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Evaluation of ISSR and RAPD Markers for the Detection of Genetic Diversity in Mulberry (*Morus* spp.)

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The present study was carried out to evaluate the ISSR and RAPD markers for their efficiency as genetic marker systems to establish the relationships between 18 mulberry genotypes. A total of 36 from 56 (64%) RAPD primers and 12 from 48 (25%) ISSR primers produced reproducible amplification patterns. A high proportion of polymorphic bands ranging from 44 to 91% was observed respectively with RAPD and ISSR markers. The average Resolving Power (Rp) of ISSR primers was higher than RAPD primers. The ISSR primers, UBC 825, 868 and 873, and RAPD primers, UBC 712, 720 and 729, possessed the highest Rp values and could in each instance distinguish all the 18 genotypes. Similarity matrix values were estimated based on Jaccards coefficient, considering 109 polymorphic ISSR and 212 polymorphic RAPD bands and two dendrograms were constructed. The dendrograms obtained with ISSR and RAPD markers distinguished the eight exotic genotypes from the ten indigenous (Indian) genotypes. A significant correlation value (r = 0.959; p = 0.001) for the cophenetic matrix between the RAPD and ISSR matrices was observed. The results indicated that the ISSR and RAPD markers could assist in the differentiation of genotypes and permit the determination of genetic distances that might be exploited by mulberry breeders in improvement programs.

Key words: *Morus* spp., ISSRs, RAPDs, Varietal identification, Genetic diversity

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Introduction

Mulberry (*Morus* spp.) is a perennial tree, belonging to the family Moraceae and is the sole food plant of the silkworm, *Bombyx mori* L., and is consequently of great importance to the silk industry. The quality and the quantity of leaf production directly influence silk production, and consequently mulberry has attracted substantial attention from plant scientists. Vavilov (1951) proposed that mulberry originated in China, which he suggested as the primary centre of origin for the genus. The majority of mulberry genotypes are "naturalized" as they have been established, adapted and persisted in areas distant from their initial origin, making their classification much difficult (Sanjappa, 1989). The identification of the species within the genus *Morus* was based on conventional studies involving differences in growth form, leaf morphology, length of style, shape of idioblasts, fruit colour and other agronomical characters (Katsumata, 1972). Sanjappa (1989) reported that the genus *Morus* comprised about 68 recognized species and hundreds of genotypes and forms. He has also stated that the nomenclature of the species was confusing and the number of species had been fluctuating. Hotta (1954) divided mulberry species into two sections, namely the Dolychocystolithiae and Brachycystolithiae, depending on the shape and position of cystolith cells of the leaf.

The assessment of genetic diversity and the management of genetic resources are pivotal for plant breeding in introgressing exotic genes into established cultivars (Tanksley and McCouch, 1997). Attempts have been made to determine the diversity of *Morus* spp. using morphological (Katsumata, 1972), cytological (Katsumata, 1979), isoenzyme (Hirano, 1982; Venkateswarlu *et al.*, 1989, 1994), RAPD (Bhattacharya and Ranade, 2001; Naik *et al.*, 2002), ISSR (Vijayan and Chatterjee, 2003) and AFLP (Sharma *et al.*, 2000) markers. As a pre-requisite for mulberry improvement programme, a large

number of morphological and agronomical characters were studied (Rajan *et al.*, 1997; Thangavelu *et al.*, 1997, 2000; Tikader and Roy, 2001; Kumar *et al.*, 2002; Suryanarayana *et al.*, 2002). Of these, information obtained through the use of molecular tools is more reliable, since such data allows more accurate assessment of the genetic constitution of the ancestral species. Over the past one and half decades, a number of molecular techniques, especially DNA-based markers, have been developed which can provide information on the extent of diversity and genetic relationships. Amplification of genomic DNA using one short arbitrary primer under conditions of low hybridisation stringency can result in the production of multiple amplification products from loci distributed throughout the genome (Williams *et al.*, 1990). This observation led to the development of genome mapping

