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Comparison of multilocus RFLPs and PCR-based marker systems for genetic analysis of the silkworm, *Bombyx mori*

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The utility of multilocus RFLPs and three PCR-based techniques, Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat-PCR (ISSR-PCR) and simple sequence repeats (SSRs) for genetic characterization was examined using 13 diverse silkworm strains. All four approaches successfully discriminated the 13 silkworm varieties but differed in the amount of polymorphism detected. The usefulness of each system was examined in terms of number of loci revealed (effective multiplex ratio, EMR) and the amount of polymorphism detected (diversity index, DI). For example, the six multilocus RFLP probes produced 180 products of which 97% were polymorphic; 15 SSR loci gave rise to an average of 8 alleles each, of which 86% were polymorphic. The ISSR-PCR produced 39 fragments of which 76.98% were polymorphic. The highest diversity index was observed for ISSR-PCR (0.957) and the lowest for RAPDs (0.744). The RAPD, ISSR-PCR and RFLP assays clearly separated the diapausing and non-diapausing silkworm varieties. These results are discussed in terms of choice of appropriate marker technology for different aspects of silkworm genome analysis.

Keywords: EMR, ISSR-PCR, non-diapausing, RAPD, RFLP, SSR.

Introduction

The detection and exploitation of naturally occurring DNA sequence polymorphisms are among the most significant developments in molecular biology. Polymorphic genetic markers have wide potential applications in animal and plant improvement programmes as a means for varietal and parentage identification, evaluation of polymorphic genetic loci affecting quantitative economic traits, and genetic mapping. RFLPs have been used extensively for genetic studies (Bishop & Skolnick, 1980; Botstein *et al.*, 1980), molecular mapping and genetic improvement programmes. However, the technical complexity of performing RFLP analysis coupled with the widespread use of short-lived radioisotopes for detection have prompted researchers to look for alternative methods.

The advent of the PCR has resulted in the development of a large number of molecular techniques, which offer an effective alternative to the hybridization methods of RFLP analysis. PCR-based approaches use only small quantities of DNA, avoid DNA blotting and use of radioactivity, and are amenable to automation. The RAPD method described by Williams *et al.* (1990) and Welsh & McClelland (1990) generates PCR products by annealing to randomly distributed homologous target sites of the template DNA. This technique mostly generates dominant markers, although length polymorphisms caused by insertions/deletions can also occur at low frequencies. Because of its relative simplicity, RAPD technology is being extensively used in genetic analysis of various plant and animal species. However, limitations in the applications of RAPDs such as the dominant nature of the markers, chance co-migration of bands at different loci, and requirement for stringent protocol standardization to ensure reproducibility have also been encountered (Black, 1993).

Another class of PCR-based markers, the microsatellites, takes advantage of the abundant and ubiquitously distributed simple sequence repeats (SSRs) in the eukaryotic genome (Hamada *et al.*, 1982; Weber & May, 1989; Dietrich *et al.*, 1992). The variation in repeat number can be visualized as differences in the length of PCR-amplified products (Tautz *et al.*, 1986; Tautz & Renz, 1984). Analysis of SSRs requires prior characterization of sequences flanking the repeats to allow the design of primers for PCR amplification. SSRs are

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codominant markers and can reveal multiple alleles at a single locus. The technique is robust in that it is highly reproducible and the primer sequence information can be exchanged between laboratories.

As PCR technology finds increased use in genetic analysis, novel variations of this technique are emerging which promise precision, economy and speed (Wu *et al.*, 1994; Zietkiecwicz *et al.*, 1994; Vos *et al.*, 1995). One such alternative is SSR-anchored PCR (or ISSR-PCR for Inter-Simple Sequence Repeat PCR) in which a stretch of microsatellite sequence, which also contains a short oligonucleotide 'anchored' sequence either at the 5′ or 3′ end, is targeted to the template DNA in question and upon PCR amplification, inter-repeat genomic regions can be visualized as discrete products (Zietkiecwicz *et al.*, 1994). The technique generates a large number of markers by simultaneously targeting multiple microsatellite loci and allows screening of a large number of samples in a single gel.

