GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Molecular characterization of sour orange (*Citrus aurantium*) accessions and their relatives using SSR and SRAP markers

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Genet. Mol. Res. 11 (3): 3267-3276 (2012)
Received November 11, 2011
Accepted June 21, 2012
Published September 12, 2012
DOI http://dx.doi.org/10.4238/2012.September.12.10

ABSTRACT. Citrus production with its many varieties is of importance since it provides economically important products for Turkish exports. Sour orange is a rootstock commonly used for propagating the different scion varieties. Knowledge of the genetic diversity of the rootstock accessions would be useful in order to improve citrus breeding programs. We studied genetic relationships and diversity of 51 accessions of sour orange (*Citrus aurantium*) and their relatives using SSR (simple sequence repeat) and SRAP (sequence-related amplified polymorphism) molecular markers. Twenty-one SRAP primer combinations were tested on these accessions and relatives, producing 167 polymorphic fragments, with a mean of 8.0 and a mean polymorphism information content

value of 0.47. Seventeen SSR primers also produced 30 polymorphic fragments, with a mean of 1.4 per primer and a mean polymorphism information content value of 0.39. The unweighted pair-group method with arithmetic average analysis using combined SSR and SRAP data showed a similarity range from 0.71 to 1.00 among the accessions. In the cluster analysis, sour orange relatives were indicated as a separate group from sour orange. 'Macrophylla' and 'Mexican lime' were the accessions most distinct (0.71) from the others. We conclude that genetic diversity in these sour orange accessions is lower and some of them were identical.

Key words: *Citrus aurantium* L.; Genetic resource; Sour orange

INTRODUCTION

Citrus is one of the major fruit crops grown in tropical and subtropical regions of the world. Citrus production in Turkey is among the most important within the Mediterranean basin countries, with production of 3.5 million tons per year (FAO, 2009). Budding a desired scion onto rootstock seedlings is a technique commonly used to propagate *Citrus*. Sour orange (*Citrus aurantium* L.) is a widely planted, high-performance rootstock (Hodgson, 1967; Castle, 1987). Sour orange has been reported to be a hybrid of mandarin and pummelo (Barkley et al., 2006), and it is one of the most widely used rootstocks in the world. The bulk of citrus production in the Mediterranean region of Turkey uses scions grafted onto sour orange (Tuzcu et al., 2001).

Citrus taxonomy and phylogeny are complicated, controversial, and lead to confusion owing to the sexual compatibility between *Citrus* and its related genera. The high frequency of bud mutations, the long history of cultivation, and wide dispersal are some of the many factors influencing taxonomic differentiation (Nicolosi et al., 2000). Another complexity in *Citrus* taxonomy is disagreement about the degree of differentiation necessary to justify species status and the consideration of hybrids as naturally occurring species forms (Roose et al., 1995). Sour orange produces seeds containing both nucellar and zygotic embryos, and therefore, uniformity is almost complete (Siragusa et al., 2006). Understanding the taxonomy, phylogenetic relationships, and genetic variability in *Citrus* is critical for the determination of genetic relationships, characterization of germplasm, control of genetic erosion, design of sampling strategies for core collections, establishment of breeding programs, and registration of new cultivars (Herrero et al., 1996).

In the past, studies on relationships between genera and species were carried out mainly using morphological characteristics. More recently, diversity studies has gained further support from various studies using molecular markers. Molecular markers have been reported to be powerful tools for elucidating genetic diversity, determining parentage, and revealing phylogenetic relationships among various *Citrus* species (Barkley et al., 2006).

Many attempts have been made to study species relationships, fingerprint accessions, evaluate phylogenetic relationships among accessions, map, and examine the level of genetic diversity in *Citrus* using DNA fingerprints. Methods such as restriction fragment length polymorphism (Fang et al., 1997; Fu et al., 2004), randomly amplified polymorphic DNA (Aka-Kacar et al., 2005; Rodriguez et al., 2005), amplified fragment length polymorphism (Fu et

al., 2004; Campos et al., 2005), simple sequence repeats (SSR) (Barkley et al., 2006, 2009), inter-simple sequence repeats (Fang et al., 1998; Capparelli et al., 2004), and sequence-related amplified polymorphism (SRAP) (Uzun et al., 2009) have been used.

SSR markers have been the most commonly implemented markers in molecular biology for mapping, genetic diversity, phylogenetic construction, and fingerprinting because they are codominant, highly polymorphic, and easy to use (Barkley et al., 2009). SRAP markers are part of a simple and efficient system that can be adapted for a variety of purposes in various crops, including map construction, gene tagging, genomic and complementary DNA fingerprinting, and map-based cloning (Li and Quiros, 2001; Comlekcioglu et al., 2010). In our study, genetic relationships and diversity were determined using SSR and SRAP molecular markers within 51 sour oranges (*C. aurantium* L.) and their relatives collected from selections and introductions.

