Indian J. Hort. 70(1), March 2013: 18-25

Assessment of genetic diversity in *khirni* [*Manilkara hexandra* (Roxb.) Dubard]: An important underutilized fruit species of India using Random Amplified Polymorphic DNA markers

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ABSTRACT

***Manilkara hexandra* (Roxb.) Dubard (*khirni*), is an important underutilized fruit species from western and central India with high potential as commercial horticultural crop. Agro-morphological characters and random amplified polymorphic DNA (RAPD) markers were used to estimate genetic variability in 23 diverse accessions of this species. Fifteen RAPD primers were used which generated a total of 119 bands, out of which, 103 bands (78%) were polymorphic revealing substantial genetic diversity within this species. Similarity value among all the *M. hexandra* accessions ranged from 0.52 to 0.82 with an average of 0.68. Dendrogram of 23 accessions revealed two main clusters. Principle Component Analysis (PCA) also showed almost similar grouping patterns with the dendrogram. The results obtained from morphological and RAPD analyseis indicated the presence of substantial genetic diversity among the *khirni* genotypes. The out-crossing nature of this species is responsible for the presence of high level of genetic diversity providing rich genepool for selecting genotypes with desirable characters for commercial exploitation. The most promising accessions identified based on desired horticultural attributes and RAPD analysis were IC-552938, IC-552940, IC-552918 and IC-552953. These accessions needs to be further evaluated and could be used either as direct selections or in the improvement of this fruit species.**

Key words: Genetic diversity, *Manilkara hexandra*, RAPD, Sapotaceae.

INTRODUCTION

Manilkara hexandra (Roxb.) Dubard is one of the important indigenous minor fruit species of India, belongs to family Sapotaceae (Stewart and Brandis, 1). It is locally known as *khirni*. In India, this species is generally distributed in western and central India especially in the parts of Madhya Pradesh, Gujarat, Rajasthan and Vidharbha region of Maharashtra as natural wild populations or grown occasionally in backyards and homestead gardens. Fruits of this tree species have high economical value as mature fresh fruits which are very sweet and consumed raw as well as after drying (Malik *et al.*, 2, 3). Fruit is a good source of minerals, sugars, protein, carbohydrates and vitamin-A. Fresh or dried fruits are consumed by local inhabitants (Malik *et al.*, 2, 3). This tree is commonly used as commercial rootstock for sapota. Its bark and leaves are also used for several medicinal purposes. The seeds contain approximately 25% oil, which is used for cooking purposes.

Germplasm utilization of underutilized fruit crops and devising vegetative propagation methods for selected high yielding genotypes have been the major constraints for popularizing these nutritious tropical fruits among the farmers (Arora and Rao, 4). Tropical fruit species are mostly heterozygous due to a high degree of out-crossing and require systematic morphological characterization backed by molecular characterization to study the extent of variability and utilization of existing germplasm. Phenotypic markers can identify accessions but most of the traits are environmentally affected. DNA based molecular markers are independent of environmental effects and provide direct information on the genome of each individual. Hence, molecular markers have been widely used for assessment of genetic diversity, cultivar identification, systematic and phylogeny in plants (Weising *et al*., 5; Bajpai *et al*., 6; Kumar *et al.*, 7; Singh and Singh, 8).

Recently, Malik *et al*. (3) characterized 47 diverse accessions of *M. hexandra* based on morphology and discussed its socio-economic importance and conservation needs. There has been no published report on molecular characterization of *M. hexandra* germplasm. Therefore, the present study has been undertaken to infer genetic diversity within *M. hexandra* using agro-morphological characters and RAPD marker.

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MATERIALS AND METHODS

Specific exploration and collection missions were undertaken in the west and central Indian states of Gujarat and Madhya Pradesh for the survey and collection of *M. hexandra* during 2008-10. A total of 23 accessions were collected from different districts of these two states (Table 1). Trees of this species are found in both wild and semi-wild states, growing in the forest areas, marginal forest lands, homestead gardens and farmer's fields. Collections were mostly made following selective sampling strategy where samples collected from single plant was given an indigenous collection number (IC number) and treated as individual accession. Leaf and fruit samples of each accession were collected from the natural population. Detailed passport information of each accession was registered in NBPGR database.

