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DETERMINATION OF GENETIC DIVERSITY AND RELATIONSHIPS WITHIN CITRUS AND RELATED GENERA USING DAMD MARKERS

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Abstract

Seventy accessions of the genus *Citrus* and related genera in *Aurantioideae* were used to better identify genetic diversity, estimate genetic similarities, polymorphism rates and relationships using amplification of minisatellite DNA (DAMD) markers. A total of 255 bands were scored from 20 DAMD-PCR markers and all (100%) of them were polymorphic. The accessions studied had similar values ranging from 0.31 to 0.84, showing a high level of variation. DAMD markers provided useful results to understand genetic basis of the citrus group. In addition, these markers revealed different knowledge from the other DNA-based marker system among the accessions. Also, DAMD-PCR markers appeared to be as useful as other for genetic analysis in citrus and its relatives.

Introduction

Aurantioideae sub-family is a quite large taxonomic group including orange (*Citrus sinensis* (L.) Osbeck), mandarin (*C. reticulate* Blanco), lemon (*Citrus limon* (L.) Burm. f.), grapefruit (*Citrus paradisi*) like economically valuable species, their relative variety and species. The sub-family is highly complicated, controversial and confusing group just because of sexual compatibility between citrus and related genera, relatively high bud mutation frequency, widespread and quite old history of cultivation (Nicolosi *et al*. 2000).

In the past, primarily morphologic and geographic data had been employed in citrus taxonomy and several systems had been suggested for citrus classification. Among them, the systems recommended by Swingle (Swingle and Reece 1967) and Tanaka (1977) are the widely used. The primary difference between these systems is the total number of identified species. While Tanaka (1977) identified 162 species, Swingle identified only 16 species. Scora (1975) recommended that there were only three 'basic' true species of *Citrus* within the sub-genus *Citrus*. Several molecular and biochemical studies have been conducted to support their thesis (Barkley *et al*. 2006, Uzun *et al*. 2009). Breeding strategies have been developed to elucidate the relationships, diversity and taxonomy of *Citrus* species and to preserve the biodiversity. Through elucidated genetic variability, it will then be possible to characterize germplasm, to control genetic erosion and to register new cultivars (Barkley *et al*. 2006).

It was carried out by several genetic researchers to evulate the genetic relationships among *Citrus* species and related genera using RFLP (Abkenar *et al.* 2004), ISSR (Gulsen and Roose 2001a), RAPD (Nicolosi *et al*. 2000, Naz *et al*. 2014), SSR (Barkley *et al*. 2006, El-Mouei *et al.* 2011) and AFLP (Pang *et al.* 2007), SRAP (Uzun *et al.* 2009) and peroxidase gene-based (Uzun *et al.* 2014). Also, minisatellites which sequentially repeated DNA of eukaryotic genomes and most of them exhibit allelic length variations because of differences in number of repeated units have used to asses genetic relationship of some plants such as direct or directed amplification of minisatellite region DNA (Heath *et al.* 1993, Jeffreys *et al.* 1985).

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The DAMD-PCR technique offers various advantages over the previous DNA-based techniques includes primers from minisatellite core sequences (Karaca *et al*. 2002). Minisatellites are the sections of a genome containing hypervariable regions (HVR) or variable number of tandem repeats (VNTR) (Jeffreys *et al*. 1985). These are tandem repetitions of a 10 - 60 bp DNA sequence motif known as the 'core' sequence, also known to occur in many diverse species of plants and animals and can be effective as PCR primers at relatively high stringencies in a wide range of organisms (Heath *et al.* 1993). DAMD-PCR technique employs minisatellite sequence-specific primers and can efficiently be implemented and yield reproducible DNA markers (Karaca and Ince 2008). However, there is limited information available about the application of DAMD-PCR technique in *Citrus* accessions.

The present study was conducted to elucidate the genetic diversity, estimate genetic similarities (GS), polymorphism rates and to assess the relationships among *Citrus* and some other genera in sub-family *Aurantioideae* by using DAMD-PCR markers.

