Fingerprinting of non-diapausing silkworm, *Bombyx mori*, using random arbitrary primers

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**Summary**  The Random Amplified Polymorphic DNA (RAPD) technique was used to study DNA profiling of 5 multivoltine silkworm genotypes. The silkworm *Bombyx mori* L, (Lepidoptera; Bombycidae) were analyzed using 30 random primers among which 18 polymorphic primers gave 73 amplified products and of which 38.3% were polymorphic. The dendrogram generated using an un-weighted pair grouped method with arithmetic averages revealed the pattern of relatedness of 5 genotypes. Genetic similarity co-efficient and cluster analysis were performed by a hierarchical clustering technique. The genetic distances between the clusters and within the clusters estimated 6% variability between the 4 races and Nistari. The results of our study indicate that RAPDs are very efficient in the estimation of genetic diversity in populations that are closely related and acclimatized to local environmental conditions. The polymorphic data obtained from the study can be further utilized for MV genome mapping research and finally to assign function to sequences through biometrical tools. Modern breeding tools like molecular markers which show easily detectable differences among different races of a species offer a wide range of applications for silkworm breeding programs. India is being a country with diverse environmental conditions, the local races are rich reservoirs of many resistant genes, and molecular markers are inevitable tools to study inheritance of such complex genes.

**Key words**  Multivoltine, Random Amplified Polymorphic DNA, Cluster analysis, Acclimatized, Marker Assisted Selection.

Domesticated insect *Bombyx mori* L is used for silk production and it comprises large number of races distributed both in tropical and temperate countries. The silkworm is raised in many countries in the world by virtue of its commercial value and has wide distribution with well-defined geographical races (Hirobe 1968). These races display genetic, physiological, ethological, morphological, biochemical and quantitative differences (Yokoyama 1979, Gamo and Ohtsuka 1980). The genotypes include univoltine (UV), bivoltine (BV) and multivoltine (MV) strains with 1, 2 and many generations respectively per year. UV and BV races yield good quality and quantity silk but are very prone to environmental changes and diseases, where as MV races are moderately resistant to diseases, less prone to environmental changes but yield silk of low quality and quantity. The relationship between voltinism, moultinism and quantitative traits has been reasonably well defined (Nagotomo 1942).

Silkworm hybrid production has been practiced in southern India from very early years and local MV races were utilized for the production of hybrids with various races obtained from temperate countries. The development of pure mysore race was a major success which adapted well to the local environment conditions than all the other races. It was moderately resistant to biotic and abiotic constraints and it converged well with all the introduced bivoltine varieties to produce cross
breeds. Many high yielding BV races were used for production of crossbreeds with only pure mysore has a female parent and such usage of a narrow genetic based female parent for many years has led to the creation of hybrid that is susceptible to various biotic and abiotic factors in dwindling situation of the environment.

Lack of assessing genetic diversity in the available germplasm, unavailability of modern tools to know the genomes at molecular level, environmental disturbances during the time of selection and polygenic control of various traits in silkworm have led to the poor selection of parents in breeding programs (Datta 1984, Datta and Ashwath 2000). It is well known that the resistance to biotic and abiotic constraints is governed by polygenes with complex inheritance patterns and with lot of environmental influences. Therefore it is required to produce genotypes for particular geographical environment by utilizing the races acclimatized to that location. Although BV races yield good quality and quantity of silk the source of resistance to environmental factors is with in the MV races. New breeding tools like molecular markers can be effectively applied with conventional breeding strategies and the genes beneath the resistance can be discovered.

The use of molecular markers is well suited to the genetic improvement of the silkworm (Nagaraju 2002, Nagaraju and Singh 1997). Such MV races acclimatized to local environmental conditions are very less studied at the DNA level. Analyzing closely related silkworm races using DNA markers helps to identify closely linked genetic markers for various constraints and allows molecular characterization of these multivoltine races, which improves the selection of polygenetic traits in breeding programs. Analyzing closely related races for more different primer sequences will reveal the sequence variation of many unknown genes and assigning functions to these sequences will help to dissect the genetics of hardiness in such highly domesticated insect species.

In the present study, we report the genetic differences among multivoltine silkworm races using the RAPD technique and demonstrate its utility for the genetic improvement of such hardy MV race.

Materials and methods

Silkworm stocks

Five non-diapausing silkworm genotypes including, Pure mysore, Nistari, Mysore princess, Kollegal jawan and Tamilnadu white were used in the present study and were obtained from the Central Sericultural Germplasm Resource Centre (Table 1). Eggs obtained from these races were allowed to hatch and larvae were reared under laboratory-controlled conditions according to standard rearing techniques as described by Krishnaswamy et al. (1978).

