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GENETIC DIVERSITY OF CITRUS GERMPLASM IN PAKISTAN BASED ON RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

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ABSTRACT

Citrus has a long history of cultivation in the world and is considered as an economically important fruit crop. In south Asia together with Pakistan many varieties are widely grown for exports as well as domestic use. Therefore considering the importance, current study is aimed to identify and evaluate the genetic diversity of 17 Pakistani Citrus cultivars with 14 RAPD (Random amplified polymorphic DNA) markers. From the screening of 30 primers, a total of fourteen decamer primers were chosen that produced 8.35±0.41 bands per primer on average with 96 polymorphic bands of 117 total bands. The phylogenetic relationship was assessed by using Jaccard's similarity coefficient. The phylogenetic tree separated 17 Citrus genotypes into 3 main groups using analysis of Unweighed Pair Group Method with Arithmetic Mean. The similarity coefficient detected across all the genotypes was 0.66 with two cultivars of mandarin (Fallglo and Murcott) and grapefruit (Chakotra and Ruby red) showing the highest similarity value of 0.92. High genetic distance illustrated difference in origin of 17 Citrus cultivars. The current study revealed the successful utilization of genetic marker i.e. RAPD to analyze the phylogenetic relationship and genetic diversity among the citrus varieties hence can be recommended as well to be used in Citrus breeding programs.

Key words: Citrus genotypes, Genetic characterization, Polymerase chain reaction, Phylogenetic relationship, RAPD.

INTRODUCTION

The genetic diversity of a crop provides a baseline to plant breeders to detect unique germplasm for the improvement of horticultural traits, introgression of disease resistance into elite cultivars and analysis of viability and purity of rootstocks which in turn can increase both quality and quantity of fruit production (Baig *et al.*, 2009; Li *et al.*, 2010). For the determination of genetic relatedness, germplasm characterization and establishment of breeding programs in Citrus, the understanding of taxonomy, phylogenetic relationships and genetic variability plays an important and key role (Herrero *et al*., 1996). Citrus germplasm characterization and improvement was carried out but with a little success and the factors may include characters associated with reproductive biology of the species like prolonged juvenile period, great interspecific fertility, apomictic reproduction and polyembryony (Corazza-Nunes *et al*., 2002). Hybrids i.e. natural as well as cultivated origin comprise important fruit such as oranges, lemons, grapefruits, mandarins.

Citrus is among the most extensively cultivated fruit crop throughout the world having a large global market. According to FAO, 2012 Pakistan stands on tenth position in the citrus annual production (1982200 tons) and a cultivation area (194500 hectares) among other countries. This marks citrus the most important fruit crop grown in Pakistan followed by other fruit crops like mango, guava and date (Shah *et al*., 2010). The global citrus market is valued more than $2.135 billion in which Pakistan's annual share has reduced to $33 million, just around 2.5 % (FAO, 2012).

Initially, classification of citrus was entirely based on geographical and morphological data that made the systematics of this fruit crop more controversial (Moore, 2001). The evaluation of genetic diversity using morphological markers alone has some limitations, particularly in species of a complex genre like citrus because of recurrent hybridization, bud mutations, polyploidy and apomixis. Different marker systems have been used to investigate DNA polymorphisms for the selection of desired parents to be used in the cultivar improvement, superior hybrid resulted from cross population (Talon and Gmitter, 2008; Sharma *et al.*, 2015) and ultimately to manage the germplasm collection (Biswas *et al.*, 2012). PCR-based markers especially RAPDs (Random Amplified Polymorphic DNA) have been in use for the identification of genetic relationship among species or cultivars (Machado *et al*., 1995; Bastianel *et al*., 1998) citrus hybrids and mutants (Elisiaro *et al*., 1999) as well as periclinal chimeras (Sugawara *et al*., 2002). RAPDs are considered as simple primers as for their designing no preceding information of DNA sequence data is needed (Hisada *et al*., 1997). These are fast, easy to be analyzed and distributed throughout the genome and also for their PCR amplification, DNA in low concentrations is required (Sarwat *et al*., 2008).

