CHARACTERIZATION OF CITRUS CULTIVARS AND CLONES IN CYPRUS THROUGH MICROSATELLITE AND RAPD ANALYSIS

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ABSTRACT

Fifty one accessions from the Citrus collection of the Agricultural Research Institute, Lefcosia, Cyprus (ARI), representing 8 Citrus species, were analyzed with 10 microsatellite and 6 RAPD markers. Both, microsatellite and RAPD analyses, allowed the discrimination of the studied accessions at species level. A low level of polymorphism was obtained among cultivars within the species. In the group of oranges (6 cultivars and their clones), only one out of ten SSR primers discriminated between two groups of cultivars: the commercial cultivar Shamouti and two local cultivars, Jaffa and Aematousiki, on one hand and Valencia orange and the local orange Shekeriko on the other hand. In the group of lemons (3 cultivars and their clones) all three studied varieties, local Polyphori, local Lapithou and commercial Lisbon, were distinguished by one SSR and two RAPD primers. The local mandarin Arakapas (7 accessions) and Willowleaf showed complete genetic similarity by using both microsatellite and RAPD markers. The SSR markers did not reveal a polymorphism among the clones of the studied cultivars. Clone-specific RAPD markers were found for one clone of Frappa (4 accessions) and one clone of Bergamot (5 accessions). On the basis of the microsatellite data the local Cyprus cultivar Koumantantas was identified as Sour orange.

Keywords: Citrus, genetic diversity, SSRs, RAPD, polymorphism

Introduction

Citrus is an ancient perennial crop widely cultivated in the tropical and subtropical regions. The long history of cultivation and dissemination, natural and human selection played an important role in the large diversity existing nowadays within the genus Citrus. The taxonomy of Citrus is complicated due to the sexual compatibility between Citrus and related genera, as well as between species within the genus Citrus, the high rate of bud mutations and the asexual reproduction through nucellar embryony, which is characteristic for several Citrus species. This led to a discrepancy between classification systems related to the number and kind of species. According to the two most accepted classification systems of Swingle (35) and Tanaka (36), there are 16 genera and correspondingly162 citrus species, while Scora (33) and Barret and Rhodes (4) determined only three true *Citrus* species, Pummelo (*C. grandis* (L) Osb.), Citron (*C. medica* L.) and Mandarin (*C. reticulata* Blanco) and suggested that all other Citrus species originated by crosses between these main species or between them and other related genera.

Molecular markers such as isozymes, RFLP and RAPD have been applied to study genetic diversity (1, 6, 7, 10, 14, 29) and relationships within the genus Citrus (15, 27) in order to complement morphological data and thus shed more light on the classification of Citrus.

During the last decade microsatellite markers (SSRs) were extensively exploited for identification of cultivars, assessment of genetic diversity, phylogenetic studies and management of germplasm resources/collections due to their high level of polymorphism, even distribution in the genome, codominant Mendelian inheritance, transferability between laboratories and conservation across taxa. In Citrus, SSRs and ISSR have been applied for identification of species/cultivars/clones (2, 8, 12, 14, 18, 20, 22, 23, 25, 28, 30), for phylogenetic studies (3,13, 32) and construction of genetic maps (5, 9, 21).

In Cyprus, Citrus have been cultivated at least since the middle ages (19, 24). Oranges, mandarins, lemons and grapefruits are used for production mainly of fresh fruit, but also byproducts, such as juice and marmalade. A large part of fruit production is based on local cultivars, most of them considered as clones of the widespread economically important cultivars, as Valencia and Shamouti oranges, Willowleaf mandarin, Clementine, Lisbon lemon and Marsh Seedless grapefruit. Within these local clones/cultivars there is a great phenotypic diversity with regard to shape of fruits and leaves, tree appearance, yield and resistance to diseases. However, these local accessions have not been characterized yet at molecular level. The Agricultural Research Institute (ARI) of Cyprus has established and is maintaining at present a collection of about 100 citrus accessions, including local and commercial citrus cultivars, which represent nearly all cultivars grown on the island.