The silkworm, *Bombyx mori*, is an excellent model genetic system and an important insect economically. Genome analysis has been initiated in the silkworm with the objectives of obtaining genetic maps using different marker systems: RFLPs (Shi *et al.*, 1995), RAPDs (Promboon *et al.*, 1995) and RAPD double primers (Yasukochi, 1998), and to characterize genetic diversity by DNA fingerprinting using ISSR-PCR (Reddy *et al.*, 1999a), a heterologous minisatellite probe, Bkm (Nagaraju *et al.*, 1995; Sharma *et al.*, 1998), microsatellite loci (Reddy *et al.*, 1999b) and RAPDs (Nagaraja & Nagaraju, 1995). In addition, more than 3000 silkworm strains which represent a repertoire of genetic differences for various complex traits such as silk fibre length, larval growth and resistance to disease, are being maintained in different countries. Molecular genetic analysis of such complex traits using DNA markers would provide valuable tools for economic improvement of this insect.

In the present study, we examined multilocus RFLPs and three PCR-based marker assays, RAPDs, SSRs and ISSR-PCR for evaluating the genetic diversity in certain representative strains of silkworm. We report results here using 13 diverse silkworm strains with a view to comparing their effectiveness in detecting genetic variation and to determine the utility of each system with respect to technical and financial considerations.

Materials and methods

Silkworm strains and DNA extraction

Thirteen diverse silkworm genotypes were used in the present study. The characteristics of the genotypes are listed in Table 1. All DNA extractions were performed

Table 1 Characteristics of silkworm strains used in the present study

Strains	Voltinism	Origin	Larval pattern	Cocoon colour	Cocoon shape	Larval duration (D : H)	Cocoon weight (gm)	Shell weight (gm)	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	Non-diapausing	India	Plain	White	Peanut	21 : 00	0.92	0.103	11.1	350
Gungnong	Non-diapausing	China	Marked	Cream	Short oval	20 : 00	1.07	0.173	15.0	480
Moria	Non-diapausing	Assam (India)	Plain	Cream	Spindle	22 : 00	1.09	0.136	12.5	375
Nistari	Non-diapausing	West Bengal (India)	Marked	Golden yellow	Spindle	22 : 00	1.10	0.151	13.7	450
Pure Mysore	Non-diapausing	Karnataka (India)	Plain	Greenish yellow	Spindle	28 : 00	1.00	0.138	13.8	425
Daizo*	Non-diapausing	Japan	Marked	Dark greenish yellow	Spindle	20 : 00	1.18	0.170	14.7	380
Sarupat	Non-diapausing	Assam (India)	Plain	Cream	Spindle	22 : 00	1.00	0.131	13.1	375

*Also lays diapausing eggs.

on liquid nitrogen frozen silk glands using the method already described (Nagaraja & Nagaraju, 1995).

RFLP analysis

High molecular weight genomic DNA was isolated from posterior silk glands of instar 5 larvae pooled from 10 individuals per strain. 10–12 μg of DNA was digested to completion with restriction enzymes, *Eco*RV or *Bam*HI (New England Biolabs) depending on the probe (Table 2). Digested samples were fractionated by 0.8% agarose gel in TAE buffer and southern transferred on to a nylon membrane (Hybond-N, Amersham) by vacuum blotting (Pharmacia Biotech). The membranes were baked at 80°C for 2 h. The anonymous multilocus probes used in the present study were obtained from a *Pst*I subgenomic library. The probes were labelled with α^{32}P dCTP using a random primer labelling kit (Amersham, UK). All other operations including pre-hybridization, hybridization and washing were performed according to Sambrook *et al.* (1989). The membranes were then exposed to X-ray film for 1–3 days. Autoradiograms were examined visually to score the hybridized bands.

RAPD analysis

RAPD analysis was carried out on 13 silkworm strains using 10-mer oligonucleotide primers (listed in Table 2) obtained from Operon Technologies, USA as described by Nagaraja & Nagaraju, (1995).

