Eur. J. Entomol. **99**: 267–276, 2002
ISSN 1210-5759

DNA fingerprinting with homologous multilocus probes and search for DNA markers associated with yield attributes in silkworm, *Bombyx mori*

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Key words. *Bombyx mori,* DNA fingerprinting, multilocus probes, Restriction Fragment Length Polymorphism, RFLP

Abstract. RFLP clones harbouring multi-copy DNA sequences were isolated from the *Pst* I sub-genomic library of the indigenous silkworm race, *Nistari*, and were used for DNA fingerprinting studies in 13 stocks of silkworm, *Bombyx mori* L. Six multilocus probes produced 180 RFLP markers that showed a high level (98%) of polymorphism and are highly useful in molecular mapping, genotype characterization and marker assisted selection (MAS). The dendrogram derived from UPGMA analysis clearly divides the 13 silkworm stocks into two major clusters: high- and low-yield stocks. Furthermore, adopting multiple regression analyses, the RFLP marker(s) associated with characters of economic importance were identified, a first of its kind for any species of insect of commercial importance. The results obtained create an opportunity of using germplasm stocks directly for isolating specific RFLP band(s) and use it for MAS in breeding programs.

INTRODUCTION

The Restriction Fragment Length Polymorphism (RFLP) profiling (Botstein et al., 1980; Tanksley et al., 1989) proved to be a very important technique for identifying genotypic differences. It is applied to both plant and animal systems (Jeffreys et al., 1985, 1991; Miller & Tanksley, 1990; Song et al., 1990; Jung et al., 1993), especially because of their reproducibility (Paull et al., 1998). The relationship between different species as well as other taxonomic issues has been examined with improved methodologies for generating RFLP profiles (Virk et al., 1996; Salimath et al., 1995; Lu et al., 1996). The polymorphic DNA markers also helped in the construction of a linkage map for various plant (Tanksley et al., 1992; Paterson et al., 1995) and insect species (O'Brien, 1993) including the silkworm, *Bombyx mori* (Shi et al., 1995). The banded krait minisatellite (Bkm-2 (8)) has also been used successfully as a probe in generating highly polymorphic RFLP profiles for several stocks of *B. mori* (Nagaraju et al., 1995). Probes were also developed from homologous genomes by the digestion of genomic DNA with specific restriction enzymes for identification of clones (Jung et al., 1993). However, such a system has not been utilised for assessing genetic variability among the different stocks and breeds of *B. mori*. The present report is based on the result of such an attempt. As indicated elsewhere, the system is based mainly on the generation of multi-copy DNA sequence probes (Sulaiman et al., 1995).

B. mori, commercially, contributes more than 90% to total silk production (Currie, 1996) and is represented by a large number of stocks having a wide range of yield potential (Chatterjee & Datta, 1992; Chatterjee, 1993). Conventional studies were made to understand the genetics of yield components, but until now, no attempt has been made to identify specific DNA marker(s) contributing to the wide variability in yield potential. Furthermore, the low yielding silkworm stocks are less susceptible to diseases and abiotic stresses, while the high yielding breeds and hybrids need strict disease management and control of abiotic factors (Goldsmith, 1991; Chatterjee et al., 1993) for fuller realization of yield potential. Indian silk-cocoon producers include a large section of small and marginal farmers, for whom strict disease management and high input of fertiliser and disinfectants are a well-defined economic constraint. Thus, molecular analysis of these yield components and identification of DNA markers associated with high germ-load tolerance and high yield potential are of great significance for establishing work on directional molecular breeding of the silkworm. The relevance of DNA markers for molecular breeding and conventional breeding has been extensively highlighted elsewhere (Mitra et al., 1999), and is of great relevance for India and other countries of the third world practicing sericulture.

The identification of specific DNA markers associated with such attributes of economic importance, including that of disease resistance, is in itself an important issue. One of the approaches that has been adopted by Michaelmore et al. (1991) was the generation of bulk-segregants based on specific yield component or disease resistance/susceptibility among the F_2 population. The F_2 population is raised from the cross between two stocks, divergent for the specific character under study. Another approach is to establish a similar association on the basis of correlation between DNA profiles and specific phenotypic characters (Virk et al., 1996; Yonash et al., 2000). The present report is based on the result of analyses fol-

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TABLE 1. List of thirteen silkworm stocks, information on their parentage and country of origin.

Stock	Parentage	Country	Reference
BBE-CHI	Unknown (Hu204)	China	Reddy et al., 1999
BBI-0095	(N122.C110).(N124.C124)	Indian breed	Chatterjee, 1993
BBE-JAP	Unknown (NB1)	Japanese breed	Chatterjee & Datta, 1992
BBI-0082	Kinshu-Showa	Indian breed	Thangavelu et al., 1997
BBI-0081	(N124.C124). (Kokko.Seihaku)	Indian breed	Thangavelu et al., 1997
BBI-0044	(N124.C124). (Kokko.Seihaku)	Indian breed	Thangavelu et al., 1997
BME-0005	Shi x Nichi (C'Nichi)	Indian race	Chatterjee & Datta,1992; Ghosh, 1949
BME-0013	Unknown (Guangnong)	China	Chatterjee et al., 1993a
BMI-0003	Unknown (Moria of Assam)	Indian race	Chatterjee & Datta, 1992
BMI-0017	Unknown (Nistari of Bengal)	Indian race	Chatterjee & Datta, 1992
BMI-0001	Unknown (Mysore, Karnataka)	Indian race	Chatterjee & Datta, 1992
BME-0052	Unknown (Diazo)	Chinese race	Chatterjee et al., 1993a
BMI-0002	Unknown (Sarupat of Assam)	Indian race	Chatterjee & Datta, 1992

BB = *Bombyx* bivoltine; BM = *Bombyx* multivoltine; I = indigenous; E = exotic; CHI = Chinese; JAP = Japanese.

lowing the second approach and is first of its kind for any species of insect having economic importance.

