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Genetic stability of mulberry germplasm after cryopreservation by two-step freezing technique

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Genetic stability of *Morus alba*, *Morus indica*, *Morus laevigata* (indigenous collection) and *Morus* species (exotic collection) have been studied in *in vitro* regenerated plants of mulberry (fresh, before and after cryopreservation) using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. This study examined the genetic stability of cryopreserved dormant buds of *Morus* germplasm that were stored in liquid nitrogen using two-step freezing, then rewarmed and regrown. Dormant buds of mulberry collected during winter period were found suitable for the cryopreservation in liquid nitrogen. In the present study, the plants were regenerated directly from dormant buds (before and after cryopreservation) without intermediary callus phase. These regenerants thus bear low risk of genetic instability. Both the single primer amplification reaction (SPAR) markers showed reproducible and well resolved banding patterns in mulberry germplasm, in which RAPD marker generated a total of 201 bands based on 15 primers; however, ISSR markers were given 145 bands using 11 primers. Both markers showed monomorphic banding patterns and did not reveal any polymorphism among the mother plant and *in vitro* regenerants before and after cryopreservation, suggesting that cryopreservation, using two-step freezing, does not affect genetic stability of mulberry germplasm. The transitory nature of these polymorphisms should be carefully considered when monitoring for genetic stability.

Key words: Cryopreservation, Genetic stability, *in vitro* culture, ISSR, mulberry, RAPD.

INTRODUCTION

Mulberry (*Morus* spp.) belongs to the family 'Moraceae', a family of deciduous or evergreen trees and shrubs, mostly of pantropical distribution and characterized by milky sap. The origins of most cultivated mulberry varieties are believed to be in the Himalayan foothills by the evidences gathered from fossils (Collinson, 1989), morphology, anatomy (Benavides et al., 1994; Hou, 1994) and molecular biological (Zerega et al., 2005) covering both temperate and sub-tropical regions of Northern hemisphere (Anonymous, 2006) and later spread to major continents including Asia, Europe, North and South America, and Africa (Machii et al., 1999). Sanjappa (1989) recognized 68 species within the genus *Morus*, out of which, *M. alba*, *M. indica*, *M. nigra*, *M. latifolia*, *M. multiculis* are cultivated for silkworm rearing, *M. rubra* and *M. nigra* for fruits (Yaltirik, 1982) and *M. laevigata* and *M. serrata* for timber (Tikader and Vijayan, 2010). Cultivation of mulberry and silkworm rearing started in China before 2200 BC (FAO, 1990) and currently mulberry is cultivated in almost all Asian countries (Vijayan et al., 2011). In India, four main species of *Morus*, namely, *M. alba*, *M. indica*, *M. laevigata* and *M. serrata* have been reported (Hooker, 1885; Tikader and Dandin, 2005; Vijayan, 2010).

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Table 1. List of samples of *Morus* species used for genetic stability.

S/N	Sample no.	Collector no.	Species
1	M1-A*, M1-B**, M1-C***	MI-0583	*Morus laevigata*
2	M2-A*, M2-B**, M2-C***	ME-0051	*Morus* sp.
3	M3-A*, M3-B**, M3-C***	MI-0622	*Morus indica*
4	M4-A*, M4-B**, M4-C***	RC-03	*Morus indica*
5	M5-A*, M5-B**, M5-C***	RC-02	*Morus laevigata*
6	M6-A*, M6-B**, M6-C***	ME-0087	*Morus* sp.
7	M7-A*, M7-B**, M7-C***	MI-0698	*Morus alba*
8	M8-A*, M8-B**, M8-C***	RC-01	*Morus alba*
9	M9-A*, M9-B**, M9-C***	ME-0099	*Morus* sp.
10	M10-A*, M10-B**, M10-C***	MI-0678	*Morus alba*

*Fresh leaf samples, ***in vitro* regenerated samples before cryopreservation and *** after cryopreservation

Tissue culture with its distinct advantages is used for short-term preservation (Withers and Engelmann, 1997) but it does not serve for long-term preservation. Hence, cryopreservation only economically viable method is adopted for long-term preservation. Under cryopreservation, plant materials are stored at ultra-low temperatures in liquid nitrogen (-196°C). At this temperature, cell division and metabolic activities remain suspended and the material remains unchanged for a long period. Thus, cryopreservation ensures genetic stability of the mulberry germplasm besides requiring only limited space and protecting material from contamination. In mulberry, the most appropriate material for cryopreservation is the winter bud (Fukui et al., 2011; Rao et al., 2009), *in vitro*-grown shoot apices in *Morus* species (Gupta, 2011; Padro et al., 2012) and *M. bombicys* (Yakuwa and Oka, 1988), though embryonic axes, pollen, synthetic seeds can also be used (Niino, 1995).

The survival rates of winter buds stored in liquid nitrogen up to three to five years did not change significantly (Rao et al., 2009). Either prefreezing at -10 or -20°C along with rapid thawing at 37°C or pre-freezing at -20 or -30°C along with slow thawing at 0°C was a suitable treatment for high percentages of survival and shoot regeneration (Rao et al., 2007). It is desirable to assess the genetic integrity of the germplasm of micropropagated plantlets with that of field plants and plants regenerated after surviving cryogenic (-196°C) storage to determine if they are true-to-type after cryopreservation. The most commonly used marker systems for genetic stability study are Random Amplified Polymorphic DNA (RAPD) (Srivastava et al., 2004), amplified fragment length polymorphism (AFLP) (Wang and Yu, 2001), and inter-simple sequence repeat (ISSR) (Vijayan et al., 2005, 2006; Zhao et al., 2006). The application of RAPD and ISSR for the characterization of genetic stability has been well documented in *Morus* species (Rao et al., 2007, 2009; Vijayan, 2004; Vijayan and Chaterjee, 2003; Vijayan et al., 2004, 2005).

In the present study, the genetic stability of the *in vitro* regenerated plants of mulberry (fresh, before and after cryopreservation) was analysed through the RAPD and ISSR markers using 10 mulberry germplasm.