Materials and Methods

Seventy *Citrus* accessions and its related genera in *Aurantioideae* (Table 1) were used. Leaf tissues were sampled for DNA extractions from "Alata Horticultural Research Institute", Erdemli-Mersin, Turkey. CTAB method was used for DNA extractions from young leaves of 70 accessions following the procedures described by Doyle and Doyle (1990). To amplify minisatellite regions of *Citrus* accessions, commercially synthesized (Iontek, Istanbul, Turkey) 20 primers, directed amplification of minisatellite DNA (DAMD) markers which based on the minisatellite regions in rice (*Oryza sativa* L.), phage M13 and human (*Homo sapiens*) genomic DNAs (Table 1) were used. The components used in 25 µl PCR mixture were as follows: 10 ng genomic DNA, 2.4 mM of each minisatellite primer, 0.28 mM of each dNTP, 3 mM $MgCl_2$, 80 mM Tris HCl (pH 8.8), 19 mM $(NH_4)_2SO_4$, 0.009% Tween-20 (w/v) and 2 units Taq DNA polymerase. Polymerase chain reactions were carried out using thermal cyclers (Senso Quest, Goettingen, Germany) using touch-down PCR reaction conditions were 3 min at 94°C, and followed by pre-PCR at 94°C for 1 min for denaturation (10 cycles), for 50 s at 50°C for annealing and for 2 min at 72°C for extension stage. For the first 10 cycles annealing temperature was reduced by 0.5°C per cycle. After that, the PCR amplification was continued for 30 more cycles at a 45°C annealing temperature and final extension was at 72°C for 10 min as Ince and Karaca, (2011). PCR products were separated using 2% agarose gel in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 120 volt for 3 hrs for DAMD-PCR products. The fragments were photographed under UV light. A 100 bp standard DNA ladder was used for DAMD-PCR analysis in order to confirm the appropriate markers.

Data were analyzed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software (Rohlf 2000). A similarity matrix was constructed by using DAMP-PCR data based on Dice (1945) coefficient. A dendrogram constructed with the help of the UPGMA (unweighted-pair group method arithmetic average) for the purpose of determining genetic relationships among *Citrus* and its related genera (Mantel, 1967).

Results and Discussion

Seventy *Citrus* accessions and related genera belonging to *Aurantioideae* were assessed through 20 DAMD-PCR primers. Of the total of 296 bands scored from 20 DAMD-PCR markers, all (100%) were polymorphic. The number of bands scored per primer combination varied between 8 (URP25F and URP32F) and 21 (URP6R) with an average value of 14.8. The least number of total bands was observed in URP32F and URP25F(8) primers (Table 1). In previous

studies, Lu *et al.* (2011) and Brown *et al.* (2009) observed 5 - 9 bands per primer and around 85% polymorphism with ISSR and RAPD markers. Creste *et al.* (2004) reported 12.8 fragments per primer with microsatellite markers. In present study, URP6R (21) yielded the greatest number of total bands. The similar polymorphism ratio for DAMD-PCR primers in *Murraya paniculata* (96.29 %) was also observed by Verma *et al.* (2009). Uzun *et al.* (2009) indicated 100% polymorphism among *Citrus* accessions and related genera with SRAP markers. Uzun *et al.* (2014) also used 14 POGP primers and reported 99% polymorphism in *Citrus* and relatives.

Kumar and Nair (2013) evulated genetic variations and phylogenetic relationships among 50 wild and cultivated accessions of 19 Indian *Citrus* genotypes to comparison using directed amplification of minisatellite DNA (DAMD) markers. DAMD-PCR analysis with four primers yielded 45 bands, of which 35 (78 %) were polymorphic. Morphometric assessments carried out with 76 morphologic characters indicated a high level variability ranging from 0.18 to 1.00 (with a mean value of 0.39) and the Jaccard's coefficient for genetic similarity calculated from DAMD data varied between 0.41 and 1.00 (with a mean value of 0.68).

