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Molecular Characterization and Analysis of the Greek Citrus Germplasm

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Abstract

Molecular markers were used to evaluate genetic similarity among 36 citrus accessions (*C. sinensis*, *C. reticulata*, *C. limon* and cultivars) of the Greek national clonal germplasm repository located in Poros. In total, nine RAPD and five ISSR primers produced 262 loci, of which 236 were polymorphic. Both techniques were proven to be equally analytical with an average discrimination power above 0.9. The RAPD and ISSR markers were highly correlated and clustering among them had high correspondence. Citrus accessions formed separate clusters according to their species, even though sweet orange and mandarin cultivars revealed high affinity, while lemons were more divergent. *C. sinensis* genotypes had the highest intragenetic affinity suggesting that cultivated oranges have a narrow genetic base. On the contrary, the *C. reticulata* group was the most variable. In general, Greek varieties exhibited a distinctive genetic background that could be discriminated from foreign established cultivars using the mentioned molecular techniques. This information could facilitate their use in future *Citrus* spp. breeding programs.

Keywords: AMOVA, *Citrus* spp., genetic diversity, ISSR, RAPD

Introduction

The *Citrus* L. genus includes several of the most important world's fruit crops, such as oranges, lemons, limes, mandarins, grapefruits, pummelos and kumquats (Mabberley, 2008). Since antiquity, citrus fruits have been widely utilised for their nutritional, medicinal, cosmetic and ornamental properties and acknowledged as a notable source of essential nutrients (Dugo and Di Giacomo, 2002). The history of citrus cultivation is extremely composite; reports place its primary origins in Persia and Middle East (4,000 BC), from where it spread eastward through the Malayan Archipelago to China and Japan and southward to Australia (Nicolosi, 2000; Pfeil and Crisp, 2008).

Though its manifold economic magnitude, citrus taxonomy still remains controversial, primarily due to the sexual compatibility and low genetic barriers between related genera that results to interspecific and intergeneric hybridization (Moore, 2001). Furthermore, bud mutations, apomixis and the long period of cultivation ads up to the genus complexity (Scora, 1988). Initially, *Citrus* spp. taxonomy was founded exclusively based on morphological and geographical data. This led to major controversy on systematics of species within the *Citrus* subgenus (Moore, 2001). Two dissimilar classifications schemes have been developed and adopted; the Swingle system that recognizes 16 species (Swingle and Reece, 1967) and the Tanaka taxonomy that superfluously splits and identifies 162 species in the genus (Tanaka, 1977). However, advanced studies based on biochemical and morphological traits, suggests that there are only three 'true' species, i.e. citron (*C. medica* L.), mandarin (*C. reticulata* Blanco), and pummelo (*C. maxima* L. Osbeck). Other mentioned cultivated *Citrus* spp. theorized to be hybrids derived as apomictically perpetuated biotypes (Barrett and Rhodes, 1976; Scora, 1988).

DNA-based markers have recently gained popularity in genetic studies among species and cultivars. Particularly in citrus, molecular markers have been implemented in large-scale genomic characterization, gene mapping, germplasm studies and assessment of intra- and intergenetic variability. Among various molecular techniques, Randomly Amplified Polymorphic DNA (RAPD; Luro *et al*., 1995; Higashi *et al*., 2000) and Inter-Simple Sequence Repeats (ISSRs) are more common in use, due to the combination of their analytical power and relative simplicity.

Moreover to the taxonomic complexity of the citrus group itself, many germplasm collections include synonyms and homonyms cultivars. In the current study we've used certified citrus genotypes from mother trees of the national *Citrus* spp. foundation collection in Poros Arboricultural Station, which is the Greek National clonal Germplasm Repository for citrus. At the present time, the level of genetic diversity in this collection remains unidentified.

The purpose of the present study was:

1. Estimate the degree of intergenetic and intragenetic variability among citrus cultivars of Greek and foreign origin.

2. Compare the capability and effectiveness of RAPD and ISSR markers on citrus genotypes.

3. Facilitate the use of related cultivars and hybrids in future breeding programs exploiting the Greek citrus germplasm.

Materials and methods

Plant materials and DNA extraction

Thirty-six citrus accessions were collected from Arboricultural Station (Poros, Greece) (Tab. 1).

