. The efficiency of the breeding and selection process can be assessed in many different ways including the ultimate success of the varieties released and the frequency with which new strains are produced.

Several studies have been conducted to determine the best strain for silkworm breeding programs [2, 3]. As a result, molecular markers have proved to be a powerful tool in replacing bioassays. The use of molecular markers to track loci and genome regions in economic characters is now routinely applied in many breeding programs.

Improvements in marker screening techniques have also been important in facilitating the tracking of genes [4]. The development of molecular markers provided a new technological base for selection of parental lines and prediction of hybrid performance and heterosis. At present, molecular markers are widely used in QTL mapping of quantitative traits [5, 6] and exploration of genetic diversity, with the latter often employed in prediction of heterosis [7-9]. Biochemical markers are useful for screening germplasm with the minimum cost in time and labour [10]. The most important factor in biological genetic resource conservation regimes is to maintain pure strains of each species and establish accurate genetic relationship among species [11].

This investigation was planned with the objective to record the genetic differences in terms of DNA profiles for the better understanding of the genetic variability among the silkworm races and their hybrids so that the knowledge gained may serve as an important yardstick in silkworm breeding.

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MATERIALS AND METHODS

Silkworm Races: Four different silkworm races (*Bombyx mori* L.) were drawn from the germplasm of Department of Studies in Sericulture Science, University of Mysore, Manasagangotri, Mysore and 12 hybrids were prepared and the characteristic features of the four races and list of crosses are given in Table 1 and 2 respectively.

After incubation of eggs at 25 ±10 °c and relative humidity of 80±5 %, block box was carried out on 8th day to achieve uniformity in hatching. Three layings of each of the pure races and its hybrids were selected. The larvae hatched from each layings were reared separately under uniform laboratory conditions as described by Yokoyama [12] and Krishnaswami [13].

Whole moth body tissue (after egg laying) was collected from all the genotypes for DNA extraction. DNA was extracted according to the procedure of described by Nagaraja [14] and Somasundaram *et al*. [15].

Oligonucleotide Primers: Particulars of primers used and their sequence and molecular weight in base pairs are enlisted in the Table 3. These random primers were dissolved in autoclaved Milli Q water (Millipore water system, USA), aliquoted and stored at-20°C for RAPD analysis according to the manufacturer's instructions.

RAPD-PCR Analysis: The primers that gave clear and polymorphic amplification patterns were used for further analysis with all the 16 genotypes. For each primer, a 25 µl

Table 1: Characteristics of four silkworm races used in present study

Strain	Voltinisim	Larval pattern	Cocoon color	Cocoon shape
C_{108}	Bivoltine	Plain	White	Oval
NB_4D_2	Bivoltine	Plain	White	Dumbble
Pure Mysore (PM)	Multivoltine	Plain	Greenish yellow	Spindle
Nistari	Multivoltine	Marked	Golden yellow	Spindle

Table 2: Regular and reciprocal crosses

SL. No.	Regular	SL. No.	Reciprocal
1	$C_{108}^{*} \times NB_4D_2$	1	$NB_4D_2 \times C_{108}$
2	$C_{108} \times$ Nistari	2	Nistari $\times C_{108}$
3	$C_{108} \times$ Pure Mysore	3	Pure Mysore $\times C_{108}$
4	$NB_4D_2 \times$ Nistari	4	Nistari $\times NB_4D_2$
5	$NB_4D_2 \times$ Pure Mysore	5	Pure Mysore $\times NB_4D_2$
6	Nistari × Pure Mysore	6	Pure Mysore × Nistari

The first parent is always the female.

Table 3: List of fifteen RAPD primers used in the present study

Sl. No.	Primer Code	Primer Sequence (5' to 3')	Molecular Weight (bp)
1	OPA-01	CAGGCCCTTC	2964
2	OPA-02	TGCCGAGCTG	3044
3	OPA-03	AGTCAGCCAC	2997
4	OPA-04	AATCGGGCTG	3068
5	OPA-05	AGGGGTCTTG	3099
6	OPA-07	GAAACGGGTG	3117
7	OPA-08	GTGACGTAGC	3108
8	OPA-09	GGGTAACGCC	3053
9	UBC-704	GGAAGGAGGG	2000
10	UBC-714	GGGTGGGTGT	3000
11	UBC-731	CCCACACCAC	3000
12	UBC-747	CCACCAACCC	3500
13	UBC-769	GGGTGGTGGG	2200
14	UBC-770	GGGAGGAGGG	3000
15	UBC-773	GGGTTGTTGG	2500

