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MOLECULAR CHARACTERIZATION AND PHYLOGENETIC RELATIONSHIP OF DIFFERENT CITRUS VARIETIES OF PAKISTAN

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ABSTRACT

Genetic relationship among 17 citrus commercially important cultivars, including local and some hybrid varieties of Pakistan was evaluated by RAPD (Random Amplified Polymorphic DNA) markers. Fifteen decamer primers were selected out of 25 primers that produced 153 polymorphic bands with 13bands per primer on an average with a total of 188 bands. To analyze the genetic similarity or dissimilarity Jaccard Coefficients was used. The dendrogram that separated 17 citrus genotypes into 4 main clusters was generated by using UPGMA. Across all the genotypes the genetic similarity value observed were 0.66 with the two cultivars of Kinnow and Seedless kinnow (Mandarin) and two cultivars of Meiwa and Marumi (Kumquat) showing maximum similarity value (0.85).Genetic difference among the citrus varieties was high that depicted that they do not belong to similar origin.

Key words: Genetic diversity, Citrus varieties, RAPD, Polymerase chain reaction, phylogenetic relationship.

INTRODUCTION

Citrus (*Citrus sinensis* L.) is one of the most significant fruit crop known from antiquity being a very good source of vitamin "C" with great antioxidant potential (Gorinstein *et al*., 2001). Citrus is the member of family Rutaceae that comprises about 158 genera and 1900 species. It includes perennials that produce fruits of variable sizes. Citrus is placed among the important fruit crops worldwide being sown in more than 125 countries between latitude 35°-36° with suitable climates and in temperature range of 4^oC-50^oC. Citrus fruit appeared on globe 30 million years ago. Citrus cultivation is considered to have started in Nippur (Mesopotamia) that dates back to 4000 B.C. (Mab-berley, 2008).

Internationally, citrus production has reached up to 122 million tons (FAO, 2008). Fruits are used to prepare many by products besides their consumption as fresh (Whitney and Rolfes, 1999). Due to its fruit quality and ornamental value citrus cultivation has flourished. In terms of production of fruit and area under cultivation, citrus has the leading position among other fruit crops in Pakistan. Most of the citrus production in Pakistan (80%) is reliant on mandarins (Kinnow) dominates production together with another cultivar as Feuterell's early. Pakistan is the sixth largest producer of Kinnow (mandarin) and oranges, generating 95% of the world Kinnow production. Pakistan citrus industry is contributing 1% of fresh fruit from 2% area of cultivation yielding approximately 12.7 tons per hectare. Citrus is contributing 25% fruit production, of which Punjab's share is 95% (Anonymous, 2010).

Molecular markers based on DNA sequences proved to be an ideal means for the identification and estimation of relatedness among genotypes of different origin. Different markers based on DNA sequences have been developed in the previous years. For DNA finger-printing and genome mapping, Restriction fragment length polymorphism (RFLPs) was considered to be first molecular marker (Nahn *et al*., 2001). By using markers based on PCR, the repertoire of genotype identification has been transformed. RAPD markers produced as a result of the DNA amplification with short oligonucleotides as primers provide a quick and relatively easy method for the identification and classification of fruit trees. This approach has been proved to be advantageous of being excluding the demand blotting and radioactive detection and requiring small amounts of genomic DNA (Cipriani *et al.*, 1996).

For the protection of the breeder's benefit lines and documentation of genetic resources, varietal identification is very essential (Selbach and Cavalli-Molina, 2000). Analysis of genetic diversity for the identification of groups with comparable genotypes is very essential to evaluate and conserve. The utilization of genetic resources as a potential basis of genesfor the germplasm identification may be capable forthe improvementin cultivars performance and for the determination of genotypic and phenotypic expression of genotypes (Subudhi*et al.,* 2002; Nemera *et al*., 2006). Diversity studies would also be desirable for the purpose of better management and conservation of the genetic resources and for planning the breeding strategies (Badenes *et al*., 2000).

Besides the Citrus being the most important fruit of Pakistan, no work has been reported on the evaluation of genetic diversity so far. Keeping in view the importance of citrus crop the presentstudy isaimed to identifythe genetic variation and evaluate the inter-

relationships among 17 commercially important citrus varieties by using RAPDs (primers).