Tab. 1. Plant materials utilized for RAPD and ISSR analyses

N$_o$	Cultivar or common name	Genotype
1	Kumquat	*Fortunella margarita x Fortunella japonica*
2	'Carrizo'	*Citrus sinensis x Poncirus trifoliata*
3	Poncirus trifoliata	*Poncirus trifoliata*
4	'Troyer'	*Citrus sinensis x Poncirus trifoliata*
5	'Citrumelo 1452'	*Poncirus trifoliata x Citrus paradisi*
6	'Mars seedless'	*Citrus paradisi*
7	'Eureka'	*Citrus limon*
8	'Interdonato'	*Citrus limon*
9	'Lisbon'	*Citrus limon*
10	'Adamopoulou'	*Citrus limon*
11	'Ermioni'	*Citrus limon*
12	'Karistino'	*Citrus limon*
13	'Lekka'	*Citrus limon*
14	'Maglino Xylokastrou'	*Citrus limon*
15	'Nova'	*Citrus reticulata* (clementine x tangelo)
16	'Page'	*Citrus reticulata* (clementine x tangelo)
17	'Marisol'	*Citrus reticulata*
18	Citron	*Citrus medica*
19	'Common mandarin'	*Citrus reticulata*
20	'Clementine Sra-63'	*Citrus reticulata*
21	'Minneola'	*Citrus reticulata x Citrus paradisi x Citrus grandis*
22	'Fisher'	*Citrus sinensis*
23	'Moro'	*Citrus sinensis*
24	'Navel Late'	*Citrus sinensis*
25	'New Hall'	*Citrus sinensis*
26	'Salustiana'	*Citrus sinensis*
27	'Manthopoulos'	*Citrus sinensis*
28	'Valencia Olinda'	*Citrus sinensis*
29	'Poros'	*Citrus sinensis*
30	'Argos'	*Citrus sinensis*
31	'Artas'	*Citrus sinensis*
32	'W. Navel'	*Citrus sinensis*
33	'Gouritsis'	*Citrus sinensis*
34	'Volkameriana'	*Citrus limon x Citrus medica*
35	'Lane Late'	*Citrus sinensis*
36	Sour orange	*Citrus aurantium*

Genomic DNA was extracted as previously described by Doyle and Doyle (1987) using the new season leaves. DNA concentration and quality was calculated spectrophotometrically and confirmed with (1%) agarose electrophoresis.

RAPD and ISSR analyses

More than 30 RAPD primers (Invitrogen) were initially tested in preliminary experiments and nine of them resulted to unambiguous polymorphic products among genotypes. For the ISSR analysis seven primers were tested and five were selected for the final reactions (Tab. 2).

Tab. 2. List of the primers used in RAPD and ISSR analyses

	Primer	Sequence (5'-3')
RAPD		
	RI-3	GTCCGTCAAC
	RAPD-1	TCCGCAACCA
	RAPD-5	AACGCGCAAC
	RAPD-14	ACGCTCCATC
	OPB-1	GTTTCGCTCC
	OPB-11	GTAGACCCGT
	OPG-5	CTGAGACGGA
	OPH-9	TGTAGCTGGG
	OPAH-17	CAGTGGGGAG
ISSR		
	825	(AC)8T
	842	(GA)8YG
	850	(GT)8YC
	856	(AC)8YA
	861	(ACC)6

The RAPD and ISSR protocols followed Despotaki *et al*. (2011). The amplified products were resolved on 1.5% and 2% agarose gels respectively, buffered with 1x TAE and stained with EtBr.

Data analysis

Reproducible DNA fragments were scored as present (1)/absent (0) for each reaction and were assembled in a binary data matrix table. Genetic similarities were calculated using the Dice's coefficient and an UPGMA dendrogram was constructed. Mantel test was used to compute the cophenetic correlation (999 permutations). All analyses were performed using the NTSYS-PC 2.01 software (Rohlf, 2000). For bootstrap analysis the FreeTree pro-

gram was employed and the dendrogram was displayed with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The level of polymorphism and informativeness were estimated following procedures previously described by Belaj *et al*. (2003). Genotypic variations were assessed across various populations by means of analysis of molecular variance (AMOVA) using GenALEx 6 (Peakall and Smouse, 2006). The significance of the resulting variance components and inter-population genetic distances were tested using 999 random permutations.

Results

Primers were carefully chosen after thorough initial screening and those that amplified fairly robust and polymorphic loci were selected. Out of 30 RAPD primers inquired, nine were employed for the full scale experiments. In addition, after a selection scheme, five out of seven ISSR primers were also utilized. The most prolific RAPD primer was OPB-11 (28 loci), while the least productive were OPG-5 and RAPD-1 (both amplified 15 loci). Respectively for the ISSR analysis, 24 loci were amplified by primer 856 and 11 loci were detected when using primer 861.

