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Genome fingerprinting of the silkworm, *Bombyx mori*, using random arbitrary primers

The random amplified polymorphic DNA (RAPD) technique was used to study DNA profiling of thirteen silkworm genotypes. The genotypes included six diapausing and seven nondiapausing varieties that represent a high degree of divergence with respect to geographic origin, and morphological, qualitative, quantitative and biochemical characters. Two hundred sixteen amplified products were generated using 40 random primers. Genotype-specific amplification products were identified. Amplification products specific to diapausing genotypes were also identified. Segregation of the RAPD marker was analyzed in a backcross population and found to be inherited as dominant Mendelian traits. Based on pairwise comparison of amplified products, the genetic similarity coefficient was calculated using the NeiLi similarity coefficient, and cluster analysis was performed by a hierarchical clustering technique. Silkworm genotypes were clustered into two groups, one consisting of six diapausing and the other of seven nondiapausing genotypes. The results of our study suggest that the RAPD technique could be used as a powerful tool to generate genetic markers that are linked to traits of interest in the silkworm.

1 Introduction

The silkworm, *Bombyx mori*, an economic silk-secreting insect, comprises a large number of ecotypes and synthetic inbred lines that are distributed in temperate and tropical countries. These genotypes differ widely in their qualitative and quantitative characters [1, 2]. The tropical genotypes (nondiapausing) are hardy and can withstand adverse eco-climatic conditions but produce very small quantities of silk of poor quality, while the genotypes of temperate origin (diapausing) produce higher quantities of good-quality silk. The latter genotypes are low silk yielders in the tropical environment because of high levels of heat, humidity, disease and inadequate sanitary conditions during silkworm rearing [3]. The classical breeding approaches, although they have increased silk productivity, have not been quite successful in integrating the high yielding traits of temperate genotypes with the low yielding disease-resistant tropical genotypes [4].

For the silkworm, the presently available linkage map is based on morphological and biochemical markers. Our earlier attempts to screen for biochemical markers resulted in the identification of only one enzyme, digestive α-amylase, which showed polymorphism between diapausing and nondiapausing genotypes [5]. A lack of information, especially at the molecular level, has limited our ability to gain deeper insight into the genome of this economic insect. So far, development of DNA-based genetic markers in the silkworm is still in its infant stage. The development of such molecular markers will be extremely useful in silkworm improvement program. Recently attempts have been initiated to construct the preliminary linkage map using RFLPs, which is one of the earliest molecular mapping techniques to be used [6]. The development of the polymerase chain reaction (PCR) technique [7] provides alternative strategies for generating molecular markers. Recently, random amplified polymorphic DNAs (RAPDs) have been developed [8, 9], which are based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequences. 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 [10]. RAPDs have been successfully used for fingerprinting of viruses, bacteria, fungi, plants, humans [9, 11, 12] and, more recently, insects [13, 14]. RAPD markers have been shown to be useful to identify specific regions of chromosomes [15], markers linked to specific disease resistance genes [16], as a tool for genetic mapping, strain identification, systematics and population studies [13, 17–22]. In the present study, we report the genetic differences among diverse silkworm genotypes using the RAPD technique and demonstrate its utility for the genetic improvement of silkworms.

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Nonstandard abbreviations: DIG, digoxigenin; **RAPD,** random amplified polymorphic DNA

Keywords: Random amplified polymorphic DNA / Genetic diversity / *Bombyx mori* / Silkworm

2 Materials and methods

2.1 Silkworm stocks and crosses

Six diapausing (HU_{204}, KA, NB_1, NB_7, NB_{18}, NB_4D_2) and seven nondiapausing (C.nichi, Gungnong, Moria, Nistari, Pure Mysore, Diazo and Sarupat) silkworm genotypes were used in the present study (Table 1). F_1 hybrid offspring were obtained by crossing a Nistari female with a male of NB_1. The F_1 female was crossesd with an NB_1 male to produce backcross (BC) offspring. DNA was extracted from the parents, the F_1 offspring and the BC offspring for use in the study.

2.2 Genomic DNA isolation

Genomic DNA was isolated from the posterior silk glands collected from day-3 fifth instar larvae [23].

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Briefly, silk glands were ground in liquid nitrogen using a pestle and mortar. Extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA and 1% SDS) and proteinase K (100 μg/mL) was added to the ground tissue and incubated at 37°C for 2 h with occasional swirling. The DNA was extracted twice with phenol-chloroform-isoamylalcohol (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.

