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Genetic diversity and relationship assessment among mulberry (*Morus spp*) genotypes by simple sequence repeat (SSR) marker profile

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Mulberry (*Morus* L.) is essential for sericulture industry as the primary source of food for silkworm *Bombyx mori* L. In India, long tradition of practising sericulture includes the use of a large number of indigenous cultivars. Since knowledge on genetic divergence of these cultivars/varieties is imperative for conservation and gainful utilization, simple sequence repeat (SSR) profiling was employed to assess genetic relatedness among 17 mulberry genotypes maintained in the Germplasm Bank of Temperate Sericulture Institute, SKUAST Kashmir, Mirgund. Six SSR primers were utilised which generates 17 alleles among the genotypes. The polymorphism information content (PIC) value varied from 0.260 (MulSTR3) to 0.623 (MulSTR4), with an average of 0.438 per locus. The highest similarity value of 0.92 was observed between Lemoncina and Kanva-2, as compared to the lowest similarity coefficient of 0.15 was between SKM-48 and Chinese white. Clustering of the genotypes was done with unweight pair group method using arithmetic average (UPGMA) which generates five clusters. Cluster-2 contained maximum (six) genotypes.

Key words: Clustering, genetic relatedness, mulberry, SSR.

INTRODUCTION

Genetic variation assessment in plant population has many potential uses for evolutionists, breeders and conservation biologists. Genetic diversity among the population reflects the intensity of interactions among various evolutionary processes such as genetic drift, mutation and natural selection (Wright, 1978; Wu et al., 1999). To overcome the evolutionary effects, it is imperative to formulate appropriate strategies for conservation and utilization. Mulberry is a perennial, deep-rooted, widely adaptable, fast growing tree plant belonging to the genus *Morus* L. under the order Utricales and the family Moraceae (Hooker, 1885). Mulberry is being extensively cultivated in sericulture important countries of both tropical and temperate regions to feed the monophagous silkworm insect *Bombyx mori* L. It is propagated in tropical countries like India, Pakistan, Bangladesh through stem cuttings, while in temperate countries the seed is the major source of propagation. In India, the cost of mulberry leaf production is reported to be nearly 60% of total expenditure of silkworm cocoon production (Das and Krishnaswami, 1965). Characterization of germplasm is essential to identify individual genotypes and also the

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extent of variability existing among the accessions. Characterization is also a process in which characters are subject to systematic data recording and analysis which finally help in elucidating the genetic diversity among accessions. The comprehensive information obtained from such exercise would help the breeder, geneticists and conservationists for effective utilization of the valuable genetic resources (Vijayan et al., 2005). Earlier classification and evaluation of the genus *Morus* were done primarily based on phenotypic expression of the plants such as growth form, leaf morphology, fruit properties and other agronomical characters (Edrogan, 2003; Koyuncu et al., 2004). Besides the availability of considerable diversity in the existing gene pool, mulberry improvement through conventional breeding is severely constrained owing to multiple factors, such as lack of genetic markers and efficient selection strategies, out crossing behavior and a long breeding cycle. These constraints call for resource to newer high genetic resolution approaches, such as DNA based markers providing means for efficient germplasm characterization. Initially all the primer pairs were tested for PCR amplification and only six working pairs were used to ascertain the genetic diversity among 17 mulberry genotypes.

MATERIALS AND METHODS

Plant material

The plant material for the present study comprised of 17 mulberry genotypes namely Goshoerami, Ichinose, Kokuso-20, Kokuso-21, Kokuso27, KNG, Kanva-2, Kasuga, Lemoncina, Chinese white, Brentul, Botatul, Chatatul Mirgund, Local mulberry, SKM-27,SKM-33 and SKM-48 (Table 1) maintained in the germplasm bank of the Temperate Sericulture Research Institute SKUAST-Kashmir, Mirgund. These varieties are cultivated extensively at different agro climatic zones by farmers of India to rear the silkworm insect, *Bombyx mori* L. Some of the leaf yield attributing traits of these varieties are given in Tables 2 and 3, and were recorded by following the standard descriptor (Thangavelu et al., 1997). Young leaves were collected for DNA extraction from each variety.