The objective of the present study was to characterize accessions of the Citrus collection of ARI by microsatellite and RAPD markers in order to determine genetic diversity and relationships among them, to discriminate the local cultivars/clones and, to establish relationships of the local cultivars with similar common commercial cultivars.

Materials and Methods

Plant material: Leaf samples were collected from 51 plants maintained in the citrus germplasm collections of ARI. The investigated citrus accessions represented eight Citrus species: sweet orange (*Citrus sinensis* (L.) Osbeck), lemon (*Citrus limon* (L.) Burm f.), mandarin (*Citrus reticulata* Blanco), Bergamot (*Citrus bergamia* Risso& Poit.), grapefruit (*Citrus paradisi* Macf.), sour orange (*Citrus aurantium* L.), Frappa (*Citrus maxima* (Burm) Merrill) and Koumantantas (*Citrus cumandatore)*. Forty three of the accessions were local or traditional cultivars/clones, whereas 8 were common citrus species/cultivars/clones (3 sweet oranges, 1 lemon, 2 mandarins, 1 grapefruit, and 1 sour orange. The list of species, cultivars and clones is presented in **Table 1**.

TABLE 1

Citrus accessions investigated in this study

No	Accession
	Sweet orange (*Citrus sinensis* (L.) Osbeck)
1	Jaffa clone 4 (sweet orange)
2	Jaffa clone 6
3	Jaffa clone 9
4	Jaffa clone 11 ME 285
5	Jaffa clone16 ME 228
6	Jaffa clone19
7	Jaffa clone 20
8	Jaffa clone 23
9	Jaffa clone 24
10	Jaffa clone 25
11	Jaffa clone 26
12	Jaffa clone 27
13	Jaffa clone 28
14	Aematousiki (sweet orange)
15	Shamouti 2000-3 (sweet orange)
16	Shamouti 2005-17
17	Valencia orange 2005-18 (sweet orange)
18	Valencia orange long 2003-19
19	Shekerico (acidless orange)
	Bergamot (*Citrus bergamia* Risso& Poit.)
20	Bergamot clone 1
21	Bergamot clone 2
22	Bergamot clone 3
23	Bergamot clone 4
24	Bergamot clone 5
	Frappa (*Citrus maxima* (Burm.) Merrill)
25	Frappa clone 1
26	Frappa clone 2
27	Frappa clone 3
28	Frappa clone 4
	grapefruit (*Citrus paradisi* Macf.)
29	Marsh seedless grapefruit
	lemon (*Citrus limon* (L.) Burm f.)
30	Lemon Lapithou clone 77
31	Lemon Lapithou clone 118
32	Lemon Lapithou clone 174
33	Lemon Lapithou clone 201
34	Lemon Lapithou clone 212
35	Lemon Lapithou clone 267
36	Lemon Lapithou clone 2003-15
37	Lisbon lemon 2005-15
38	Polyphori lemon ME 52
39	Polyphori lemon 2000-12
40	Polyphori lemon 2004-5
	mandarin (*Citrus reticulata* Blanco),
41	Mandarin willowleaf 2005-16
42	Mandarin Arakapas ME 30
43	Mandarin Arakapas ME 38
44	Mandarin Arakapas ME 41
45	Mandarin Arakapas ME 42
46	Mandarin Arakapas ME 48
47	Mandarin Arakapas ME 67
48	Mandarin Arakapas ME 84
	Citrus deliciosa
49	Clementine
	sour orange (*Citrus aurantium* L.*)*
50	Sour orange
	Koumantantas (*Citrus cumandatore)*
51	Koumantantas

DNA extraction: Collected leaf samples were ground to fine powder in liquid nitrogen. DNA extraction was performed with Genomic Prep Cells and Tissue DNA Isolation Kit (General Electric Healthcare).