MATERIALS AND METHODS

Silkworm stocks

The present study was based on thirteen races and breeds of Indian, Chinese and Japanese origin (Table 1), and these stocks are maintained in the lab for more than 10 generations. The mean estimates of ten yield components and the status of voltinism for the thirteen stocks utilized, given in Table 2, are taken from published manual of Central Silkworm Germplasm Resource Centre (Thangavelu et al., 1997).

DNA extraction

The genomic DNA was isolated from posterior silk glands of a minimum of ten four days old, fifth instar larvae for each of the thirteen stocks mentioned earlier. To study inheritance, the genomic DNA was extracted from individual moths of BMI-0017 (low yielding Indian race) and BBE-JAP (high yielding Japanese breed) as well as their F_1 hybrid progeny. The silk-glands or the moths were crushed in liquid nitrogen, subjected to lysis in the presence of proteinase-K at 37°C and followed by the normal method of phenol-chloroform extraction with ethanol precipitation. The DNA initially obtained was re-extracted in the same way after RNase A treatment and finally dissolved in 10mM TE buffer [Tris-Na_2EDTA (ethylene-diamine-tetra-acetic acid, di-sodium salt)].

TABLE 2. Average estimates (Thangavelu et al., 1997) of yield components of thirteen silkworm stocks used for generating RFLP profiles.

Stock	Origin	EGG (No)	TLD (Hrs)	VLD (Hrs)	LWT (gm)	PWT (gm)	CWT (gm)	SHWT (cg)	SR (%)	YLD (No)	YLD WT (kg)	VOLT
BBE-CHI	Chinese	481	590	138	2.77	1.07	1.29	0.22	15.8	6303	8.03	D
BME-0013	Chinese	417	558	135	2.43	0.96	1.13	0.17	14.6	7592	8.57	ND
BME-0052	Chinese	393	564	162	2.17	0.83	0.96	0.13	13.8	7062	6.72	D
BME-0005	Indian(1)*	397	539	112	2.10	0.88	1.00	0.12	12.0	7923	8.11	ND
BMI-0003	Indian(1)	388	564	145	2.69	0.97	1.13	0.16	14.7	8045	9.72	ND
BMI-0017	Indian(1)	381	557	143	2.10	0.89	1.02	0.13	13.0	7972	7.72	ND
BMI-0001	Indian(1)	457	626	186	1.82	0.86	1.00	0.14	14.1	7410	7.15	ND
BMI-0002	Indian(1)	405	569	149	2.52	0.99	1.16	0.17	14.9	7717	9.02	ND
BBI-0095	Indian(2)*	412	602	174	3.52	1.25	1.50	0.25	16.8	8246	11.92	D
BBI-0082	Indian(2)	424	599	165	3.42	1.28	1.56	0.28	18.0	8038	11.59	D
BBI-0081	Indian(2)	395	600	166	3.61	1.30	1.57	0.27	17.2	8292	13.42	D
BBI-0044	Indian(2)	479	646	206	3.54	1.47	1.82	0.36	19.6	8342	16.12	D
BBE-JAP	Japanese	524	600	167	3.54	1.69	1.95	0.26	17.3	6198	9.98	D
Standard deviation		44.2	30.4	24.3	0.66	0.27	0.33	0.07	2.16	710.5	2.73	

*(1) and (2) indicate Indian race and Indian breed, respectively.
EGGNO = mean number of eggs laid by one mother moth; TLD = total larval duration; VLD = duration of fifth instar; LWT, PWT, CWT, and SHWT denote weight of single matured larva, pupa, cocoon, and cocoon shell, respectively; SR% = ratio of weight of shell to the weight of cocoon; YLDNO and YLDWT = number of cocoons realized /10,000 larvae reared and their weight. VOLT = voltinism; ND and D denote non-diapause and diapause, respectively.