DNA extraction

Total genomic DNA was isolated using the modified cetyl trimethyl ammonium bromide (CTAB) protocol of Murray and Thompson (1980). Young leaves were harvested randomly from five to six plants of each genotype (~ 5 to 7 g of fresh weight). Harvested leaves were placed in polybags and stored in ice . Five hundred milligram (500 mg) of leaf lamina was ground to fine powder using prechilled pestle and mortar after adding liquid nitrogen to make leaves brittle as well as to stop DNase activity. The powder was transferred immediately to 50 ml autoclaved polypropylene centrifuge tube containing 10 ml of extraction buffer pre-warmed at (65°C).The extraction buffer comprised 1% CTAB, 1.5% PVP, 1.4M sodium chloride, 50 mM EDTA, 50 mM Tris HCL and 0.1% mercaptoethanol. The powder was suspended in the buffer by inverting and rotating the tubes properly. Tubes were incubated at 65°C for 45 to 60 min in a water bath. Samples were mixed occasionally while maintaining at 65°C. After incubation, 10 ml of chloroform; isoamyl alcohol (24:1) was added to each tube and tubes were swirled, till it made a dark green emulsion. The tubes were placed on a rotator shaker for 30 to 45 min and centrifuged at 5000 rpm for 30 min at room temperature. The supernatant was transferred to a clean sterile 50 ml falcon tube and was added equal or ⅔ volume of ice cold isopropyl alcohol and tubes were inverted gently several times. The DNA formed white cotton like precipitate and good quality DNA floated top. The floating DNA was hooked out using a sterile hooked Pasteur pipette. The hooked or pelleted DNA was transferred into a clean sterile 2 ml microfuge tubes and rinsed with 70% ethanol for 5 min so as to remove any residual salt followed by recentrifugation. Pellet was collected and leftover ethanol was dried up completely by turning down microfuge tubes on a blotting paper and was allowed to air dry at room temperature for overnight and was added 200 to 300 µl volume of 1x Tris-EDTA (TE) buffer (10 mM Tris HCL, 1 mM EDTA, pH 8.0). The tubes were left for few hours at room temperature to allow DNA to dissolve. Purification of DNA was done following the phenol chloroform extraction method (Sambrook et al., 1989). Quantity of DNA was checked by loading the samples on 0.8% agarose gel. Along with the DNA sample, marker of known concentration (uncut λ DNA of 30 ng/µl concentration) was also run along with the samples. The gel was run for about 1 to 2 h at voltage of 80 V/cm. The gel was visualized under ultraviolet (UV) transilluminator using photo gel documentation system (BioRad) and DNA samples were photographed.

PCR amplification with simple sequence repeat (SSR) primers

A total of six working SSR primers were selected to ascertain the genetic diversity of 17 genotypes. PCR amplification was performed in a 96 well micro titer plate in a MJ research PTC 200 or Eppendorf master cycler. The PCR amplification were carried out in a final volume of 20 µl reaction using 20 ng of genomic DNA with 2 µl of 1x PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2 mM $MgCl_2$, 1 pmol of each primer, 0.2 mM of each of dATP, dTTP, dCTP and dGTP, and 1 U of Taq DNA polymerase. Amplification reaction was carried out by following cycle profiles: 1 cycle at 94°C for 3 min followed by 45 cycles at 94°C for 1 min, 53°C for 1 min, 72°C for 2 min and a final 5 min extention at 72°C. PCR products were electrophoreses on 3% agarose gel in 0.5X Tris acetate EDTA (TAE) buffer stained in ethidium bromide. The gel was run at 60V visualized under UV light and photographed using UV Photo gel documentation system (Model GDS 7600) with GRAB-IT software programme (Annotating Graber 32 bits). Scoring of bands was on the basis of presence (1) or absence (0) of a particular band.