Microsatellite analysis and data analysis: The plants were analyzed at the following 11 microsatellite loci: CMS-3, CMS-4, CMS-7, CMS-8, CMS-19, CMS-20, CMS-23, CMS-24, CMS-30, CMS-47-1, CMS-4 7-2 (2). PCR reaction was carried out in GeneAmp PCR System 9700 (Applied Biosystem) in 20 µl reaction mixer containing 25 ng DNA, 1 µM of each primer, 200 nmol of each dNTPs, 1.5 mM $MgCl_2$ and 1U of Taq polymerase (General Electric Healthcare). In all cases, the forward primer was Cy-5 fluor labeled. The thermal cycling used for amplification followed the protocol of Ahmad et al. (2): 35 cycles comprising 1 min denaturing at 94 ^{0}C, 1 min annealing at 55-60 ^{0}C depending on the primer, 2 min extension at 72 ^{0}C, followed by final extension at 72 ^{0}C for 30 min. Fragment analysis of the obtained PCR products was carried out on an ALF Express II sequencer (General Electric Healthcare), and alleles were sized with the software Allele Locator 1.03. Internal standards were produced by

amplification of PUC19 fragments with sizes 100, 150, 200, 250, 300, 350, 400, 450, 500 bp. Allele frequencies, expected (He) and observed heterozygosity, (Ho), probability of identity (PI) and probability of null alleles were calculated with software Identity 1.0 (37). The dendrogram was constructed by Microsat software (2) for the calculation of genetic distances in [-log (proportion of shared alleles)]. The distance matrix obtained from Microsat was processed with KITSCH from the PHYLIP package (16) and TREEVIEW (31).

RAPD analysis: Six 10-mer primers obtained from Ready-To-Go-RAPD Analysis Kit (General Electric Healthcare) were used for RAPD analysis. PCR reactions were carried out in a Ready-To-Go RAPD Analysis Beads (General Electric Healthcare) in a 25 µl reaction mixture containing 10 ng of template DNA and 25 pmoles of primer. The following protocol was used for the amplification: 95 °C for 5 min, followed by 45 cycles of 1 min at 95 °C, 1 min at 36 °C and 2 min at 72 °C. The amplification products were separated by electrophoresis in 2% agarose gel and visualized by staining with ethidium bromide. The size of the PCR products was scored according to a 100 bp ladder as a size standard.

Results and Discussion

Microsatellite analysis

Fifty one accessions from the Citrus collection of ARI were genotyped by 10 nuclear microsatellite markers (**Table 1**). The set of markers used in this study successfully amplified all citrus species which is in accordance with the earlier reported high level of conservation of SSR primers across Citrus species and related genera (2, 8, 20, 22, 29, 30).

The comparison of the obtained microsatellite allelic profiles allowed the discrimination of the studied accessions at species level. All studied species were found to have unique allelic profiles. A low level of polymorphism was found among cultivars within the species and lack of polymorphism was observed among clones by using this set of microsatellite markers.

The applied microsatellite markers were assessed in relation to their possibility to reveal polymorphism in the investigated species and cultivars. Two of the microsatellite markers, CMS 3 and CMS 8, were not informative, resulting in amplification of one allele with an identical size in all investigated accessions. CMS 47 detected two microsatellite loci, indicatively CMS47-1 and CMS47-2. In total 39 alleles were obtained in all cultivars and species investigated at all 11 microsatellite loci (**Table 2**). The number of alleles ranged from 1 allele per loci CMS 3 and CMS 8 to 6 alleles per locus CMS 24, with an average number of 3.5 alleles per locus. The average value is lower than the one obtained by Ahmad et al. (2) (4.5) and quite lower than that obtained by Barkley et al. (3) (11.5). The possible reason for this difference could be the higher number of both SSR markers applied and accessions analyzed in these studies.

Comparison of SSR markers with regard to their information content (number of alleles and PI values) showed that the most informative loci for the investigated set of species were loci CMS24 (6 alleles and PI value 0.12) and CMS47-1 (4 alleles and PI value 0.20), whereas the less informative ones were CMS 3 and CMS8 (one allele and PI 1.0). The values of PI varied between markers from 0.12 to 1.0 with a total value $1.03x10^{-5}$.