Besides the fact that Citrus is among the most economically important fruit of Pakistan more research work is needed yet including the estimation of genetic diversity. Hence, considering the importance of genetic diversity of citrus the current study is planned for the identification and assessment of genetic variation and establishment of the inter-relationships among seventeen citrus cultivars by using RAPDs.

MATERIALS AND METHODS

Plant material: In current research work, 17 citrus cultivars (Table 1) were analyzed after collecting them from Citrus Research Institute, Sargodha, Pakistan at latitude 32.08 ^{0}N and 72.67 ^{0}E. The plants were grown on less saline but well drained soil and were mainly grafted on *Citrus jambhiri*. The plants were cultivated with a planting distance of 5.4 meters between the plants and of 8.5 meters between the rows.

DNA extraction procedure: The genomic DNA was extracted from young fresh leaves following CTAB (hexadecyltrimethyl ammonium-bromide) method reported by Murray and Thompson, (1980) with some modifications. The detail of modifications was described earlier by Shahzadi *et al*., (2014).

RAPD analysis: RAPD marker system gave an easy and quick technique for the identification of citrus cultivars as well as their taxonomic studies. In the current study after screening out 30 decamer primers having oligonucleotides of arbitrary sequences (Invitrogen USA) 14 primers were selected for the analysis of genetic diversity of 17 citrus varieties.

PCR scheme for RAPD primers: The DNA samples were diluted to10 ng/µl by adding deionized water in it for RAPD analysis. The PCR procedure was accomplished in a volume of 15 µl taking 10 ng μL^{-1} DNA sample, 0.20 µM dNTPs, 1.5mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.4 µM of primer and 0.6U Taq DNA polymerase (Invitrogen, USA) (Williams *et al*., 1990).

The PCR conditions for DNA amplification was optimized in Primus-96 Thermocycler. The optimized protocol was initial denaturation of 94°C for 4 minutes and then a total of 35 cycles including denaturation for 30 seconds at 94°C, primer annealing for 1 minute at 47°C and extension for 1 minute and 30 seconds at 72°C. Additional time i.e. 7 minutes at 68°C was also given as final extension period.

Gel Electrophoresis: The amplified PCR products were separated on horizontal gel electrophoresis (ELITE 300 PLUS) by using 0.8% agarose gel stained with 0.55 µg/ml ethidium bromide. The gel visualization was carried out on UV-trans illuminator (MD-20-312nm) to detect and record the banding profiles and then photographed with Dolphin gel documentation system. Each PCR reaction was repeated at least three times to check the reproducibility and reliability of banding patterns and finally only stable bands were scored.

Data analysis: Clear and repeatable amplified products were scored as 0 (absent) and 1 (present) band to make a data matrix. The identification of different varieties was carried out by naming specific bands with the estimated size of the amplified fragment along with primer number. The genetic polymorphism was calculated based on the absence and presence of bands. The data matrix (0 or 1) was used to analyze and calculate the genetic distance and genetic similarity with Simqual (sub-program NTSYS-PC) (Rohlf, 1998). The phylogenetic tree was made using distance matrix with unweighed pair group method with arithmetic average (UPGMA) i.e. a subprogram of NTSYS-PC.

RESULTS

Genetic polymorphism among Citrus cultivars: After screening 30 RAPD primers, a total of 14 primers were found to generate reproducible polymorphic amplified products. The banding profile and genetic polymorphism of primer CP-04 andCP-13 are presented in Fig. 1 and 2. A total of 117 amplified products were producedfrom the extracted DNA of 17 citrus cultivars, of which 96 bands were polymorphic and 21 bands were monomorphic (Table 2). Maximum polymorphic bands (12) were produced by Primer CP-09 while primer CP-05 and CP-08 produced the lowest number (4). The variation in total bands was recorded from 5 (CP-05) to 12 (CP-09) with an average of 8.35±0.41 bands per primer. The polymorphism percentage ranged from 62.50% (CP-07) to the highest 100% for primers CP-09 and CP-11. Among 17 citrus genotypes, an average polymorphism of 80.63% was calculated. The amplified polymorphic bands ranged in size from 350bp - 2500 bp.