Genomic DNA isolation

Genomic DNA was isolated from the moths of all the 5 silkworm races following the method of Suzuki et al. (1972). Two or 3 moths of each races were grinded separately in liquid nitrogen using a mortar and pestle. Extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA and 1% SDS) and proteinase K (100 μg/ml) were added to the grinded tissue and incubated at 37°C for 2 h with occasional swirling. The DNA was extracted twice with phenol-chloroform-isoamylo alcohol (24 : 24 : 1) and once with chloroform. The supernatant DNA was ethanol-precipitated, resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and incubated at 37°C for 1 h after addition of RNase A (100 μg/ml), DNA was reextracted with phenol-chloroform and ethanol-precipitated as described earlier. The genomic DNA was quantified on 0.8% agarose gels and diluted to a uniform concentration (10 ng/μl) for the RAPD study.

DNA amplification and separation

The amplification of genomic DNA was performed according to Nagaraja and Nagaraju
(1995) with slight modifications. The amplification was carried out in a 20 μl reaction volume containing 50 ng of template DNA, 1×PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂ and 50 mM KCl), 0.15 μM primer, 100 μM each of dATP, dCTP, dGTP and dTTP, 0.3 units of Taq DNA polymerase (Boehringer Mannheim). The random sequence 10-mer primers were obtained from Operon Technologies. Each reaction was overlaid with 15 μl mineral oil (Sigma). For each primer examined, negative control was maintained which contained all the components except genomic DNA. Amplification was performed in Eppendorf thermo cycler (25 wells) and programmed for initial denaturation: 94°C for 5 min, followed by 34 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min and final extension of 72°C for 5 min. The amplified products were separated according to molecular size on 1.5% agarose on TAE buffer (40 mM Tris, 20 mM acetic acid, 10 mM EDTA) and detected by staining with ethium bromide. The DNA fragments amplified by a given primer were scored as present (1) or absent (0) for all the genotypes studied. The RAPD marker showing monomorphic pattern or inconsistent amplification bands were excluded from the final analysis.

**Statistical analysis**

Statistical analysis was conducted using ‘STATISTICA’ computer Package. The program used

<table>
<thead>
<tr>
<th>Strains</th>
<th>Voltinism</th>
<th>Origin</th>
<th>Larval pattern</th>
<th>Cocon color</th>
<th>Cocon shape</th>
<th>Larval duration (H)</th>
<th>Cocon weight (g)</th>
<th>Shell weight (g)</th>
<th>Shell ratio (g)</th>
<th>Filament length (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nistari</td>
<td>Non diapausing</td>
<td>West Bengal (India)</td>
<td>Marked</td>
<td>Golden yellow</td>
<td>Spindle</td>
<td>557</td>
<td>1.017</td>
<td>0.132</td>
<td>12.996</td>
<td>399</td>
</tr>
<tr>
<td>Kollegal Jawan</td>
<td>Non diapausing</td>
<td>Karnataka (India)</td>
<td>Plain</td>
<td>White</td>
<td>Oval</td>
<td>549</td>
<td>1.276</td>
<td>0.192</td>
<td>5.121</td>
<td>698.5</td>
</tr>
<tr>
<td>Mysore Princes</td>
<td>Non diapausing</td>
<td>Karnataka (India)</td>
<td>Plain</td>
<td>White</td>
<td>Oval</td>
<td>558</td>
<td>1.164</td>
<td>0.182</td>
<td>15.836</td>
<td>326</td>
</tr>
<tr>
<td>Tamilnadu White</td>
<td>Non diapausing</td>
<td>Tamilnadu (India)</td>
<td>Plain</td>
<td>White</td>
<td>Oval</td>
<td>566</td>
<td>1.121</td>
<td>0.187</td>
<td>15.703</td>
<td>428.5</td>
</tr>
<tr>
<td>Pure Mysore</td>
<td>Non diapausing</td>
<td>Karnataka (India)</td>
<td>Plain</td>
<td>Light green</td>
<td>Spindle</td>
<td>626</td>
<td>0.995</td>
<td>0.139</td>
<td>14.073</td>
<td>307</td>
</tr>
</tbody>
</table>

![Fig. 1. Five silkworm genotypes with ladder, Lane: M: Molecular weight marker; Lane: 1 Nistaru; Lane: 2 Kollegal Jawan; Lane: 3 Mysore Princes; Lane: 4 Tamil Nadu While; Lane: 5: Pure Mysore, White arrow mark towards right side indicate polymorphic band and towards left side indicate monomorphism bands.](image-url)
was cluster analysis joining (tree clustering) with raw input data. The main parameters, which guided the joining process of linkage was ‘Unweighted Pair Group method with Arithmetic mean’ (UPGMA) and the distance was computed using Euclidean distance.

