

Genetic Diversity and Population Structure of Traditional Greek and Cypriot Melon Cultigens (*Cucumis melo* L.) Based on Simple Sequence Repeat Variability

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Abstract. Seventeen simple sequence repeat (SSR) markers were used to assess the genetic diversity and population structure among traditional Greek and Cypriot melon cultigens (*Cucumis melo* L.). All SSR markers were polymorphic with a total number of 81 alleles, whereas all cultigens could be distinguished with at least one SSR, except cultigens 43 and 41. Reference accessions showed larger genetic variability with an average of four alleles per locus and 0.65 gene of diversity compared with an average of 2.47 alleles per locus and 0.30 of gene diversity for the Greek/Cypriot cultigens. Observed heterozygosity was very low, indicating a lack of outcrossing, at least in recent times. Unrooted neighbor-joining tree analysis and population structure analysis clustered the cultigens and the reference genotypes into five groups. All cultigens could be distinguished; the Cypriot cultigens were more closely related to the inodorus 'Piel de Sapo', whereas the Greek cultigens were located in an intermediate position between the inodorus 'Piel de Sapo' and the cantalupensis 'Védrantais'. The cultigen 'Kokkini' was the most divergent among the Greek and Cypriot cultigens. This association between geographic origin and genetic similarity among Greek and Cypriot cultigens indicates geographic isolation. Most of the cultivars from the same cultivar group (i.e., inodorus, cantalupensis) clustered together, but some exceptions were found, suggesting that former inodorus landraces would have been transformed to cantalupensis as a result of intercrossing and further selection by farmers. Results of population structure analysis support mixing between cantalupensis and inodorus. 'Agiou Basileiou', an inodorus cultigen, was assigned to the subpopulation IV/II of which II is a pure cantalupensis subpopulation. Greek and Cypriot melon cultigens were developed from a broader germplasm base than western Mediterranean cultivars and exhibited useful for melon breeding programs genetic variability.

Melon (*Cucumis melo* L.) origin was traditionally believed to be African (Robinson and Decker-Walters, 1997), although recent

molecular systematic studies suggest that it may be Asian (Renner et al., 2007). Currently, the distribution of wild and cultivated melons

is worldwide. A high level of molecular and morphological variability in leaf, plant, and fruit characteristics has been described in melon species (Akashi et al., 2002; Burger et al., 2006; Goldman, 2002; Kirkbride, 1993; Monforte et al., 2003; Stepansky et al., 1999). Melon classification has been progressively evolving by adopting the propositions of many researchers (Jeffrey, 1980; Kirkbride, 1993; Munger and Robinson, 1991; Naudin, 1859; and more recently, Pitrat et al., 2000). Today, it is as follows: there are two major subspecies, *melo* and *agrestis*, which are divided even further; the subspecies *agrestis* into the *varietas* (varieties): *conomon*, *makuwa*, *chinensis*, *acidulus*, and *momordica*; and the subspecies *melo* into the *varietas*: *cantalupensis*, *reticulatus*, *adana*, *chandalak*, *amer*, *inodorus*, *flexuosus*, *chate*, *tibish*, *dudaim*, and *chito*. The subspecies *agrestis* is distributed mainly in central and Oriental Asia, whereas the subspecies *melo* is distributed mainly in the Near East and Mediterranean regions. The primary diversity center of melon is located in central Asia, Iran, Afghanistan, India, Transcaucasia, Turkmenistan, Tajikistan, and Uzbekistan. Far-East Asia and the Mediterranean regions are secondary centers of diversity.

Apart from the significant amount of genetic variability observed among melon germplasm, occidental modern melon cultivars have a relatively narrow genetic base because they belong to a limited number of *varietas* (mainly *inodorus*, *reticulatus*, and *cantalupensis*) (Silberstein et al., 1999).