SSR analysis

Simple Sequence Repeat (SSR) polymorphism analysis was performed using 15 primer sets for different microsatellite motifs listed in Table 2 on 13 silkworm genotypes. The SSR loci have been cloned and characterized from the subgenomic library constructed from the Nistari silkworm strain as described by Reddy *et al.* (1999b). PCR was performed using a Perkin Elmer 480 Thermal cycler. Typical PCR reactions (20 μL) were performed in 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl and 1.0% Triton X-100), 1 mM of dATP, dGTP, dTTP each and 0.25 mM dCTP, 4 μM of each primer, 2 μCi of α^{32}P-dCTP (1000 Ci/mmol), 1 unit of Taq DNA polymerase (Perkin-Elmer Cetus) and 20 ng of template DNA. Different concentrations of $MgCl_2$ (1–4 mM) were used depending on the locus amplified. Samples were overlaid with 20 μL of mineral oil. PCR cycles for microsatellite loci included a 2-min at 94°C initial denaturation step, followed by 30 cycles of 94°C (30 s), annealing at 42°C to 55°C (30 s) and extension at 72°C (45 s). The final elongation step was extended to 10 min at 72°C. Aliquots of amplified DNA from individual PCR reactions were mixed with formamide stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue and 0.05% xylene cyanol in the ratio of 3 : 2). Samples (4 μL) were denatured at 75°C for 2 min, chilled on ice and electrophoresed on standard sequencing gels (6% acrylamide, 8 M urea, 1× TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.3). An M13 sequence ladder was used on each gel as a size marker. After electrophoresis, the gels were fixed for 2 × 20 min with 10% acetic acid, dried at room temperature and exposed to X-ray film for 4–12 h.

SSR-anchored PCR

A range of primers was synthesized on an Applied Biosystems DNA synthesizer based on the core repeats, anchored either at the 5′ end or 3′ end (Table 2). These primers were used to screen the silkworm strains as described by Reddy *et al.* (1999a). The PCR reaction was carried out in a total volume of 20 μL containing 20 ng of genomic DNA, 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl and 1.0% Triton X-100), 10× dNTP stock (2 mM), 10 μM of anchored primer (a mixture of 1 : 4 labelled primer using γ^{32}P ATP (6000 Ci, BRIT, JONAKI, Hyderabad) and cold primer. The samples were overlaid with 10 μL of mineral oil and processed using a Perkin-Elmer Cetus model 480 Thermal Cycler. The cycling conditions were: initial denaturation of 2 min at 94°C, 27 cycles of 30 s at 94°C, 45 s at 52°C and 2 min at 72°C, and one last cycle of 7 min at 72°C. On completion of the PCR, the reaction was stopped using 6 μL of stop solution as indicated previously. A 4 μL aliquot of each of the reaction products was denatured at 75°C for 2 min, chilled on ice and then run on a sequencing gel containing 6% polyacrylamide, 8 M urea and 1× TBE, at 1500 V of constant power for 10–12 h. Sequencing reactions of M13mp18 DNA were used as molecular weight standards to determine the exact length of the PCR products. After electrophoresis, gels were fixed for 2 × 20 min in 10% glacial acetic acid, dried at room temperature and applied to autoradiographic film for 2–10 h at room temperature.

Data analysis

In the RFLP assay, hybridized bands and in PCR-based assays specific amplified products that were reproducible in successive amplifications were identified as marker bands defined by their molecular weights estimated from the size standards. Polymorphisms were scored for presence (1) or absence (0) of the bands. The proportion

Table 2 A list of RAPD, ISSR and SSR primers and multilocus RFLP probes used in the study

