DNA Fingerprint Variability Within and Among the Silkworm *Bombyx mori* Varieties and Estimation of Their Genetic Relatedness Using Bkm-Derived Probe

A. Sharma, M. P. Niphadkar, P. Kathirvel, J. Nagaraju, and L. Singh

Genetic diversity within and among 13 silkworm varieties (6 diapausing and 7 nondiapausing) that differ in various quantitative and qualitative characters of economic importance was determined by DNA fingerprinting using Bkm-derived 2(8) probe. A high degree of genetic similarity was observed within each variety studied. Based on fingerprints of pooled DNA, the genetic similarity among various varieties was calculated. The dendrogram constructed using UPGMA resulted in the 13 varieties resolving into two major clusters. These two clusters were comprised of five nondiapausing as one group and five diapausing varieties as the other. The genetic similarity estimated within and among silkworms is consistent with the pedigrees and geographical distribution of the varieties. Our study has demonstrated that the variability of DNA fingerprints within and among silkworm can provide an essential basis on which breeders may plan crossbreeding strategies to produce potentially heterotic hybrids.

The domesticated silkworm (*Bombyx mori*) comprises a large number of ecotypes and synthetic inbred lines that are distributed in temperate and tropical countries. These different varieties differ in their qualitative and quantitative traits that affect silk yield (Gamo 1983). The nondiapausing varieties available in tropical countries are poor silk yielders, although they are rapid breeders (Polyvoltine) and are hardy, that is, known to survive and reproduce efficiently under tropical conditions. The temperate varieties are invariably diapausing (uni- or bivoltine) and are endowed with higher silk yield of better quality. However, they fail to attain normal yield levels under tropical conditions (Nagaraja and Nagaraju 1995; Nagaraju et al. 1995). In other words, diapausing varieties are low silk yielders in the tropical conditions because of high levels of heat, humidity, diseases, and inadequate sanitary conditions during silkworm rearing (Datta and Nagaraju 1993; Goldsmith 1991). Although the classical silkworm breeding approaches, particularly crossbreeding of tropical and temperate varieties, have resulted in an overall increase in silk productivity, they have been unsuccessful in integrating the high-yielding traits of temperate varieties with the robustness of low-yielding tropical varieties.

Molecular marker-assisted breeding is expected to increase the speed and precision in silkworm breeding processes to integrate the desired characters from tropical and temperate varieties into elite varieties. However, until recently, there was a complete lack of information on the molecular analysis of the silkworm genome. In recent years attempts have been initiated to construct molecular linkage maps based on random amplified polymorphic DNAs (RAPDs; Pramboon et al. 1995) and restriction fragment length polymorphic DNAs (RFLPs; Shi et al. 1995).

DNA fingerprinting, first described by Jeffreys et al. (1985), is now commonly used to study genetic variability and to analyze pedigree relationships in a wide variety of organisms including insects (Blanchetot and Gooding 1994; Dallas 1988; Georges et al. 1988; Nybom 1991). It has been proven that PCR-based DNA fingerprinting using random arbitrary primers is a powerful tool in investigating the genetic diversity of silkworm varieties (Nagaraja and Nagaraju 1995). In addition, the potential use of a minisatellite probe, banded krait minor satellite DNA [Bkm-2(8); Aggarwal et al. 1994; Lang et al. 1993; Singh 1995; Singh and Jones 1986; Singh et al. 1980, 1984, 1988] in generating DNA fingerprints in silkworm has also been demonstrated (Nagaraju et al. 1995). Characterization and quantification of genetic diversity, both within and between populations, has long been a major goal in crop improvement programs. In silkworm breeding programs, information concerning the genetic diversity within a variety is essential for a rational use of genetic resources. It is particularly useful in the characterization of individual varieties and various ecotypes in detecting duplications in germplasm collection and serves as a general guide in the choice of parents for producing heterotic hybrids. The objectives of the present study were to examine genetic diversity within 13 silkworm varieties and to estimate genetic relatedness among them. The silkworm genotypes studied differ in the following characteristics: larval duration, cocoon weight, cocoon shell weight, silk filament length, and voltinism (refers to the number of life cycles in a year) (Nagaraja and Nagaraju 1995).

Materials and Methods

Silkworm Varieties

Six diapausing (Hu_{204}, Ka, NB_1, NB_7, NB_{18}, and NB_4D_2) and seven nondiapausing (C. nichi, Gungnong, Moria, Nistari, Pure Mysore, Diazo, and Sarupat) silkworm varieties, which differ from each other in a number of characteristics, were used in the present study. The characteristics of the varieties used are discussed in detail in Nagaraja and Nagaraju (1995).

