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Genetic Relatedness among Cultivars of the Greek Plum Germplasm

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Abstract

Genetic diversity of the Greek plum germplasm collection was investigated using a combined RAPD and ISSR molecular markers approach. Twenty-six genotypes held at NAGREF-Naoussa were analyzed, producing in total 150 loci, of which 116 were polymorphic. Both techniques were highly informative and had a discrimination power greater than 0.9. RAPD and ISSR dendrograms were fairly correlated. The accessions were clustered according to ploidy and species. All *Prunus domestica* genotypes were grouped together and showed greater similarity to *P. insititia* and *P. cerasifera* genotypes compared to *P. salicina*, which was found genetically diverged. Bayesian structural analysis revealed significant admixture among genotypes. Greek varieties *P. domestica* 'Goulina' and 'Asvestochoriou' exhibited a distinctive genetic background, differentiating them from foreign varieties. This feature could make them attractive for breeding programs, since they can increase genetic diversity.

Keywords: AMOVA, ISSR, *Prunus* spp., RAPD, STRUCTURE

Introduction

Plum species belong to genus *Prunus* L. (Rosaceae), and are naturally distributed in the temperate regions of the Northern Hemisphere (Mabberley, 2008). This taxa is a diverse group of plants with various botanical species that have been cultivated for 2000-4000 years (Banegal, 1954). The most economically important plum species are generally classified into two groups: the European (*Prunus domestica* L.) and the Japanese (*Prunus salicina* Lindl.) plum.

It has been proposed that *P. domestica* originated in Southern Europe or Western Asia around the Caucasus Mountains and the Caspian Sea (Cullinan, 1937). However, it is also widespread in the Balkans and the Mediterranean countries, including Greece. *Prunus domestica* has been cultivated in Europe for at least 2000 years, but and up to now no distinctly wild form is documented (Westwood, 1993). Thus the evolution of the European plum remains a controversial matter. Crane and Lawrence (1952) suggested that *P. domestica*, a hexaploid (2n = 6x = 48), originated through an interspecific cross between the diploid (2n = 2x = 16) *P. cerasifera* Ehrh. (Myrobalan plum) and the tetraploid (2n = 4x = 32) *P. spinosa* L., either through chromosome doubling of the hybrid triploid or through the action of unreduced parental gametes. On the contrary, Zohary (1992) proposed that *P. domestica* was an autopolyploid derived from *P. cerasifera* – rather than an allopolyploid, while Eryomine (1991) suggested that a number of species, such as *P. microcarpa* C.A. Mey., *P. salicina*, *P. armeniaca* L., and *P. persica* L., could have participated in the lineage of *P. domestica*.

Prunus salicina, a diploid (2n = 2x = 16) species domesticated in China from ancient times (its wild forms are believed to thrive in the regions of Shensi and Kansu), was introduced in Japan 200-400 years ago (Ramming and Cociu, 1990). These plums were initially improved in Japan and later, to a much greater extent, in the United States (Okie and Ramming, 1999) where subsequent breeding resulted in larger fruit cultivars (latter half of the 19th century).

Traditionally, classification within the genus *Prunus* was mostly based on fruit morphology and thus being debatable (Aradhya *et al*., 2004). Furthermore, phenotype is influenced by environmental factors, mainly as a result of the long generation time and large size of the trees. Therefore, precise characterization for *Prunus* spp. and germplasm evaluation is a prerequisite, in order to develop effective conservation and breeding strategies. In order to indisputably explore the genetic diversity of these numerous plum varieties and their interrelationships, molecular marker technologies can prove valuable.

In the present study, two different molecular marker approaches (RAPD and ISSR) were employed in order to evaluate the degree of genetic diversity of the Greek National Plum Collection held at NAGREF-Naoussa, and to further analyze the genetic structure of three related plum species: *P. domestica*, *P. salicina* and *P. insititia* (L.) C.K. Schneid. Additionally, the elucidation capability and effectiveness of RAPD and ISSR markers on genetic relationships among plum genotypes was examined.

Materials and methods

Plant material and DNA extraction

Twenty-two prune varieties (*P. domestica* and *P. salinica*), three *P. insititia* and one *P. cerasifera* accessions held at NAGREF-Naoussa, were included in the present study (Tab. 1).