Primer code	Primer sequence (5′ to 3′)	Annealing temperature (°C)	No. alleles	Allele size	Heterozygosity
RAPD:					
OPA	20 numbers	36			
OPB	20 numbers	36			
ISSR:					
5′-anchored-SSR	BDB(CA)7	52			
	VHVG(TG)7	52			
	DBDA(CA)7	52			
	HVH(TG)7	52			
3′-anchored-SSR	(GT)8RG	52			
	(GT)8RTCY	52			
SSR:					
sat158	cttccagacaaccattagat cagcatccatccttatttat	49	11	190–246	0.76
sat211	gatcgaactacgcaattacg cagcatccatccttatttat	49	6	6–146	0.89
sat256	attgcctcggacaggagtgt tgtgaggacaggccgtta	53	7	111–194	0.82
sat346	gaagacagagcgaagtgga atggattcctgctggtagat	53	6	139–189	0.83
sat892	caataaatgcttacgagtttaa tatcggtagttccttgactt	47	3	175–187	0.66
sat951	attgtaaccgatttgagaga attcgcacaataagttcact	48	5	107–122	0.68
sat962	cataataaataaataataat tgtagtgggatagtcagtat	42	6	108–114	0.78
sat1013	aacagatgctgcggactggt tgccattcacaatacaacat	50	5	135–162	0.80
sat1411	gaatgttctgctggtgg taatgtttttatactttattatatg	45	8	109–162	0.68
sat1423	ctttcgatcaccgcgttctc cgctacgaaataccattatctgaca	55	9	130–176	0.82
sat1893	aatgcagaatcgtaattttt tttgaccacagacaataagc	45	7	98–158	0.85
sat2550	ggtcccttgaaactgcgat cagagacctgccggttgtcgtt	53	9	121–168	0.87
sat2604	gctcgccatatgcaatcctc cgtcattgccttcatttcagttc	53	8	143–186	0.86
sat2763	acgcgctctacaaaataccatta gatcaccggttctgctctcg	53	17	105–179	0.90
sat3513	cgcaattctgtattagataa aaaggtattattcttattcg	46	6	134–223	0.84
RFLP:	Probe number	Enzyme used for digestion			
	pBmN 138	*Eco*RV			
	pBmN 319	*Eco*RV			
	pBmN 445	*Eco*RI			
	pBmN 803	*Bam*HI			
	pBmN 948	*Eco*RV			
	pBmN 966	*Eco*RV			

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of bands that were shared between any two screened varieties averaged over the total number of loci was used as the measure of similarity for all marker types. This corresponds to using simple matching coefficients of similarity. The data was analysed using Nei and Li coefficients (Nei & Li, 1979).

Cluster analyses were based on similarity matrices using the unweighted pair group method analysis (UPGMA) program in WIN BOOT software (Yap & Nelson, 1996). The relationships between varieties were represented graphically in the form of dendrograms. In order to determine the utility of each of the marker systems, diversity index (DI), effective multiplex ratio (EMR) and marker index (MI) were calculated according to Powell *et al.* (1996).

DI for genetic markers was calculated from the sum of the squares of allele frequencies:

$$\mathrm{DI_n} = 1 \quad \sum pi^2,$$

where ‘pi’ is the allele frequency of the ith allele.

The arithmetic mean heterozygosity, $\mathrm{Di_{av}}$, was calculated for each marker class:

$$\mathrm{Di_{av}} = \sum \mathrm{Di_{n/n}},$$

where ‘n’ is the number of markers (loci) analysed.
The DI for polymorphic markers is:

$$(\mathrm{Di_{av}})_p = \sum \mathrm{Di_n}/\mathrm{n_p}$$

where ‘n_p’ is the number of polymorphic loci and n is the total number of loci.

EMR (E) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay.

$$\mathrm{EMR\ (E)} = \mathrm{n_p}(\mathrm{n_p}/\mathrm{n})$$

MI is defined as the product of the average diversity index for polymorphic bands in any assay and the EMR for that assay, $\mathrm{MI} = \mathrm{DI}_{\mathrm{avp}} \times \mathrm{E}$.

Results

The multilocus RFLPs and all three PCR assays reliably discriminated among the 13 silkworm varieties examined (Fig. 1a–1d). However, each of the PCR and multilocus RFLP-based techniques differed in the type and degree of polymorphism detected. Moreover, the total number of assays varied for each of the marker systems. It was 40 primers for RAPDs, 15 primer pairs for dinucleotide SSR loci, only six anchored primers for ISSR–PCR and six multilocus probes for RFLPs (Table 2). As a result, the total number of bands scored for each of the assays varied, ranging from 122 for SSR to 239 for ISSR-PCR. In the RAPD analysis, using 40 random primers, a total of 216 discrete amplified products (5.4 bands/primer) were obtained, of which 204 were polymorphic (94.4%) in at least one pair-wise comparison between silkworm varieties, and the remaining 12 were monomorphic. Four 5′-anchored and two 3′-anchored SSRs revealed a total of 239 amplification products (39.8 bands/primer) of which 184 were polymorphic (76.98%). Primer pairs designed for 15 SSR loci yielded 122 PCR products (8.1 products per assay) of which 105 were polymorphic (86%). Using six multilocus probes, a total of 180 different bands were scored (an average of 30 bands/probe), of which (97.77%) were polymorphic. These data are summarized in Table 3.