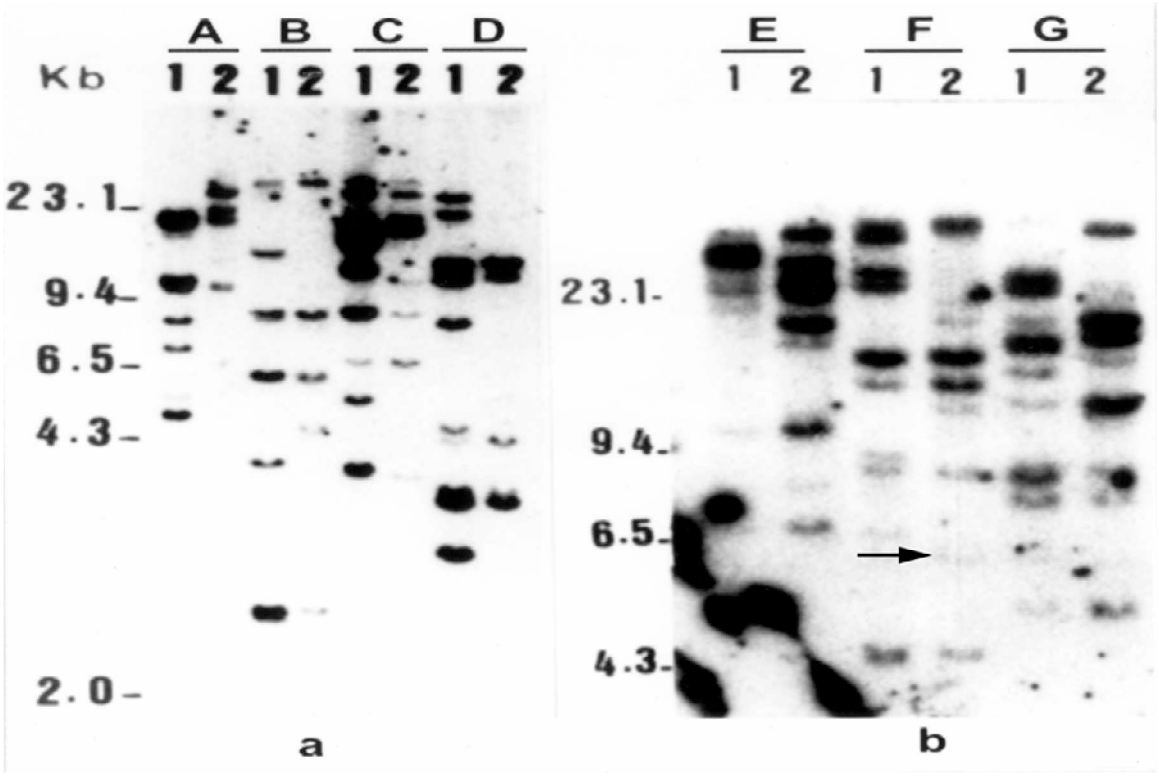

Fig. 1. Polymorphism detected by multilocus probe, p*Bm*N 138(a) & 319(b) on two selected divergent stocks, BMI-0017 (1) and BBE-JAP (2), when digested with different restriction enzymes, *Bam* HI (A), *Eco* RI (B&E), *Eco* RV (C&F), and *Hind* III (D&G). The arrow marks the position of bands not properly labeled in the autoradiogram, but clearly visible in Fig 2b.

DNA probes

A size selected (0.5–2 kb) *Pst* I silkworm genomic library was prepared in the pUC_{18} using genomic DNA from BME0017 (*Nistari*), one of the oldest Indian races of silkworm. Multiple copy recombinant clones were selected by colony hybridization using ^{32}P-labelled total silkworm genomic DNA as a probe (Sulaiman et al., 1995). For Southern hybridization, probes with insert length varying between 0.8 to 1.8 kb, were labeled with α^{32}P CTP using a random primer labeling kit (Amersham Int., Buckinghamshire, UK) (Sambrook et al., 1989).

Restriction digestion, electrophoresis and Southern hybridization

10–12 µg of DNA per sample was digested to completion with different restriction enzymes (New England Biolabs, Hertfordshire, England). Four restriction endonucleases such as *Bam* HI, *Eco* RI, *Eco* RV and *Hind* III were used for the DNA fingerprinting studies. Digested DNA was separated by electrophoresis on 0.8% agarose gel in 1 X TAE (40mM Tris, 20 mM acetate and 1mM EDTA) buffer. The gel fractionated DNA samples were vacuum blotted to a Hybond-N Nylon membrane (Amersham Int., Buckinghamshire, UK). Subsequent to baking at 80°C for 2 h, the membranes were hybridized (Sambrook et al., 1989) at 65°C for 16 h, and washed several times at 65°C in descending concentration of 2.0 X to 0.1 X SSC (sodium chloride/sodium citrate buffer) containing 0.1% SDS (sodium dodecyl sulphate). Subsequently, the blots were exposed with Kodak X-ray film with intensifying screens for 1–3 days at –70°C.

Data analysis

Binary scoring (0 and 1) was done from RFLP profiles observed for 13 different silkworm stocks using six different probes in autoradiograms. For all DNA profiles, only distinguishable fragments greater than 2.0 kb were scored. Pairwise comparison of DNA fingerprint lanes was done only within a gel.

Estimation of similarity index. For clustering, the similarity index (S) was calculated after Nei and Li (1979), as the fraction of shared fragments between pairs of silkworm stocks. For silkworm stocks, x and y, $S = 2Nxy/Nx+Ny$ where Nxy was the number of common fragments in the two silkworm stocks, while Nx and Ny are the number of fragments scored in stocks x and y, respectively. For the thirteen different genotypes, S was converted to a genetic distance as $D = 1–S$. The dendrogram was constructed using the "UPGMA" (Unweighted pair-group method using arithmetic average) method in the PHYLIP programme (Felsenstein, 1989).

Correlation and multiple regression analysis. All DNA bands scored were considered as independent variables while yield components of silkworm stocks were taken as dependent variables. First, Pearson correlation coefficients were determined for all DNA markers. DNA markers showing positive correlation with yield components were identified and analyzed using multiple regression analyses. The provision of stepwise multiple regression analysis available with SPSS 10.0 program was used with ≥ 0.45 and ≤ 0.099 as F-value to enter and remove, respectively.

