INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0903/2018/20–7–1497–1504
DOI: 10.17957/IJAB/15.0652
http://www.fspublishers.org

Full Length Article

Genetic Divergence of *Moringa oleifera*, Economically Important, Yet An Endangered Species from Pakistan

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Abstract

Moringa oleifera Lam. (*M. oleifera*) is an economically important tree species of the Moringaceae family, having various medicinal uses and edible leaves and pods. It is found in regions of Pakistan and India and is acclimatized to hot and dry weather conditions. Despite its importance, conservation based on genetic divergence is lacking. In this study, RAPD markers were developed and shown to be highly polymorphic in genetic variation analyses of *M. oleifera* accessions. In total, 90 *M. oleifera* accessions were assessed using 9 RAPD primers to produce thirty-one distinct DNA bands. The polymorphic information content (PIC) value ranged from 0.39 to 0.57, with an average of 0.44. The GLK-11, marker was found to be the most informative marker on baes of highest PIC value. Clustering patterns revealed that eight growing districts in Pakistan that share their geographical boundaries enable the exchange of *M. oleifera* germplasm. Movement of *M. oleifera* accessions from one geographical region to another is demonstrated, except for the Faisalabad district, which is quite distant from the other 8 districts studied. The accessions were divided into four groups on the basis of principal component analysis (PCA). The similarity index of 10% between, and 90% within the Moringa accessions was observed using AMOVA. In conclusion, the genetic variation patterns present in regions of Moringa cultivation, which could benefit the selection of distinct accessions for maintenance and improvement of the breeding germplasm collections. The accessions having widespread geographical distribution had more genetic variation as compared to the closely situated accessions. Therefore, it is needed to conserve the diminishing germplasm from its area of origin.

Keywords: *Moringa oleifera*; Genetic diversity; RAPD; Molecular markers; Crop improvement

Introduction

Moringa oleifera Lam. (*M. oleifera*) is a versatile, diploid (2n=28) tree with both self- and cross-pollinates (Ramachandran *et al.*, 1980) and is well adjusted to the warm and dry weather condition of the peninsular, central as well as north-western regions of India (Pandey *et al.*, 2011) and Pakistan (Shahzad *et al.*, 2014). *M. oleifera* has been used as animal fodder, medicine, as well as edible leaves and pods for more than last 400 years. Ain-i-Akbari, the earliest data about north-Indians, documents the use of Moringa in pickle and food products (Watt, 1892). In the last two centuries, it has been used in other parts of the globe as a multi-purpose goods (Morton, 1991; Folkard and Sutherland, 1996).

The Moringaceae family comprises of 13 species. These are spread all over the world according to their subjugation and weather. Moringaceae is now getting famous in the Cambodia, and the Philippines Caribbean Islands, North and South America, and Central America (Anwar *et al.*, 2007). *Moringa oleifera* is gaining attention as the natural nutrition of the tropics. Due to its multiple products, nutritional importance and economical potential, it has attracted both the business and scientific communities. The leaves, fruit, flowers and immature pods of this tree are highly enriched with nutrients and used as vegetable in about all parts of the world, most commonly in India, Africa, Pakistan, Hawaii and Philippines (Anwar and Bhanger, 2003). Moringa leaves are enriched with important biomolecules such as vitamin C, protein, potassium, calcium and β-carotene. Moreover, they serve as an important source of natural antioxidants, including flavonoids, phenolics, ascorbic acid and carotenoids which can be used to increase the shelf-life of fat containing foods items (Siddhuraju and Becker, 2003).

To cite this paper: Shahzad, U., M.J. Jaskani, F.S. Awan, M. Shahjahan, S.A. Naqvi and A. Wasaya, 2018. Genetic divergence of *Moringa oleifera*, economically important, yet an endangered species from Pakistan. *Int. J. Agric. Biol*., 20: 1497–1504

Despite the considerable importance of Moringa species, its cultivation and germplasm characterization studies were not up to the mark. Detailed studies of the dissemination and genetic variability of Moringa species are limited. However considerable variations in quantitatively inherited characters have been acknowledged in natural populations from India. Genetic diversity and the population history of a species were studied by using DNA based markers. These genetic markers are used to detect genetic variation within and between individuals, of a species by screening (Collard *et al.,* 2005). Moreover, these DNA markers can be used to identify desirable genetic characters for crop improvement programs (Schulman, 2007). Genetic characterization of *M. oleifera* germplasm using AFLPs discovered significant differences among populations of different regions (Ulloa, 2005). Similarly, genetic variability between domestic and wild type population of *M. oleifera* L. was also identified using Twelve RAPD markers (Mgendi *et al.,* 2010). Another study used RAPD primers to study the genetic diversity of 16 Moringa accessions (Da Silva *et al.,* 2012). Microsatellite markers were also successfully used to unravel the genetic diversity of Moringa (Shahzad *et al.,* 2013). There is a great apprehension in the protection of the Moringa species from biodiversity, dietary, ethno-botanical and pharmacological perspectives. Continued agricultural yield is expected because of marvelous potential opportunities linked with *M. oleifera* and the development of cash crops in semi-arid regions of the world.