MATERIALS AND METHODS

Dormant buds of different *Morus* species were collected from the field genebank of Central Sericultural Germplasm Resources Centre (CSGRC), Hosur, Tamil Nadu (Table 1). Three accessions were collected from bio-diversity garden of National Bureau of Plant Genetic Resources (NBPGR), New Delhi. Buds attached to twigs wrapped in cotton bags were air lifted to cryolab at NBPGR reaching within 48 h of harvesting. After receipt, the twigs were wrapped in polyethene bags and kept in refrigerator at 10 to 15°C temperature and used for experimentation within 25 days of harvest.

Cryopreservation using two-step freezing

Descaled buds of mulberry were tied in muslin cloth and put in charged silica gel for 4 to 7 h at room temperature for desiccation. The desiccated buds were packed in 1.0 ml polypropylene cryovials. These vials were shifted sequentially at 5, -5, -10, -15, -20, -25 and -30°C keeping at each of the temperatures for a minimum of 24 h. The cryovials were held at -30°C for 48 h and then directly plunged in the liquid nitrogen at -196°C. Cryopreserved buds were thawed by slow thawing and transferred in sterile moist moss for rehydration. The viability of fresh, desiccated and cryopreserved dormant buds of *Morus* species was tested *in vitro* culturing method. For recovery growth of the cryostored dormant buds, 1 to 2 outer scales of the rehydrated buds were further removed followed by washing with Tween 20 for 15 min. Tween 20 was rinsed off with running tap water. These buds were then surface sterilized with 0.1% mercuric chloride for 9 min, rinsed three times with sterile water washes using autoclaved distilled water (5 min each). The sterilized buds were cultured on basal MS medium (Murashige and Skoog, 1962) with 3% sucrose (w/v) and solidified with 0.8% agar. MS medium was supplemented with 1 mgl^{-1} BAP initially for bud sprouting. The cultures raised from cryopreserved buds were maintained in culture room in dark for seven days.

After dark incubation, these cultures were shifted in diffused light for 3 days. After 10 days of culturing, these cultures were exposed

to normal culture room light intensity (3000 lux/ 36 µ mol $^{-1}$ sec^{2}). The sprouted buds were sub-cultured on the MS medium supplemented with 1.0 mgl^{-1} BAP and 0.2 mgl^{-1} GA_3 for elongation. The elongated plants were further sub-cultured and transferred to multiplication medium (0.5 mgl^{-1} BAP + 0.5 mgl^{-1} Kn + 0.1 mgl^{-1} IAA) and finally transferred to rooting medium (half MS + 0.5 mgl^{-1} IBA). Fresh leaf tissues of these mulberry samples were cleaned with water, air dried and stored in -80°C refrigerator for further experiments (Table 1).

DNA isolation

Total genomic DNA was isolated from leaf tissues using the cetyl trimethyl ammonium bromide method with few modifications (Doyle and Doyle, 1990). DNA concentration was determined spectrophotometrically at 260 nm. Quality of genomic DNA was determined through electrophoresis on 0.8% agarose gel.

RAPD-PCR amplification

The RAPD primers of Operon Technologies Alameda, CA, USA were used for molecular analysis. A total of 40 primers were screened in *Morus* species, of which 15 primers were selected for final profiling based on banding patterns and reproducibility. The basic protocol of RAPD-PCR reported by William et al. (1990) was followed for PCR amplification in a total reaction volume of 15 ml, containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.6 U Taq DNA polymerase (Life Tech, India), 0.2 mM of dNTP each, 10 pmoles of RAPD primer and 20 ng of DNA template. DNA amplification was carried out in a PTC-200 TM thermocycler and the thermal cycler conditions for PCR reactions were an initial denaturation cycle for 3 min at 94°C followed by 40 cycles comprising 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. An additional cycle of 5 min at 72°C was used for final extension. Amplification products were separated by electrophoresis in 1.8% agarose gels and stained in ethidium bromide. A photographic record was taken under UV gel doc system (Alpha Innotech, USA).

ISSR-PCR amplification

A total of hundred primers of University of British Columbia (UBC) procured from Geno Biosciences Pvt. Ltd. were used for ISSR-PCR optimization trials. Eleven primers, which gave the best amplification results with the sample DNA, were selected for final ISSR-PCR analysis. PCR-amplification was carried out in 25 µl reaction volume containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.0 to 2.5 mM $MgCl_2$, 0.2 mM dNTP each, 1.0 U *Taq* DNA polymerase (Bangalore Genie, India), 0.2 µM primer and 20 ng genomic DNA. The amplification was performed in a PTC-200 thermocycler (MJ Research, Massachusetts, USA), with reaction conditions programmed as initial pre-denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min and final extension at 72°C for 7 min. Amplification products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide, and bands were visualized and documented in UV gel doc system (Alpha Innotech Corporation).

Data analysis

Amplified bands were scored as present (1) or absent (0) homologous bands across all the accessions studied. Molecular weight of the amplified bands was estimated using 1 kb DNA ladder (Gibco BRL Life Technologies, NY, USA) as a standard. A pairwise similarity matrix of all the accessions was estimated based on Jaccard's coefficient (Jaccard, 1908) and a dendrogram was generated based on the unweighted pair-group method for arithmetic mean (UPGMA) using the software NTSYS version 2.10e (Rohlf, 2000). Principal component analysis was also carried out to study relationships among accessions using the same software. The test for association was conducted based on two-way Mantel test (Mantel, 1967).

RESULTS

The genetic stability of the fresh, *in vitro* raised plants before and after cryopreservation was studied through the ISSR and RAPD markers using 10 mulberry accessions comprising four different species of *Morus* (Table 1). The PCR amplification products of control (unfrozen) *in vitro* and cryopreserved (frozen in liquid nitrogen) samples were plotted together for comparison.

