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Assessment of genetic diversity by DNA profiling and its significance in silkworm, *Bombyx mori*

Silkworm genetic resources that are being maintained in different countries are yet to be adequately tapped to develop elite varieties that are suited to different agro-eco-climatic conditions of countries like India. This is mostly due to unavailability of efficient protocols that could uncover usable genetic variability in silkworms. Molecular markers are known to provide unambiguous estimates of genetic variability of populations since they are independent of confounding effects of environment. The DNA fingerprinting assays, based on random amplified polymorphic DNA (RAPD) and banded krait minor satellite DNA (Bkm) 2(8) multilocus probes, which successfully characterise the diverse silkworm genotypes at their DNA level, are described. The use of these two DNA fingerprinting assays in estimation of within- and between-population genetic diversity is discussed.

1 Introduction

The assessment of genetic variation is a major concern in plant and animal improvement programmes. This is important for several reasons: first, the ability to distinguish reliably different genotypes is important for designing the breeding programmes and population-genetic analysis. Second, an estimation of the amount of variation within genotypes and between genotypes is useful for predicting potential genetic gain in a breeding programme and in setting up appropriate cross-breeding strategies. Traditionally, morphological and phenotypic characteristics have been used for these purposes. Since such characteristics are often controlled by multiple genes and subject to varying degrees of environmental modification and interaction, the differences between genotypes are not always absolute and reliable [1]. Selectively neutral molecular markers are considered to provide the best estimates of genetic diversity since they are independent of the confounding effects of environmental factors [2]. DNA fingerprinting analysis has proved to be a sensitive method for determining genetic polymorphism. In the first report of DNA fingerprinting, Jeffreys *et al.* [3] demonstrated that highly variable repetitive DNA sequences could be detected by hybridization with specific probes. Since then, a large number of variations of southern hybridization has been published [4–7].

The advent of polymerase chain reaction (PCR) also resulted in the development of a large number of molecular techniques which have already made rapid impact in population genetics, analysis of biodiversity, genetic mapping, and studies of relationships among populations at different levels. One of the PCR approaches is the application of random amplified polymorphic DNAs (RAPD) in which genomic DNA is amplified with single short (*ca.* 10 nucleotides) primers of arbitrary nucleotide sequence [8, 9]. Different sequences may be amplified in different organisms, depending on the nucleotide sequences of the primer used and the source of template DNA, resulting in a multilocus DNA fingerprint patterns which could be scored for their genotype specificity. Because of technical advantages, RAPD markers have been extensively used in genetic analysis of various plant and animal species [10]. However, there are limitations in the applications of RAPD technology that include the dominant nature of the RAPD markers, chance comigration of bands at different loci and requirement of stringent standardisation of protocol to ensure reproducibility [11, 12]. Another class of PCR-based markers, the microsatellite markers, take advantage of the abundant and ubiquitously distributed simple sequence repeats (SSR) in the eukaryotic genomes [13–17]. This technique, however, requires prior characterisation of sequences flanking the repeats to allow primer design for PCR amplification. As PCR technology is finding its increased use in genetic analysis, more and more novel variations of this technique are emerging, which promise precision, economy and speed [18–20].

The silkworm *Bombyx mori*, an economic silk-secreting insect, comprises a large number of ecotypes and inbred lines that are distributed in temperate and tropical countries. These different genotypes display large differences in their qualitative and quantitative traits that ultimately control silk yield. The non-diapausing varieties that are available in tropical countries are poor silk yielders although they are known to survive and reproduce efficiently under tropical conditions. On the other hand, the temperate varieties are invariably diapausing and are endowed with higher silk yield of better quality. However, they fail to attain normal yield levels under tropical conditions because of high levels of heat, humidity, diseases, and inadequate sanitary conditions during silkworm rearing [21–23]. Although classical silkworm breeding approaches have resulted in an overall increase in silk productivity by virtue of harnessing hybrid vigour in the hybrids of tropical and temperate silkworm strains, they have been unsuccessful in complementing high-yielding traits of temperate genotypes with the robustness of low-yielding tropical genotypes. It is esti-

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Nonstandard abbreviations: Bkm, banded krait minor satellite DNA; **RAPD,** random amplified polymorphic DNA

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mated that more than 3000 silkworm genotypes, which comprise geographically distinct ecotypes and inbred lines derived from cross-combinations of several silkworm genotypes of diverse origin, are being maintained in six countries [24]. The countrywise silkworm genetic stocks are given in Table 1.