Tab. 1. Plant material utilized for RAPD and ISSR analyses, including the number assigned, cultivar or common name, species and assignment of each variety to clusters based on Bayesian simulations (K = 2, K = 5)

Nº	Cultivar or common name	Species	Assignment to clusters	
			K = 2	K = 5
1	'Goulina'	*Prunus domestica*	1	1
2	'Asvestochoriou'	*Prunus domestica*	1	1
3	'Bluefree'	*Prunus domestica*	1	1
4	'Black Beauty'	*Prunus salicina*	2	Admixed
5	'Friar'	*Prunus salicina*	2	2
6	'Calita'	*Prunus salicina*	Admixed	Admixed
7	'Stanley'	*Prunus domestica*	1	1
8	'Angeleno'	*Prunus salinica*	2	Admixed
9	'Anna Späth' Oradea	*Prunus domestica*	1	Admixed
10	'Anna Späth' Pitesti	*Prunus domestica*	1	1
11	'President'	*Prunus domestica*	1	1
12	'Tuleu gras' bistro	*Prunus domestica*	1	1
13	'Kisnanai'	*Prunus domestica*	1	Admixed
14	'Tuleu dulce'	*Prunus domestica*	1	Admixed
15	'Koromilo roumanias'	*Prunus insititia*	1	3
16	'Feher besztercei'	*Prunus domestica*	1	1
17	'Kesley'	*Prunus salicina*	Admixed	Admixed
18	'11/11'	*Prunus insititia*	1	3
19	'Scoldus' SS	*Prunus domestica*	1	1
20	'Mirabelle de Nancy'	*Prunus domestica*	1	Admixed
21	'Gilej'	*Prunus domestica*	1	Admixed
22	'Daw dean'	*Prunus insititia*	1	Admixed
23	'Myrobalanos'	*Prunus cerasifera*	Admixed	Admixed
24	'Santa Rosa'	*Prunus salicina*	2	Admixed
25	'Black Diamond'	*Prunus salicina*	2	Admixed
26	'Black Gold'	*Prunus salicina*	Admixed	Admixed

Young leaves were collected, cleaned with moist paper towels and flash-frozen in liquid nitrogen. The tissue was stored at -80 °C. Genomic DNA was extracted as previously described by Doyle and Doyle (1987). DNA concentration and quality was calculated spectrophotometrically (Unicam Helios; OD260nm/OD280nm ratios were above 1.8) and confirmed with 1% agarose electrophoresis using standard λ-phage molecular weights. All samples were diluted at a concentration of 5 ng/μL by adding T.E., for use in PCR reactions, and stored at -20oC.

RAPD and ISSR reactions

More than 30 RAPD primers (Invitrogen) were initially tested in preliminary experiments of which ten resulted in unambiguous polymorphic products among genotypes. For the ISSR analysis ten primers were tested and five were selected (Tab. 2).

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

Primer	Sequence (5-3)	Primer	Sequence (5-3)
RAPD		ISSR	
OPB-1	GTAGACCCGT	818	$(CA)_8G$
OPB-11	GTTTCGCTCC	825	$(AC)_8T$
OPH-13	GACGCCACAC	844	$(CT)_8RC$
OPH-18	GAATCGGCCA	861	$(ACC)_6$
OPAH-17	CAGTGGGGAG	889	$DBD(AC)_7$
OPBD-7	GAGCTGGTCC		
OPA-9	GGGTAACGCC		
RAPD-3	AGAACCGAGG		
RAPD-5	TCCAACGGCT		
RAPD-20	TCCGGGTTTG		

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

The RAPD/ ISSR banding patterns were visualized and photographed using a Canon A630 and a UV table (Serva). Reproducible fragments were scored as present (1)/absent (0) for each reaction and were assembled in a binary data matrix table. Comparison of the discriminating capacity, level of polymorphism and informativeness were estimated following the procedure described by Belaj *et al*. (2003). Genetic similarities between taxa were calculated using the Dices coefficient and an UPGMA dendrogram was constructed. Mantel test was used to compute the cophenetic correlation, i.e., to test the goodness of fit of the cluster analysis to the similarity matrix and the goodness of fit of cluster analysis to the similarity matrices (999 permutations). All of the above analyses were performed using the NTSYS-PC 2.01 software (Rohlf, 2000). Bootstrap analysis was performed using the FreeTree program (Pavlicek *et al*., 1999) and displayed with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