MATERIALS AND METHODS

Plant material: In present study 17 citrus varieties including local and hybrid (Table 1) were collected from the Citrus Research Institute Sargodha, Pakistan.

DNA isolation: The extraction of genomic DNA was done by using young leaves following the CTAB (hexadecyl trimethyl ammonium-bromide) method (Murray and Thompson, 1980) with some modifications. Fine powder was made by grinding 1g leaves in liquid nitrogen. Extraction buffer [EDTA (20 mM), NaCl (1.4 M), CTAB (2% (wv^{-1}), Tris–HCl (100 mM), pH 8.0,2–mercaptoethanol(2%)] was added to this powder and incubated at 65°C for 30 min. With chloroform–octanol (24: 1) the DNA extraction and later on washing with 70% ethanol was done. Then T.E buffer Tris–HCl (10 mM) pH 8.0, EDTA (1mM) was added to dissolve DNA pellet. The determination of DNA concentration was done by using UV-spectrophotometer (at 260 nm) and diluted up to 20ng/µL when in use. The storage of DNA samples (Stock) was done at –20°C.

PCR Procedure: The primers (RAPDs) were obtained from the Invitrogen, USA. For the amplification 25 decamer RAPDs (primers) were tested. The reaction mixture for PCR in a volume of 15 µl, comprising of template DNA (20 ngμL^{-1}), Taq DNA polymerase (0.6U), Single primer (0.4 µM), dNTPs (0.20 µM), 1.5mM $MgCl_2$, Tris-HCl (10 mM) and KCl (50 mM) was performed (William *et al.*,1990).

The amplification of DNA was done inThermal cycler and the settings for PCR were the first denaturation cycle of 94°C (4 min) followed by 35 cycles comprising of 30 sec at 94°C, 1 min at 36°C and 2 min at 72°C.The final extension was done at 68°C for 7 min. Agarose gel (1.0 %) stained in ethidium bromide was used to separate the amplified products by electrophoresis. Under UV illumination a photograph was taken and was kept in record.

Data analysis: The scoring of repeatable and clear amplified products was done as 0 for absent bands and 1 for present ones. To identify the different varieties, specific bands with the estimated size of the amplified fragment and primer number were named. The presence or absence of bands was the basis for calculation of polymorphism. The data matrix (0 or 1) used for the analysis of the genetic distance and to calculate genetic similarity was done with Simqual (sub-program NTSYS-PC) (Rohlf, 1998). The dendrogram was made with UPGMA (unweighed pair group method with arithmetic average) a subprogram of NTSYS-PC by the using distance matrix.

RESULTS

The polymorphic and repeatable amplified products were produced by 15 primers after screening 25 primers. The pattern of bandingand polymorphism generated with the primer (CP-10) (Figure 1) is presented. DNA amplification extracted from selected citrus varieties (17) produced 188 amplified products, among them153 were polymorphic and 35 were monomorphic products (Table 2). DNA bands amplified ranged between 10 (Primer CP- 2, CP-3 and CP-5) to 16 (Primer CP-10) with the average of 13bands for each primer. The number of maximum (15) and minimum (9) polymorphic bands (15) obtained waswith primer CP-10and primer CP-06 respectively. Average polymorphism calculated across all 17 varieties was 81%. The amplified product size ranged from 100 to 4600 base pairs. Polymorphism study was carried out for 3 mandarin cultivars (Kinnow, Seedless Kinnow and Feuterell's early) that resulted in 98 amplified products. The no. of monomorphic and polymorphic was 22 and 76, respectively. Primer CP-12 generated maximum polymorphic bands and showed 95% polymorphism. Polymorphism analysis was done for 3 sweet oranges (Mosambi, Parson Brown and Salustiana) resulting in 112 amplified products, out of them69 and 43 were monomorphic and polymorphic respectively. The maximum no. of bands produced was with Primer CP-4 whereas Primer CP-5 gave the minimum number of bands among sweet oranges. The highest polymorphism level (88%) observed was with primer CP-4whereas primer CP-03 produced the minimum polymorphism (47%).