Cophenetic correlation between ultrametric similarities of tree and similarity matrix was found to be high (r = 0.86), suggesting that the dendrogram strongly represented the similarity matrix calculated according to Dice's coefficient (Dice 1945). The accessions studied had similarity values ranging from 0.31 to 0.84, showing a high level of variation (Fig. 1). Among the accessions, *Pamburus missionis* (Wight) Swing. *Aegle marmelos* (L.) Corrêa and *Glycosmis pentaphylla* (Retz.) Corr. was the most distinct with a similarity value of 0.31, which was consistent with previous reports (Morton *et al.* 2003, Uzun *et al.* 2009). *Clausen alansium, Murraya paniculata* (subtribe Clauseninae and tribe Clauseneae) and *Hesperethus acrenulata* (subtribe Citreae, tribe Citrinae, group primitive citrus fruit trees) were placed in the same cluster. *Pamburus missionis* was also distinct from therest of the samples with a similarity value of 0.32. *Glycosmis pentaphylla* (subtribe Clauseninae and tribe Clauseneae), nested alone in the dendrogram. Also, *Citropsis gilletiana* Swingle & M. Kell nested alone in the dendrogram. *Atalantia ceylanica* (Arn.) Oliv and *Severinia buxifolia* Tenore nested together at same group. *Eremocitrus glauca* Swing. was alone in group of *C. sudachi* Hort. ex Shirai, *C. natsudaidai* Hay., *Aeglopsis chevalieri* Swing. And *C. hystrix* DC. Prodr., Citrumelo 1452, 'Sacaton' Citrumelo WN, 'C-32' Citrange, 'Carrizo' Citrange, 'Troyer' Citrange 3360, and 'C-35' Citrange which the members of the subtribe Citrinae 'C-32' were the same groups except Citromen 1449 (Fig. 1). *Pleiospermium alatum* (subtribe Citreae and tribe Citrinae, group primitive citrus fruit trees) nested with *Severinia buxifolia* Tenore and *Atalantia ceylanica* (Arn.) Oliv. *Severinia buxifolia* and *Atalantia ceylanica* belonging to the subtribe Citrinae were similar with a value of 0.60, which was also consistent with the previous studies (Uzun *et al*. 2009, Zhen-hua *et al*. 2011; Uzun *et al*. 2014). Yamamoto *et al*. (2008) discussed that primitive citrus fruit trees and near citrus fruit trees were closely related based on chromosome types between them such as present results indicated among *Citrus* species, only *C. Micrantha* and *C. tachibana* (Mak.) Tan. were closer to *Microcitrus australasica* (F. Muell. Swing) than other *Citrus* spp. This result is consistent with the findings of Uzun *et al*. (2014). *Microcitrus* species are native to Australia and New Guinea (Pang *et al*. 2007). Perhaps, their geographic conditions caused genetic differentiation of this genus from *Citrus* and this stuation is consistent with previous studies (Nicolosi *et al.* 2000, Pang *et al.* 2007). It was reported that *Severinia* was closer to *Citrus* than the other genera except *Fortunella* (Federici *et al*. 1998). Nevertheless, it was observed in this study that *Microcitrus* was closer to *Citrus* than *Severinia*. Such a finding is supported by Uzun *et al*. (2009) and Uzun *et al*. (2014). *Microcitrus*, *Eremocitrus* and Citrus were classified under 'true citrus fruit trees', whereas *Severinia* was in 'primitive citrus fruit trees' (Swingle and Reece 1967). The *Poncirus* group and their hybrids (except citranges) were clustered with *Citrus* with a similarity level of 0.65 (Fig. 1).

This finding is also consistent with findings of Uzun *et al.* (2014). In this group, Citrumelo 1452 and 'Sacaton' Citrumelo WN were closer to each other than the others since they are derived from *P. trifoliata* × *C. paradise* hybrid. 'Troyer' Citrange 3360 (*P. trifoliata* × *C. sinensis*) was closer to *Poncirus trifoliata* (L.) Raf. But according to Uzun *et al.* (2014) and Uzun *et al*. (2009), citrangedina complex hybrid between three genera, was closer to *Poncirus* than the other ancestors. But citranged is nested with Ichangpapeda at same group. The *Poncirus* group was separated from *Citrus*, which was consistent with some previous studies (Barkley *et al.* 2006, Pang *et al.* 2007, Uzun *et al.* 2009b, Uzun *et al.* 2014). 'Schaub Rough lemon', 'Kutdiken' limon, 'Interdonato' limon and 'Limoneira 8A' limon nested at same group. But improved 'Meyer' lemon separated from lemon group and nested with West Indian Lime, Australian sour orange, Yuzu, Rangpur, Meyer lemon West Indian lime and clustered together with a similarity level of 0.68, being consistent with previous study except Meyer lemon (Uzun *et al*. 2014). Meyer lemon clustered with Interdonato and Limoneira 8A as indicated by Uzun *et al.* (2014).

