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Investigation of genetic relationships among trifoliata oranges and their hybrid relatives based on ISSR markers

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Abstract

Diversity and genetic relationships were investigated in 19 trifoliata oranges [(Poncirus trifoliata (L.) Raf] and their 27 hybrids including 20 citranges (P. trifoliata X Citrus sinensis), five citrumelos (P. trifoliata X C. paradisi) and two citremons (P. trifoliata X C. limon) based on inter-simple sequence repeat (ISSR) markers. Twelve ISSR primers produced a total of 120 fragments and 82 of them were polymorphic (68%). The unweighted pair group method arithmetic average (UPGMA) analysis as assessed with ISSR data demonstrated that the accessions had a similarity range from 0.67 to 1.00. In the dendrogram there were three main groups including trifoliata, citremon and citrange–citrumelo groups. The trifoliata orange, citrange, citrumelo and citremon groups were separated. The citrumelo group was nested in the citrange branch. Genetic diversity among the trifoliata accessions was low probably because of their mutation origin. However all trifoliata accessions were distinguished except for two of them. The citrange group had higher polymorphism than the other trifoliata hybrids. Our study indicated that ISSR markers were useful to determine genetic diversity of related citrus groups such as trifoliata oranges and their intergeneric hybrids.

Key words: *Poncirus trifoliata,* citrus, citremon, citrange, citrumelo, rootstock

Introduction

Poncirus trifoliata (L.) Raf., is known as trifoliata orange, native to central or northern China, and widely distributed. The first known description of trifoliata orange as a rootstock occurs in Han Yen-chih's *Chü Lu*, written in 1178 A.D., and translated in 1923. As an outdoor ornamental, this plant is commonly grown in the warm temperate regions of China, Japan, Western Europe, and Eastern United States. It is sometimes used as a hedge, for which it is very effective. It has been the most important rootstock in Japan, primarily for the satsuma mandarins, and increasingly is being employed in Australia, California, and Argentina (HODGSON, 1967 [1]). Trifoliata orange belongs to 'true citrus fruit trees' in subtribe Citrinae tribe, Citreae subfamily Aurantioideae (SWINGLE & REECE 1967 [2]), a close relative of the genus *Citrus* and is widely used as a rootstock now for citrus trees in citrus growing countries. It is preferred because of its resistance to citrus tristeza virus (CTV), *Phytophthora* root rot [(*Phytophthora citropthora* (Sm. and Sm.) Leonian] and to citrus nematode [(*Tylenchus semipenetrans* (Cobb)]. But it is susceptible to citrus exocortis viroid (CEVd) and to iron chlorosis on calcareous soil (FANG & al. 1997 [3]; NOVELLI & al. 2000 [4]).

Despite remarkable differences from *Citrus* in nearly all respects, *Poncirus* hybridizes freely with the citrus species. Because of its outstanding cold-hardiness, it was early used in

the citrus breeding program beginning in Florida in 1897, and continuing for several decades, many crosses have been made between the trifoliata orange and citrus species and some with other genera around the world. From this work came a series of bigeneric hybrids including the citranges (*P. trifoliata* X *Citrus sinensis)*, citrumelos (*P. trifoliata* X *C. paradisi)*, citrandarins (*P. trifoliata* X *C. reticulata)*, citremons (*P. trifoliata* X *C. limon)* which are of horticultural importance or promise (HODGSON, 1967 [1]). Use of trifoliata hybrids especially citranges and citrumelos as citrus rootstock has increased for their tolerance to tristeza disease and cold (BENSON & al. 1997[5]). Most of citrus cultivars including many sweet oranges, grapefruit selections, and rootstocks such as many cultivars of trifoliata oranges originated from mutation. Identifcation of these scion cultivars in nursery situations is particularly difficult because some cultivars are distinguishable only by fruit traits. It is also difficult to distinguish some rootstock cultivars after trees are planted in the field. This may become an issue if a grower suspects that a nursery sold trees on a rootstock other than ordered. The ability to identify citrus cultivars using a small amount of leaf or other vegetative tissue would be helpful in protecting the rights of citrus breeders, growers and nurseries (FANG & ROOSE, 1997 [6]).