The marker index (MI) was used as an estimate of the utility of each system. MI is the product of the diversity index (DI) and effective multiplex ratio (EMR). The arithmetic mean of DI for polymorphic markers for each system is presented in Table 4. The highest level of polymorphism as measured by DI was detected using SSR–PCR (DI 0.957), followed by RFLP (0.936), SSR (0.79) and RAPD (0.744). Also presented in Table 4 is the arithmetic mean for EMR for each marker system. SSRs were assigned an EMR of 1 on the assumption that they reveal a single locus (which may not always be true). The average EMR is highest for RFLPs (28.68) which was slightly higher than that of ISSR-PCR (23.58) and sixfold that of RAPDs (4.81). The mean MI for each marker system calculated from the experimental data is also listed in Table 4. The distinctively higher EMRs for RFLPs and ISSR–PCR are reflected in the calculated MI for these two systems (26.67 and 23.58, respectively).

Spearman’s rank correlations were used to rank the similarities generated using different marker assays and linear correlation regression analysis was used to compare individual similarity coefficients from two marker systems. The correlations are given in Table 5. Both tests indicated the best correspondence between RFLP and SSR (0.796), followed by SSR and ISSR-PCR (0.685) and RAPD and ISSR-PCR (0.682).

Silkworm germplasm can be divided into two gene pools, diapausing and non-diapausing, based on voltinism (number of generations in a year). The diapausing strains are mostly distributed in temperate zones and are characterized by longer larval life, higher body weight, higher fecundity, longer silk fibre and greater susceptibility to viral diseases as compared to the non-diapausing varieties, which are distributed mostly in tropical