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. Bayesian model-based clustering approach to identify the genetic structure in the plum germplasm was performed using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) based on the combined RAPD and ISSR data. The STRUCTURE algorithm was run using both the admixture model and the no admixture model, with 10 independent replicate runs per K value (number of clusters) ranging from 1 to 10. Each run involved a burning period of 20000 iterations, and a post burning simulation length of 20000. Validation of the most likely number of clusters K was performed using the Structure Harvester online application (http://taylor0.biology.ucla.edu/structureHarvester).

Results and discussion

Within *Prunus*, genetic relationships between *P. domestica*, *P. insititia* and *P. cerasifera*, are far from clarified. Still, delineation of the diverse taxa involved and information on their morphological and genetic diversity is an indispensable and crucial tool for their proper utilization. Extensive polyploidisation (Zohary, 1992; Woldring, 2000), hybridization, introgression (Stace, 1975; Woldring, 2000) and a long cultivation history (Zohary and Hopf, 1994; Woldring, 2000; Nielsen and Olrik, 2001) led the European *Prunus* taxa into a complex biological species concept with overlapping species relationships (Depypere *et al*., 2009). Furthermore, it resulted in a broad genetic variation and transitional states between and within the different taxa.

In the present study, we adopted an integrated method using the resolving power of molecular markers (RAPD and ISSRs) to validate genetic relationships among plum species and cultivars, and to determine the genetic diversity of the Greek plum germplasm. The final aim is to discover the genetic composition and the possible overlapping of the plum genepool which still remains vastly uncharted. Out of 30 RAPD and 10 ISSRs primers tested, 10 RAPD and 5 ISSRs primers were retained for their ability to produce unambiguous and polymorphic bands (Tab. 2). In total 106 RAPD markers were amplified of which 80 were polymorphic (Tab. 3).

Each primer amplified six to 16 loci (three to 12 polymorphic) resulting to an average of 10.6 bands (8 polymorphic) per primer. The most prolific RAPD primer was OPAH-17, amplifying 16 DNA bands, while the least productive was OPA-9, detecting only six loci. The most polymorphic RAPD markers were amplified by RAPD-20 and RAPD-5 (90%) and the least were produced by OPBD-7 and OPA-9 primers (50%). Respectively, for the ISSR analysis, in total 44 loci were detected (36 polymorphic). Each primer produced eight to 11 DNA bands (six to eight polymorphic), and on average 8.8 loci were amplified (7.2 were polymorphic). The most prolific ISSR primer was 889 that amplified 11 loci and the least were 818, 825 and 861, producing eight loci. Strikingly, all loci amplified by primers 818 and 825 were polymorphic. On the contrary, primer 889 revealed the lowest polymorphic proportion (54%).

Tab. 3. Levels of polymorphism and comparison of the discriminating capacity of RAPD and ISSR markers in 26 *Prunus* spp. genotypes

Index with their abbreviations		RAPD	ISSR
Number of assay units	U	10	5
Number of non-polymorphic bands	n_{np}	26	8
Number of polymorphic bands	n_p	80	36
Average number of polymorphic bands/assay unit	n_p/U	8	7.2
Number of loci	L	106	44
Number of loci/assay unit	n_u	10.6	8.8
Number of Banding pattern	T_p	147	76
Number of patterns/assay unit	I	14.7	15.2
Average Confusion Probability	C	0.07	0.09
Average discriminating power	D	0.93	0.91
Average limit of discriminating power	D_L	0.90	0.88
Effective number of patterns/assay unit	P	9.81	8.33

Generally, ISSR data revealed lower similarity values than RAPD. The Mantel matrix correspondence test was used to compare the goodness of the fit of each similarity matrix. Both techniques had well-fitted cluster analysis to their corresponding similarity matrix; 0.89 for RAPD and 0.80 for ISSR. Likewise, the correlation coefficients were statistically significant for both marker systems. RAPD and ISSR similarity matrices were moderately correlated (0.60). However, correlation between their corresponding dendrograms was significantly higher (0.79).