amplification reaction contained 2.5 µl of 10 X PCR buffer with $MgCl_2$, 2.5 µl of 1 mM dNTP,1.0 µl of 0.2 mM of primer, 1.0 µl of 1 mM Spermidine, 15 ng of genomic DNA and 0.8 units of Taq DNA polymerase (Perkin Elmer,USA). PCR amplifications were performed in a Perkin Elmer Cetus 9700 thermal cycler (P.E. Biosystems, USA). The PCR conditions included initial denaturation at 94 °C for 4 min, followed by 5 cycles at 92 °C for 30 s, 2 min at 35 °C, 90 s at 72 °C; 35 cycles of denaturation at 92 °C for 5 s, annealing at 40 °C for 20 s, extension at 92 °C for 90 s with final extension at 72 °C for 5 min.

RESULTS

PCR was performed as per standard protocol and the resulting PCR products were electrophorectically analyzed through 2% agarose gels containing ethidium bromide (5 µg/ml) in 1X TAE buffer (PH 8.0) and documented in the gel documentation system. A molecular weight marker (Lambada with EcoR1/Hind III double digest) was used for analysis of the fragment size. All amplification reactions were carried out at least thrice in order to make sure consistency and repeatability of fingerprints generated using selected RAPD primers. All the DNA bands are found to be spread over the molecular weights ranging from 831 bp to 2126 bp depending on the amplification of DNA. The relevant data on the polymorphic and monomorphic bands are given in Table 4. It is evident that fifteen primers were used and ten have generated bands. These ten RAPD primers amplified a total of 422 bands out of which 262 bands were polymorphic in nature.

Data in Table 4 also indicate that the percentage of polymorphism ranged from 0.0% to 100.0%. High degree of polymorphism of 100% was detected in case of 4 primers, *viz*., UBC-704, UBC-731, UBC-747 and OPA-05. The lowest percentage of polymorphism (0.0%) was observed when amplified by OPA-01 and OPA-02. The overall polymorphism observed with 10 primers among the all genotypes was found to be 62.1%. The highest number of bands (75) was obtained with primer UBC-747 (Figure 1), while the lowest number (12) was obtained with

Table 4: Summary of PCR assay among the sixteen genotypes

Sl. No.	Primer Code	No. of Bands Scored	No. of Monomorphic Bands	No. of Polymorphic Bands	% of Polymorphism
1	UBC-704	12	0	12	100.0
2	UBC-714	55	16	39	70.9
3	UBC-731	68	0	68	100.0
4	UBC-747	75	0	75	100.0
5	UBC-769	70	32	38	52.3
			Percentage for UBC primers		84.64 %
6	OPA-01	16	16	0	0.0
7	OPA-02	48	48	0	0.0
8	OPA-03	35	32	3	8.6
9	OPA-05	15	0	15	100.0
10	OPA-09	28	16	12	42.9
			Percentage for OPA primers		30.30 %
11	Total	422	160	262	62.1

