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GENETIC DIVERSITY ANALYSIS OF MULBERRY SILKWORM (*BOMBYX MORI*) STRAINS USING RAPD MARKERS

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ABSTRACT

The Mulberry Silkworm Moth (*Bombyx mori*) is an important commercial insect species having wide genetic diversity. For conservation and breeding programmes, genetic diversity among 18 silkworm strains was analyzed through PCR amplification of the genomic DNA using five Random Amplified Polymorphic DNA (RAPD) primers. The five tested primers generated a total of 368 amplified bands with 48 polymorphic (13.21%). The highest number of polymorphic bands (17.14%) was produced by primer CA-9.The data unraveled a mean genetic similarity distance of 0.546 ranging from 0.110 to 0.991. The mean genetic similarity distance among inbred lines was ranged from 0.124 to 0.943, while the mean genetic similarity distance among hybrids was ranged from 0.129 to 0.984. The highest genetic similarity distance was between C102 and 205PO*J101, while the lowest genetic similarity distance was between 205PO and 205MKD*205PO. The strains were clustered into four groups at 66% similarity level based on UPGMA with complete linkage and squared Euclidean Distance, while they were clustered into two main groups at 33% similarity level. The 206MKD*205PO and J101 were remotely related. Based on these findings it is concluded that the strains have enough genetic diversity which may be conserved in stage of diapause eggs and manipulated for hybrid synthesis breeding programmes.

Key words: Genetic Diversity, Mulberry Silkworm, PCR, Random Markers, Genetic Relatedness.

INTRODUCTION

Sericulture is an agro-based industry which provides job opportunities to low income strata of rural communities. The rearing of the Mulberry Silkworm Moth (*Bombyx mori* L.) has a long history spreading over a period of 5000 years (Shivashankar *et al*., 2012), during which this insect is completely domesticated. Despite complete domestication, the Mulberry Silkworm Moth has wide genetic variability that is distributed from temperate to tropical zones. These races exhibit divergent morphological, biochemical and biometric traits (Srivastava *et al*., 2005; Awasthi *et al*., 2008). The races with wide geographical ranges, especially in tropical areas have greater tolerance to adverse eco-climatic conditions but are less productive. There are several environmental factors affecting adaptation and shaping up of mulberry silkworm strains in populations of different races. Similarly, polyvoltine strains are hardy but less productive, while the bivoltines strains are susceptible to diseases under adverse biotic and abiotic conditions. These factors lead to breeding continuous for strains with hardiness of polyvoltines and productivity of bivoltines (Kumaresan *et al*., 2000).

The DNA genome of silkworm rearing stocks provides empirical building blocks for breeding programmes. For developing a potential high yielding and disease resistant silkworm strain, selection and breeding of parental stock based on molecular markers can be very effective and efficient. Random Amplified Polymorphic DNA (RAPD) primers are considered the most effective tools for assessing insect genetic diversity between individuals or within and between related species or populations (Yoon and Aquadro, 1994; Salvato *et al*., 2002; Llewellyn *et al*., 2003; Ellegren, 2004; Speight *et al*., 2005). Importantly, these RAPD markers have abundant polymorphism and are independent of environmental conditions (Behura, 2006).

For genetic studies, both single and multiple loci DNA markers are available. The selection of a particular genomic DNA marker depends upon the objective of the study and is usually trade-off between practicality and precision of genetic marker (Nagaraju and Goldsmith, 2002). In recent past, different DNA markers have been developed for studying genetic diversity, genetic relatedness, phylogeny and population dynamics in insects (Bardakci, 2001; Behura, 2006). Among these, PCR-RAPD markers are a simple technique for assessing genetic diversity of organisms. Several workers have used PCR-RAPD markers successfully for estimation of genetic diversity and relatedness of the mulberry silkworm strains (Eroğlu and Arica, 2009; Reddy *et al*., 2009; Moorthy *et al*., 2013).

Conservation and maintenance of silkworm genetic resources has paramount importance for meeting the desired objectives of the silkworm breeders for immediate and long-term utilization in silkworm breeding

programmes. Assessment of genetic resources is an essential prerequisite for effective use of the silkworm resources. Thus, the silkworm breeders ought to identify the genetic variability between silkworm strains (Jingade *et al*., 2011). The genetically distant strains have potential of evolution of hybrids that are higher yielding and resistant to microbial diseases. The present study was, therefore, conducted to assess (i) genetic diversity among 18 mulberry silkworm strains, and (ii) their genetic relatedness.