In spite of such a wide silkworm genetic base, hardly any information is available either on the unique features of many of these genotypes, or on the extent of genetic diversity between or within the genotypes, which, in turn, limit their use in the production of elite stocks for the benefit of improving quality and productivity of silk. Besides, many of the genotypes phenotypically look alike although they are endowed with unique genetic features. Conversely, the same genotype when raised under different eco-climatic conditions also gives rise to many significantly different phenotypic features. These kinds of phenotypic similarity of divergent genotypes and dissimilarity of closer genotypes prove to cause difficulties for the silkworm breeders when choosing parental genotypes for cross-breeding programmes. Hence it is important that the vast genetic resources of silkworms available in different countries are genetically characterised and optimally used for synthesising elite genotypes to upgrade the productivity levels in the sericulture industry. In addition, precise characterisation of genotypes is extremely important in sericulture to protect the silkworm breeders' rights. To date, new varieties are usually described on the basis of their morphological characteristics which, as stated earlier, are highly variable and environmentally dependent, thus requiring reliable techniques for genotype characterisation. These limitations call for harnessing the recent developments in molecular biology for rational utilization of silkworm genetic resources. Such an attempt would also enhance our ability to gain deeper insight into the genome of this economic insect. Earlier attempts to screen for allozyme variations in silkworm resulted in the identification of the polymorphic form of only one enzyme, digestive alpha amylase. This enzyme is polymorphic only between diapausing and non-diapausing genotype groups and different genotypes within the group show little variation [25]. Only recently have attempts been initiated to construct molecular linkage maps based on RAPD [26] and RFLP [27] and to the fingerprint silkworm genome using the M13 probe [28, 29], silkworm transposons [30], RAPDs [21], and a banded krait minor satellite DNA (Bkm)-2(8) minisatellite probe [31]. In the present communication we review the work on DNA fingerprinting and its significance in silkworm improvement programmes.

Table 1. Countrywise silkworm genetic stocks

Country	Silkworm genetic stocks
China	600
France	53
India	550
Italy	123
Japan	1672
Republic of Korea	286

Source: [24]

2 RAPD-based silkworm characterisation

Williams *et al.* [8] and Welsh and McClelland [9] described the use of short oligonucleotide primers of arbitrary sequence for the amplification of genomic DNA. These techniques are based on the amplification of short inverted repeats scattered throughout the genome and have already provided innovative methods for DNA profiling and related research [10, 32–34]. RAPDs have been used to examine genetic variation in both geographically distinct ecotypes and highly inbred lines of silkworm accessions [21]. The study, interestingly, resulted in diapausing and non-diapausing genotype-specific PCR products. The dendrogram based on data from amplification using 40 oligonucleotides in thirteen highly divergent silkworm genotypes was consistent with the known geographical and breeding history of silkworm populations. The 40 primers tested revealed 216 PCR products, of which 206 were polymorphic. The primers OPA-01 and OPA-02 detected diapausing and non-diapausing genotype-specific amplified products (Fig. 1). Mendelian inheritance of the polymorphic RAPD markers was confirmed using the segregating backcross progeny. The RAPD-based dendrogram resulted in a clear separation of two groups, one comprising diapausing and the other non-diapausing genotypes [Fig. 2]. Among the diapausing genotypes, all the 'Chinese type' genotypes such as KA, NB_7 and NB_1, which spin oval cocoons, grouped separately while the 'Japanese