Various PCR are available for assessing phylogenetic relationships and genetic studies in plants. For example, inter-simple sequence repeat (ISSR) markers involve amplification of DNA segment between two identical microsatellite repeat regions. ISSRs have high reproducibility possibly due to the use of longer primers (16–25 mers) as compared to RAPD primers (10- mers) which permits the subsequent use of high annealing temperature (45–60 ^{o}C), leading to higher stringency. This technique overcomes most of limitations such as low reproducibility and high cost (ZIETKIEWICZ & al. 1994 [7]; PRADEEP REDDY & al. 2002 [8]). It is rapidly being used by the research community in various fields of plant improvement. The technique is useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of crop species (PRADEEP REDDY & al. 2002 [8]). ISSRs have been used to determine the genetic diversity, characterization, phylogenetic relationships among *Citrus* and related genera (GULSEN & ROOSE, 2001a [9]; SHAHSAVAR & al. 2007 [10]; UZUN & al. 2009 [11]; MARAK & LASKAR, 2010 [12]; UZUN & al. 2010 [13]) and trifoliata orange accessions (FANG & al. 1997 [3]). There are different marker systems which were compared for detecting diversity and relationships in *Cynodon* accessions (GULSEN & al. 2010a [14]). In that study, the combined analysis of SRAP, POGP, ISSR, and RAPD markers indicated that correlation coefficients of so-called Mantel test among the GS similarity matrices produced with SRAP, POGP, and ISSR markers were similar (r = 0.85, 0.86, and 0.87, respectively). However, correlation between RAPD and the other markers was low (0.71). In the present study, we have examined the diversity and phylogenetic relationships in trifoliata oranges and their hybrids including citranges, citrumelos and citremons using ISSR markers. This study increases our understanding of diversity and relationships among the trifoliata oranges and their hybrids, which includes more diverse accessions.

Materials and Methods

Plant Materials

Nineteen trifoliata orange, 20 citrange (*P. trifoliata* X *C. sinensis)*, five citrumelo (*P. trifoliata* X *C. paradisi)* and two citremon (*P. trifoliata* X *C. limon)* accessions were used for this study (Table 1). All accessions were obtained for DNA extractions from 'Tuzcu Citrus Collection', University of Cukurova, Adana, Turkey.

Table 1. Plant materials, their cultivar or common name and species name or origin