Table 1. Details of eighteen mulberry genotypes, their origin, species, ploidy level, sex and leaf lobation

Sl. no.	Genotype	Origin	Species	Ploidy & chromosome no. (2n)	Sex	Leaf lobation
1.	K_2	OPH[1] selection India	*M. indica*	28 Diploid	Female	entire
2.	S_{54}	Induced mutant India	*M. indica*	28 Diploid	Female	entire
3.	S_{13}	OPH[1] selection India	*M. indica*	28 Diploid	Male	entire
4.	S_{34}	CPH[2] selection India	*M. indica*	28 Diploid	Male	entire
5.	V1	CPH[2] selection India	*M. indica*	28 Diploid	Male	entire
6.	Bilidevalaya	Selection India	*M. indica*	28 Diploid	Female	lobed
7.	RFS-175	OPH[1] Selection India	*M. indica*	28 Diploid	Male	entire
8.	English Black	CPH[2] selection France	*M. alba*	28 Diploid	Female	entire
9.	Ichihei	OPH[1] selection Japan	*M. bombycis*	28 Diploid	Female	lobed
10.	PKS-1-4	Selection Pakistan	*M. alba*	28 Diploid	Male	entire
11.	Cattaneo	Selection Italy	*M. latifolia*	28 Diploid	Monoecious	entire
12.	Tsuka-	Collection saguwa Japan	*M. latifolia*	28 Diploid	Female	entire
13.	Kokuso-27	CPH[2] selection Japan	*M. alba*	28 Diploid	Male	lobed
14.	Kairo Roso	Selection Japan	*M. latifolia*	28 Diploid	Female	entire
15.	*M.rotundiloba*	Collection Burma	*M. rotundiloba*	28 Diploid	Female	lobed
16.	MR-2	Clonal selection India	*M. sinensis*	28 Diploid	Monoecious	heterophyllus
17.	RFS-135	OPH[1] selection India	*M. indica*	28 Diploid	Male	entire
18.	Birds Foot	Collection India	*M. laevigata*	42 Triploid	Female	lobed

[1]Open pollinated hybrid; [2]Controlled pollinated hybrid.

(Williams *et al.*, 1990) and fingerprinting (Welsh and McClelland, 1990) applications with Random Amplified Polymorphic DNA (RAPD) and other PCR based techniques. Tandemly repetitive DNA sequences, such as mini- and micro- satellites, are a major component of all eukaryotic genomes. Wang *et al.* (1994) found that dinucleotide microsatellites were prevalent in plants while mono-, tri-, tetra-, and penta- nucleotide repeats were less common. Recently, a relatively novel molecular technique that permits the detection of polymorphism in microsatellite and inter- microsatellite loci without prior knowledge of DNA sequence has been described as inter-simple sequence repeat PCR (ISSR-PCR) (Zietkiewicz *et al.*, 1994). This technique involves the use of a single primer consisting of a microsatellite sequence plus a short arbitrary sequence (anchor), which targets a subset of microsatellites and amplifies the region between two closely spaced and oppositely oriented simple sequence repeats. The production of large number of fragments, reproducibility and low cost are the advantages of these ISSR markers (Salimath *et al.*, 1995; Fang *et al.*, 1997; Moreno *et al.*, 1998). ISSR markers have proven valuable for fingerprinting studies and genetic diversity investigations in rice (Qian *et al.*, 2001; Nagaraju *et al.*, 2002), *Nothofagus* (Mattioni *et al.*, 2002), chikpea (*Cicer* spp.) (Iruela *et al.*, 2002), chest nut (Casasoli *et al.*, 2001), mangrove (Ge and Sun, 1999) and grapevine (Moreno *et al.*, 1998). The present study was undertaken to determine genetic diversity and to establish relationships between different genotypes of mulberry using RAPD and ISSR techniques.