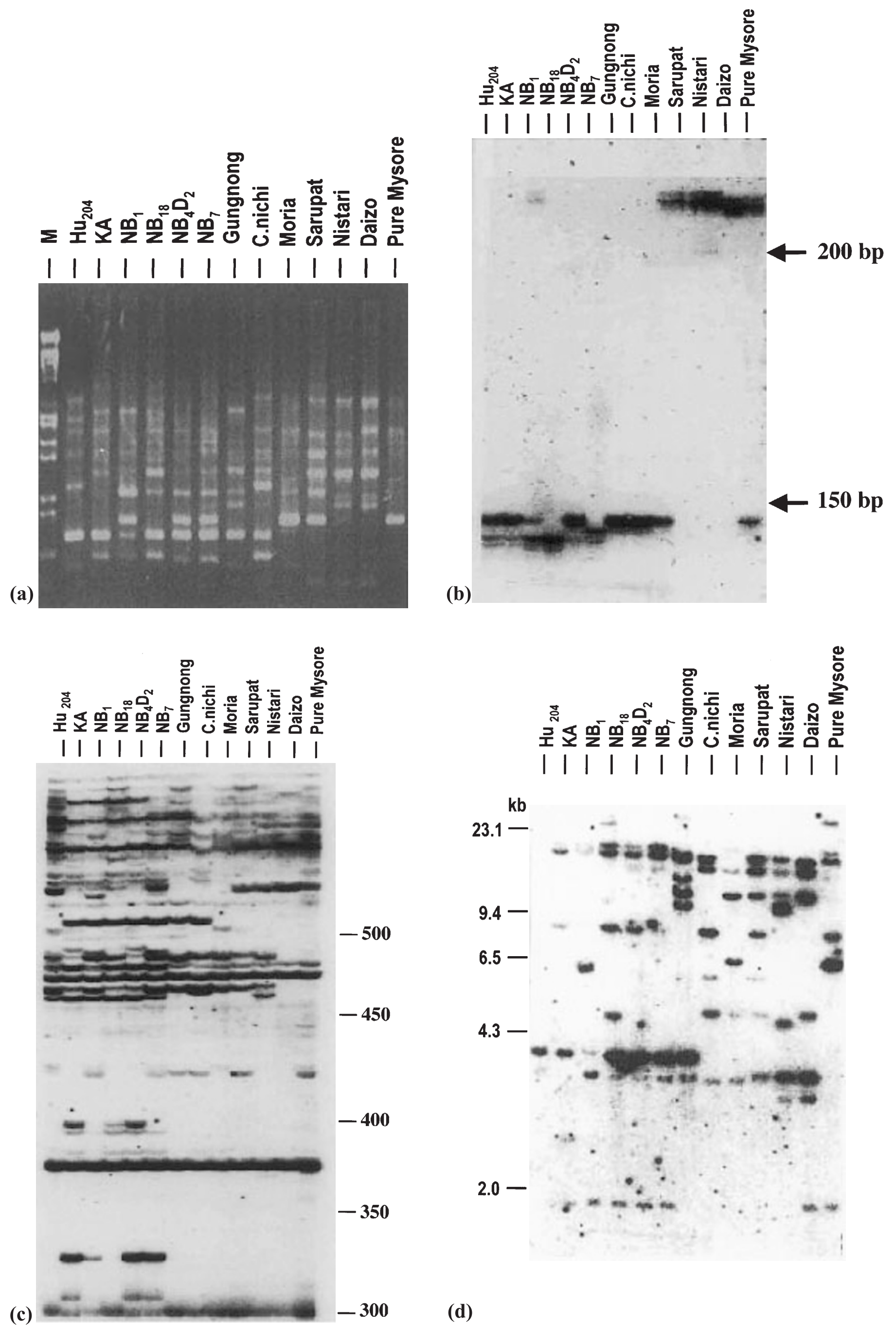

Fig. 1 Polymorphisms detected in 13 silkworm strains using (a) RAPD (OPA-03), (b) SSR (sat3513), (c) ISSR-PCR (GAT GCT GATA (CA)7) and (d) RFLP (pBmN138).

countries, have shorter larval span, attain smaller body size, secrete short and thick fibre, and are resistant to viral diseases. Genetic similarities revealed by each of the marker systems were calculated using the Nei and Li coefficients and the dendrograms produced from similarity matrices for each marker system are shown in Figs 2(a)–2(d).

A clear separation of the two sets of diapausing and non-diapausing gene pools was evident using SSR, RAPD and ISSR-PCR assays. Two RAPD primers, OPA-01 and OPA-02, generated products that were specific to all diapausing and non-diapausing strains; one of the SSR loci (sat 211) also produced diapause and non-diapause strain diagnostic alleles. Only in the SSR assay, the non-diapausing strain, *C. nichi*, was found clustered with the diapausing strains. Similar results were also obtained by Nagaraju *et al.* (1995) and Sharma *et al.* (1998) using a heterologous multilocus probe,

Table 3 A summary of each type of marker assay performed on 13 silkworm strains

	Total number of assays	Total number of products	Total polymorphic products	Mean number of products per assay	Percentage of polymorphic products
SSR	15 (primer pairs)	122	105	8.10	86.00
Inter-SSR	6 (primers)	239	184	39.83	76.98
RAPD	40 (primers)	216	204	5.40	94.44
RFLP	6 (probes)	180	176	30.00	97.77

Table 4 A summary of mean DI, EMR and MI for different marker assays of silkworms

Marker assay	Mean DI	Mean EMR	Marker Index
SSR	0.795	1.0	0.795
Inter-SSR	0.957	23.6	22.570
RAPD	0.744	4.8	3.580
RFLP	0.930	28.7	26.670

Bkm. In addition, the cluster analysis produced a strong evidence for a subgroup based on similar pedigree (NB_{18} and NB_4D_2), in all the four marker assays.

Discussion

To our knowledge, this is the first report of a comparison in animal genetics involving the four genetic marker systems. The current availability of several molecular techniques for the detection of polymorphisms has prompted us to explore their utility in the silkworm with a view to using them for genome mapping, germplasm screening and strain improvement programmes. Three PCR-based techniques, RAPD, SSR and ISSR-PCR, as well as RFLP methods, were examined for their ability to generate useful polymorphisms using 13 silkworm varieties. Each technique differed not only in its underlying principle, but also in the informativeness in regard to type and amount of polymorphisms detected. The highest level of polymorphism was detected using multilocus RFLP probes (97.77%) and the lowest was associated with ISSRs (76.8%). However, the ISSR revealed an average number of 39 bands per lane or per assay compared to eight bands for SSRs in the pooled DNA samples. Powell *et al.* (1996) introduced the concept of MI as an overall measure of efficiency of different marker assays. We utilized this concept to measure MI and DI in the silkworm. When the overall DIs of the four techniques were compared, ISSR had the highest value (0.957) followed closely by multilocus RFLPs (0.930).

