Distinguishing Grapefruit and Pummelo Accessions using ISSR Markers

Aydin UZUN[1], Osman GULSEN[1], Turgut YESILOGLU[2], Yildiz AKA-KACAR[2] and Onder TUZCU[2]

[1]Department of Horticulture, Faculty of Agriculture, Erciyes University, Kayseri, Turkey;
[2]Department of Horticulture, Faculty of Agriculture, Cukurova University, Adana, Turkey

Abstract: Grapefruit is the fourth economically most important citrus fruit in the world. In this research Inter-Simple Sequence Repeat (ISSR) markers were used to distinguish twenty-nine grapefruit (*Citrus paradisi* Macf.), five pummelo (*Citrus maxima* (Burm.) Merr.) and one *Citrus hassaku* Hort. Ex Tanaka accessions. Twelve ISSR primers produced a total of 100 fragments and 62 of them were polymorphic. The number of average polymorphic fragments per primer was 5.2. The mean polymorphism information content (PIC) was 0.37. The unweighted pair group method arithmetic average (UPGMA) analysis demonstrated that the accessions had a similarity range from 0.79 to 1.00. The accessions were separated into two main clusters; group A with five pummelos and group B with grapefruits. In the pummelo cluster, all pummelos were distinguished whereas in the grapefruit cluster some accessions were not clearly separated. There was a low level of variation in the grapefruits due to their mutation origin.

Keywords: accession; *Citrus maxima*; *Citrus paradisi*; germplasm characterization; molecular markers

Because of sexual compatibility between *Citrus* and related genera coupled with the high frequency of bud mutations, long history of cultivation and wide dispersion, taxonomy and phylogeny of *Citrus* are very complicated, controversial and complex (Nicolosi *et al.* 2000). Many of citrus cultivars are very closely related, apparently having diverged by mutations that alter specific horticultural traits. These mutations can be maintained because the citrus is usually propagated vegetatively by grafting the scion cultivar onto a rootstock. In addition, many citrus cultivars produce apomictic seedlings through nucellar embryony, and nucellar seedlings that differ in horticultural traits or lack pathogens present in their parent have often been selected, being named as cultivars. Thus, using morphological traits, it can be difficult to distinguish between many citrus cultivars such as grapefruits (Fang & Roose 1997).

The grapefruit (*Citrus paradisi* Macf.) was notified as a natural hybrid between pummelo (*Citrus maxima* (Burm.) Merr.) and sweet orange (*C. sinensis* L. Osb). It originates from Barbados in the Caribbean islands and was first named as *Citrus paradisi* Macf. by James Macfedyan in 1837 (Scora *et al.* 1982; Scora 1988). Grapefruits are highly polyembryonic, therefore they are of nucellar origin. Genetic variation among common grapefruit cultivars was reported to be very low due to their nucellar origin (Fang & Roose 1997; Corazza-Nunes *et al.* 2002). After having been introduced into Florida, grapefruit became an economically important *Citrus* species and to date many grapefruit cultivars arose as mutations (Hodgson 1967; Moore 2001). The United States is the major producer country of grapefruits in the world.

The pummelo is native to tropical and subtropical regions in Asia and has been cultivated in

China for over 2000 years (Corazza-Nunes *et al.* 2002; Yong *et al.* 2006). Barrett and Rhodes (1976) reported that pummelo was one of the three true citrus species and most of subsequent studies were in agreement with this statement (Federici *et al.* 1998; Nicolosi *et al.* 2000; Barkley *et al.* 2006; Uzun *et al.* 2009a). Pummelo has played an important role as a parent of many citrus fruits, such as lemons, oranges and grapefruits. Molecular studies on pummelo genetic diversity are scarce. Randomly amplified polymorphic DNA (RAPD) (Corazza-Nunes *et al.* 2002) and simple sequence repeats (SSR) (Yong *et al.* 2006) markers have been used to determine pummelo genetic diversity. They demonstrated a considerable variation probably due to their zygotic origin.