Both marker techniques proved to be highly effective in discriminating the 26 genotypes analysed, since the majority of bands were polymorphic amongst genotypes (more than 75%). On average, RAPD produced more polymorphic bands per assay (80 over 36 for ISSR; Tab. 3). However, ISSR were proven to be more prolific in terms of banding patterns per assay; 15.2 (ISSR) versus 14.70 (RAPD), on average. RAPD were also more informative since they produced 9.81 effective patterns per assay against 8.33 produced by ISSRs. Nonetheless, both techniques were highly informative, since they both had an average discrimination power higher than 0.91. The number and percent of polymorphic loci, diversity index, effective multiplex ratio, and marker index were higher for RAPD than for ISSR markers, similar to previous reports by Kumar *et al*. (2009) and Aran *et al*. (2012) for plum germplasm. In total, 150 loci were detected, which is comparable to results reported by Shimada *et al*. (1999), Casas *et al*. (1999) and Hend *et al*. (2009) using 20, 13 and 10 RAPD primers, respectively. The high discriminating

power of the primers used shows their efficiency and confirms the genetic diversity of the accessions studied, a result supported by the distance matrix among genotypes (0.50-0.97). Hend *et al*. (2009) obtained a 97.3% polymorphism and a range of genetic similarity between 0.18-0.80 among genotypes. However, Shimada *et al*. (1999) also studying genetic variation of plum cultivars using RAPD markers, reported a 24% polymorphism and a similarity index that ranged from 0.62 to 1. It is noteworthy that the Hend *et al*. (2009) study focused on indigenous plant material, while samples for the present study and that of Shimada focused on commercial genotypes. The higher diversity among indigenous genotypes compared to that of commercial ones, can be addressed by the genetic drift due to selection within commercial cultivars (Aran *et al*., 2012).

Both markers showed a high degree of similarity in topologies (data not shown), though with some minor differences in the positioning of some genotypes at the main groups. Clustering reflected relationships among most of the accessions, upon their species genotype/ploidy. Cluster analysis was carried out by combining the two sets of marker profiling data and a consensus phenogram was constructed (Fig. 1).

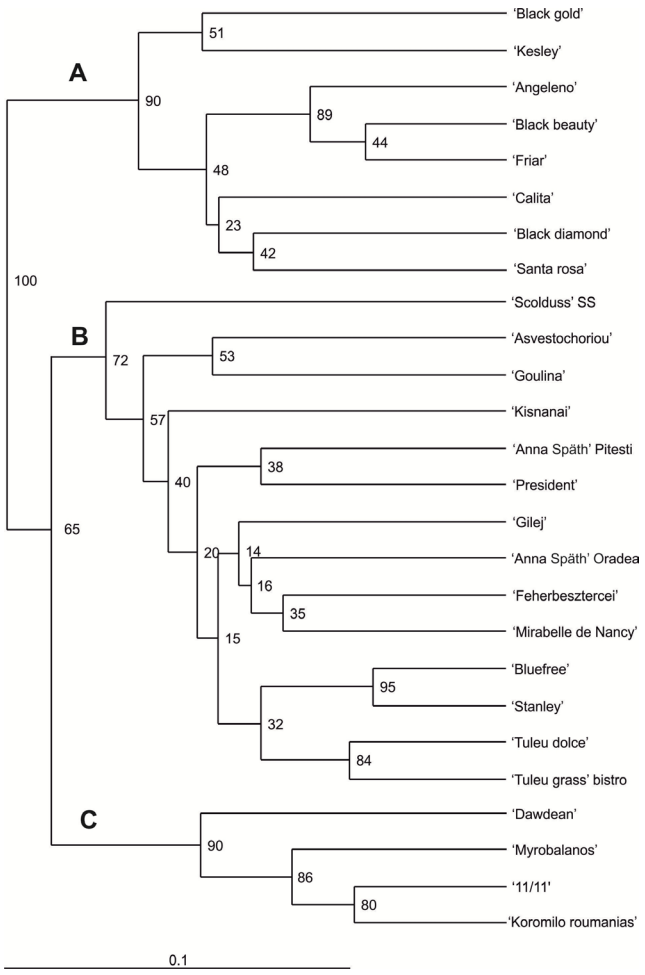

Fig. 1. RAPD and ISSR consensus dendrogram and bootstrap analysis. Bootstrap support values, based on 100 replications are presented on nodes

Combined RAPD and ISSR analysis broadly grouped the 26 accessions into three distinct clusters showing relation on the basis of species genotype. The bootstrap values were relatively high within and between species. Species were grouped into three clusters.