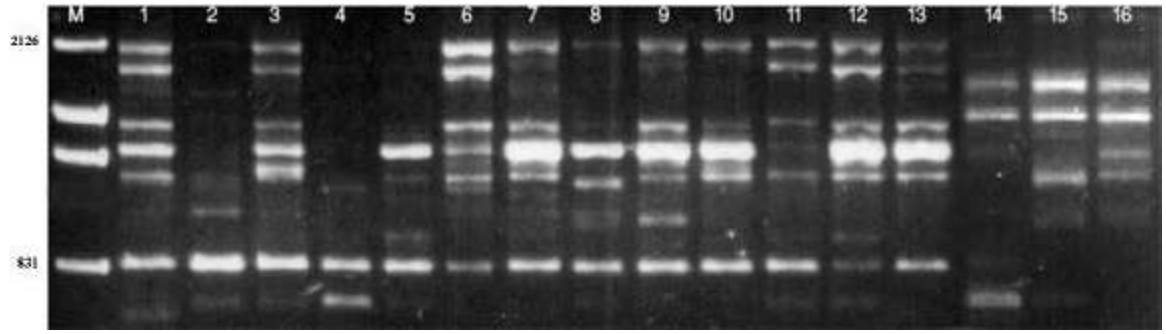

Fig. 1: RAPD amplification pattern using the primer UBC-747
M: Marker, 1: C_{108}, 2: NB_4D_2, 3: Pure Mysore, 4: Nistari, 5: $C_{108} \times NB_4D_2$, 6: $C_{108} \times$ Pure Mysore, 7: $C_{108} \times$ Nistari, 8: $NB_4D_2 \times C_{108}$, 9: $NB_4D_2 \times$ Pure Mysore, 10: $NB_4D_2 \times$ Nistari, 11: Pure Mysore $\times C_{108}$, 12: Pure Mysore $\times NB_4D_2$, 13: Pure Mysore × Nistari, 14: Nistari $\times C_{108}$, 15: Nistari $\times NB_4D_2$, 16: Nistari × Pure Mysore.

Table 5: Distance of dissimilarity index among the sixteen genotypes

	C108	NB4D2	PM	Nistari	CNB	CP	CNi	NBC	NBP	NBNi	PC	PNB	PNi	NiC	NiNB
NB4D2	0.27														
PM	0.59	0.44													
Nistari	0.43	0.51	0.52												
CNB	0.36	0.44	0.30	0.24											
CP	0.23	0.43	0.20	0.14	0.32										
CNi	0.26	0.40	0.23	0.18	0.39	0.26									
NBC	0.34	0.42	0.21	0.15	0.27	0.21	0.14								
NBP	0.19	0.45	0.31	0.25	0.33	0.23	0.23	0.25							
NBNi	0.26	0.48	0.24	0.18	0.29	0.12	0.19	0.17	0.27						
PC	0.26	0.48	0.24	0.18	0.33	0.12	0.19	0.17	0.27	0.19					
PNB	0.18	0.35	0.25	0.20	0.24	0.21	0.28	0.09	0.25	0.21	0.25				
PNi	0.18	0.35	0.15	0.09	0.27	0.15	0.11	0.09	0.18	0.21	0.21	0.27			
NiC	0.51	0.45	0.32	0.27	0.45	0.08	0.44	0.40	0.25	0.43	0.25	0.27	0.13		
NiNB	0.41	0.42	0.43	0.38	0.46	0.48	0.31	0.30	0.36	0.28	0.40	0.30	0.33	0.44	
NiP	0.33	0.44	0.34	0.30	0.37	0.40	0.23	0.25	0.27	0.31	0.16	0.32	0.29	0.39	0.29

CNB: $C_{108} \times NB_4D_2$, CP: $C_{108} \times$ Pure Mysore, CNi: $C_{108} \times$ Nistari, NBC: $NB_4D_2 \times C_{108}$, NBP: $NB_4D_2 \times$ Pure Mysore, NBNi: $NB_4D_2 \times$ Nistari, PC: Pure Mysore $\times C_{108}$, PNB: Pure Mysore $\times NB_4D_2$, PNi: Pure Mysore × Nistari, NiC: Nistari $\times C_{108}$, NiNB: Nistari $\times NB_4D_2$, NiP: Nistari × Pure Mysore.

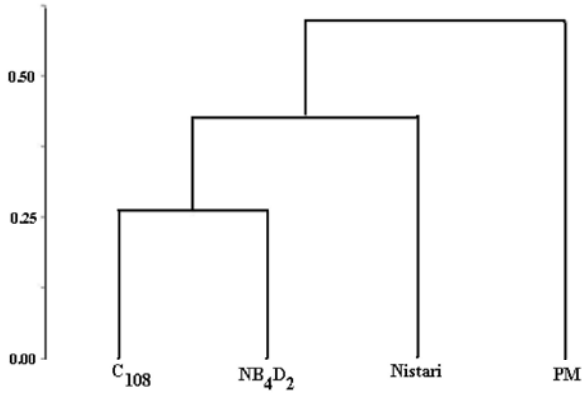

Fig. 2: Dendrogram showing the relationships among pure races based on RAPD.