Genetic stability analysis in mulberry

RAPD analysis

Fifteen (15) primers were selected for the RAPD analysis based on the reproducibility and banding patterns. A total of 201 bands were generated from 15 RAPD primers, of which 169 bands were polymorphic (84.08%) with an average of 11.27 polymorphic bands per primer. The fragment size ranged from 200 to 3000 bp (Table 2). A representative gel profiles generated using primers OPA-02 and OPA-04 are shown in Figure 1. Each primer amplified at a range of 4 to 20 bands with an average of 13.40 bands per primer. OPA-02 primer amplified the maximum number of 20 bands, whereas OPA-10 primer generated the minimum of 4 bands. The polymorphism percentage ranged from 53.85 (primer OPE-03) to 100% (OPA-06 and OPA-11) with an average of 84.08% polymorphism (Table 2). Pattern of distribution of amplified bands across all the accessions revealed that some primers generated unique bands, namely OPA-02 in M1 (400 bp in *M. laevigata*) or M4 (2200 bp in *M. indica*), OPA-04 in M1 (870 bp in *M. laevigata*), OPA-06 in M1 (750 bp in *M. laevigata*), OPA-13 in M4 (650 bp in *M. indica*), OPA-17 in M1 (250 bp in *M. laevigata*), OPA-18 in M9 (870 bp in *Morus* sp), and OPE-04 amplified a single fragment in M9 (2000 bp in *Morus* sp).

A pairwise Jaccard's similarity values ranged from 0.37 to 0.83 (average 0.60) among the 10 accessions of mulberry (Table 3). A maximum similarity value of 0.83 was observed between M1 and M5 samples (both belong to *M. laevigata*), whereas M2 and M9 (*Morus* sp) showed least similarity coefficient of 0.37. All the three samples (fresh, *in vitro* raised before and after cryopreservation) of each *Morus* species showed 100% similarity among the treatments.

A dendrogram generated based on UPGMA method grouped all the 10 accessions into two major clusters

Table 2. Details of the RAPD and ISSR primers, their sequence and number of amplified bands used in analysis of *Morus species.*

Primer	Sequence (5'-3')	Total no. of bands	PB[a]	PPB[b]	Unique bands	Range of fragment size (bp)
OPA01	CAGGCCCTTC	12	10	83.33	0	550-1500
OPA02	TGCCGAGCTG	20	16	80.00	2	400-2500
OPA04	AATGGGGCTG	18	15	83.33	1	500-3000
OPA06	GGTCCCTGAC	08	08	100.00	1	650-920
OPA08	GTGACGTAGG	12	11	91.67	0	450-2000
OPA09	GGGTAACGCC	13	13	100.00	0	500-2000
OPA10	GTGATCGCAG	04	03	75.00	0	450-950
OPA11	CAAATCGCCGT	09	09	100.00	0	570-1000
OPA13	CAGCACCCAC	17	15	88.23	1	250-1400
OPA17	GACCGCTTGT	13	12	92.31	1	250-1400
OPA18	AGGTGACCGT	15	12	80.00	1	200-2500
OPC02	GTGAGGCGTC	17	14	82.35	0	300-2500
OPE03	CCAGATGCAC	13	07	53.85	0	350-2000
OPE04	GTGACATGCC	15	11	73.33	1	350-2000
OPE20	AACGGTGACC	15	13	86.67	0	350-2500
		201	**169**	**84.08**	**8**	
	ISSR					
UBC-807	AGAGAGAGAGAGAGAGT	16	16	100.00	1	200-1100
UBC-808	AGAGAGAGAGAGAGG	12	12	100.00	1	350-990
UBC-810	GAGAGAGAGAGAGAT	14	11	78.57	1	200-1000
UBC-811	GAGAGAGAGAGAGAC	11	07	63.63	1	370-960
UBC-812	GAGAGAGAGAGAGAA	15	14	93.33	1	300-1140
UBC-825	ACACACACACACACT	12	11	91.67	0	375-2000
UBC-827	ACACACACACACACG	14	14	100.00	0	400-1100
UBC-841	GAGAGAGAGAGAGAYC	15	14	93.33	0	300-2000
UBC-855	ACACACACACACACYT	15	14	93.33	1	250-1100
UBC-858	TGTGTGTGTGTGTGRT	11	11	100.00	2	300-1000
UBC-864	ATGATGATGATGATG	11	10	90.90	0	200-1100
		146	**134**	**91.78**	**8**	

[a] Total Polymorphic Bands; [b] Percentage of polymorphic bands

(Figure 2A). First cluster was the largest one and divided into two sub-clusters (II-a and II-b). Sub-cluster II-a, again sub-divided into II-a1 and II-a2 group. II-a1 group comprising 03 samples, namely, M1 (*M. laevigata*), M5 (*M. laevigata*) and M6 (*Morus* sp.). Within this cluster, the M1 and M5 showed 83% genetic similarity. Group II-a2 comprised of three samples naming M2 (*Morus* sp.), M3 (*M. indica*) and M4 (*M. indica*) in which, M3 and M4 were closely related with similarity value of 0.81. Sub-cluster II-b comprised of three samples namely, M7, M8 and M10 (all belong to *M. alba*) in which, M7 and M8 were closely related with similarity value of 0.75. Second cluster consisted only one sample M9 (*Morus* sp.) which was distinct from all other samples with similarity value of 0.46. Based on Mantel Z-statistics (Mantel, 1967), the correlation coefficient (r) was estimated to be 0.77. 2-D (Figure 2B) generated from PCOA of RAPD data was also in coherence with the clustering pattern of UPGMA dendrogram. First and second principal components accounted for 27.93 and 17.26%, respectively of the total variation.

