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Analysis of molecular diversity in okra (*Abelmoschus esculentus*) genotypes using RAPD markers

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ABSTRACT

Okra [*Abelmoschus esculentus* (L.) Moench] genotypes were sown during *kharif* 2011-12 at Horticultural Research Centre, Department of Horticulture, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, India, developed and maintained for using conventional agronomically practices to keep the crop in good condition. Study of morphological and molecular diversity in landrace collections was carried out during 2011-12. Germplasm collections of all the 20 diverse genotypes of okra were screened with 20 RAPD primers in order to determine genetic identities, genetic diversity and genetic relationships. On the basis of molecular characterization, the number of amplified alleles observed ranged from minimum 2 alleles to maximum 9 alleles. Similarity value for all the 20 genotypes of okra ranged from 0.486 to 0.669. The lowest similarity was displayed by Azad bhindi1 (AB 1) and VRO5 with the similarity coefficient value 0.486. Out of these samples analyzed, genotypes Azad Bhindi 2 and FB 10 displayed the greatest genetic similarity, with a similarity coefficient value of 0.669. All genotypes were distributed into four main distinct clusters. Cluster analysis clearly showed the genetic diversity among the genotypes under study.

Key words: Alleles, Cluster analysis, Genetic diversity, Okra, PCR, RAPD Primers

Okra [*Abelmoschus esculentus* (L) Moench] is also called bhindi and lady's finger is an upright annual grown in *kharif* and *zaid* seasons, herbaceous 3 to 8 ft tall plant. It is always an interesting crop for breeder to work. Its chromosome number 2n=130 and belongs to the Malvaceae family. Okra requires a long, warm and humid growing period. It can be successfully grown in hot humid areas. It is sensitive to frost and extremely low temperatures. For normal growth and development a temperature between 24° and 28°C is preferred. A pH of 6.0–6.8 is ideally-suited. All soils need to be pulverized, moistened and enriched with organic matter before sowing (GOI 2011). Almost all parts of okra plant are consumed, like fresh okra fruits are used as vegetable, eaten boiled or in culinary preparations as sliced and fried pieces, roots and stem are used for clearing the cane juice for making of gur and leaves and stems are used for making fiber and ropes. Okra seeds containing good quality edible oil and high protein are used to complement other protein sources. The okra pods contain mucilage, which is comprised of a mixture of pectin and carbohydrates, which is used as a thickener in food industries. Okra flour is an effective food additive in wheat flour for baking bread with good technological and sensory characteristics. Its stem is also used for paper making in paper industries. It is also used in thickening of soups and gravies because of its high mucilage content. Okra fruits are also sliced and sun-dried or canned and pickled for off-season use. It has good nutritional value, particularly rich in vitamins (30 mg/100 g), calcium (90 mg/100 g) and iron (1.5 mg/100 g). It is an excellent source of iodine and useful for the control of goiter disease (Singh *et al.* 2013, Gul *et al*. 2011, Goswami *et al*. 2015 and Saifullah 2009). The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics. Various types of molecular markers differing in their principles and methodologies are available such as AFLP, SSR, RFLP and RAPD (Kaur *et al*. 2013 and Kong *et al*. 2011). Molecular markers have proven to be powerful tools in the assessment of genetic variation and evaluation of genetic relationships within and among species (Azmat and Khan 2010, Semagn *et al*. 2006). A DNA marker derived from a small region of DNA that shows sequence polymorphism between individuals within or between species. DNA markers which are phenotypically neutral have allowed scanning of the whole genome in many crop plants including okra. Study of phenotypic and genetic diversity in germplasm collection is

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important for germplasm conservation. In addition, the characterization of much diversified materials with molecular markers offers a unique opportunity to define significant marker trait association of biological and agronomic interest but these markers are highly influenced by environmental factors (Saifullah *et al.* 2010). To our knowledge, scarcely studies have been focused on studying the genetic diversity at DNA level of okra benefiting from the advantages of RAPD. Therefore this research aims to estimate the genetic relationship between okra genotypes from different agro-ecological regions using the RAPD primers (Azmat 2012). Knowledge about the nature and magnitude of variation existing in available breeding materials are requisite to choose the characters for effective selection of desirable genotypes to undertake planned breeding programme. Further, to improve the productivity, information about the nature and magnitude of genetic divergence would help selection of diverse effective gene recombination. The available literature reveals that breeding programme on the basis of variability and diversity can help in okra improvement. The present investigation was, therefore, undertaken to evaluate the genetic variability for different characters and diversity of genotypes for identification of suitable parents for the okra improvement (Pradip *et al*. 2010, Upadhyay 2004 and Singh 2012).