Genomic DNA Extraction

For studying genetic variability within a variety, DNA from 8 male and 8 female moths of each of the 13 varieties was extracted separately. For analyzing genetic relatedness among the 13 varieties, DNA was isolated from silk glands of day 3 fifth instar larvae of each of the varieties (Suzuki et al. 1972). In brief, silk glands were ground in liquid nitrogen using a mortar and pestle. A buffer containing 100 mM Tris-HCl pH 8.0, 50 mM NaCl, 50 mM EDTA, and 1% SDS was added to it. The mixture was incubated at 37°C for 2 h with occasional swirling. The DNA was extracted once each with phenol, phenol : chloroform : isoamyl alcohol, and chloroform : isoamyl alcohol, ethanol precipitated, and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was treated with RNase A (100 μg/ml final concentration) at 37°C for 2 h, following which it was extracted with organic solvents, precipitated, and dissolved in TE as described above. DNA was quantified, using a known standard, on an agarose gel stained with ethidium bromide.

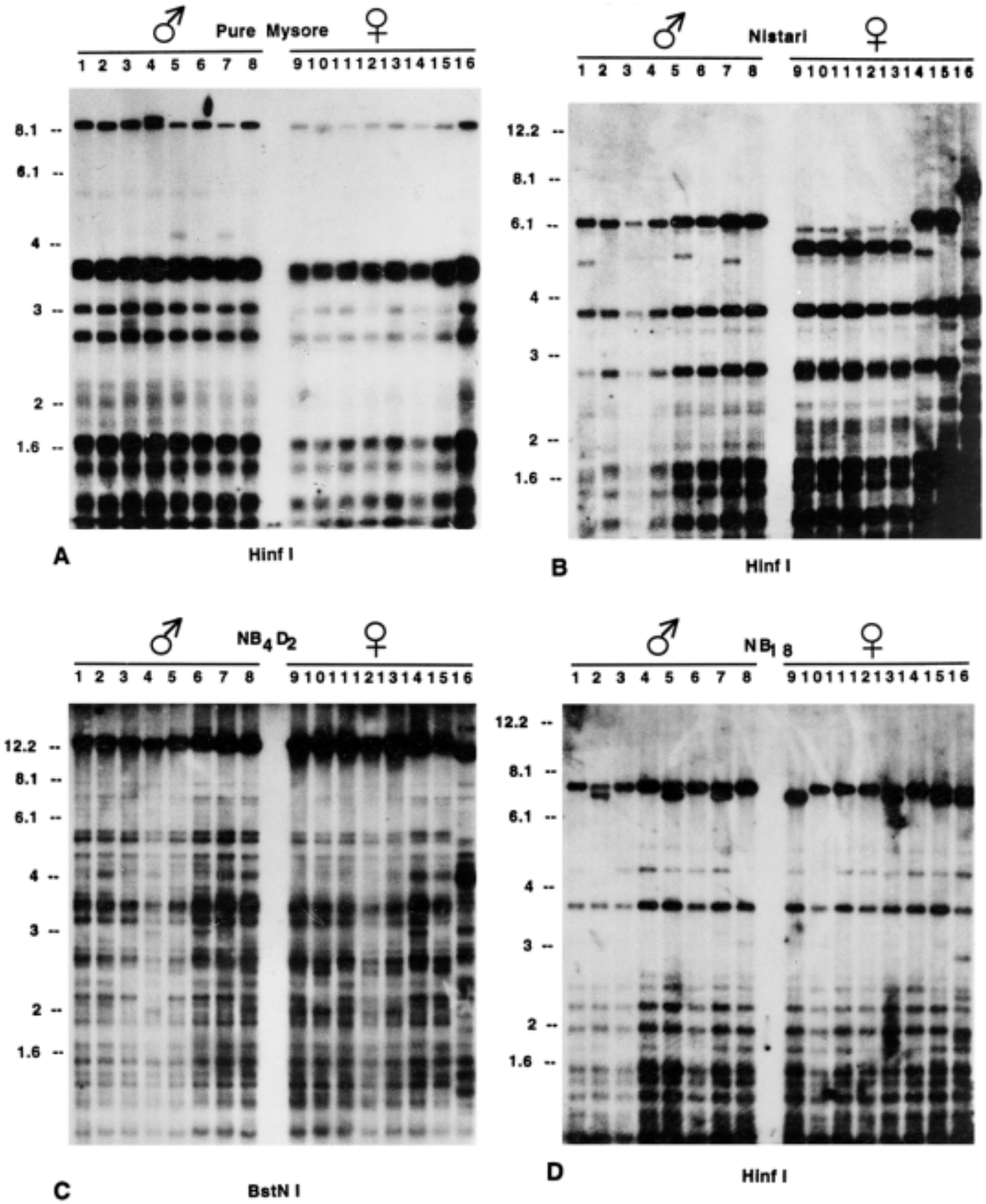

Figure 1. Bkm 2(8) hybridization pattern of DNA from **(A)** Pure Mysore, **(B)** Nistari, **(C)** NB_4D_2, and **(D)** NB_{18} silkworm varieties. Eight male and eight female moths of each variety were fingerprinted in a single gel. Males and females are indicated in the photograph. Restriction enzymes used were *Hin*fI (A, B, and D) and *Bst*NI (C). Note that individuals within a variety show more or less similar fingerprint profiles. Also note the absence of sex-specific hybridized bands. Each lane contains 8–10 μg of completely digested DNA. Numbers on the left indicate DNA fragment size in kilobase pairs.

Table 1. Similarity coefficients within various diapausing and nondiapausing varieties of *Bombyx mori* with respect to sex as well as restriction enzyme, sex, restriction enzyme, and irrespective of sex and restriction enzyme (mean)

