The Journal of Animal & Plant Sciences, 24(6): 2014, Page: 1752-1757
ISSN: 1018-7081

ASSESSING GENETIC DIVERSITY OF PAKISTANI CITRUS VARIETIES USING MICROSATELLITE MARKERS

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ABSTRACT

Ten microsatellite markers i.e. Expressed Sequence Tag Simple Sequence Repeat (EST-SSR) markers were used to develop finger print profiles of sixteen Citrus cultivars. The DNA fingerprints were used to characterize the samples and detection of different level of polymorphisms. The total alleles identified were 64 with an average of 6.4 alleles / locus. Two markers i.e. TAA33 and BQ624307 gave minimum number of alleles (4) while one marker naming TAA41 detected the maximum alleles (11) and hence was the most polymorphic marker. The highest no. of alleles were observed in Tahiti lime where ten markers resulted in a total of 24 alleles. The lowest number of alleles was detected in two cultivars namely Meiwa and Eustis lime with 13 alleles each. To show the genetic relationship among varieties, the genetic dissimilarity values calculated by Ward's minimum variance method were used to develop a dendrogram with DARwin software package v. 5.0.158.

Kew words: diversity; genetic markers; phylo-genetic relationship; microsatellites

INTRODUCTION

Citrus is one of the most widespread fruit crop in the international market, with pronounced economic and health value. It is one of the most challenging plants to improve through traditional methods of breeding. Generally, Citrus includes species of six closely linked genera, *Citrus*, *Microcitrus*, *Eremocitrus*, *Clymenia*, *Poncirus* (trifoliate oranges) and *Fortunella* (also known as Kumquat); belong to the family Rutaceae (Swingle *et al.*, 1967). These genera include mostly diploid (2n=2x=18) species with relatively small genome size, eg., *Citrus siensis* L. Osbeck (Sweet orange) has a genome size of about 367Mb (Talon and Gmitter, 2008). The systematics and taxonomy of the genus *Citrus* are complex and as many of species are hybrids of different species from two different genera, the exact number of natural species is not clear yet. There is ample genetic evidence of hybrid origin of some wild, true-breeding species (Nicolosi *et al.*, 2000) in the genus Citrus. Genetic diversity and relatedness among *Citrus* species have been conducted using morphological, biochemical and genetic markers (Koehler-Santos *et al.*, 2003).

Molecular markers compared with morphological and biochemical markers are not environmentally influenced and hence are effective tools for explaining genetic diversity and revealing phylogenetic relationship which is an advantage in distinguishing between closely related individual (Biswas *et al.*, 2012; Warburton *et al.*, 2005). These genetic markers included AFLP (Amplified Fragment Length Polymorphism), RFLP (Restriction Fragment Length polymorphism), ISSR (Inter-Simple Sequence Repeat), RAPD (Random Amplified Polymorphic DNA) and SSR (Simple Sequence Repeat) (Nakano *et al.*, 2013).

SSR (Simple sequence repeat) markers contain tandem repeats having short nucleotide motifs with a length of between one and six nucleotides are very important in plant genetics. They have many important genetic characteristics i.e., multi-allelic nature, wide-ranging genomic distribution, high polymorphism and reproducibility for networking activities. They are abundant and distributed in mitochondrial, chloroplast and nuclear genomes (Barkley *et al.*, 2009). SSR primer pairs are transportable across species within a genus or between related genera, as sequences flanking microsatellite regions are highly conserved (Oliveira *et al.*, 2006). These markers have proved to be valuable in assessing the genetic diversity in citrus (Barkley *et al.*, 2006; Novelli *et al.*, 2013) and to investigate the apomictic or sexual origin of plant seedlings (Aleza *et al.*, 2010). For the identification of closely linked plant genotypes, numerous microsatellite markers were developed during the last decade. In citrus, previous microsatellite markers were developed from genomic libraries mostly located in non-coding region of DNA (Park *et al.*, 2009).