2.3 DNA amplification and separation

The amplification of genomic DNA was performed according to [8]. The amplification reaction was carried out in a 25 μL reaction volume containing 25 ng of template DNA, 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 50 mM KCl), 0.2 μM primer, 100 μM each of dATP, dCTP, dGTP and dTTP, and 0.75 units of Taq DNA polymerase (Boehringer Mannheim). The random sequence 10-mer primers were purchased from Operon Technologies Inc. Alameda USA. Each reaction sample was overlaid with 15 μL mineral oil (Sigma). For each primer examined, negative control was maintained which contained all the components except the genomic DNA. Amplification was performed in a Pharmacia Thermal Cycler (Gene ATAQ Controller) and programmed for initial denaturation: 94°C for 2 min, followed by 45 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. The amplified products were separated according to molecular size on 1.5% agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA) and detected by staining with ethidium bromide [24].

2.4 Hybridization analysis

Amplified DNA was transferred to a Hybond N (Amersham, UK) membrane according to the manufacturer's instructions. PCR products were separated on 3.5% polyacrylamide gel and the specific RAPD fragment was excised and isolated [24]. The isolated RAPD fragment was suspended in 10 μL of TE buffer and diluted 10 000-fold before reamplification. The probes were labeled using the nonradioactive DIG random prime labeling kit (Boehringer Mannheim). Prehybridization, hybridization and development of the membrane were carried out according to the manufacturer's instructions.

2.5 Data analysis

Each genotype was scored for the presence (1) or absence (0) of the amplified products. Similarity index matrices were generated based on the number of shared amplified products according to the method of [25]. $F = 2\ Nxy/Nx + Ny$, where F is the mean of genetic similarity between a pair of lanes; 2 Nxy is the number of shared fragments between x and y populations. Nx and Ny are the number of scored fragments in two populations. The data were analyzed using the subsets of data derived from duplicate experiments and the resultant dendrogram was tested for congruity. Goodness-of-fit to the 1:1 pattern of segregation for RAPD marker, as predicted for Mendelian characters in a backcross population, was determined using the Chi-square test.

3 Results

3.1 Optimization of amplification conditions for silkworm DNA

The silkworm DNA was amplified with slight modifications according to the protocol of [8]. An initial denaturation of 2 min resulted in better separation of DNA strands allowing binding of random 10-mers to template DNA for sequence-specific PCR amplification. Each DNA sample was pooled from 15–20 larvae of the representative ecotypes and inbred lines. Amplification of silkworm genomic DNA with random oligonucleotide primers resulted in a series of discrete bands of varying

Table 1. Characteristics of silkworm genotypes

Strains	Voltinism	Origin	Larval pattern	Cocoon color	Cocoon shape	Larval duration (D:H)	Cocoon weight (g)	Shell weight (g)	Shell ratio %	Filament length (m)
Hu204	Diapausing	China	Plain	White	Peanut	24:00	1.43	0.240	16.7	760
KA	Diapausing	India	Plain	White	Oval	24:00	1.71	0.289	17.0	804
NB1	Diapausing	Japan	Plain	White	Oval	25:00	1.95	0.410	21.0	970
NB7	Diapausing	India	Plain	White	Oval	25:00	1.78	0.364	20.5	906
NB18	Diapausing	India	Plain	White	Peanut	25:00	1.93	0.402	20.8	904
NB4D2	Diapausing	India	Plain	White	Peanut	25:00	1.99	0.420	21.1	934
C.nichi	Nondiapausing	India	Plain	White	Peanut	21:00	0.92	0.103	11.1	350
Gungnong	Nondiapausing	China	Marked	Cream	Short oval	20:00	1.07	0.173	15.0	480
Moria	Nondiapausing	Assam (India)	Plain	Cream	Spindle	22:00	1.09	0.136	12.5	375
Nistari	Nondiapausing	West Bengal (India)	Marked	Golden yellow	Spindle	22:00	1.10	0.151	13.7	450
Pure-Mysore	Nondiapausing	Karnataka (India)	Plain	Greenish yellow	Spindle	28:00	1.00	0.138	13.8	425
Diazo	Nondiapausing	Japan	Marked	Dark greenish yellow	Spindle	20:00	1.18	0.170	14.7	380
Sarupat	Nondiapausing	Assam (India)	Plain	Cream	Spindle	22:00	1.00	0.131	13.1	3.75