Materials and Methods

Details of eighteen mulberry genotypes selected for the present study, their origin, species, ploidy level, sex and leaf lobation are presented in Table 1. Freshly opened young leaves of the genotypes were collected and stored at -80°C. The frozen leaves were ground to a fine powder in liquid nitrogen and DNA was extracted using a modification of the method of Dellaporta (Dellaporta *et al.*, 1983; Venkateswarlu *et al.*, 2002). A working solution of DNA (10 ng/μl) was prepared in sterile double distilled water. 56 RAPD primers and 48 ISSR primers were tested for amplification. 36 RAPD primers and 12 ISSR primers gave reproducible amplification products (Table 2). The protocols of Williams *et al.* (1990) for RAPD analysis and Zietkiewicz *et al.* (1994) for ISSR were adapted. DNA amplifications were performed in a 20 μl reaction volume containing approximately 40 ng template DNA, 0.5 μM of a single primer chosen from the #9 of the University of British Columbia Biotechnology Laboratory (UBC, Vancouver, Canada), 200 μM each of dNTPs (USB, Amersham Lifesciences, Ohio, USA) and 1 Unit of Taq DNA Polymerase in 1 × PCR buffer and 2 mM $MgCl_2$ provided by the manufacturers of the enzyme (MBI, Fermentas, Vilnius, Lithuania) for ISSR. For RAPD, the conditions are the same as those of ISSR except that 25 ng of template DNA were utilized. RAPD primers were obtained from UBC, Canada and Operon Technologies Inc., California, USA (Table 2). PCR amplifications of the genomic DNA with different primers were carried out using an MJ Research Thermo-cycler PTC 200. The PCR schedules were adapted as follows:

1. For ISSR primers: 94°C for 2 min. followed by 35 cycles of 94°C for 30 sec., 50°C for 30 sec., 72°C for 2 min. and a final extension of 10 min. at 72°C.
2. For RAPD primers: 93°C for 2 min. followed by 40 cycles of 93°C for 1 min., 35.5°C for 1 min. 72°C for 2 min. and a final extension for 10 min. at 72°C.

The PCR amplified products were run with gel electrophoresis (BRL Unit) on a 1.5% agarose gel (Life Technologies Inc.) at 70 V until completion (about 4 hrs) in 1 × TBE buffer and stained with ethidium bromide (0.5 μg/ml) for 30 min. A standard molecular weight marker (Mass ruler DNA ladder, MBI Fermantas, Vilnius, Lithuania) was used in each electrophoretic run and the UV transilluminated gels were photographed with Nikon (FM2) Camera using Kodak 400 ASA film. The amplified bands were scored as present (1) or absent (0) across the 18 genotypes for each primer separately. The scoring was repeated three times over by three independent scorers. Each reaction was repeated three times and only reproducible bands were included in the analysis. According to Prevost and Wilkinson (1999) the resolving power (Rp) of a primer is $Rp = \sum I_b$ where I_b (band informativeness) takes the value of: $1 - [2 \times (0.5 - p)]$, p being the proportion of the 18 genotypes containing the band. The Genetic Similarity (GS) between pairs of accessions was estimated according to Jaccards coefficient (Jaccard, 1908). The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), number of different genotypes (NG), Resolving power (Rp) and number of exclusive bands (NEB) were recorded. Dendrograms were constructed by employing Jaccards coefficient of similarity and the genotypes were grouped by using Hierarchical cluster programme of SPSS version 10.0 (SPSS Inc., Chicago, USA) to group different genotypes into discrete clusters. Estimates of the differences between the dendrograms based on RAPD- and computing the cophenetic values and constructing the relative cophenetic matrices for each marker type obtained

Table 2. RAPD and ISSR primers, sequence details, PCR product size range, Total number of bands (TNB), Number of polymorphic bands (NPB), Percentage of polymorphic bands (P%), number of different genotypes (NG), Resolving Power (Rp) and Number of exclusive bands (NEB) obtained