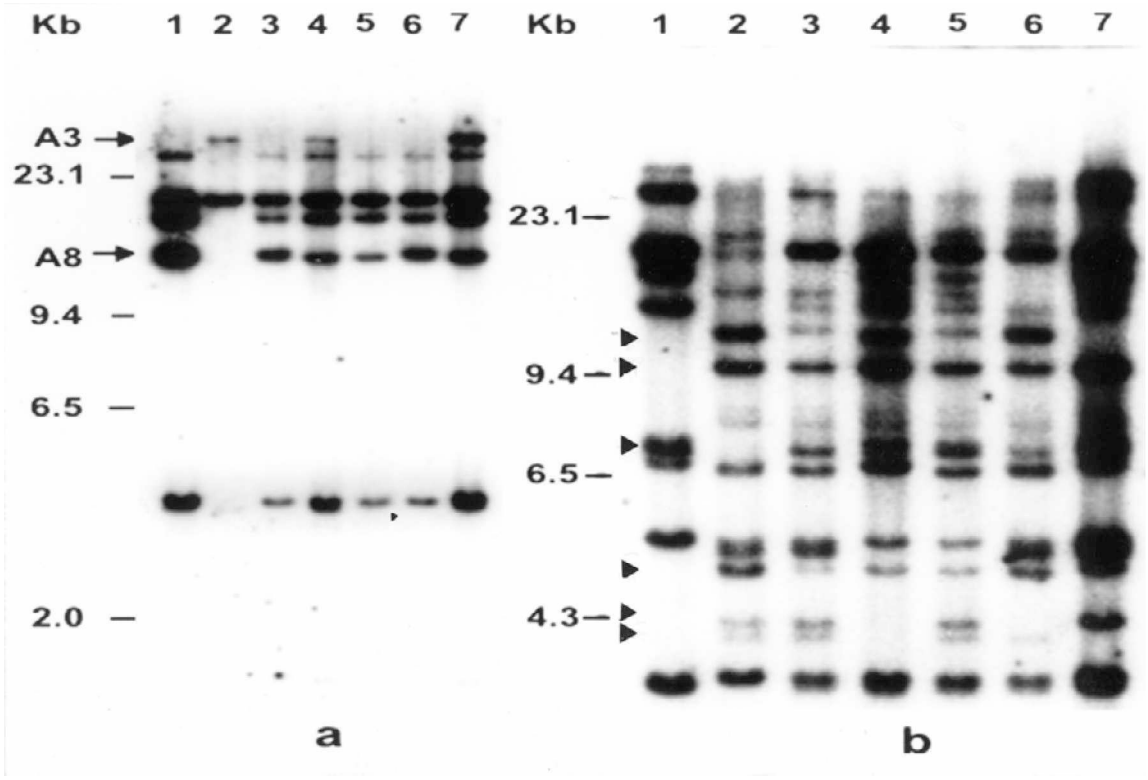

Fig. 2. The hybridization pattern of *Eco* RV digested DNA from parents, BMI-0017 (lane-1) and BBE-JAP (lane-2) silkworm stocks and their F_1 offspring (lanes 3–7) with the multilocus probe, p*Bm*N 138. a – bands A13 and A8 marked with arrows are two of the eight bands projected in Table 6 for their significant association with LWT and SR%, respectively; b – the arrow-heads point out the polymorphic bands between the two parents and illustrate the detection of homo- or heterozygous status from the segregation of the marker(s) in F_1 individuals. The duplex- band of ~ 5.0 kb in lane 2 appears very prominent in comparison to the faint band in lane-2 of F in Fig. 1b.

RESULTS

DNA fingerprinting analysis

A total of 1056 recombinant plasmids were obtained from the partial genomic library developed from *Pst* I digest of silkworm genomic DNA. On dot blot screening, 245 recombinant plasmids showing signal of moderate intensity were selected as putative multiple copy clones. Of these, twenty-five clones were randomly selected and screened for their polymorphism on two selected divergent silkworm stocks, BMI-0017 (low yielding) and BBE-JAP (high yielding). Four restriction enzymes, *Bam* HI, *Eco* RI, *Eco* RV and *Hind* III were used for initial screening (Fig. 1a, b). Among the above four restriction enzymes, *Eco* RI and *Eco* RV produced maximum polymorphism (85%) when compared to *Bam* HI or *Hind* III (60%). Southern hybridization showed that the cloned fragments of all 25 recombinant plasmids were distributed in the size range of 2 to 23 Kb (Fig.1a,b). However, six of these p*Bm*N clones (138(A), 319(B), 445(C), 803(D), 948(E), and 966(F)), with an insert size varying from 0.8 to 1.8kb and a high level of polymorphic pattern between these BMI-0017 and BBE-JAP stocks, were subsequently selected for fingerprint analysis in the thirteen silkworm stocks (Fig. 3a,b).

The six multilocus probes produced 180 RFLP markers across the 13 silkworm stocks and of these, 38 fragments were not present in more than one stock. The number of unique fragments was found to be highest for BME-0005, while, no unique fragment could be detected for BBI0081 and BBI0082. Inheritance pattern for the few selected multilocus probes such as p*Bm*N 138, 319, 803 and 966 were studied in the F_1 progeny of BMI-0017 X BBE-JAP cross (Fig. 2a,b). The pattern presented in Fig. 2a,b, clearly substantiates the inheritance of the RFLP markers from parents to offspring. From the profiles of F_1 individuals and their parents, presented in Fig 2a,b, it is also evident that before planning to utilize specific markers for MAS program, it is possible to determine whether a marker in a parent is homo- or heterozygous. For example, the presence of the marker A-8 in all F_1 individuals suggests that it is homozygous in the parent-1. On the other hand, the absence of the band A-3 in three of the five F_1 individuals tested indicated it to be in heterozygous condition in parent-2. Likewise, the first two markers at the top of Fig 2b (indicated with arrowhead), could be detected in all the offspring, showing its homozygous status in parent 2 because this is the only parent possessing these markers. The distribution of the last two marked fragments exhibits their heterozygous status in