intensity. To achieve good reproducibility and strong signal in the RAPD assay, one of the most important parameters is the concentration of genomic DNA. Thus, DNA template concentration assays were performed over a range of 10–100 ng of DNA per 25 μL reaction mixture (Fig. 1; lanes 1–7). A template DNA concentration of 20–70 ng (lanes 2–6) was found to generate a consistent amplification profile but lower (lane 1) and higher (lane 7) concentrations appeared to be inhibitory. At optimum template DNA concentration (25 ng/25 μL reaction volume), the RAPD profile remained consistent and repeatable even when the template DNA was prepared by different extraction methods, which included both crude extraction and the use of a column purification kit (data not shown).

The reproducibility of the RAPD assay was tested by performing duplicate reactions at different times using identical genotypes and primer combinations. In these amplification reactions RAPD patterns were consistent and reproducible for any given genotype and primer combination; however, the relative intensity of some bands was sometimes variable. A strictly controlled temperature profile and reliable Taq DNA polymerase were needed for consistent amplification patterns. Occasionally we observed some amplified products in control reaction samples which contained no template DNA. If such products were present in the experimental samples these were not included in the analysis.

3.2 Sensitivity of the RAPD assay

DNA samples from the two distinct genotypes Nistari and NB_1 were artificially mixed in various ratios. A distinct polymorphic marker of 1300 bp in Nistari (Fig. 2; lane 1) and 700 bp in NB_1 (Fig. 2; lane 6) could be obtained with the random primer OPA-01. The PCR performed on DNA mixtures of these two genotypes with

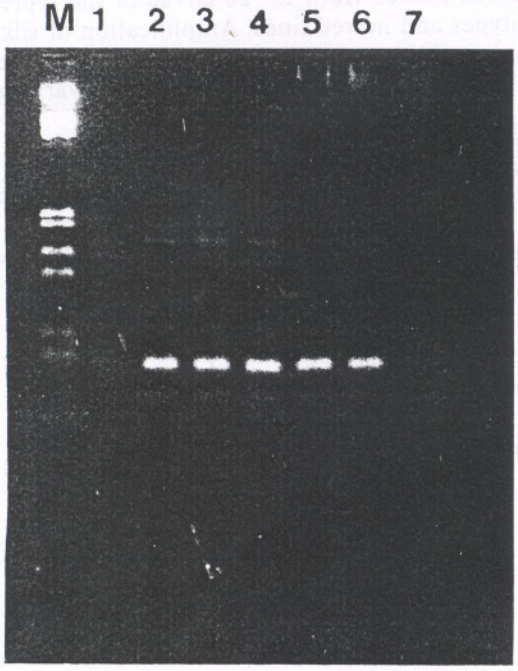

Figure 1. PCR amplification of different amounts of NB_1 DNA with primer OPA-01. Lanes (1)–(7) contain 10, 20, 30, 40, 50, 70 and 100 ng of genomic DNA. M is a lambda *Eco*RI-*Hin*dIII digest.

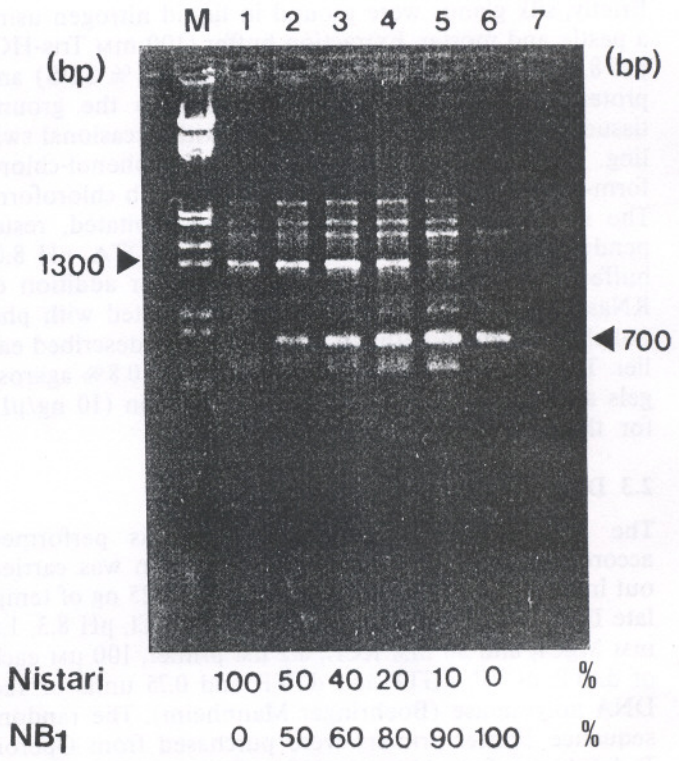

Figure 2. PCR amplification of DNA from Nistari and NB_1 genotypes mixed in various ratios (indicated at the bottom). M is a lambda *Eco*RI-*Hin*dIII digest. Bands unique to a particular template are shown by arrow.