Twenty three *khirni* accessions have been characterized for fruit, seed and pulp characters to analyze the existing variability in this species. Data of fruit characters were taken in the field before bringing germplasm to the laboratory for recording of seed and pulp characters. Mean and standard error values for quantitative data were calculated using five fruits and seeds per accession (Table 2).

Total genomic DNA was extracted from young healthy leaves of all accessions using a modified protocol of Saghai-Maroof *et al.* (9). Quality and quantity of genomic DNA was estimated through 0.8% agarose gel electrophoresis and spectrophotometry. Polymerase chain reaction (PCR) conditions were optimized by verifying the concentration of template DNA, *Taq* DNA polymerase and $MgCl_2$. A total of 50 RAPD primers (Operon Technologies Alameda, USA) were screened in the *khirni* germplasm. Of these, 15 primers were selected for final analysis based on reproducibility and polymorphism. Reproducibility of the fingerprinting patterns was tested by running the reaction in duplicates. Each 25 µl reaction mixture contained 1x reaction buffer (100 mM Tris-HCl, pH

Table 1. Collection details of *M. hexandra* accessions used for morphological characterization and RAPD analysis.

IC No.	Locality	District	State	Latitude	Longitude	Altitude (m)
IC-552911	Choti Koral	Vadodara	Gujarat	21.83	73.20	27
IC-552914	Shukle Tirth	Bharuch	Gujarat	21.75	73.12	27
IC-552926	Gyanpura	Dhar	M.P.	22.38	75.40	134
IC-552927	Gyanpura	Dhar	M.P.	22.38	75.40	134
IC-552928	Gyanpura	Dhar	M.P.	22.38	75.40	134
IC-552931	Nandlal pura	Dhar	M.P.	22.32	75.40	573
IC-552935	Phulpura	Neemach	M.P.	24.47	75.28	487
IC-552938	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552939	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552940	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552941	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552942	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552943	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552944	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552945	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552946	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552947	Rampura	Neemach	M.P.	24.47	75.43	403
IC-552953	Palli	Dahod	Gujarat	22.82	73.98	504
IC-552957	CHES, Godhra	Godhra	Gujarat	22.68	73.52	123
IC-552961	Bhadurna	Vadodara	Gujarat	22.55	73.22	68
IC-552917	Bhalod	Bharuch	Gujarat	21.82	73.17	27
IC-552918	Bhalod	Bharuch	Gujarat	21.82	73.17	27
IC-552916	Bhalod	Bharuch	Gujarat	21.82	73.17	27

(M.P. - Madhya Pradesh)

Table 2. Details of some important morphological characters of 23 *M. hexandra* accessions.