All the wild Moringa species could become victims of the constant decline in biodiversity. Sexual propagation of these species would not be possible without having sufficient plants for cross-pollination. Since flowering of a number of the large tree species of Moringa does not even originate until a critical size is achieved, it would be impractical in a glasshouse, and tissue culture may be the only useful means to cultivate these trees other than tropics. The conventional system of asexual propagation of *M. oleifera* is in continuous progress without knowing its genetic basis and varietal differences. But the genetic basis and differences of *M. oleifera* are not yet known in its area of origin, no prior study was conducted to unravel the genetic diversity of the different accessions present in Pakistan. The aim of the present study was to unravel the genetic diversity of the most cultivated and easily propagated species of *M. oleifera* and to promote their use in the diet of underdeveloped countries to fulfill their nutritional deficiencies.

Material and Methods

Plant Collection and Sample Preparation

A total of 9 districts listed as Bahawalpur, Layyah, Faisalabad, Dera Ghazi Khan, Rajanpur, Muzaffar Garh, Khanewal, Multan and Lodhran from Punjab, Pakistan were nominated for collection of Moringa accessions germplasm (Fig. 1). Fresh leaves from the new branches were collected in plastic bags and stored temporarily in ice box and finally stored in -80^{o}C freezer for further use in lab for DNA extraction. In an initial study, five different places (10–15 km distance) in each district were selected to collect the plant samples and each site was replicated twice. Plant collection sites are shown in Fig. 1 and Table 1. Genomic DNA isolation from fresh leaves of ninety Moringa accessions was done by using the CTAB method as described by (Doyle and Doyle, 1987) with minor changes. The isolated genomic DNA was quantified using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). 0.8% gel was used to check the quality of DNA by running all DNA samples on gel. Good quality DNA samples were chosen, and remaining samples were again treated. The working dilutions of all DNA samples were made using the DNA stock.

RAPD (Random Amplified Fragment Length Polymorphism) Marker Analysis

The 20 RAPD markers were designed and synthesized by Gene link were used in this study. Before use, RAPD decamers were optimized. Finally nine polymorphic RAPD primers were selected as shown in Table 2.

Data Analysis

All observable and robust polymorphic RAPD bands were counted in case of presence of band pointed as (1) while in case of absence pointed as (0). The frequency of polymorphic bands for all accession were calculated as 'n/N' where 'n' is the number of plants having specific band and 'N' stands for total number of bands on all genotype plants. The percentage of polymorphic bands for each genotype was calculated. The polymorphic information content was calculated by using the formula (PIC) = 1 - Σ $(Pij)^2$, where P_{ij} is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns (Botstein *et al.,* 1980).

Genetic similarity between all Moringa genotypes was determined by using Nei's and Li'ss genetic similarity indices and genetic relationship within the genotypes was accessed by clustering all the accessions with UPGMA (Unweighted paired group of arithmetic means). Principal component analysis (PCA) was also done by using the PAST, software Version 1.25 (Hammer and Khoshbakht, 2005) for the determination of genetic variability which helped to accurately understand the grouping of all 90 Moringa genotypes. Genetic distances were calculated by using analysis of molecular variance (AMOVA) with GenALEx V.6.1 (Peakall and Smouse, 2006). AMOVA divided the molecular variance among and within the population and also tested for its significance.

Table 1: Plant material collection sites with location district, location name and plant identity

