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RAPD Analysis of Genetic Variability among Silkworm Breeds

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ABSTRACT

Four mulberry silkworm breeds viz., Pure Mysore, Nistari, NB_4D_2 & CSR2 and two hybrid (Pure Mysore x CSR_2 and Nistari x NB_4D_2) silkworms were used for the present study. Genomic DNA was isolated from samples and loaded on agarose for checking the quality. PCR amplification was performed using RAPD primer. The PCR products were loaded on 2 % agarose gel. The results obtained by RAPD analysis software including System Software Aligner, Weighbor Tree, Jukes-Cantor Correction, Bootstrap. The RAPD profile obtained on agarose gel as well as phylogenetic tree showed Sample CSR_2, NB_4D_2, Nistari X NB_4D_2, Nistari, Pure Mysore and Pure Mysore X CSR_2 are different from each other. Also the phylogenetic relationship presented branching-out CSR_2 and Pure Mysore together and bootstrap value between the two strains is 61. Sample NB_4D_2 also branching from the root but is different from CSR_2 and Pure Mysore.

Key words: *Bombyx mori, genetic variability, hybrid, RAPD analysis*

INTRODUCTION

Due to their great economic value, more than 3000 genetically different silkworm (*Bombyx mori*) strains, some of which produce different qualities and yields of the silk, are maintained in Europe and Asia[18]. Recently, genetic markers are used in animal and plant improvement programmes for varietal and parentage identification, construction of linkage maps and evaluation of polymorphic genetic loci affecting quantitative economic traits[16]. Development of molecular markers is important for construction of linkage map and fingerprinting of silkworm strains for breeding[16]. Also, PCR based techniques have been widely used to detect the polymorphic genetic markers in the silkworm[16, 17]. Random amplified polymorphic DNA (RAPD) is one of the PCR- based techniques used as a tool for genetic mapping and strain identification[15,16,24]. Because of its relative simplicity, RAPD method is being extensively used in genetic analysis[17]. The most important factor in biological genetic resource conservation regimes is to maintain pure strains and establish accurate genetic relationship among species[21].

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The use of different markers to determine the intraspecific biodiversity and better understanding of genetic polymorphism of silkworm have always been within the range of researcher's interest[19,6,8]. Identification of biodiversity in the silkworm is also important for designing better breeding programs. RAPDs have been successfully used for fingerprinting of viruses, bacteria, fungi, plants, humans[5,10,26] and, more recently, insects[3,9]. In the present study, the genetic diversity and genetic structure of the four pure and two hybrids of silkworm *Bombyx mori* L., were estimated simultaneously using RAPD markers. The aim of this study is to assess the genetic diversity of the silkworm races in a parallel study and to investigate the molecular variation among them, so that the knowledge gained may serve as an important yardstick in silkworm breeding.

MATERIAL AND METHODS

Silkworm races

Four mulberry silkworm races *viz*., Pure Mysore, Nistari, NB_4D_2 & CSR_2 and two hybrid (Pure Mysore × CSR_2 and Nistari × NB_4D_2) silkworms were used for the present investigation. The silkworm rearing was conducted in the laboratory following the method described by Krishnaswamy[12]. The larvae on third day of fifth instar were collected and the midgut tissue was obtained by dissecting the larvae in ice cold water and the gut contents were removed. The midgut tissue was collected from all the genotypes for DNA extraction. DNA was extracted according to the procedure of described by Nagaraja[14] and Somasundaram[22].

Genomic DNA Isolation

100mg of tissue material was taken in a mortar and pestle. 750 µl of suspension buffer was added, homogenized and pipetted into a 2ml vial. 5 µl of the RNase solution was added to the above and then mixed 5-6 times by inverting the vial and placed it at 65°C for 10 min with intermittent mixing. 1ml lysis buffer was added to the above, mixed 5-6 times, incubated the mixture at 65°C for 15 min. Cooled to Room Temperature (RT), spun at 13,000g for 1min at RT and the clear supernatant collected in a 2ml vial. Supernatant was loaded on the spin column (600 µl each time) and they were spun at 13,000g for 1min at RT, discarded the contents of the collection tube and placed the spin column back in the same collection tube. 500 µl of wash buffer was add to the column, spun at 13,000g for 1min at room temperature, discarded the contents of the collection tube, placed the spin column back in the same collection tube and repeated again. The empty column was spinned with the collection tube at 13,000g for 2 min at RT, and placed the spin column in a fresh vial. 50 µl of warm elution Buffer (already kept at 65°C) was added into the spin column. The vial along with the spin column was kept at 65°C for 1 min, spun at 13,000g for 1 min at room temperature. Two steps back were repeated, eluted and collected in the same vial. DNA concentration was determined by both UV-spectrophotometer and quantitative analysis on agarose gel. Genomic DNA was isolated from samples using Chromous Bacterial genomic DNA isolation kit (RKT09). Genomic DNA isolated was loaded on agarose for checking the quality.