The DNA samples of 4 grape fruit cultivars namely Chakotra, Ruby red, Marsh seedless and Star Ruby produced a total of 150 amplified products. Of these, 66 and 94 bands were monomorphic and polymorphic respectively. The maximum polymorphic bands were produced by primer CP-07 while no primer CP-01 failed to amplify the DNA samples.The genetic analysis was studied for 1 genotype of Kumquat group i.e., Nagami that generated 29 and 15 polymorphic and monomorphic bands respectively hence making a total 44 amplified products. Primer CP-04 produced the highest number (9) whereas Primer CP-02 yielded the lowest number (3) of polymorphic alleles.

The genetic analysis of polymorphism was also carried out for 3 cultivars i.e. Jatti Khatti (Rough lemon) Lakeland and Mesero (Lemon group) producing a total of

104 amplified products. Out of these the polymorphic and monomorphic bands generated were 74 and 30, respectively. Primer CP-11 gave the maximum (11) and Primer CP-04 produced the minimum (2) of polymorphic bands. Primer CP-13 produced the highest (85%) while Primer CP-01 produced the least polymorphism level (15%).

A total of 192 amplified products were generated from the DNA amplification of 5 Sweet orange genotypes i.e. Pineapple, Olinda Valencia, Succari, Hamlin and Marrs early. Of these 192, the monomorphic and polymorphic bands were 65 were 127 respectively. Primer CP-13 generated the highest number of polymorphic bands (10) whereas only one polymorphic band was produced by three primers i.e. CP-01, CP-04 and CP-11. The analysis of Polymorphism was also studied for 3 genotypes i.e. Fallglo, Kinnow seedless and Nagpuri santra (mandarin group) that generated a total of 96 amplified products and the polymorphic and monomorphic bands were 85 and 11, respectively. Primer CP-09 yielded the maximum polymorphic alleles (12) whereas the minimum polymorphic alleles (3) were produced by two primers i.e. CP-06 and CP-08. Primer CP-11 identified the highest i.e. 100% whereas the lowest level which was 34.8% was detected by CP-05.

Phylogenetic relationship among Citrus cultivars: The similarity coefficients were calculated using the data obtained from DNA amplification of 17 Citrus genotypes with 14 selected RAPD primers. A maximum similarity coefficient i.e. 0.92 was found in two genotypes of mandarin group i.e. Fallglo and Murcott and two cultivars of Chakotra and Ruby red of grape fruit group. Fallglo and Nagami were detected to be most dissimilar (0.40) and an average similarity coefficient of 0.66 was found across all the Citrus cultivars.

The phylogenetic tree (Fig. 3) showed that selected 17 Citrus genotypes were mainly separated into three major groups indicating mandarin, sweet orange and grape fruit. It is obvious that Nagami did not fall in any of the main clusters and appeared as a separate branch at similarity coefficient of 0.68 so is the most distant of all. Based on the similarity coefficients, the three groups were further divided into sub groups. Three major clusters at a similarity coefficient of 0.74 splitted into two main sub-groups. The first main cluster at the coefficient value of 0.80 included two sub groups. Fallglo and Murcott, very close to one another having a similarity coefficient of 0.92 together with Nova and Nagpuri Santra presented the mandarins as one of the subgroups. Four grapefruit genotypes i.e. Chakotra, Ruby red, Star Ruby and Marsh seedless having a similarity coefficient of 0.91 comprised the other subgroup. Olinda Valencia and Succari i.e. sweet orange cultivar joined the subgroup at similarity coefficient of 0.88. Pineapple i.e. another sweet orange variety merged at a similarity coefficient of 0.86 to the subgroup.