Tree No.	District Name	Plant ID	Location	PPB
1	Bahawalpur	BR1	Basti Ramaan 1	0.71
2		BR2	Basti Ramaan 2	0.81
3		BC1	Basti Chandrani 1	0.94
4		BC3	Basti Chandrani 3	0.68
5		BI1	Tibbi Izzat 1	0.84
6		BI2	Tibbi Izzat 2	0.87
7		BK1	Kheeri 1	0.90
8		BK3	Kheeri 3	0.84
9		BF1	Fatto Wali 1	0.84
10		BF2	Fatto Wali 2	0.81
11	Rajanpur	RM1	Machi Wala 1	0.87
12		RM2	Machi Wala 2	0.94
13		RH1	Hamoon Wala 1	0.84
14		RH3	Hamoon Wala 3	0.90
15		RB1	Basti Bhanjar 1	0.90
16		RB3	Basti Bhanjar 3	0.84
17		RA1	Akil Pur 1	0.90
18		RA2	Akil Pur 2	0.87
19		RM1 (b)	Meeran 1	0.84
20		RM2 (b)	Meeran 2	0.84
21	Khanewal	KK1	Kabir Wala 1	0.94
22		KK2	Kabir Wala 2	0.90
23		KP1	Pull Akil 1	0.87
24		KP2	Pull Akil 2	0.94
25		KY1	Yousaf Wala 1	0.97
26		KY2	Yousaf Wala 2	0.94
27		KM1	Miyan Chanoo 1	0.90
28		KM2	Miyan Chanoo 2	0.94
29		KA1	Abidabad 1	0.87
30		KA2	Abidabad 2	0.97
31	Muzaffar Garh	MM1	Mehmood Kot 1	0.81
32		MM2	Mehmood Kot 2	0.81
33		MT1	Taleeri 1	0.94
34		MT2	Taleeri 2	0.90
35		MB2	Bukhi Chowk 1	0.90
36		MB3	Bukhi Chowk 3	0.84
37		MK1	Kenal Mastoi 1	0.97
38		MK2	Kenal Mastoi 2	0.90
39		MS1	Sarai Kotadu 1	0.87
40		MS2	Srai Kotadu 2	0.74
41	Layyah	LK1	Khokhran 1	0.94
42		LK3	Khokhran 3	0.94
43		LW1	Basti Wig 1	0.84
44		LW2	Basti Wig 2	0.87
45		LS1	Sayyadan 1	0.90
46		LS2	Sayyadan 2	0.90
47		LA1	Ahad Nagar 1	0.90
48		LA2	Ahad Nagar 2	0.90
49		LI1	Ilyani 1	0.84
50		LI3	Ilyani 3	0.81
51	Dera Ghazi Khan	DG2	Basti Gilani 2	0.87
52		DG3	Basti Gilani 3	0.87
53		DK1	Khajji Wala 1	0.74
54		DK3	Khajji Wala 3	0.74
55		DN1	Nawan Khoh 1	0.74
56		DN2	Nawan Khoh 2	0.94
57		DB1	Chah Bol 1	0.87
58		DB2	Chah Bol 2	0.74
59		DJ1	Jaskani Wala 1	0.81
60		DJ2	jaskani Wala 2	0.81
61	Multan	MNB1	Pull Barar 1	0.81
62		MNB2	Pull Barar 2	0.84
63		MNS1	Shujabad 1	0.90
64		MNS3	Shujabad 3	0.84
65		MNP1	Phatak Baag 1	0.90
66		MNP2	Phatak Baag 2	0.90
67		MNA1	Aara Pull 1	0.87
68		MNA3	Aara Pull 3	0.90
69		MNC1	CCRI 1	0.87
70		MNC2	CCRI 2	0.84
71	Lodhran	LOA1	Arif Wala 1	0.81
72		LOA2	Arif Wala 2	0.77
73		LOM1	Malkaan 1	0.84
74		LOM3	Malkaan 2	0.87
75		LOR1	Raja pur 1	0.87
76		LOR2	Raja pur 2	0.84
77		LOK1	Kasai wala 1	0.90
78		LOK2	Kasai wala 2	0.81
79		LOD1	Dunya pur 1	0.94
80		LOD2	Dunya pur 2	0.81
81	Faisalabad	FF1	UAF 1	0.77
82		FF2	UAF 2	0.81
83		FA1	AARI 1	0.81
84		FA3	AARI 3	0.77
85		FS1	Samndri 1	0.77
86		FS2	Samandri 2	0.84
87		FG1	Gutt Wala 1	0.71
88		FG2	Gutt Wala 2	0.74
89		FJ1	Jaran wala 1	0.84
90		FJ2	Jaran Wala 2	0.94