Both marker techniques proved to be highly effective in discriminating the 36 genotypes analysed. The total amount of amplified loci was 171 (RAPD) and 91 (ISSR), while the majority of bands was polymorphic amongst genotypes; approximately 90% for both techniques (Tab. 3).

Tab. 3. The level of polymorphism and comparison of the discriminating capacity, of RAPD and ISSR markers in 36 citrus genotypes

Index & Abbreviations		RAPD	ISSR
Number of assay units	U	9	5
Number of non-polymorphic bands	n_{np}	17	9
Number of polymorphic bands	n_p	154	82
Average number of polymorphic bands/assay unit	n_p/U	17.11	16.40
Number of loci	L	171	91
Number of loci/assay unit	n_u	19	18.2
Number of Banding pattern	T_p	199	119
Number of patterns/assay unit	I	22.11	23.80
Average Confusion Probability	C	0.07	0.05
Average discriminating power	D	0.93	0.95
Average limit of discriminating power	D_L	0.90	0.92
Effective number of patterns/assay unit	P	10.06	12.71

On average RAPD detected more polymorphic bands per assay (17.11 over 16.4 for ISSR). However, ISSR were proven to be more informative; 23.80 (banding patterns per assay) over 22.11, and 12.71 (effective patterns per assay) against 10.06, respectively. The effective number of patterns specifies the population size, where genotypes can be distinguished in one assay (Belaj *et al*., 2003). In the present study it was shown that one RAPD primer could discriminate almost ten citrus genotypes, while almost 13 genotypes could be distinguished using just one ISSR primer set. Nonetheless, both techniques were highly informative, since they both had an average discrimination power higher than 0.9.

Comparison of informativeness exhibited by the marker systems used is presented on Tab. 4.

Tab. 4. Comparison of infromativeness obtained with RAPD and ISSR markers in 36 citrus genotypes

Index & Abbreviations		RAPD	ISSR
Average number of alleles per locus	n_{av}	2	2
Expected heterozygosity of the polymorphic loci	H_{ep}	0.41	0.47
Fraction of polymorphic loci	β	0.90	0.90
Expected heterozygosity	H_e	0.37	0.42
Effective number of alleles per locus	n_e	1.72	1.76
Total number of effective alleles	N_e	264.88	144.32
Assay efficiency index	A_i	29.43	28.87
Effective multiplex ratio	E	14.63	12.56
Marker index	MI	6.08	6.01

Expected heterozygosity of the polymorphic loci ranged from 0.37 (for RAPD) to 0.42 (for ISSR). The effective number of alleles per locus was 1.72 for RAPD, while ISSR showed slightly higher values (1.76). In general, RAPD markers were equally polymorphic as ISSR, since the fraction of polymorphic loci was 0.90 for both techniques. The highest assay efficiency and marker index values were observed for RAPD (29.43 and 6.08) and the lowest for ISSR (28.87 and 6.01, respectively). The relatively high value of the marker index for both techniques is the result of a high multiplex ratio component (14.63 for RAPD and 12.56 for ISSR). High values of assay efficiency and marker indexes also highlight the distinctive nature of these markers; this was due to the simultaneous detection of several polymorphic markers per single reaction.

In general, ISSR data gave slightly lower similarity values than RAPD (data not shown). The Mantel matrix correspondence test was used to compare the accuracy of the fit of each similarity matrix. Both techniques had well-fitted cluster analysis to their corresponding similarity matrix; 0.94 for RAPD and 0.89 for ISSR. Likewise, the correlation coefficients were statistically significant for both marker systems. Similarity matrices among the RAPD and ISSR were fairly correlated (0.87). Moreover, correlation between their corresponding dendrograms was

even higher (0.93). Both markers showed a high degree of similarity in topologies (data not shown), though with minor differences in the positioning of some genotypes at the main groups. Clustering reflected depending relationships among most of the accessions, upon their species background.

Cluster analysis was carried out on the two sets of marker profiling data and a consensus phenogram was constructed (Fig. 1).