The second subgroup comprised of two branches at a similarity value of 0.83, one of which has two sweet orange cultivars i.e. Hamlin and Mars early at a similarity coefficient of 0.89 and the second branch further divided into two sub branches at a coefficient value of 0.88. One sub-branch comprised Jatti Khatti and Lake land i.e. two cultivars of Lemon group at a similarity coefficient of 0.92 and Mesero another lemon variety merged at a similarity value of 0.86 with the other two lemon cultivars.

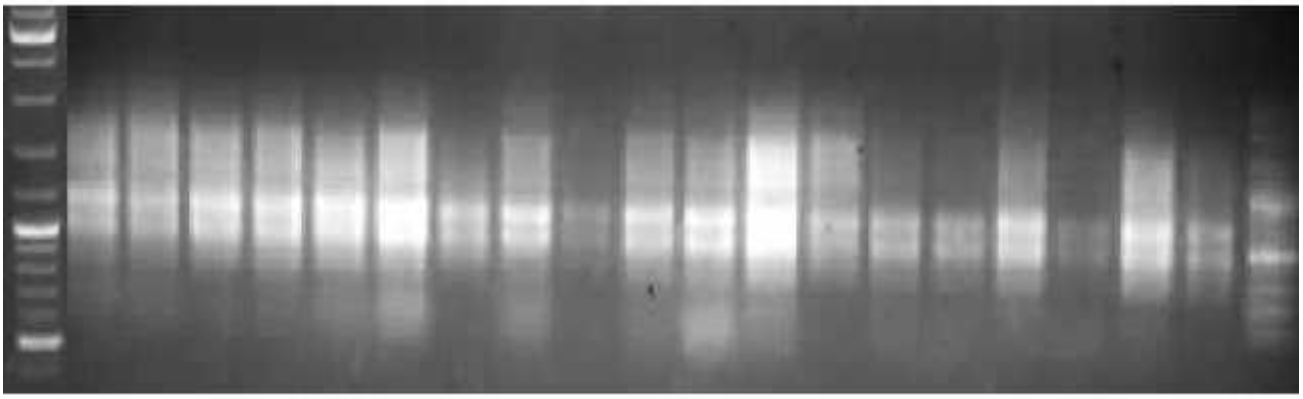

Figure 1. Agarose gel presenting RAPD banding pattern produced by Primer CP-13. L, Ladder; 1) Marsh seedless; 2) Chakotra; 3) Star ruby; 4) Ruby red; 5) Jatti Khatti;6) Lakeland lemon; 7) Mesero lemon; 8) Succari; 9) Pineapple; 10) Marrs early; 11) Olinda valencia; 12) Hamlin; 13) Fallglo; 14) Nova; 15) Nagpuri Santra;16) Murcott; 17)Nagami