As the knowledge about genetic relationship and variation among the genotypes is very important to classify and utilize germplasm resources. So the aim of present work was to identify the genetic variability and assessment of the genetic relationships among Citrus cultivars using microsatellite markers. By studying the

genetic variability within species would greatly assist the breeders in cultivar identification to develop fingerprint database and maintain the germplasm repositories of Pakistani Citrus varieties. It would also be helpful to provide diagnostic service to the industry stakeholders to rule out potential variety identity mistakes at nursery stage.

MATERIALS AND METHODS

Plant material: A total of 16 Citrus varieties (7 Lemon, 2 Sweet Lime, 3 Acid Lime, 3 kumquats and 1 root stock) including local and hybrids (Table 1) used in the present study were collected from the Citrus Research Institute, Sargodha, Pakistan.

DNA isolation: The genomic DNA from fresh and healthy leaves was extracted by following the modified CTAB method (Murray and Thompson, 1980). About 1g young and healthy leaves were taken in liquid nitrogen with a pestle and mortar. The ground tissue was added to Extraction buffer [20mM EDTA, 1.4 M NaCl, (2%CTAB, Tris–HCl (100 mM), pH 8.0, 2% of 2–mercaptoethanol] and incubated at 65 °Cfor 30 min. The samples were extracted with a mixture of chloroform: isoamyl alcohol (24:1, v/v) and centrifuged for 5 min at 5,000 rpm. The DNA was precipitated by adding two-third volumes of ice-cold isopropanol followed by centrifugation for 5 min at 5,000 rpm. The DNA pellet was washed with 70% ethanol and after air drying suspended in 200ml TE buffer [10 mMTris–HCl (pH 8.0), (1mM) EDTA].The amount of DNA was estimated by measuring absorbance at 260 nm using UV-spectrophotometer. The isolated DNA was diluted upto a final concentration of 20 ngμL^{-1}in TE buffer for each sample. The storage of DNA samples (Stock) was done at –80 °C.

Table 1. Citrus varieties used to study phylogenetic relationship by microsatellites (EST-SSRs)

Genotypes	Botanical names
Lemon group	
• Eureka Lemon	*Citrus limon* "Eureka"
• Volkamer Lemon	*Citrus* volkameriana *Tan. & Pasq.*
• Desi Lemon	*Citrus limon* "Desi lemon"
• Bara Masi	*Citrus limon (Brum).*
• Lake-Land Lemon	*Citrus limon* "Lakeland lemon"
• Mesero Lemon	*Citrus limon*'Mesero'
• Lisbon Lemon	*Citrus limon* "Lisbon"
Sweet Lime	
• Peshawari Mittha	*Citrus limettioides* Tan.
• Local Mittha	*Citrus limettioides* Tan
Acid Lime	
• Tahiti Lime	Citrus *X latifolia Tan* 'Tahiti lime'
• Persian Lime	Citrus *X latifoliaTan.* 'Persian lime'
• Eustis Lime	*Citrus aurantifolia*
Kumquat	
• Marumi	
• Meiwa	*Fortunella japonica Thunb cv* 'Marumi'
• Naghmi	*Fortunella crassifolia Thunb cv'* Meiwa'
Root stock	*Fortunella japonica Thunb cv* 'Naghmi'
• Rough Lemon	
	Citrus jhambheri Lush cv 'Jatti Khatti'

Microsatellite Markers: Among 14 EST-SSR primers used for the DNA analysis, only 10 were able to identify polymorphism between the samples (Table 2). Four microsatellite markers used in the present study have been previously published (Kijas *et al*., 1997) and the rest are not yet published. The primers were synthesized by Operon Technologies (Alameda, Calif., USA).