Combined analysis broadly grouped the 36 accessions into three distinct clusters showing relation on the basis of species genotype. The bootstrap values were relatively high within species and in a lesser extent between species. Species were grouped into three clusters. Cluster I had a bootstrap value of 44%. Within this cluster, all *C. reticulata* and *C. sinensis* L. accessions (17 in total) were grouped together with *C. reticulata* 'Minneola' as an outgroup. The sub-clustering of sweet orange had a strong bootstrap

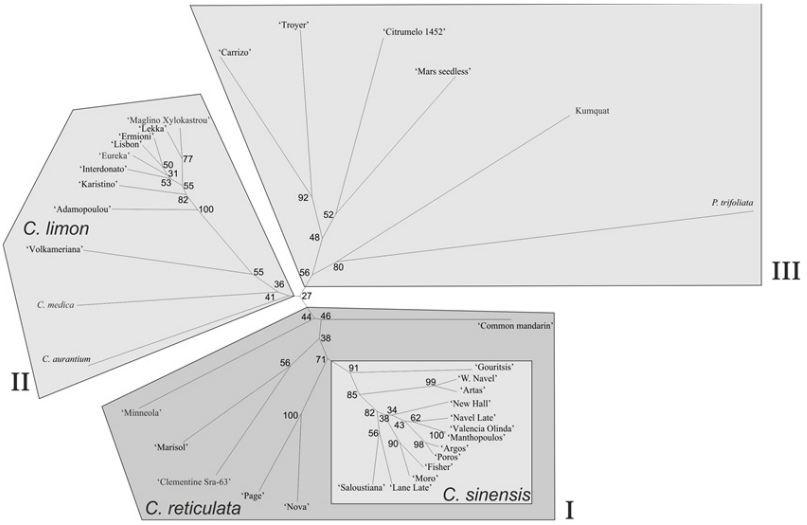

Fig. 1. RAPD and ISSR consensus phenogram generated after bootstrap analysis. Bootstrap support values, based on 100 replications are presented on nodes. Clusters are indicated in roman numerals. High affinity among the *C. sinensis* and *C. reticulata* species is depicted

of 91%. Within the subgroup high affinity was observed (bootstrap values from 34% up to 100%) as depicted from the genetic distances among sweet oranges. Cultivars 'Valencia Olinda' and 'Manthopoulos' were almost identical (100% bootstrap value), while high affinity was observed among the cultivars 'Artas' and 'Washington Navel' (99%), 'Poros' and 'Argos' (98%), 'Fisher' and 'Moro' (90%) and in a lesser extent among 'Lane Late' and 'Saloustiana' (56%). Furthermore, within this cluster the Tangelos x Clementine hybrids 'Nova' and 'Page' were grouped together (100%) with 71% bootstrap separation from *C. sinensis* samples. Clementine clone 'sra-63' and 'Marisol' were also clustered together (56%) although having low affinity to the mandarin core (38%). The Greek 'Common mandarin' had little similarity to the other mandarin accessions reflected by moderate bootstrap support (46%). In general, the mandarin group did not have strong bootstrap support like sweet oranges, perhaps due to the large proportion of interspecies hybrids in this data set.

Cluster II comprised all *C. limon* L., as well as, *C. aurantium* L., *C. medica* and a 'Volkameriana' lemon accession. Within the lemon cluster bootstrap values from 31% to 100% were detected, supporting clear differentiation of lemons from related *Citrus* spp. As in the sweet orange cluster, lemon varieties revealed clear discrimination. Interestingly, the Greek cultivars 'Adamopoulou', 'Karistino', support 'Lekka' and 'Maglino Xylokastrou' are disjointed from the core of the lemon group comprised of 'Interdonato', 'Eureka', 'Lisbon' and 'Ermioni' revealing genetic differences from foreign vegetative material.

Group III contained all *Poncirus* hybrids and a *C. paradisi* accession ('Mars seedless'). Kumquat which is a *Fortunella margarita x Fortunella Japonica* hybrid had low relatedness to *Citrus* spp clusters; *P. trifoliate* shared the lowest affinity to all accessions supporting of monophyl for the trifoliate species.

Integrated Principal Coordinate Analysis (PCOORDA) resulting from RAPD and ISSR combined data matrices showed a clear separation of the three *Citrus* species (Fig. 2).