IC No.	Fruit			Seed wt. (g)	Pulp wt. (g)
	L × W (cm)	Wt. (g)	TSS (°B)		
IC-552911	2.17 (±0.07)	1.40 (±0.10)	25.20 (±0.33)	0.18 (±0.01)	1.22 (±0.09)
IC-552914	5.08 (±0.40)	1.12 (±0.07)	30.20 (±0.08)	0.23 (±0.01)	1.29 (±0.25)
IC-552916	4.54 (±0.41)	1.11 (±0.12)	26.00 (±0.63)	0.20 (±0.01)	0.92 (±0.11)
IC-552917	2.36 (±0.08)	1.84 (±0.07)	29.00 (±0.40)	0.15 (±0.01)	1.69 (±0.06)
IC-552918	2.21 (±0.03)	1.64 (±0.10)	26.20 (±0.95)	0.14 (±0.00)	1.50 (±0.10)
IC-552926	0.54 (±0.10)	0.97 (±0.09)	23.00 (±2.28)	0.17 (±0.01)	0.80 (±0.09)
IC-552927	3.88 (±0.32)	1.20 (±0.08)	27.00 (±0.63)	0.17 (±0.01)	1.03 (±0.08)
IC-552928	2.47 (±0.29)	1.86 (±0.31)	28.00 (±1.10)	0.24 (±0.01)	1.62 (±0.30)
IC-552931	5.08 (±0.22)	1.12 (±0.08)	30.00 (±0.33)	0.23 (±0.01)	1.29 (±0.08)
IC-552935	6.18 (±0.47)	1.19 (±0.23)	28.00 (±1.10)	0.15 (±0.02)	1.04 (±0.22)
IC-552938	2.79 (±0.06)	1.82 (±0.14)	21.80 (±2.22)	0.15 (±0.01)	1.67 (±0.13)
IC-552939	2.17 (±0.40)	1.03 (±0.08)	30.00 (±0.00)	0.20 (±0.00)	0.83 (±0.08)
IC-552940	3.73 (±0.44)	0.95 (±0.06)	30.00 (±0.00)	0.14 (±0.01)	0.81 (±0.07)
IC-552941	3.29 (±0.30)	0.86 (±0.06)	19.00 (±1.67)	0.17 (±0.01)	0.69 (±0.06)
IC-552942	5.25 (±0.52)	1.02 (±0.14)	20.40 (±1.08)	0.11 (±0.02)	0.91 (±0.13)
IC-552943	2.33 (±0.20)	0.61 (±0.07)	30.00 (±0.00)	0.16 (±0.02)	0.49 (±0.07)
IC-552944	4.53 (±0.53)	1.78 (±0.26)	29.20 (±0.33)	0.22 (±0.01)	1.56 (±0.25)
IC-552945	2.04 (±0.06)	1.32 (±0.06)	29.40 (±0.54)	0.18 (±0.01)	1.14 (±0.05)
IC-552946	3.84 (±0.30)	1.41 (±0.20)	20.40 (±1.08)	0.18 (±0.01)	1.23 (±0.19)
IC-552947	2.63 (±0.22)	0.77 (±0.13)	29.40 (±0.36)	0.15 (±0.01)	0.62 (±0.12)
IC-552953	2.97 (±0.18)	0.74 (±0.09)	28.00 (±1.10)	0.15 (±0.01)	0.59 (±0.08)
IC-552957	5.68 (±0.19)	1.96 (±0.09)	29.20 (±0.33)	0.22 (±0.01)	1.74 (±0.09)
IC-552961	2.17 (±0.07)	1.40 (±0.10)	25.20 (±0.33)	0.18 (±0.01)	1.22 (±0.09)

8.3 and 500 mM KCl), 2 mM $MgCl_2$, 1U *Taq* DNA polymerase, 0.2 mM dNTP each (Promega, USA), 0.6 µM of primer and 20 ng template. PCR reactions were carried out in a DNA thermalcycler (Gene amp 9600 PCR system Perkin-Elmer Cetus, Norwalk CT). The PCR amplification conditions were as follows: Initial denaturation step at 94°C for 3 min. followed by 40 cycles of denaturation at 94°C for 1 min., annealing at 35°C for 1 min., and extension at 72°C for 1 min. followed by final extension at 72°C for 5 min. The amplified products were electrophoresed on 1.8% agarose gel at 100 v followed by staining with ethidium bromide and photographed on Polaroid 667 film under ultra-violet light.

Amplified bands were scored as present (1) or absent (0) across all the accessions. Molecular weight of the amplified bands was estimated by using 1 kb DNA ladder (Gibco BRL Life Technologies New York, USA) as standard. A pairwise similarity matrix of all the accessions was estimated based on Jaccard's coefficient (Jaccard, 10) and a dendrogram was generated based on the unweighted pair group method for arithmetic mean (UPGMA) using software NTSYS ver. 2.10e (Rohlf, 11). Principal Component Analysis (PCA) was also carried out to study relationship among accessions using same software. Bootstrap analysis of the dendrogram was done using software FreeTree ver. 0.9.1.50 (Pavlicek *et al.*, 12).

