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Characterization and Estimation of Genetic Diversity in Citrus Rootstocks

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ABSTRACT

Thirty one genotypes representing 10 rootstocks of *Citrus* sp. were analyzed using 10 microsatellite and 17 Operon primers. The DNA analysis allowed the characterization of all samples and the detection of different level of polymorphisms. Specific markers, differentiating the rootstocks, were identified and could be used as marker assisted selection in breeding programs. The genetic diversity and relationships between the rootstocks revealed the highest value in sour orange, while the lowest one in Cleopatra mandarin. Data based on SSR and RAPD markers were used to estimate the genetic dissimilarity and to establish a dendrogram reflecting the genetic relationship between the different rootstocks. The dendrogram showed that all rootstocks were classified into tow main groups, the first formed of *P. trifoliata* and its hybrids, and the other consists of the rest of rootstocks. © 2011 Friends Science Publishers

Key Words: *Citrus* rootstock; Molecular markers; Genetic diversity; Syria

INTRODUCTION

Citrus genus is an economically important fruit crop in the world, mainly in Mediterranean region. The taxonomy and phylogeny of *Citrus* are complicated due to the high rate of bud mutation and the sexual compatibility between *Citrus* and related genera and the asexual reproduction through nucellar embryony, which is characteristic in several *Citrus* species and most rootstock (Frost, 1943; Cameron, 1979).

Citrus is usually propagated vegetatively by grafting the scion cultivars onto a rootstock; therefore, it was possible to keep the mutations. Uniformity and purity of rootstock genotypes, essential for orchard establishment and increase both quantity and quality of fruit production. Based on morphological traits, it is difficult to distinguish between many *Citrus* cultivars such as many sweet orange, and some rootstocks such as many cultivars of trifoliate orange (Fang & Roose, 1997). In addition, morphological traits are highly influenced by the environment conditions (Fang *et al*., 1998).

A variety of DNA markers have been used to study the *Citrus* taxonomy and phylogenetic relationships within *Citrus* and with related genera. Random Amplified Polymorphic DNA (RAPD) markers have been used for taxonomy studies (Luro *et al*., 1992), for genetic diversity (Abkenar & Isshiki, 2003; Das *et al*., 2004), for differentiating hybrids in *Citrus* breeding program (Bastianel *et al*., 1998) and for phylogenetic analysis (Federici *et al*., 1998; Cabrita *et al*., 2001).

During the last decade, microsatellite markers (SSR) have been extensively exploited. They have been applied in *Citrus* for identification of species and cultivars (Novelli *et al*., 2000; Corazza-Nunes *et al*., 2002; Koehler-Santos *et al*., 2003), for phylogenetic studies (Pang *et al*., 2003; Barkley *et al*., 2006), for zygotic and nucellar seedlings identification (Oliveira *et al*., 2002; Ruiz *et al*., 2000) and for the linkage maps (Kijas *et al*., 1997; Cristofani *et al*., 2003).

The objectives of the present study were the identification of ten *Citrus* rootstocks present in the Germplasm pool at the Department of *Citrus* Research in Tartous, Syria, then the evaluation of their genetic diversity and the establishment of genetic relationships between them using microsatellite and RAPD markers.

MATERIALS AND METHODS

Plant material: A total of 31 samples for 10 rootstocks, representing all rootstocks in the Germplasm pool at the Department of *Citrus* Research, were used in this study. 9 rootstocks belonging to the genus *Citrus* and one [trifoliate orange (*Poncirus trifoliate*)] is from *Poncirus* (Table I). Three samples of different trees (genotypes) were used for each rootstock, except for sour orange, where 8 genotypes were analysed, as it is the only rootstock used in Syria, and one sample from each of rough lemon (*Citrus jambhiri* Lush.) and rangpur (*Citrus limonia* Osbeck).