Various DNA molecular markers have been used to characterize the genetic diversity of melons, including restriction fragment length polymorphism (Zheng et al., 1999), amplified fragment length polymorphism (Garcia-Mas et al., 2000), random amplified polymorphic DNA (RAPD) (Sensoy et al., 2007; Staub et al., 2004), and simple sequence repeat (SSR) (Danin-Poleg et al., 2001), using diverse germplasm from different locations worldwide. Recently, several research groups have focused on the genetic variability among Mediterranean landraces (López-Sesé et al., 2003; Lotti et al., 2008; Sensoy et al., 2007; Staub et al., 2004), which belong to the *varietas* *inodorus*, *cantalupensis*, *reticulatus*, and *flexuosus*. These reports have shown among traditional landraces an important amount of genetic variability, which has to be systematically classified before their potential use like their introduction in breeding programs.

Among the Mediterranean melon landraces, Greek and Cypriot landraces are not well known. Staub et al. (2004) studied the diversity among Greek melon accessions using RAPD markers, concluding that the Greek germplasm is genetically unique.

In the present work, we used a set of published SSR markers (Danin-Poleg et al., 2001; Fernandez-Silva et al., 2008; Gonzalo et al., 2005) to genetically assess a number of traditional Greek and Cypriot melon cultigens, not tested before, and compare these cultigens with a set of reference accessions to

increase our understanding of the distribution of genetic variability of melon germplasm in the Mediterranean area.

Materials and Methods

Plant material and DNA extractions. In this study, 21 different accessions were used: seven Greek and five Cypriot traditional cultigens from geographically diverse areas, two popular in the Cyprus market commercial varieties, and seven reference genotypes. Traditional cultigens were obtained on excursions to small farms in Greece and Cyprus. The two commercial varieties, designated C1 and C2, were obtained from a local store in Cyprus. The seven reference genotypes were selected to include a broad spectrum of genetic variability. Details about the origin and classification of accessions into *varietas* groups are given in Table 1.

Seeds were germinated at 30 °C in petri dishes containing wet paper for 6 d. Germinated seeds were planted in pots containing standard pot mixture and transferred into the greenhouse until seedlings developed two or three true leaves, which subsequently were used for DNA extraction.

DNA was extracted as described by Rogers and Bendich (1988) from 10 individual plants per accession. Extracted DNA was quantified with a Hitachi U-2001 spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) and mixed equally at an amount of 50 ng/μL. Basic DNA manipulations and molecular techniques were performed according to Sambrook et al. (1989).

Simple sequence repeat genotyping. In this study, 17 SSR markers all previously developed by Danin-Poleg et al. (2001), Fernandez-Silva et al. (2008), or Gonzalo et al. (2005) were used. All SSR markers are found in Table 2. Two sequencing analyzers were used for SSR visualization: the ABI 310 Prism Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and LI-COR R2 (LI-COR Inc., Lincoln, NE) sequencers.

For ABI 310 visualization, polymerase chain reactions (PCRs) were performed using a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Forward primers were labeled with a fluorescent dye, 6-FAMTM, TETTM, or HEXTM (PE Applied Biosystems Inc., Foster City, CA). All oligos were purchased from MWG-Biotech AG (Ebersberg, Germany). The PCR reactions comprised 60 ng of genomic DNA, 166 μM dNTPs, 0.6 μM of each primer, 1 U of Taq polymerase, 1.2 μM of 10× PCR, and 2 mM MgCl₂ buffer and were performed in a final volume of 15 μL. The cycling conditions were as follows: an initial cycle at 94 °C

for 4 min followed by 35 cycles at 94 °C for 30 s, 40 to 60 °C (depending on primer) for 30 s, and 72 °C for 1 min. A final cycle was allowed at 72 °C for 15 min. Samples were run on an ABI Prism 310 Genetic Analyzer. Uncoated capillaries, 47 cm long with an internal diameter of 50 μm, and denaturant gel POP-4TM (Applied Biosystems Inc., Foster City, CA) were selected for separation of fragments, whereas the Virtual Filter Set C of the GeneScan3.1 software (Applied Biosystems Inc., Foster City, CA) was used for equipment calibration. Amplified PCR products were diluted 1:7 in distilled water. Two microliters of the diluted PCR product were subsequently mixed with 13 μL formamide and 0.5 μL of molecular standard GeneScanTM 500 TAMRA (Applied Biosystems Inc., Foster City, CA) denatured at 95 °C for 3 min and kept on ice until loading. Loading of the amplified fragments was achieved with 15.0 kV for 5 s. The fluorochrome-labeled PCR products were electrophoresed at 15.0 kV, laser power 9.8 mW, and a temperature of 60 °C. Electrophoretic data were analyzed with the standard GeneScan3.1 software (Applied Biosystems Inc.).