RESULTS AND DISCUSSION

A large variability within *khirni* germplasm was recorded in almost all the characters studied (Table 2). Fruit size (length × width) ranged from lowest value of 0.85 cm × 0.28 cm (IC-552926) to highest values of 2.5 cm × 2.82 cm (IC-552935). Fruit weight of smallest fruit was 0.61 g (IC-552943) and highest was more than three times, 1.96 g (IC-552957). TSS value ranged from 19° (IC-552941) to 30.2°B (IC-

522914), which is twice that of lowest value. Seed weight showed variation as smallest seeds weighed 0.11 g (IC-552942) and heaviest weighed 0.24 g (IC-552928). The pulp weight had large variation as it ranged from 0.49 (IC-552943) to 1.74 g (IC-552957), a variation which is about seven times (Table 2).

In the RAPD marker analysis, a total of 119 bands were generated in 23 accessions of *M. hexandra* based on 15 RAPD primers. Of these, 103 bands were polymorphic (78%) (Table 3). Polymorphism within the *khirni* germplasm was found to be significantly high, which reveals a substantial amount of genetic diversity within this species. Representative gel profiles of *M. hexandra* generated based on RAPD primers. Size of amplified fragments within the *khirni* germplasm ranged from 250-2300 bp. The range of amplified bands was between 2 to 11 with an average number of 7.39 bands per primer. OPA-13 primer gave maximum number of amplified bands (11), while lowest number (4) was generated by OPM-6. Average number of polymorphic bands per primer was 6.87, while highest polymorphism (100%) was revealed by OPA-11 and OPA-13 primers. OPO-16 primer was able to generate accession specific unique band (1150 bp) in IC-552941 collected from Rampura, Neemach district of Madhya Pradesh (MP). This accession is more genetically divergent from the rest of the accessions and can be utilized for broadening the genetic base of this species through crop improvement programmes.

Similarity values among all the *M. hexandra* accessions ranged from 0.52 to 0.82 with an average of 0.68 (Table 4). Maximum similarity (0.82) was recorded between accessions IC-552941 and IC-552942. Both accessions were collected from the same localities of Rampura, Neemach district of MP. However, minimum genetic similarity (0.52) was found between IC-552935 and IC-552938 accessions, which were collected from the Phulpura and Rampura, respectively of Neemach district of MP. In the UPGMA dendrogram, all the 23 accessions of *khirni* divided into two main clusters with high bootstrap value (82%) (Fig. 1). The first cluster contained 19 accessions which were divided into two sub-clusters at a similarity value of 0.67. In sub-cluster I, two accessions, *i.e.* IC-552941 and IC-552942 showed the highest similarity (82%).

Both accessions also have lowest TSS values. Similarly, IC-552926 and IC-552927 accessions grouped together with 81% similarity in the dendrogram showing close genetic relationship. These accessions were similar in seed weight (0.17 g). In sub-cluster II, accession IC-552940 collected from Rampura of Neemach district was very distinct from the other accessions, had high TSS value (30°B). Cluster II

Table 3. Details of amplified bands generated in 23 *M. hexandra* accessions using 15 RAPD primers.

Primer	Sequence 5' to 3'	Total No. of bands	Total No. of polymorphic bands	Polymorphism (%)	Fragment size range (bp)
OPA-11	CAATCGCCGT	8	8	100	400-1400
OPA-13	CAGCACCCAC	11	11	100	600-1800
OPA-17	GACCGCTTGT	7	6	85.71	560-1300
OPC-1	TTCGAGCCAG	10	8	80.00	350-1350
OPC-11	AAAGCTGCGG	10	8	80.00	400-1700
OPC-12	TGTCATCCCC	11	10	90.90	470-1300
OPC-18	TGAGTGGGTG	10	9	90.00	600-1300
OPD-2	GGACCCAACC	9	6	66.67	250-1350
OPD-5	TGAGCGGACA	9	8	88.89	400-2300
OPD-15	CATCCGTGCT	7	4	57.14	400-1100
OPM-5	GGGAACGTGT	5	4	80.00	300-900
OPM-6	CTGGGCAACT	4	2	50.00	620-1200
OPM-7	CCGTGACTCA	7	3	42.86	1000-2000
OPO-12	CAGTGCTGTG	10	7	70.00	375-1100
OPO-16	TCGGCGGTTC	11	9	81.82	520-1300
Total		129	103		
Avg.		8.6	6.87	79.84	

Table 4. A pairwise similarity matrix of 23 *M. hexandra* accessions based on RAPD data.