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DNA isolation: Young leaves (3-4 weeks old) were collected and used for DNA extraction. 200 mg of fresh leaves were ground with 1 mL of preheated (65°C) extraction buffer (2% hexadecyltrimethylammonium bromide [CTAB: Sigma H-5882], 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). Mixtures were incubated at 60°C for 30 min. then extracted with chloroform-isoamyl alcohol (24:1). Aqueous phases containing DNAs were separated by centrifugation for 10 min, 4000 t/min, at room temperature, then transferred to 2 mL clean tubes. Nucleic acids were precipitated by adding 2/3 of their volumes of cold isopropanol. Nucleic acids were then washed twice with 76% ethanol with 10 mM ammonium acetate. After brief air drying, DNA pellets were resuspended in 300 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and kept at -20°C until use. The analysis was conducted in the laboratory of Molecular Genetic in the Faculty of Agriculture, Tishreen University, Lattakia, Syria.

PCR Amplification and Electrophoresis

RAPD markers: Thirty eight decamer primers obtained from Operon Technologies Inc., were tested and seventeen of them produced polymorphic fragments (Table II).

Each PCR reaction contained 15 ng of DNA template, 200 µM of dNTPs, 15 pg of Operon primer, 1 X of PCR buffer with 1.5 mM of $MgCl_2$ and one unit of *Taq* DNA polymerase, in a final volume of 15 µL. PCR program conditions consisted of 94°C for 5 min, then 35 cycles of 94°C for 1 min followed by 37°C for 1 min and 72°C for 3 min. and one cycle at 72°C for 5 min. Electrophoresis was carried out on 1.75% agarose gel and stained with 0.5 mg. L^{-1} of ethidium bromide.

SSR markers: Out of 14 SSR primer pairs used in the analysis of DNA, 10 were able to detect polymorphism between the samples (Table II). The PCR amplifications were conducted in a total volume of 10 µL solution containing 35 ng of genomic DNA, 1 X of PCR buffer, 200 µM of dNTPs, 3 µM of each primer and 0.5 unit of *Taq* DNA polymerase. PCR program used for the amplification consisted of a cycle of 95°C for 5 min, 45 cycles of 95°C for 30 s followed by annealing step at 65°C for 30 s with -0.7°C/cycle for 15 cycles, then at 54°C for 30 cycle, then at 72°C for 10 min., and one cycle at 72°C for 7 min. The PCR products were separated on 6% acrylamide gel and stained with silver nitrate (Bassam & Caetano-Anollés, 1993).

Data analysis: The amplification products were scored as 1 and 0 for present and absent bands or alleles, respectively. The data matrix was analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 3.2 (Rohlf, 1993). Dendrograms were generated by the Unweighted Pair Group Method with Arithmetic Average (UPGMA) (Sneath & Sokal, 1973). Analysis of genetic diversity (GD) was calculated according to the following formula of Nei (1987):

$$GD = n(1 - \Sigma p^2)/(n - 1)$$

Where (n) is the number of samples and (p) is the frequency of one allele.

Gene diversity was calculated as following:

$$H = (1 - \Sigma p_{ij}^2)$$

Where (p_{ij}) is the frequency of the jth allele generated with the primer i. (Weir, 1990).

RESULTS

RAPD analysis: The analysis of 31 DNA samples by 17 Operon primers showed 143 different bands. Out of them 119 bands (fragments) were polymorphic between the different samples and 24 ones were monomorphic (Fig. 1). The number of amplified fragments varied from 4 (primer OPS-04), to 12 (primer OPB-20) with the average of 8.4 fragments per primer. High level of polymorphism was detected with the different primers, it ranged from 40% (OPB-04, OPE-13) to 100% (OPM-08, OPK-03 & OPG-14) (Table III).

Microsatelitte analysis: the ten SSR primers used in this study have successfully allowed genotyping all accessions and detecting polymorphisms among the Syrian *Citrus* rootstocks. The total number of alleles produced by the ten primer pairs was 48 alleles, ranging from 2 alleles (Org-8, Org-29 & Org-31) to 9 alleles (Org-23) per locus, with an average of 4.8 alleles/locus (Table IV). The highest number of alleles (22) was detected in trifoliate while the lowest number (16) was detected in Cleopatra mandarin (Table V). The maximum polymorphic information contents (PIC) was detected by the primer pair Org-20 (0.77) and the minimum (0.39) was revealed by the primer pair Org-8 (Table V).

Specific markers and genetic diversity: Specific primers were identified in this study. They were able to produce unique and specific fragments or alleles in a determinate genotype (Table IV). Genetic diversity (H) is evaluated for each rootstock (Table V). The lowest value was detected in Sanki, while the highest level was revealed in Sour orange.