Inter-Simple Sequence Repeat (ISSR) markers involve the amplification of DNA segments 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°C) leading to higher stringency. This technique overcomes most limitations such as low reproducibility and high cost (Zietkiewicz *et al.* 1994; Pradeep Reddy *et al.* 2002). It is widely used by the research community in various fields of plant science such as breeding, germplasm conservation and genetic mapping (Pradeep Reddy *et al.* 2002). ISSRs have been used to determine genetic diversity, characterization, phylogenetic relationships among the *Citrus* and related genera (Gulsen & Roose 2001; Shahsavar *et al.* 2007; Uzun *et al.* 2009b; Marak & Laskar 2010).

Genetic diversity of several *Citrus* species has been well evaluated, particularly of mandarins, but species with non-hybrid origin such as sweet oranges and grapefruits and with hybrid origin such as pummelos lag behind (Corazza-Nunes *et al.* 2002). The objective of this study was to estimate genetic polymorphism and relationships among grapefruit and pummelo accessions based on ISSR markers.

MATERIALS AND METHODS

Plant materials

Twenty-nine grapefruit, five pummelo and one *Citrus hassaku* accessions were used for this study (Table 1). Leaf samples of all accessions were obtained from the Tuzcu Citrus Collection, University of Cukurova, Adana, Turkey.

Table 1. *Citrus paradisi* and *C. maxima* accessions used in the study of germplasm characterization

Species name	Accession name
C. paradisi Macf.	Cocktail
C. paradisi Macf.	Pernambuco
C. paradisi Macf.	Mc Carty
C. paradisi Macf.	Grapefruit SRA 640
C. paradisi Macf.	Frost Marsh
C. hassaku Hort ex Tanaka	*Citrus hassaku*
C. paradisi Macf.	Sweetie SRA 602
C. paradisi Macf.	Oroblanco
C. paradisi Macf.	Davis Seedless
C. paradisi Macf.	Duncan
C. paradisi Macf.	Flame
C. paradisi Macf.	Foster B 6/5 28-12
C. paradisi Macf.	Foster B 6/5 28-16
C. paradisi Macf.	Foster B 6/5 29-16
C. paradisi Macf.	Henderson (Adana)
C. paradisi Macf.	Henderson SRA 336
C. paradisi Macf.	Little River
C. paradisi Macf.	Frost Marsh
C. paradisi Macf.	J BC 430 Marsh
C. paradisi Macf.	Marsh Seedless
C. paradisi Macf.	Ray Ruby (Adana)
C. paradisi Macf.	Redblush (3191 R,N)
C. paradisi Macf.	Redblush
C. paradisi Macf.	Reed
C. paradisi Macf.	Rio Red (Adana)
C. paradisi Macf.	Ruby SRA 287
C. paradisi Macf.	Ruby SRA 286
C. paradisi Macf.	Shambar SRA 22
C. paradisi Macf.	Star Ruby (Adana)
C. paradisi Macf.	Whenny
Citrus maxima (Burm.) Merr.	Pummelo Reinking
Citrus maxima (Burm.) Merr.	Pummelo Pink
Citrus maxima (Burm.) Merr.	Pummelo Kao Panne
Citrus maxima (Burm.) Merr.	Pummelo Red
Citrus maxima (Burm.) Merr.	Pummelo WN

DNA extraction and ISSR analysis

Genomic DNA was extracted from young leaves by the CTAB method as described by Doyle and Doyle (1990). DNA concentration was measured with a microplate spectrophotometer (BioTek Instruments, Inc., Vinooski, USA), and 10 ng/µl DNA templates were made using TE (10mM Tris-HCl, 0.1mM EDTA, pH 8.0). A total of 12 ISSR primers previously evaluated by Fang and Roose (1997) and Gulsen *et al.* (2010) were used for all clones (Table 2). PCR reaction components and PCR cycling parameters were performed as described by Uzun *et al.* (2009b). PCR products were separated on 2% agarose gel in 1× TBE buffer (89mM Tris, 89mM Boric acid, 2mM EDTA) at 115 V for 2.5–3 h. The fragment patterns were photographed under UV light for further analysis. A 100 bp standard DNA ladder (GeneRuler, Fermentas) was used for ISSR analysis as the molecular standard in order to confirm the appropriate markers.