Statistical analysis

Only consistent, bright, reproducible (that is, band absence will be randomly verified) SSR bands were scored as present (1) or absent (0), and each character was treated independently. The polymorphic information content (PIC) values described by Botstein et al. (1980) were used to refer to the relative value of each marker with respect to the amount of polymorphism exhibited. PIC values for each of the six primers was estimated using formula given by Nei (1973).

$$PIC = 1- \sum_{i=1}^{n} (P_{ij})^2$$

PIC is synonymous with the term ‘gene diversity’ as described by Weir (1990). The PIC takes into account not only the number of alleles that are expressed but also the relative frequencies of those alleles (Smith et al., 1997). Genetic similarity and cluster analyses was performed by subjecting character state data to empirical examination using the NTSYS-pc software version 2.0e (Rohlf,

Table 1. List of the mulberry genotypes maintained in the Germplasm Bank of Temperate Sericulture Research Institute Mirgund, SKUAST- Kashmir.

S. No.	Genotype	Origin	S. No.	Genotype	Origin
1.	Goshoerami	Japan	11.	Brentul	Kashmir (India)
2.	Ichinose	Japan	12.	Botatul	Kashmir (India)
3.	Kokuso-20	Japan	13.	Chatatul Mirgund	Kashmir (India)
4.	Kokuso-21	Japan	14.	Local Mulberry	Kashmir (India)
5.	Kokuso-27	Japan	15.	SKM-27	TSRI (Mirgund, Kashmir)
6.	KNG	Japan	16.	SKM- 33	TSRI (Mirgund, Kashmir)
7.	Kanva-2	Karnataka(India)	17.	SKM- 48	TSRI(Mirgund, Kashmir)
8.	Kasuga	Japan			
9.	Lemoncina	Italy			
10.	Chinese white	China			

Table 2. Performance of 17 mulberry genotypes (pooled over Spring).

Genotype	Number of shootlets plant^{-1}	Length of longest shoot (cm)	Total shoot length plant^{-1} (m)	Leaf yield plant^{-1} (kg)
Goshoerami	79.85	66.75	34.19	4.751
Ichinose	45.55	51.00	28.25	3.233
Kokuso-20	53.16	59.50	27.70	3.575
Kokuso-21	60.90	52.65	29.74	3.530
Kokuso-27	50.66	57.80	28.60	3.058
KNG	52.94	50.04	35.95	3.728
Kanva-2	43.54	42.50	26.90	1.443
Kasuga	41.50	52.00	32.50	3.255
Lemoncina	51.99	66.24	32.60	2.528
Chinese white	49.70	91.90	28.15	2.051
Brentul	51.89	97.19	32.61	3.301
Botatul	46.85	72.15	26.21	3.593
Chatatul Mirgund	48.10	58.33	32.25	3.351
Local-mulberry	54.15	64.12	27.00	2.076
SKM-27	44.89	66.00	26.84	2.435
SKM-33	77.40	67.66	31.24	4.100
SKM-48	46.10	75.95	28.90	2.820
Mean	52.89	64.22	29.98	3.10
CD at 5%	0.713	0.854	0.3875	0.133

1997), where the SIMQUAL used to calculate Jaccard's coefficients. In addition, genetic relationships were compared by visual examination of Dendrogram derived from clustering procedures using similarity matrices. Dendrogram were constructed using the unweighted pair group method with arithmetic average (UPGMA) procedure (Rohlf, 1997).

RESULTS

The morphological traits showed significant variations among genotypes for number of branches, length of longest shoot, total shoot length and leaf yield plant^{-1} (Tables 2 and 3). It is evident from Table 2 that number of shootlets plant^{-1} was recorded the highest (79.85) in Goshoerami followed by SKM-33 (77.40), and Kokuso-21 showed lowest (60.90) with an average of (53.00). Length of the longest shootlet varied from (42.50 cm) in Kanva-2 to (97.19 cm) in Brentul. Total shoot length was found highest (35.95 m) in KNG followed by Goshoerami (34.19 m) and lowest (26.21 m) in Botatul with an average of (29.98 m). Leaf yield plant^{-1} was recorded maximum (4.75 kg) in Goshoerami and minimum (1.44 kg) in Kanva-2 with an average of (3.10 kg).