MATERIALS AND METHODS

The experimental material of the present investigation comprised 20 genetically diverse genotypes of okra. All cultivars were collected from core collection maintained and grown at Horticultural Research Centre, Department of Horticulture, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut (UP), India. Premature leaves were collected from all plants during 2011-12 *kharif* season. Then, leaf samples were packed into sterilized plastic polybags and stored at -80ºC in deep freezer for the purpose of isolation of genomic DNA and molecular analysis was done in Molecular Laboratory and for confirmation of results the experiment maintain in next year 2012-13. The details of the genotypes are given in Table 1.

Table 1 The details of all twenty diverse genotypes of okra included in the present study

Variety	Source	Variety	Source
IC218872	IIVR, Varanasi	C11, HR4	IIVR, Varanasi
IC306053	IIVR, Varanasi	FB10	IIVR, Varanasi
IC11527	IIVR, Varanasi	VRO1668	IIVR, Varanasi
EC169367	IIVR, Varanasi	VRO238	IIVR, Varanasi
SC108	IIVR, Varanasi	Parbhani Kranti (PK)	Parbhani
VRO5	IIVR, Varanasi	SKY/TD/RS113	NBPGR, New Delhi
C7801	IIVR, Varanasi	KS442	CSAUAT, Kanpur
BO2	IIVR, Varanasi	Azad bhindi1 (AB1)	CSAUAT, Kanpur
VRO3	IIVR, Varanasi	Azad bhindi-3 (AB3)	CSAUAT, Kanpur
IC69302	IIVR, Varanasi	KS310	CSAUAT, Kanpur

After attaining the proper growth the fresh leaves of each variety ware collected and wrapped in an aluminum foil. The packed leaf tissue were then stored in (-80^{0}C), deep freezer. These samples were maintained under appropriate conditions in the lab, for extraction of DNA for further experiments (by Doyle and Doyle 1990). For isolation of DNA, 3 g fresh leaf tissue was taken and grounded to fine powder with a chilled mortar and pestle using liquid nitrogen and transferred to a centrifuge tube containing 15 ml of pre-warmed (60ºC) CTAB-extraction buffer. The sample was mixed well by inverting the tubes several times and incubated for 1 hr in a shaking water bath (NSW, India) at 65ºC. An equal volume of Chloroform: Isoamylalcohol (24:1) was added to the tube and mixed gently for 15 min. The tubes were centrifuged in cooling centrifuge (CPR 24, Remi, India) at 8 000 rpm for 10 min at room temperature using a R-242, fixed angle rotor (Remi, India). After centrifugation, aqueous phase was transferred to a fresh tube and again extracted with an equal volume of Chloroform: Isoamylalcohol (24:1). Aqueous layer was transferred to the fresh tube and 1.5 ml of NaCl (5 M) was added and mixed gently. RNase treatment was performed by adding 5 µl of RNase (10 mg/ml) and carrying out incubation for 30 minutes at room temperature. For precipitating DNA, 0.6 volume of chilled Isopropanol was added to the tube and incubated for 2 h. The tubes were then centrifuged at 10 000 rpm for 10 min. The supernatant was discarded and 5 ml of 70% ethanol was added for washing. The tubes were centrifuged at 7 500 rpm for 10 min. The supernatant was discarded and pellet was air-dried and dissolved in 100 µl of TE buffer. The extracted DNA was subjected to an additional step of purification. For this 1 µl of RNase A (10 mg/ml) was added to 100 µl of DNA solution, and incubated at 37ºC for 1 hr. The DNA was again extracted with equal volume of Chloroform : Isoamylalcohol (24:1). The aqueous layer was separated and 0.6 volume, chilled solution of ethanol-NaOAc was added. The reaction mixture was incubated for 10 min at –20ºC deep freezer, followed by centrifugation in a cooling centrifuge (CPR 24, Remi, India) at 10 000 rpm for 10 min using a fixed angle rotor R-248 (Remi, India). The pellet was washed with 70% ethanol, air-dried and dissolved in 100 µl TE buffer and stored at 4ºC for further experiments.