	Cultivar or common name	Species name or origin
1	Trifoliata Benecke	*Poncirus trifoliata* (L.) Raf.
2	Trifoliata Rubidoux (3376R)	*Poncirus trifoliata* (L.) Raf.
3	Trifoliata Town	*Poncirus trifoliata* (L.) Raf.
4	Trifoliata Luisi	*Poncirus trifoliata* (L.) Raf.
5	Trifoliata Dwarf	*Poncirus trifoliata* (L.) Raf.
6	Trifoliata Rich	*Poncirus trifoliata* (L.) Raf.
7	Trifoliata Pomeroy	*Poncirus trifoliata* (L.) Raf.
8	Trifoliata Krider	*Poncirus trifoliata* (L.) Raf.
9	Trifoliata English	*Poncirus trifoliata* (L.) Raf.
10	Trifoliata Christiansen	*Poncirus trifoliata* (L.) Raf.
11	Trifoliata SEAB	*Poncirus trifoliata* (L.) Raf.
12	Trifoliata Davis	*Poncirus trifoliata* (L.) Raf.
13	Trifoliata Ferme Blanche	*Poncirus trifoliata* (L.) Raf.
14	Trifoliata Flying Dragon	*Poncirus trifoliata* (L.) Raf.
15	Trifoliata Rubidoux (L. Alfred)	*Poncirus trifoliata* (L.) Raf.
16	Trifoliata Jacobson	*Poncirus trifoliata* (L.) Raf.
17	Trifoliata Yamaguchi	*Poncirus trifoliata* (L.) Raf.
18	Trifoliata Yerli (Rize)	*Poncirus trifoliata* (L.) Raf.
19	Trifoliata Menager	*Poncirus trifoliata* (L.) Raf.
20	Citrange Morton	(*Poncirus trifoliata* X *Citrus sinensis)*
21	Citrange Troyer	(*Poncirus trifoliata* X *Citrus sinensis)*
22	Citrange Savagee	(*Poncirus trifoliata* X *Citrus sinensis)*
23	Citrange Rusk	(*Poncirus trifoliata* X *Citrus sinensis)*
24	Citrange Cunningham	(*Poncirus trifoliata* X *Citrus sinensis)*
25	Citrange Etonia	(*Poncirus trifoliata* X *Citrus sinensis)*
26	Citrange Kindia	(*Poncirus trifoliata* X *Citrus sinensis)*
27	Citrange Montauban	(*Poncirus trifoliata* X *Citrus sinensis)*
28	Citrange Tuzcu M-2	(*Poncirus trifoliata* X *Citrus sinensis)*
29	Citrange 8C15.7	(*Poncirus trifoliata* X *Citrus sinensis)*
30	Citrange 11C80.8	(*Poncirus trifoliata* X *Citrus sinensis)*
31	Citrange 8C15.5	(*Poncirus trifoliata* X *Citrus sinensis)*
32	Citrange 8C15.16	(*Poncirus trifoliata* X *Citrus sinensis)*
33	Citrange 8C14.7	(*Poncirus trifoliata* X *Citrus sinensis)*
34	Citrange 11C80.7	(*Poncirus trifoliata* X *Citrus sinensis)*
35	Citrange Uvalde	(*Poncirus trifoliata* X *Citrus sinensis)*
36	Citrange Coleman	(*Poncirus trifoliata* X *Citrus sinensis)*
37	Citrange C32	(*Poncirus trifoliata* X *Citrus sinensis)*
38	Citrange C35	(*Poncirus trifoliata* X *Citrus sinensis)*
39	Citrange Carrizo	(*Poncirus trifoliata* X *Citrus sinensis)*
40	Citrumelo Swingle	*(Poncirus trifoliata* X *Citrus paradisi)*
41	Citrumelo 4475	*(Poncirus trifoliata* X *Citrus paradisi)*
42	Citrumelo 1452	*(Poncirus trifoliata* X *Citrus paradisi)*
43	Citrumelo Sacaton	*(Poncirus trifoliata* X *Citrus paradisi)*
44	Citrumelo Sacaton (Newcomb)	*(Poncirus trifoliata* X *Citrus paradisi)*
45	Citremon 1449	(*Poncirus trifoliata* X *Citrus limon)*
46	Citremon 1449 CRC	(*Poncirus trifoliata* X *Citrus limon)*

DNA extraction and ISSR analysis

Genomic DNA was extracted from young leaves of 46 accessions by the CTAB method as described by DOYLE & DOYLE (1990) [15]. DNA concentration was measured with a microplate spectrophotometer (BioTek Instruments, Inc. Vinooski, USA), and 10 ng/µL DNA templates were made using TE (10 mM Tris– HCl, 0.1 mM EDTA, pH 8.0). A total of 12 ISSR primers previously evaluated by FANG & ROOSE, (1997) [6] and GULSEN & al. (2010b [16]) were used for PCR amplifications (Table 2). PCR reaction components and cycling parameters were performed as described by UZUN & al. (2009 [11]). PCR products were separated on 2% agarose gel in 1X TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) at 115 V for 3 h. The fragment patterns were photographed under UV light for further analysis. A 100 bp standard DNA ladder (GeneRuler, Fermentas) as the molecular standard in order to confirm the appropriate markers were used for ISSR analysis.

Data Analysis

A similarity matrix using the similarity coefficient of simple matching was constructed for the ISSR data. Cluster analysis was performed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software package (ROHLF, 2000 [17]). The genetic similarity matrix and ultrametric distance matrix produced from UPGMA-based dendrogram with COPH module nested in the same software was compared using Mantel's matrix correspondence test (MANTEL, 1967 [18]). The result of this test is a cophenetic correlation coefficient, *r*, that indicates how well dendrogram represents similarity data. Polymorphism information content (PIC) values were calculated ISSR markers according to SMITH & al. (1997 [19]), using the following formula for all primer combinations:

$PIC = 1-\sum fi^2$

Where fi^2 is the frequency of the i^{th} allele. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles (SMITH & al. 1997 [19]). PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles in equal frequencies).