Results

In the present investigation, 73 distinct DNA bands were generated with 30 decamer RAPD primers in 5 silkworm races. Among the 30 RAPD primers used in the study, 18 primers gave polymorphic bands. Out of total 73 bands, 28 bands (38.3%) were polymorphic and 45 bands (61.64%) were monomorphic. For each primer evaluated, a multiple band profile comprising 1 to 7 major amplifications and a varying number of weak products or a faintly smeared region were observed. Highest numbers of bands (6) were produced by primers OPM-12 and OPN-13 (Fig. 1) and this indicates that more amplification sites were available corresponding to the nucleotide sequence of these particular primers. Primers OPN-16 and OPK-17, which had three polymorphic bands, in contrast OPM-20, OPN-4, OPO-3 and OPK-1 had 10 monomorphic bands. The relationships between the 5 multivoltine silkworm races are portrayed in the dendrogram (Fig. 2). The Bengal race, Nistari, which produces golden, colored silk, was distinct from the other races, which were more closely related to one another.

UPGMA cluster initially showed 2 main clusters with Pure mysore, Mysore princess, Kollegal jawan and Tamilnadu white on 1 cluster and Nistari on other cluster. The group of all 4 genotypes shared 3% dissimilarity and 97% similarity. Nistari formed the other separate main cluster at 6.2% dissimilarity. Mysore princess and Kollegal jawan shared only 1% dissimilarity and Tamilnadu white showed 1.5% dissimilarity. Pure mysore formed a separate cluster at 3% dissimilarity, while remaining 97% was similar.

Discussion

Four genotypes, Pure mysore, Tamilnadu white, Mysore princess and Kollegal jawan are of south Indian origin and are geographically distinct from the East Indian race Nistari and even the RAPD data obtained in the present study showed the same pattern of diversity. Among the 5 races studied closer similarity was obtained between the Tamilnadu white, Mysore princess and Kollegal jawan races. These 3 high-yielding multivoltine races were originally developed during 1965 by applying a double cross method in Mysore and Madras province with Pure mysore has one of the parent (Chatterjee et al. 1993). Larval color, cocoon color and cocoon shape of all these 3 races are also similar and they differ in resistance to various stress. The Pure mysore race shared 3% dissimilarity with its progenies and showed some uniqueness which may be due to its source of resistance. Just with random sequence analysis these four closely related south Indian races are showing variation and this difference may be because of differences in the sequences for various biotic and Abiotic stresses.

The MV strains have been studied much through classical genetics but there study at nucleotide level is still in infant stage. The variation obtained at sequence level through various kinds of PCR amplification really accounts for variation in many important phenotypic traits. It is proven fact that the MV races are more resistant than BV races, and molecular studies of such races will help to understand the genetics of resistance in these domesticated insects, and the molecular studies on prevailing indigenous races will throw light on complex Genotype x environment (GXE) in-
interactions. For sustain of the industry such races are very important and their utilization in silk-worm breeding is inevitable for the development of region specific races. Studies on such races are very much necessary to develop a farmer preferred; resistant variety which will thrive very well at the prevailing local conditions of Indian villages.

The amplification products resulting from the RAPD assay vary between genotypes and hence can be used as genetic markers as well as to construct linkage maps. Modern tools like molecular markers offer a wide range of applications for silkworm breeding programs. India being a country with diverse environmental conditions the local races are rich reservoirs of many resistant genes and molecular markers are inevitable tools to study inheritance of such complex traits. The results of our study indicate that RAPDs are very efficient in the estimation of genetic diversity in populations that are closely related and acclimatized to local environmental conditions. The polymorphic data obtained from the study can be further utilized for MV genome mapping research and finally to assign function to sequences through biometrical tools. Extensive classical breeding programs with available modern breeding tools, such has DNA markers will help to dissect the genetics of hardiness in tropical environment in such races. In future studies of the silkworm we anticipate increasing the variety of molecular studies and increase the number of species examined to provide a greater understanding of the genetic resources of silkworm. To develop races for a particular geographic region and constraint the study on such tropical races are very much obligatory and present work was a beginning with DNA fingerprinting.

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References