The obtained microsatellite allelic profiles were used for assessment of genetic diversity among the studied species and cultivars. The estimated values of genetic diversity (expected heterozygosity-He) of the studied genotypes varied between 0.0 for loci CMS 3 and CMS 8 to 0.8 for locus CMS 24, with a mean value of 0.53. The observed heterozygosity (Ho-the

TABLE 2

Genetic parameters of 11 microsatellite loci used for analysis of 51 Citrus accessions: number of alleles, observed (Ho) and expected (He) heterozygosity, probability of identity (PI) and probability of null alleles

Number of locus	Locus	Number of alleles	Expected heterozygosity	Observed heterozygosity	Probability of identity	Probability of null alleles
1	CMS 3	1	0.0000	0.0000	1.0	0.0000
2	CMS 4	3	0.4863	0.5625	0.4802	0.0512
3	CMS 7	4	0.7011	0.5625	0.2534	0.0815
4	CMS 8	1	0.0000	0.0000	1.0000	0.0000
5	CMS 19	5	0.6484	1.0000	0.3073	-0.2132
6	CMS 20	3	0.5371	0.4375	0.3906	0.0648
7	CMS 23	4	0.6542	0.8750	0.3027	-0.1334
8	CMS 24	6	0.8046	0.7500	0.1250	0.0303
9	CMS 30	5	0.7031	0.2500	0.2401	0.2660
10	CMS 47-1	4	0.7460	0.7500	0.2065	-0.0022
11	CMS 47-2	3	0.5644	0.8125	0.3753	-0.1585
Total		**39**			**$1.030773x10^{-5}$**	
Average		**3.5**	**0.53**	**0.54**		

percentage of heterozygous individuals among all tested ones) ranged from 0 for loci CMS 3 and CMS 8 to 1.0 for locus CMS 19 with a mean value of 0.54. The obtained zero values of He and Ho at loci CMS 3 and 4 could be explained with the only one allele detected at these loci. The observed heterozygosity was higher than the expected one at 5 loci, equal at two loci and lower at 3 loci and significantly lower at the remaining 1 locus. The lower values of Ho at four loci could be explained with the higher positive values of the estimated probability of null alleles for these loci. The heterozygote deficiency could also be a consequence of the constraint of breeding techniques (34) that employ the selection of the given horticultural traits and their maintenance by asexual propagation.

Within each species the level of polymorphism was found to be quite low and is discussed herebelow.

Oranges

The group of sweet oranges was represented by 13 clones of the local Cyprus cultivar Jaffa, three local cultivars of Aematousiki, Valencia long and Shekeriko, respectively, two clones of the commercial cultivar Shamouti and one clone of Frost Valencia orange. The local Jaffa orange was introduced in the 19th century from Israel and is similar to the commercial Shamouti, which was introduced from California, whereas Aematousiki is a red-flesh sweet orange with elongated fruits and tree appearance similar to Jaffa. Shekeriko is an acidless local orange with round fruits and an appearance similar to Valencia. The clones of Jaffa show diversity in their morphological characteristics, such as fruit size and shape, thickness of fruit albedo and time of ripening. All analyzed orange cultivars and their clones were found to have identical microsatellite profiles at 9 out of 10 SSR loci. At locus CMS 7 cultivars Aematousiki , Valencia long, Shamouti and all clones of the cultivar Jaffa were homozygous with one allele 152 bp in size, whereas Frost Valencia orange and Shekeriko were heterozygous possessing two alleles, 150 and 152 bp in size (**Fig. 1A**). Thus, the detected polymorphism among orange cultivars discriminated the Frost Valencia orange and the local Shekeriko on one hand, and cultivars Jaffa, Aematousiki, Shamouti, and the Valencia long on the other hand. This shows that the local variety termed "Valencia long" may actually be another clone of Jaffa orange. The high level of similarity of genotypes of the investigated cultivars and clones is in contrast with the observed phenotypic variability among them, indicating that the local cultivars were possibly derived through mutations which are not detectable by the used SSRs or they are clones of the same original cultivars. This is in accordance with the view that most of the orange cultivars were derived through mutations which affect mostly fruit traits (17). In addition, nucellar seedlings, which are characteristic for oranges, may bear new traits and for this reason could be selected and named as different cultivars (12, 23). This may lead to confusion relevant to the identification of cultivars and clones in the Citrus collections.