Pooled over autumn (Table 3) revealed that number of shoots plant^{-1} was found maximum (28.86) in Goshoerami and minimum (18.40) in Chinese white with an ave-

Table 3. Performance of 17 mulberry genotypes (pooled over Autumn).

Genotype	Number of shoots plant^{-1}	Length of longest shoot (cm)	Total shoot length plant^{-1} (m)	Leaf yield plant^{-1} (kg)
Goshoerami	28.86	181.66	27.51	4.863
Ichinose	23.48	154.83	24.83	3.580
Kokuso-20	27.95	183.33	32.10	4.075
Kokuso-21	26.70	131.83	27.85	3.970
Kokuso-27	24.93	148.00	29.00	3.970
KNG	23.15	161.16	21.66	4.618
Kanva-2	25.80	162.50	27.80	1.685
Kasuga	24.75	157.83	25.03	3.560
Lemoncina	27.85	175.50	23.71	2.776
Chinese white	18.40	242.66	25.73	2.525
Brentul	25.25	208.16	26.91	4.348
Botatul	23.08	183.33	24.15	4.378
Chatatul Mirgund	25.85	186.00	27.03	4.080
Local-mulberry	26.90	173.50	29.71	2.735
SKM-27	20.70	179.50	23.90	3.821
SKM-33	25.86	109.50	26.06	4.450
SKM-48	23.93	120.66	23.21	3.893
Mean	24.90	168.23	26.25	3.72
CD at 5%	0.868	11.852	0.7673	0.060

Table 4. List of Simple Sequence Repeats (SSR) Primers used in the study.

S/N	Primer	Repeat motif	No. of alleles	PIC value
1	MulSTR1	(GTT)6 + (GTT)4	3	0.551
2	MulSTR2	(GTT)11	2	0.415
3	MulSTR3	(GA)33	2	0.260
4	MulSTR4	(GAA)6	4	0.623
5	MulSTR5	(CCA)8	3	0.364
6	MulSTR6	(GT)15	2	0.415
Total		-	17	-
Average		-	2.83	0.438

rage of (24.90). Length of longest shoot recorded highest (242.66 cm) in Chinese white and lowest (109.50 cm) in SKM-33. Total shoot length was found maximum (32.10 m) in Kokuso-20 and minimum (23.21 m) in SKM-48 with an average of 26.25 m. Leaf yield plant^{-1} was recorded highest (4.86 kg) in Goshoerami followed by KNG (4.61 kg) with an average of (3.72 kg). The molecular analysis of these genotypes also showed significant variability. A total of 17 alleles were detected across the seventeen genotypes through the use of six SSR markers (Table 4). Each of the loci differed significantly in their ability to determine variability among the genotypes. The number of alleles per loci generated by these SSR markers varied from 2 (MulSTR3) to 4 (MulSTR4). No significant correlation was observed between the number of alleles detected and the number of sequence repeated in SSR motif of these SSR markers. The PIC value varied from 0.260 (MulSTR3) to 0.623 (MulSTR4), with an average of 0.438 per locus (Table 4). Significant correlation existed between the PIC values and number of alleles at SSR loci. PIC values of >0.40 was revealed by the SSR markers viz. MulSTR1, MulSTR2, MulSTR4 and MulSTR6. PIC values of <0.40 was found by SSR markers MulSTR3 and MulSTR5.The pair wise genetic similarity coefficient varied from 0.15 to 0.92 (Table 5). The highest similarity value of 0.92 was observed between Lemoncina and Kanva-2, as compared to the lowest similarity coefficient of 0.15 was between SKM-48 and Chinese white.

The allelic diversity data was used to produce a Dendrogram (Figure 1) by using distance matrix-UPGMA; thus revealing the genetic relationship among mulberry genotypes/selections. Dendrogram are efficient means of summarizing microsatellite data and can reveal relationship

Table 5. Similarity coefficient matrices of 17 genotypes of mulberry using SSR markers (UPGMA, Jaccard's coefficient).