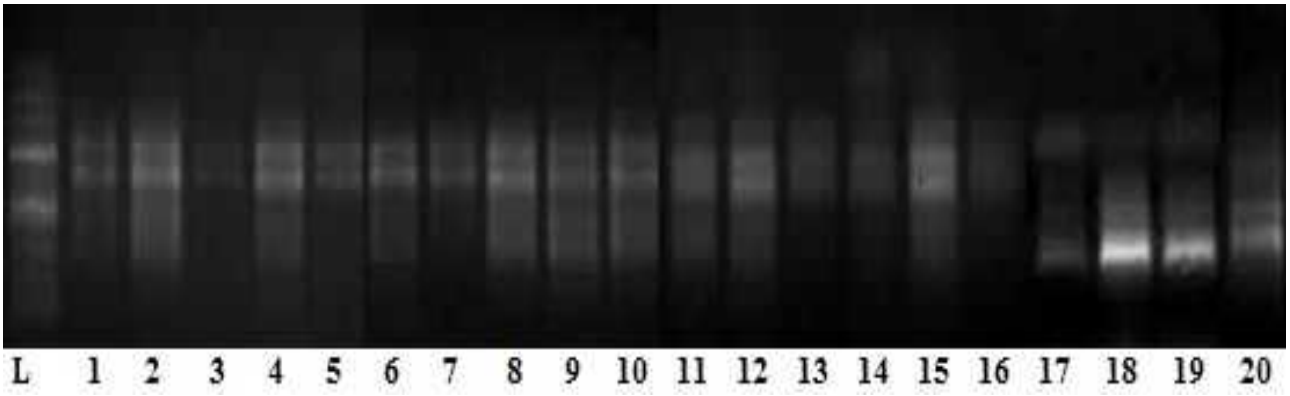

Figure 2. Agarose gel presenting RAPD banding pattern produced by Primer CP-13. L, Ladder; 1) Marsh seedless; 2) Chakotra; 3) Star ruby; 4) Ruby red; 5) Jatti Khatti;6) Lakeland lemon; 7) Mesero lemon; 8) Succari; 9) Pineapple; 10) Marrs early; 11) Olinda valencia; 12) Hamlin; 13) Fallglo; 14) Nova; 15) Nagpuri Santra;16) Murcott; 17)Nagami