ISSR analysis

Eleven primers were selected for the ISSR analysis based on the reproducibility and banding patterns. A total of 146 bands were generated, of which 134 bands were polymorphic (91.78%). Each primer amplified 7 to 16 polymorphic bands with an average of 12.18 bands per primer (Table 2). UBC-807 primer amplified the maximum number of 16 bands, whereas UBC-811 amplified the lowest number of polymorphic bands 7. The polymerphism percentage ranged from 63.63 (primer UBC-811) to 100% (UBC-807, UBC-808, UBC-827 and UBC-858). Average polymorphism across all the 10 accessions was

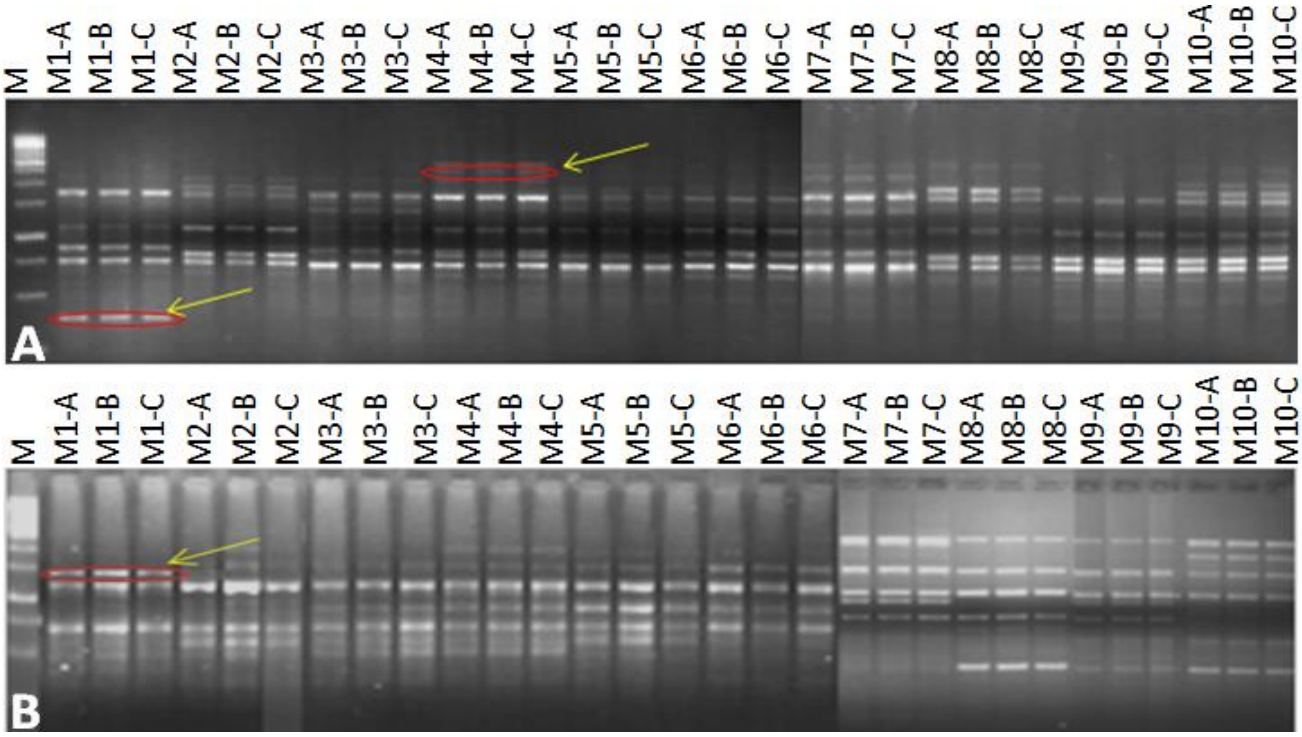

Figure 1. Gel profiles of the 10 mulberry accessions (ten control and ten *in vitro* regenerated samples before and after cryopreservation) generated with the RAPD primers: [A] OPA-2 and [B] OPA-4. M is the λ DNA marker. Arrows shows the unique band in red circle.

91.78%. Overall size of the PCR amplified fragments ranged from 200 to 2000 bp (Table 2). Pattern of distribution of bands across all accessions of mulberry revealed that the primer UBC-807 (1100 bp for *M. alba*), UBC-808 (990 bp for *M. laevigata*), UBC-810 (1000 bp *M. alba*), UBC-811 (960 bp for *M. laevigata*), UBC-812 (1000 bp for *M. laevigata*), UBC-855 (1100 bp for *M. alba*) and UBC-858 (300 bp for *M. laevigata* and 1000 bp for *Morus* sp.) amplified a unique DNA fragment which distinguished one species from the others (Figure 3). All cryopreserved and fresh samples showed 100% similarity among the treatments (fresh, *in vitro* raised before and after cryopreservation). A pairwise similarity values among all the 10 accessions of mulberry ranged from 0.41 to 0.97 (Table 4). The maximum similarity of 0.97 was observed between M3 (*M. indica*) and M4 (*M*. *indica*) accessions and showed close genetic similarity, whereas M9 (*Morus* sp.) showed least similarity coefficient of 0.41 with M1 and M5 (*M. laevigata*). Average similarity across all the cultivars was 0.69.

In the dendrogram, all the 10 accessions were grouped into three major clusters (Figure 4A). First cluster comprised of two accessions, namely M1 and M5 (both are *M. laevigata*) which were closely related with similarity value of 0.95. Second cluster was again divided into two sub-clusters (II-a and II-b). Sub-cluster II-a was the biggest comprising of the 4 accessions namely M2, M3, M4 and M6, in which M3 and M4 (both *M. indica*) were genetically most similar showing 97% similarity. The sub-cluster II-b comprised of three accessions that is, M7, M8 and M10 (all *M. alba*), in which, M7 and M10 were showing 98% genetic similarity to each other. In the third cluster, while M9 (*Morus* sp.) was diverse from other samples of this cluster with similarity value of 0.49. Based on Mantel Z-statistics (Mantel, 1967), the correlation coefficient (r) was estimated to be 0.93. 2-D plot generated from PCOA of ISSR data also in coherence with the clustering pattern of UPGMA dendrogram. The first five principal components accounted for 91.14% of the total variation and the first three accounted for 70.74% of the variation, in which maximum variation was contri-buted by first component (30.86%) followed by second component (23.91%), and third component (15.97%). 2-D plot generated through PCOA also showed the same grouping pattern as the UPGMA dendrogram (Figure 4B).