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17
G1	1.00																
G2	0.73	1.00															
G3	0.33	0.55	1.00														
G4	0.50	0.55	0.33	1.00													
G5	0.50	0.36	0.83	0.17	1.00												
G6	0.67	0.73	0.50	0.83	0.33	1.00											
G7	0.50	0.73	0.50	0.67	0.33	0.67	1.00										
G8	0.50	0.73	0.67	0.50	0.50	0.67	0.67	1.00									
G9	0.62	0.83	0.62	0.62	0.46	0.77	0.92	0.77	1.00								
G10	0.57	0.77	0.57	0.57	0.43	0.71	0.71	0.71	0.80	1.00							
G11	0.33	0.55	0.50	0.67	0.33	0.50	0.67	0.83	0.62	0.57	1.00						
G12	0.46	0.50	0.77	0.31	0.77	0.31	0.31	0.46	0.43	0.40	0.46	1.00					
G13	0.73	0.60	0.36	0.55	0.55	0.55	0.73	0.55	0.67	0.62	0.55	0.33	1.00				
G14	0.73	0.60	0.36	0.55	0.55	0.55	0.73	0.55	0.67	0.62	0.55	0.33	0.90	1.00			
G15	0.55	0.40	0.16	0.73	0.17	0.55	0.36	0.18	0.33	0.31	0.36	0.17	0.40	0.40	1.00		
G16	0.60	0.44	0.20	0.60	0.40	0.60	0.60	0.40	0.55	0.50	0.40	0.18	0.89	0.89	0.44	1.00	
G17	0.36	0.20	0.18	0.36	0.18	0.36	0.18	0.36	0.17	0.15	0.36	0.17	0.20	0.20	0.60	0.22	1.00

G1, Chatatul Mirgund; G2, Ichinose; G3, Kokuso-20; G4, Kokuso-21; G5, Kokuso-27; G6, KNG; G7, Kanva-2G8, Kasuga; G9, Lemoncina; G10, Chinese white; G11, Brentul; G12, Botatul; G13, Goshoerami; G14, SKM-33; G15, SKM-27; G16, Local mulberry; G17, SKM-48.

among genotypes. The 17 genotypes were classified into five clusters (Table 6) using UPGMA cluster method. cluster-1 had four genotypes (Chatatul Mirgund, Goshoerami, SKM-33, Local mulberry), cluster-2 consisted of six genotypes (Ichinose, Kanva-2, Lemoncina, Chinese white, Kokuso-21, KNG),Cluster-3 comprised of 2 genotypes (Kasuga and Brentul), cluster-4 had three genotypes (Kokuso-20, Kokuso-27, Botatul), cluster-5 consisted of two genotypes (SKM-27 and SKM-48).

DISCUSSION

Genetic relationships assessment of a crop species has important consequences in plant breeding and genetic resources conservation. It is useful particularly in selecting parents for breeding purposes as genetically divergent but sexually compatible parents produce greater heterosis. In case of mulberry the sexual incompatibility among various species was reported to be very little as most of the interspecific hybridization produced good seed fertility (Das and Krishnaswami, 1965; Dandin et al., 1987; Tikader and Rao, 2003). This obviously indicates that inter specific hybridization in mulberry is quite possible. Analysis of the growth and yield attributing traits revealed significant genetic variation for all the characters in both seasons. The present findings are in concurrence with the study of Das and Krishnaswami (1969), Bari et al. (1990) and Rahman et al. (1994). They also reported that significant genotypic differences were found regarding leaf yield and its contributing characters in mulberry. Polymorphic information content (PIC) varied from 0.260 (MulSTR3) to 0.623 (MulSTR4) with an average of 0.438 per locus. PIC values of >0.40 were revealed by the SSR markers viz. MulSTR1, MulSTR2, MulSTR4 and MulSTR6. Similarity coefficient values ranged from 0.15 to 0.92. The highest similarity value (0.92) was recorded between Lemoncina and kanva-2 and the lowest (0.15) was found between SKM-48 and Chinese white. High similarity coefficients indicate genotypes belong to similar genetic background which was clearly indicated by the present study that *Morus alba* and *Mangifera indica* cannot be separated out due to the mixing of gene pool of these two species due to lack of reproductive isolation as reported by Das and Krishnaswami (1965) and Dwivedi et al. (1989). Contrarily, magnitude and spectrum of lower value similarity coefficients among genotypes is indicative of higher genetic diversity among the genotypes. The cluster analysis of the present study reveals that the genotypes belong to *M. indica* and *M. alba* grouped in a single group that is, cluster-2 (Ichinose, Kanva-2, Lemoncina, Chinese white, KNG). From the present study, it is once again confirmed that genotypes of *M. indica* and *M. alba* are genetically very close and *M. indica* should be treated as synonymous of *M. alba* as suggested by Gururajan (1960) and Hirano (1977).