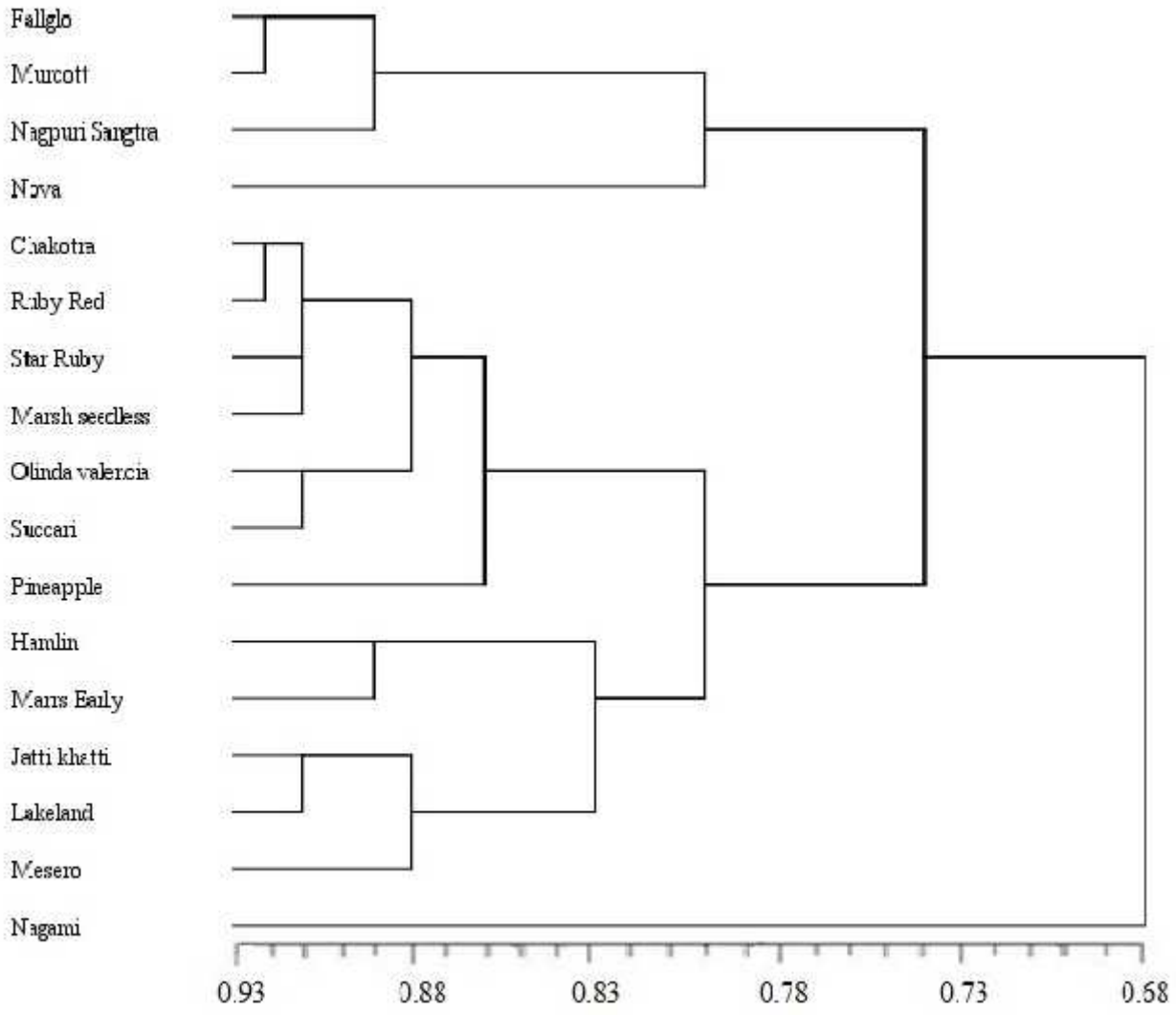

Figure 3. Dendrogram showing genetic relationship among 17 citrus cultivars produced by cluster tree analysis (UPGMA) using Jaccard's similarity coefficients

Table 1. List of 17*Citrus* genotypes used in the present study

S.#	Common Name	Scientific Name (Swingle system)
Grape fruit		
1.	Marsh seedless	*Citrus* × *paradisi* 'Marsh'
2.	Chakotra	*Citrus* × *paradisi* 'Chakotra'
3.	Star ruby	*Citrus* × *paradisi* 'Star ruby'
4.	Ruby red	*Citrus* × *paradisi*'Ruby red'
Kumquat		
5.	Nagami	*Fortunella margarita* (Lour.) Swing.
Lemon		
6.	Lakeland	*Citrus* x *limon* 'Lakeland'
7.	Mesero	*Citrus* x *limon* 'Mesero'
8.	Jatti khatti	*Citrus jambhiri* Lush. cv 'Jatti khatti'
Sweet Oranges		
9.	Succari	*Citrus* x *sinensis* 'Succari'
10.	Pineapple	*Citrus* x *sinensis* 'Pineapple'
11.	Marrs early	*Citrus* x *sinensis* 'Marrs early'
12.	Olinda valencia	*Citrus* x *sinensis* 'Olinda valencia'
13.	Hamlin	*Citrus* x *sinensis* 'Hamlin'
Mandarin		
14.	Nova	*Citrus reticulata*Blanco'Nova'
15.	Fallglo	*Citrus reticulata*Blanco'Fallglo'
16.	Murcott	*Citrus* x *reticulata* Blanco 'Murcott'
17.	Nagpuri santra	*Citrus* x *reticulata* 'Nagpuri santra

Table 2. Amplified DNA bands and %age polymorphism produced by 14 RAPD primers among 17 *Citrus* genotypes.

S.No.	Primer code polymorphism	Primer sequence	Total bands	Polymorphic bands	%age
1	CP-1	TGCCGAGCTG	7	6	85.70
2	CP-02	AATCGGGCTG	8	7	87.50
3	CP-03	AGGTGACCGT	7	5	71.42
4	CP-04	GTTTCGCTCC	9	8	88.88
5	CP-05	GGACTGGAGT	5	4	80.00
6	CP-06	TGCGCCCTTC	7	5	71.42
7	CP-07	CTGCTGGGAC	8	5	62.50
8	CP-08	AGGGAACGAG	7	4	57.10
9	CP-09	CCACAGCAGT	12	12	100.00
10	CP-10	GTGAGGCGTC	8	6	75.00
11	CP-11	CCGCATCTAC	11	11	100.00
12	CP-12	GATGACCGCC	9	7	77.77
13	CP-13	TGGACCGGTG	10	8	80.0
14	CP-14	AAAGCTGCGG	9	8	91.66
	Total		117	96	----
	Mean		8.35±0.41	6.85±0.37	80.63