DNA amplification for RAPD primers were performed a total volume of 20 µl reaction volume containing 5µl DNA, 2µl Taq buffer 10x with $MgCl_2$, 4µl dNTP mix (10mM), 4µl primer (10mM), 2µl of Taq DNA polymerase (3u/µl) and 3µl double distilled water. The mixture was mixed by spinning for a short time and placed in Eppendorf Master cycler gradient for amplification. The cycling conditions were- 1 cycle (initial denaturation) 94^{0}C for 5 min., 45 cycles of 94^{0}C for 1 min. (denaturation), annealing of primers at 36^{0}C for 1 min., extension at 72^{0}C for 2 min. and a final extension at 72^{0}C for 10 min. (Annealing

temperature differed primer to primer). RAPD-PCR products were run for electrophoretic analysis on 1.2% agarose gel stained with Ethidium bromide and analysis of gel was done under gel-doc system. Twenty okra genotypes were used to estimate genetic diversity. Polymorphic products from RAPD primers were calculated for presence (1) or absence (0) across all okra genotypes for each primer. The pair-wise genetic similarities among all pairs of samples were estimated with Jaccard's coefficient (Jaccard 1908). The statistical analysis were carried out and RAPD data used to make pair-wise comparison of the genotypes based on shared and unique amplification products to generate a similarity matrix by using NTSYS-PC software (version 2.11s) (Rohlf 2000). In order to group genotypes into discrete clusters a dendrogram was constructed by employing UPGMA method (Sneath and Sokal 1973).

RESULTS AND DISCUSSION

DNA based markers are effective and reliable tools for measuring genetic diversity in crop germplasm and studying evolutionary relationship. Molecular genetics techniques using DNA polymorphism is increasingly used to characterize and identify a novel germplasm for uses in the crop breeding process. But there is a lack of information on comparative evaluation of molecular markers in vegetable crops. For DNA fingerprinting, the most important consideration is the marker system to be used. The present study addresses potential use of PCR-based molecular markers (RAPD) for assessing the genetic diversity among all genotypes of okra. The genomic DNA from all diverse genotypes of okra was isolated using the CTAB method. For estimating the size of total genomic DNA the standard molecular weight marker of 100bp was loaded. Though the plant species are same, the variation in molecular weight of genomic DNA was observed. This variation may be due to hardness and dryness of leaf material. One possible reason for this variation may be due to incomplete homogenization. The variation in molecular weight values in different genotypes is quite understandable, as each genotype is known to have its own unique length of DNA.

Molecular profiling: To assess the genetic diversity of all 20 genotypes of okra, 20 RAPD primers were used to amplify the genomic DNA. Maximum primers resulted in polymorphic, scorable and reproducible results whereas some primers showed lower amplification or no amplification. The following two reasons may be attributed for the lower amplification of primers (i) the appropriate complementary sequence may occur infrequently in okra genome; or (ii) the corresponding primer sites may be distantly located in okra DNA in such a way that lower amplification occurred. All of them were found to be polymorphic hence considered for genetic diversity analysis. The number of polymorphic primers and fragments generated were not in similar range for all genotypes. They show vary significantly in different genotypes. This was understandable as product amplified depends upon the sequence of RAPD primers and their compatibility within genomic DNA. The number of markers detected by each primer depends on primer sequence and the extent of genetic variation, which was genotype specific (Azmat 2012 and Upadhyay *et al*. 2004).