MATERIAL AND METHODS

Plant material

Forty-two *C. aurantium* L. accessions and those of 9 relatives were used (Table 1). All plant material for genetic studies was obtained from the Tuzcu Citrus Collection (University of Cukurova, Adana, Turkey).

DNA extraction

Total genomic DNA was extracted from young leaves using the cetyltrimethylammonium bromide method as described by Doyle and Doyle (1990).

SRAP analysis

Twenty-one primers in high-ratio polymorphic bands were amplified with the rest of the accession DNAs (Table 2). Each 15-µL reaction consisted of 1.33 mM primers, 200 mM of each deoxyribonucleotide triphosphate (Biorun, France), 1.5 µL 10X polymerase chain reaction (PCR) buffer (Biorun), 2 mM $MgCl_2$, 0.8 mg/µL bovine serum albumin (Biological Industries, Israel), 5.8 mL double-distilled water, 1 U *Taq* polymerase (Biorun), and 20 ng template.

A DNA Thermal Cycler (Nyx Technik) was used, and cycling parameters, included 2 min of denaturing at 94°C and 5 cycles of 3 steps: 1 min of denaturing at 94°C, 1 min of annealing at 35°C, and 1 min of elongation at 72°C. In the next 35 cycles, the annealing temperature was increased to 50°C, and for extension, 1 cycle of 5 min at 72°C was performed.

SSR analysis

Twenty-six primers (Barkley et al., 2006; Roose, 2009) were used to amplify the DNA. Seventeen primers producing scorable polymorphic bands were used to amplify all of the accessions (Table 3). Each 10-µL reaction consisted of 1.0 µL primers, 200 mM of each deoxyribonucleotide triphosphate, 1.0 µL 10X PCR buffer , 1.0 µL 2.5 mM $MgCl_2$ 4.8 µL double-distilled water, 0.2 µL 0.6 U Taq DNA polymerase, and 1.0 µL 20 ng DNA. A DNA Thermal

Cycler (Bio-Rad) was used, and the cycling parameters included 3 min of denaturing at 94°C, 35 cycles of 3 steps [30 s of denaturing at 94°C, 30 s of annealing at 50°C or 40°C (depending on the primer), and 1 min of elongation at 72°C], and 1 cycle of 10 min at 72°C for extension.

Table 1. Plant materials used in this study were identified by Tanaka species name, common or cultivar names and origin or country names.

Cultivar or common name	Species name (Tanaka system)	Origin or country introduced
Apepu Azaguie sour orange	*Citrus aurantium* L.	Ivory Coast
Bouquetier de Nice sour orange	*Citrus aurantium* L.	Morocco
Ferando sour orange	*Citrus aurantium* L.	France
Petit Pierre sour orange	*Citrus aurantium* L.	Tunisia
Santucci sour orange	*Citrus aurantium* L.	France
Menton sour orange	*Citrus aurantium* L.	France
Alibert sour orange	*Citrus aurantium* L.	Tunisia
Cardosi sour orange	*Citrus aurantium* L.	France
Luisi sour orange	*Citrus aurantium* L.	France
Alibert Hybrid sour orange	*Citrus aurantium* L.	Tunisia
Spain Genest sour orange	*Citrus aurantium* L.	Spain
Australian sour orange	*Citrus aurantium* L.	Morocco
Florida sour orange	*Citrus aurantium* L.	USA
Tulear sour orange	*Citrus aurantium* L.	Madagascar
Curacao sour orange	*Citrus aurantium* L.	Trinidad and Tobago
Brasil sour orange	*Citrus aurantium* L.	USA
Granito sour orange	*Citrus aurantium* L.	Algeria
Daidai SEAB sour orange	*Citrus aurantium* L.	Tunisia
Smooth Seville sour orange	*Citrus aurantium* L.	Pakistan
Tuzcu M 36 sour orange	*Citrus aurantium* L.	France
Paraguay sour orange	*Citrus aurantium* L.	USA
GouTou sour orange	*Citrus aurantium* L.	South Africa
Chinotto (CUZF)	*Citrus myrtifolia* Raf.	USA
Chinotto (BATEM)	*Citrus myrtifolia* Raf.	USA
Tosu sour orange	*Citrus aurantium* L.	USA
Tuzcu 33-11 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 33-6 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 33-32 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 31-28 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 01-23 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 01-17 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 01-24 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 31-25 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 31-30 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 01-20 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu K 35 sour orange	*Citrus aurantium* L.	TRNC
Tuzcu K 34 sour orange	*Citrus aurantium* L.	TRNC
Tuzcu 31-33 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu M 37 sour orange	*Citrus aurantium* L.	France
Tuzcu 893 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 892 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 39 sour orange	*Citrus aurantium* L.	Turkey
Tuzcu 40 sour orange	*Citrus aurantium* L.	Turkey
Brazilian 3372 sour orange	*Citrus aurantium* L.	USA
Yuzu	*C. junos* Sieb ex Yan.	USA
Bergamot	*C. bergamia* Risso & Poit	Italy
Bergamotto Commune	*C. bergamia* Risso & Poit	Italy
Rangpur lime	*C. limonia* Osbeck	USA
Mexican lime	*C. aurantifolia* (Christm) Swing.	USA
Macrophylla	*C. macrophylla* Wester	USA
Taiwanica	*C. taiwanica* Tan. and Shim.	USA