A 20-nucleotide sequence from the M13 cloning vector (CACGACGTTGTAAACG ACC) was attached to the 5-end of the forward primers for visualization using the LI-COR IR2 sequencer. The M13 primer was labeled with either IRD-700 or IRD-800 dyes. PCR reactions were performed in a final volume of 15 μL with 1 × Taq buffer [10 mM Tris-HCl, 50 mM KCl, 0.001% gelatin (pH 8.3)], 1.5 to 3.5 mM MgCl₂, 166 μM dNTPs, 5 pmol of forward and reverse primer, 0.66 pmol of M13-labeled primer, 2 U Taq DNA polymerase, and 20 ng of genomic DNA. The cycling conditions were as follows: an initial cycle at 94 °C for 4 min followed by 35 cycles at 94 °C for 30 s, 40 to 60 °C (depending on primer) for 30 s, and 72 °C for 1 min. A final cycle was allowed at 72 °C for 15 min. Five microliters of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were added to the PCR mix and samples were denatured at 96 °C for 2 min. Electrophoresis was performed in denaturing conditions at 50 °C in TBE (90 mM Tris-borate, 2 mM EDTA pH 8.0, and 7.5 M urea) buffer using 6% polyacrylamide gels (AA:BIS = 19:1). Microsatellite allele sizes were estimated comparing their migration pattern with the IRD-700 or -800, 50- to 350-bp size standards (LI-COR).

Genetic variability analysis. Analysis of the pooled DNA samples was based on the assumption that the observation of two or more SSR alleles in a single genotype could have resulted from the presence of several heterozygous plants, homozygous plants for the alternative alleles, or a combination of both. Experimental conditions during the PCR amplification did not permit the quantification of the frequency of an SSR allele in the sample based on the band intensity. Therefore, all detected alleles were assumed to have a frequency of 1/n (n = number of

alleles). Number of alleles, allele frequency, major allele frequency, polymorphism information content (PIC), gene diversity, genetic distances according to Nei et al. (1983), and neighbor-joining (NJ) trees were calculated with PowerMarker 3.25 (Liu and Muse, 2005). The NJ tree was plotted with MEGA 3.0 (Kumar et al., 2004).

The number of populations in our collection was inferred using STRUCTURE 2.2 (Falush et al., 2007; Pritchard et al., 2000). Briefly, several population numbers (from $K = 1$ to 11) were tested with the software and the α posterior probability for each K recorded. The total number of populations was set for K when the probability reached a plateau for higher K .

Results

Polymorphism among all accessions. All SSR markers were polymorphic with a total number of 81 alleles ranging from two (ECM231) to nine (CMAT89) with an average of 4.76 alleles per locus (Table 2). Major allele frequency had an average of 0.65 (ranging from 0.88 for CMAT35 to 0.31 for CMAT89). Similar trends were observed for gene diversity and PIC variables (Table 2). The number of within-accession polymorphic loci ranged between zero and three (data not shown). All within-accession polymorphic loci showed only two alleles within the accession. One or two polymorphic loci were observed in 23.8% of the accessions and three alleles only in 5.8% of the accessions (data not shown). The low variability within accession is reflected by the observed heterozygosity ranging between 0 and 0.20 with an average of 0.052, being lower than expected for all markers. For five markers (CMCTN35, CMCTN38, ECM122, ECM88, and TJ2), the observed heterozygosity was 0.