A low level of polymorphism between sweet oranges was reported in several studies. Ahmad et al. (2) could not distinguish among 39 oranges by using 26 SSRs. According to the above authors, only one microsatellite distinguished between Moro red-flesh orange and Valencia orange. Roose and Fang (12) distinguished 14 out of 41 sweet orange cultivars by ISSR markers. Novelli et al. (29) obtained 8 different genotypes when investigating 41 sweet arranges with 50 SSR markers.

Lemons *(C. limon (L.)Burm f.))*

The group of lemons studied included the commercial cultivar Lisbon and two local cultivars, Lapithou and Polyphori, and their clones. The common commercial cultivar Lapithou is the main_lemon cultivated in Cyprus for centuries, as is well adapted to local conditions, produces good quality fruit and is tolerant to mal secco (caused by *Phoma tracheiphila*). Polyphori is cultivated mainly in the back yard of houses, bears fruit several times a year, but it is sensitive to mal secco. Nine out of 10 microsatellite markers could neither distinguish between these 3 cultivars, nor among the clones of Lapithou and Polyphori. At locus CMS 23 all Lapithou clones were

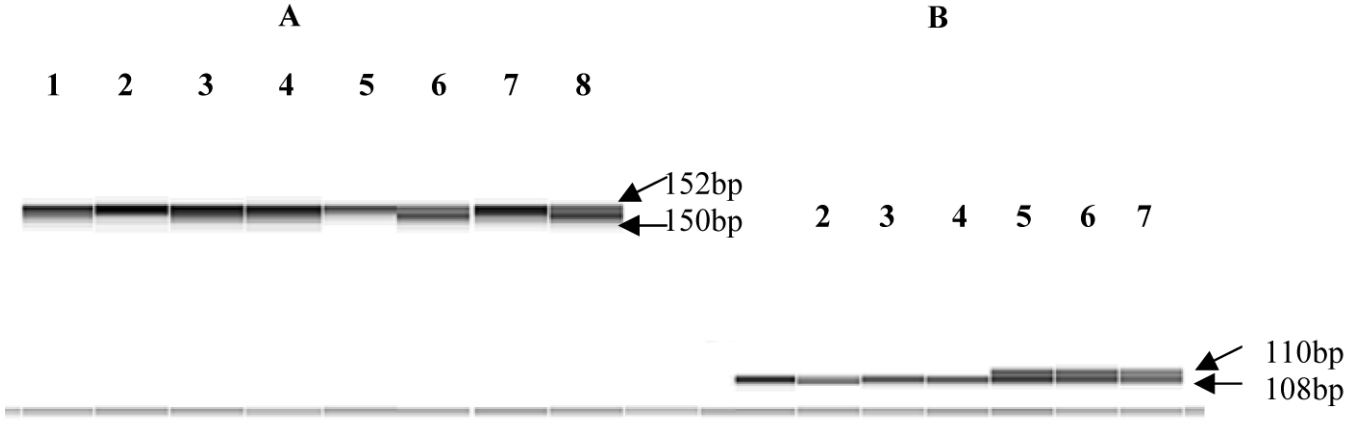

Fig. 1. A. Microsatellite profile of 8 Citrus accessions at locus CMS 7. 1-3 clones of Jaffa, 4-cv.Aematousiki, 5-Shamouti, 6-cv. Frost Valencia, 7-- cv.Valencia long, 8-cv. Shekeriko, **B.** Microsatellite profile of 7 Citrus accessions at locus CMS 23. 1-4 -clones of cv. Lapithou, 5-cv. Lisbon, 6, 7 -cv. Polyphori

homozygous with alleles 108 bp in length, while Lisbon and the clones of Polyphori possessed two alleles with the size of 108 and 110 bp (Fig. 1B). On the basis of these data it could be concluded that the local cultivar Polyphori and its clones were probably derived from Lisbon by mutation, which was maintained by propagation through the nucellar embryo. Lapithou lemon proved not to be identical to Lisbon, as had been earlier believed by citriculturists (Economides, personal communication, 1985). This is in accordance with the long life of Lapithou on the island. In contrast to our results which showed low polymorphism among the studied cultivars, Fang and Roose (12) obtained high genetic diversity among seven lemon cultivars by using ISSR markers, which distinguished 5 out of 7 cultivars.