Results and discussion

ISSR amplification

A total of 12 ISSR primers were screened and a total of 120 bands with high intensity were scored. The number of bands scored per primer ranged from 3 ($HVH(CA)_7T$) to 19 ($(GA)_8YG$), with a mean of 10.0. A picture of primer $(GACA)_4$ was presented in Fig. 1. Polymorphic fragment number varied between 1 ($HVH(CA)_7T$) and 13 ($(GA)_8$ YG), with a mean of 6.8, totaling 82 (68%). FANG et al. (1997) obtained higher band number (as mean 58) from ISSR markers in trifoliata orange owing to use of nondenaturing polyacrylamide gel.

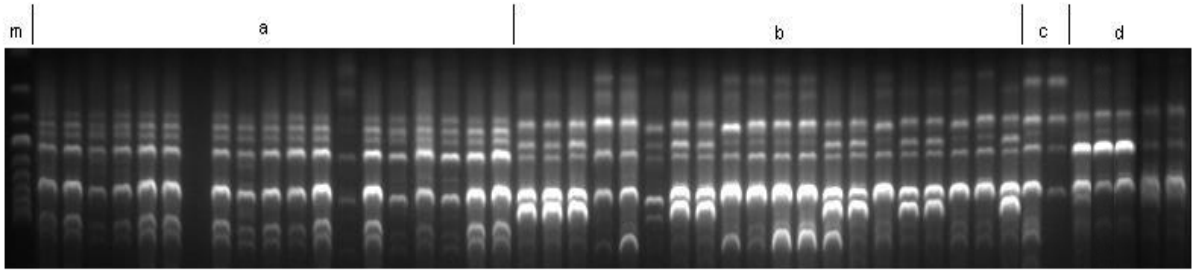

Figure 1. ISSR profiles amplified from DNA of accessions studied using primer $(GACA)_4$ (m= 100 bp DNA marker, a: trifoliatas, b: citranges, c: citremons, and d: citrumelos)

On the other hand they found lower polymorphism (10.2%) probably because of studying only trifoliata oranges, not their hybrids. The PIC values for the 12 primers ranged from 0.17 $(CAC)_6$ to 0.68 $(TCC)_5$ RY), with an average of 0.41 in our study (Table 2).

Table 2. List of the ISSR primers, their numbers of total and polymorphic fragments, percentage of polymorphism, and polymorphism information contents used in this study

ISSR Primers	Total Fragments	Polymorphic Fragments	Polymorphism (%)	PIC
$(CAC)_6$	6	2	33	0.17
$(GA)_8YG$	19	13	68	0.52
$(GAA)_6$	14	11	79	0.50
$(GACA)_4$	10	8	80	0.57
$(GT)_8YA$	10	7	70	0.38
$(TAA)_8$	8	6	75	0.31
$(TCC)_5RY$	10	9	90	0.68
$BDB(CA)_7C$	9	7	78	0.41
$DBDA(CA)_7$	11	7	64	0.43
$HVH(CA)_7T$	3	1	33	0.25
$HVH(TCC)_7$	10	3	30	0.20
$VHV(GT)_8G$	10	8	80	0.46
Mean	10.0	6.8	68	0.41
Total	120	82	-	-

Phylogenetic analysis

The dendrogram was constructed based onthe UPGMA procedure using 120 ISSR markers. The Mantel test indicated that cophenetic correlation between ultrametric similarities of tree and similarity matrix was high ($r = 0.92$, $P < 0.01$). This suggests that the cluster analysis strongly represents the similarity matrix. The accessions studied had similarity values ranging from 0.67 to 1.00 (Fig. 2). Dendrogram was divided into three main clusters: citremons, trifoliatas and citranges-citrumelos. The citremons were the most distant from all of other accessions with similarity level of 0.67 and genetic similarity between two citremons was very high. Citremon was reported as a hybrid between trifoliata orange and lemon whereas citrange between trifoliata orange and orange, citrumelo between trifoliata orange and grapefruit (HODGSON, 1967 [1]). Half of the genome of lemon was contributed by citron (*C. medica* L.) (GULSEN & ROOSE 2001a, b [9], [20]). It can be suggested that citrange and citrumelo are genetically closer each other than citremon because orange and grapefruit are closely related. Thus it may explain that why citremons were distant from citrange and citrumelo in the dendrogram.