The first two components, which accounted for 37.31% of the total eigenvalues, were used to construct a plot (dimention-1 and dimention-2 represented respectively 11.59% and 8.82% of the total variation). Genotypes of Group I, were clustered separately from Group II in the first axis, while *C. medica*, *C. aurantium* and the 'Volkameriana' accession remained unrelated, and were plotted in the middle position, in respect to the other two clusters. PCOORDA depicts relationships among *C. reticulata* and *C. sinensis* species more comprehensively than the phenogram; since a clear separation is evident (discrimination occurs in two dimensions). As also observed in the

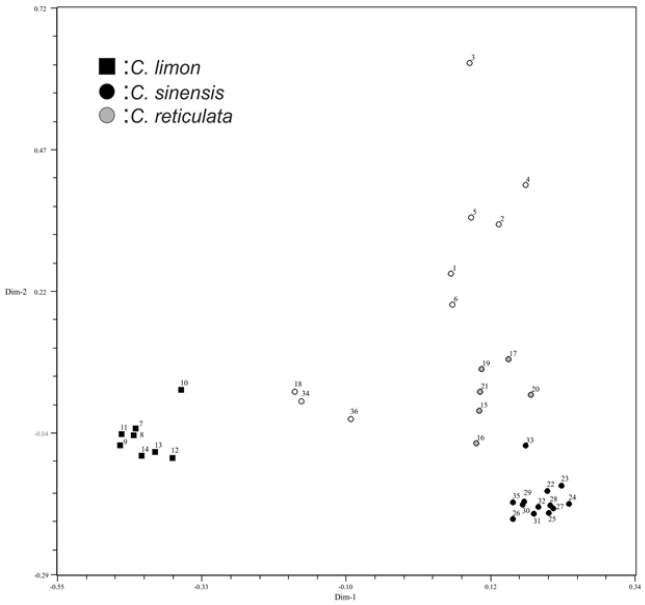

Fig. 2. Principal Coordinate Analysis based on integrated RAPD and ISSR data from *Citrus* spp. as mentioned in Tab. 1

unrooted tree, mandarins had a greater variability within as compared to the sweet oranges group. Group III was also separated (as shown in dimention-2) although placed in proximity to the Group I, since some of the encompassed hybrids have a partial *C. sinensis* origin. Especially for *P. trifoliate* it was observed that was the most distant accession of all and remained unrelated.

Analysis of molecular variance revealed that a high proportion (47%) of the total genetic diversity was present within the three types (Tab. 5).

Tab. 5. Results of AMOVA analysis of the three *Citrus* species

Source of variation	*df*	Sum of squares	% variance	Φst	p-value
Among populations	2	355.302	52.69	0.527	0.001
Within populations	23	410.544	47.31		

The highest variability was recorded for the *C. sinensis* group (SS=207.8), followed by *C. reticulata* (SS=124.4) and *C. limon* (SS=78.4). AMOVA also revealed that the genetic distance between clusters was significant (Fst=0.527, p=0.001). Fst values suggested the presence of divergence between *Citrus* types (Tab. 6).

C. limon group was clearly distinguished from *C. sinensis* (Fst=0.628, p=0.001) and *C. reticulata* (Fst=0.556, p=0.001). The closest genetic proximity was found among the *C. sinensis* and *C. reticulata* species (Fst=0.304, p=0.001) as it was observed by genetic distances, corresponding phenogram and the PCA plot.

Tab. 6. Species differentiation based on RAPD and ISSR analyses and 999 permutations (lower diagonal matrix, p values, Φst values) and level of confidence (upper diagonal matrix)

	C. limon	*C. reticulata*	*C. sinensis*
C. limon	-	0.003	0.001
C. reticulata	0.556	-	0.001
C. sinensis	0.628	0.304	-

Discussion

Phylogenetic relations for certain citrus taxa have been fairly controversial in the past; nevertheless, molecular marker technologies elucidate some of these ties. The difficulty in classifying *Citrus* spp. is mainly due to repeated interspecies crosspollination and adventitious nucellar embryony that stabilizes and perpetuates hybrid taxa (Scora, 1975). Frequent hybridization, introgression, bud mutations and polyploidy have created numerous hybrids and mutant varieties throughout the citrus belt of the world. An additional difficulty in *Citrus* taxonomy is incongruity on what degree of genetic divergence validates species status, and whether apparent hybrids should be considered at species status.

Those who followed the biological species concept, do not coincide that crossbreeds are true species (Swingle and Reece, 1967; Mabberley, 2008), while those that concur to the horticultural concept (Tanaka 1977), regard any notable variants of *Citrus* as a true species. While both the above approaches have their own arguments, a proper circumscription and citrus classification is still awaited (Kumar *et al*., 2013).