Data analysis

Each band was scored as present (1) or absent (0) and data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc version 2.1) software package (Rohlf 2000). A similarity matrix was constructed based on Dice's coefficient (Dice 1945) which considers only one to one matches between two taxa for similarity. The similarity matrix was used to construct a dendrogram using the unweighted pair group method arithmetic average (UPGMA) to determine genetic relationships among the germplasm studied. The representativeness of dendrograms was evaluated by estimating cophenetic correlation for the dendrogram and comparing it with the similarity matrix, using Mantel's matrix correspondence test (Mantel 1967). The result of this test is a cophenetic correlation coefficient, *r*, indicating how well the dendrogram represents similarity data. Polymorphism information content (PIC) values were calculated according to Smith *et al.* (1997), using the algorithm for all primer combinations as follows:

$$PIC = 1 - \Sigma f_i^{\,2}$$

where:
$f_i^{\,2}$ – 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

Table 2. Results on ISSR primers used in *Citrus paradisi* and *C. maxima*

ISSR Primers	Fragment No.		Polymorphism range (%)	PIC
	total	polymorphic		
BDB$(CA)_7$C	7	4	57	0.38
$(CAC)_6$	5	2	40	0.06
DBDA$(CA)_7$	7	5	71	0.50
$(GA)_8$YG	14	9	64	0.45
$(GAA)_6$	9	6	67	0.37
$(GACA)_4$	7	4	57	0.49
$(GT)_8$YA	7	5	71	0.29
HVH$(CA)_7$T	4	2	50	0.33
HVH$(TCC)_7$	13	6	46	0.31
$(TAA)_8$	9	7	78	0.53
$(TCC)_5$RY	10	7	70	0.44
VHV$(GT)_8$G	8	5	63	0.34
Mean	8.3	5.2	62	0.37
Total	100	62	–	–

PIC – Polymorphism information content

the relative frequencies of those alleles (Smith *et al.* 1997). PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles in equal frequencies). The principal components analysis (PCA) of the original binary data matrix was also performed using NTSYS-pc version 2.1.

RESULTS AND DISCUSSION

ISSR amplification

A total of 12 ISSR primers were screened and a total of 100 bands with high intensity were scored. The number of bands scored per primer combination ranged from 4 (HVH$(CA)_7$T) to 14 $(GA)_8$YG), with a mean of 8.3. The polymorphic fragment number varied between 2 (HVH$(CA)_7$T;$(CAC)_6$) and 9 $(GA)_8$YG), with a mean of 5.2, 62 in total. Corazza-Nunes *et al.* (2002) obtained 4.6 polymorphic fragments per primer for grapefruit and pummelos according to their RAPD data. On the other hand, they found lover polymorphism (49%) than was found in our study. The PIC values for the 12 primers ranged from 0.06 $(CAC)_6$ to 0.53 $(TAA)_8$, with an average of 0.37 in our study (Table 2).

Analysis of genetic relationships

A similarity matrix was calculated using ISSR data according to Dice's coefficient (Dice 1945). Similarity dendrogram was constructed using the UPGMA cluster analysis (Figure 1). Cophenetic correlation between ultrametric similarities of tree and similarity matrix was found to be high