DISCUSSION

Phylogenetic relationships of some citrus groups have been controversial in the past; yet, technologies based on molecular marker reveal many of these links. The complications in Citrus classification are mostly due to frequent inter-species cross pollination that propagates and stabilizes hybrid taxa, bud mutations, adventitious nucellar embryony, polyploidy (Scora, 1975). The introgression and recurrent hybridization have created many hybrids and mutant varieties all over the citrus belt globally.

In the present study RAPD markers proved their potential as a rapid and valuable method for the differentiation and clustering 17 different *Citrus* cultivars. Traditionally the identification of Citrus varieties is based on morphological characters (Malik *et*

al., 2012; Martasari, 2012) but as it can be done at particular reproductive stage only and is highly environmentally influenced. Therefore identification and characterization based on data produced by molecular markers is the need of the hour and more reliable.

The role and use of RAPD primers in identification of phylogenetic relationship among *Citrus* cultivars was also studied by Nhan *et al*., 2002; Malik *et al*., 2012; Naz *et al*., 2014. In the present study, the screened 14 RAPD primers amplified 117 products, out of them 96 were polymorphic with 8.3 bands per primer on average. El-mouei *et al*., (2011) also reported similar results in the analysis of 31 Citrus accessions where a total of 143 bands were detected using 17 RAPD primers and out of which119 were polymorphic with an average of 8.4 bands per primer. Across selected 17 Citrus genotypes the average polymorphism of 80.63% was detected. The high polymorphism level attained in the current investigation shows higher genetic diversity among 17 Citrus cultivars including 5 major groups i.e. Grape fruit, Kumquat, Lemon, Sweet orange and Mandarin. Similar results were obtained by Nicolosi *et al*., (2000) and Abkenar *et al*., (2004) where RAPD markers permitting the distinction of some citrus genotypes were identified.

Mandarin is one of the Citrus true species and all mandarin cultivars are resulted from mutation of it or as a result of hybridization between it and other species. This is proved in the current study where among 17 Citrus cultivars four mandarin genotypes i.e. Fallglo, Murcott, Nova and Nagpuri santra fall in the same clade and two of the four showed the highest similarity coefficient of 0.92 quite relevant to the results of Tripolitsiotis *et al*., (2013). It is obvious because of the fact that all the four have the same origin. Fallglo and Nagami revealed a similarity value of 0.44 showing the unrelatedness of both. Similar results were reported for 41 samples of 33 cultivars by Fang *et al*., (1997). Nagami separated from other citrus genotypes as a distinct variety in the present study which might be due to the fact that it is the close relative to citrus i.e *Fortunella margrita* (Barret and Rhodes, 1976) and not a true citrus genotype.

Sweet orange, lemon and grape fruit genotypes fall in the same large clade but in different subgroups which is quite evident as the three groups have different parentage and origin. To support the clade containing sweet orange and grape fruit, different marker systems have been used that included RAPD and SCAR (Nicolosi *et al*., 2000) RFLP (Federici *et al*., 1998) and SSCP (Luro *et al*., 2012).

Our results demonstrated the feasibility of using molecular markers to differentiate and between within the different *Citrus* taxa. RAPD fingerprinting has a number of potential applications in the efficient use and management of genetic resources collection, determination of cultivar purity and particularly in the identification of mislabeled accessions (Ahmad *et al*., 2003). Understanding the genetic diversity of Citrus genotypes using molecular data is very critical for planning, germplasm management and application of Citrus breeding program in Pakistan. RAPD markers used in the present study allowed the identification and differentiation within and between species. They could also be used as marker assisted selection in the citrus breeding programs.

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