The high MI as well as DI is a reflection of the efficiency of the ISSR-PCR and multilocus RFLP markers to simultaneously analyse a large number of bands rather than of the level of polymorphism detected. The results show that ISSR-PCR and multilocus RFLP markers are the most powerful of the four techniques for fingerprinting silkworm varieties. The large number of PCR products observed in the ISSR-PCR assay is not surprising since the *B. mori* genome is abundant with dinucleotide repeat motifs, particularly CA repeats (Reddy *et al.*, 1999b) and ISSR primers are designed to anchor such microsatellite motifs to amplify the genomic sequences lying between them. Many of the anonymous multilocus RFLP probes used in the present study are probably derived from genomic regions containing transposable elements or minisatellite-like sequences which account for several thousand copies per haploid genome of the silkworm (Nagaraju *et al.*, 1995; Robertson & Asplund, 1996). Although most ISSR loci are dominant, rather than codominant, ISSR-PCR markers offer several advantages over RFLPs for genotyping, the major one being the rapid production of a large number of markers in a cost-effective manner. Moreover, the ISSR markers offer higher reproducibility due to the use of longer primers and a higher annealing temperature than those used for RAPDs. Because of these advantages, we believe that the ISSR-

Table 5 Correlations obtained between the genetic similarities in silkworms calculated using ISSR, SSR, RAPD and RFLP techniques, based on comparison of similarity matrices (Spearman's rank correlation [SRC] and regression analysis) r^2

	ISSR		SSR		RAPD		RFLP	
	SRC	r^2	SRC	r^2	SRC	r^2	SRC	r^2
ISSR	—	—						
SSR	0.685	0.294	—	—				
RAPD	0.682	0.411	0.679	0.325	—	—		
RFLP	0.612	0.430	0.796	0.286	0.66	0.558	—	—