PPB= percentage of polymorphic bands

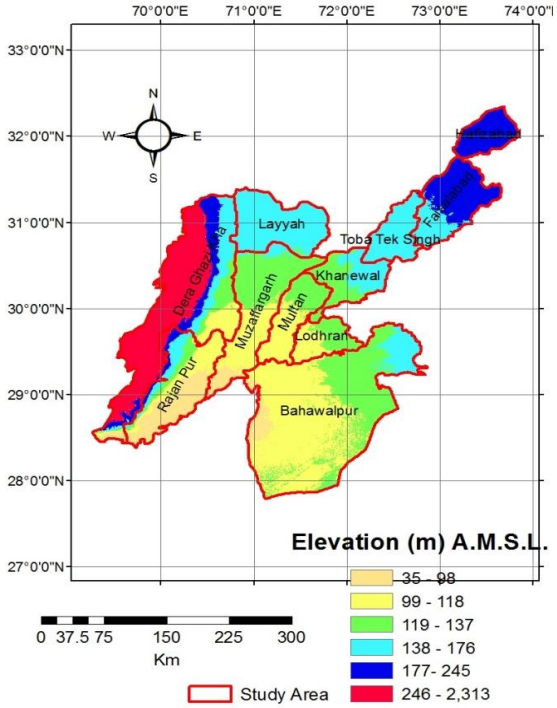

Fig. 1: Study area of germplasm collection of Moringa with the longitude, latitude and the elevation in Punjab (Pakistan)

Results

A total of 31 fragments were amplified, using nine polymorphic RAPD primers for the determination of genetic variability among 90 members of *Moringa*. On an average

two to five (2–5) bands were amplified with an average of 3.44 bands per marker. All polymorphic primers revealed genetic variation in the selected germplasm.

The mean band frequency (MBF) of the polymorphic band ranges from 0.76–0.93 with an average of 0.86. The minimum mean band frequency was detected in GLK-07 primer while maximum band frequency was detected in primer GLK-12 (Table 2).

The polymorphic information content (PIC) gave the information of the occurrence of the polymorphic alleles with respect to the specific markers. The PIC value ranges from 0.39 to 0.57 with an average value of 0.44. Primer GLK-11 was highly informative for the detection of polymorphic alleles with the maximum PIC value 0.57 followed by marker GLK-15, GLK-12, GLJ-01 with PIC values of 0.47, 0.45 and 0.44 respectively. Two primers, GLK-20 and GLK-02, provided a similar PIC value of 0.42. The other three primers (GKL-04, GLK-07 and GLK-16) showed a slightly lower PIC value i.e., 0.39 (Table 2).

The percentage of polymorphic bands was calculated for specific germplasm. The range of polymorphic bands was 0.97 to 0.68 with mean value of 0.86 within all the 90 Moringa plants samples. Three Moringa genotypes KA2, KY1, and MK1 had the highest ratio of polymorphic bands of 0.97, while the minimum ratio of polymorphic bands (0.68) was observed in BC3 genotype followed by 0.71 (BR1) and 0.74 (MS2, DK1, DK3, DN1, DB2 and FG2) (Table 1). The AMOVA showed that the variance factor among and within the population was 10 and 90% respectively. These values are significant at a probability of less than 0.001 (Table 3). The genotypes with close association were clustered in one group, while the remaining genotypes were spread into 3 clusters, illustrating the presence of genetic variability among these genotypes (Fig. 2 and Table 4). Genotypes that were collected from D.G. Khan Muzaffar Garh, Multan and Lodhran districts were grouped in same cluster. This group has 11 members on basis of similarity index of different genotypes. Two genotypes from Multan district were named as MNS1 and MNS3, five genotypes from Muzaffar Garh district were named MK1, MT1, MT2, MM2 and MK2, and 2 genotypes from district Dera Ghazi Khan were named as DB1 and DN1, two other genotypes from Lodhran, district were named LOM1 and LOA2. The second group comprised of ten genotypes, three from Rajanpur (RB1, RH1 and RM2b), two from Khanewal (KA1 and KY1) two from Bahawalpur and Dera Ghazi Khan (BR1 and DK1, respectively) and 3 genotypes from district Faisalabad (FA1, FG1 and FG2). Group three comprised of five genotypes 2 from Rajanpur (RM1, RM2), 2 from Dera Ghazi Khan (DG2 and DG3 respectively), and 1 from Faisalabad district (i.e., FJ1).

Table 2: RAPD Primers along with their amplification details used in Moringa germplasm diversity analysis

Primer Name	Sequence (5'-3')	TNB[a]	NPB[b]	PIC[c]	MBF[d]
GL Decamer K-02	GTCTCCGCAA	3	3	0.42	0.86
GL Decamer K-04	CCGCCCAAAC	4	4	0.39	0.91
GL Decamer K-07	AGCGAGCAAG	5	5	0.39	0.90
GL Decamer K-11	AATGCCCCAG	5	5	0.57	0.76
GL Decamer K-12	TGGCCCTCAC	2	2	0.45	0.93
GL Decamer K-15	CTCCTGCCAA	3	3	0.47	0.86
GL Decamer K-16	GAGCGTCGAA	4	4	0.39	0.85
GL Decamer K-20	GTGTCGCGAG	2	2	0.42	0.89
GL Decamer J-01	CCCGGCATAA	3	3	0.44	0.80
	Total	31	31	3.94	7.75
	Mean	3.44	3.44	0.44	0.86

a= Total Number of Bands. b= Number of Polymorphic Bands. c= Polymorphic Information Content. d= Mean Band Frequency