Same SSR markers utilized by Ramesh et al. (2004), produced a polymorphic information content that varied from 0.85 to 0.90 among the 43 genotypes. Ruiz et al. (2011) studied 57 accessions of walnut using 32 pairs of SSR markers and obtained similarity coefficient ranging

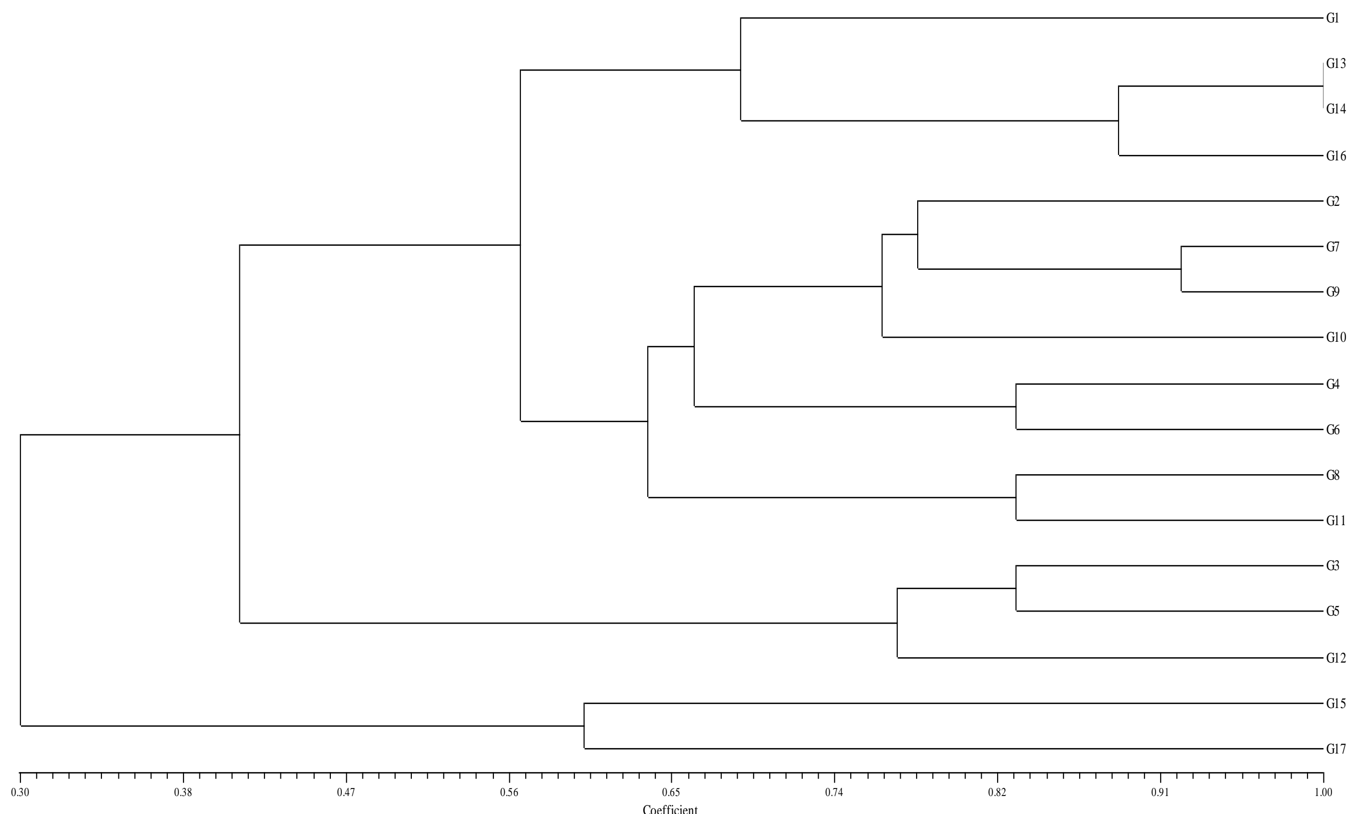

Figure 1. Dendrogram depicting the genetic relationship between seventeen genotypes of mulberry using SSR markers (Jaccards Coefficient, UPGMA).

Table 6. Clustering pattern of 17 mulberry genotypes based on SSR marker.

Cluster no.	Number of genotypes	Name of genotype/selection
I	4	Chatatul Mirgund (G1), Goshoerami (G13), SKM-33 (G14), Local mulberry (G16)
II	6	Ichinose (G2), Kanva-2 (G7), Lemoncina (G9), Chinese white (G10), Kokuso-21 (G4), KNG (G6)
III	2	Kasuga (G8), Brentul (G11)
IV	3	Kokuso-20 (G3), Kokuso-27 (G5), Botatul (G12)
V	2	SKM-27 (G15), SKM-48 (G17)

from 0.18 to 0.76 and obtained two main clusters based on UPGMA. Genetic relationships among apple cultivars and landraces from several geographical regions of Iran were evaluated by Farrokhi et al. (2011) using simple sequence repeat (SSR) markers. Forty five alleles were generated at 16 SSR loci. Polymorphism information content (PIC) was varied from 0.18 to 0.76. The mean PIC value for all loci was 0.49. Markers with high PIC values such as CH03c02, CH03g12z, CH05d04, Hi01d06y and Hi02d04 could be effectively used in genetic diversity studies of apple. Mohamed and Salah (2011) revealed that eight cultivars got grouped into two subclusters with 21% dissimilarity by SSR markers. One subcluster includes Superior and Early Superior, with 94% genetic similarity. The other subcluster included five cultivars (Thompson seedless-1, Thompson seedless-2, Thompson