DISCUSSION

The aim of this study was to develop a simple and rapid technique to assist post-cryo assessment of genetic stability in vegetatively propagated germplasm. The results clearly demonstrate the application of molecular

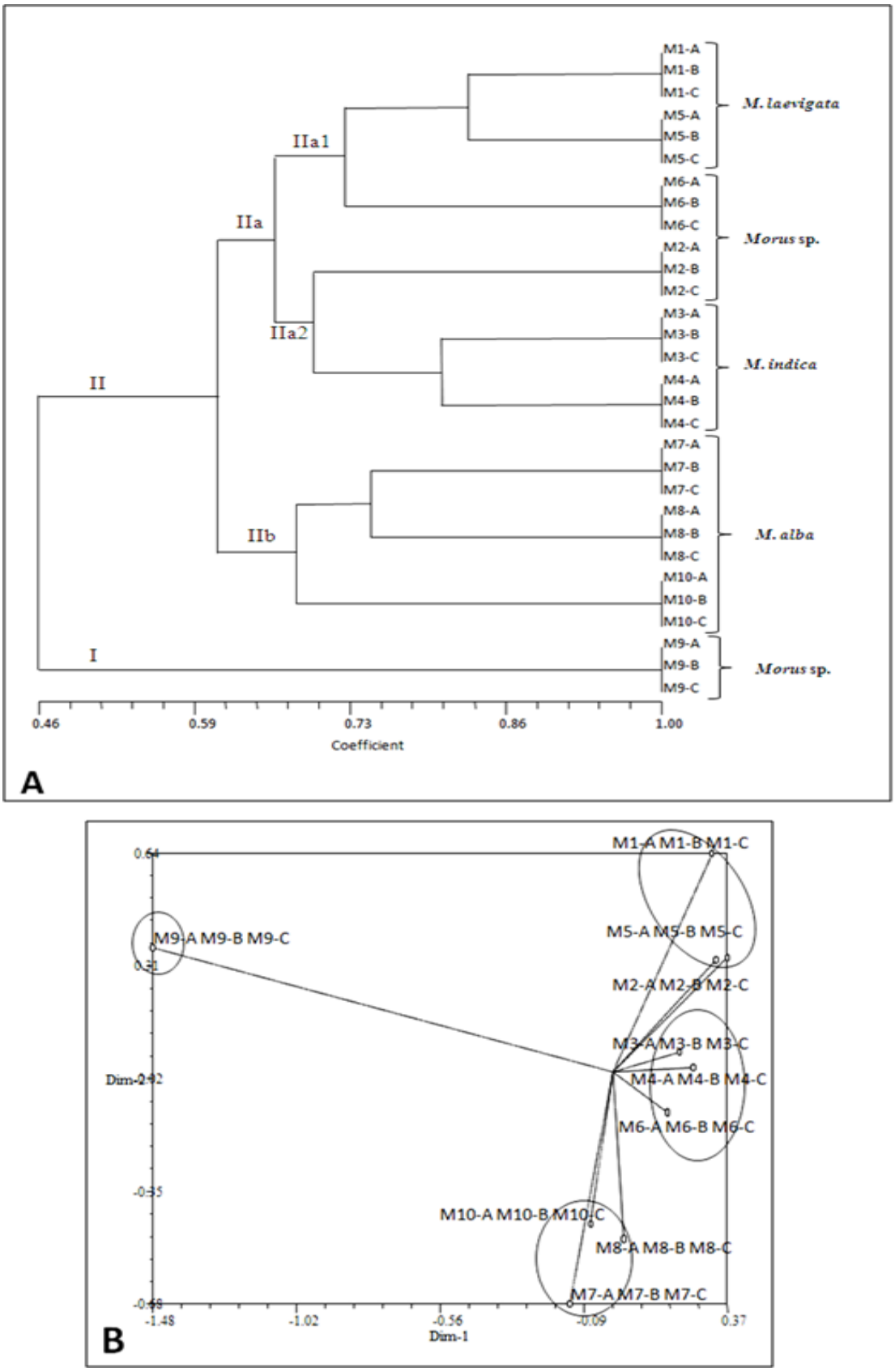

Figure 2. Mulberry [A] UPGMA dendrogram and [B] 2-D plot of 10 mulberry cultivars (ten control and 10 *in vitro* regenerated samples before and after cryopreservation) generated based on RAPD data.

Table 3. A pairwise similarity matrix of 10 samples (fresh, *in vitro* raised before and after cryopreservation) of different *Morus* species based on RAPD data.