Table 3: Summary of Analysis of Molecular Variance for the genetic diversity among moringa genotypes

Source	d.f	SS	MS	Est. Var.	%
Among Pops	8	91.356	11.419	0.600***	10%
Within Pops	81	439.100	5.421	5.421***	90%
Total	89	530.456		6.021	100%

$P < 0.001$***

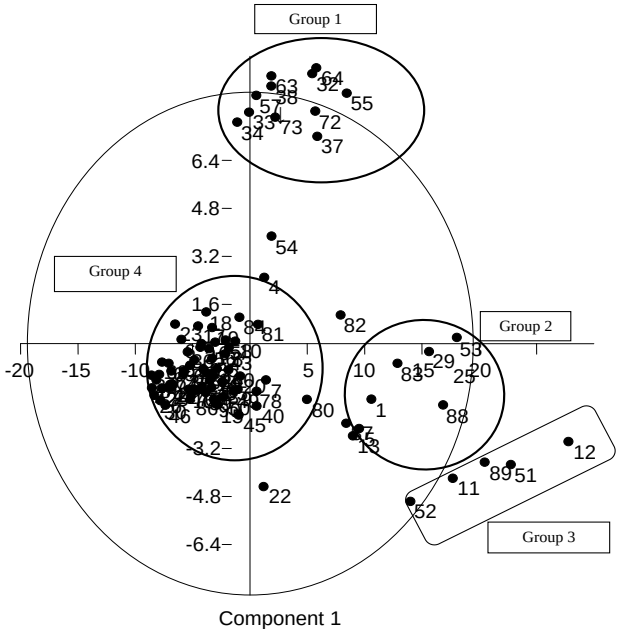

Fig. 2: Principal Component Analysis (PCA) of Moringa

Genetic Similarities between Moringa Genotypes

Genetic Similarity within all genotypes revealed that the highest similarity (96.77%) was found in 24 genotypes. The lowest similarity (48.39%) was observed between the FA3 and BR1 genotypes. Most of the genotypes fall into the similarity indices of 51.61 to 58.08% (Table 5).

Genetic Relationship Assessment within Moringa Genotypes

Moringa genotypes were clustered into different groups to access the genetic association using the coefficient indices described by "Nei's" that was purely based on the

Table 4: Principal Component Analysis (PCA) of Moringa with area codes

Label	Area code	District
PCA Group 1		
63	MNS1	Multan
64	MNS3	Multan
32	MM2	Muzafargarh
55	DN1	D.G. Khan
38	MK2	Muzafargarh
57	DB1	D.G. Khan
34	MT2	Muzafargarh
33	MT1	Muzafargarh
73	LOM1	Lodhran
72	LOA2	Lodhran
37	MK1	Muzaffargarh
PCA Groupe 2		
53	DK1	D.G. khan
29	KAI	Khanewal
83	FAI	Faisalabad
15	RB1	Rajanpur
1	BR1	Bahawalpur
88	FG2	Faisalabad
87	FG1	Faisalabad
20	RM2 (b)	Rajanpur
25	KY1	Khanewal
13	RH1	Rajanpur
PCA Groupe3		
12	RM2	Rajanpur
51	DG2	D.G. Khan
89	FJ1	Faisalabad
11	RM1	Rajanpur
52	DG3	D.G. Khan

similarities among the genotypes. According to the cluster analysis and genetic relationship among the Moringa genotypes, there were 4 core groups represented as A, B, C and D. In first cluster indicated as Group A consisted of genotypes as KA2, BF1, BR2, MS1, BK3, MK2, LS2, KY2, MT2, KK1 and KK2. The distinct genotype of Group A is BF2 that is separated from all other genotypes that were presented in that group (Fig. 3).

Most of the genotypes fall in Group B, including LW1, LW2, LA2, LK3, RB1, RH1, RH3, BC1, KP1, KA1, KM2, KM1, MNC1, MNC2, MNP1, MNS1, MNA1, MNA3, MB2, MNS3, MB2, LOK1, MM1, MM2 and DB1. Interestingly, LA2 and MNS1 showed inclusive similarity.

Cluster C comprised of fourteen genotypes, including LK1, LA1, LS1, KP2, MK1, B12, KY1, LOM1, MNP2, BK1, LOR1, MT1, DN2 and RM2. The remaining genotypes make up group D.