Polymorphism within Greek and Cypriot cultigens and comparison with reference accessions. All SSRs, except TJ31, ECM122 and ECM231, were polymorphic between Greek and Cypriot cultigens. All Greek and Cypriot cultigens could be distinguished with at least one SSR, except 43 and 41, which were different only in one allele for locus ECM81; 43 was heterozygous for this locus, whereas 42 was homozygous.

Table 2 also shows the genetic variability comparison between the Greek and Cypriot traditional cultigens together as a group (Greek/Cypriot) versus the reference accessions. As expected, reference accessions shows a larger genetic variability with an average of four alleles per locus and average gene diversity of 0.65 than the Greek/Cypriot cultigens with 2.47 alleles per locus and gene diversity of 0.30. Moreover, allelic frequencies were more balanced among reference accessions, which had an average major allele frequency of 0.45 compared with 0.78, which was the average major allele frequency of Greek/Cypriot cultigens.

Phylogenetic relations among melon accessions. The unrooted NJ tree based on the genetic distance by Nei et al. (1983) is

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Table 1. Genetic material used in the study.^z

Plant designation	Type of accession ^y	Code	Origin	Varietas	Seed donor ^w
Piel de Sapo	Ref. gen.	PS	Spain	Inodorus	Semillas Fitó
Védrantais	Ref. gen.	VED	France	Cantalupensis	INRA
Ein Dor	Ref. gen.	EIN	Israel	Cantalupensis	NCRPIS
Flexuosus	Ref. gen.	FLEX	Iraq	Flexuosus	NCRPIS
PI161375	Ref. gen.	SON	Korea	Chinensis	NCRPIS
PI124112	Ref. gen.	INB	India	Momordica	NCRPIS
Ames 24297	Ref. gen.	TRI	India	Wild melon	NCRPIS
C1 Prasinokitrinio	Ref. gen.	C1	Commercial	ND ^x	LS
C2 Kanarini	Ref. gen.	C2	Commercial	ND	LS
Arakapas	Tra. cult.	29	Cyprus	ND	LF
Orounta	Tra. cult.	41	Cyprus	ND	LF
Astromeritis 42	Tra. cult.	42	Cyprus	ND	LF
Astromeritis 43	Tra. cult.	43	Cyprus	ND	LF
Kokkini	Tra. cult.	Koed	Cyprus	ND	LF
Thrakiotiko	Tra. cult.	Thrak	Greece	Inodorus	NAGREF
Kokkini Banana	Tra. cult.	Koka	Greece	Cantalupensis	NAGREF
Episkopi Pediados	Tra. cult.	ZK1	Greece	ND	LF
Anopoleos	Tra. cult.	Anoos	Greece	ND	LF
Zakynthos	Tra. cult.	Zakos	Greece	Cantalupensis	NAGREF
Amindaio	Tra. cult.	Amio	Greece	Inodorus	NAGREF
Agiou Basileiou	Tra. cult.	Agleiou	Greece	Inodorus	NAGREF

^zPlant designation indicates the common name or accession number followed by type of accession, the code used in the figure, the country of origin, the *varietas* according Pitrat et al. (2000), when known, and the seed donor.

^yRef. gen. = reference genotype; Tra. cult. = traditional cultigen.

^xND = not determined.

^wSeed donors: Semillas Fitó S.A. (Barcelona Spain); INRA = l'Institut National de la Recherche Agronomique (Avignon, France); NCRPIS = North Central Regional Plant Introduction Station (Ames, IA); LS = local store; LF = local farmer; NAGREF = National Agricultural Research Foundation (Gastouni, Greece).

Table 2. Genetic variability parameters for each simple sequence repeat marker used in this study and their mean calculated with Powermarker 3.25 (Liu and Muse, 2005).^z