The number of amplified products obtained from 8 Lime and lemon genotypes (Mitha, Eurika, Desi Lemon, Bara masi, Meyer lemon, Tahiti Lime, Kaghzi Lemon and Jatti Khatti) was 201. The number of polymorphic and monomorphic bands was 128 and 73 respectively. Primer CP-11 and Primer CP-06 produced maximum and minimum number of polymorphic alleles. Primer CP-05 generated 82% polymorphism.Two varieties of Kumquats (Meiwa and Marumi) were also analyzed for polymorphism resulting in 152 products; among them the number of polymorphic and monomorphic alleles was 99 and 53, respectively. Primer CP-10 and Primer CP-8 gave maximum and minimum number of polymorphic alleles respectively among kumquat group.

Genetic relationship between citrus genotypes: The similarity coefficient was used to generate the data with 15 reproducible primers (RAPDs) among 17 citrus genotypes. The maximum similarity value (0.84) was shown between 4 cultivars of Marumi and Meiwa as well as in kinnow and Seedless kinnow. The average similarity coefficient value among all the genotypes was 0.66. Mitha (sweet lime) was closely related with Desi Lemon (Lemon) with a value of 0.81. Feuterell's early

showed its similarity with Kinnow and Seedless kinnow at a value 0.82. A similarity value of 0.76 was observed in Kaghzi lemon with Eurika and Desi lemon.

Table 1. Citrus varieties used to study phylogenetic relationship by RAPD markers:

S. No.	Common Name	Scientific name (Swingle system)
	Mandarin	
1	Seedless Kinnow	*Citrus sinensis* Osbeck.
2	Kinnow	*Citrus reticulate Blanco*
3	Feuterell's Early	*Citrus reticulata Blanco cv Feuterell's early*
	Sweet Oranges	
4	Mosambi	*Citrus sinensis cv mosambi*
5	Parson Brown	*Citrus X sinensis cv 'Parson'*
6	Salustiana	*Citrus x sinensis cv 'Salustiana'*
	Limes and Lemons	
7	Mitha	*Citrus limettioides* Tan.
8	Eurika	Citrus *limon* L. Burm.
9	Desi Lemon	*Citrus limon (swingle)*
10	Bara masi	*Citrus clemendien (Brum).*
11	Meyer lemon	*Citrus× meyeri*
12	Tahiti Lime	Citrus *aurantifolia*
13	Kagzi Lemon	Citrus *limon*
14	Jatti Khatti	*Citrus jhambheri Lush cv JattiKhatti*
	Kumquat	
15	Marumi	*Fortunella japonica* Thunb *cv Marumi*
16	Meiwa	*Fortunella crassifolia* Thunb *cv Meiwa.*
	Grapefruit	
17	Shamber	*Citrus paradise*

Table 2. DNA bands amplified and polymorphism generated in 17 citrus genotypes using 15 RAPD markers

S. No.	Primer Code	Primer Sequence	Total Bands	Polymorphic bands	% age of Polymorphism
01	CP-01	TGCCGAGCTG	13	10	76.9
02	CP-02	AATCGGGCTG	12	09	75.0
03	CP-03	AGGTGACCGT	14	08	57.1
04	CP-04	GTTTCGCTCC	13	11	84.6
05	CP-05	GGACTGGAGT	10	08	80.0
06	CP-06	TGCGCCCTTC	12	09	81.8
07	CP-07	CTGCTGGGAC	13	11	84.6
08	CP-08	AGGGAACGAG	12	10	80.0
09	CP-09	CCACAGCAGT	12	10	83.3
10	CP-10	GTGAGGCGTC	16	15	93.7
11	CP-11	CCGCATCTAC	12	10	83.3
12	CP-12	GATGACCGCC	14	12	85.7
13	CP-13	TGGACCGGTG	11	9	81.8
14	CP-14	AAAGCTGCGG	13	11	84.6
15	CP-15	AGTCAGCCAC	14	12	86.6
Total			190	155	81.5%