Including DG2, DJ1, DJ2, DB2, LOA1, LOA2, FS1, FS2, FJ2, LI1, LOD1, LOD2, RA1, RA2, LOR2, MNB2, MNB1, RM1(b), LI3, LOK2, FA3, RM1, DG3, LOM3 and MS2. The genotype DN1 was identified as a distinct member of cluster D.

In the group D the two genotypes MNB1 and DJ2 were more alike from other genotypes in the group. However, RM2 noticeably showed distinct behavior from all other genotypes. The two genotypes that do not form group with others were BR1 and BI1. Also, genotypes FA1, FF2 and FF1 all clustered together but 3 genotypes including FJ1, FG2 and FG1 grouped and exhibited distinctiveness from the rest of the genotypes. DK1 appeared as the most distinct genotype that did not grouped with other genotype and appeared separately. The most dissimilar genotype was DK1, which did not cluster with any genotype. Similarly, genotypes BC3 and DK3 grouped with one another as they did not form group with rest of the genotypes.

Discussion

The present study used RAPD markers to reveal the inherited diversity amongst 90 (ninety) Moringa genotypes collected from nine (9) different districts of Punjab, Pakistan. Recent investigations of genetic diversity amongst Moringa genotypes revealed that RAPD DNA markers were effectively exploited in Moringa for the assessment of genetic relatedness, recognition of cultivated and non-cultivated plants, and to find the genetic relationship using phylogenic analysis of Moringa genotypes (Mgendi *et al.*, 2010; Abubakar *et al.*, 2011; Da Silva *et al*., 2012). This study unraveled the genetic diversity among the studied Moringa germplasm that was collected from different locations in Punjab (Pakistan province) (Anwar *et al*., 2007). Nine polymorphic RAPD primers were used to genetically characterize the ninety trees of Moringa that amplified 31 fragments and bands produced by all primers, ranging from 2–5, with an average band of 3.44 per primer. Mgendi *et al*. (2010), Abubakar *et al*. (2011), Da Silva *et al*. (2012) also amplified RAPD markers for the assessment of Moringa germplasm.

This polymorphic information measure indicated the allelic numbers as well as the relative frequency of amplified alleles within the population. In our results, the average Polymorphic Information Content (PIC) was 0.44 that ranged from minimum 0.39 to maximum 0.57. The GLK-11 marker appeared as most informative one because it reveals the largest PIC among all the markers tested here. Da Silva *et al*. (2012) reported PIC value ranged from minimum 0.00 to maximum 0.41 by applying RAPD markers that is of less range compared with present study. This differs from the results of Abubakar *et al*. (2011), who reported much higher PIC values of 0.46 to 0.95.

The ninety Moringa genotypes investigated here formed 4 clusters. This indicates that the genotypes present among these 4 groups are more genetically identical than with members of the other groups. Cluster A comprised of genotypes from Bahawalpur, Khanewal, and Muzaffar Garh, with one genotype from Rajanpur and one genotype from Layyah. Cluster B contained the highest number of populations with genotypes from Bahawalpur, Khanewal, Layyah, Lodhran, Multan, Rajanpur, Dera Ghazi Khan and Muzaffar Garh districts. Cluster C comprised of genotypes from Bahawalpur, Dera Ghazi Khan, Rajanpur, Multan, Muzaffar Garh, Lodhran and Layyah districts.