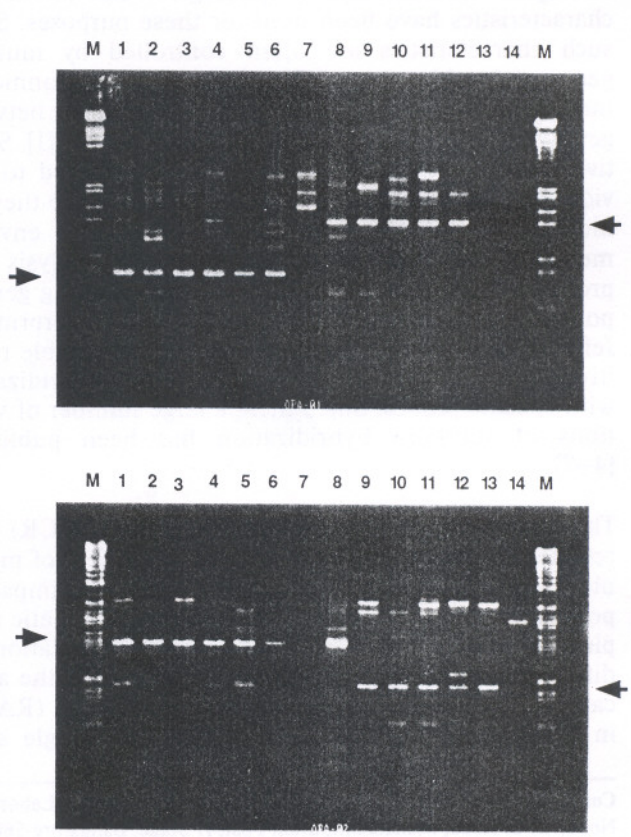

Figure 1. RAPD fingerprints obtained for diapausing and non-diapausing silkworm genotypes using (a) OPA-01 and (b) OPA-02. Lanes (1) to (14) are HU_{204}, KA, NB_1, NB_{18}, NB_4D_2, C.nichi, Gungnong, Moria, Nistari, Pure Mysore, Diazo, Sarupat and control, respectively. M is a lambda *Eco*RI- *Hin*dIII digest. Arrows indicate diapausing and non-diapausing genotype-specific amplified products.

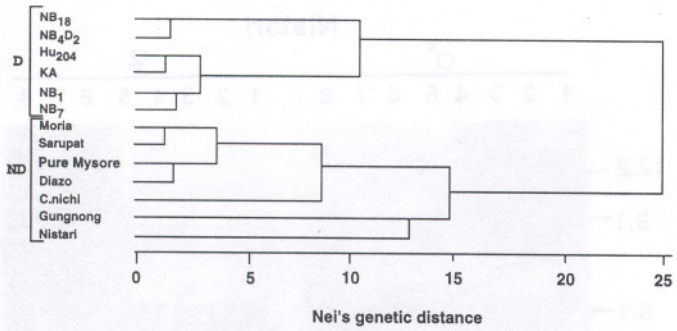

Figure 2. Dendrogram of diapausing (D) and non-diapausing (ND) silkworm genotypes based on RAPD analysis of pooled DNA samples.

type' genotypes NB_{18} and NB_4D_2, which spin peanut-shaped cocoons, were found in another group. The tropical genotypes, Sarupat and Moria, which share the same geographical origin (Assam, India) were grouped in the same cluster. RAPD analysis therefore is powerful in resolving the genotypes based on yield, geographical origin, voltinism and morphological differences. RAPD analysis thus shows potential to become a valuable tool for analysis of genetic variation among populations and for identifying molecular markers for economic traits of silkworm.

3 Bkm 2(8)-based silkworm DNA profiling

A banded krait minor (Bkm) satellite DNA, consisting of highly conserved GATA repeats, has been found to be scattered all over the genome of a wide range of higher eukaryotes and to reveal extensive RFLP [35–37]. It has been used as a multilocus probe in DNA fingerprinting of humans, crocodilians, *etc.* [38–40]. Using the Bkm 2(8) probe we demonstrated the utility of this probe in generating DNA fingerprints of 13 divergent genotypes of silkworm and also examined the intra- and inter-population genetic diversity. The DNA fingerprinting revealed 9–31 discrete bands depending on the two restriction enzymes, *Bst*NI and *Hin*fI used. The *Hin*fI profiles revealed well-resolved DNA fragments in the molecular weight range of 9–1.6 kbp and were more informative than the *Bst*NI-generated DNA profiles. The genotype-specific fingerprint profile was evident in almost all thirteen genotypes examined. A sample of DNA fingerprint profiles of four genotypes is shown in Fig. 3. For example Pure Mysore, Nistari, and NB_{18} revealed major diagnostic bands of 8, 6.3 and 7.4 kbp after digestion with *Hin*fI enzyme, whereas NB_4D_2 showed a 12.5 kbp band in the *Bst*NI profile.

The phenetic analysis of RFLP data separated thirteen strains into three groups (Fig. 4). One group consisted of all the diapausing genotypes (except HU_{204}); the second group consisted of all non-diapausing genotypes (except Pure Mysore and Nistari) and also the diapausing genotype HU_{204}. The third group consisted exclusively of Pure Mysore and Nistari. The clustering pattern obtained was comparable to the phenogram resulting from RAPD analysis. The sister lines of NB_4D_2 and NB_{18} genotypes, which are derived from the same Japanese double-cross hybrid, shared the same group. Similarly, Moria and Sarupat, which share the same geographical locale, were grouped in the same cluster as observed in the RAPD analysis. Nistari and Pure Mysore were also grouped in the same cluster as observed in the RAPD analysis, but unlike RAPD assay, they were delineated into separate clusters. These studies show that Pure Mysore is possibly evolved from Nistari, which was introduced to India through Thailand almost 500 years ago [41].