Phylogenetic analysis: The information based on RAPD and SSR data were used to calculate the similarity and the genetic distance between the different rootstocks, according to Nei and Li (1979). The obtained values of similarity were used to establish the dendrogram generated by UPGMA showing the relationships among the different rootstocks. In the dendrogram based on SSR and RAPD data (Fig. 2), two distinct branches were revealed. The first branch contains trifoliate orange and its hybrids Citromello and Carrizo and the second one contains the rest of rootstocks. The second branch is divided into two distinct clusters, the first one includes genotypes of sour orange and mandarin (M. sanki & M. Cleopatra), while the second cluster regrouped all the rest of genotypes (Palestine sweet lime, Volkamer lemon, Rough lemon & Rangpur lime Fig. 2).

Table I: List of rootstock samples used in the analysis

Common name	Scientific name (Tanaka system)
Trifoliate orange	*Poncirus trifoliata* (L.) Raf.
Citromello	*C. paradisi* × *P. trifoliata*
Carrizo	*C. sinensis* × *P. trifoliata*
Palestine sweet lime	*C. limettioides* Tan.
Cleopatra mandarin	*C. reshni* Hort.ex Tan
Sanki mandarin	*C. sunki* Tan.
Sour orange	*C. aurantium* L.
Volkamer lemon	*C. volcameriana* Ten. & Pasq
Rough lemon	*C. jambhiri* Lush
Rangpur lime	*C. limonia* Osbeck

Table II: List of Operon primers used in the analysis and the polymorphisms detected

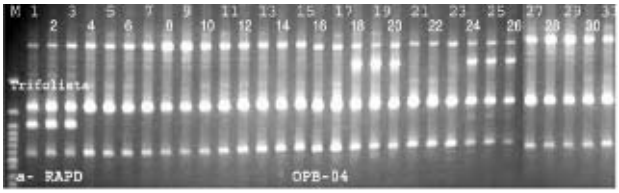
Primers	Number of total bands	Number of monomorphic bands	Number of polymorphic bands	% Polymorphism
OPB01	10	1	9	90
OPB04	5	3	2	40
OPB20	12	1	11	91.66
OPF15	10	1	9	90
OPG14	10	-	10	100
OPH07	8	2	6	75
OPH08	11	1	10	90.90
OPH11	14	5	9	64.28
OPJ15	6	1	5	83.33
OPK03	7	-	7	100
OPK09	7	1	6	85.71
OPM08	8	-	8	100
OPQ14	7	2	5	71.42
OPR05	10	1	9	90
OPR20	9	1	8	88.88
OPS09	4	1	3	75
OPE13	5	3	2	40
Total	143	24	119	

DISCUSSION

The analysis of our rootstock samples with molecular markers proved that RAPD and SSR markers were very useful and informative in the differentiation and estimation of genetic diversity within and between the different rootstocks collected from the Department of *Citrus* Research in Tartous. These results are in accordance with other studies using molecular markers to differentiate the different species of *Citrus* (Federici *et al*., 1998; De Pasquale *et al*., 2006; Hvarleva *et al*., 2008).

Specific fragments or alleles were identified with RAPD and SSR markers respectively, they were able to differentiate some rootstocks. RAPD markers OPF-15, OPR-20, OPB-04 and OPS-09 were able to differentiate the genotypes of Mandarin group, while OPB-04 produced a specific band, which was present only in trifoliate orange, and OPK-03 was able to distinguish trifoliate orange, Citrange and Citromello. Specific SSR markers were also identified Org-14 detected a single allele in Sanki mandarin and a single allele in sour orange ,Org-23 could discriminate Carrizo Citrange and Org-31-F-98, which generated the same allele in trifoliate orange, Carrizo, Citromello, was able to discriminate the trifoliate orange and its hybrids.

Fig. 1: Analysis of DNA samples and detection of specific markers with RAPD primer



These primers are very useful in a breeding program since they can help to follow some specific fragments in the generations and could be used as marker-assisted selection.