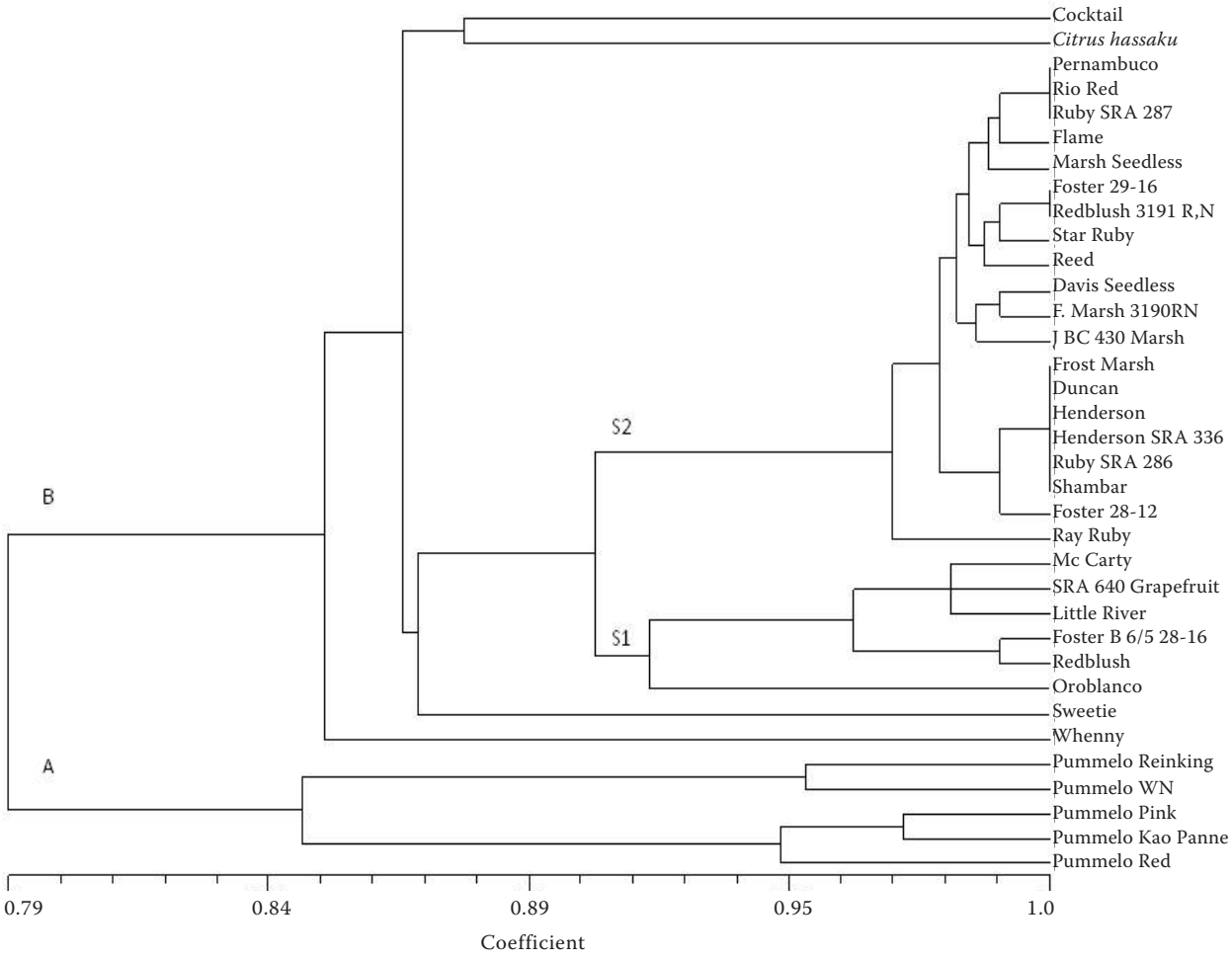

Figure 1. Dendrogram of 35 accessions of pummelo and grapefruit based on the ISSR markers using the UPGMA method

(r = 0.95, P < 0.01), suggesting that the cluster analysis strongly represents the similarity matrix. The accessions studied had similarity values ranging from 0.79 to 1.00. Pummelo and grapefruit groups were separated clearly (Group A and B). Group A consisted of 5 pummelos with a similarity value of 0.79 to grapefruits. All pummelos were distinguished clearly from each other. Yong *et al.* (2006) also distinguished pummelo accessions using SSR markers. Genetic similarities of pummelos used in our study were equal or higher than 0.85. Corazza-Nunes *et al.* (2002) found a similarity level of three pummelos ~0.90 based on RAPD and SSR data. This report was consistent with our results. Pink, Kao Panne and Red pummelos nested in the same subcluster whereas Reinking and Pummelo WN were included in another subcluster.