RAPD-PCR Analysis

Genomic DNA isolated was PCR amplified under below conditions:

DNA: 1µl, RAPD primer: 2 µl, dNTPs(10mM) 2 µl, Taq DNA polymerase, Assay buffer (10X) 5 µl, Taq DNA Polymerase 0.5 µl, Water 39.5 µl. Total volume was made 50 µl. The PCR conditions included initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94°C for 1 min, 55 °C for 1 min, 72 °C for 2 min with final extension at 72 °C for 2 min. Polymorphic RAPD Primer Used in the Study was 5'-AGGACTCGATAACAGGCT-3'. PCR amplification was performed using RAPD primer. The PCR products were loaded on 2 % agarose gel. 100bp Ladder (Chromous Biotech, India) was used as a molecular weight marker.

RAPD analysis software details

Phylogentic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-

Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000. For estimating the sampling distribution by resampling with replacement from the original sample Bootstrapping was used. In making phylogenetic trees, the approach is to create a pseudoalignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudoalignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The GeneScan™ 500 LIZ® Size Standard is a fifth dye-labeled size standard for the reproducible sizing of fragment analysis data. This size standard is used for fragments between 35 and 500 bp and marker fragments can be labeled with the dyes FAM™, VIC™, NED™ or PET®.

RESULTS

Figure 1 shows Genomic DNA fractionated on 1% agarose. Lanes L,1, 2, 3, 4, 5 and 6 as 500 bp Ladder, CSR_2, NB_4D_2, Nistari x NB_4D_2, Nistari, Pure Mysore, Pure Mysore x CSR_2 respectively. PCR was performed as per standard protocol. Figure 2 shows PCR products which were electrophorectically analyzed through 2% agarose gels containing ethidium bromide (5 µg/ml) in 10X Assay buffer (PH 8.0) and documented in the gel documentation system. A molecular weight marker was used for analysis of the fragment size. It includes seven lanes L, 1, 2, 3, 4, 5 and 6 as 100 bp Ladder, RAPD profile of CSR_2, NB_4D_2, Nistari x NB_4D_2, Nistari, Pure Mysore, Pure Mysore x CSR_2. 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 weight ranging from 100 bp to 1000 bp depending on the amplification of DNA. The genetic distance estimated from the RAPD markers, following the methods of Nei and Li/Dice [16], varied from 0.23404 (Pure Mysore and Nistari × NB_4D_2) to 0.52941 (CSR_2 and Pure Mysore x CSR_2) (Table 1). The cluster analysis based on RAPD analysis software details and based on Phylogenetic Tree has generated the dendrogram which has clearly separated the races. From the RAPD profile obtained on agarose gel as well as phylogenetic tree, it is observed that the Sample CSR_2, NB_4D_2, Nistari x NB_4D_2, Nistari, Pure Mysore and Pure Mysore x CSR_2 are different from each other. The phylogenetic relationship shows branching out of CSR_2 and Pure Mysore together and Bootstrap value between the two strains is 61. Sample NB_4D_2 also branching from the root but it is different from CSR_2 and Pure Mysore. Also RAPD peak data files(Figures 4 - 9) show that RAPD primer is labeled with FAM which gives blue color in Genescan analysis and Orange peaks are internal size standard.

Table 1: Distance Matrix Table: (Nei and Li/Dice method), Tree-construction method: Neighbor-joining

RACES	CSR2	NISTARI x NB_4D_2	NB_4D_2	NISTARI	PURE MYSORE x CSR_2	PURE MYSORE
CSR2	-	0.38776	0.45833	0.5	0.52941	0.26087
NISTARI x NB_4D_2	0.38776	-	0.38776	0.26316	0.38462	0.23404
NB_4D_2	0.45833	0.38776	-	0.464	0.529	0.391
NISTARI	0.5	0.26316	0.464	-	0.322	0.333
PURE MYSORE x CSR_2	0.52941	0.38462	0.529	0.322	-	0.387
PURE MYSORE	0.26087	0.23404	0.391	0.333	0.387	-