Marker	Sample size	Allele number	Genotype number	Major allele frequency	Gene diversity	Observed heterozygosity	Polymorphism information content
CMAG128	21	4	5	0.65	0.52	0.10	0.46
CMTCN41	21	8	7	0.62	0.59	0.05	0.57
CMTA134	21	6	7	0.67	0.53	0.10	0.50
CMAT89	21	9	10	0.31	0.80	0.05	0.78
TJ31	21	4	4	0.83	0.29	0.05	0.28
CMCTN35	21	4	4	0.45	0.63	0.00	0.55
CMCTN38	21	6	6	0.62	0.58	0.00	0.55
CMAT35	21	4	4	0.88	0.22	0.05	0.21
CMAGN75	21	5	5	0.67	0.51	0.05	0.47
ECM122	21	4	4	0.86	0.26	0.00	0.25
CMAGN79	21	7	7	0.55	0.65	0.05	0.61
CMTCN6	21	3	4	0.58	0.53	0.11	0.43
ECM231	21	2	3	0.88	0.22	0.05	0.19
ECM178	21	3	4	0.64	0.49	0.05	0.41
ECM81	21	4	6	0.55	0.57	0.20	0.49
ECM88	21	3	3	0.50	0.55	0.00	0.46
TJ2	21	5	5	0.81	0.34	0.00	0.32
Mean	21	4.76	5.18	0.65	0.49	0.05	0.44
Subdivision							
Greek/Cypriot	14	2.47	2.82	0.78	0.30	0.06	0.26
Reference	7	4.00	4.06	0.45	0.65	0.04	0.59

^zIn the bottom of the table, genetic variability parameters of the Greek/Cypriot traditional cultigens versus the reference accessions are also shown.

shown in Figure 1A. The tree depicts the clustering of the Greek cultigens within the *Cucumis melo* subsp. *melo* accessions, clearly different from the subsp. *agrestis* accessions ('SON' and 'INB'). All genotypes were divided into five groups (I to V). Greek and Cypriot cultigens were spread into two subpopulations (IV and V), whereas reference genotypes were divided into subpopulations I, II, and III with the exception of the reference accessions 'EIN' and 'PS', which were grouped into the V group and the Cypriot cultigen 'Koed' in Group I. Most of the Cypriot cultigens were closely related and close to the inodorus reference genotype 'PS'. Greek accessions were generally lo-

cated in an intermediate position between the inodorus 'PS' and the cantalupensis 'VED'.

The population structure was studied with the software STRUCTURE 2.2 (Falush et al., 2007; Pritchard et al., 2000). To estimate the number of populations (K), an analysis was performed with a predefined population number ranking from $K = 2$ to 11 whereby the logarithm of the probability of the data $\{\ln[\Pr(X/K)]\}$ was recorded for each K . The maximum $\ln[\Pr(X/K)]$ was observed for $K = 8$, although the differences with $K = 5$ to 7 were small (data not shown), i.e., a plateau for $\ln[\Pr(X/K)]$ was observed at $K = 5$. A larger K did not result in any further division within Greek and Cypriot accessions, only

between reference genotypes. Thus, $K = 5$ was chosen as the final estimated number of populations. The subpopulations inferred by population analysis are depicted in Figure 1B. The x axis represents the Q value (the estimated membership coefficients for each individual in each subpopulation). Different color segments within each individual indicate the percent representation of each corresponding subpopulation. Y-axis bars represent each accession. Five subpopulations were inferred (I to V); the assignment of individuals to each subpopulation was the same as was shown by the NJ tree. There were three cultigens that had a coefficient $q > 0.3$ for two subpopulations and therefore