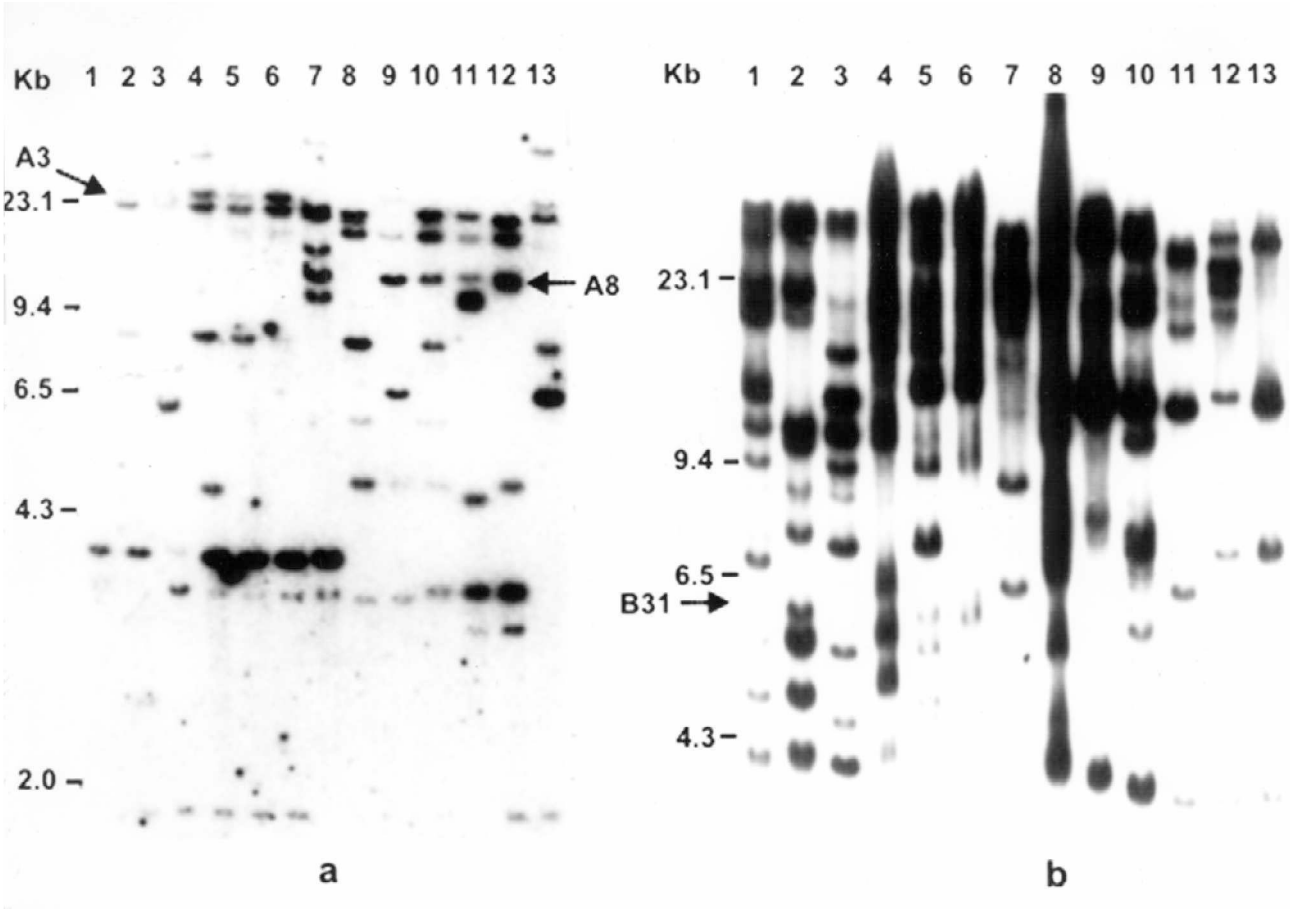

Fig. 3. Polymorphism generated with multilocus probes, p*Bm*N 138(a) and 319(b) using pooled template DNA of 13 silkworm stocks, digested with *Eco* RV. Lane 1-13 represents profiles for BBE-CHI, BBI-0095, BBE-JAP, BBI-0082, BBI-0081, BBI-0044, BME-0005, BME-0013, BMI-0003, BMI-0017, BMI-0001, BME-0052, and BMI-0002, respectively. The three RFLP-markers, A3, A8, and B31 (arrows), designate the bands selected for their significant association with LWT, SR%, and YLDWT, respectively (Tables 5 and 6).

parent 2. However, mention should be made of the very prominent duplex-band of ~ 5.0kb in lane 2 of Fig. 2b. The band in the same position in Fig. 1b (lane 2 of F) appears very faint, possibly due to a bleaching effect in the autoradiogram as evident from black smear in the left corner. However, it may be also noted that DNA template used for work presented in Fig. 1 is from a minimum of 10 individuals, isolated *en-masse*, while that in Fig. 2 is from a single parent of the particular cross.

Genetic variation

Each hybridising fragment from the DNA fingerprint for 13 silkworm stocks (Fig. 3a,b) was scored as a piece of independent data and a genetic similarity index (S) for each pair of silkworm stocks was calculated (Table 3), which revealed the similarity index ranging from 0.23 to 0.75 with the average of 0.40. It is also of interest to note that the highest similarity was found between the Chinese stock, BME-0052, and the Indian stock, BMI-0002.

Genetic relationship among silkworm stocks

The dendrogram derived from UPGMA analysis clearly depicts the presence of two major clusters (Fig. 4). The Cluster-A includes all the five Indian stocks of low yield potentials (Table 2) and two Chinese stocks, almost of similar yield status. The Cluster-B includes all improved Indian breeds and one Chinese stock, BBE-CHI, of medium yield of 8.03 kg cocoons/100 broods. The dendrogram further reveals that BME-0005 enters the cluster combination at the eleventh (last) step of agglomeration while in the preceding step, cluster-pair of BBI-0095 and BBE-CHI, joined the small sub-cluster of BBI-0044: BBI-0081: BBI-0082: BBE-JAP.

Association between DNA markers and yield components

Correlation analysis between 180 alleles and ten yield components revealed significant (P = 0.05 and 0.01) association for 70 alleles, and the r-values ranged from a minimum of 0.555 to 0.811. The results (Table 4) revealed that 18 alleles bear significant correlation with not more than one yield trait. Eleven of these alleles, A2, A7, B8, B18, C6, D20, E6, E7, E11, E14, and E18 showed significant correlation with VLD only. C7, E23, E27, and D10 were associated with EGGNO, TLD, LWT, and YLDWT, respectively. The remaining three, A12,