Table 5: Genetic Similarity matrix of moringa with highest and lowest values

Highest value				Lowest value			
Sr. #	Similarity index value	Plant combination/ID		Sr. #	Similarity index value	Plant combination/ID	
		1	2			1	2
1	0.9677	22 (KK2)	21 (KK1)	1	0.4839	84 (FA3)	1 (BR1)
2		25 (KY1)	24 (KP2)	2	0.5161	74 (LOM3)	DK3 (54)
3		28 (KM2)	14 (RH3)	3	0.5484	50 (LI3)	4 (BC3)
4		29 (KA1)	27 (KM1)	4		58 (DB2)	4 (BC3)
5		30 (KA2)	26 (KY2)	5		59 (DJ1)	4 (BC3)
6		48 (LA2)	28 (KM2)	6		59 (DJ1)	53 (DK1)
7		26 (KY2)	30 (KA2)	7		74 (LOM3)	4 (BC3)
8		34 (MT2)	21 (KK1)	8		78 (LOK2)	54 (DK3)
9		34 (MT2)	26 (KY2)	9		82 (FF2)	54 (DK3)
10		35 (MB2)	28 (KM1)	10		82 (FF2)	55 (DN1)
11		35 (MB2)	29 (KA1)	11		83 (FA1)	4 (BC3)
12		38 (MK2)	39 (MS1)	12		83 (FA1)	40 (MS2)
13		46 (LS2)	26 (KY2)	13		83 (FA1)	54 (DK3)
14		56 (DN2)	25 (KY1)	14		83 (FA1)	55 (DN1)
15		63 (MNS1)	28 (KM2)	15		87 (FG1)	43 (LW1)
16		65 (MNP1)	28 (KM2)	16		88 (FG2)	55 (DN1)
17		67 (MNA1)	64 (MNS3)	17		88 (FG2)	58 (DB2)
18		68 (MNA3)	23 (KP1)	18	0.5806	40 (MS2)	1 (BR1)
19		68 (MNA3)	28 (KM2)	19		53 (DK1)	49 (LI1)
20		69 (MNC1)	64 (MNS3)	20		62 (MNB2)	53 (DK1)
21		77 (LOK1)	28 (KM2)	21		70 (MNC2)	4 (BC3)
22		77 (LOK1)	42 (LK3)	22		72 (LOA2)	4 (BC3)
23		77 (LOK1)	67 (MNA1)	23		73 (LOM1)	4 (BC3)
24		77 (LOK1)	69 (MNC1)	24		74 (LOM3)	58 (DB2)
25	0.9355	16 (RB3)	9 (BF1)	25		76 (LOR2)	4 (BC3)
26		24 (KP2)	12 (RM2)	26		76 (LOR2)	53 (DK1)
27		26 (KY2)	3 (BC1)	27		76 (LOR2)	54 (DK3)
28		28 (KM2)	3 (BC1)	28		78 (LOK2)	1 (BR1)
29		26 (KY2)	21 (KK1)	29		80 (LOD2)	1 (BR1)
30		27 (KM1)	25 (KY1)	30		81 (FF1)	40 (MS2)
31		28 (KM2)	23 (KP1)	31		81 (FF1)	54 (DK3)
32		28 (KM2)	26 (KY2)	32		81 (FF1)	55 (DN1)
33		27 (KM1)	13 (RH1)	33		83 (FA1)	72 (LOA2)
34		30 (KA2)	25 (KY1)	34		87 (FG1)	4 (BC3)
35		32 (MM2)	31 (MM1)	35		87 (FG1)	31 (MM1)
36		34 (MT2)	9 (BF1)	36		87 (FG1)	44 (LW2)
37		34 (MT2)	22 (KK2)	37		87 (FG1)	57 (DB1)
38		34 (MT2)	30 (KA2)	38		87 (FG1)	58 (DB2)
39		35 (MB2)	25 (KY1)	39		88 (FG2)	8 (BK3)
40		35 (MB2)	27 (KM1)	40		88 (FG2)	16 (RB3)
41		36 (MB3)	35 (MB2)	41		88 (FG2)	76 (LOR2)

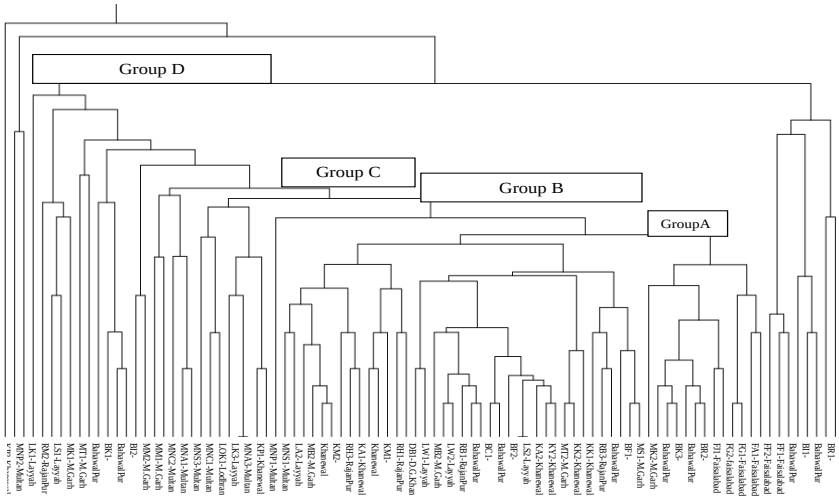

Fig. 3: Cluster analysis of 90 Moringa accessions

The members of groups B and C consist of genotypes from all districts of Punjab, except district Faisalabad. This might happen due to runaway of genotype from the indicated cultivated area, but they could not make genetically distinct themselves until now as most of the germplasm in cluster B is from Multan (seven genotypes) followed by Khanewal (four genotypes) and Muzaffar Garh (four genotypes) districts while in cluster C, 15 genotypes with maximum genotypes (three) from district Layyah. The cluster D, is most populated having 26 genotypes. The maximum number of genotypes (seven) was from the Lodhran district, followed by Dera Ghazi Khan (five) genotypes.