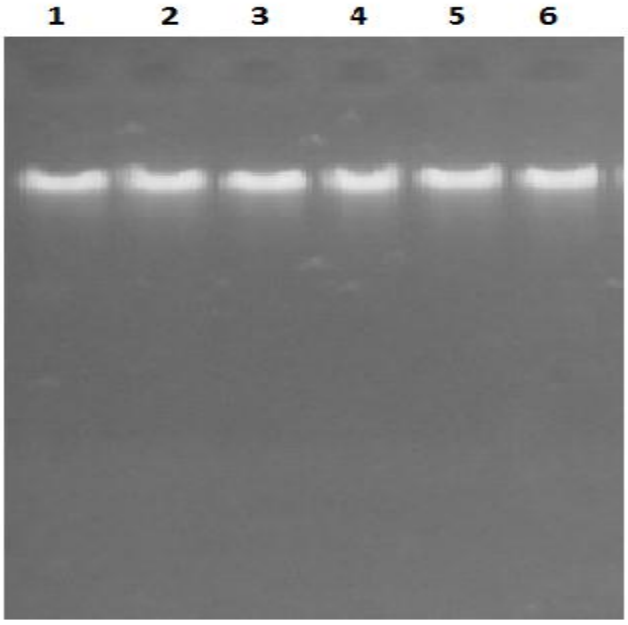

Figure 1: Genomic DNA extracted from 6 samples run on 1% agarose gel
Lane description: Lane 1: CSR_2, Lane 2: NB_4D_2, Lane 3: Nistari x NB_4D_2, Lane 4: Nistari, Lane 5: Pure Mysore, Lane 6: Pure Mysore x CSR_2

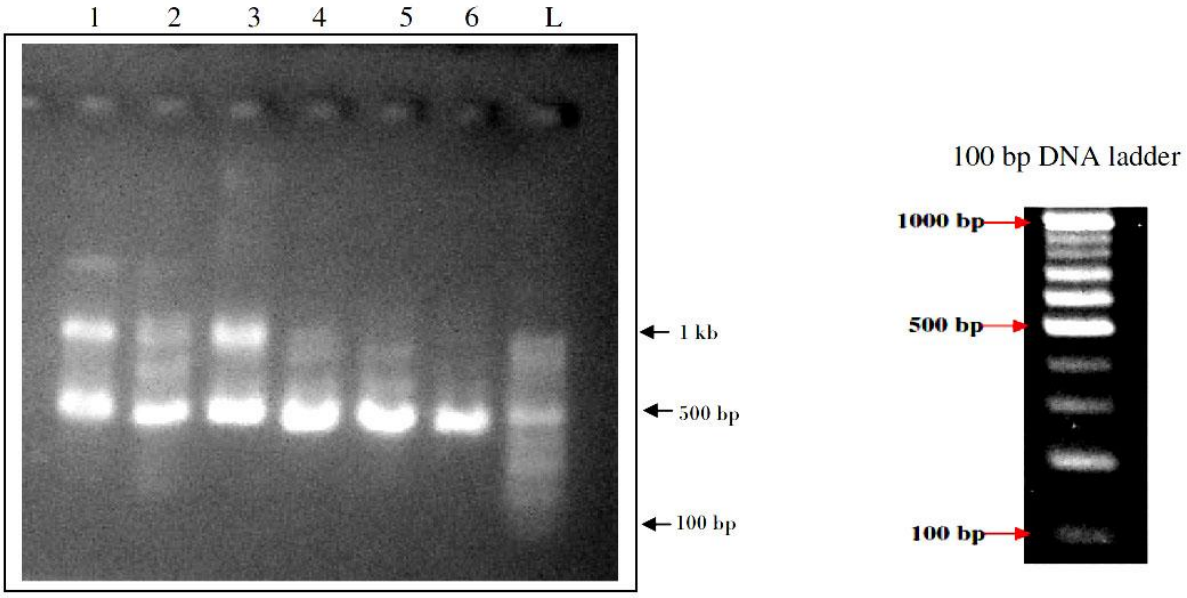

Figure 2: RAPD RESULTS
Lane description: Lane 1: RAPD profile of CSR_2, Lane 2: RAPD profile of NB_4D_2, Lane 3: RAPD profile of Nistari x NB_4D_2, Lane 4: RAPD profile of Nistari, Lane 5: RAPD profile of Pure Mysore, Lane 6: RAPD profile of Pure Mysore x CSR2, Lane L: 100 bp Ladder.