Figure 2 showed the cluster tree analysis in which 17 citrus varieties were divided broadly into 4 groups expressing mandarin, Lime and Lemon, Sweet orange and Kumquats. Four main groups were fu divided further into many subgroups according to their similarity values. Three groups were branched at a similarity coefficient value of 0.69. First major cluster comprised of two sub-clusters in which the highest similarity coefficient value was detected in Kinnow and Seedless kinnow (0.85). Feuterell's early combined into this sub-group at different similarity coefficient of 0.82. The second cluster comprised Eurika that indicated genetic

similarity with Bara masi at a coefficient value of 0.84 and Meyer lemon was observed to be closed to Kaghzi lemon at the similarity value of 0.79 that combined with the above cluster at similarity coefficient of 0.76. The second major cluster was comprised of Salustiana that showed 84% closeness with Mosambi and Parson Brown was combined with the both at a similarity value of 0.81. Shamber was combined in the cluster at a coefficient value of 0.72.Third major cluster included two sub groups. Marumi and Meiwa (Kumquat group) were closed to each other and indicated a similarity value of 0.85 represented one sub group. The other subgroup included Mitha & Desi lemon (2 genotypes of Lime and Lemon group) showed a similarity value of 0.81 and merged with Kumquat subgroup at a similarity value of 0.69.

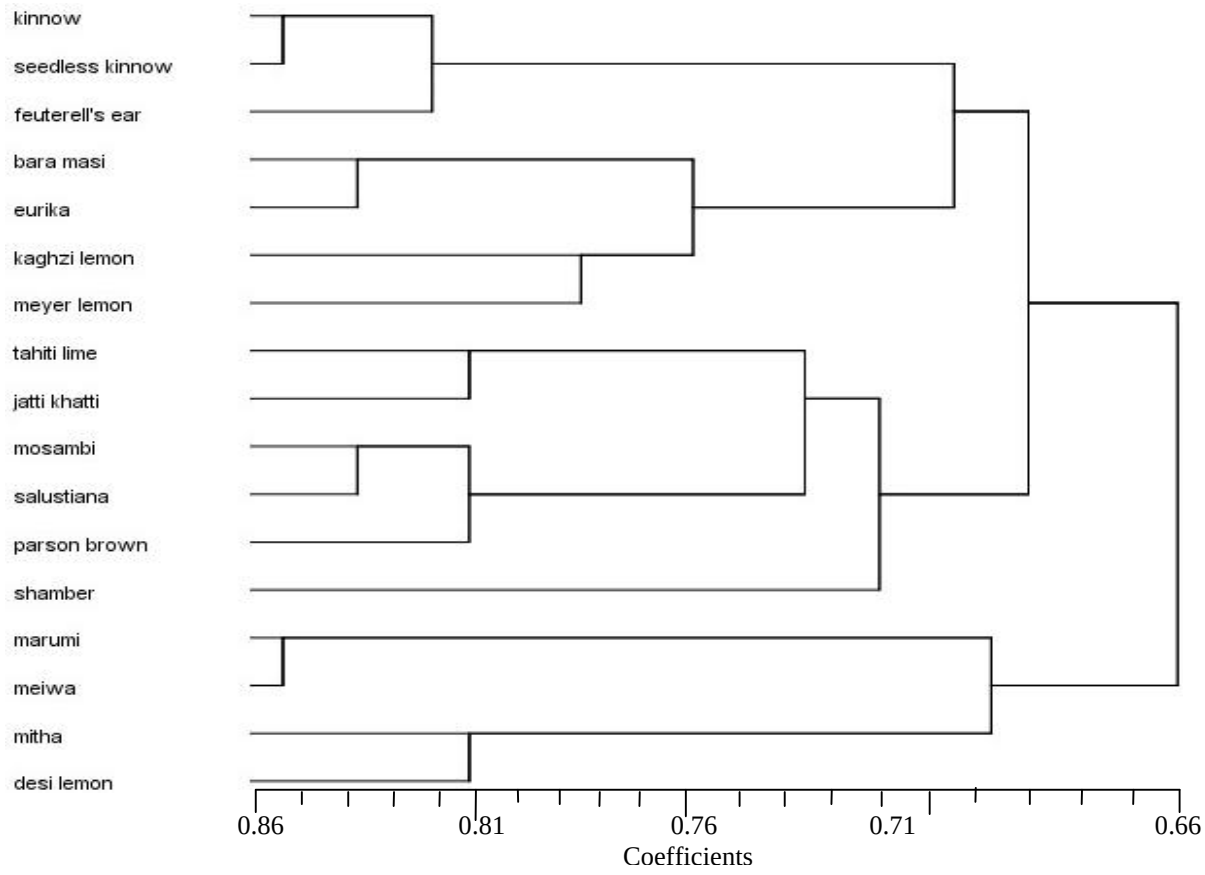

Figure 2. Dendrogram illustrating genetic relationship among 17 citrus genotypes, generated by UPGMA cluster tree analysis