Group E consisted of 4 genotypes from the Faisalabad district, whereas the other three groups did not have any member from Faisalabad. The genotypes from Faisalabad district were clustered separately into two sub-groups; one consisted of FF1, FF2 and FA1 and the other consisted of FG1, FG2 and FJ1. Both sub-groups showed close similarity with each other. Interestingly, similar clustering and genetic relationships were reported in Nigerian Moringa populations (Abubakar *et al.*, 2011).

Clustering in the present study revealed that the eight districts that share geographic boundaries are exchanging their germplasm. It also revealed the movement of Moringa genotypes from one area to another, except for the Faisalabad district, which is far away from the other eight districts and clustered differently from the other groups.

By using PCA a principal component analysis, the 90 Moringa accession could be divided into four main clusters. Most of the genotypes were grouped into cluster 1, while cluster 2 had comprised genotypes from Lodhran, Multan, Muzaffar Garh and Dera Ghazi Khan. These districts like Multan, Dera Ghazi Khan, Lodhran and Muzaffar Garh are geographically close to each other. The Moringa genotypes included in cluster 3 were from Bahawalpur, Dera Ghazi Khan, Rajanpur and Khanewal, and 3 genotypes from district Faisalabad (FA1, FG1 and FG2). Group 4 consisted of only 5 genotypes from Dera Ghazi Khan and Rajanpur, as well as one from Faisalabad district. Rajanpur and Dera Ghazi Khan are neighboring districts and both genotypes were isolated from one location in each district.

Genetic similarity was detected among the Moringa genotypes, with a range of 48.39 to 96.77%. Most of them were found between the plant genotypic combination of BR1 and FA3 which were collected from Bahawalpur and Faisalabad districts, respectively, were found to be most divers one, and both districts are in different environmental and geographical zones in the Punjab Province (Pakistan).

The total area examined here was around 200 to 1,500 km, with 8 districts situated closer than the one in Faisalabad, which does not share any borders with the other districts. About 10% between the population, and 90 % within the subjected Moringa population, genotypic similarity was obtained by AMOVA (analysis of molecular variance) analysis. This indicates that the phenomenon of selfing, as well as wide crossing, between these genotypes is, supporting the current studies. whereas 26% of *Moringa oleifera* genotypes were self-fertilized, as estimated using AFLP Primers (Muluvi *et al.*, 2004). However, some gene flow may occur due to cross-pollination, resulting in the addition or deletion of some alleles in the genome.

These results disclose genetic variability patterns in Moringa farming and could also be useful for the detection of diverse genotypes for maintenance and their expansion through breeding programs. The genotypes having varied ecological distribution, and with cross-fertilization, have much more genetic variability as compared to self-fertilizing Moringa species (Casiva *et al.*, 2002).

Cross fertilization is an important method for improving adaptability of a species. The variations within and between any species are calculated by reproduction approaches. There may be a limited gene flow from one region to another region due to some topographical limitations (Schaal *et al.*, 1998). Less genetic variation indicated that individual plants were genetically more alike; with restricted gene flow happen during succeeding propagation in these regions (Punjab).

Transfer of Moringa genotypes from one place to another may cause mixing of the genetic material in the area. Moringa trees are not presently being cultivated as a marketable crop and there is no deliberate farming system in the areas selected in this study. The results obtained from this study, both PCA and the cluster analysis support each other. The individual genotype revealed a traditional form of genotype mixing from neighbouring districts, while genotypes from the district Faisalabad clustered discretely due to their isolated whereabouts. In this study, the *M. oleifera* members demonstrate 64% cross pollination and 26% self-pollination respectively. The occurrence of selfing and early sexual maturity offer the opportunity for the production of inbred lines and future hybridization as well as breeding programs (Muluvi *et al.*, 2004). It is expected that the development of such program may enhance the cultivation of these species in future and *M. oleifera* proved to be good for biodiversity concerns and to use this genetic variability for the conservation and development of new commercial variety in this region.

Conclusion

This species is indigenous to the southern part of Punjab province and this research also identified the genetic differences among the studied accessions. Therefore, the genetic divergence may provide the basis to develop a new cultivated variety with desirable characteristics and can boost the economic development of the country.

Acknowledgements

We are thankful to the Higher Education Commission (HEC) of Pakistan for the financial assistance to conduct

this research work under HEC indigenous research grant No.106-2001-AV6-014. We want to pay our special thanks to Dr. Mansoor Hameed for the taxonomic identification and development of Moringa descriptor.

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(Received 11 August 2017; Accepted 26 February 2018)