the same arbitrary primer resulted in a multiple band profile in which both 1300 bp and 700 bp bands, which were unique to Nistari and NB_1, respectively, could be unambiguously discriminated (Fig. 2; lanes 2–5). The DNA profile of the two genotypes could be distinguished even when the template DNA was mixed at a ratio of 10:1 (lane 5). Most polymorphic bands were found to be amplified in proportion to the amount of template DNA present in the reaction mixture. These results clearly demonstrate the feasibility of the RAPD assay, even when the template DNA sample is composed of more than one genome, and that it is possible to differentiate each genome without resorting to its physical separation.

3.3 Inheritance analysis

Selected random primers, which were known to generate polymorphic markers, were used for amplification to monitor the segregation of the RAPD markers among the F_1 hybrid and 80 BC offspring. The arbitrary primer OPA-01 produced an RAPD marker of 1300 bp only in Nistari genotype (Fig. 3; lane 1), which was inherited to F_1 offspring (Fig. 3; lane 3) in Mendelian fashion and segregated as dominant marker in BC offspring (Fig. 3; lanes 4–12). Among 80 BC offspring, the marker ($OPA\text{-}01_{1300}$) was present in 36 offspring and absent in 44 offspring, which fits into the 1:1 expected Mendelian ratio with the Chi-square value of 0.8 ($P \geq 0.1$).

3.4 Marker identification

The RAPD pattern was assessed in genomic DNA from thirteen silkworm genotypes using 40 random primers.

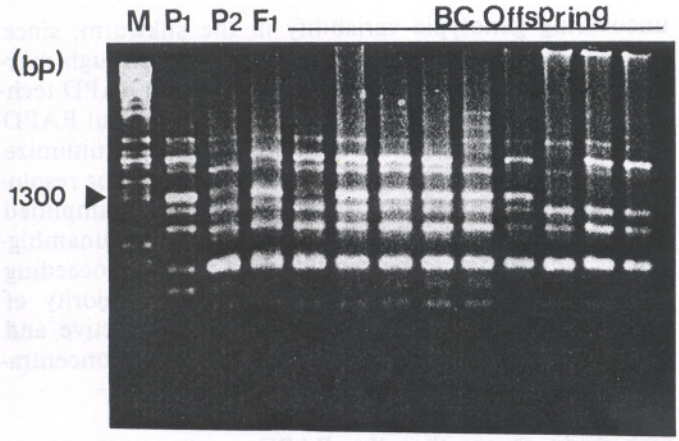

Figure 3. Inheritance of RAPD marker (OPA-01_{1300}) to F_1 (Nistari × NB_1) and backcross (F_1 × NB_1) offspring. P1, Nistari, P2, NB_1. M is a lambda *Eco*RI-*Hin*dIII digest. Segregating amplified product, OPA-01_{1300} is shown by arrow.

For each primer evaluated, a multiple band profile comprising one to five major amplification products and a varying number of weak products or a faintly smeared region was observed. The number and size of amplified products varied depending upon the sequence of random primers and genotypes used; a total of 216 discrete amplified products were produced. The size of the amplified fragments ranged from 200 to 3000 bp. Of 216 products, 204 were polymorphic in at least one pairwise comparison between silkworm genotypes, and the remaining 12 were monomorphic. Diapausing and nondiapausing silkworm genotypes could be distinguished by five specific markers. The markers OPA-01_{700} (Fig. 4a; lanes 1–6) and OPA-02_{1300} (Fig. 4c; lanes 1–6) were specific for diapausing genotypes, while OPA-01_{1300} (Fig. 4a; lanes 8–12), OPA-02_{800} (Fig. 4c; lanes 9–13) and OPB-10_{700} (Fig. 4d; lanes 7–11) were nondiapausing genotype-specific markers. Besides, two diapausing sister-lines showed a unique product for marker OPB-10_{900} (Fig. 4d; lanes 5 and 6).