Group B included all grapefruits and grapefruit or pummelo hybrids. Similarity values in this group were between 0.85 and 1.00. Whenny was separated clearly from the others with the similarity level of 0.85. It is notified that Whenny originated as a chance seedling in Australia and under heat-deficient climatic conditions in Australia and New Zealand it is a summer-maturing variety. While the fruit is grapefruit-like in most respects, the monoembryonic nature of seeds and some of the other characters suggest that it is probably a pummelo hybrid (Hodgson 1967). *Citrus hassaku* and Cocktail nested in the same subcluster and were separated from the other grapefruits at the similarity level of 0.87. *C. hassaku* was reported as an independent species (*Citrus hassaku* Hort. Ex Tanaka) and originated as a chance seedling in Japan and its characteristics strongly suggest the pummelo-mandarin parentage with pummelo predominant (Hodgson 1967). At the same time Kahn *et al.* (2001) reported that *C. hassaku* was a pummelo hybrid. In the dendrogram this species was distinguished from pummelos and grapefruits. It is assumed that Cocktail is a hybrid between Frua mandarin and low acidity pummelo (Kahn *et al.* 2001). According to our ISSR data Cocktail

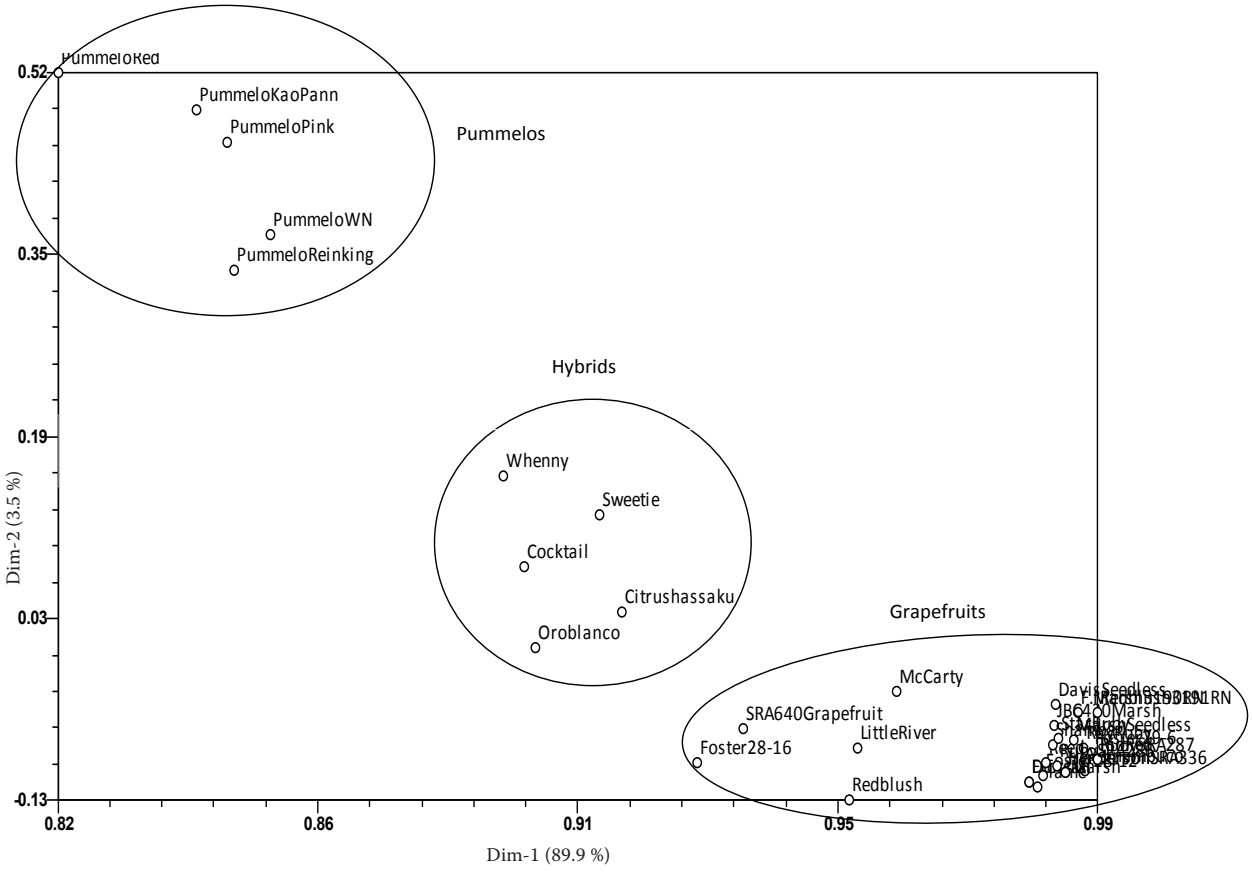

Figure 2. Two-dimensional plot of the principal components analysis of ISSR data includes grapefruits and pummelos

is different from the other grapefruits, and was probably derived as a hybrid of pummelo. On the other hand, Sweetie was found to be distinct from the true grapefruits. Sweetie was reported as a synonym of Oroblanco (Cottin 2002). Concordantly with this report Sweetie was clustered as closely related with Oroblanco in our study.

The rest of grapefruits were divided into two subgroups at a similarity level of ~0.91. The first subgroup (S1) included Oroblanco, Redblush, Foster 28-16, Little River, SRA 640 Grapefruit and McCarty. In this subgroup, Oroblanco was the most distinct accession. On the other hand, Oroblanco was clustered with grapefruits in a previous study (Yong *et al.* 2006). This accession was reported as a hybrid between acidless pummelo and grapefruit (Kahn *et al.