The number of SSR alleles/locus varied from 2 to 9 in our samples (Table III). These results are consistent with other analysis when they have got 2 to 9 alleles by analyzing their *Citrus* samples with SSR markers (Golein *et al*., 2006; Li *et al*., 2006; Zerihun *et al*., 2009). The highest number of alleles was found in trifoliate orange with 22 alleles (for 9 samples), whereas the lowest one was observed in Cleopatra mandarin with 16 alleles (for 3 samples). Although the number of alleles alone is not enough, it gives an indication about the level of genetic diversity in a species or a variety.

We could notice from the dendrogram, that all samples belonging to the same rootstock regrouped together in one cluster. Although all samples were different and each one had its own unique pattern, but the genetic diversity in the whole collection was not high. The highest level of genetic diversity was detected in sour orange (0.197), while the lowest one (0.11) was in Sanki mandarin. Similar results were obtaine by analyzing DNA of sour orange with SSR and RAPD markers (Siragusa *et al*., 2006; Ming *et al*., 2009). The level of genetic diversity in our mandarin cultivar (Sanki) was low and close to the values detected in other studies (Coletta Filho *et al*., 2000; Hvarleva *et al*., 2008). Although the genetic diversity within each mandarin cultivar was low, the level of genetic diversity over the whole species of mandarin was higher than other species (*C. medica* & *C. maxima*) (Coletta Filho *et al*., 1998).

The dendrogram based on genetic dissimilarity showed that Trifoliate orange (*Poncirus trifoliata*), Carrizo and Citromello samples were clustered together in one branch, distant from other samples. Considering the phylogeny of Carrizo and Citromello as hybrids from crosses between *P. trifoliata* and *C. sinensis,* and *P. trifoliata* and *C. paradisi*, respectively. The position of these hybrids (close to trifoliate orange) could be justified. These results are in accordance with those already described the relationships between *P. trifoliata* and its hybrids (Herrero *et al*., 1996; Federici *et al*., 1998; Schäfer *et al*., 2004).

Rangpur lemon and Rough lemon were clustered together with Palestine sweet lime and Volkamer lemon (Fig. 2). The presence of these rootstocks together in one cluster could be explained by the fact that all these rootstocks are hybrids and one of the suggested parents is the Citron (*C. medica*) (Nicolosi *et al*., 2000). Rangpur

Table III: Sequences of SSR primers, number of alleles detected, GD and PIC values

Primers (Locus)	Primer sequences 5 to 3'	N. of alleles	GD	PIC
Org-1	5'-TTTGACATCAACATAAAACAAGAAA/5'-TTTTAAAATCCCTGACCAGA	3	0.421	0.432
Org-2	5'-AAAGGGAAAGCCCTAATCTCA/5'-CTTCCTCTTGCGGAGTGTTC	7	0.433	0.447
Org-3	5'-TTCCTTATGTAATTGCTCTTTG/5'-TGTGAGTGTTTGTGCGTGTG	6	0.597	0.616
Org-8	5'-AGAAGCCATCTCTCTGCTGC/5'-AATTCAGTCCCATTCCATTCC	2	0.390	0.412
Org-14	5'-CGCCAAGCTTACCACTCACTAC/5'-GCCACGATTTGTAGGGGATAG	8	0.528	0.545
Org-20	5'-GGATGAAAAATGCTCAAAATG/5'-TAGTACCCACAGGGAAGAGAGC	6	0.775	0.800
Org-23	5'-AGGTCTACATTGGCATTGTC/5'-ACATGCAGZTGCTATAATGAATG	9	0.399	0.412
Org-28	5'-ATGAAGGCTTTTTAGAGCCGAGTT/5'-ATAATAGGGGCCCACTTGACTTG	3	0.571	0.532
Org-29	5'-GTTCGCTCCACGCGATTTAT/5'-TGTGAAGAAAGATTTGGTGGGTTT	2	0.312	0.322
Org-31-F-98	5'-ATATAGCCCCTAGGCCTCCCTATC/5'-GAGTAACCATGGGAGGAGAAAGGA	2	0.413	0.426
total		48		
Average		4.8	0.434	0.484