Mandarins *(Citrus reticulate Blanco)*

The group of mandarins included 7 clones of the local variety Arakapas, and two commercial cultivars Willowleaf and Clementine. The local Arakapas mandarin is similar to Willowleaf, but produces larger and tastier fruits, while the different clones of Arakapas showed differences in fruit size and shape. The clones of Arakapas and Willowleaf had identical microsatellite profiles at all studied microsatellite loci, suggesting that the observed morphological differences between these two cultivars must be associated with somatic mutations, which were not detectable with the used SSR markers. The differences between the groups of Clementine and Satsuma mandarins were determined by Ahmad et al. (2), as well as by Fang and Roose (12), but cultivars within these groups remained undistinguishable.

The microsatellite profile of Clementine differed from that of Willowleaf at 7 alleles at 7 out of 11 analyzed microsatellite loci. Clementine shared half of its alleles with Willowleaf, and half with sweet oranges, suggesting the proposed origin of Clementine from a cross between mandarin and orange (11, 27).

The comparison of microsatellite profiles allowed to determine the identity of some accessions and to propose some relationships between the investigated species. Koumantantas was a local species/variety, phenotypically similar to sour orange, but with bigger leaves and bigger and more rough fruits. Our data showed that the microsatellite profile of Koumantantas is identical to that of Sour Orange. Therefore Koumantantas may be considered as Sour Orange.

Cv. Frappa is also a local citrus species/cultivar, grown traditionally mainly for the preparation of sweets and considered to belong to *C. maxima*. Phenotypically it is similar to grapefruit, but it produces larger fruit with thicker skin. Our microsatelllite data indicated that Frappa is not identical to Grapefruit, but shared half of its alleles with Grapefruit. This caused a speculation for parent/offspring relationship between Frappa and Grapefruit, a refined species introduced in the island in the early 20th century (24).

In order to analyze the genetic relationship between the studied species and cultivars, a dendrogram showing genetic distances was constructed (**Fig. 2**). The dendrogram demonstrated the distribution of the studied species/cultivars in two main clusters, on the basis of their similarity, calculated as a proportion of shared alleles. The first cluster contains the mandarin and orange cultivars. Oranges were separated in one subcluster, including two groups with identical genotypes. Clementine was plotted more closely to the one of its proposed parents, the sweet orange, than to the other one, mandarin Willowleaf. This is because the dendrograms are constructed on the basis of shared alleles and thus reflects more similarity than kinship (34). In this case Clementine had more common alleles with sweet orange (77%) than with Willowleaf (68%).