PCR Procedure: PCR amplifications were accomplished in 20 µl reaction mixture using thermal cycler (Perkin Elmer Model 480). For genotyping, labeled (6-FAM, HEX) primers were used. The PCR reaction mixture contained template DNA (20ng), 1µM each primer, 200 µM of dNTPs, and 1X gold PCR buffer, 0.5U Taq (AmpliTaq Gold DNA polymerase) and $MgCl_2$ solution (2mM). The optimization of conditions was done separately for each marker. All markers were tested on

DNA samples at the annealing temperature of 56ºC, while all other conditions of procedure were kept constant i.e. 5 min at 94ºC; 35 cycles of 30 sec at 94ºC, 45 sec at 56ºC and 2 min at 72ºC and final extension was done for 4 min at 72ºC. The confirmation of all amplifications was performed by running PCR product (5µl) on agarose gels (2%).

Fluorescently labeled PCR products for markers of different allele sizes or of the same size but with different labels were pooled together. Capillary electrophoresis of the PCR products was accomplished on a 3500 × l DNA Analyzer (Applied Biosystems Inc.). Gene Mapper V. 3.7 software package was used to examine all polymorphic sites in the 16 Citrus cultivars.

Table 2. Primer sequences, repeat motifs, number of putative alleles and their size revealed by 10 microsatellites in Citrus cultivars

Marker		Sequence (5'-3')	Repeat motif	No. of Loci	No. of alleles	Allele size (bp)
BQ623065	F	GGTGTTGTTCTCGCAACAGA	GAA9	1	5	269,275,278,281,284
	R	CGGCAGCCTATTGCTACTTC				
BQ624307	F	TTCAAGCCAAAGCAAGAGGT	AGC8	1	4	133,136,139,145
	R	ACCCAAATGCTCAAAACACC				
BQ624796	F	ACGATGACCAAGAATCCAGC	TCT8	1	5	240,244,247,250,253
	R	AAGATCCCACAAGCCATCAC				
C24033	F	GCAGCAATTCTGAAGGAAGG	TAA9	1	8	256,260,264,266,267,
	R	ACGGCCTCAATGGAACCTAT				270,273,277
C24317	F	ACTGCTGTTCACCCTGTTCC	CTT10	1	5	131,134,137,143,146
	R	GAGAGCTTTCGAGCCTTTGA				
CTG1006372	F	TCAGCACTGAATCCAATCCA	TAA16	1	9	269,273,276,287,290
	R	GTGAGAGCTTGAGGCTGACC				296,297,299,303
TAA15	F	GAAAGGGTTACTTGACCAGGC	TAA	1	5	147,159,162,183,199
	R	CTTCCCAGCTGCACAAGC				
TAA33	F	GGTACTGATAGTACTGCGGCG	TAA	1	4	112,115,118,121
	R	GCTAATCGCTACGTCTTCGC				
TAA41	F	AGGTCTACATTGGCATTGTC	TAA	1	12	124,128,130,132,133,136
	R	ACATGCAGTGCTATAATGAATG				138,141,143,146,150,165
TAA45	F	GCACCTTTTATACCTGACTCGG	TAA	1	8	120,121,127,129
	R	TTCAGCATTTGAGTTGGTTACG				130,133,136,142

Data analysis: To determine thegenetic diversity and relationship of the citrus cultivars, SSR profile was scored at 10 loci. For the identification of different varieties, the estimated size of the amplified alleles and primer number were recorded. The estimation of genetic distance among all Citrus genotypes was done using the Dice dissimilarity index. Hierarchial clustering method was used to make a dendrogram. The analysis was done with the "DARwin" software package Version 5.0.158 developed by CIRAD (Perrier and Jacquemoud-Collet, 2006).

RESULTS

In the present work, a total of fourteen EST - SSR primers were used to evaluate the genetic diversity of 16 Citrus cultivars. Out of them, ten primers revealed polymorphic loci while no amplified products were observed with the other four primers. The total number of alleles produced on these ten loci was 64 alleles with an average of 6.4 alleles per primer combination. The

number of alleles ranged from a minimum of 4 for Locus BQ624307 and TAA33 to a maximum of 11 for the most polymorphic markerTAA41 (Table 2). Most microsatellites could very easily differentiate at the species level but individual cultivars within Lemon group, Acid lime and Sweet lime which were thought to be evolved by mutation, were not distinguishable. The highest number of alleles were detected in the Tahiti Lime where the ten primers resulted in 24 alleles. The lowest number was detected in the Meiwa and Eustis lime with 13 alleled each. The dendrogram based on the genetic dissimilarity values was obtained (Fig 1).