	Sex and restriction enzyme								
	*Bst*NI		*Hin*fI		Sex		Restriction enzyme		
Varieties	Male	Female	Male	Female	Male	Female	*Bst*NI	*Hin*fI	Mean
HU_{204}	0.970 ± 0.02	0.968 ± 0.02	0.875 ± 0.05	0.866 ± 0.06	0.923 ± 0.06	0.917 ± 0.07	0.966 ± 0.02	0.870 ± 0.07	0.918 ± 0.06
KA	0.748 ± 0.14	0.774 ± 0.13	0.760 ± 0.11	0.905 ± 0.05	0.754 ± 0.13	0.837 ± 0.12	0.741 ± 0.14	0.805 ± 0.09	0.773 ± 0.12
NB_1	0.764 ± 0.12	0.801 ± 0.09	0.839 ± 0.12	0.725 ± 0.11	0.802 ± 0.13	0.763 ± 0.11	0.780 ± 0.10	0.739 ± 0.14	0.759 ± 0.12
NB_7	0.881 ± 0.05	0.908 ± 0.04	0.922 ± 0.04	0.954 ± 0.02	0.906 ± 0.05	0.928 ± 0.04	0.887 ± 0.05	0.923 ± 0.04	0.904 ± 0.05
NB_{18}	0.973 ± 0.02	0.989 ± 0.01	0.924 ± 0.04	0.900 ± 0.05	0.994 ± 0.04	0.945 ± 0.06	0.979 ± 0.02	0.908 ± 0.04	0.944 ± 0.05
NB_4D_2	0.885 ± 0.07	0.886 ± 0.07	0.946 ± 0.05	0.778 ± 0.34	0.916 ± 0.07	0.832 ± 0.25	0.872 ± 0.06	0.863 ± 0.26	0.867 ± 0.19
C. nichi	0.961 ± 0.03	0.947 ± 0.03	0.978 ± 0.02	0.980 ± 0.03	0.970 ± 0.03	0.964 ± 0.04	0.950 ± 0.03	0.970 ± 0.04	0.960 ± 0.03
Gungnong	0.886 ± 0.06	0.829 ± 0.09	0.900 ± 0.09	0.774 ± 0.17	0.893 ± 0.08	0.802 ± 0.14	0.848 ± 0.08	0.831 ± 0.14	0.840 ± 0.11
Moria	0.917 ± 0.05	0.826 ± 0.13	—	0.881 ± 0.09	0.917 ± 0.05	0.853 ± 0.12	0.835 ± 0.13	0.881 ± 0.08	0.847 ± 0.12
Nistari	0.841 ± 0.10	0.771 ± 0.15	0.887 ± 0.05	0.640 ± 0.31	0.864 ± 0.08	0.706 ± 0.25	0.717 ± 0.16	0.672 ± 0.24	0.695 ± 0.20
Pure Mysore	0.968 ± 0.01	0.962 ± 0.03	0.953 ± 0.04	0.895 ± 0.07	0.961 ± 0.03	0.929 ± 0.06	0.948 ± 0.03	0.921 ± 0.06	0.935 ± 0.05
Diazo	1.000 ± 0.00	0.722 ± 0.16	0.915 ± 0.06	0.728 ± 0.16	0.958 ± 0.06	0.725 ± 0.16	0.787 ± 0.16	0.796 ± 0.15	0.791 ± 0.16
Sarupat	0.925 ± 0.05	0.902 ± 0.04	0.836 ± 0.08	0.873 ± 0.10	0.880 ± 0.08	0.888 ± 0.08	0.888 ± 0.05	0.831 ± 0.08	0.860 ± 0.07