Data analysis

A similarity matrix using the similarity coefficient of simple matching was constructed

for SSR and SRAP data based on the presence (1) or absence (0) of fragments for each primer. Cluster analysis was performed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software package (Rohlf, 2000). The genetic similarity matrix and ultrametric distance matrix produced from unweighted pair group method arithmetic average-based dendrograms using the cophenetic value matrix module nested in the same software was compared using the matrix correspondence Mantel (1967) test. The result of this test is a cophenetic correlation coefficient, *r*, that indicates how well the dendrogram represents similarity data. Polymorphism information content (PIC) values were calculated for SSR and SRAP markers according to the method of Smith et al. (1997).

RESULTS

Twenty-one SRAP primer combinations were screened, and 192 bands displaying polymorphism with high intensity were assessed for discrimination on accessions. The number of bands scored per primer combination ranged from 4 (em11/me1) to 20 (em2/me3), with a mean of 9.1. The number of total polymorphic fragments was 167, with a mean of 8.0. All fragments scored for each primer combination were polymorphic. The PIC values for the 21 primer combinations ranged from 0.14 (em2/me5) to 0.69 (em5/me12), with a mean of 0.47 (see Table 2).

Table 2. Diversity statistics for 21 sequence-related amplified polymorphism (SRAP) primer combinations studied in 51 sour orange rootstocks.

SRAP primers	Allele sizes (bp)	Total band (No.)	Polymorphic band (No.)	Polymorphism (%)	PIC
em1 me4	320/1270	11	10	91	0.58
em2 me3	120/1050	20	19	95	0.56
em2 me5	120/780	6	5	83	0.14
em2 me8	260/850	7	6	86	0.34
em3 me3	130/1250	10	7	70	0.52
em4 me5	280/750	8	7	88	0.52
em4 me6	200/650	12	10	83	0.56
em5 me10	380/880	6	6	100	0.55
em5 me12	300/560	9	9	100	0.69
em6 me6	180/600	8	6	75	0.44
em7 me8	200/880	11	10	91	0.57
em7 me9	280/820	10	8	80	0.36
em9 me3	80/600	9	9	100	0.60
em9 me11	140/880	9	7	78	0.27
em11 me1	320/640	4	3	75	0.27
em10 me11	150/1000	11	11	100	0.34
em13 me4	80/1600	10	10	100	0.59
em14 me1	220/640	7	6	86	0.60
em15 me6	100/720	10	7	70	0.45
em15 me10	300/850	5	3	60	0.41
em16 me12	80/860	9	8	89	0.48
Total		192	167		
Mean		9.1	8.0	87	0.47

PIC = polymorphism information content.

Seventeen of the 26 SSR primers produced well-resolved band fragments. A total of 17 SSR primers were screened, and 44 bands were scored. The number of bands scored per primer ranged from 1 (CAC39.cAGG9) to 5 (CAC19), with a mean of 2.1. The PIC values for the 17 primers ranged from 0.00 (TAA27 and CAGG9) to 0.99 (CAC39), with a mean of 0.39 (see Table 3). A similarity matrix based on SRAP and SSR data was calculated according to

coefficient of Dice (1945). Dice's similarity was used to the cluster analysis and to generate a dendrogram showing the relationship among the accessions situated as shown in Figure 1. The cophenetic correlation between ultrametric similarities of the tree and the similarity matrix was high ($r = 0.98$, $P < 0.01$), suggesting that the cluster analysis strongly represents the similarity matrix. In sour oranges, the 'Australian' and 'Smooth Seville' accessions separated from the others and showed a high level of similarity (0.96).