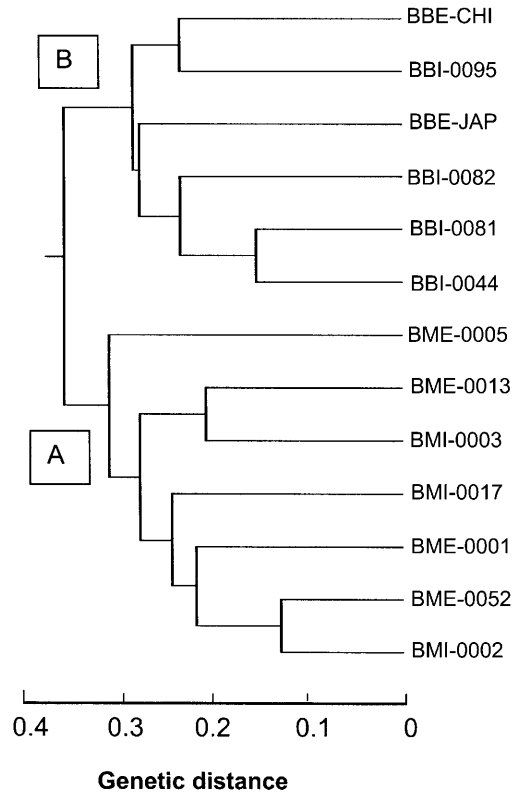

Fig. 4. The dendrogram based on RFLP markers illustrating the classification of silkworm stocks into two well defined sub-clusters, comprising of stocks having low (sub-cluster-A) and high yield (sub-cluster-B) potential.

B16 and F20 were correlated to YLDNO alone. However, no fragment showed significant correlation, either negative or positive, with all the yield parameters. It is of further interest to note that, except in the case of EGGNO, the fragment showing negative correlation were more in number than those revealing positive correlation, with respect to any dependent variable.

Results of stepwise regression analyses with the selected 70 fragments and eleven dependent variables (ten yield components and voltinism) are presented in Table 5. It is evident that within the limit fixed for F-to enter and F-to remove, the analysis selected only three fragments for EGGNO, CWT and SHWT while for TLD, the number of alleles contributing positive effect is 9, which is the maximum. Some fragments have significant contributory effect in the formation of regression equation for more than one character. For example, D5 was selected for EGGNO, TLD and VLD while F24 appeared significant for TLD and LWT. Further, the relative importance of some bands differs for different yield components, e.g., D5 was selected at step-2 for EGGNO and TLD, while it was selected at the step-1 for VLD. Summarizing the data in Table 5, it may be said that 27 fragments are being selected out of 70 fragments analyzed for multiple regression, three of which are marked in Fig. 3a,b.

To test the efficacy of the selection of fragments through MRA, the status of marker(s) selected at the first step of stepwise regression in the thirteen silkworm stocks was examined by t-test (Table 6). Result for all the yield components, except EGGNO, show that the group mean for those stocks where the band is absent significantly differs from the group mean for those stocks where the band is present.

DISCUSSION

Level of polymorphism

Shi et al. (1995) showed that the silkworm genome with comparatively higher GC content can produce high polymorphism when restriction enzymes with six base pair recognition sequences are used. The presented results corroborate the above finding. Similar level of polymorphism was also observed in plants (Neuhusen, 1992; Bark & Havey, 1995) and in insects (*Musca domestica*; Blanchetot, 1991).

The level of polymorphism (98%) presently realized is higher than that realised with various PCR based markers such as RAPD (94%; Nagaraja & Nagaraju, 1995), ISSR (86%; Reddy et al., 1999b) and SSR (77%; Reddy et al., 1999a) or that realised with the use of Bkm-2(8) probe (Nagaraju et al., 1995). Landry et al. (1987) comparing various sources of probes for their efficiency in detecting RFLP in lettuce concluded that an increased detection of polymorphism in multiple copy sequences could be the

TABLE 3. Genetic similarity index (S) between pairs of silkworm genotypes.

	BBI-0095	BBE-JAP	BBI-0082	BBI-0081	BBI-0044	BME-0005	BME-0013	BMI-0003	BMI-0017	BMI--0001	BME-0052	BMI-0002
BBE-CHI	0.553	0.444	0.396	0.455	0.468	0.328	0.333	0.333	0.367	0.239	0.259	0.207
BBI-0095	-	0.435	0.450	0.518	0.487	0.320	0.239	0.261	0.269	0.237	0.260	0.260
BBE-JAP		-	0.426	0.505	0.478	0.421	0.377	0.358	0.373	0.280	0.404	0.316
BBI-0082			-	0.529	0.575	0.392	0.277	0.319	0.283	0.232	0.294	0.333
BBI-0081				-	0.706	0.393	0.323	0.323	0.324	0.300	0.374	0.393
BBI-0044					-	0.400	0.348	0.391	0.308	0.280	0.340	0.360
BME-0005						-	0.404	0.456	0.365	0.365	0.443	0.426
BME-0013							-	0.604	0.508	0.411	0.439	0.404
BMI-0003								-	0.525	0.449	0.491	0.526
BMI-0017									-	0.538	0.556	0.508
BMI--0001										-	0.591	0.574
BME-0052											-	0.754

TABLE 4. List of RFLP markers showing significant positive and negative correlation with selected yield parameters.