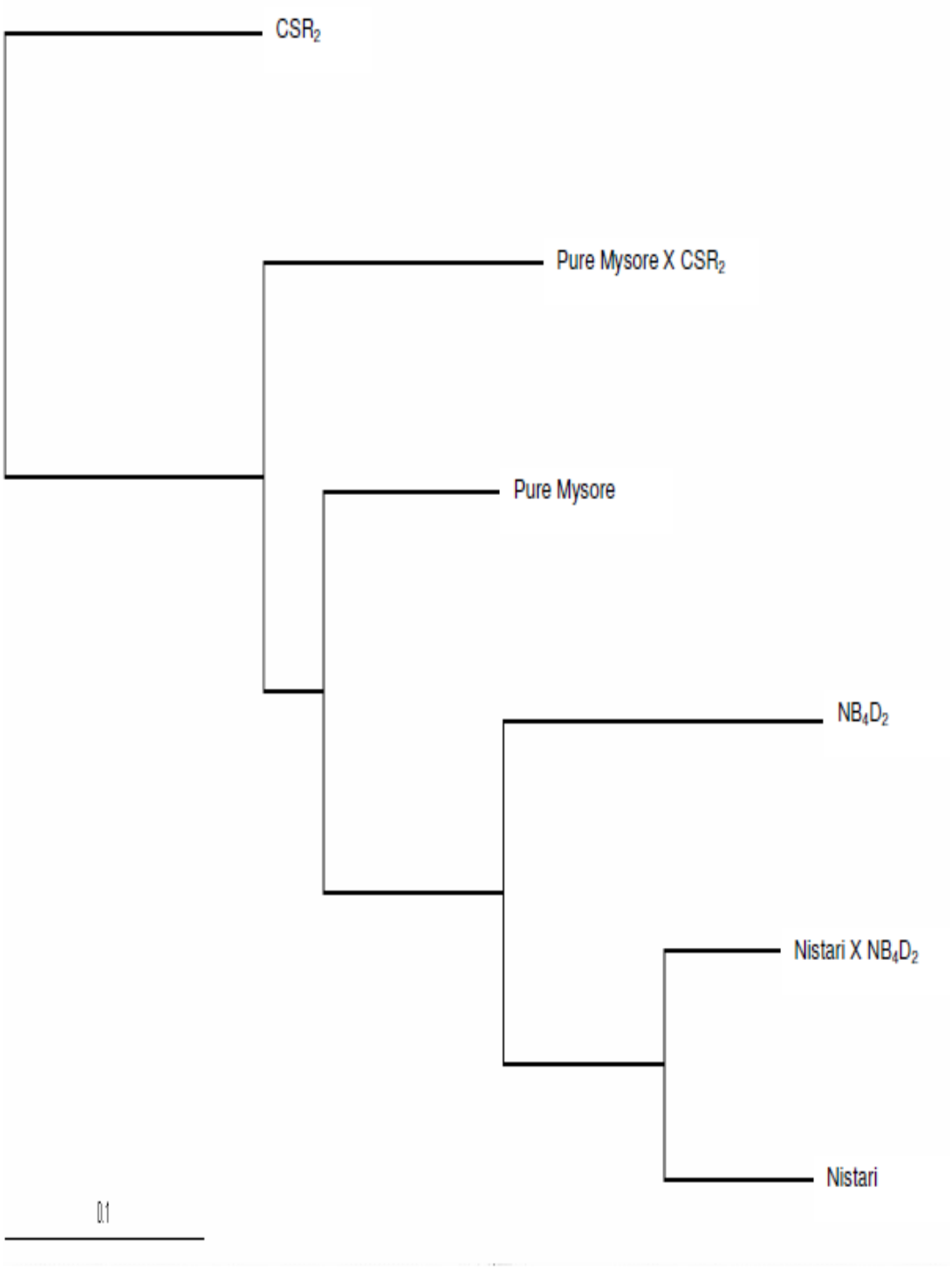

Figure 3: PHYLOGENETIC TREE

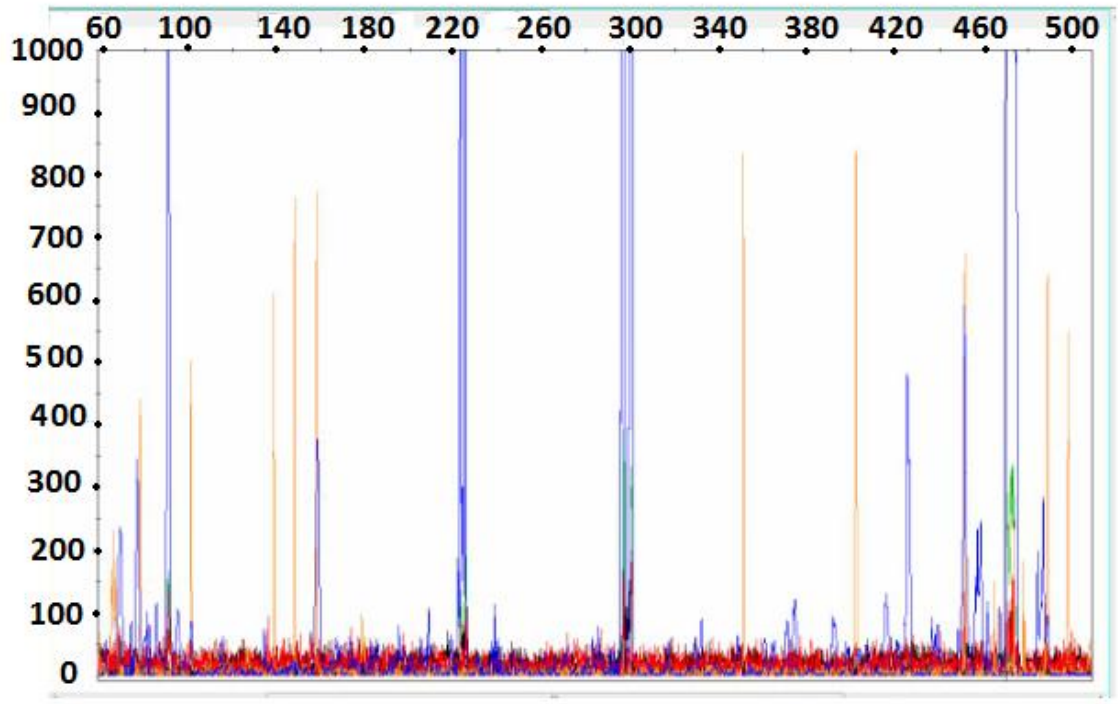

Figure 4: CSR_2 RAPD peak data file

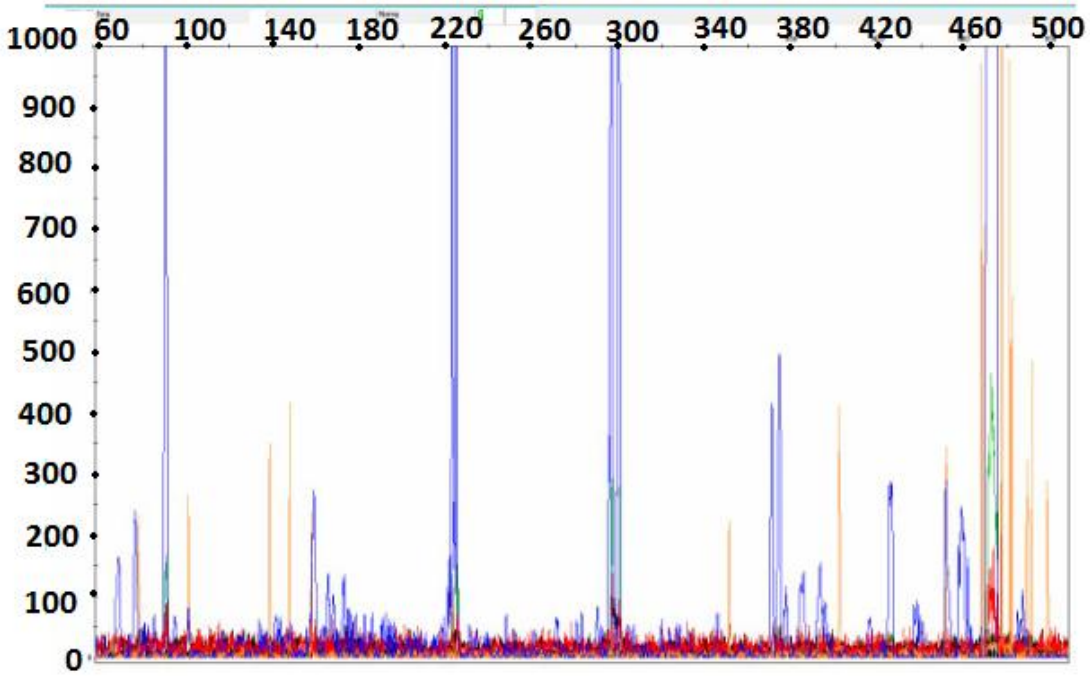

Figure 5: NB_4D_2 RAPD peak data file

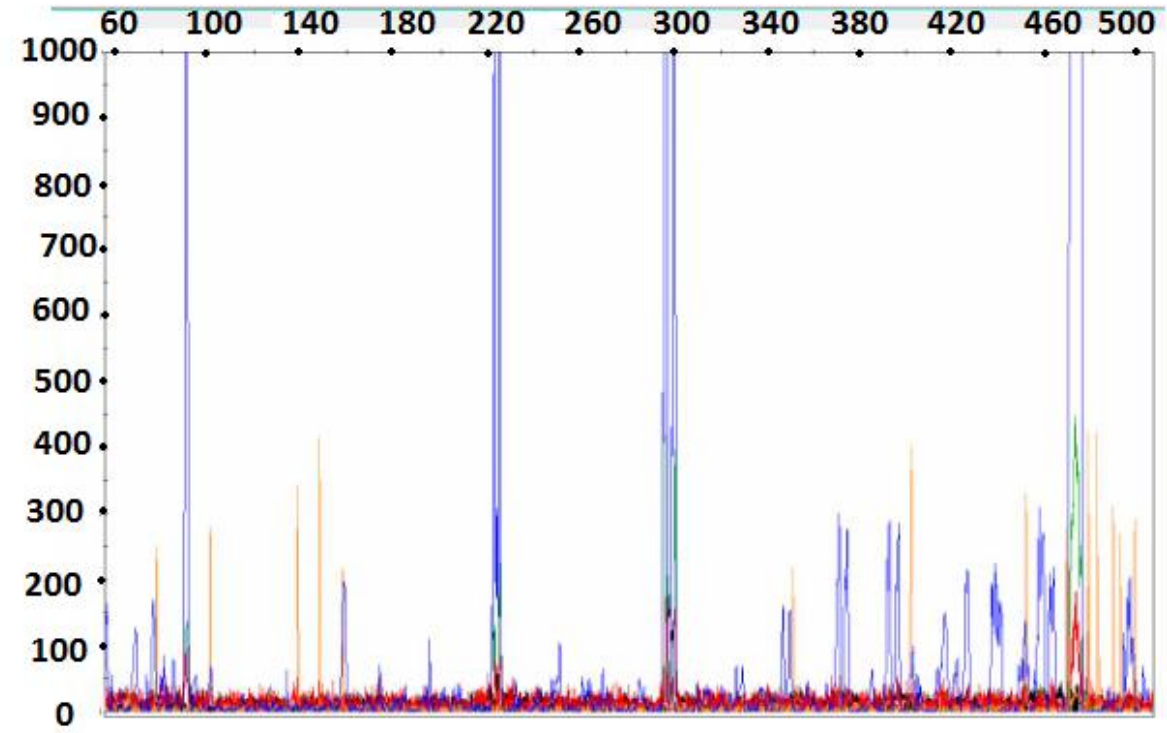

Figure 6: Nistari RAPD peak data file

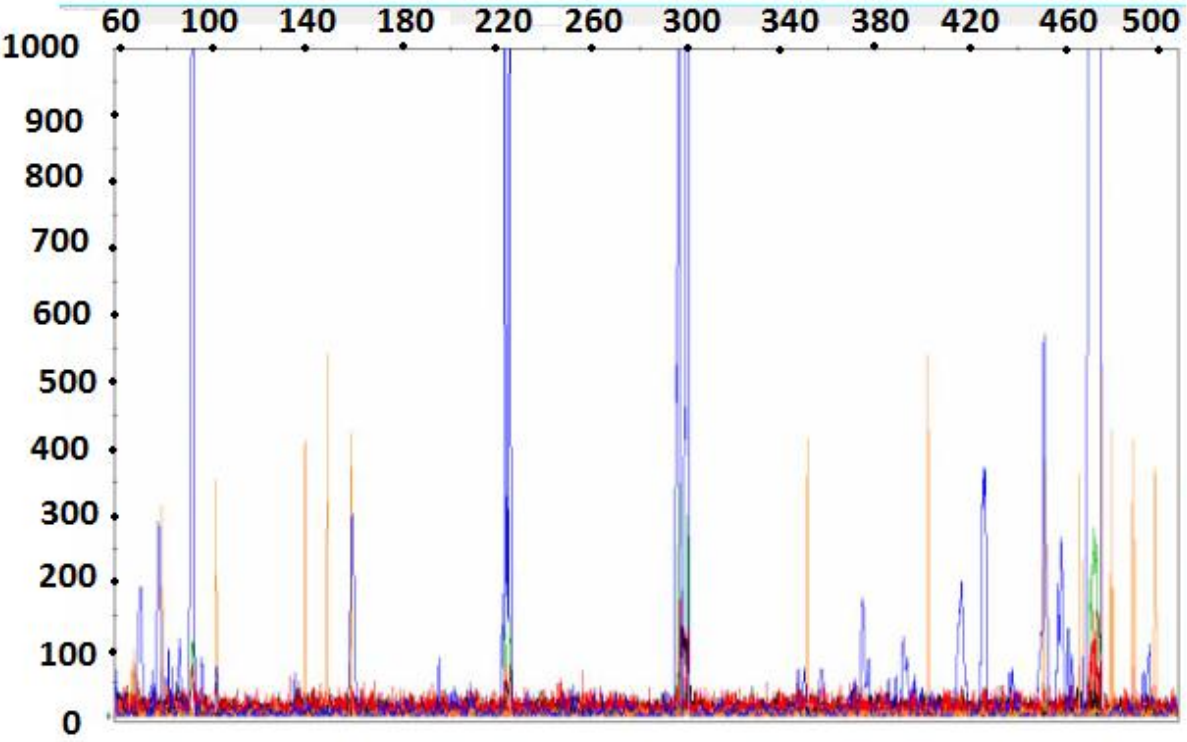

Figure 7: Pure Mysore RAPD peak data file

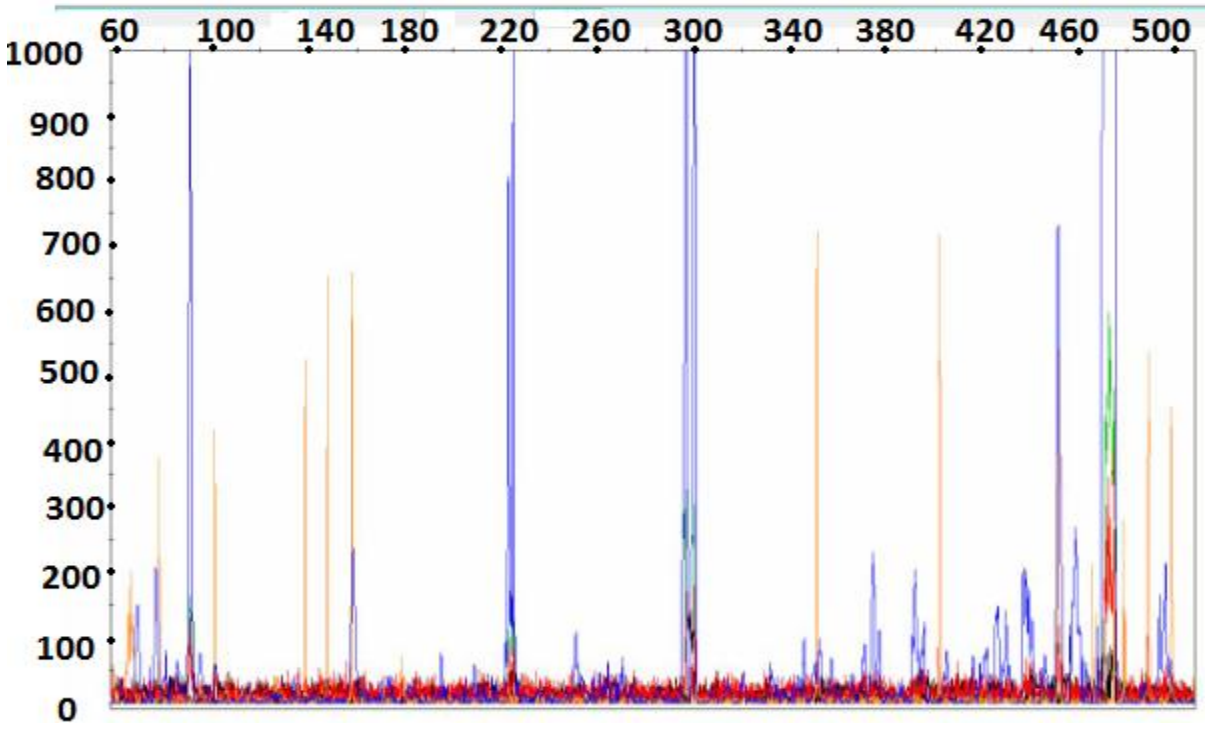

Figure 8: Pure Mysore x CSR_2 RAPD peak data file

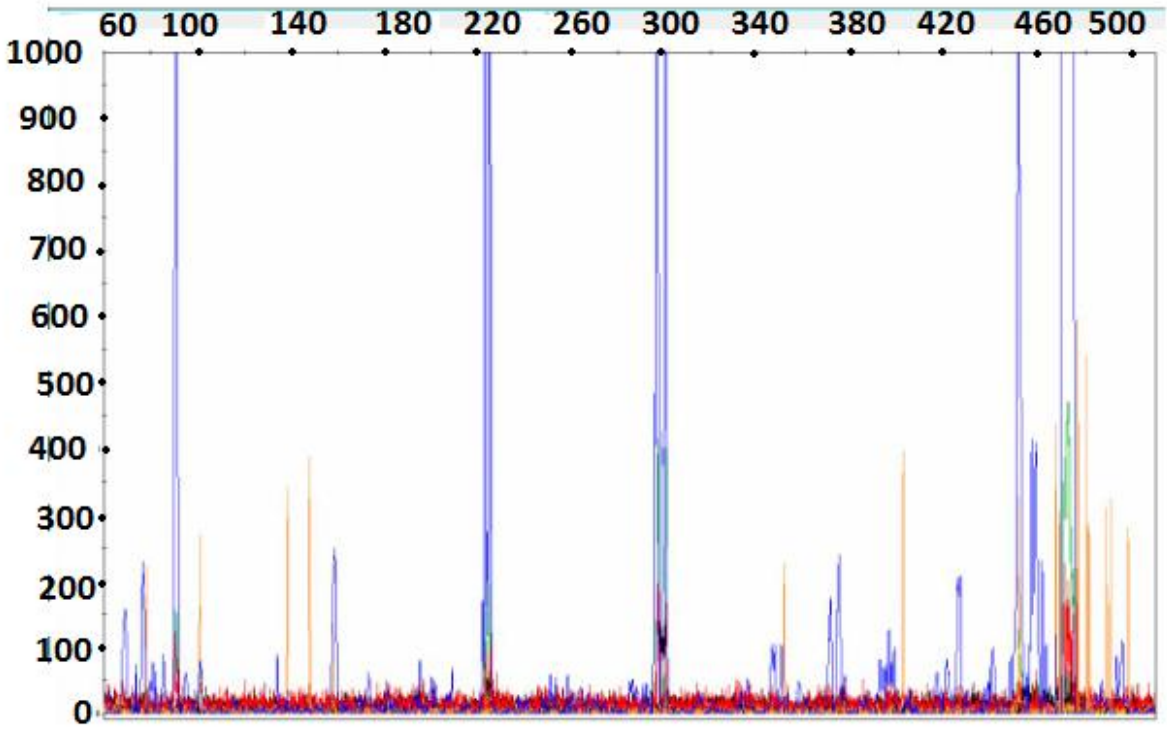