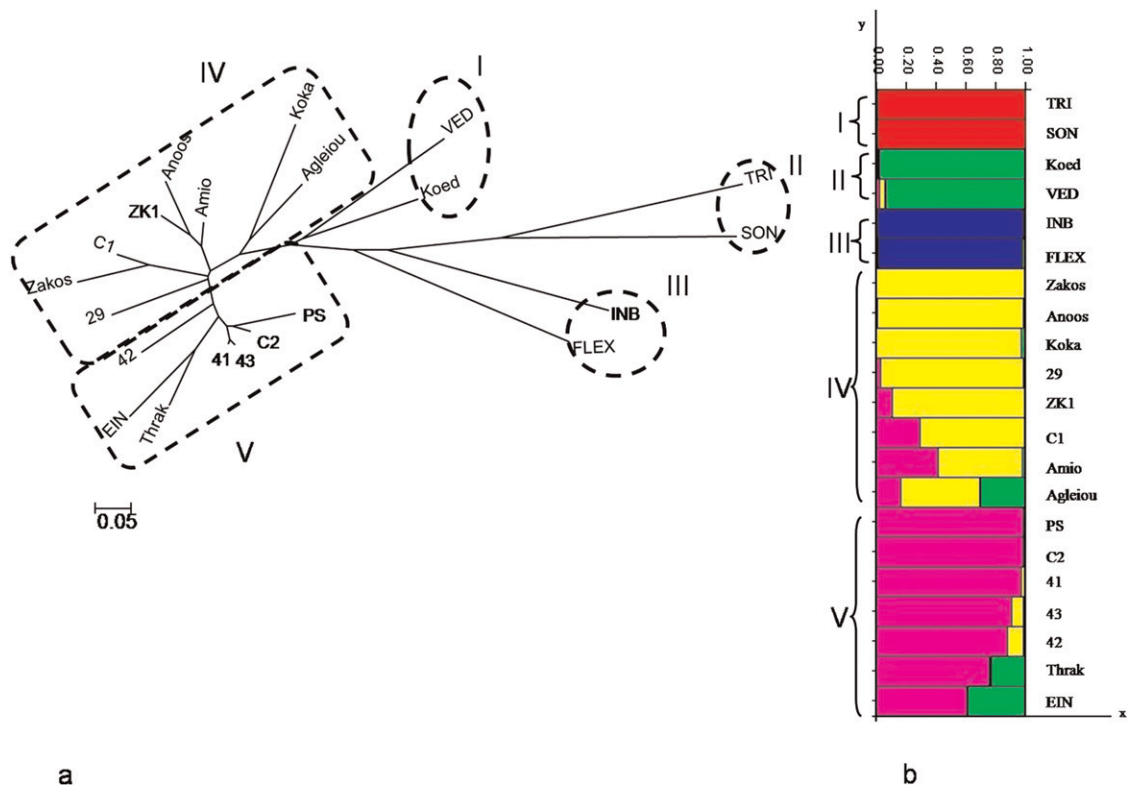


Fig. 1. (A) The neighbor-joining (NJ) tree based on genetic distance according to Nei et al. (1983). The distance scale is shown at the bottom of the tree. The accessions are coded according to Table 1. (B) Bar plot of population structure estimates of 21 accessions of melon assessed with 18 simple sequence repeat markers. Each accession is represented by a single horizontal bar (y-axis) broken into K colored segments (x-axis) with lengths proportional to q of the K -inferred subpopulations. The sum of q values for each accession is one. Accession code names corresponding to each bar are shown on the right side. Latin numbers (I to V) correspond to the predefined subpopulations ($K = 5$).

were assigned into mixed subpopulations. The cultigen 'EIN' was assigned to the subpopulation V/II, the cultigen 'Agleiou' was assigned to the subpopulation IV/II, and the cultigen 'Amio' was assigned to the subpopulation V/IV.

Discussion

In previous studies, López-Sesé et al. (2003) and Staub et al. (2004) used RAPD markers to characterize a large number of Greek melon germplasm. In this study, we also assessed the genetic diversity of Greek germplasm but focused only on cultigens, including some from Cyprus. All Greek and Cypriot cultigens used in this study were assessed for the first time, except for 'Agleiou' and 'Zakos', which were used as internal references.

For our assessment, the molecular marker-type SSR was used, which to our knowledge is the first time this has been done for either Greek or Cypriot germplasm accessions. The selected SSRs, even relatively small in number (17), were sufficient to distinguish all the tested cultigens. This demonstrates the usefulness of the chosen marker set to study the genetic variability among the analyzed cultigens. The tested cultigens have been primarily developed and maintained by local farmers and, hence, cross-pollination with landraces would be

expected, resulting in appreciable levels of heterozygosity. Our results showed very low observed heterozygosity among Greek and Cypriot traditional cultigens indicating lack of intercrossing either between them or with other landraces in recent times. This unexpected result is in agreement with the results of Staub et al. (2004), which are attributed to the small sample sizes examined and/or the use of similar seed lot among related growers. To their assumptions, we add the hypothesis that if outcrossing occurred, farmers have made efforts to maintain the genetic originality of the cultigens, probably to satisfy regional consumer preferences.