Primer	Sequence	Size range bp	TNB	NPB	P%	NG	Rp	NEB
RAPD UBC	**5' - 3'**							
704	GGA AGG AGG G	500-2000	10	5	50	17	7.984	1
711	CCC TCT CCC T	800-1900	8	5	62	9	6.288	1
712	GGG TGT GGG T	250-2000	11	10	91	18	8.684	2
720	GGG AGG GAG A	250-2000	11	10	91	18	8.596	1
723	CCC TCT CCT C	700-2200	9	6	67	14	6.786	1
729	CCC AAC CCA C	600-2000	12	7	58	18	8.698	2
732	CAC CCA CCA C	300-1900	9	6	67	15	6.829	1
733	GGG AAG GGA G	300-1900	9	6	67	16	6.963	1
736	GAG GGA GGA G	350-2000	9	7	78	16	7.576	1
747	CCA CCA ACC C	400-2200	11	8	73	18	8.396	2
750	GGG TGG TGT G	300-2000	9	6	67	14	6.863	1
751	CCC ACC ACA C	300-2000	8	5	62	12	6.692	1
753	GGG AGG AGG A	750-1900	7	6	86	9	6.236	1
754	GGG TGG TGG T	500-2200	11	7	64	18	8.586	1
755	CCC ACC ACC A	600-2000	8	6	75	8	6.183	2
756	CCC TCC TCC T	600-1500	10	6	60	16	7.423	1
762	GTG TGG TGG G	400-2500	10	7	70	14	6.798	1
764	CTC TCC TCC C	500-2500	7	5	71	8	6.169	2
770	GGG AGG AGG G	350-2200	7	5	71	7	5.961	1
771	CCC TCC TCC C	500-2700	8	6	75	9	6.250	1
774	GGT GTG TGG T	600-2000	8	5	62	14	6.763	2
778	CCA CAC CAC A	350-2200	9	6	67	8	6.208	1
781	GGG AAG AAG G	500-2000	9	5	56	16	7.534	1
782	GGG AAG AGA G	300-2000	8	4	50	10	6.423	2
792	CAA CCC ACA C	400-2200	11	6	55	18	8.463	2
RAPD OPERON	**5' - 3'**							
OPW04	CAG AAG CGG A	400-2500	8	5	62	10	6.458	2
OPW05	GGC GGA TAA G	600-2000	9	5	56	10	6.463	2
OPW06	AGG CCC GAT G	700-2500	8	5	62	9	6.296	2
OPW17	GTC CTG GGT T	500-2500	12	6	50	18	8.316	1
OPW18	TTC AGG GCA C	400-1900	9	5	56	12	6.768	2
OPW19	CAA AGC GCT C	600-2500	7	4	57	8	6.238	2
OPW20	TGT GGC AGC A	900-2300	9	4	44	12	6.664	1
OPY11	AGA CGA TGG G	500-2100	8	5	62	9	6.264	1
OPY12	AAG CCT GCG A	800-2000	10	5	50	16	7.687	2
OPY13	GGG TCT CGG T	300-2500	8	6	75	10	6.417	1
OPY20	AGC CGT GGA A	700-1500	12	7	58	18	8.363	1
		Total	329	212	65	472	255.286	50