MATERIALS AND METHODS

Eighteen silkworm strains including six inbred pure lines and 12 hybrids of the Mulberry Silkworm Moth (*Bombyx mori* L.) were collected from Sericulture Division, Pakistan Forest Institute, Peshawar during Autumn (September-November) Silkworm Rearing Season 2014 (Table 1). A total of 42 probes consisted of male and female moths of F_1 and F_2 generation were used for DNA isolation and DNA amplification.

Table 1: Origin and commercial traits of 18 silkworm strains

S. No.	Strain	Origin	Larval pattern	Cocoon Shape	Fec	CW (g)	SW (g)	C/SR (%)	Yield
1	205MKD	Japan	Marked	Peanut	397.3	1.16	0.23	20.09	14.1
2	205MKD*205PO	Pakistan	Marked	Peanut	399.3	1.17	0.24	20.44	14.7
3	205MKD*206PO	Pakistan	Marked	Peanut	426.0	1.21	0.26	21.22	15.1
4	205MKD*C102	Pakistan	Marked	Peanut	344.0	1.18	0.24	20.34	15.0
5	205PO	China	Plain	Oval	384.0	1.09	0.24	21.84	13.6
6	205PO*206MKD	Pakistan	Plain	Oval	372.3	1.22	0.26	21.43	15.2
7	205PO*J101	Pakistan	Marked	Oval	386.8	1.33	0.28	20.95	16.7
8	206MKD	Japan	Marked	Peanut	383.4	1.2	0.25	20.72	15.0
9	206MKD*205PO	Pakistan	Marked	Peanut	415.0	1.44	0.3	21.10	14.4
10	206MKD*C102	Pakistan	Marked	Peanut	419.0	1.24	0.25	20.40	15.4
11	206PO	China	Plain	Oval	427.0	1.13	0.23	20.69	14.1
12	C102	China	Plain	Oval	393.6	1.23	0.25	20.33	15.4
13	C102*205MKD	Pakistan	Plain	Oval	410.0	1.18	0.25	21.23	14.6
14	C102*206MKD	Pakistan	Plain	Oval	438.0	1.27	0.27	21.49	15.9
15	C102*J101	Pakistan	Plain	Oval	426.0	1.17	0.25	21.56	15.7
16	H*H	Pakistan	Marked	Oval	398.0	1.5	0.31	21.01	15.0
17	J101	Japan	Marked	Peanut	356.5	1.18	0.22	18.76	13.8
18	J101*205PO	Pakistan	Marked	Peanut	393.0	1.24	0.26	20.85	15.5

Fec= Fecundity eggs/female; CW= Cocoon weight; SW=Cocoon shell weight; C/SR=Cocoon shell ratio; Yield=Kg/10,000 larvae; H*H= C102*206MKD*205PO*J101

DNA Isolation: Three silkworm moths were used for each probe for DNA isolation. The DNA was extracted and purified using Thermo Genomic DNA Purification Kit (Catalogue No. K0512). The moths were crushed in liquid nitrogen using 400 µl of Lysis Solution (10-mM Tris-HCl, pH 7.8, 150 mM NaCl, 1-mM ethylenedia-amine tetra acetic acid (EDTA), 0.5% NP-40) containing 0.2 mg/ml RNase A + protease + phosphate inhibitor) in mortar and pestle. The crushed probes were transferred to Eppendorf tube and incubated at 65°C for ten minutes using hot water bath. After incubation, 600 µl of chloroform was added in the solution and emulsified gently by inverting 3-5 times. The samples were centrifuged at 14,000 rpm for 15 minutes. Prepared precipitation solution was added by mixing 720 µl of sterilized deionized water with 80 µl of supplied 10 times concentrated precipitation. The upper aqueous phase containing DNA was transferred into a new Eppendorf tube, where 800 µL of freshly prepared precipitation solution was added. The solution was mixed gently by several inversions for two minutes and centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed completely and DNA pellet was dissolved in 100 µl of NaCl solution (1.2 M) by gentle vortexing. Cold ethanol (300 µl) added and DNA was allowed to precipitate. The precipitate was centrifuged at 14,000 rpm for 30 minutes. The ethanol was removed and the pellets were washed once with 70% cold ethanol. The DNA was dissolved in 50 µl of TE Buffer by proper mixing. The isolated DNA was stored at 4°C. DNA was quantified by 1.1% agarose gel and diluted to uniform concentration for the RAPD analysis. The method was followed as described by Suzuki *et al*. (1972).