DNA Fingerprinting

For each gel lane, 8–10 μg of *Bst*NI- or *Hin*fI-digested DNA was loaded. Digested samples were electrophoresed in 30 cm long, 5 mm thick, 0.8% agarose gels at 60 V for 16–18 h in TPE buffer (15 mM Tris-HCl, 18 mM NaH_2PO_4, 0.5 mM EDTA, pH 7.8). Marker X (Boehringer Mannheim) was used as molecular weight markers. For analyzing similarity within a variety, DNA of all the 16 individuals (8 males and 8 females), digested with either *Bst*NI or *Hin*fI, were run in a single gel. Similarly, pooled DNA samples digested with either *Bst*NI or *Hin*fI were run in a single gel to estimate among variety differences. Gel fractionated DNA samples were transferred onto Hybond-N membrane (Amersham, UK) using a vacuum blotting assembly at 30 mm Hg (Olszewska and Jones 1988). The membranes were baked at 80°C for 2 h under vacuum. The blots were prehybridized in 7% SDS, 0.5% sodium phosphate buffer (pH 7.5) at 60°C for 2–3 h, and then hybridized with 1–2 × 10^6 cpm/ml of Bkm probe in the same but fresh buffer at 60°C for 14–18 h. The Bkm-2(8) DNA (Aggarwal et al. 1994; Lang et al. 1993; Singh 1995; Singh and Jones 1986; Singh et al. 1980, 1984, 1988) containing a 545 bp sequence consisting mainly of GATA repeats was used as a probe. Single-stranded ^{32}P-labeled probe was prepared to a specific activity of 0.7–3.0 × 10^8 cpm/μg (Hu and Messing 1982), using ^{32}P-dATP (specific activity 3000 Ci/mmol; Jonaki, BARC, India). After hybridization, blots were washed in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 sodium citrate, pH 7.2) con-