The occurrence of common or unique amplified fragments in the diapausing and nondiapausing genotypes indicated that useful phylogenetic information can be obtained by analysis of unique bands. However, it is important to demonstrate the homology of comigrating RAPD bands before venturing into such comparisons. This is especially important when a large number of bands are produced. Identification and characterization of common bands are currently underway in our laboratory. The initial results reveal that homology exists between common RAPD fragments. A sample of band OPA-01_{700} (Fig. 4a; lanes 1–6, shown by arrow), which was diagnostic to diapausing strains, was investigated by Southern analysis of RAPD gels using a reamplified single band as probe in order to generate information on the degree of homology between common bands. The probe OPA-01_{700} hybridized to the diapause strain specific bands (Fig. 4b; shown by arrow), indicating the homology between the comigrating amplified products in the diapausing silkworm genotypes.

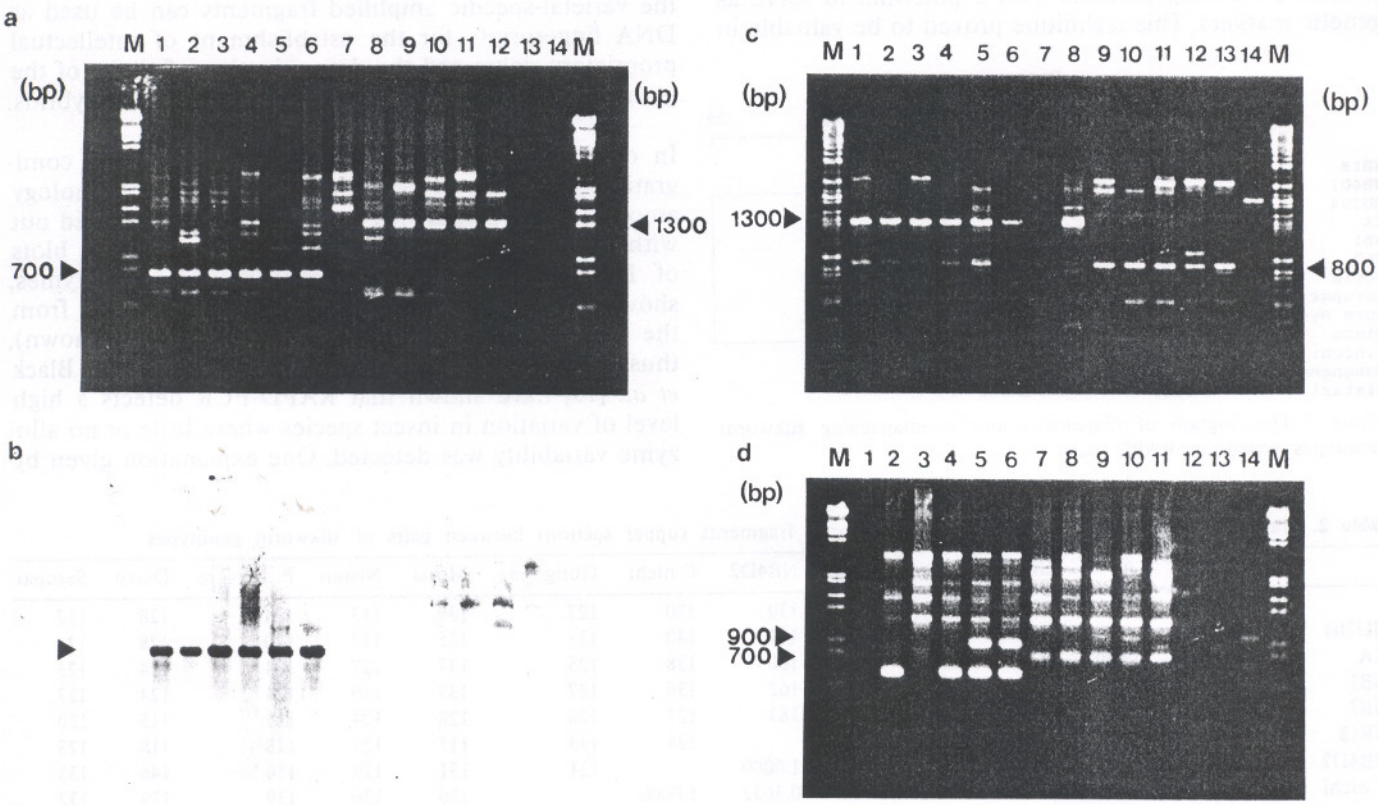

Figure 4. RAPD fingerprints obtained for diapausing and nondiapausing silkworm genotypes using (a) primer OPA-01. Lanes (1)–(14) are HU_{204}, KA, NB_1, NB_{18}, NB_4D_2, C.nichi, Gungnong, Moria, Nistari, Pure Mysore, Diazo, Sarupat and control, respectively. M is a lambda *Eco*RI-*Hin*dIII digest. Arrows indicate diapausing and nondiapausing genotype-specific amplified products. (b) Southern blot prepared from gel in (a) probed with band OPA-01_{700} excised from lane (1). (c) RAPD fingerprints using OPA-02. Details as in (a). Arrows indicate diapausing and nondiapausing genotype-specific amplified products. (d) RAPD fingerprints using OPB-10. Details as in (a). Arrows indicate diapausing and nondiapausing genotype-specific amplification products.

3.5 Relationship among genotypes

The similarity index values (1-*F*) obtained for each pairwise comparison of RAPD scored bands among the thirteen silkworm genotypes are shown in Table 2. The mean 1-*F* value from these comparisons was 0.2593. The highest 1-*F* value of 0.4428 was observed between the genotypes Pure Mysore and NB_{18}. The genotype Pure Mysore showed the highest 1-*F* value of 0.4428, when compared to all other genotypes (mean 1-*F* = 0.2593) and appeared to be the most divergent genotype. The lowest 1-*F* value of 0.0065 was recorded between diapausing genotypes KA and HU_{204}. A dendrogram generated by the agglomeric method of the hierarchical clustering technique is shown in Fig. 5. Cluster analysis is a standard method to study relatedness and genetic diversity among genotypes. The thirteen silkworm genotypes were resolved into two clusters based on RAPD analysis, one group comprised of seven nondiapausing and the other group of six diapausing silkworm genotypes. It is clear from the dendrogram that the genotypes NB_4D_2 and Diazo are most divergent. The closest nearest-neighbor genotypes were found to be NB_{18} and NB_4D_2, HU_{204} and KA, Moria and Sarupat.