RAPD Amplification: Polymerase Chain Reactions (PCR) were carried out by a BIO-RAD (T 100) Thermal Cycler and consisted of a total reaction of volume 25 µl containing: 12.5 µl PCR Buffer, 0.2 µl forward and reverse primers each, 0.2 µl of enzyme, 1.0 µl Taq DNA polymerase and 10.9 µl of sterile deionized water. PCR

Thermo profiling for promoter amplification was: 1st cycle at 96°C for 1 minute, 96°C for 20 minutes followed by 40 cycles each of 55°C for 20 seconds, 72°C for 30 seconds and 72°C for 5 minutes. The reaction was cooled and held at 12°C. Amplification products (3 µl) were separated by electrophoresis on a 1.0% agarose gel, stained with ethidium bromide and visualized under Gel Documentation System (Unitec). The method was used as described by Sambook *et al*. (1989).

Data Analysis: Genetic diversity was assessed using RAPD markers. All the reproducible bands amplified by five primers were counted. Similarity index matrices were generated based on standardized variables following complete linkage of the number of shared amplified fragments. Following methodology described by Bukhari *et al*. (2010), a dendrogram was constructed based on squared Euclidean distance matrix data sets by applying un-weighted pair group method of arithmetic average (UPGMA) cluster analysis using Minitab ver.17 software. The method described by Bukhari *et al*. (2010) was used.

RESULTS AND DISCUSSION

A total of five RAPD primers were tested for DNA amplification. All the primers generated amplification of DNA genome. However, in some cases, bands stained poorly, unique bands were of very low frequency and were excluded for further analysis. The five primers generated 368 bands among those 48 were polymorphic (13.21%). The highest number of polymorphic bands was 17.14% generated by primer CA-9 (Forward: CTTCCAGACAACCATTAGAT; Reverse: CAGCATCCATCCTTATTTAT) followed by the primer GA-9. The primer GA-12 and Sat-3513 generated the lowest number of polymorphic bands. The number of bands generated by each primer varied from 32 to 40. The polymorphic bands ranged between four and six in each primer. The sequence, total number of bands and polymorphic bands from each individual primer are presented in Table 2.

The level of polymorphism revealed in this study was low, however, all tested primers showed polymorphism in amplified loci. Previously, variable number of polymorphic bands in different silkworm strains with Random Amplified Polymorphic DNA (RAPD) primers have been reported by Awasthi *et al.* (2008) and Eroğlu and Arica (2009). Some of the percentage polymorphic loci amplified in the present study are comparable with those observed by Eroğlu and Arica (2009). RAPD profiles generated for 42 probes with primer CA-9 are reproduced in figure 1. One kilo bite (1 kb) plus ladder was run for size confirmation of the bands. The results of molecular analysis did not reveal considerable genetic differences among silkworm strains.

The band size ranged between 100 base pair (bp) and 400 bp. The maximum number of silkworm strains produced band size of 400 bp, while the two strains produced band size of 200 bp. One hybrid (205PO*J101) produced band size of 100 bp. Similarly, one hybrid (J101*205PO) produced band size of 300 bp. The variation in band size with varying silkworm strains and RAPD markers has also been reported by Nagaraja and Nagaraju (1995). They found amplified band size ranging from 200 bp to 1300 bp in different silkworm strains. Similarly, Eroğlu and Arica (2009), and Akkir *et al.* (2010) observed fragmented band size between 200 bp and 2,000 bp, and 200 bp and 1,500 bp, respectively in three Turkish silkworm strains by using different RAPD markers.