Understanding taxonomy, phylogenetic relationships, and genetic variability in *Citrus* spp., is a necessity towards

determining genetic relationships, characterizing germplasm, controlling genetic erosion, designing sampling strategies or core collections, establishing breeding programs, and the registration of new cultivars (Herrero *et al*., 1996). In this study, we adopted an integrated approach using the resolving power of RAPD and ISSR markers in order to verify possible relationships amongst the *Citrus* species and cultivars and to explore the genetic composition of the Greek citrus reservoir.

High levels of polymorphism were recorded for both marker systems and elevated values of the effective number of patterns per assay units were recorded. This is vital for the controlling of germplasm collections where numerous cultivars need to be precisely characterized and identified (Belaj *et al*., 2003). Mantel test revealed the high association between the two techniques. Biswas *et al*. (2010) utilised RAPD, ISSR, IRAP and REMAP markers for the genetic analysis of *Citrus* spp. and also recorded that the highest correlation between the four different markers was among RAPD and ISSR. High intra- and inter-genetic affinities were verified between *Citrus* spp. The construction of an integrated phenogram and a PCOORDA plot clustered all genotypes into distinct groups.

Within the *Citrus* genus, such analyses clearly differentiated species with sub-elliptic fruit (*C. limon*, and *C. medica*) from those with flattened or sub-spherical shaped fruit at the ends (*C. sinensis* and *C. reticulata*). Furthermore, *C. sinensis* and *C. reticulata* species were clustered together in the UPGMA tree but were clearly discrete in the plot. Clustering association of sweet oranges and mandarins has been recorded before (Gulsen and Roose, 2001; Shahsavar *et al*., 2007; Marak and Laskar, 2010). It has been proposed and adopted, supported by morphological, phytochemical and molecular data that sweet orange derived from a pummelo and mandarin cross (Scora, 1975; Barrett and Rhodes, 1976; Nicolosi *et al*., 2000). In addition, Barkley *et al*. (2006) suggested that sweet orange inherited the majority of its genetic makeup from mandarin and only a small proportion from pummelo. Sweet orange is the most broadly grown and consumed citrus fruit, but still surrounded by a veil of a mystery. In the present study sweet oranges did not cluster according to their type or origin. It seems that morphological traits, responsible for cultivar discrimination in sweet oranges, have a narrow genetic base or do not fully correspond to the genetic variability. Possibly most of the current orange varieties are selections from earlier established cultivars and developed deviant morphological and/or biochemical properties. This could be the case for the Greek cultivar 'Manthopoulos', evidently originated from a Valencia type cultivar and also the cultivar 'Artas', visibly affiliated to 'W. Navel'. On the contrary, the only cultivar that clearly differentiates in the orange cluster is 'Gouritsis' cultivar, something that proves that its morphological characteristics correspond to genetic divergence.

This narrow genetic base for oranges was also observed by other scientists. Barrett and Rhodes (1976) found low intraspecific phenotypic affinity in this *Citrus* type when correlated to other citruses considered as facultative apomicts (sour orange, grapefruit and lemon). Furthermore when SSR moleculars markers were employed no major differences were detected among orange cultivars (Luro *et al*., 1995), hence it was pointed out (considering the morphological – genetic incongruity) that sweet orange varieties are monophyletic, resulting from a single ancestor through mutation and selection of desirable clones (Luro *et al*., 1995; Fang and Roose, 1997). A possible explanation for some of the uniformity of *C. sinensis* is that some of the early populations may have passed through severe bottlenecks resulting to genetic reconstruction. If so, the low effective size of these populations would have increased genetic drift and have lost allelic diversity; thus narrowing down genetic variability. This would have resulted in a new random allelic composition of populations, independent of their geographical location (Yang *et al*., 2010).

In the current study it was determined (based on the similarity matrix, the bootstrap values and the PCOORDA plot) that sweet oranges share the highest intragenetic affinity among the *Citrus* taxa. If the sweet orange was derived from one or more backcrosses to the mandarin, and due to vegetative propagation and intervarietal diversification without sexual recombination, *C. sinensis*, could display very low or no intraspecific molecular polymorphism. This would explain why sweet oranges had the lowest observed heterozygosity of all the *Citrus* accessions. Barkley *et al*. (2006) and Uzun *et al*. (2009) also reported the close molecular relationship amongst sweet oranges.