Table 2. Continued

ISSR UBC	Y = (C or T)							
808	AGA GAG AGA GAG AGA GC	500-2000	11	9	82	15	6.768	2
812	GAG AGA GAG AGA GAG AA	600-3000	12	10	83	17	8.064	2
818	CAC ACA CAC ACA CAC AG	400-2500	12	9	75	16	7.640	2
823	TCT CTC TCT CTC TCT CC	700-2300	10	8	80	14	6.809	1
825	ACA CAC ACA CAC ACA CT	400-2500	13	10	77	18	8.734	3
826	ACA CAC ACA CAC ACA CC	500-2400	8	7	87	13	6.653	2
827	ACA CAC ACA CAC ACA CG	600-2800	11	8	73	16	7.458	2
834	AGA GAG AGA GAG AGA GYT	400-2200	10	8	80	10	6.496	2
836	AGA GAG AGA GAG AGA GYA	500-2000	12	10	83	16	7.540	2
864	ATG ATG ATG ATG ATG ATG	500-2200	11	9	82	16	7.534	2
868	GAA GAA GAA GAA GAA GAA	600-2500	12	10	83	18	8.738	2
873	GAC AGA CAG ACA GAC A	400-2500	14	11	79	18	8.764	3
		Total	136	109	80	187	91.198	25

by ISSR-marker analyses. A cophenetic matrix was derived from the similarity matrix to test the goodness of fit of the clusters by comparing the RAPD and ISSR matrices using Mantel nonparametric test calculator (Mantel, 1967; Liedloff, 1999).

Results

The present study with 12 ISSR primer sequences indicated that microsatellites are more frequent in mulberry containing the repeated di- nucleotides (AG)n, (CA)n,

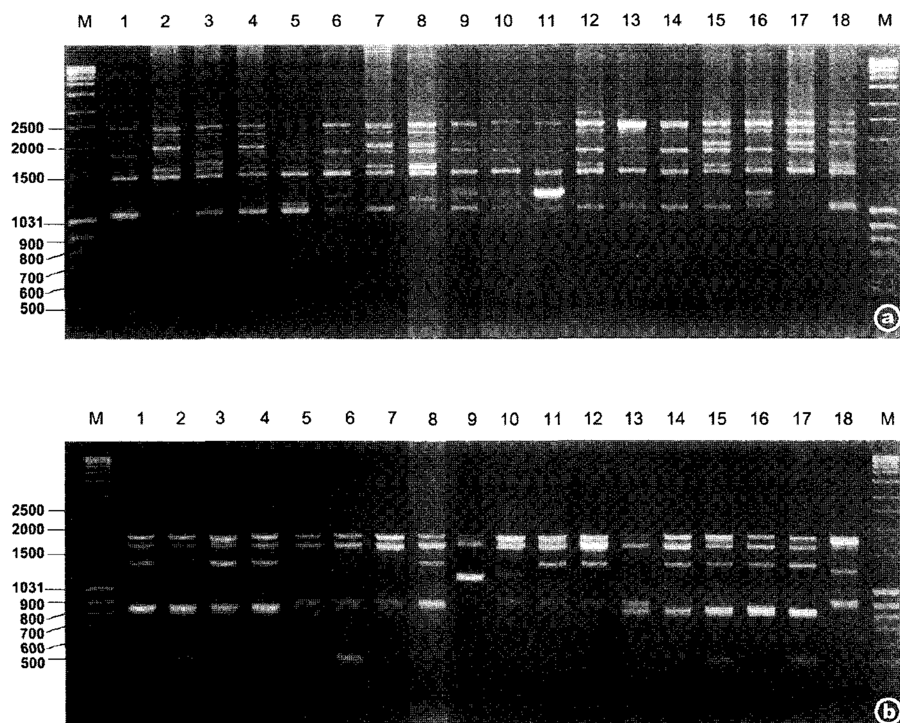

Fig. 1. Profiles of a) ISSR primer UBC 826 and b) RAPD primer UBC 778 in 18 mulberry genotypes. The lanes represent: M- molecular weight marker, 1- K2, 2- S54, 3- S13, 4- S34, 5- V1, 6- Bilidevalaya, 7- RFS-175, 8- English Black, 9- Ichihei, 10- PKS 1-4, 11- Cattaneo, 12- Tsukasagawa, 13- Kokuso-27, 14- Kairo Roso, 15- *M. rotundiloba*, 16- MR-2, 17- RFS-135 and 18- Birds Foot.