It was indicated that rangpurs were more similar to mandarins, but they were probably the hybrids between limes and mandarins or the hybrids of limes and sour orange; therefore, the origin of the rangpurs has been unclear, but they have been generally classified with mandarins with previous studies (Barkley *et al.* 2006). Origin of Bergamot was unclear as Hodgson (1967), but probably related to sour orange. Bergamot was defined as a hybrid of citron and sour orange (Nicolosi *et al.* 2000) and clustered with sour orange (Federici *et al.* 1998). But, although there is no clear relationship among rangpur-mandarin and Bergamot-sour orange, in present study rangpurs nested with 'Australian' sour orange at same clustur with a similarity level of 0.71. Rangpurs was also at same cluster with Meyer lemon and West Indian lime. There are some differences among the results of SRAP, peroxidase gene profiles and DAMP-PCR markers. These differences may be resulted from different marker analysis of the accessions. Calamondin (*C. mitis)* nested at same cluster with mandarins. Such a finding is consistent with SRAP markers (Uzun *et al.* 2009) but not consistent with peroxidase gene profiles (Uzun *et al.* 2014) since *C. mitis, C. ichangensis* and *C. webberi* grouped together in the dendrograms in their study. *C. mitis* called as Calamondin was a hybrid between *Citrus* and *Fortunella* (Swingle and Reece 1967). *Citrus ichangensis* and *C. webberi* were classified in the genus Papeda within *Citrus*. 'Pink' Pummelo,'Kao Panne' Pummelo, 'Reinking' Pummelo (*C. maxima)* were in the same cluster with Oroblanco and the hybrid derived from (*C. maxima* × *C. paradisi*) were clearly separated from the other accessions with a similarity level of 0.65. Similarity values among the pummelos were over 0.80. The similar results were also reported by Uzun *et al*. (2014).

Genetic relationships between pummelos and grape fruits were higher in previous studies with different marker systems than the present study (0.64) using DAMP-PCR markers. In previous studies, similarity level of pummelos and grape fruits was 0.83 for SRAP data (Uzun *et al.* 2009) and 0.79 for ISSR data (Uzun *et al.* 2010) and 0.68 for peroxidase gene profiles (Uzun *et al.* 2014). These results may be explained by differences in diversification of marker systems used in these studies.

C. micrantha Wester (Small-flowered papeda), *C. tachibana* (Mak.) Tan *Microcitrus australasica* (F. Muell. Swing.) and *C. webberi* nested in the same cluster. But these results were different from the findings of Uzun *et al.* (2009, 2014)."Citron" and 'Etrog' citron belong to *C. medica* L. separated from *Poncirus trifoliata* (L.) Raf. and it was consistent with Uzun *et al*. (2014). Kutdiken and Interdonato were close to each other and nested at the same cluster with Schaub' Rough lemon. Also, Macrophylla (Alemow) nested at close cluster with lemons. Volkamer lemon (*C. volkameriana*) nested with Cocktail and Star Ruby. Although ''Curacao'' sour orange and ''Gou Tou Cheng" belong to *C. aurantium* L. species, ''Curacao'' sour orange nested with 'Commune' Bergamot and ''Gou Tou Cheng separated from the other groups.

However, Uzun *et al*. (2014) reported that Gou Tou Cheng nested with Yuzu and ‘‘Curacao’’ sour orange nested with Citromen 1449. All lemons and limes nested at same cluster with a similarity value of 0.60 in present study. In previous studies, citron, lemon, rough lemon and *C. volkameriana* were in the same group (Uzun *et al*. 2009; Uzun *et al*. 2014). In this study, citrons was apart from lemons and *C. volkameriana.*