Yield trait	Positively correlated alleles	Negatively correlated alleles
EGGNO	A.14, B.12, B.22, C.3, C.12, C.25, D.25, D.26, E.25, F.14	C.7, D.12, F.22
TLD	B.31, C.5, C.12, C.19, D.5, E.24,	D.9, D.12, E.21, E.23, F.11, F.22, F.24, F.25, F.26
VLD	A.23, B.8, B.31, D.5,	A.2, A.7, B.13, B.18, C.6, D.20, E.6, E.7, E.11, E.14, E.18, E.21, F.11, F.24, F25, F.26
LWT	A.3, A.18, A.23, B.22, B.31, B.35, C.12, C.19, E.24, F.14,	A.8, A.9, B.39, D.3, D.4, D.8, D.9, D.11, D.12, D.14, D.17, D.24, D.28, E.9, E.10, E.15, E.17, E.27, F.15, F.22,
PWT	A.3, A.18, A.23, B.22, B.31, B.35, C.12, C.19, E.25, F.8,	A.8, A.9, B.39, D.3, D.4, D.8, D.9, D.11, D.12, D.14, D.17, D.24, D.28, E.9, E.10, E.15, E.17, F.24, F.25, F.26.
CWT	A.3, A.14, A.18, A.23, B.12, B.22, C.12, C.19, D.15, D.17, D.26, E.24, E.25, F.8, F.14	A.8, B.39, D.4, D.8, D.9, D.11, D.12, D.14, D.24, D.28, E.9, E.15, E.17, F.22, F.24, F.25, F.26.
SHWT	A.3, A.18, A.23, B.15, B.22, B.31, B.35, C.5, C.12, C.19, D.15, D.24, E.8, E.24, F.14	A.8, B.39, D.4, D.8, D.9, D.11, D.12, D.14, D.17, D.28, E.9, E.15, E.17, F.11, F.22, F.24, F.25, F.26
SR%	A.3, A.18, A.23, B.22, B.31, B.35, C.4, C.19, E.8, E.24, F.14,	A.8, D.9, D.12, D.17, D.28, E.9, E.15, E.21, F.11, F.24, F.25, F.26,
YLDNO		A.12, A.14, B.12, B.16, C.3, C.25, D.25, D.26, E.25, F.20
YLDWT	A.3, A.18, A.23, B.15, B.31, B.35, C.4, C.12, C.19, E.8, E.24, F.14	A.8, B.39, D.8, D.9, D.10, D.11, D.14, D.17, D.24, D.28, E.15, F.22

result of independent mutation in the different copies of the gene in the presence of a relevant selection pressure. Noli et al. (1997) and Hahn et al. (1995) also observed a higher level of polymorphism in barley and maize, with homologous probes against that realised with RAPD markers.

Genetic diversity

Genetic similarity estimates based on RFLPs have been shown to be good in accordance with pedigrees in several crops (Smith et al., 1990) and have been used to identify divergent parents for F_1 hybrid production (Powel et al., 1996). Reliable estimates of genetic diversity within and between silkworm stocks, as revealed by the RFLP analysis (Powel et al., 1996), are important for the maintenance of germplasm stocks and selection of parents for the development of elite hybrids with higher yield potential.

Earlier studies showed that the number of probes required for generating sufficient number of markers for assessing genetic similarity varies from 15 (Dos Santos et al., 1994) to 100 (Messmer et al., 1993). However, in the present approach substantial numbers of bands were generated with only six probes. The same has been considered as sufficient to estimate genetic similarly as it was projected by Tivang et al. (1994) in maize, that the number of bands required for a CV of 10% was 388, 150 and 38 for closely, intermediately, and distantly related inbreeds, respectively. Pejic et al. (1998) while using the bootstrap procedure suggested that 150 bands are sufficient for reliable estimates of genetic similarity.

Genetic relationship among silkworm stocks

As indicated in an earlier paragraph the clustering or grouping on the basis of RFLP marker clearly demarcates the yield potential of 13 silkworm stocks studied. In addition, the closer association between BBI-0044 and BBI-0081 is substantiated with the information on the origin of these two breeds, both of which were developed from the commercially available double hybrid of (Kokko x Seihaku) × (N_{124} × C_{124}). The greater distance of BBI-0082 from the above two breeds is supported by the fact that the latter was developed from a different Japanese hybrid namely Kinshu x Showa (Chatterjee, 1993). On the other hand, BBI-0095 was developed (Thangavelu et al., 1997) from another Japanese double hybrid [(N_{122} × C_{110}) × (N_{124} × C_{124})]. However, it is difficult to explain the association of BBE-CHI with BBI-0095. It is possible that sometime during the breeding process a common parent was used to infuse certain specific characters.

With regard to the relationship between the low yielding stocks, certain aspects need to be highlighted. Though BME-0005 is very close to BMI-0017 or BMI-0001 with regard to the economic character, they do not occupy close position in the dendrogram. This is not unusual if one considers the origin of these races. BMI-

TABLE 5. Result of multiple regression analyses (Stepwise method) for identifying significant association between yield components and specific RFLP markers.

DNA marker	R^2	R^2-Change	F-change	Sig. of F-change
EGGNO				
C3	0.572	----	14.694	0.003
+D5	0.882	0.310	26.232	0.000
+F14	0.951	0.069	12.487	0.006
TLD				
F24	0.543	---	13.094	0.004
+D5	0.808	0.265	13.778	0.004
+E9	0.946	0.138	23.114	0.001
+C7	0.974	0.028	8.392	0.020
+E27	0.992	0.018	15.190	0.006
+D10	0.999	0.007	37.813	0.001
+D8	1.000	0.001	11.741	0.019
+A9	1.000	0.000	19.000	0.012
VLD				
D5	0.494	----	10.759	0.007
+A23	0.847	0.353	23.098	0.001
+F4	0.946	0.946	16.662	0.003
+E17	0.979	0.032	12.087	0.008
+E8	0.992	0.013	11.334	0.012
+D26	0.998	0.006	18.125	0.005
LWT				
A3	0.658	----	21.199	0.001
+B17	0.901	0.242	24.342	0.001
+F24	0.969	0.069	20.119	0.002
+E23	0.989	0.020	14.111	0.006
+C19	0.994	0.006	7.001	0.033
PWT				
F14	0.625	----	18.306	0.001
+C19	0.912	0.287	32.575	0.000
+A3	0.953	0.041	7.895	0.020
+A14	0.979	0.026	10.147	0.013
CWT				
F14	0.630	----	18.710	0.001
+C19	0.930	0.301	43.193	0.000
+A3	0.974	0.043	14.909	0.004
SHWT				
C12	0.624	----	18.253	0.001
+F14	0.881	0.257	21.689	0.001
+B35	0.943	0.062	9.740	0.012
+B35(C12#)	0.939	0.004	0.594	0.460
+A3	0.970	0.031	9.372	0.014
SR%				
A8	0.582	----	15.305	0.002
+F14	0.782	0.200	9.153	0.013
+B31	0.902	0.121	11.091	0.009
+A2	0.953	0.051	8.588	0.019
+D17	0.979	0.026	8.640	0.022
YLDNO				
C3	0.738	----	31.052	0.000
+D17	0.899	0.161	15.878	0.003
+B32	0.939	0.040	5.954	0.037
+E23	0.970	0.030	7.982	0.022
YLDWT				
B31	0.685	----	23.957	0.000
+F14	0.896	0.211	20.231	0.001

C12 was removed from the regression equation at this stage.
+ indicates markers selected up to the preceding step in the equation.