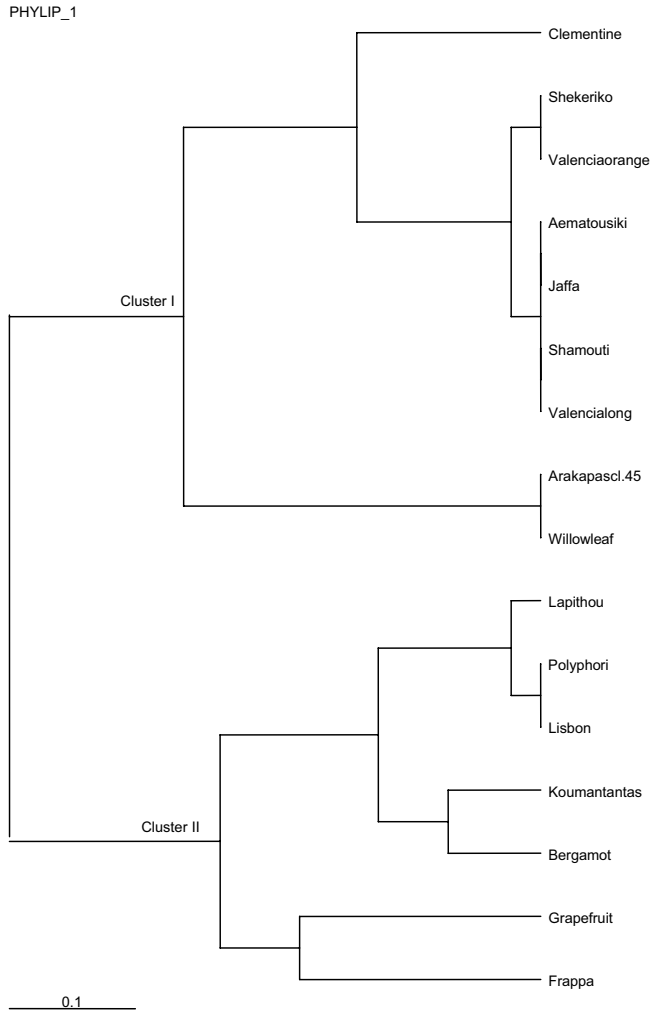

Fig. 2. Dendrogram of 16 Citrus species/cultivars

The second cluster consists of two subclusters-one containing lemon cultivars, Sour Orange and Koumantantas, being identical, and Bergamot. The other group included Grapefruit and Frappa. Frappa was plotted more closely to Grapefruit, which is in agreement with the phenotypic similarity between them and the proposed kinship.

Lack of polymorphism was obtained among 5 clones of Bergamot and 4 clones of Frappa.

RAPD analysis

To further assess the genetic diversity between cultivars and clones the 51 accessions were analyzed with 6 RAPD primers. The number of amplified fragments per primer varied from 4 to 15. The size of fragments ranged between 400 and 3000.

The chosen set of RAPD markers allowed discrimination of the studied genotypes at species level. The investigated citrus species of Orange, Lemon, Mandarin, Grapefruit, Bergamot, Frappa and Koumantantas were found to have unique RAPD profiles.

Three out of six primers (primers 2, 3 and 6) did not detect any polymorphism between cultivars, or between clones. The other three primers (1, 4 and 5) allowed the revelation of polymorphism in the groups of Lemon, Frappa and Bergamot.

Two cultivar-specific markers, PL1-600 and PLT5-550, corresponding to fragments of 600 and 550 bp, which were generated by RAPD primers 1 and 5, respectively, were found in lemons.

Primer 1 discriminated between Polyphori lemon on the one hand and Lisbon and Lapithou lemons on the other hand. Primer 1 produced in the clones of Polyphori a band of 0.6 kbp in length, which was absent in the RAPD profiles of Lisbon and Lapithou clones. Primer 5 discriminated between the 7 Lapithou clones on one hand and Lisbon and the 3 Polyphori clones on the other hand, as a result of the amplification of 550 bp fragment in Lisbon and Polyphori accessions, that was absent in the Lapithou lemons (**Fig. 3**).

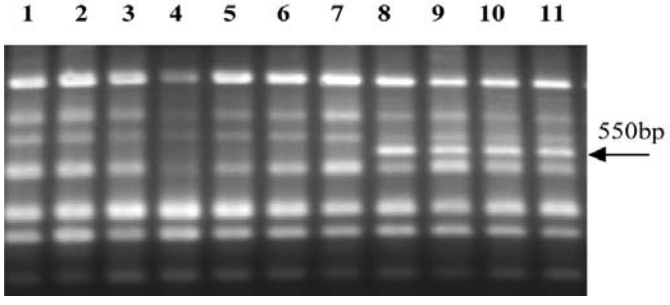

Fig. 3 RAPD profile of lemon accessions generated by primer 5. Lines 1-7-clones of cv. Lapithou, line 8-cv. Lisbon, lines 9-12 clones of cv. Polyphori

Lack of polymorphism was detected between Willowleaf and the local mandarin Arakapas as well as among clones of the local mandarin Arakapas. This result indicated that the local variety Arakapas is most probably a clone of Willowleaf, derived from somatic mutations that were not detected by the molecular markers used. Colleta Filho et al. (6, 7) detected a very low polymorphism among Ponkan mandarins by using 25 random primers. In contrast, Das et al. (10) obtained a high genetic diversity among 25 mandarin plants collected from different regions of India by using 15 RAPD markers.

Clone-specific markers were also found for one clone of Frappa and one clone of Bergamot.