* 2001). The rest of 20 grapefruits nested in the other subgroup (S2). In this subgroup the similarity value varied between 0.98 and 1.00. Two Henderson, Frost Marsh, Duncan, Ruby SRA 286 and Shambar grapefruits were genetically identical. On the other hand, Pernambuco, Rio Red and Ruby SRA 286 showed complete genetic similarity. Based on our results it can be concluded that mutations play an important role in the origin of grapefruits. Cultivars with distinct morphological characters (pigmented or yellow flesh colour, seedy and seedless fruits) such as Henderson, Ruby, Duncan showed complete genetic similarity. This result supports previous research (Corazza-Nunes *et al.* 2002).

The principal components analysis (PCA) was performed for better visualisation of relations among the accessions studied. The classical principal components analysis (PCA) is likely an example of dimensionality reduction. Therefore it is important that the required information is strongly related to the variance in the data (Scholz & Selbig 2006). The PCA revealed some aspects of interrelations among the studied materials that were not discernable by the cluster analysis (Marak & Laskar 2010). The results of PCA are demonstrated in Figure 2. PCA-1 and PCA-2 represented 89.9% and 3.5% of the variation in the binary data matrix, respectively. It implies that 93.4% of the total variation in the original dimensions could be represented by just two dimensions defined by the first two PCs. Two-dimensional dispersion showed that five pummelos were distinguished and nested clearly apart from grapefruits. Accessions that are of hybrid origin such as Whenny, Cocktail, Oroblanco, Sweetie, and *C. hassaku* were between pummelos and grapefruits on the dispersion graphic. Most grapefruits constituted an intensive group due to their low genetic variation concordantly with the dendrogram.

Yong *et al.* (2006) concluded that pummelos were monoembryonic and that there was a high level of polymorphism in the pummelos. All pummelos used in this study were clearly separated and they might be of zygotic origin. The low level of polymorphism found in most grapefruits in our study was reported previously in various studies. Barrett and Rhodes (1976) speculated that within the group variation in orange, lemon, grapefruit and sour orange originated from a single tree. Fang and Roose (1997) found a very low polymorphism in grapefruits based on ISSR data and stated that all grapefruits were derived from the same ancestral tree by mutation. There was no variation in grapefruits in previous studies based on isozyme (Roose 1988) and SSR (Luro *et al.* 2000) data. In a study of 23 grapefruits Corazza-Nunes *et al.* (2002) detected a similarity level ranging from 0.98 to 1.00, agreeing with our results.