	M1-A	M1-B	M1-C	M2-A	M2-B	M2-C	M3-A	M3-B	M3-C	M4-A	M4-B	M4-C	M5-A	M5-B	M5-C	M6-A	M6-B	M6-C	M7-A	M7-B	M7-C	M8-A	M8-B	M8-C	M9-A	M9-B	M9-C	M10-A	M10-B	M10-C
M1-A	1.00																													
M1-B	1.00	1.00																												
M1-C	1.00	1.00	1.00																											
M2-A	0.65	0.65	0.65	1.00																										
M2-B	0.65	0.65	0.65	1.00	1.00																									
M2-C	0.65	0.65	0.65	1.00	1.00	1.00																								
M3-A	0.66	0.66	0.66	0.70	0.70	0.70	1.00																							
M3-B	0.66	0.66	0.66	0.70	0.70	0.70	1.00	1.00																						
M3-C	0.66	0.66	0.66	0.70	0.70	0.70	1.00	1.00	1.00																					
M4-A	0.62	0.62	0.62	0.69	0.69	0.69	0.81	0.81	0.81	1.00																				
M4-B	0.62	0.62	0.62	0.69	0.69	0.69	0.81	0.81	0.81	1.00	1.00																			
M4-C	0.62	0.62	0.62	0.69	0.69	0.69	0.81	0.81	0.81	1.00	1.00	1.00																		
M5-A	0.83	0.83	0.83	0.62	0.62	0.62	0.70	0.70	0.70	0.71	0.71	0.71	1.00																	
M5-B	0.83	0.83	0.83	0.62	0.62	0.62	0.70	0.70	0.70	0.71	0.71	0.71	1.00	1.00																
M5-C	0.83	0.83	0.83	0.62	0.62	0.62	0.70	0.70	0.70	0.71	0.71	0.71	1.00	1.00	1.00															
M6-A	0.69	0.69	0.69	0.61	0.61	0.61	0.69	0.69	0.69	0.72	0.72	0.72	0.76	0.76	0.76	1.00														
M6-B	0.69	0.69	0.69	0.61	0.61	0.61	0.69	0.69	0.69	0.72	0.72	0.72	0.76	0.76	0.76	1.00	1.00													
M6-C	0.69	0.69	0.69	0.61	0.61	0.61	0.69	0.69	0.69	0.72	0.72	0.72	0.76	0.76	0.76	1.00	1.00	1.00												
M7-A	0.52	0.52	0.52	0.50	0.50	0.50	0.65	0.65	0.65	0.63	0.63	0.63	0.58	0.58	0.58	0.68	0.68	0.68	1.00											
M7-B	0.52	0.52	0.52	0.50	0.50	0.50	0.65	0.65	0.65	0.63	0.63	0.63	0.58	0.58	0.58	0.68	0.68	0.68	1.00	1.00										
M7-C	0.52	0.52	0.52	0.50	0.50	0.50	0.65	0.65	0.65	0.63	0.63	0.63	0.58	0.58	0.58	0.68	0.68	0.68	1.00	1.00	1.00									
M8-A	0.56	0.56	0.56	0.62	0.62	0.62	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.65	0.65	0.73	0.73	0.73	0.75	0.75	0.75	1.00								
M8-B	0.56	0.56	0.56	0.62	0.62	0.62	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.65	0.65	0.73	0.73	0.73	0.75	0.75	0.75	1.00	1.00							
M8-C	0.56	0.56	0.56	0.62	0.62	0.62	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.65	0.65	0.73	0.73	0.73	0.75	0.75	0.75	1.00	1.00	1.00						
M9-A	0.42	0.42	0.42	0.37	0.37	0.37	0.47	0.47	0.47	0.45	0.45	0.45	0.42	0.42	0.42	0.47	0.47	0.47	0.52	0.52	0.52	0.50	0.50	0.50	1.00					
M9-B	0.42	0.42	0.42	0.37	0.37	0.37	0.47	0.47	0.47	0.45	0.45	0.45	0.42	0.42	0.42	0.47	0.47	0.47	0.52	0.52	0.52	0.50	0.50	0.50	1.00	1.00				
M9-C	0.42	0.42	0.42	0.37	0.37	0.37	0.47	0.47	0.47	0.45	0.45	0.45	0.42	0.42	0.42	0.47	0.47	0.47	0.52	0.52	0.52	0.50	0.50	0.50	1.00	1.00	1.00			
M10-A	0.59	0.59	0.59	0.54	0.54	0.54	0.63	0.63	0.63	0.62	0.62	0.62	0.62	0.62	0.62	0.67	0.67	0.67	0.69	0.69	0.69	0.68	0.68	0.68	0.51	0.51	0.51	1.00		
M10-B	0.59	0.59	0.59	0.54	0.54	0.54	0.63	0.63	0.63	0.62	0.62	0.62	0.62	0.62	0.62	0.67	0.67	0.67	0.69	0.69	0.69	0.68	0.68	0.68	0.51	0.51	0.51	1.00	1.00	
M10-C	0.59	0.59	0.59	0.54	0.54	0.54	0.63	0.63	0.63	0.62	0.62	0.62	0.62	0.62	0.62	0.67	0.67	0.67	0.69	0.69	0.69	0.68	0.68	0.68	0.51	0.51	0.51	1.00	1.00	1.00

Table 4. A pairwise similarity matrix of 10 samples (fresh, *in vitro* raised before and after cryopreservation) of different *Morus* species based on ISSR data.