Genetic similarity matrix and cluster analysis

The total of 20 primers used for amplification of 20 diverse genotypes of okra. The entire fragments generated in this way were polymorphic (Table 2). Thus, the higher percent polymorphism suggested that the RAPD procedure is a viable approach for the examination of genetic diversity. The number of amplified alleles observed ranged from minimum 2 alleles to maximum 9 alleles. Similarity value for all the 20 genotypes of okra ranged from 0.486 to 0.669. The lowest similarity was displayed by Azad Bhindi 1 (AB 1) and VRO5 with the similarity coefficient value 0.486. Out of 20 samples analyzed, genotypes Azad Bhindi 2 and FB 10 displayed the greatest genetic similarity, with a similarity coefficient value of 0.669 (Table 3). The resultant dendrogram grouped the 20 genotypes into four main distinct clusters. The cluster-I grouped into six genotypes, viz. IC 218872, Azad Bhindi 2, IIHR 4, SKY/TD/RS113, FB 10 and IC 69302. The main cluster was subdivided into two sub-clusters, first sub cluster show two genotypes namely IC 218872 and Azad Bhindi 2. Second sub-cluster showed four genotypes namely IIHR 4, SKY/TD/RS113, FB 10 and IC 69302. The cluster-II grouped into eight genotypes, viz. EC 169367, C7801, Parbhani Kranti, VRO238, VRO3, BO2, VRO5 and VRO1668. The main cluster was subdivided into two sub-clusters, first sub cluster divided into six

Table 2 List of 20 RAPD Primers that showed polymorphism (%) in okra genotypes

Primers name	No of alleles	Polymorphic band	Polymor-phism (%)	Total no. of bands
OPM-16	6	20	60.60	33
OPM-18	3	9	45.00	20
OPX-14	3	9	34.61	26
OPX-17	7	24	42.10	57
OPX-18	2	10	83.33	12
OPY-02	6	16	27.58	58
OPY-03	2	2	12.50	16
OPY-04	3	12	54.54	22
OPY-17	3	8	28.57	28
OPY-18	5	12	32.43	37
OPD-05	4	9	23.68	38
OPD -07	5	16	34.78	46
OPD -08	5	8	19.04	42
OPD -09	4	9	22.50	40
OPD -11	3	3	8.57	35
OPB-15	2	3	12.50	24
OPA-17	3	5	20.83	24
OPC-11	5	10	27.02	37
OPJ-01	5	11	28.20	39
OPJ-07	9	17	25.37	67
Average	4.25	10.65	32.1875	35.05

Table 3 Pairwise similarity matrix of 20 genotypes of okra

	IC 218872	IC 306053	IC 11527	EC 169367	SC 108	VR O5	C7 801	BO2	VRO3	KS 310	C11 HR4	IC 69302	SKY/TD/ RS113	FB 10	KS 442	VRO 1668	VRO 238	PK	AB1	AB3
IC218872	1.000																			
IC218872	0.579	1.000																		
IC218872	0.532	0.601	1.000																	
IC218872	0.586	0.583	0.608	1.000																
IC218872	0.540	0.601	0.662	0.622	1.000															
IC218872	0.547	0.594	0.525	0.601	0.568	1.000														
IC218872	0.610	0.563	0.574	0.646	0.653	0.625	1.000													
IC218872	0.540	0.536	0.554	0.594	0.547	0.576	0.606	1.000												
IC218872	0.554	0.543	0.554	0.565	0.532	0.554	0.625	0.576	1.000											
IC218872	0.572	0.504	0.543	0.561	0.514	0.543	0.603	0.565	0.550	1.000										
IC218872	0.597	0.579	0.583	0.615	0.568	0.590	0.606	0.561	0.568	0.608	1.000									
IC218872	0.594	0.540	0.565	0.604	0.558	0.507	0.578	0.536	0.529	0.576	0.615	1.000								
IC218872	0.633	0.565	0.583	0.601	0.561	0.597	0.588	0.554	0.525	0.529	0.633	0.658	1.000							
IC218872	0.619	0.522	0.583	0.586	0.518	0.583	0.574	0.547	0.540	0.500	0.633	0.601	0.669	1.000						
IC218872	0.615	0.532	0.579	0.576	0.594	0.550	0.603	0.550	0.514	0.540	0.594	0.590	0.579	0.637	1.000					
IC218872	0.604	0.550	0.561	0.565	0.547	0.626	0.596	0.561	0.554	0.507	0.590	0.565	0.604	0.561	0.608	1.000				
IC218872	0.543	0.576	0.594	0.604	0.529	0.565	0.592	0.586	0.601	0.583	0.558	0.568	0.586	0.572	0.590	0.622	1.000			
IC218872	0.576	0.514	0.554	0.622	0.576	0.583	0.661	0.590	0.626	0.565	0.568	0.572	0.612	0.576	0.622	0.633	0.622	1.000		
IC218872	0.550	0.518	0.536	0.568	0.522	0.486	0.560	0.493	0.572	0.561	0.536	0.511	0.536	0.522	0.568	0.543	0.547	0.622	1.000	
IC218872	0.558	0.532	0.529	0.619	0.536	0.572	0.603	0.572	0.572	0.547	0.550	0.518	0.586	0.586	0.619	0.536	0.576	0.601	0.633	1.000