Figure 9: Nistari x NB_4D_2 RAPD peak data file

DISCUSSION

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 phylogenetic control of various traits have been reported [7,28,2,25]. New tools like molecular markers can be effectively applied with conventional breeding strategies and the genes for the resistance can be discovered[16]. 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[29]. 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 [23]. Arunachalam[1] reported that there is an optimum level of genetic divergence between parents to obtain heterosis in F1 generation and they also reported that it may not be logical to advocate the use of extremely divergent parents to obtain heterotic combinations. The average genetic distance from analyzed samples proved to be relatively high, which can be due to the fact that hybrids are from two different species and also to the distant origin of these species. With RAPDs it is possible to assess genetic similarity amongst a range of silkworm genotypes on the basis of band sharing analysis. In conclusion, RAPD analysis promises to become a valuable tool for analysis of genetic variation, estimating genetic distance among populations and generating molecular markers for economic traits of the silkworm.

From the DNA analysis it is clear that the samples are different from each other, and each sample has individual genetic characters. The hybrids showed average values in Phylogenetic Tree. To achieve good reproducibility and strong signal in the RAPD assay, one of the most important parameters is the concentration of genomic DNA. Thus it was done carefully based on standard protocol. It was demonstrated in the present study that the RAPD technique can be successfully applied to the silkworm study to identify useful DNA polymorphism with a potential to serve as genetic markers. Black *et al*.,[3] have shown that RAPD-PCR detects a high level of variation in insect species where little or no allozyme variability was detected. RAPD markers have been shown to be useful as a tool for genetic mapping, strain identification, systematics and population studies[3,4,27] which was proved in our study also.

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