Grapefruits, despite considerable variation in morphological characters such as rind and flesh colour or fruit size, were genetically nearly identical. Contrasting with this diversity of agronomic traits, very low genetic variability was also found in cultivated citrus by use of molecular markers (Breto *et al.* 2001). Luro *et al.* (2000) reported that the microsatellites could not distinguish mutation-derived species such as sweet and sour orange. Gulsen and Roose (2001) found similar results in lemons (*C. limon*) based on isozyme, SSR, and ISSR data. Molecular markers are powerful tools for elucidating genetic diversity, determining parentage, and revealing phylogenetic relationships among various *Citrus* species. However, accessions arising from spontaneous mutation are often difficult to distinguish as discussed by Barkley *et al.* (2006). In the present study, we distinguished some grapefruit accessions with ISSR markers. So this marker system might be useful to detect cultivars obtained by mutation. Similar results were obtained in lemons derived from clonal selections and in four of 12 lemon accessions distinguished using ISSR markers (Uzun *et al.* 2009b). Based on our results, it can be concluded that variations in the agronomical characters are mainly due to mutations in grapefruits.

References

Barkley N.A., Roose M.L., Krueger R.R., Federici C.T. (2006): Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theoretical and Applied Genetics, **112**: 1519–1531.

Barrett H.C., Rhodes A.M. (1976): A numerical taxonomic study of affinity relationships in cultivated *Citrus* and its close relatives. Systematic Botany, **1**: 105–136.

Breto M.P., Ruiz C., Pina J.A., Asins M.J. (2001): The diversification of *Citrus clementina* Hort. Ex Tan., a vegetatively propagated crop species. Molecular Phylogenetics and Evolution, **21**: 285–293.

Corazza-Nunes M.J., Machado M.A., Nunes W.M.C., Cristofani M., Targon M.L.P.N. (2002): Assessment of genetic variability in grapefruits (*Citrus paradisi* Macf.) and pummelos (*C. maxima* Burm. Merr.) using RAPD and SSR markers. Euphytica, **126**: 169–176.

Cottin R. (2002): *Citrus* of the World. A Citrus Directory. Version 2. SRA INRA CIRAD, San Giuliano.

Dice L.R. (1945): Measures of the amount of ecologic association between species. Ecology, **26**: 297–302.

Doyle J.J., Doyle J.L. (1990): Isolation of plant DNA from fresh tissue. Focus, **12**: 13–15.

Fang D.Q., Roose M.L. (1997): Identification of closely related *Citrus* cultivars with inter-simple sequence repeat markers. Theoretical and Applied Genetics, **95**: 408–417.

Federici C.T., Fang D.Q., Scora R.W., Roose M.L. (1998): Phylogenetic relationships within the genus *Citrus* (*Rutaceae*) and related genera as revealed by RFLP and RAPD analysis. Theoretical and Applied Genetics, **96**: 812–822.

Gulsen O., Roose M.L. (2001): Lemons: diversity and relationships with selected *Citrus* genotypes as measured with nuclear genome markers. Journal of American Society for Horticultural Science, **126**: 309–317.

Gulsen O., Uzun A., Canan I., Seday U., Canihos E. (2010): A new *Citrus* linkage map based on SRAP, SSR, ISSR, POGP, RGA and RAPD markers. Euphytica, **173**: 265–277.

Hodgson R.W. (1967): Horticultural varieties of citrus. In: Reuther W., Webber H.J., Batchelor L.D. (eds): The Citrus Industry. Vol. 1. University of California Press, Berkeley, 431–591.

Kahn T.L., Krueger R.R., Gumpf D.J., Roose M.L., Arpaia M.L., Batkin T.A., Bash J.A., Bier O.J., Clegg M.T., Cockerham S.T. *et al.* (2001): *Citrus* genetic resources in California: Analysis and recommendations for long-term conservation. Report No. 22. University of California Division of Agriculture and Natural Resources, Genetic Resources Conservation Program, Davis CA, USA.