GD: Genetic diversity; PIC: Polymorphic Info Contents

Table IV: Specific fragments and alleles detected by RAPD and SSR primers

RAPD Primers	Total number of fragments	Specific fragments	Distinguished rootstocks with specific markers
OPF15	10 (fragments)	1	Cleopatra mandarin, Sanki mandarin
OPS09	4 (fragments)	1	Cleopatra mandarin, Sanki mandarin
OPR20	9 (fragments)	1	Cleopatra mandarin, Sanki mandarin
OPR20	9 (fragments)	1	Cleopatra mandarin
OPK03	7 (fragments)	1	Trifoliate orange, Citromello, Carrizo
OPB04	5 (fragments)	1	Trifoliate orange
OPB04	5 (fragments)	1	Cleopatra mandarin, Sanki mandarin
SSR Primers	**No. of alleles**	**Specific alleles**	
Org-23	9 (Alleles)	1	Carrizo
Org-31-F-98	2 (Alleles)	1	Trifoliate orange, Citromello, Carrizo
Org-14	8 (Alleles)	1	Sanki mandarin
Org-14	8 (Alleles)	1	Sour orange

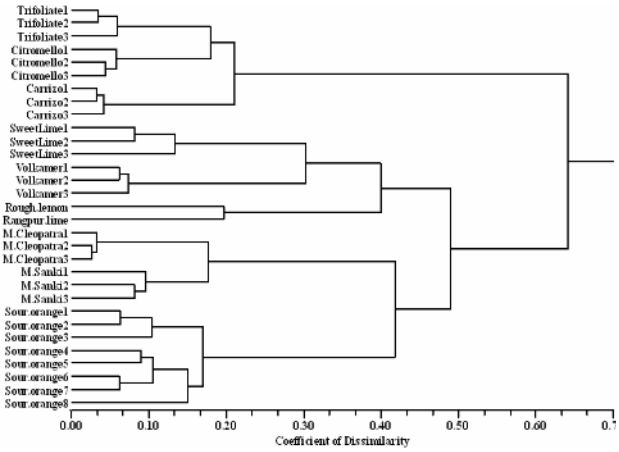
Table V: Value of genetic diversity (H) based on SSR results

No. of rootstocks	Trifoliate orange	Citromello	Carrizo	Sweet lime	Cleopatra	Sanki	Sour orange	Volkamer lemon
No. of alleles	22	17	17	20	16	17	20	20
H	0.156	0.145	0.156	0.168	0.14	0.11	0.197	0.133

lemon and rough lemon are hybrids between citron and mandarin, and citron was the male parent for Palestine sweet lime (Nicolosi *et al*., 2000).

Volkamer lemon regrouped in the same branch with sour orange and mandarin, and it's position is justified.

Fig. 2: Dendrogram of genetic distance (Dissimilarity) based on RAPD and SSR data



Some studies considered it as a hybrid between citron and sour orange (Deng *et al*., 1996), while others suggested Mandarin as one of its parents (Luro *et al.,* 1995; Nicolosi *et al*., 2000). In our study, Volkamer lemon is as close to sour orange as to Mandarin group, which did not support any of the two suggestions about its second parent (Mandarin or sour orange). Rough lemon is the closest rootstock to Volkamer lemon in the cluster (Hussein *et al*., 2003) and with the phylogeny suggesting the same putative parents for the two hybrids (Volkamer lemon & Rough lemon) (Luro *et al*., 1995).

Cleopatra mandarin and Sanki mandarin were differentiated from each other and were clustered together in one cluster in the same branch with Sour orange. It is known that Mandarin is one of the three true species of *Citrus* (Scora, 1975; Barrett & Rhodes, 1976) and that Sour orange (*C. aurantium*) is considered as a hybrid between Pummelo and Mandarin (Scora, 1975; Luro *et al*., 1995). Therefore, the presence of a part of the Mandarin genome [as it's considered as a putative parent of sour orange (Luro *et al*., 1995)] in sour orange genome can explain the position of sour orange in the cluster closer to Mandarin than to other species or rootstocks.

In conclusion, we have confirmed that RAPD and SSR markers can detect enough polymorphism to differentiate and characterize all samples (genotypes) and the different rootstocks. The combination of different kinds of molecular markers proved to be a powerful tool in carrying out a more complete analysis of *Citrus* phylogeny and origin.

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