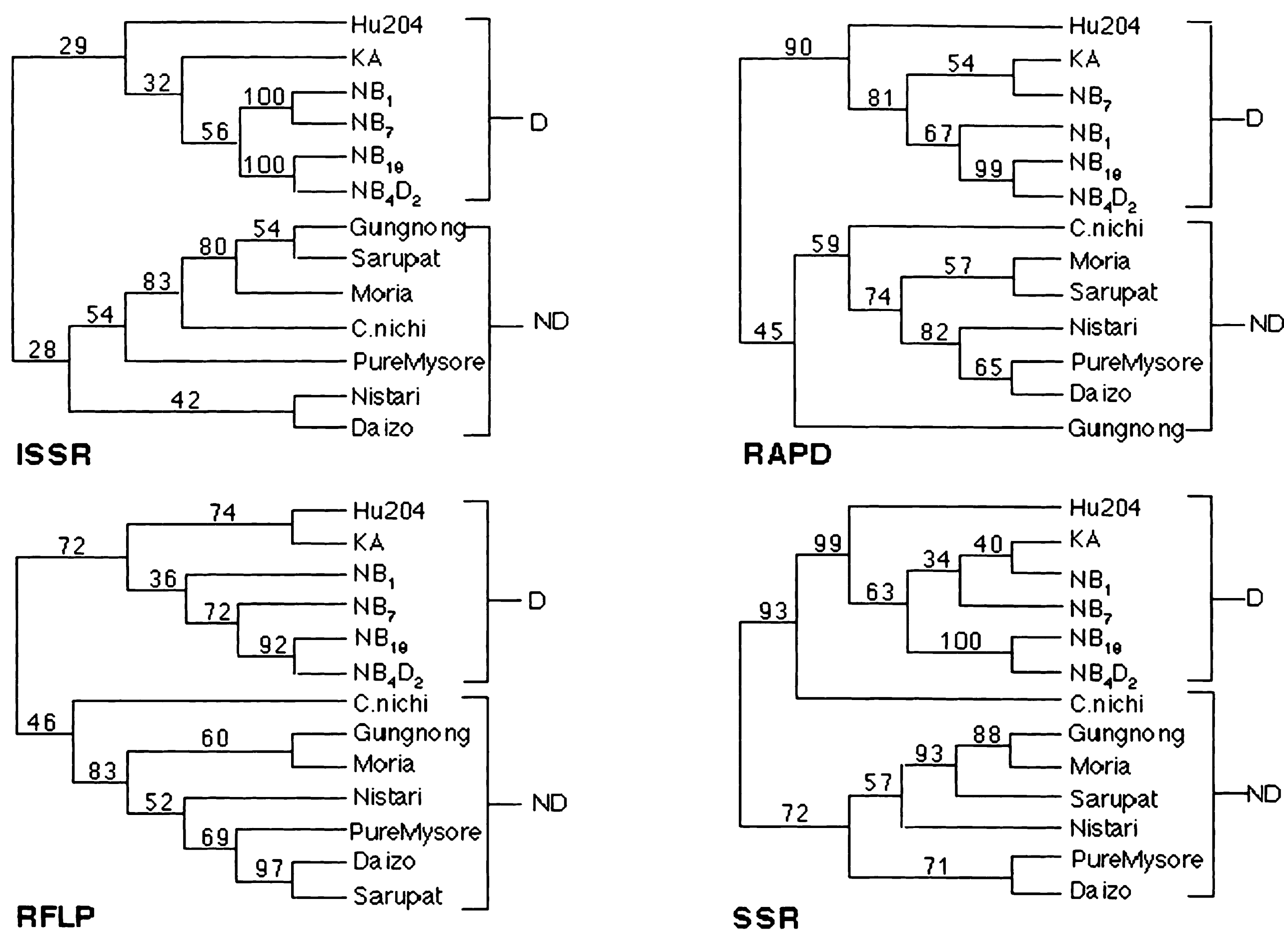

Fig. 2 Dendrograms generated from genetic similarity matrices calculated using Nei and Li coefficients for ISSR-PCR, RFLP, RAPD and SSR marker assays. D, diapausing silkworm strains; ND, non-diapausing silkworm strains. Numbers on the nodes indicate the number of times a particular branch was recorded per 100 bootstrap replications following 1000 replications.

PCR technique has great potential in silkworm breeding, germplasm evaluation and genetic mapping. Recently we have automated the ISSR-PCR assay by incorporating the fluorescent nucleotide in the PCR reaction and electrophoresing the PCR products on an ABI 377 automated sequencer. This method (called FISSR-PCR) requires very little quantity of template DNA (3–5 ng) and further enhances the sensitivity and resolution of the markers, rendering the method very suitable for high throughput genotyping and genetic mapping experiments (Nagaraju *et al.* in preparation).

The multilocus RFLP probes also offer features such as high reproducibility and unambiguous marker scoring similar to ISSR-PCR, but require radioactive labelling and hybridization, which are labour intensive and are not cost-effective because of the large quantities of DNA needed for blotting and the longer preparation time. On the other hand both ISSR-PCR and multilocus markers are scored as dominant markers since neither of them reflect the allelic status of the polymorphic bands, and hence homozygosity levels cannot be determined in population studies. Although the RAPD technique is widely used, problems of reliability and repeatability have been reported. Many factors such as concentration of Mg^{2+}, quality of the template, thermal cycler, and the source of polymerase affect amplification. Although in our study, RAPD markers reliably discriminated the silkworm varieties, the technique has to be approached with caution. In these aspects, SSRs are more useful than the other three techniques since they reveal codominantly inherited, multiallelic products of loci that can be readily used for genetic diversity, pedigree evaluation and genetic mapping. However, the SSR technology is expensive considering that prior sequence information is required to design the locus-specific primers. But the advantages that they are robust and are amenable to automation compensate the cost inputs and increase the cost–benefit ratio.

The major goals envisaged for molecular markers in the silkworm are the construction of linkage maps to understand the genetics of simple as well as complex traits, and for their use in marker-assisted selection. In this respect, SSR markers should serve as markers of choice. Studies have shown that the silkworm genome contains $(CA)_n$ and $(GA)_n$ repeats at almost every 40 kb and 100 kb, respectively (Reddy *et al.*, 1999b). Such abundantly distributed microsatellite loci would provide the required markers for intensive molecular mapping of the silkworm genome. More than 3000 silkworm varieties maintained in different countries, and which represent an array of differences for various traits, remain to be characterized genetically. In countries like China, India, Thailand, South Korea, and Japan, where sericulture is being practised, silkworm breeders develop new silkworm varieties which must be characterized for their DNA profiles for production of heterotic hybrids, protection of intellectual property rights and other statutory, regulatory and legal applications. Based on the observed EMR, ISSR-PCR is best suited for generation of the volume of information required for performance of such a task.

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