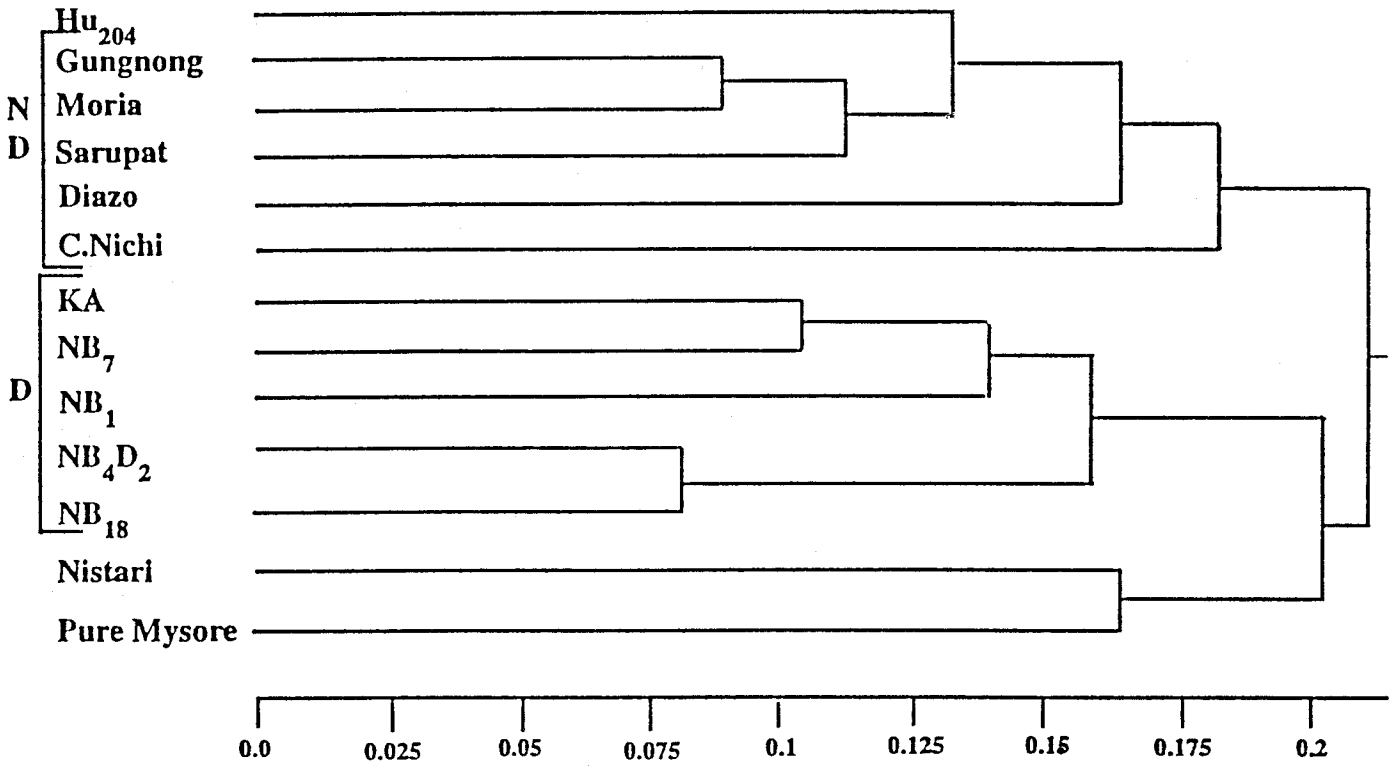

Figure 2. UPGMA phenogram showing relationships among various diapausing (D) and nondiapausing (ND) silkworm varieties. The phenogram is based on Bkm 2(8) fingerprinting of pooled DNA samples. Scale shows probable degree of divergence. See text for details.

taining 0.1% SDS for 15 min each, once at room temperature and twice at 60°C. The membranes were then exposed to x-ray films for 2–3 days.

Fingerprint Analysis

Autoradiographs were examined visually to score the number of hybridized bands. All bands showing similar molecular weights were considered to be identical. Each lane was scored for the presence or absence of a particular fragment. For all the DNA fingerprints analyzed, only distinguishable bands in the size range of 1.0–23 kb were scored. The similarity index (S) matrices were generated based on the number of shared fragments between each pair of fingerprints; $S = 2\ N_{AB}/(N_A + N_B)$ where N_{AB} is the number of bands shared by both lanes A and B, respectively, and N_A and N_B represented the total number of bands present in lanes A and B (Nei and Li 1979; Wetton et al. 1987). Mean and standard deviations were calculated (Blanchetot and Gooding 1994) using all possible pairwise combinations, that is, irrespective of sex and restriction enzyme as well as with respect to sex (male and female individuals), to restriction enzyme (*Bst*NI and *Hin*fI) and both. Using fingerprint data of pooled DNA samples digested with *Bst*NI and *Hin*fI, the genetic relatedness among the 13 silkworm varieties was estimated by calculating the difference value D in all possible pairwise combinations. The D between any two DNA fingerprint profiles was calculated as the number of bands that were different divided by the total number of fragments present in the two varieties (Gilbert et al. 1990). The D values were used to construct a phylogenetic tree using the UPGMA (unweighted pair group method with arithmetic means) option in the "neighbor" program (PHYLIP software, version 3.41; Felsenstein J, University of Washington, Seattle). Separate dendrograms were first constructed based on fingerprints obtained with the two restriction enzymes used, that is, *Bst* NI and *Hin*fI. As the two types of dendrograms were found to be similar, the fingerprint data resulting from the two enzymes were pooled together to construct the final dendrogram. The reliability of the dendrogram was also tested using other options (neighbor-joining and Fitsch–Margoliash) in the PHYLIP software. The UPGMA dendrogram was representative of all the dendrograms.