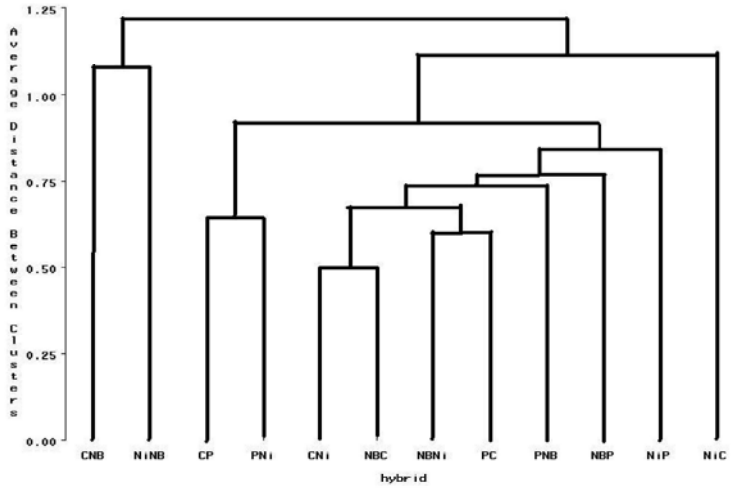

Fig. 3: Dendrogram showing the clustering among F_1 hybrids based on RAPD
CNB= $C_{108} \times NB_4D_2$, NiNB= Nistari $\times NB_4D_2$, CP= $C_{108} \times$ Pure Mysore, PNi= Pure Mysore × Nistari, CNi= $C_{108} \times$ Nistari, NBC= $NB_4D_2 \times C_{108}$, NBNi= $NB_4D_2 \times$ Nistari, PC= Pure Mysore $\times C_{108}$, PNB= Pure Mysore $\times NB_4D_2$, NBP= NB4D2 × Pure Mysore, NiP= Nistari × Pure Mysore, NiC= Nistari $\times C_{108}$

primer UBC-704. It is evident from Table 4 that the PCR products of the primers UBC 704, 714, 731, 747, 769 resolved on 1.5 % agarose gel and stained with Ethidium bromide depicts considerable variation. The number of bands produced by UBC-704 is twelve, UBC-714 is fifth five, UBC-731 is sixty eight, UBC-747 is seventy five and UBC-769 is seventeen and the concordant monomorphic bands are ranges from 0-32. The UBC primers revealed a total polymorphism of 84.64%.

The genetic distance estimated from the RAPD markers, following the methods of Nei [16], varied from 0.09 (Pure Mysore × Nistari and Nistari; Pure Mysore × NB_4D_2 and NB_4D_2 × C_{108}) to 0.59 (Pure Mysore and C_{108}) (Table 5).

The cluster analysis based on UPGMA method based on similarity index values (Figure 2) has generated the dendrogram which has clearly separated the bivoltine and multivoltine races into two groups. Pure Mysore is genetically further away from the former groups. When the silkworms were taken as two separate groups, the average genetic distance among the multivoltine and bivoltine silkworm was 0.52 and 0.27, respectively; the maximum genetic distance was 0.59 and the minimum genetic distance 0.27, respectively. The above results indicate that the two multivoltine silkworm races are genetically more distant than the two bivoltine silkworm races. Figure 3 represents dandogram based on Nei [16] algorithms differential clustering data were recorded with proximity values ranging from 0.02 to 1.25. Minimum genetic distance of 0.13 was observed in the hybrids of Nistari × NB_4D_2 and C_{108} × Pure Mysore.

DISCUSSION

The genetic architecture of traits is of key concern to evolutionary geneticists. Many of these architectural issues can be addressed by analysis of a collection of tightly linked markers and the appropriate experimental design [17]. The detection and exploitation of naturally occurring DNA sequence polymorphisms are among the most significant developments in molecular biology. Polymorphic genetic markers have wide potential applications in animal and plant improvement programmes as a means for varietal and parentage identification, evaluation of polymorphic genetic loci affecting quantitative economic traits and genetic mapping [4]. Most traits in nature and of importance to sericulture and agriculture are quantitatively inherited. These traits are difficult to study due to the complex nature of their inheritance. However, recent developments of genomic technologies provide a revolutionary means for unraveling the secrets of genetic variation in quantitative traits. Genomic technologies allow the molecular characterization of polymorphic markers throughout the entire genome that are then used to identify and map the genes or quantitative trait loci (QTLs) underlying a quantitative traits based on linkage analysis [18]. Over a long period, hybrid cultivars with superior performance and strong heterosis have been developed through extensive field trials. This has involved high cost and considerable time. Therefore, it is very useful to find a simple and reliable method that could predict heterosis prior to expensive field testing.