The matrices of genetic distance index values estimated for each pair-wise comparison of RAPD scored bands among 18 silkworm strains and presented in Table 3 eventually indicated a high degree of genetic diversity among all silkworm strains. The similarity genetic distance ranged between 0.110 and 0.991 with a mean of 0.546. The mean genetic similarity distance among the inbred lines ranged between 0.124 and 0.943 with a mean of 0.512, while the similarity distance among the hybrids ranged between 0.129 and 0.984 with a mean of 0.562. The highest genetic similarity distance among the silkworm strains was between C102 and 205PO*J101, while the lowest genetic similarity distance was between 205PO and 205MKD*205PO (Table 3).The highest genetic similarity distance in inbred lines was between 205MKD and J101, while the lowest similarity distance in inbred lines was between 205PO and 206PO. The present genetic diversity results in inbred lines showed promising potential of breeding for hybrid vigour. The highest genetic similarity distance in hybrids was between 206MKD*205PO and C102*205MKD, while the lowest genetic similarity distance in hybrids was between 205MKD*C102 and 206MKD*C102. The range of genetic diversity in hybrids was relatively greater compared to the inbred lines. The genetic diversity indicated by RAPD markers was in corroboration with commercial traits of the silkworm strains reproduced in Table 1.

The mean fecundity rate was 398.3 eggs per female and 402.3 eggs per female for inbred lines and hybrids, respectively. The mean cocoon weight, cocoon shell weight, cocoon shell ratio and cocoon yield was 1.23 g, 0.26 g, 20.80% and 14.96 kg/10k larvae, respectively. The commercial traits were relatively superior in hybrids as compared to inbred lines (Table 1). The superior commercial growth performance traits of hybrids compared to inbred lines were in conformity of greater genetic diversity unraveled through PCR amplification of the genomic DNA using five RAPD markers. Srivastava *et al*. (2005) associated commercial traits of silkworm with genetic diversity determined using RAPD markers in 20 silkworm strains differing in

voltinism and nutrigenomic. Similarly, traits in silkworm strains using polymerase chain reaction have been established (Srivastava *et al*., 2005; Ramesha *et al*., 2010).

Table 2: Primers and banding profiles by selected RAPD markers

Primer		Nucleotide sequence 5′→3′	Total bands	Polymorphic bands	%age
GA-12	F	GGTCGCCATAGCAATCCTC	39	4	10.25
	R	CGTCATTGCCTTCATTTCAG	39	4	10.25
GA-9	F	GAAGACAGAGCGAAGTGGA	32	5	15.62
	R	ATGGATTCCTGCTGGTAGAT	32	5	15.62
CT-9	F	GCTCGCCATATGCAATCCTC	40	5	12.50
	R	CGTCATTGCCTTCATTTCAGTTC	40	5	12.50
CA-9	F	CTTCCAGACAACCATTAGAT	35	6	17.14
	R	CAGCATCCATCCTTATTTAT	35	6	17.14
Sat-3513	F	CGCAATTCTGTATTAGATAA	38	4	10.52
	R	TAAAGGTATTATTCTTATTCG	38	4	10.52
		Total	368	48	-
		Mean	36.8		13.21