0017 is a race used on the Gangetic plain for not less than 100 years (Mukherjee, 1912), whereas BMI-0001 is a race used in Southern peninsula where sericulture developed later. Thus, the distance reflected in the dendrogram appears justified. The position of C'Nichi is quite interesting. Chatterjee & Datta (1992) indicated that this race is a segregant from the original hybrid Shi.Nichi. This hybrid was introduced during early part of the last century (Ghosh, 1949). Other studies in this laboratory (unpublished) also show the presence of DNA markers specific to high cocoon weight and high shell weight in this race, though the expression of high cocoon weight and high shell weight could not be realised.

Association between DNA markers and yield components

Different groups of scientists have utilized different statistical approaches to ascertain the association(s) between molecular markers and yield attributes. For instance, Barbosa-Neto et al. (1996) and Virk et al. (1996) adopted linear regression, while Lynch (1999) and Yonash et al. (2000) utilized genetic correlation and single band/multiband analysis, respectively.

The correlation analysis enabled us to find 70 RFLP markers showing significant association with ten yield components. Pearson correlation estimates do not take into account the multiple interactions between independent variables on one hand and that between independent and dependent on the other. Multiple regression analysis is a relevant tool in this context, as projected by the "multiband" analysis for establishing linkage between RFLP markers and antibody response in meat-type chickens (Yonash et al., 2000). Virk et al. (1996) also adopted a similar approach in analyzing quantitative variation within rice germplasm using RAPD markers. The application of t-test (Table 6) attests significance to the difference between the two means for all components except EGGNO, thereby strengthening the adoption of MRA for establishing association between DNA markers and yield attributes.

Thus the result obtained creates an opportunity for isolating specific bands for further characterisation of the fragment(s). Moreover, such selected markers can help in marker assisted selection (MAS) program for molecular breeding (Stomberg et al., 1994). The success of such selection programs depends exclusively on the extent of linkage between markers and the relevant loci such as QTLs (quantitative trait loci). This requires further study with the specific markers selected from such analyses.

Abbreviations used

BBE = *Bombyx* bivoltine exotic; BBI= *Bombyx* bivoltine indigenous; BME = *Bombyx* multivoltine exotic; BMI = *Bombyx* multivoltine indigenous; CHI = Chinese; CV = coefficient of variance; EDTA = ethylene diamine tetraacetic acid; EGGNO = mean number of eggs laid by one mother moth; ISSR = inter simple sequence repeat; JAP = Japanese; LWT, PWT, CWT, and SHWT, denote weight of single matured larva, pupa, cocoon, and cocoon shell, respectively; MAS = marker assisted selection; MRA = multiple regression analysis; ND & D = non-diapause and diapause; PHYLIP = phylogeny inference

TABLE 6. Results of t-test for the difference in the mean values realized from a particular yield component in stocks showing the presence and absence of the band reflected at the first step of MRA.

Parameters	Band selected	Mean and SD of yield components		t -value	Significance
		Present	Absent		
Egg Number (EGGNO)	C3	413.4 ± 30.2	502.5 ± 30.4	12.7	0.08(NS)
Total Larval Duration (TLD)	F24*	602.5 ± 25.9	558.8 ± 11.7	2.2	0.002
V instar Larval Duration (VLD)	D5	150.5 ± 18.4	196.0 ± 14.1	4.3	0.05
Larval Weight (LWT)	A3	22.3 ± 3.3	32.6 ± 4.6	2.2	0.001
Pupal Weight (PWT)	F14	1.0 ± 0.2	1.4 ± 0.2	2.6	0.01
Cocoon Weight (CWT)	F14	1.2 ± 0.2	1.8 ± 0.2	2.8	0.01
Shell weight (SHWT)	C12*	27.6 ± 5.2	16.0 ± 4.5	2.3	0.003
Shell Ratio (SR%)	A8*	16.8 ± 1.6	13.5 ± 1.0	2.2	0.001
Yield Number (YLDNO)	C3*	7876.3 ± 397.4	6250.5 ± 74.2	2.2	2.1e-07
Yield Weight (YLDWT)	B31	8.7 ± 1.5	13.8 ± 2.1	3.2	0.03

*Band showing association with higher yield components.

package; QTL = quantitative trait loci; RFLP = restriction fragment length polymorphism; SDS = sodium dodecyl sulphate; SR% = ratio of weight of shell to the weight of cocoon; SSC = saline sodium citrate; TAE = Tris acetic acid; TE = Tris-EDTA; TLD = total larval duration; TRIS= hydroxymethyl-aminomethane; UPGMA = unweighted pair-group method using arithmetic average; VLD = duration of fifth instar; VOLT = voltinism; YLDNO and YLDWT = number of cocoon realized /10,000 larvae reared and their weight in kg.

ACKNOWLEDGEMENTS. This work was supported, in part, by a grant (No. 2666/95) from the Dept. of Biotechnology, Govt. of India. The financial support of the Dept. of Biotechnology and Central Silk Board is greatly appreciated. The authors thank Mr. C.V. Nair and Mr. P. M. Rajendiran for their assistance in silkworm rearing and maintenance.

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Received November 8, 2001; revised April 2, 2002; accepted May 8, 2002