Accn. No.	IC552911	IC552914	IC552926	IC552927	IC552928	IC552931	IC552935	IC552938	IC552939	IC552940	IC552941	IC552942	IC552943	IC552944	IC552945	IC552946	IC552947	IC552953	IC552957	IC552961	IC552917	IC552918	IC552916
IC552911																							
IC552914	0.78																						
IC552926	0.70	0.65																					
IC552927	0.69	0.68	0.81																				
IC552928	0.65	0.63	0.69	0.71																			
IC552931	0.66	0.66	0.69	0.76	0.69																		
IC552935	0.65	0.68	0.60	0.66	0.55	0.76																	
IC552938	0.63	0.57	0.52	0.57	0.55	0.53	0.52																
IC552939	0.77	0.68	0.69	0.70	0.66	0.66	0.63	0.69															
IC552940	0.69	0.63	0.64	0.65	0.57	0.65	0.67	0.64	0.71														
IC552941	0.77	0.65	0.74	0.75	0.67	0.71	0.70	0.59	0.79	0.74													
IC552942	0.76	0.69	0.70	0.71	0.66	0.72	0.72	0.59	0.75	0.71	0.82												
IC552943	0.67	0.64	0.69	0.71	0.65	0.68	0.66	0.62	0.73	0.70	0.71	0.79											
IC552944	0.70	0.63	0.68	0.68	0.70	0.66	0.65	0.59	0.75	0.71	0.76	0.75	0.75										
IC552945	0.69	0.64	0.71	0.76	0.65	0.74	0.74	0.54	0.71	0.69	0.76	0.78	0.74	0.77									
IC552946	0.67	0.63	0.69	0.75	0.65	0.74	0.72	0.53	0.59	0.68	0.70	0.69	0.66	0.67	0.74								
IC552947	0.63	0.60	0.67	0.72	0.64	0.65	0.61	0.59	0.71	0.62	0.69	0.68	0.71	0.74	0.75	0.66							
IC552953	0.60	0.58	0.63	0.64	0.60	0.63	0.71	0.55	0.68	0.65	0.66	0.68	0.62	0.70	0.69	0.70	0.67						
IC552957	0.68	0.62	0.68	0.72	0.67	0.75	0.72	0.55	0.72	0.64	0.77	0.71	0.69	0.75	0.78	0.75	0.75	0.71					
IC552961	0.67	0.63	0.71	0.74	0.74	0.67	0.66	0.57	0.72	0.64	0.74	0.75	0.72	0.73	0.75	0.66	0.74	0.66	0.75				
IC552917	0.65	0.60	0.64	0.67	0.62	0.69	0.67	0.63	0.66	0.61	0.64	0.69	0.67	0.69	0.72	0.67	0.71	0.67	0.76	0.71			
IC552918	0.62	0.59	0.63	0.62	0.61	0.64	0.61	0.58	0.67	0.61	0.65	0.67	0.62	0.67	0.62	0.56	0.66	0.66	0.65	0.67	0.75		
IC552916	0.64	0.61	0.66	0.70	0.77	0.73	0.62	0.55	0.70	0.63	0.70	0.71	0.68	0.73	0.67	0.66	0.67	0.64	0.67	0.69	0.67	0.71	