Genetic variability was studied within silkworm genotypes based on DNA fingerprinting with Bkm-2(8). The DNA profile of individuals within a given genotype shared more or less identical patterns (Fig. 3, Table 2). These results on molecular similarity are highly valuable in view of the fact that in silkworm, hybrids of inbred lines or ecotypes of diapausing and non-diapausing genotypes are reared for silk production where high genetic similarity among individuals of each of the parental genotypes is required.

4 Discussion

The bulk of various synthetic inbred lines of silkworm available today are evolved by means of classical breeding, either for specific traits or for an index of silk yield attributes of economic importance. Reliable estimates of genetic diversity within and between genotypes are important both for the maintenance of genotypes and germplasm stocks and selection of parents for the development of elite hybrids. We have shown that such inter- and intra-population similarity can be measured in silkworm by using RAPD and Bkm-based DNA fingerprinting analysis. We realise that the estimates of genetic relatedness may not be fully accurate for several reasons: for example, a Bkm probe detects multiple bands simultaneously to form a complex DNA fingerprint. Therefore, any fragments that are allelic can not be determined, which introduces obstacle to the interpretation of DNA fingerprint data [42–44]. Hence isolation of single-locus markers might help to solve the problem [43]. For example, the genotype NB18 reveals a single-locus marker at 7 kbp position, which could serve as an ideal single-locus marker (Fig. 3D). Our initial study with the inheritance pattern of this marker on a small number of progeny was not adequate to determine the allelic status of this marker. Since a single silkworm brood yields a 400–500 progeny, it may be essential to determine the allelic status of such markers using at least 50–60 progeny.

In our hands, RAPD assay was reliable and gave consistent results in grouping the genotypes. However, considering the inherent disadvantage of low annealing temperature used in the RAPD techique and inconsistent results encountered by many investigators when different thermal cyclers, different sources of Taq polymerases, and different concentrations of DNA were used, it may be worthwhile to convert the RAPD markers into sequence-characterised amplified regions (SCARs) by sequencing the two ends of the RAPD fragments of interest and synthesising two longer primers (24 mers) homologous to each end [45]. Such primer information can be readily exchanged between laboratories and reliably used for quick genetic analysis.