Results and Discussion

Genetic Variability Within Varieties

Genetic variability within silkworm varieties was analyzed based on DNA fingerprints using Bkm-2(8) derived probe on *Bst*NI- or *Hin*fI-digested DNA from male and female individuals of 13 silkworm varieties. A few representative examples of such profiles are shown in Figure 1A–D. DNA fingerprints of 104 male and 104 female individual silkworms revealed the hybridizable bands ranging from 1 to 23 kb. The DNA profile of individuals within a given variety showed a more or less identical pattern (Figure 1A–D, Table 1). Comparisons of the fingerprints were made irrespective of sex and restriction enzyme as well as with respect to sex and restriction enzyme (Table 1). No sex-specific DNA fingerprint pattern was observed (Figure 1). The similarity coefficient within a given variety did not show any variation with respect to sex or restriction enzyme (Table 1). In general, a high degree of similarity in Bkm DNA hybridization pattern of individuals within a variety was observed. These results on molecular similarity are highly valuable in view of the fact that in silkworm, only hybrids are reared for commercial silk production and high genetic similarity among individuals of each of the parental varieties involved in the hybrid is known to result in uniform, heterotic hybrids (Nagaraju et al. 1996).

Relationship Between Varieties

DNA fingerprinting with pooled DNA samples was carried out to study the genetic variation among the 13 silkworm varieties. Based on data from DNA profiles generated by Bkm 2(8)-derived probe, we constructed a dendrogram (Figure 2) that resolved the 13 silkworm varieties into two major clusters. These two clusters were comprised of five nondiapausing varieties and five diapausing varieties. The power of DNA fingerprinting in estimating the genetic relationship of populations in various species has been well demonstrated (Castagnone-Sereno et al. 1993; Meng et al. 1996; Nagaraja and Nagaraju 1995). The silkworm varieties (Moria and Sarupat) that shared the same geographical distribution are in the same cluster. Similarly the silkworm varieties (NB_4D_2 and NB_{18}) that are derived from the common pedigree are grouped in the same cluster. These studies clearly reveal the power of DNA fingerprinting in grouping silkworm varieties based on voltinism, geographical distribution, and pedigree relationships.

The results presented here demonstrate that DNA fingerprinting using multilocus Bkm 2(8)-derived probe offers a reliable and effective way of assessing genetic variability within and between the populations. However, what remains to be demonstrated is the association of such DNA profile-based genetic distance and the degree of heterosis and hybrid performance, which would provide a reliable avenue for crossbreeding programs in silkworm.

From the Centre for Cellular and Molecular Biology (CCMB), Hyderabad 500 007, India (Sharma, Niphadkar, Kathirvel, and Singh) and the Centre for DNA Fingerprinting and Diagnostics, CCMB Campus, Hyderabad 500 007, India (Nagaraju). Address correspondence to L. Singh at the address above. This work is supported by the Central Silk Board (Ministry of Textiles), Government of India. We thank Mr. Venugopalan Nair and Mrs. G. R. Savithri for technical assistance.

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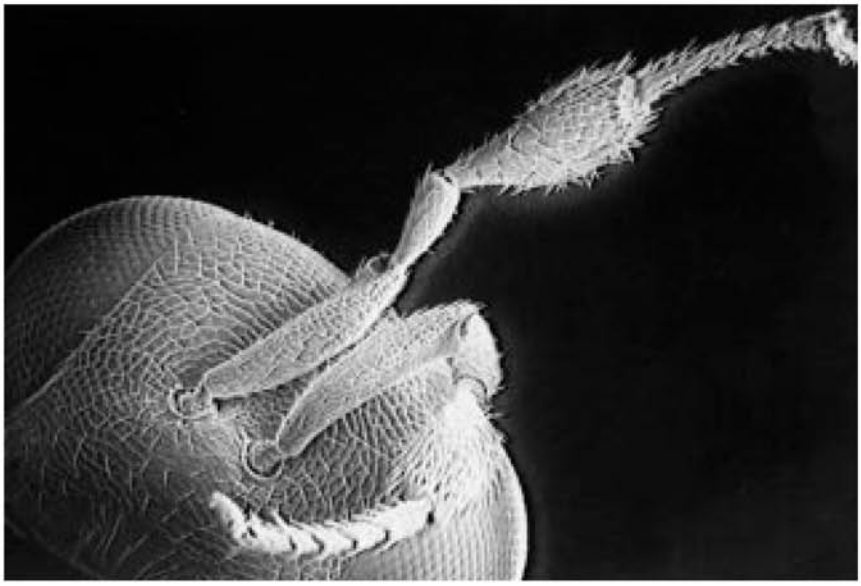

Figure 1. Scanning electron micrograph of the distantennapedia mutation. Abnormal swelling of the third antennal segment is apparent, as are leglike distal segments (tarsal segments) and presence of the tarsal claws.

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