(TC)*n*, tri- nucleotides (GAA)*n* and repeated tetra- nucleotides (GACA)*n* (Table 2). The total number of amplified products were 136 (an average of 11.33 bands per primer) ranging from 400 to 3000 bp of which 109 (80%) were polymorphic. The genotypes showed a total of 25 exclusive bands. The resolving power (Rp) of the 12 ISSR primers ranged from 6.496 for UBC 826 primer to 8.764 for UBC 873 primer. The UBC 873, 868 and 825 ISSR primers depicted the highest Rp values of 8.764, 8.738 and 8.734 respectively and each of the three primers was able to distinguish all the 18 mulberry genotypes (Table 2 and Fig. 1a).

The SPSS dendrogram obtained based on Jaccards coefficient of similarity showed two main clusters distinguished as A and B (Fig. 2a). Cluster A contained all the 8 exotic genotypes while cluster B contained all 10 indigenous (Indian) genotypes. Cluster A had 2 sub clusters (A1 and A2), A1 with four genotypes and A2 with four genotypes. Cluster B had 2 sub clusters (B1 and B2), B1 containing 9 genotypes and B2 containing a single genotype, Birds Foot.

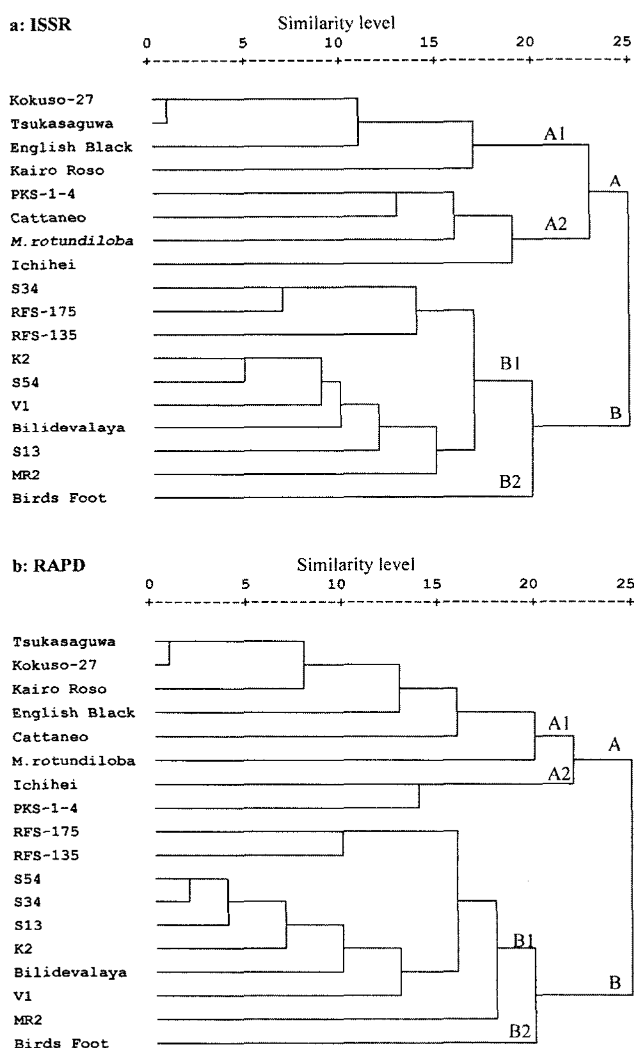

Fig. 2. Dendrograms derived from a) ISSR markers and b) RAPD markers depicting the relationship between the mulberry genotypes (The bars on the top represents similarity matrix based on Jaccards coefficient).