Marker PF4-580 allowed distinguishing one out of 5 clones of Bergamot. Comparison of RAPD profiles of 5 clones of Bergamot, amplified with primer 4, showed the presence of a band of 580 bp in length in the profile of Bergamot clone 2 that was not observed in the profiles of the other Bergamot clones (**Fig. 4**).

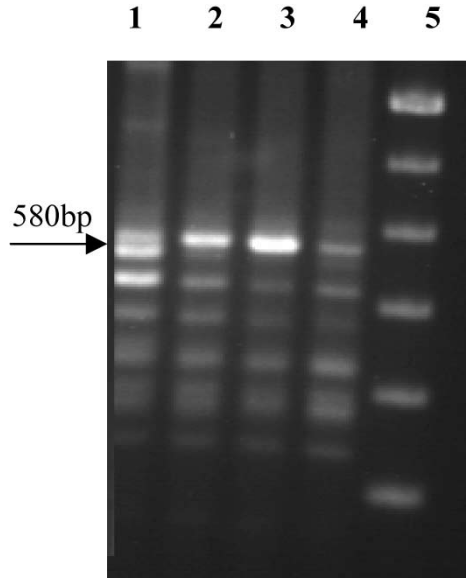

Fig. 4. RAPD profile of Bergamot accessions generated by primer 4. Lines 1-4-clones of Bergamot, M –size marker 100 bp ladder

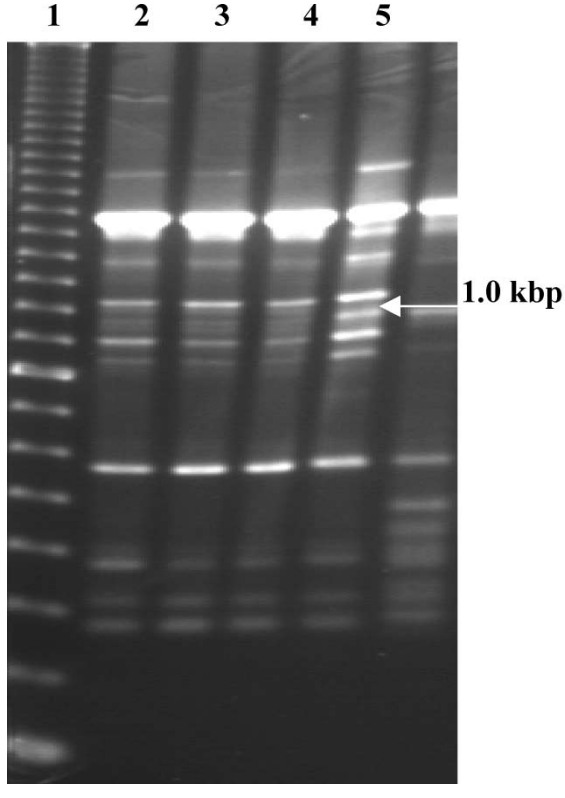

Fig. 5. RAPD profile of Frappa accessions generated by primer 4. Lines 1-4-clones of cv. Frappa, M-size marker-100bp ladder

Marker PB4-1000 corresponded to a fragment of 1 kbp in length that was generated by RAPD primer 4 only in the accession Frappa clone 4, but not in the other Frappa clones (**Fig. 5**).

Conclusions

With this study a molecular characterization of 51 accessions in the Citrus collection of Cyprus was initiated. Despite the morphological diversity of the investigated cultivars and their clones, a low DNA polymorphism was detected by using SSRs and RAPD markers

Nevertheless, with the present study it was shown that the traditional lemon cultivar of the island "Lapithou" is distinguishable from the commercial cultivar Lisbon and also from another local cultivar "Polyphori", whereas the traditional Cyprus mandarin "Arakapas" was indistinguishable from Willowleaf mandarin at molecular level. In addition, molecular markers were identified for discrimination of the orange and lemon cultivars and also of Frappa and Bergamot clones. The analysis of additional loci is necessary to identify and discriminate further the investigated accessions that differ in their phenotypic characteristics.

The data obtained in this study provide genetic information, which can assist the management of Citrus collections in Cyprus and the selection programs for further improvement of Citrus as well as the establishment of relations between Citrus species and cultivars worldwide.

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