The next major group was the mandarins, which did not form a well-defined clade, as sweet oranges did. Federici *et al*. (1998) also established that *C. reticulata* accessions do not form a unified clade when hybrid accessions are included. As a result, the 'Nova' and 'Page' cultivars that come from 'Clementine' x 'Tangelo' hybrids were not directly correlated to the 'Clementine SRA-63' and 'Marisol' subcluster. Furthermore, the Greek 'Common mandarin' was linked as an outgroup of the orange-mandarin cluster, revealing surprisingly low genetic affinity and stressing the tremendous genetic intra-variability of mandarins (Uzun *et al*., 2009). It has been suggested that mandarins are the most phenotypically heterogeneous group among apomictic types of *Citrus* spp.; both monoembryonic and polyembryonic clones exist, as do self-fertile and self-incompatible types (Swingle and Reece, 1967).

C. reticulata is one of the three citrus types (alongside to *C. medica* and *C. grandis*) that Barret and Rhodes (1976) proposed as true species. Coletta Filho *et al*. (1998) used RAPD markers to evaluate genetic similarity among mandarin accessions and recorded high genetic similarity between them. However, genetic variability in *Citrus* is associated to the high number of taxonomic units (species

and hybrids), as well as to common bud mutations and limb sports. Contrasting the sweet orange group, where practically all cultivars arose by somatic mutations, genetic variation in *C. reticulata* is associated with hybridization among species and intraspecific hybrids (Coletta Filho *et al*., 1998). Genetic compatibility and disparity among mandarins challenge the taxonomy of the group. Tanaka (1977) classified the mandarins into 36 species, whereas Swingle and Reece (1967) classified them as one species. Barkley *et al*. (2006) found that the mandarins were the most polymorphic among the ancestral species. Moreover, high phenotypic and genetic variation reflects the long history of cultivation during which natural hybridization and mutations created deviation within the mandarin group. Therefore it was proposed that mandarins are really composed of many different taxonomic groups that all belong to a single biological species (Coletta Filho *et al*., 1998).

Lemons were the third cluster according to analyses and relatively high intraspecific affinity was detected. Barrett and Rhodes (1976) also proposed that lemons have quite high genetic uniformity. In addition, they conjecture that a specific biotype was selected and minor mutational variations contributed to lemon heterogeneity. This was supported by molecular markers (Nicolosi *et al*., 2000); however phylogenetic studies concluded that lemons have possibly polyphyletic origin (Fang *et al*. 1998). On the other hand, Herrero *et al*. (1996) established that limes and lemons show a high percent of heterozygosity due to sexual hybridization, which seems to be the main cause of variability. Adding up, it was suggested that this unique apomictic citrus type was a complex *C. medica x C. aurantium hybrid* (with sour orange being the maternal parent), but share a larger proportion of citron genes (Nicolosi *et al*., 2000). This is in agreement with the result of the present study since both *C. medica* and *C. aurantium* had high affinity to the lemon cluster.

Within the subcluster, lemon cultivars were organised according to their origin, with moderate bootstrap support. It is likely that all lemons with high similarity in the main cluster are clonally derived from a single ancestor. The major commercially important cultivars 'Lisbon' and 'Eureka' did not shape separate clusters. Most of these cultivars originated as selections by growers, allegedly on or after other cultivars of the same form. This was strong evidence that differences among these accessions originated by mutation, not by sexual recombination. Also, one possible explanation for this incongruity is that molecular markers might evolve too quickly and unevenly into recently diverged genotypes (Gulsen and Roose, 2001).

The rest of the lemons were easily distinguishable, suggesting that they have different origins. Lemons having independent origins were 'Interdonato', and the Greek cultivars 'Lekka', 'Maglino Xylokastrou', 'Karistino' and 'Adamopoulou'. Deng *et al*. (1995) also established 'Interdonato' as quite distinct lemon cultivar. The overall impression of evolution and variability in lemons depicted from this study is that most lemon cultivars have been obtained by mutation. However, there are additional lemon cultivars with more diverse origins, that could possibly arose by selfing and/or independent hybridization of citron with other citrus taxa (Gulsen and Roose, 2001). Especially the Greek lemon cultivars due to their genetic variability in relation to established varieties, could offer a valuable reserve for breeders concerned in improving lemon traits.