F= Forward; R= Reverse

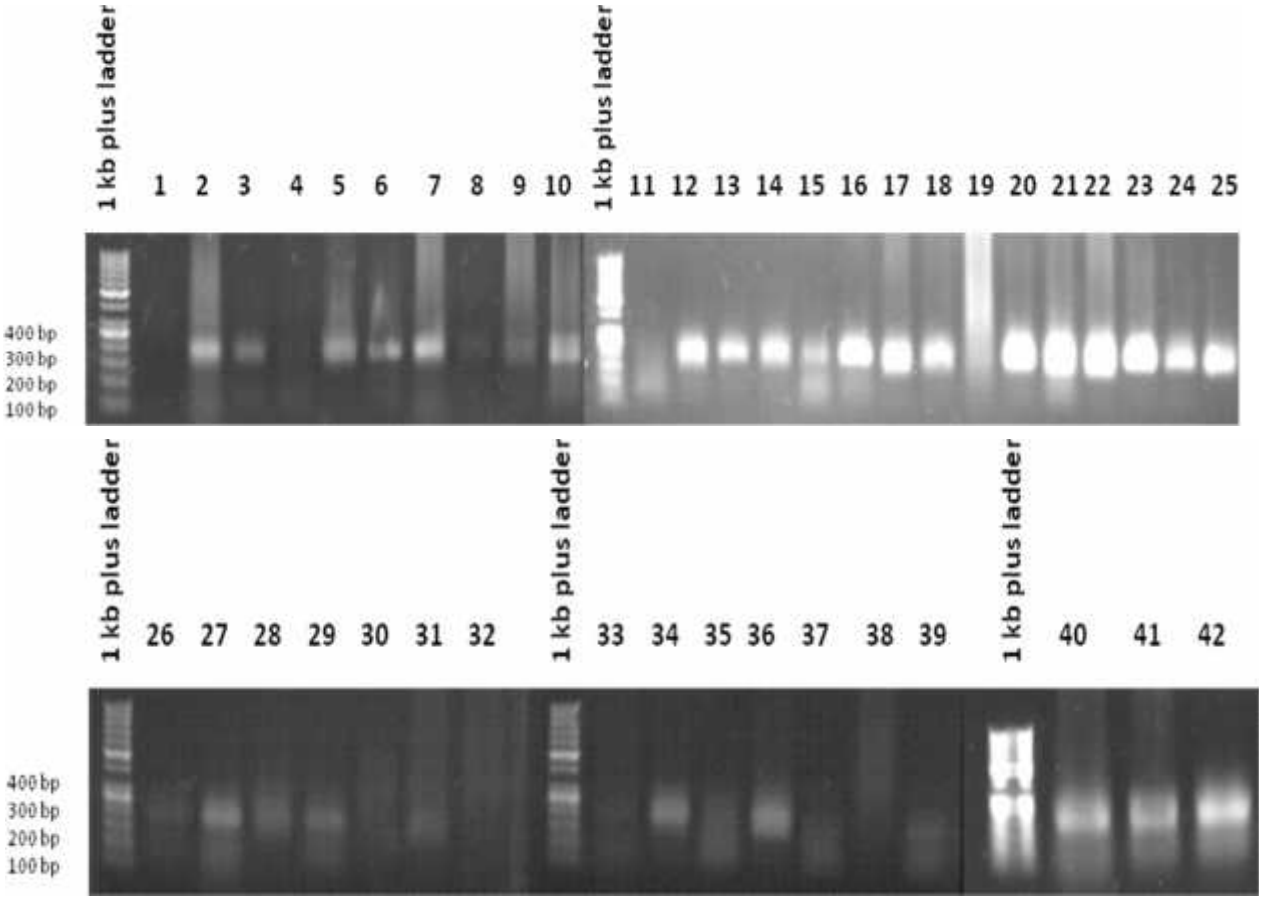

Figure 1: A PCR DNA fingerprinting of mulberry silkworm strains observed using CA-9 primer

Table 3: Genetic similarity distance matrix of inbred lines and hybrids of the silkworm strains

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	0																	
2	0.542	0																
3	0.375	0.539	0															
4	0.562	0.568	0.187	0														
5	0.749	0.110	0.374	0.187	0													
6	0.937	0.668	0.562	0.375	0.187	0												
7	0.951	0.382	0.141	0.902	0.973	0.956	0											
8	0.420	0.124	0.239	0.667	0.510	0.768	0.234	0										
9	0.499	0.420	0.124	0.937	0.750	0.562	0.973	0.442	0									
10	0.686	0.526	0.311	0.124	0.937	0.749	0.802	0.568	0.186	0								
11	0.873	0.641	0.499	0.311	0.124	0.937	0.841	0.610	0.375	0.185	0							
12	0.060	0.765	0.686	0.499	0.311	0.124	0.991	0.567	0.562	0.375	0.187	0						
13	0.741	0.962	0.967	0.780	0.592	0.405	0.984	0.480	0.843	0.656	0.468	0.281	0					
14	0.622	0.973	0.248	0.660	0.873	0.686	0.898	0.520	0.223	0.937	0.749	0.562	0.281	0				
15	0.869	0.622	0.173	0.931	0.895	0.765	0.969	0.499	0.419	0.623	0.439	0.467	0.587	0.242	0			
16	0.797	0.768	0.622	0.635	0.648	0.560	0.980	0.641	0.499	0.311	0.324	0.937	0.656	0.375	0.342	0		
17	0.943	0.865	0.988	0.813	0.739	0.567	0.895	0.531	0.489	0.811	0.659	0.516	0.321	0.362	0.284	0.134	0	
18	0.519	0.370	0.524	0.424	0.680	0.766	0.752	0.798	0.820	0.587	0.548	0.542	0.719	0.626	0.511	0.424	0.653	0