4 Discussion

We demonstrate in the present study that the RAPD technique can be successfully applied to the silkworm to reveal useful DNA polymorphisms, with many random primers producing patterns with a potential to serve as genetic markers. This technique proved to be valuable in uncovering genotypic variability in the silkworm, since presently such variability is estimated only through morphological and yield attributes. However, the RAPD technique should be approached with care. Successful RAPD analysis requires replication of PCR runs to minimize mis-scoring of polymorphic products due to poor resolution and occasional occurrence of sporadic amplified products. Hence, the RAPD profile should be unambiguously determined to be reproducible before proceeding to use unique products as markers. The majority of primers used in the present study gave distinctive and consistent amplification with a template DNA concentration ranging from 20 to 70 ng.

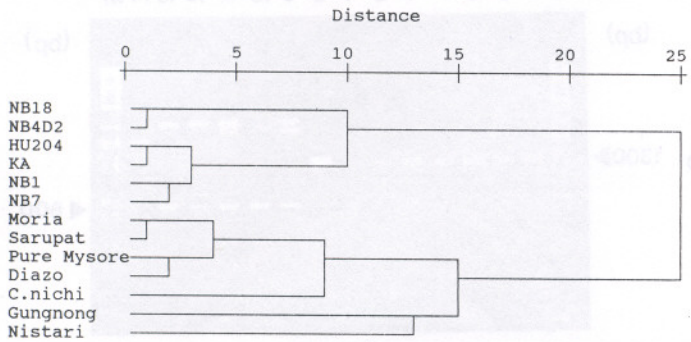

Figure 5. Dendrogram of diapausing and nondiapausing silkworm genotypes based on RAPD data.

Our study shows that the RAPD marker ($OPA\text{-}01_{1300}$) that is polymorphic in the Nistari parent was inherited as dominant marker in F_1 and showed 1:1 segregation in the BC offspring. Such a Mendelian pattern of segregation is expected in BC when the maternal parent is heterozygous for the allele (presence of the DNA band) and the paternal parent is homozygous recessive for the allele (*i.e.*, lacking the DNA band). The RAPD assay generated many diapause- and nondiapause-specific and varietal-specific products. Such kinds of diapause- and nondiapause-specific markers can be of immense value since these two groups of silkworm genotypes differ in a large number of quantitative traits of economic value such as disease resistance, larval duration and silk fiber length. The possible linkage of these markers with these traits could be exploited to practice "marker tagged breeding" to develop elite stocks. In addition, many of the varietal-specific amplified fragments can be used as DNA fingerprints for the establishment of intellectual proprietary rights and the determination of purity of the germplasm in both inbred line/ecotypes and F_1 hybrids.