A total of 329 bands were amplified with an average of 9 bands per primer. 212 bands resulted polymorphic (65%), ranging from 250 to 2700 bp. The genotypes showed 50 exclusive bands. The resolving power (Rp) of the 36 RAPD primers ranged from 5.961 for UBC 770 primer to 8.698 for UBC 729 primer. The RAPD primers, UBC 712, 720 and 729 possessed the highest Rp values of 8.684, 8.596 and 8.698 respectively and each of them could distinguish all the 18 mulberry genotypes (Table 2 and Fig. 1b).

As observed for ISSR analysis the SPSS dendrogram constructed based on Jaccards coefficient of similarity depicted two main clusters, A and B (Fig. 2b). Cluster A contained the 8 exotic genotypes and cluster B contained the 10 genotypes from India. Tsukasaguwa and Kokuso-27 were closer in cluster A while S54 and S34 were closer in cluster B. Birds Foot out grouped itself within cluster B. The correlation between the matrices of cophenetic values for the dendrograms obtained based on the RAPD and ISSR data was quite significant ($r = 0.959$; $P = 0.001$) which indicates a good fit to the cluster analysis (Rohlf, 1998).

Discussion

The occurrence of exclusive bands confirms that the 18 genotypes studied are different from each other. Further, it can be inferred that ISSR fingerprinting was more efficient than the RAPD assay, as it detected 80% polymorphic DNA markers in comparison to 65% in the case of RAPD fingerprinting. Similar results were obtained for several other plant species (Rus-Kortekaas *et al.*, 1994; Nagaoka and Ogihara, 1997; Raina *et al.*, 2001). This higher variability agrees with the general observation that woody perennial cross pollinating species maintain most of their variation within a population (Hamrick and Godt, 1989; Lambouy *et al.*, 1996; Bartish *et al.*, 2000; Gauer and Cavalli-Molina, 2000; Oraguzie *et al.*, 2001). The average Resolving Power (Rp) of ISSR primers (7.600) was higher than RAPD primers (7.091). The mean number of amplification products obtained in respect of ISSRs (11.33 per primer) is greater than that of RAPDs (9 per primer) indicating the greater efficiency of the ISSR marker system in revealing several informative bands in a single amplification. The RAPD technique has been widely used to study both wild (Yeh *et al.*, 1995; Khasa

and Dancik 1996; Owuor *et al.*, 1997; Fornari *et al.*, 1999; Nebauer *et al.*, 1999) and cultivated (Sharma *et al.*, 1995; Divaret *et al.*, 1999; Moeller and Schaal, 1999) plants. In contrast, the ISSR technique was mainly used for cultivated species (Moreno *et al.*, 1998; Wang *et al.*, 1998; Blair *et al.*, 1999). During the ISSR screening, good amplification products were obtained from primers based on (GA)*n*, (CA)*n* and (TC)*n* repeats, while (AT)*n* primers gave no amplification products, despite the fact that poly (AT) dinucleotide repeats are thought to be the most abundant motifs in plants (Morgante and Olivieri, 1993; Depeiges *et al.*, 1995). Similar results were obtained in rice (Blair *et al.*, 1999), chestnut (Casasoli *et al.*, 2001), grapevine (Moreno *et al.*, 1998) and wheat (Nagaoka and Ogihara, 1997). A possible explanation for these results is that ISSR primers based on AT motifs are self-annealing due to sequence complementarities and they form dimers during PCR amplification (Blair *et al.*, 1999). The ISSR markers are found to be powerful tools for the generation of fingerprinting in mulberry genotypes. Thus it is confirmed that the ISSR and RAPD primers can be used as a powerful tools to assess the genetic distance between different mulberry genotypes, which can be utilized in selecting the donors ensuring maximum heterosis in mulberry improvement programmes.

The similarity in the clusters obtained through these biotechnological tools provides clues for selecting the required quantitative and qualitative traits for breeding programmes. The differences found between the dendrograms generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analysed reinforcing the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among mulberry genotypes. Thus these ISSR and RAPD markers, coupled with appropriate data handling and analysis, can work as efficient tools for the evaluation of the mulberry germplasm.

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