To study the genetic relatedness a dendrogram was constructed based on squared Euclidean distance matrix data sets by applying UPGMA is shown in figure 2. The dendrogram indicated divergent genotype of the silkworm strains. All silkworm strains were grouped into four clusters at 66% similarity level. Among the four clusters, Cluster-I was the smallest which comprised two strains including a hybrid J101*205PO and a double hybrid H*H. The Cluster-II was the largest and consisted of eight strains including: two inbred lines and six hybrids. The hybrid 206MKD*205PO and inbred line J101 were at the distant ends in cluster-II. The Cluster-III contained three strains, one inbred line and two hybrids. The Cluster-IV was the 2nd largest and contained five strains. The cluster analysis showed that 206MKD*205PO and inbred line J101 were remotely related. All the strains grouped into two clusters at 33% similarity level. The Cluster I and Cluster II were clubbed with Cluster III (Figure 2).

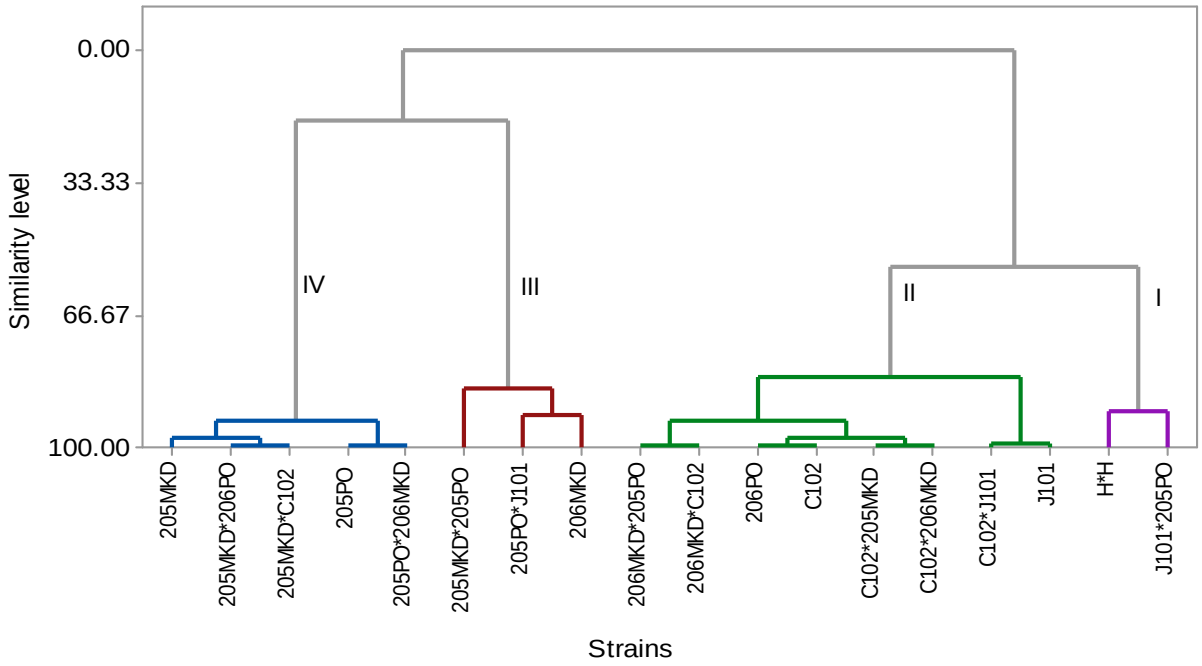

Figure 2: Dendrogram with complete linkage and squared Euclidean distance constructed using UPGMA

Conclusion: Genetic diversity and polymorphism among strains is evident through amplification with tested primers and promising parents for productive and disease resistant silkworm strains can be obtained.

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