Table 3. Diversity statistics for 17 simple sequence repeat (SSR) markers studied in 51 sour oranges rootstocks.

SSR loci	Allele sizes (bp)	Total band (No.)	Polymorphic band (No.)	Polymorphism (%)	PIC
CT21	155/160	2	2	100	0.30
AC01	150/160	2	2	100	0.62
CAG01	150/170/350	3	2	67	0.35
CAC19	200/230/240/2607350	5	3	60	0.23
TAA33	180/250	2	1	50	0.42
CAC39	195	1	1	100	0.99
CCT01	210/490	2	2	100	0.97
TAA1	195/198	2	1	50	0.17
TAA27	195/200	2	0	0	0.00
TAA45	145/150/200	3	3	100	0.60
CAC33	150/200/240/350	4	3	75	0.54
ATC09	190/210	2	1	50	0.10
CAT01	120/170/180/190	4	4	100	0.58
TAA15	195/200	2	1	50	0.41
TAA52	90/110	2	1	50	0.02
CAC23	150/260	2	1	50	0.32
cAGG9	110	1	0	0	0.00
Total		44	30		6.97
Mean		2.1	1.4	65	0.39

PIC = polymorphism information content.

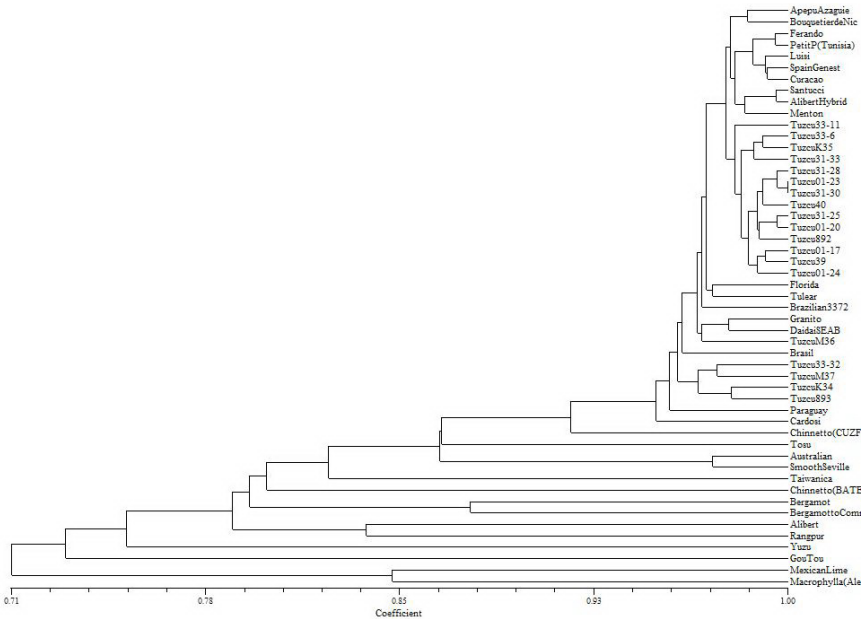

Figure 1. Dendrogram of 51 accessions based on SSR and SRAP markers.

DISCUSSION

The rise of a possible mutation related to most sour oranges can be assumed. Our findings indicate polymorphism resulting from each primer combination of SSR and SRAP markers, which can be associated with genetic diversity on accessions (see Tables 2 and 3). Bud mutations often occur in citrus trees and are generally detected by growers in branches of trees displaying altered horticultural traits, such as maturity and flowering time, or fruit characteristics. Contrasting with this diversity for agronomic traits, very low genetic variability has been found in cultivated citrus using molecular markers (Breto et al., 2001). Unlike previous studies (Luro et al., 2000), our studies showed that microsatellites can distinguish mutation-derived species such as sour oranges. The use of multi-locus, PCR-based SSR and SRAP markers allowed efficient differentiation of tightly linked accessions. The SSR and SRAP analyses of 51 sour orange accessions revealed a high degree of genetic diversity in foreign accessions, whereas a low variability was detected in local accessions. SSR and SRAP molecular markers seem to be convenient for the exact identification of plants with tight genetic relations. These results can be evaluated in future studies for their usefulness in improving new citrus rootstock.