Phylogenetic analysis: The information based on microsatellites (EST-SSRs) data was used to calculate the genetic dissimilarity and distance among Citrus cultivars. The obtained dissimilarity values were used to establish a dendrogram generated by Ward's minimum variance method to show the genetic relationship among varieties (Fig 1). In the dendrogram, two distinct main branches were revealed. The first main branch is separated into two different groups, the first one comprises 2 varieties of Lemon group (Lisbon and Mesero) and the subsequent is further separated into two sub-clusters. The first sub-group of this clade is consisted of 2 Lemon genotypes (Baramasi and Lakeland) and 1 Acid Lime (Persian lime) and the second clade is consisted of 2 Acid Lime varieties (Tahiti and Eustis) that further branches into two sub-clusters. The first sub-cluster consisted of 3 lemon (Desi, Volkamer and Eureka) and two sweet lime cultivars (Local and Peshawari Mittha). The second main branch is divided into two distinct clusters; one of which consisted of Kumquat group (Marumi, Meiwa and Nagmi) and the second contained the root stock variety (Jatti khatti).

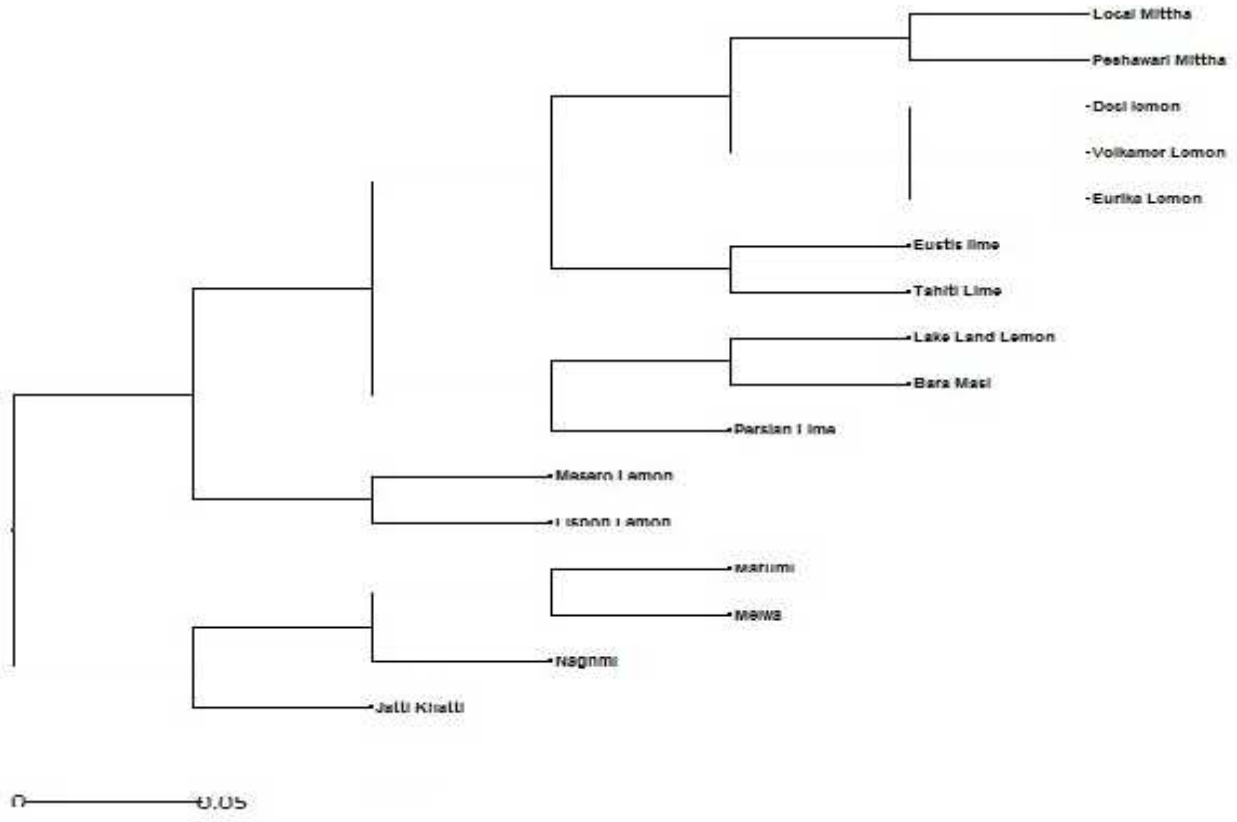

Fig 1: Dendrogram demonstrating the genetic diversity and relationships among 16 citrus genotypes based on EST-SSR data using Hierarchial clustering method with the "DARwin" software.

DISCUSSION

The analysis of genetic diversity is a classical application of SSR markers. The present study reveals the abundance of microsatellites in the Citrus genome and evaluates the use of EST-SSR markers to for systematic studies. All microsatellites produced amplification products in the expected size range in all Citrus cultivars representing different species, which indicated the existence of sequence conservation within the primer sites flanking the microsatellites in the Citrus genome (Kijas *et al.*, 1995). Jarrell *et al.*, (1992) suggested structural as well as functional homology in the Citrus genome. According to Kijas *et al.*, (1995), SSR primer

conservation was found to exist across a wide range of Citrus and related species. The analysis of 16 genotypes with microsatellite markers proved that most microsatellites were very informative and valuable in the evaluation of genetic diversity within and among the different cultivars collected from Citrus Research Institute, Sargodha, Pakistan. These 16 citrus genotypes included 7 Lemon, 2 Sweet Lime, 3 Acid Lime, 3 kumquats and 1 root stock. Among the loci analyzed by 14 microsatellite markers, only 10 showed polymorphism.