seedless-3, Bez El-Anza, and Roumi Ahmer), with similarity which starts from 82% until 96%. While the other cultivar Fayomi was falling outside of both of two subclusters. These results demonstrate that Fayomi cultivar is genetically faring from the other seven grape cultivars; while the five cultivars, (Thompson seedless-1, Thompson seedless-2, Thompson seedless-3, Bez El-Anza and Roumi Ahmer), although they belong to different farms, but they came from the same genetic origin. Also (Superior and Early Superior) cultivars despite collected from different farms but they showed the highest similarity (96%). Ercisli et al. (2011) studied 18

wild sweet cherry genotypes by SSR markers collected from diverse environments in the upper Coruh Valley in Turkey generated 46 alleles, the number of alleles per primer ranged from 3 to 7, with a mean of 4.6. The primer PS12A02 gave the highest number of polymorphic bands (N = 7), while CPSCT010, UDAp-401 and UDAp-404 gave the lowest number (N = 3). Seven groups were separated in the dendrogram, although most of the genotypes did not cluster according to phenological and morphological traits.

The present study highlights the utility of microsatellite markers in mulberry for genotype characterization. It is also concluded that polymorphic microsatellite markers are efficient tools for genetic assessment of the available mulberry gene pool for its conservation and gainful utilization. Goshoerami, SKM-33, KNG and Ichinose showed high yielding potential, were as SKM-48 and Chinese white which were found genetically diverse genotypes on the basis of molecular analysis and should be utilised in future breeding programmes.

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