Reference accessions showed higher genetic variability compared with the Greek/Cypriot cultigens. Nevertheless, the genetic variability observed among Greek/Cypriot cultigens is relatively significant compared with reference accessions, because the reduction of gene diversity is only $\approx 50\%$. These results demonstrate that the Greek and the Cypriot germplasm retain important levels of genetic variability.

The Greek and Cypriot traditional cultigens examined in the present report can be undoubtedly classified within the subspecies *melo*, very close to western Mediterranean cultivars and different from the flexuosus accession tested. In previous studies, when RAPD markers were used (Staub et al., 2004), Greek flexuosus landraces did not clearly show distinction from Greek inodorus

landraces. This difference might be a result of the different germplasm used or the higher power of discrimination of SSR markers compared with RAPDs.

The unrooted NJ tree (Fig. 1A) clustered the tested material into five population groups (I to V). The more populated among the five groups were: Group V, including mostly accessions from Cyprus, which were closely related to the inodorus 'Piel de Sapo', and Group IV, including mostly accessions from Greece, which were in an intermediate position between the inodorus 'Piel de Sapo' and the cantalupensis 'Védrantais', indicating that some of them are closer to inodorus types, whereas some were closer to cantalupensis types. This association between geographic origin and genetic affinity among Greek and Cypriot cultigens gives evidence of geographic isolation.

Our results also show that most of the accessions from the same *varietas* (i.e., inodorus, cantalupensis) cluster together, but some exceptions were found. Among the tested cultigens is the inodorus cultigen 'Agiou Basileiou', which is phenotypically very similar to 'Piel de Sapo', another inodorus cultigen. However, our results, in accordance with the work of López-Sesé et al. (2003) and Staub et al. (2004), placed it relatively distant to the 'Piel de Sapo' types and closer to cantalupensis cultigens. Apparently, farmers selected independently in Spain and Greece for the same phenotypic

characteristics from a broad ancestral gene pool, producing similar phenotypes obtained from divergent selection. One of the major differences among inodorus and cantalupensis germplasms is the fruit climacteric behavior: inodorus types are nonclimacteric and cantalupensis are climacteric. Recent studies have showed that the manipulation of very few genes can produce severe changes in climacteric behavior (Moreno et al., 2008; Obando-Ulloa et al., 2008; Périn et al., 2002a), opening up the possibility that former inodorus landraces would have been transformed to cantalupensis as a result of intercrossing and further selection by farmers. This transformation cannot be proven by this study. However, the results of STRUCTURE support mixing between cantalupensis and inodorus. 'Agiou Basileiou', an inodorus cultigen, was assigned to the subpopulation IV/II of which II is a pure cantalupensis subpopulation.

Currently, most of the mapping populations used to study the genetic control of fruit traits in melon have been produced using very distant melon genotypes (Eduardo et al., 2007; Monforte et al., 2004; Périn et al., 2002b). Our results suggest the idea that mapping populations from crosses between inodorus and cantalupensis cultivars would reveal whether the differences between these melon types are actually caused by a low number of genes.

In the study by López-Sesé et al. (2003), it was shown that inodorus accessions from Crete are more diverse than Spanish accessions. The genetic relationships among Greek and Cypriot cultigens as depicted in Figure 1 also agree with that observation. One possible explanation is that Greek and Cypriot melon accessions were developed from a broader germplasm base. This is probably a result of a number of flexuosus introductions from the remaining east Mediterranean countries, which, since antiquity, have been known to be an important cultivation center, especially for flexuosus cultigens (Janick et al., 2007).

In summary, the current report using SSR markers reinforces the previous studies of López-Sesé et al. (2003) and Staub et al. (2004) indicating that Greek and Cypriot traditional melon cultigens exhibit unique genetic variability and geographical division.

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