Cluster I had a bootstrap value of 90%. Within this cluster all *P. salicina* accessions (eight in total) were grouped together with cultivars 'Black Gold' and 'Kesley', which formed an outgroup. The sub-clustering of the Japanese plums had a strong bootstrap support since high affinity was observed (bootstrap values from 23% up to 89%) as supported from genetic distances among plums. High relatedness was depicted within the 'Angeleno', 'Black Beauty' and 'Friar' cultivars that formed a subgroup and within cultivars 'Calita', 'Black Diamond' and 'Santa Rosa' that grouped together and parted from the former subgroup by a 48% bootstrap value.

Group II contained all hexaploid *P. domestica* accessions with cultivar 'Scoldus' SS as an outgroup. Furthermore, within this cluster the Greek cultivars 'Asvestochoriou' and 'Goulina' were grouped together with 57% bootstrap separation from the hexaploid plum core. The other cultivars 'Anna Späth' (Oradea) and 'Anna Späth' (Pitesti) had little affinity among them and were clustered together with foreign cultivars. In particular, cultivar 'Anna Späth' (Pitesti) had moderate homology to 'President' cultivar (38% bootstrap support) and 'Anna Späth' (Oradea) was clustered together with cultivars 'Gilej', 'Feher besztercei' and 'Mirabelle de Nancy'. The highest homology between this group was detected among cultivars 'Bluefree' and 'Stanley' (95% bootstrap support) and 'Tuleu dulce' and 'Tuleu gras' bistro (84% bootstrap support). Finally, Cluster III comprised all *P. insititia* accessions and the *P. cerasifera* genotype. All genotypes were highly affiliated having bootstrap support more than 80%.

Analysis of molecular variance for the plum entries revealed that the highest proportion (64.05%) of the total genetic diversity was present within the three types. The highest variability was recorded for the *P. domestica* group (SS = 210.786), followed by *P. salicina* (SS =116.000) and *P. insititia* (SS =24.667). AMOVA also revealed that the genetic distance between clusters was significant (Fst = 0.359, p = 0.001). Fst values suggest the presence of divergence between *Prunus* types. *Prunus domestica* group is clearly distinguished from *P. salicina* (Fst = 0.358, p = 0.001) and *P. insititia* (Fst = 0.321, p = 0.001). The lowest genetic affinity was found among the *P. salicina* and *P. insititia* species (Fst = 0.411, p = 0.007) as it was also observed based on the genetic distances and the corresponding dendrogram.

The Bayesian analysis of genetic structure revealed interesting results. Based on the Evannos delta-K method, maximum ΔK values were obtained for K = 2 and K = 5 for the admixture model; while for the no-admixture

model maximum ΔK values were obtained only for K = 5 (data not shown). For this value (K = 2, no admixture model), *P. domestica* and *P. insititia* grouped together and *P. salicina* made up the second cluster (Fig. 2a).