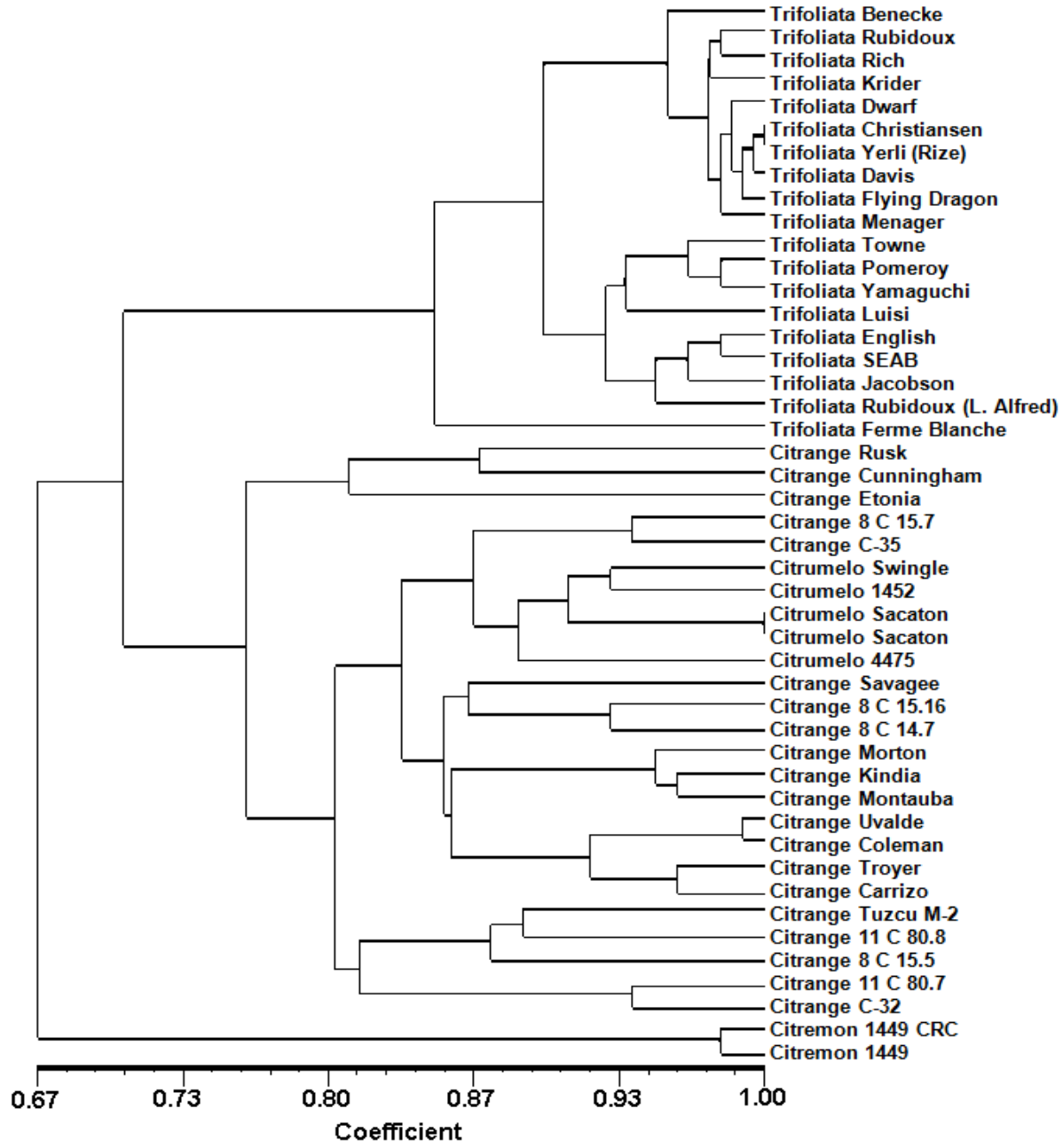

Figure 2. UPGMA cluster obtained from ISSR data showing the relationships in trifoliata oranges, citranges, citrumelos and citremons