Luro F., Rist D., Ollitrault P. (2000): Sequence tagged microsatellites polymorphism: an alternative tool for cultivar identification and evaluation of genetic relationships in *Citrus*. In: Proc. 9th Int. Citrus Congress. Florida, 170–171.

Mantel N. (1967): The detection of disease clustering and a generalized regression approach. Cancer Research, **27**: 209–220.

Marak C.K., Laskar M.A. (2010): Analysis of phenetic relationship between *Citrus indica* Tanaka and a few commercially important citrus species by ISSR markers. Scientia Horticulturae, **124**: 345–348.

Moore G.A. (2001): Oranges and lemons: Clues to the taxonomy of *Citrus* from molecular markers. Trends in Genetics, **17**: 536–540.

Nicolosi E., Deng Z.N., Gentile A., La Malfa S., Continella G., Tribulato E. (2000): *Citrus* phylogeny and genetic origin of important species as investigated by molecular markers. Theoretical and Applied Genetics, **100**: 1155–1166.

Pradeep Reddy M., Sarla N., Siddiq E.A. (2002): Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica, **128**: 9–17.

Rohlf F.J. (2000): NTSYS-pc, Numerical Taxonomy and Multivariate Analysis System. Version 2.11. New York, Exeter, Setauket.

Roose M.L. (1988): Isozymes and DNA restriction fragment length polymorphisms in *Citrus* breeding and systematics. In: Goren R., Mendel K. (eds): Proc. 6th Int. Citrus Congres. Margraf Scientific Books, Weikersheim, 155–165.

Scholz M., Selbig J. (2006): Visualization and analysis of molecular data. In: Weckwerth W. (ed): Metabolomics: Methods and Protocols. Methods in Molecular Biology, Vol. 358, Humana Press, New York, 87–104.

Scora R.W., Kumamoto J., Soost R.K., Nauer E.M. (1982): Contribution to the origin of the Grapefruit, *Citrus paradisi* (*Rutaceae*). Systematic Botany, **7**: 170–177.

Scora R.W. (1988): Biochemistry, taxonomy and evolution of modern cultivated *Citrus*. In: Goren R.K. Mendel K. (eds): Proc. 6th Int. Citrus Congress. Margraf Scientific Books, Weikersheim, 277–289.

Shahsavar A.R., Izadpanah K., Tafazoli E., Tabatabaei B.E.S. (2007): Characterization of *Citrus* germplasm including unknown variants by inter-simple

sequence repeat (ISSR) markers. Scientia Horticulturae, **112**: 310–314.

Smith J.S.C., Chin E.C.L., Shu H., Smith O.S., Wall S.J., Senior M.L., Mitchel S.E., Kresorich S., Tiegle J. (1997): An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigree. Theoretical and Applied Genetics, **95**: 163–173.

Uzun A., Yesiloglu T., Aka-Kacar Y., Tuzcu O., Gulsen O. (2009a): Genetic diversity and relationships within *Citrus* and related genera based on sequence related amplified polymorphism markers (SRAPs). Scientia Horticulturae, **121**: 306–312.

Uzun A., Gulsen O., Kafa G., Seday U. (2009b): Field performance and molecular diversification of lemon selections. Scientia Horticulturae, **120**: 473–478.

Yong L., De-Chun L., Bo W., Zhong-Hai S. (2006): Genetic diversity of pummelo (*Citrus grandis* Osbeck) and its relatives based on simple sequence repeat markers. Chinese Journal of Agricultural Biotechnology, **3**: 119–126.

Zietkiewicz E., Rafalski A., Labuda D. (1994): Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, **20**: 176–183.

Received for publication August 24, 2010
Accepted after corrections November 15, 2010

Corresponding author:

Assist. Prof. Dr. Aydin Uzun, Erciyes University, Faculty of Agriculture, Department of Horticulture, 38039 Kayseri, Turkey
tel.: + 90 352 437 17 90, fax: + 90 352 437 62 09, e-mail: uzun38s@yahoo.com