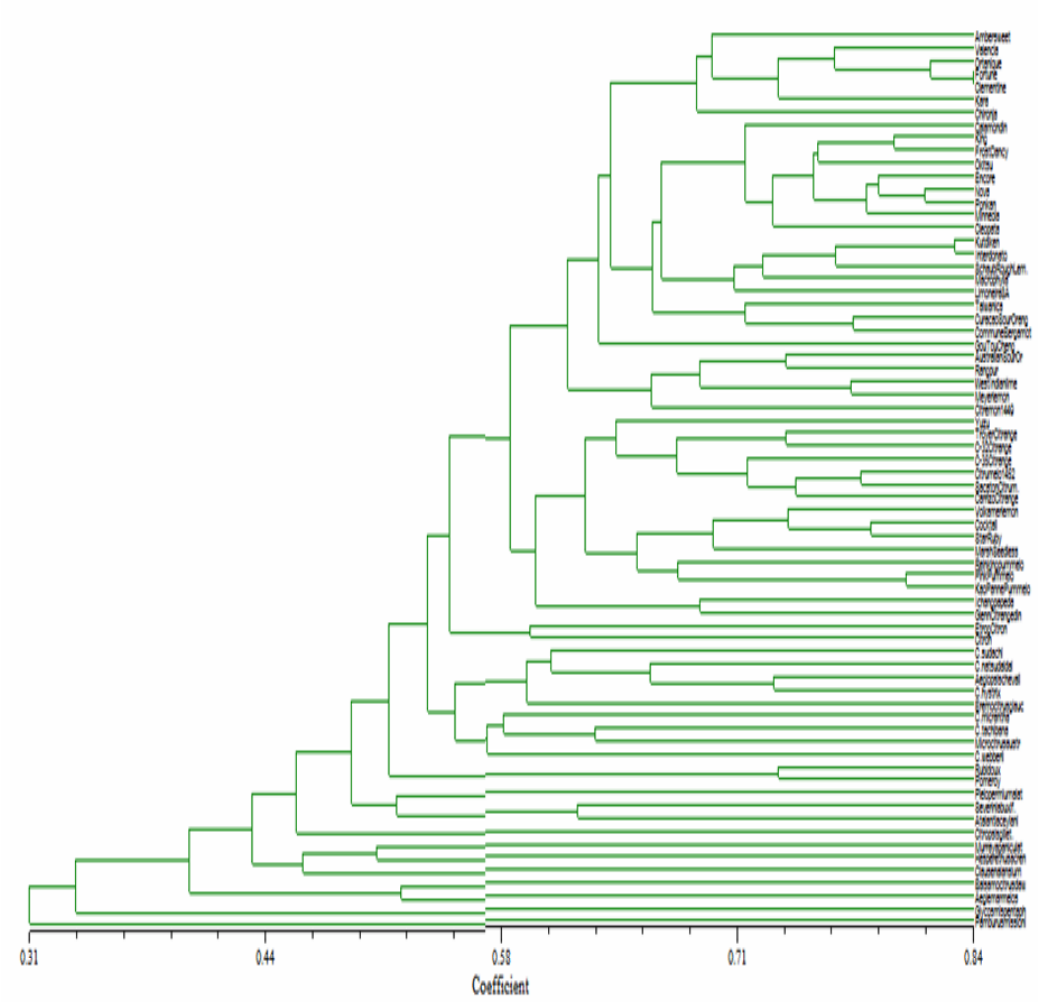

Fig. 1. Dendrogram of the 70 citrus and related genera genotypes using UPGMA method obtained from DAMP-PCR markers.

C. hystrix DC. Prodr. (Mauritius papeda), Mushiyukaku (*Eremocitrus glauca* Swing.), Natsumikan (*C. natsudaidai* Hay) and *Aeglopsis chevalieri* Swing nested at the same cluster with a similarity value of 0.58. All of them are relative of citrus. According to Uzun *et al.* (2009) based on SRAP markers and Uzun *et al.* (2014) based on peroxidase gene marker, *Citrus tachibana* and ‘Cleopatra’ mandarin were in the same branch, but in present study, Cleopatra nested with mandarin groups and *Citrus tachibana* nested with *Microcitrus australasica* (F. Muell. Swing.). In this study, there was no clear separation between Papeda and Citrusas. It was in Uzun *et al.*

(2014). Papeda group did not form a single cluster, which agreed with the results of RFLP markers (Federici *et al*. 1998), cpDNA (Nicolosi *et al.* 2000), AFLP (Pang *et al.* 2007) and SRAP data (Uzun *et al.* 2009). In the dendrogram, 'Pomeroy' trifoliata and 'Rubidoux' trifoliata are *Poncirus trifoliata* (L.) Raf. And Citrumelo 1452, 'Sacaton' citrumelo, 'C-32' citrange WN, 'Carrizo' Citrange, 'Troyer' Citrange 3360, 'C-35' citrange, citremon 1449 are hybrid of *Poncirus trifoliata* (L.) Raf. nested in the same branch with a similarity level of 0.80. According to Uzun *et al.* (2009), four citranges ("Carrizo", "Troyer", "C-32" and "C-35"), one sour orange (*C. aurantium* var. 'Australian'), one lemon (Kutdiken) and one citron nested in the same branch with a similarity level of 0.79 based on SRAP data. Citranges was reported as hybrid of orange and *P. trifoliata* (Hodgson 1967). Although *Citrus taiwanica* nested closely with 'Australian' sour orange in Uzun *et al.* 2009 and Uzun *et al.* (2014) in present study, Curacao' sour orange and *C. taiwanica* were clustered in the same group based on DAMP-PCR markers. "Star Ruby", "Marsh Seedless" and "Cocktail" grape fruits, volkamer lemon were closely clustered (Fig. 1). Results were consistent with the findings of Uzun *et al.* (2014). Grape fruit was reported as a hybrid of pummelo and sweet orange (Nicolosi *et al*. 2000).