In our study, we demonstrate that most of the comigrating bands in different genotypes share homology among the genotypes. The preliminary study, carried out with a few RAPD products as probe on Southern blots of DNA digested with different restriction enzymes, showed that many of the RAPD products originate from the repetitive region of the genome (data not shown), thus making them unscorable as RFLP probes. Black *et al.* [13] have shown that RAPD-PCR detects a high level of variation in insect species where little or no allozyme variability was detected. One explanation given by

Table 2. Matrix of 1-*F* values (lower section) and shared fragments (upper section) between pairs of silkworm genotypes

	HU204	KA	NB1	NB7	NB18	NB4D2	C.nichi	Gungnong	Moria	Nistari	P. Mysore	Diazo	Sarupat
		154	160	152	135	130	130	127	139	113	124	128	137
HU204	1.0000		154	166	153	154	140	133	135	117	120	128	121
KA	0.0065	1.0000		158	161	160	138	125	137	127	124	124	123
NB1	0.0429	0.1221	1.0000		153	162	130	127	137	119	124	124	127
NB7	0.0904	0.0743	0.1568	1.0000		183	127	126	128	124	115	115	120
NB18	0.2267	0.1823	0.1728	0.2320	1.0000		124	133	117	129	118	118	123
NB4D2	0.2515	0.1722	0.1737	0.1813	0.1055	1.0000		121	151	129	150	146	133
C.nichi	0.2147	0.2035	0.2473	0.3189	0.3508	0.3632	1.0000		136	136	139	139	132
Gungnong	0.2560	0.2281	0.3369	0.3526	0.3724	0.3333	0.3797	1.0000		144	161	155	164
Moria	0.1543	0.2281	0.2486	0.2772	0.3421	0.3968	0.1934	0.2957	1.0000		165	155	144
Nistari	0.3855	0.3883	0.3687	0.4129	0.4106	0.3835	0.3586	0.3399	0.2792	1.0000		176	159
P.Mysore	0.2948	0.3571	0.3594	0.3846	0.4428	0.4250	0.2448	0.3198	0.1832	0.2163	1.0000		163
Diazo	0.2075	0.2560	0.3090	0.3370	0.4011	0.3817	0.2678	0.2678	0.1525	0.2113	0.0904	1.0000	
Sarupat	0.1007	0.2468	0.2798	0.2749	0.3333	0.3125	0.2601	0.2601	0.0419	0.2337	0.1292	0.0305	1.0000

Gawel and Bartlett [13] for such an enhanced ability to detect variation with RAPD-PCR may lie in the physical requirements for the reaction to amplify DNA, *i.e.*, if a region is to be amplified, primers must anneal within approximately 2 kbp. Thus, regions of the genome that contain repetitive sequences or inverted terminal repeats may contain a greater number of sites amenable to RAPD amplification than coding regions. Perhaps it is the variability of these repetitive sequences that is the source of variation detected by the RAPD technique. Regardness of the molecular nature of the RAPD marker, their Mendelian inheritance will make them a valuable tool for genetic analysis of silkworms where there is a pausity of suitable genetic markers.

With RAPDs it is possible to assess genetic similarity amongst a range of silkworm genotypes on the basis of band sharing analysis. As expected from the RAPD profile, diapause and nondiapause genotypes grouped into two clusters. An interesting feature which gave credence to the use of the RAPD assay in the silkworm is that the two nondiapausing ecotypes, Moria and Sarupat, which are distributed in Assam, India, showed close similarity values. Similarly, two diapausing inbred lines, NB_{18} and NB_4D_2, which have been derived from a common double cross, also showed a close similarity index. Our study thus suggests the possibility of assessing RAPD-based genetic divergence for predicting hybrid performance in the silkworm.

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. The varietal-specific amplification of distinct bands permits their use in genetic fingerprinting of silkworm ecotypes and inbred lines. However, the technique requires stringent standardization before it can be used reliably and consistently.

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