To the best of our knowledge, no data on the assessment of relationships and diversity of accessions using SSR and SRAP molecular markers are available for *Citrus*. Nevertheless, the differentiation of *Citrus* cultivars using SSR markers to measure genetic diversity and phylogenetic relationships among 83 lemons, related taxa, and 3 proposed ancestral species has been reported (Gulsen and Roose, 2001). Correspondingly, genetic diversity statistics have also been calculated for each individual SSR marker, the entire population, and specified *Citrus* groups (Barkley et al., 2006). The efficiency of these markers for the characterization of sweet orange has been tested and evaluated using SSR markers in citrus (Novelli et al., 2006). Four microsatellite loci have been suggested to be useful tools for DNA typing in sweet orange. In another study, 51 accessions representing 8 citrus species from the citrus collection have also been analyzed using 10 microsatellite and 6 RAPD markers (Hvarleva et al., 2008). SRAP markers have been used to evaluate the genetic diversity within 86 citrus and their relatives in Aurantioideae. SRAP analysis has also correlated well with the findings of previous studies on subfamily Aurantioideae that have included many genera (Uzun et al., 2009).

Interestingly, 2 bergamot accessions evaluated in our study were placed in the same genetic cluster, which was associated with the existence of citron and sour orange hybridization (Nicolosi et al., 2000). The belief that citron is a parent of bergamot has been supported by SSR data (Barkley et al., 2006). Sour orange was suggested to most likely be a maternal parent, however, with citron being the paternal parent of bergamot (Li et al., 2010). 'Chinotto (University of Cukurova Faculty of Agriculture - CUZF)', 'Chinotto (Bati Akdeniz Agricultural Research Institute - BATEM)', and 'Taiwanica' (*Citrus taiwanica*) were more closely related to sour orange than were other relatives. This result was consistent with that of previous studies. Sour orange and *C. taiwanica* were clustered in the same group based on inter-simple sequence repeat data (Fang et al., 1998). Swingle and Reece (Swingle, 1967) have reported that *C. taiwanica* is a hybrid of sour orange. 'Smooth Seville' has been reported as a likely hybrid between sweet orange and grapefruit (Hodgson, 1967).

'Tosu', 'Chinotto (CUZF)', and 'Cardosi' also form a separate cluster, which is different from other sour orange cultivars. These accessions are probably not true sour orange, but they may be sour orange hybrids or mutants. Indeed, 'Tosu' has been suggested to be a hybrid

of sour orange and citron or sour orange and mandarin (Jackuemond, 2010). Conversely, 'Chinotto' has been assumed to originate from a sour orange mutation (Hodgson, 1967).

In our study, SSR and SRAP analyses revealed a high level of polymorphism among the accessions studied. Sour orange relatives nested distantly from the sour orange group. 'Macrophylla' and 'Mexican lime' were the most distinct accessions, with a similarity level of 0.71. These 2 accessions also showed a genetic relationship, which agrees with data from previous studies (Federici et al., 1998). Conversely, 'Gou Tou' and 'Yuzu' separated from the rest of the accessions at 0.72 and 0.74, respectively. Confusion about the taxonomy of 'Yuzu' has persisted for many years. Swingle (1967) has hypothesized that 'Yuzu' is a hybrid between Ichang papeda (*Citrus ichangensis*) and mandarin, whereas Tanaka (1954) described 'Yuzu' as a high-quality species and claimed that it was not a hybrid. Hirai et al. (1986) have reported that 'Yuzu' is not a hybrid between Ichang papeda and mandarin (Rahman et al., 2001). 'Alibert' and 'Rangpur' were clustered in the same branch at a similarity level of ~0.78. 'Alibert' has been classified as a sour orange (Cottin, 2002). It has been reported that 'Rangpur' lime, despite its name, is morphologically and genotypically quite different from limes and is listed under *Citrus reticulata* (Torres et al., 1978). Conversely, Nicolosi et al. (2000) has indicated that 'Rangpur' is a hybrid of citron and mandarin.

Tuzcu series sour oranges were selected for their favorable characteristics as rootstocks in Turkey (Okyay, 1987). Although these sour oranges are morphologically distinct, the genetic diversity among them was very low.

Our data confirmed that SSR and SRAP methods are useful tools for the identification of closely related accessions. The combination of SSR and SRAP marker methods also guarantees some additional benefits. The SRAP and SSR molecular markers seem to be suitable for the finely tuned identification of tightly related plants, and the results presented here can form the basis for the design of future *Citrus* rootstock genetic improvement projects.

ACKNOWLEDGMENTS

Research supported by the Scientific and Technological Research Council of Turkey, the General Directorate of Agricultural Research of Ministry of Food and Agriculture, and the Scientific Research Project Unit of the University of Cukurova, Adana, Turkey. We thank Dr. Ben Faber and Dr. Ömür Baysal for advice and helpful comments on earlier versions of this manuscript.

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