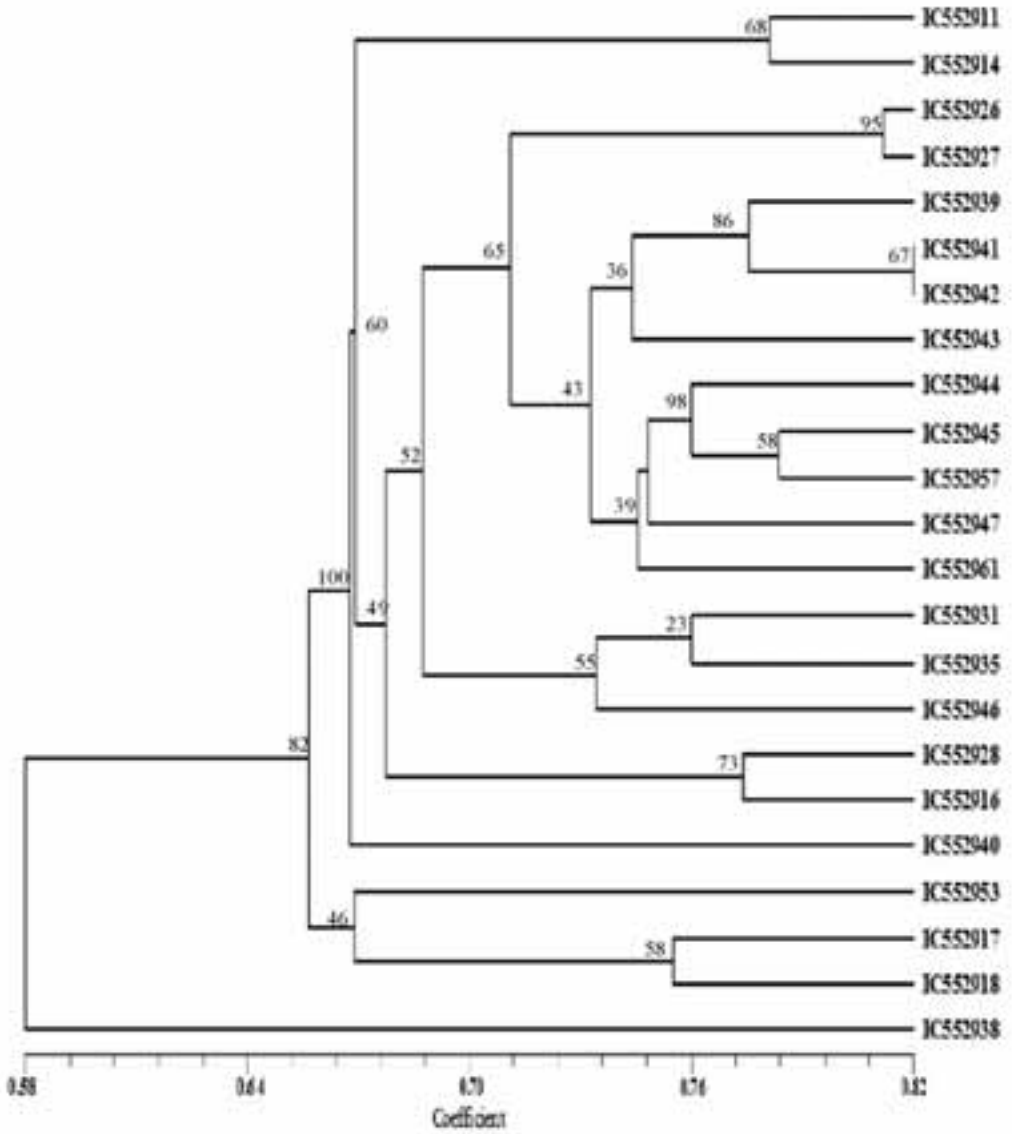

Fig. 1. UPGMA dendrogram of 23 *M. hexandra* accessions generated based on RAPD data. Numbers at the node are bootstrap values based on 500 resampling.

consisted of three accessions (IC-552917, IC-552918 and IC-552953) which were collected from Dahod and Bharuch districts of Gujarat. Accession IC-552938 was distinctly separated from all accessions in the RAPD dendrogram with similarity value of 0.58. It was collected from Rampura, Neemach district of MP, and was the most distinct from other accessions. With respect to morphology its fruit weight was the fourth highest (1.82 g) and pulp weight is also third highest (1.67 g).