	M1-A	M1-B	M1-C	M2-A	M2-B	M2-C	M3-A	M3-B	M3-C	M4-A	M4-B	M4-C	M5-A	M5-B	M5-C	M6-A	M6-B	M6-C	M7-A	M7-B	M7-C	M8-A	M8-B	M8-C	M9-A	M9-B	M9-C	M10-A	M10-B	M10-C
M1-A	1.00																													
M1-B	1.00	1.00																												
M1-C	1.00	1.00	1.00																											
M2-A	0.52	0.52	0.52	1.00																										
M2-B	0.52	0.52	0.52	1.00	1.00																									
M2-C	0.52	0.52	0.52	1.00	1.00	1.00																								
M3-A	0.58	0.58	0.58	0.67	0.67	0.67	1.00																							
M3-B	0.58	0.58	0.58	0.67	0.67	0.67	1.00	1.00																						
M3-C	0.58	0.58	0.58	0.67	0.67	0.67	1.00	1.00	1.00																					
M4-A	0.59	0.59	0.59	0.66	0.66	0.66	0.97	0.97	0.97	1.00																				
M4-B	0.59	0.59	0.59	0.66	0.66	0.66	0.97	0.97	0.97	1.00	1.00																			
M4-C	0.59	0.59	0.59	0.66	0.66	0.66	0.97	0.97	0.97	1.00	1.00	1.00																		
M5-A	0.95	0.95	0.95	0.51	0.51	0.51	0.59	0.59	0.59	0.60	0.60	0.60	1.00																	
M5-B	0.95	0.95	0.95	0.51	0.51	0.51	0.59	0.59	0.59	0.60	0.60	0.60	1.00	1.00																
M5-C	0.95	0.95	0.95	0.51	0.51	0.51	0.59	0.59	0.59	0.60	0.60	0.60	1.00	1.00	1.00															
M6-A	0.52	0.52	0.52	0.73	0.73	0.73	0.76	0.76	0.76	0.76	0.76	0.76	0.51	0.51	0.51	1.00														
M6-B	0.52	0.52	0.52	0.73	0.73	0.73	0.76	0.76	0.76	0.76	0.76	0.76	0.51	0.51	0.51	1.00	1.00													
M6-C	0.52	0.52	0.52	0.73	0.73	0.73	0.76	0.76	0.76	0.76	0.76	0.76	0.51	0.51	0.51	1.00	1.00	1.00												
M7-A	0.60	0.60	0.60	0.62	0.62	0.62	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.58	0.58	0.58	1.00											
M7-B	0.60	0.60	0.60	0.62	0.62	0.62	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.58	0.58	0.58	1.00	1.00										
M7-C	0.60	0.60	0.60	0.62	0.62	0.62	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.58	0.58	0.58	1.00	1.00	1.00									
M8-A	0.56	0.56	0.56	0.60	0.60	0.60	0.64	0.64	0.64	0.63	0.63	0.63	0.54	0.54	0.54	0.57	0.57	0.57	0.89	0.89	0.89	1.00								
M8-B	0.56	0.56	0.56	0.60	0.60	0.60	0.64	0.64	0.64	0.63	0.63	0.63	0.54	0.54	0.54	0.57	0.57	0.57	0.89	0.89	0.89	1.00	1.00							
M8-C	0.56	0.56	0.56	0.60	0.60	0.60	0.64	0.64	0.64	0.63	0.63	0.63	0.54	0.54	0.54	0.57	0.57	0.57	0.89	0.89	0.89	1.00	1.00	1.00						
M9-A	0.41	0.41	0.41	0.66	0.66	0.66	0.63	0.63	0.63	0.64	0.64	0.64	0.41	0.41	0.41	0.61	0.61	0.61	0.61	0.61	0.61	0.57	0.57	0.57	1.00					
M9-B	0.41	0.41	0.41	0.66	0.66	0.66	0.63	0.63	0.63	0.64	0.64	0.64	0.41	0.41	0.41	0.61	0.61	0.61	0.61	0.61	0.61	0.57	0.57	0.57	1.00	1.00				
M9-C	0.41	0.41	0.41	0.66	0.66	0.66	0.63	0.63	0.63	0.64	0.64	0.64	0.41	0.41	0.41	0.61	0.61	0.61	0.61	0.61	0.61	0.57	0.57	0.57	1.00	1.00	1.00			
M10-A	0.60	0.60	0.60	0.61	0.61	0.61	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.56	0.56	0.56	0.99	0.99	0.99	0.89	0.89	0.89	0.62	0.62	0.62	1.00		
M10-B	0.60	0.60	0.60	0.61	0.61	0.61	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.56	0.56	0.56	0.99	0.99	0.99	0.89	0.89	0.89	0.62	0.62	0.62	1.00	1.00	
M10-C	0.60	0.60	0.60	0.61	0.61	0.61	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.56	0.56	0.56	0.99	0.99	0.99	0.89	0.89	0.89	0.62	0.62	0.62	1.00	1.00	1.00

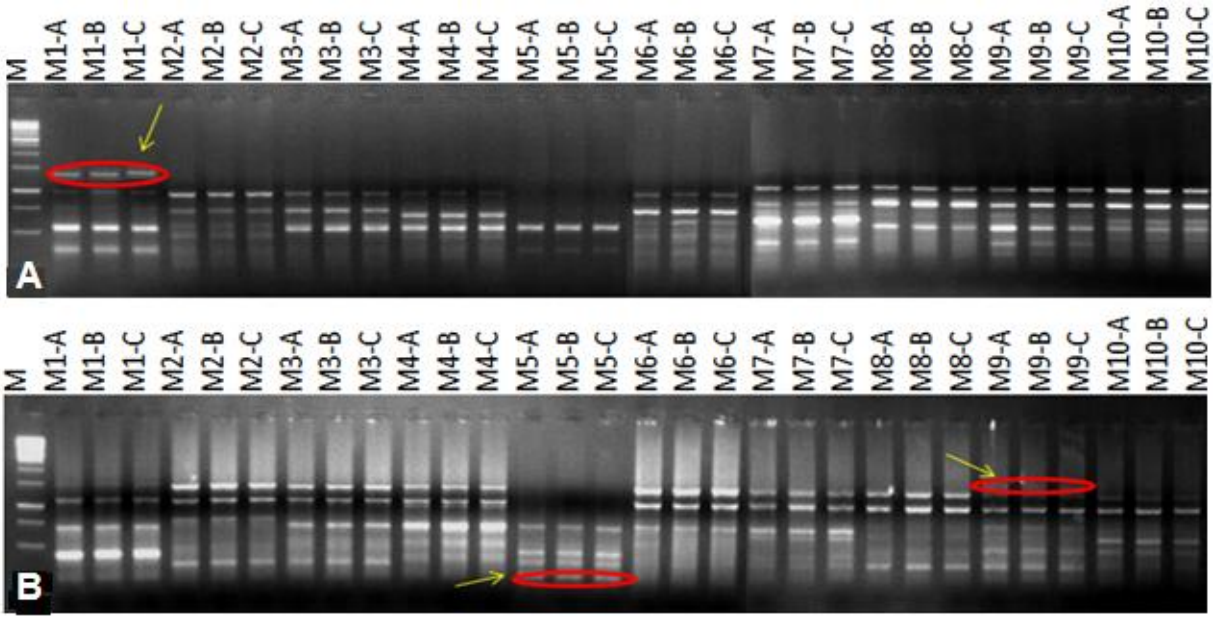

Figure 3. Gel profile of 10 mulberry accessions (ten controls, ten *in vitro* regenerated samples before and after cryopreservation) generated by ISSR primers: [A] UBC- 808 and [E] UBC-858. M is the λ DNA marker. Red circles showing the unique bands.

techniques to examine genetic stability in plants regenerated from cryopreserved dormant buds of mulberry germplasm. Genetic stability is the norm in most studies of possible plant genetic variation following cryopreservation (Harding, 2004). In the present studies, no differences were observed in mulberry between mother plants and *in vitro* regenerated before and after cryopreserved plants using RAPD and ISSR primers. This lack of variation suggests that there were no changes in the genetic fidelity of the plants due to cryopreservation. Cryopreserved yam (*Dioscorea*) shoots were genetically stable when compared to the original *in vitro* cultures (Mandal et al., 2008), and similar results were seen for apple shoot cultures (Liu et al., 2008). In the present study, the RAPD method of assessing genetic stability appeared simple and the reproducible results. However, there is little documentation on the effects of cryopreservation on the genetic stability and agronomic and/-genetic stability of plants regenerated from frozen explants.