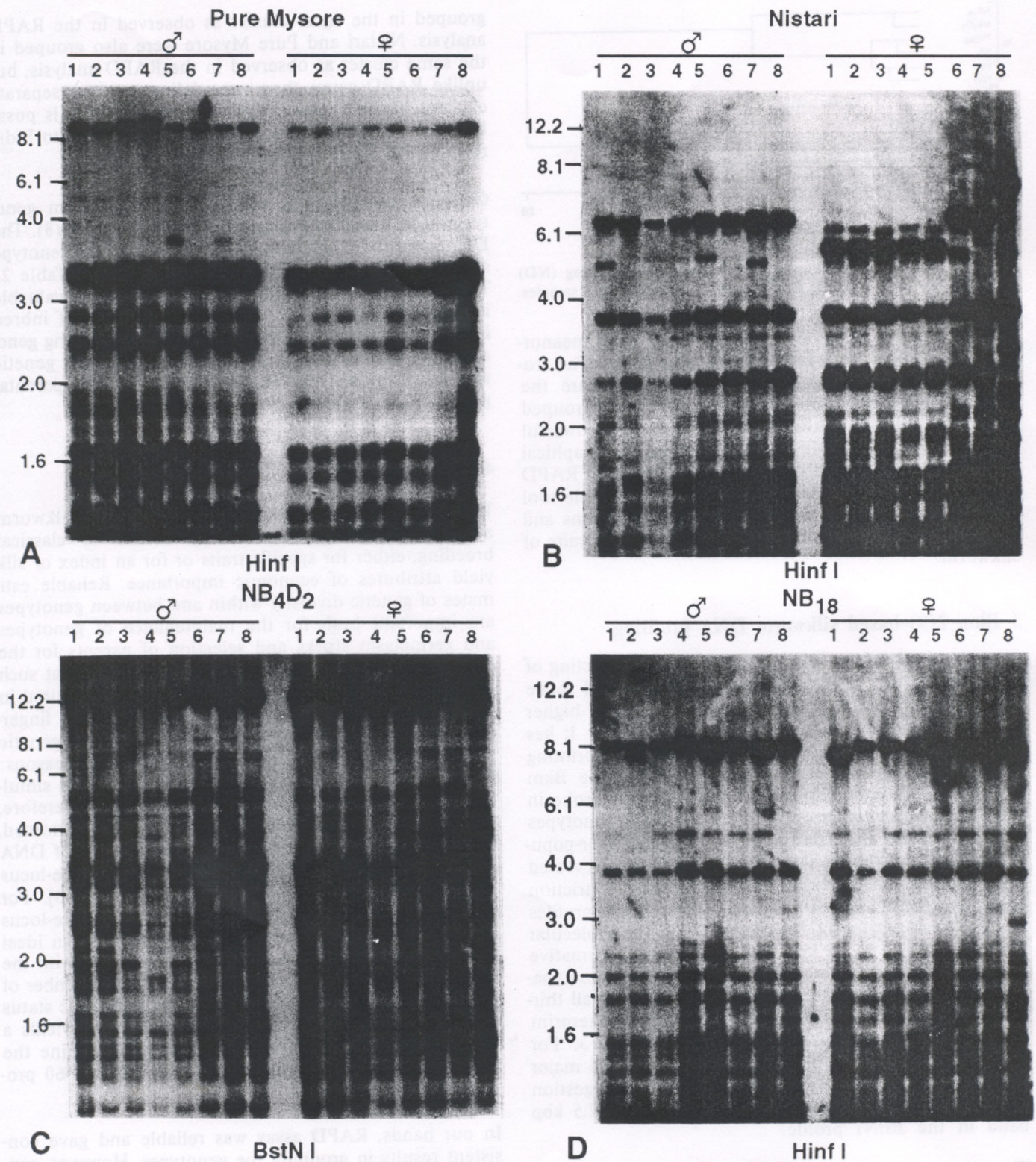

Figure 3. Bkm 2(8) hybridization pattern of DNA from (A) Pure Mysore, (B) Nistari, (C) NB_4D_2 and (D) NB_{18} silkworm genotypes. Eight male and eight female moths of each genotype were fingerprinted in a single gel. Restriction enzymes used were *Hin*fI (A, B and D) and *Bst*NI (C). Note that individuals within a genotype show more or less similar fingerprint profiles. Numbers on the left indicate DNA fragment size in kbp.

Although the accuracy in estimating the genetic relatedness remains to be determined, the intra- and inter-population genetic distance estimates obtained from the DNA fingerprint data do reflect the morphological, geographical, and pedigree history of the genotypes. Thus, we believe that the dendrograms depicted in Figs. 2 and 4 delineate, in many respects, a genetic relationship of these genotypes. Thus, DNA fingerprinting should be considered a worthwhile approach for studying genetic diversity in silkworm genotypes and to address questions relevant to systematics and breeding. It has been shown in silkworms that both dominance and epistasis are important in heterosis for traits of silk production [46]. Compared to related genotypes, genetically distant geno-

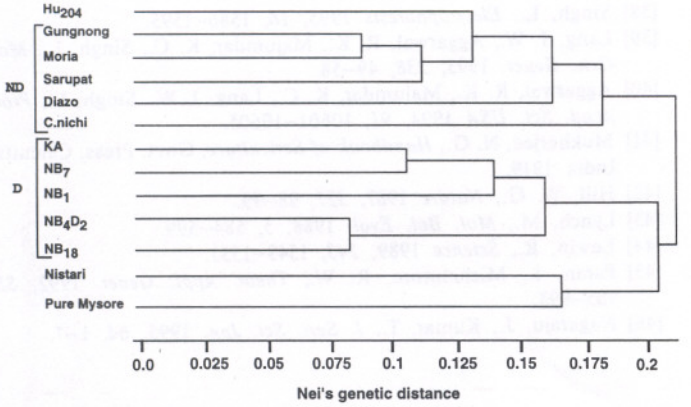

Figure 4. Dendrogram of diapausing (D) and non-diapausing (ND) silkworm genotypes based on Bkm 2(8) fingerprinting of pooled DNA samples.

types are more likely to have different fixed alleles at the same loci and hence their crosses should give a higher degree of heterosis. Furthermore, heterosis is higher if both the parental genotypes involved in the hybrids have a higher degree of homozygosity. In light of this, the information obtained by DNA fingerprinting could be valuable in silkworm to plan cross-breeding strategies. Besides, some of the genotype-specific markers, as reported in this review, could provide additional markers to increase the density of the molecular map of the silkworm genome map which is being undertaken by different groups. If the economically important traits are found to have close linkage with the DNA fingerprint pattern, it could be useful in marker-assisted selection.

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Table 2. Similarity coefficients within various diapausing and non-diapausing genotypes of *Bombyx mori* with respect to sex as well as restriction enzyme, sex, restriction enzyme and irrespective of sex and restriction enzyme (mean)

Genotypes	Sex and restriction enzyme				Sex		Restriction enzyme		Mean
	*Bst*NI		*Hin*fI		Male	Female	*Bst*NI	*Hin*fI	
	Male	Female	Male	Female					
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

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