In Citrus, the genetic variability is linked to the high number of hybrids and species (taxonomic units) as well as to limb sports and bud mutations. In the current study the varieties belonging to the lemon, acid lime and sweet lime groups clustered together. The same pattern of clustering was also achieved in the earlier studies (Federici *et al.*, 1998; Nicolosi *et al.*, 2000). This might be due to the fact of their common origin from the same parent i.e. citron. Lemons are thought to be natural hybrids of a citron (*C. medica*) and a lime (Barrett and Rhodes, 1976; Scora, 1975) or a hybrid of citron and sour orange (Gulsen and Roose, 2001; Nicolosi *et al.*, 2000). Limes are apparent hybrids of citron and papedas (Scora, 1975) or trihybrid cross of citron, pummelo and Microcitrus (Barrett and Rhodes, 1976).

The kumquats cultivars i.e. Meiwa and Marumi were clustered in the separate clade and Nagami appeared to be a separate branch of the clade. To appear in the separate clade might be due to the reason that *Fortunella spp.* is considered to be close relative to the genus Citrus as compared to the *Poncirus spp* (Xiao-Ming *et al.*, 2003). The appearance of Meiwa with other two cultivars is justified by the fact that Meiwa (*Fortunella crassifolia*) is a natural hybrid of Marumi (*Fortunella japonica*) and Nagami (*Fortunella margarita*). Meiwa has close proximity with Marumi in terms of their morphological characters so as is evident by their presence in same branch which is in accordance to the work reported by Naz *et al*., 2014. This clustering approach identified and affirmed the previous suspected parentage. For example, rough lemon was confirmed by this analysis as being natural hybrid between mandarin and citron as in the present study Jatti Khatti (a rough lemon) separated distinctly from the other cultivars. The usage of rough lemon as a root stock could also be the reason of its appearance in a separate branch with the kumquat genotypes. This study reveals the abundance of microsatellites in the Citrus genome. Our results showed conservation of microsatellite loci among the most important Citrus species. Microsatellites were able to efficiently identify the cultivars at the species level but individual cultivars within each species which were thought to be evolved by mutation, were undistinguishable.

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