RAPD analyses of cryopreserved *in vitro* grown shoot tips of *Prunus* and potato have shown no polymorphism between different amplified DNA patterns (Helliot, 1998; Hirai and Sakai, 2000). Similar results were found in the present study with mulberry dormant buds. The RAPD profiles were reproducible and no differences were found between the DNA patterns obtained with plantlets regenerated from control and cryopreserved plantlets. The RAPD technique therefore appears to be a fast, simple and efficient method for evaluating genetic stability of cryopreserved material, which can be used rapidly after the completion of a freezing experiment and will efficiently complement other genetic stability evaluation methods. Similar results were observed by Zhai et al. (2003) in grape and kiwi cryopreserved plants and found highly reproducible DNA pattern obtained with plantlets regenerated from control and cryopreserved plantlets. Condello et al. (2009) also found similar results after cryopreservation of pear germplasm using RAPD. ISSR markers were successfully applied for detection of genetic similarities or dissimilarities (Vijayan et al., 2006; Lakshmanan et al., 2007). The eleven ISSR primers generated high level of genetic diversity (91.78% polymorphism) in mulberry. Similar results were found in their studies by several researchers (Vijayan, 2004; Vijayan and Chatteerjee, 2003; Naik and Dandin, 2005; Vijayan et al., 2006; Rao et al., 2007, 2009).

Maintenance of genetic stability of cryopreserved germplasm has been reported in Melia (Scocchi et al., 2004); Dioscorea (Dixit et al., 2003); Grape and Kiwi (Zhai et al., 2003). Similarly in our study, plants regenerated from cryopreserved dormant buds were 100% genetically similar. Any accumulative DNA polymorphism may not be induced by cryopreservation (Harding, 2004). Maintenance of true-to-type clonal fidelity is one of the important aspects to be looked into in conservation activities of vegetatively propagated species.

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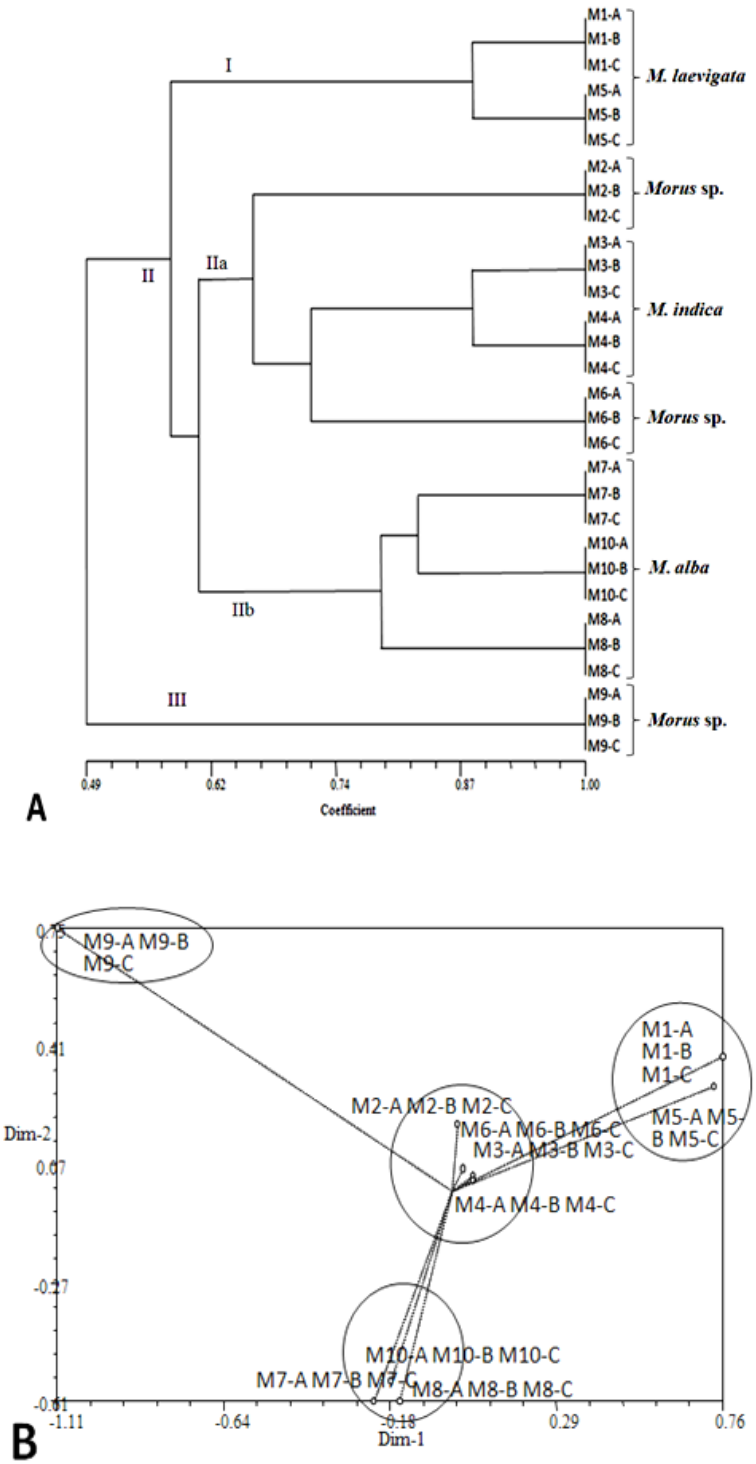

Figure 4. Mulberry [A] UPGMA dendrogram and [B] 2-D plot of 10 mulberry cultivars (ten control and ten *in vitro* regenerated samples before and after cryopreservation) generated based on ISSR data.

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