Generally, hybrids from two parents with a distant genetic background (diverse in relatedness, ecotype, geographic origin etc) have high heterosis and therefore the genetic diversity between two parents has been proposed as a possible predictor of heterosis. Zhu and Zhang [19] examined the correlation between heterosis and parental diversity for eight isozymes in seedlings of hybrid rice and found that diversity for esterase was more closely related with heterosis than diversity for the other isozymes. Chen [20] demonstrated that the greater the difference between two parental isozymes spectra, the higher the heterosis of the F_1 hybrid. Peng *et al*. [21] found no correlation between heterosis and isozymes variation in 75 F_1 hybrids derived from eighteen parental lines. Yang *et al*. [22] also demonstrated that it was difficult to predict heterosis using an index based on isozymes zymograms. Arunachalam *et al*. [23] reported that there is an optimum level of genetic divergence between parents to obtain heterosis in F_1 generation and they also reported that it may not be logical to advocate the use of extremely divergent parents to obtain heterotic combinations.

The results of this experiment discussed in the light of the findings of the above authors yields important information in the four pure races and twelve hybrids. It is important to note that the UBC primers revealed highest amount polymorphic bands ranging from twelve (UBC-704) to seventy five (UBC-747) thereby the percentage of polymorphism in three out of five primers are cent percent revealing high amount of DNA polymorphism among sixteen genotypes (four pure races and twelve hybrids). The present results clearly indicated that the four races and their derived regular and reciprocal crosses exhibited highest polymorphism. Table-4 also represents genomic DNA samples of sixteen genotypes utilizing OPA primers group namely OPA-01, OPA-02, OPA-03, OPA-05 and OPA-09. It is interesting to note that OPA-05 primer

produced a minimum of 15 bands, whereas OPA-02 produced a maximum of 48 bands. OPA-05 primer though revealed 100 % polymorphisms and the numbers of monomorphic bands are nil. The overall picture that emerges on the percent of polymorphism from the OPA primer was 50.5 % which is comparatively lesser than the polymorphism revealed by UBC primers. This kind of higher polymorphism was reported by Nagaraju [24] utilizing the two races of silkworm *Bombyx mori*. He has underlined the sensitivity of RAPD assay using arbitrary primers in Nistari and NB series. In detailed results Nagaraju *et al.* [4], Nagaraju and Singh, [25] and Chatterjee and Datta, [26] demonstrated that RAPD is one of important tool to differentiate each genome without resolving to its physical formation where higher polymorphism exists. This kind of higher polymorphism among closely related insects is also reported in *Antheraea mylittae* [27-29] using different primers. The present findings corroborates with findings of Srivastava *et al.,* [30] in *Bombyx mori* through RAPD-PCR.

The genetic distance estimated from the PCR markers following the methods of Nei [16] is presented in the Table-5. Perusal of the data among the pure races clearly indicated the genetic distance of 0.27 between NB_4D_2 and C_{108} and a highest of 0.59 between Pure Mysore × C_{108} Based on the above values a Dendrogram is clearly established (Figure-2), wherein the two multivoltine races are clustered together and the two bivoltine races are grouped in another cluster. Based on the findings utilizing similarity co-efficient to estimate the genetic distance, it is possible to say that considerable amount of genetic distance exist among four pure races and RAPD method utilized in the present experiment can be conveniently used to understand genetic distance of races at the molecular level which may be exploited for hybridization experiment for the breeders.

Differential clustering data was recorded with proximity values ranging from 0.02 to 1.25, the minimum genetic distance of 0.13 was observed in the hybrids of Nistari × C_{108} and PM × Nistari and a maximum of 0.48 in the in the hybrids of Nistari × NB_4D_2 and C_{108} × PM. The Dendrogram based on the Nei [16] algorithms has clustered the 12 hybrids into 4 major clusters (Figure-3).It is important that the contributions made by the two multivoltines PM and Nistari has resulted in the production of hybrids with different genotypes. Thus, based on the findings of the author through RAPD analysis and UPGMA analysis it is found that RAPD markers could discriminate the breeds or races based on their parentage and origin. The very fact that the pure races and the 12 hybrids cluster entirely in two groups clearly indicates how the molecular markers can be used as an important yardstick in silkworm breeding and genetics.

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