As mentioned, the citron, the sour orange and the 'Volkameriana' lemon accessions were clustered as an outgroup to the lemon core. *C. medica* is one of the core citrus species and has constantly contributed as male parent in the origin of *Citrus* hybrids (Federici *et al*., 1998; Nicolosi *et al*., 2000), while the sour oranges are thought to be natural hybrids of a mandarin and a pummelo cross (Scora, 1975; Barrett and Rhodes, 1976). This same clustering pattern has also been observed in previous studies (Federici *et al*., 1998; Nicolosi *et al*., 2000). Lemons are thought to be natural hybrids of a citron and a lime (Scora, 1975; Barrett and Rhodes, 1976), or a hybrid between citron and sour orange (Gulsen and Roose, 2001). 'Volkameriana' is correspondingly a hybrid and has morphological traits resembling lemons; its flower buds, outer surface of petals and flushes are purple (Shahsavar *et al*., 2007). Furthermore, Golein *et al*. (2012) proved affinity among sour orange and 'Volkameriana' lemon and confirmed sour orange as a maternal parent of 'Volkameriana' lemon (Nicolosi *et al*., 2000; Barkley *et al*., 2006).

The last group comprised of two Citranges (*P. trifoliata x C. sinensis*) accessions (sharing more than 90% bootstrap support), a Citrumelo (*P. trifoliata x C. paradisi*) and *C. paradisi* 'Mars seedless' (having a moderate bootstrap value of 52%). Citranges are used as rootstocks and are intergenic hybrids of *P. trifoliata* and orange. Based on their genetic distances, the phenogram and their plot topology it was concluded that the major portion in their genomes must come from *P. trifoliata*. This is in accordance to its morphological traits since Citranges are trifoliate *Citrus* spp. 'Carrizo' and 'Troyer' are considered as the same clone derivatives from the CPB 4-5019 seedling produced by a 'W. Navel' *x P. trifoliata* crossing (Savage and Gardner, 1965).

In the current study the only species that had little affinity to the *Citrus* spp. were the 'Kumquat' hybrid (*F. margarita x F. japonica*) and *P. trifoliate*. Great inconsistencies still exist regarding the genetic relationship among *Citrus* and *Fortunella* species. Herrero *et al*. (1996) using RFLPs observed that *Fortunella* spp. can nest within *Citrus clusters* eventhough 'Kumquat' is well-differentiated from *Citrus* on the basis of detailed morphological studies; *Fortunella* spp. share similar morphological characteristics with *Citrus* (floral and fruit) but they differ in quantitative characters (Swingle and Reece 1967). Pang *et al*. (2003) proposed that *Fortunella* was the most primitive genus, in contrast to *Citrus*. However, molecular markers showed that it has relative proximity to *Citrus* (Federici *et al*.,

1998; Nicolosi *et al*., 2000; Abkenar *et al*., 2004), which is consistent with the previous cpDNA analysis (Green *et al*., 1986). Apparently, there has not been the same level of divergence at the molecular level as it is phenotypically. Conversely, Araújo *et al*. (2003) illustrated that *Fortunella* was diverged from *Citrus* spp. The differences between cpDNA and other analyses can possibly be explained by the proposal that *Fortunella* is of hybrid origin and *Citrus* might be a putative paternal parent.

Poncirus was believed to be morphologically distant from *Citrus*, presenting deciduous trifoliolate leaves and its flowering time not being coincident with that of *Citrus* (Swingle and Reece, 1967). It was proposed by Huang (1997) that *Poncirus* was perhaps a *Citrus* species, and then evolved into *Poncirus* when spreading from south to north. Morphological characters (Zhou, 1991) and biochemical records (Herrero *et al*., 1996) suggested that *Poncirus* was the first deviant species from the *Fortunella* and *Citrus* cluster. Other studies employing molecular markers like RFLP (Green *et al*., 1986; Yamamoto *et al*., 1993), SSR (Pang *et al*., 2003), RAPD and SCAR data (Federici *et al*., 1998; Nicolosi *et al*., 2000) also displayed that *Poncirus* had little affinity with *Citrus*, as supported by the separation of this genus from the other genera in this study.

Conclusions

In conclusion, the present study highpoints the equal efficacy of RAPD and ISSR markers for the exploration of the Greek citrus germplasm diversity and its respective affinity to foreign species and cultivars. Assessment of genetic relationships based solely on morphological traits has serious restrictions, particularly in a complex genus like *Citrus*, whose taxonomy is controversial due to repeated hybridization, apomixis, and mutations. High inter- and intra- genetic variability was recorded among *Citrus* spp. and related genera; especially in Greek originated lemon, mandarin and, at a lesser extent, orange cultivars. It seems that the assessment of diversity among these genotypes would be of profound importance for the selection of clones prioritized for conservation and the proper utilization as genetic resources in future breeding programs.

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