Table 4 Cluster analysis based on genetic distance and UPGMA dendrogram on Jaccard's similarity coefficient, from RAPD analysis of 20 okra genotypes

Cluster	No of genotypes	Genotypes code	Name of genotypes
I	6	1, 15, 11, 13, 14 and 12	IC 218872, Azad Bhindi 2, IIHR 4, SKY/TD/RS 113, FB 10 and IC 69302
II	8	4, 7, 18, 17, 9, 8, 6 and 16	EC 169367, C 7801, Parbhani Kranti, VRO 238, VRO 3 and BO 2VRO 5 and VRO 1668
III	3	2, 3 and 5	IC 306053, IC 11527 and SC 108
IV	3	19, 20 and 10	Azad Bhindi 1 and Azad Krishna KS 312

genotypes namely EC169367, C7801, Parbhani Kranti, VRO238, VRO3 and BO2. Second sub-cluster divided into two genotypes namely VRO5 and VRO1668. The cluster-III grouped into three genotypes, viz. IC 306053, IC 11527 and SC 108. The main cluster was subdivided into three genotypes namely IC 306053, IC 11527 and SC 108. The cluster-IV grouped into three genotypes, viz. Azad Bhindi 1, Azad Krishna and KS 312. The main cluster was subdivided into two sub-clusters, first sub-cluster was divided into two genotype namely Azad Bhindi1 and Azad Krishna. Second sub cluster was divided into one genotypes namely KS312. The genotypes namely Azad bhindi1 (AB1) and VRO5 showed significant genetic diversity with a coefficient value of 0.486. Similar results show by Schafleitner *et al.* (2013), Aladele *et al.* (2008), Kaur *et al.* (2013), Salameh and Kasrawi (2011), Reddy *et al.* (2012) and Salameh (2014) in okra.

Genetic similarity matrix value for all the 20 genotypes of okra ranged from 0.486 to 0.669. The lowest similarity was displayed by Azad bhindi 1 (AB 1) and VRO5 with the similarity coefficient value 0.486. Out of 20 samples analyzed, genotypes Azad Bhindi2 and FB10 displayed the greatest genetic similarity, with a similarity coefficient value of 0.669. Thus, from the present study it can be concluded that genotypes namely Azad bhindi1 (AB1) and VRO5 showed significant genetic diversity. These genotypes may be exploited by effective crossing between these genotypes to obtain desirable segregates for further selection of superior lines in early stages of crop growth itself by exploiting the genetic distance from molecular marker data which helps to identify genotypes for mapping populations and also to identify molecular markers linked to desirable traits by marker assisted selection (MAS) (Aladele *et al.* 2008).This showed that climatic conditions and physical parameters may affect the plant genome as the plant is adapted and these changes are inherited through genome to the next generation. The range of genetic diversity values broadly indicates the degree of heterogeneity or homogeneity in different genotypes of the plant species (Prakash *et al.* 2011). Cluster analysis clearly indicated that Human intervention, which makes partitioning and distribution of variability complex is cited as reason for the grouping of samples to one cluster suitable for different regions. The present study suggested that RAPD is appropriate for analysis of genetic variability in closely related genotypes. Moreover, RAPD could able to amplify the different loci of all the 20 genotypes of okra. Respective genes responsible for some crucial components of quality and adaptation greatly influenced clustering of varieties and seem to be tightly linked with molecular markers. Similar results show by Schafleitner *et al.* (2013), Aladele *et al.* (2008), Kaur *et al.* (2013), Salameh and Kasrawi (2011), Reddy *et al.* (2012), Salameh (2014) in okra.

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