The genetic relationships among the 23 genotypes were also revealed by Principle Component Analysis (PCA), which showed almost similar grouping patterns with dendrogram (Fig. 2). In 2-D plot, IC-552938 was the most diverse as it was distinctly separated in the dendrogram. The results obtained from RAPD analysis clearly confirmed the substantial genetic diversity occurred among the *khirni* genotypes. The out-crossing nature of *khirni* should be responsible for high levels of genetic diversity detected by RAPD markers. The most promising accessions identified based on desired horticulture attributes and RAPD analysis, were IC-552938, IC-552940, IC-552918 and IC-552953. These accessions need to be further evaluated and could be used either as direct selections or in the improvement programme of this fruit species.

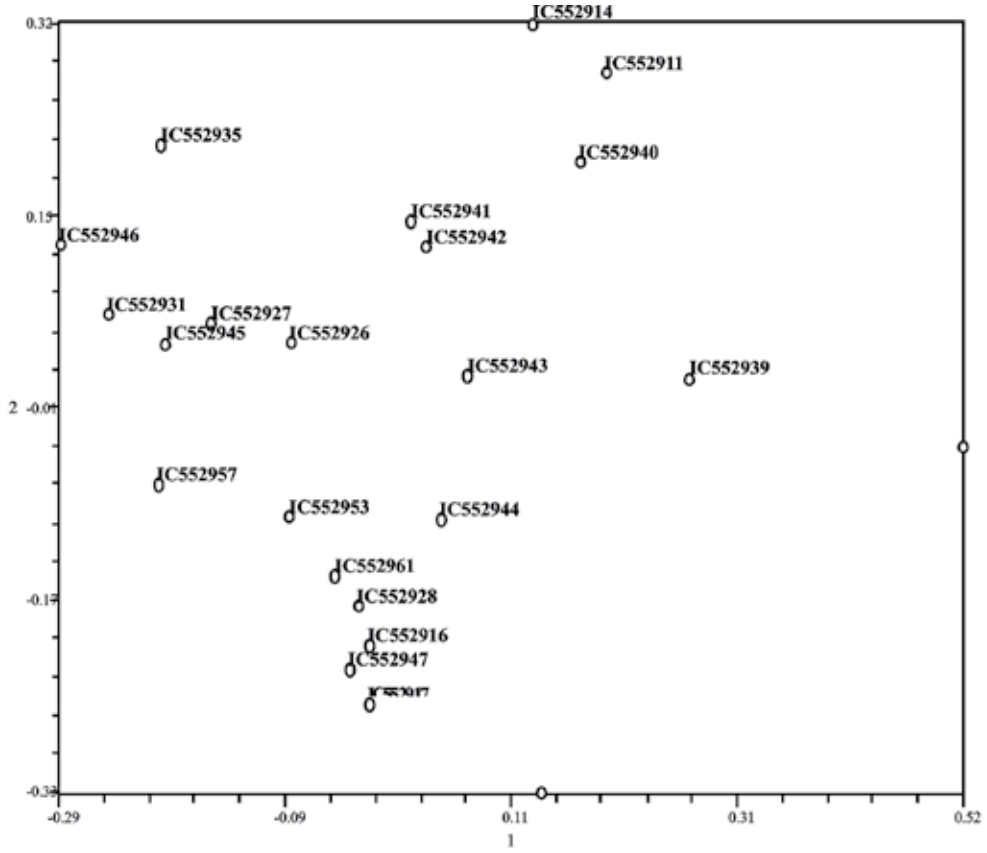

Fig. 2. 2-D plot of 23 *M. hexandra* accessions generated based on Principle Component Analysis.

Substantial genetic diversity was found within *M. hexandra* accessions revealed by RAPD markers. The information obtained from this study may be useful for better management of genetic resources of this important fruit species, identification of promising genotypes for popularization and understanding the extent of genetic diversity present in this species.

ACKNOWLEDGEMENTS

Authors are thankful to the Director, National Bureau of Plant Genetic Resources, New Delhi for support.

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Received: October, 2011; Revised: November, 2012;
Accepted: December, 1012