The citrange and citrumelo groups separated from the trifoliata oranges with similarity level of ~0.75. The similar results was also obtained by SCHAFER & al. (2004 [23]) based on RAPD markers. In this group, all 20 citranges were clearly separated from each other and there were three subclusters in the citranges. The citranges had the higher level of polymorphism contrasting the other trifoliata hybrids. It may be due to maternal origin of citranges was not the same orange. For example 'Troyer' citrange originated from 'Washington' navel orange for maternal parentage (HODGSON, 1967 [1]), 'C-35' citrange originated from 'Ruby' orange (CAMERON & SOOST, 1986 [21]), while 'Tuzcu M-2' citrange originated from 'Alanya Dilimli' orange (TUZCU & YILDIRIM, 2000 [22]). In the citranges, the subcluster of 'Rusk', 'Cunningham' and 'Etonia' citranges was the most distant

from the others. Besides, 'Uvalde' and 'Coleman' citranges showed the highest similarity at about 0.99. FANG & ROOSE (1997 [6]) also reported high level of diversity in the citranges and all accessions were distinguished except for 'Carrizo' and 'Troyer' citranges. In our results, these two citranges reported as important citrus rootstocks by HODGSON (1967 [1]), were distinguished but were highly similar since they had the same parentage. On the other hand, SCHAFER & al. (2004 [23]) reported high level of polymorphism in the citranges. Five citrumelos nested in the citrange group as a small branch. Genetic similarities of these citrumelos were between ~0.89-1.00 and two 'Sacaton' citrumelos were identical. 'Citrumelo 4475' was the most distinct from the other citrumelos.

In the trifoliata subgroup, 'Ferme Blanche' was the most distant accession with similarity value of 0.85 from the other trifoliatas. Geographic origin of this accession was reported as Algeria (TUZCU & al.1984 [24]). The similarity levels of other 18 trifoliata accessions were between ~0.90-1.00. Based on the dendrogram, the rest of accessions except for two can be separated into two groups. 'Christiansen' and 'Yerli (Rize)' accessions were genetically identical based on our ISSR data. 'Benecke', 'Robidoux 3376', 'Rich', 'Krider', 'Dwarf', Christiansen', 'Yerli (Rize)', 'Davis', 'Flaying Dragon', 'Manager' nested in the same group while the other group consisted of 8 trifoliatas. 'Flying Dragon', has curved shoots and thorns, which are morphologically different from the other accessions. This trifoliata acts as a strongly dwarfing rootstock for citrus and, reportedly, these characters arose by mutations in this accession (FANG & al. 1997 [3]). In the dendrogram, trifoliata accessions had the lower genetic diversity than the trifoliata hybrids. The similar results were reported by previous studies. FANG & al. (1997 [3]) found low polymorphism (10.2%) among the trifoliatas. SCHAFER & al. (2004 [23]) notified high level of similarities among the trifoliata accessions. PANG & al. (2007 [25]) reported low level of polymorphism in three *P. trifoliata* accessions based on AFLP markers. AKA-KACAR & al. (2009 [26]) also reported low genetic polymorphism in trifoliata oranges by SSR markers. There was low level of genetic diversity in trifoliata oranges based on some facts. *P. trifoliata* flowers about 20-30 days earlier than *Citrus*. Because of this, natural hybridization between *P. trifoliata* and *Citrus* rarely occurs. So diversity observed in trifoliata is not caused by hybridization. Low diversity among the accessions may be due to mutations coupled with polyembryoni. Most of the modern trifoliata accessions originated from old lines by the selection of mutations or by self-pollination. The trifoliata oranges produce polyembryonic seeds containing both sexual and apomictic embryos. About 80-90% of seedlings from open-pollinated trifoliata orange seeds develop from nucellar (apomictic) embryos that are genetically identical to their mother tree in all characters (KHAN & ROOSE, 1988 [27]; FANG & al. 1997 [3]).

Principal component analysis (PCA) was performed for better presentation of relation among the accessions studied. The classical PCA is more likely an example of dimensionality reduction. It is, therefore, important that the required information is strongly related to the variance in the data (SCHOLZ & SELBIG, 2006 [28]). The PCA revealed some aspects of interrelation among materials that were not discernable by the UPGMA analysis (MARAK & LASKAR, 2010 [12]). PCA-1 and PCA-2 represented 77.9% and 8.2% of the variation, respectively (Fig. 3). Based on this, 86.1% of the total variation in the original dimensions could be represented by just the two dimensions. The two dimensional dispersion indicated that trifoliata oranges and their hybrids were separated from each other. Trifoliatas, citremons and citranges-citrumelos were clustered in different groups. Citrumelos were closer to citranges. In the citrange group 'Rusk' and 'Etonia' nested distantly from the other accesions. The result of the PCA was in agreement with the dendrogram.