Table 1. Observed polymorphism with 20 DAMD-PCR primers in different citrusgenotypes.

Primer ID	Source	References	FS	TF	PF	P(%)
URP2F	Rice (*Oryzasativa* L.)	Kang *et al*. (2002)	1000-175	15	15	100
URP4R	”	”	1000-120	18	18	100
URP6R	"	"	1000-125	21	21	100
URP9F	”	”	1000-250	15	15	100
URP13R	"	"	950-275	18	18	100
URP17R	”	”	1000-200	14	14	100
URP25F	"	"	1000-400	8	8	100
URP30F	”	”	1000-300	9	9	100
URP32F	”	”	1000-470	8	8	100
URP38F	"	"	1000-280	14	14	100
FVIIEX8	Human (*Homo sapiens*)	Murray *et al*. 1988)	1000-200	17	17	100
FVIIEX8C	”	"	980-170	12	12	100
33.6	"	Jeffreys *et al*. (1985)	1000-200	18	18	100
14C2	”	Vergnaud (1989)	1000-170	14	14	100
HBV3	”	Nakamura *et al*. (1987)	1000-180	17	17	100
HBV5	"	"	1000-150	13	13	100
M13	Phage M13	Vassaet *et al*. (1987)	1000-200	17	17	100
6.2H(-)	Human (*Homo sapiens*)	Jeffreys *et al*. (1985)	1000-180	15	15	100
6.2H(+)	”	"	950-200	17	17	100
YNZ22	"	Nakamura *et al*. (1987)	1000-150	16	16	100
Mean				14.8	14.8	
Total				296	296	

FR: Fragment size (bp), TF: Total fragments, PF: Polymorphic fragments, P: Polymorphisim.

Mandarins separated into two clusters. Ortanique, Fortune, Clementine and Kara nested at same cluster. King, Frost Dancy, Okitsu, Encore, Nova, Ponkan, Minneola and Cleopatra nested in the same cluster with a similarity level of over 0.80 in this study. "Cleopatra" was the most distinct in this group. "Minneola" tangelo, a hybrid of "Duncan" grape fruit and "Dancy"

mandarin (Hodgson 1967) clustered with mandarins. "Valencia" were close to Ortanique, Fortuna and Clementine which agree with the findings of Uzun *et al.* (2014). Parental sweet orange tree was a hybrid of pummelo and mandarin (Scora 1975), which was later supported by Nicolosi *et al.* (2000). It was suggested that sweet orange has a majority of its genetic makeup from mandarin and only a small proportion from pummel (Barkley *et al*. 2006). Such findings are consistent with the results of present study. 'Chironja' was reported as a hybrid between sweet orange and grape fruit (Hodgson 1967). Genetic background of "Amber sweet" orange was complex and possibly came from orange, mandarin and grape fruit (Jackson and Futch 2003). In the dendrogram, these two cultivars were closer to orange than the other parents. There was consistency with Uzun *et al.* (2014).

Directed amplification of minisatellite DNA (DAMD) markers were used to estimate diversity, genetic relationship and population structure of *Citrus* and related genera in the present study. They provided useful results to understand genetic basis of the *Citrus* group. In addition, these markers revealed different knowledge from the other DNA-based marker system among the accessions studied. All of these diverse results indicated that directed amplification of minisatellite DNA (DAMD) marker construction of these accessions, although some results may not be similar to the results of the other DNA markers, some results were similar with Uzun *et al.* (2009) and Uzun *et al.* (2014). Differences may result in estimating diversity and relationships. With previous studies, the reproducibility of DAMD-PCR technique was investigated by using Bermuda grass (*Cynodon dactylon*), tomato (*Solanum lycopersicum*) and pepper (*Capsicum)* genomic DNAs (Karaca and Ince 2008, Ince *et al.* 2009). Analyses indicated that the touchdown PCR profile and the optimized chemical concentrations resulted in reproducible and reliable DNA amplifications (Karaca and Ince 2008). It was also noted that in some cases the DAMD-PCR produced RAPD-like results but the number of bands was sharp and clear. The relatively high PCR stringencies in DAMD-PCR application effectively limited the PCR artifacts which commonly occur in RAPDs (Karaca and Ince 2008). This study may offer new and distinct stand point to elucidate genetic background of *Citrus* and its relatives. It can be concluded the DAMD-PCR markers appeared to be as useful as SRAP and POGP markers for genetic analysis in *Citrus* and relatives.

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