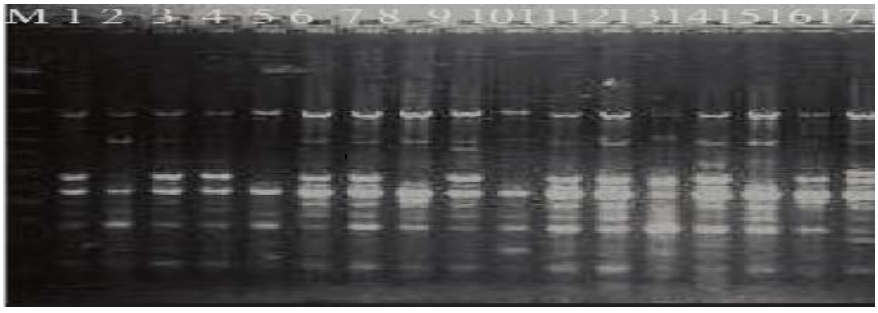

Fig. 1-RAPD Profile of 17 genotypes of Citrus spp.: RAPD amplification generated with Primer CP-10. [Lane 1, seedless kinnow; Lane 2, Kinnow; Lane 3, Feuterell's early; Lane 4, Mosambi; Lane 5, Parson Brown; Lane 6, Salustiana; Lane 7, Mittha; Lane 8, Eurika Lemon; Lane 9,Desi Lemon; Lane 10, Bara Masi; Lane 11,Meyer Lemon; Lane 12, Tahiti Lime; Lane 13, Kaghzi Lemon; Lane 14, JattiKhatti; Lane 15, Marumi; Lane 16, Meiwa; Lane 17, Shamber; & M, Marker (100bp-3 Kb).

DISCUSSION

The present study with citrus varieties has shown the potential of RAPD markers as a rapid, reproducible and useful method for distinguishing and clustering different genotypes in Citrus spp. The role of RAPD markers and its application to varietal identification and analysis of genetic relationships in citrus varieties were investigated (Nhan *et al.*, 2001).They could also be used as marker assisted selection in the citrus breeding programs.Similar results were obtained by Nicolosi*et al*. (2000) and Abkenar and Isshiki (2003) where RAPD markers allowing the distinction of some citrus genotypes. By using 15 RAPD primers, 190 amplified products were obtained which is similar with that 212 RAPDs generated by using 23 primers reported by Coletta Filho *et al.* (1998) among 25 mandarin accessions. Among 17 citrus genotypes, the average polymorphism was 81.5%. In the present study the high level of polymorphism shows greater genetic diversity among the selected citrus varieties including genotypes of 5 major categories i.e. Mandarins, Sweet oranges, Lemon and Lime, Kumquats and grapefruit. RAPD amplification among 3 genotypes of mandarins resulted in 98 amplified products which is quite consistent to the findings in which by using 21 primers, 111 amplification productswere obtained among 39 mediterranean genotypes (Machado *et al*., 1996). The no. of polymorphic and monomorphic bands was 76 and 22 respectively, with polymorphic percentage (77.5%).

Fifteen primers produced 201 amplified products among 8 genotypes of Lime and Lemon group, out of which 128 were polymorphic. Clear and repeatable bands (3-9) were obtained for every primer which is like the results (1-9 bands) obtained by Abnekar and Isshiki (2003)in 31 acid citrus species. DNA fragments amplified varied from 10-16 bands per primer. High similarity coefficient (0.85) was found among two cultivars of Kumquat group namely Marumi and Meiwa and two genotypes of Mandarin group including Kinnow and Seedless kinnow, similar to the results obtained (0.82-0.88) by Gulsen and Roose (2001) among 95 accessions of citrusfor ISSR, SSR and Isozyme analysis. The common origin by mutation is probably the reason of the high similarity (0.85) between the varieties.

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