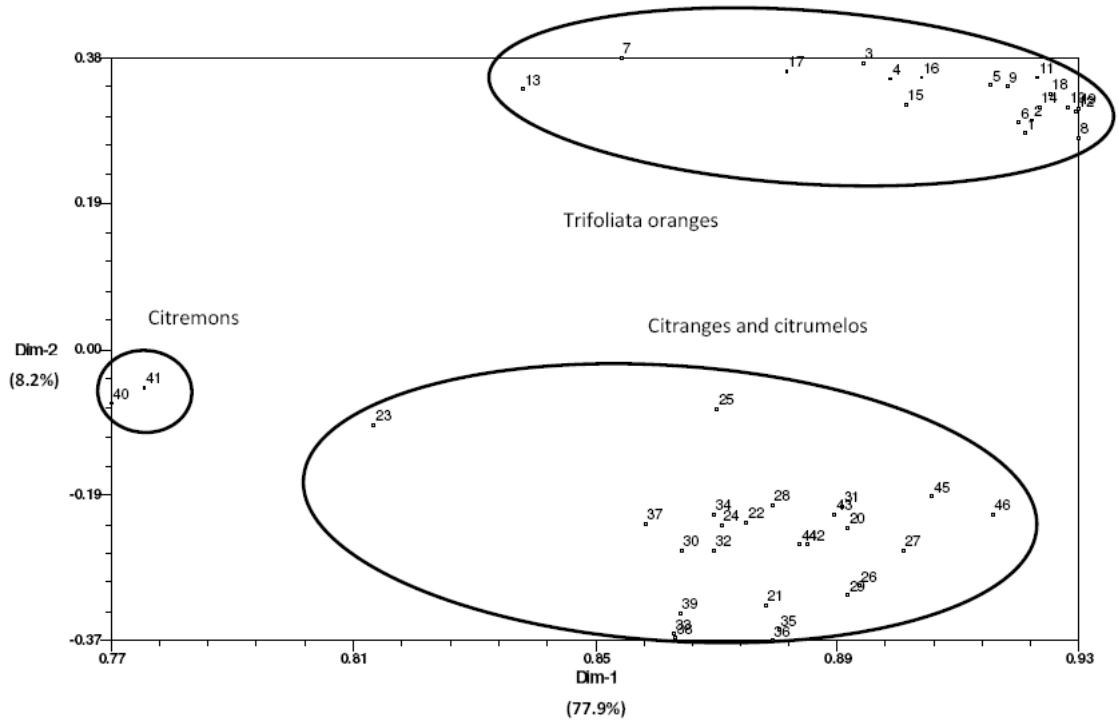

Figure 3. Plot of the first two principal components in Principal Components Analysis of ISSR data. The accession numbers were shown in Table 1.

We succesfully differentiated trifoliata oranges and their hybrids by using ISSR markers. The ISSR analysis was a powerful technique for fingerprinting closely related accessions such as those of trifoliata orange. The ISSR analysis was useful because rapid production of large number of markers in a cost-effective manner is possible. The ISSR markers have great potential in plant breeding and germplasm evaluation for fingerprinting narrow-based germplasms like those present in trifoliata orange (FANG & al. 1997 [3]), grapefruit-pummelo (UZUN & al. 2010 [13]) and for constructing linkage maps (GULSEN & al. 2010b [16]).

Conclusion

Trifoliata orange is cold hardy and highly resistant to root rot disease and citrus nematode. Moreover, it is generally immune to citrus tristeza virus that is widely distributed in citrus producing areas in the world. For this reason, the trifoliata orange has been widely used as parental material for rootstock breeding (YOSHIDA & al. 1999 [29]). Therefore understanding of the genetic diversity in trifoliata orange accessions should allow more efficient exploitation of this material by citrus breeders. This study indicated that the ISSR markers represent a powerful tool for investigation of closely related citrus accessions such as the trifoliata oranges.

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