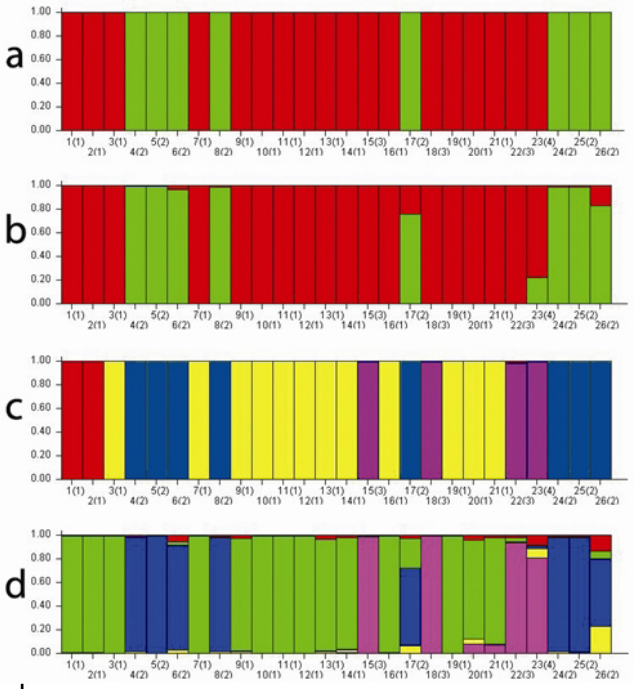

Fig. 2. Bar plot of the results from the Bayesian analysis on plum species genotypes indicated in Tab. 1. On the horizontal axis (1): *Prunus domestica*; (2): *Prunus salicina*; (3): *Prunus insititia* (4): *Prunus cerasifera*. a: K=2, no admixture model. b: K = 2, admixture model. c: K = 5, no admixture model. d: K=5, admixture model

However, in the admixture model, some *Prunus* spp. individuals were admixed between the two populations (Fig. 2b; Tab. 1). Considering a greater number of populations, *P. insititia* appeared as a new population (Fig. 2d) and *P. salicina* revealed more complex genetic structure showing the highest proportion of admixed individuals. Strikingly, for the no admixture model and for K = 5, the Greek cultivars 'Goulina' and 'Asvestochoriou' revealed a unique genetic profile, separating them from the other *P. domestica* accessions (Fig. 2c).

In both the UPGMA clustering and the Bayesian approach, classification was according to species genotype and ploidy level. European and Japanese plums formed different clusters and a third cluster contained the *P. insititia* and *P. cerasifera* genotypes. The latter species had a higher affinity to *P. domestica* rather than to *P. salicina*. The close relationship between *P. cerasifera*, *P. domestica* and *P. insititia* has been demonstrated based on morphological studies (Woldring, 2000; Nielsen and Olrik, 2001) and confirmed in several genetic analyses (Aradhya *et al*., 2004; Shaw and Small, 2004; Katayama and Uematsu, 2005). Furthermore, according to Woldring (2000), both morphological similarities between *P. insititia* and *P. domestica* and the incidence of a wide variety of forms with many overlapping structures suggest a close relationship. Overlapping characters between *P. insititia* and related taxa are probably due to the long cultivation history (Gleason, 1958).

In our analysis, as well as in the analysis of Depypere *et al*. (2009), both species displayed high levels of variation in spite of the relatively low sample sizes, and a tendency towards genetic differentiation. The observed intensive genetic diversity can be explained by a large and frequent recombination within the genus *Prunus* (Ortiz *et al*., 1997). Moreover, the high variability and recombination is favoured by the partial self-incompatibility present in plum genotypes and particularly by the capacity of *P. domestica* to intercross and form hybrids with other hexaploid *Prunus* species as mentioned by Ortiz *et al*. (1997). Still, the RAPD-ISSR UPGMA clustering and the Bayesian inference (at K=2) grouped accessions of *P. insititia* and *P. domestica* to nearby/same clusters. This concurs with other studies (Shaw and Small, 2004; Katayama and Uematsu, 2005), where *P. insititia* and *P. domestica* are shown to have similar cpDNA patterns. Nevertheless, in the AMOVA analysis, as well as, in the Bayesian inference when larger number of populations are simulated (i.e. K=5), individuals of *P. insititia* and *P. domestica* are clustered separately. Even though *P. insititia* is more primitive than *P. domestica* (Depypere *et al*., 2009), both taxa originated most probably from a single or very similar ancestral line, differentiated due to human selection and domestication (Woldring, 2000), which could explain their high genetic similarity. Furthermore, Stace (1975) and Zohary (1992) suggest a close relationship amongst the diploid *P. cerasifera* and the hexaploid *P. domestica*. Zohary (1992) also states that *P. domestica* plums, and predominantly *P. insititia*, are morphologically comparable to *P. cerasifera*; this is in accordance with the possibility of a polyploid *P. cerasifera* origin for *P. domestica* (Depypere *et al*., 2009). In a recent study based on cpDNA sequences, Reales *et al*. (2010) showed that hexaploid plums "group together with 100% posterior probability and 87% bootstrap support in a clade containing *P. cerasifera*, *P. divaricata* and *P. ursina*". Also, Horvath et al. (2011) confirmed this result since most *P. domestica* cpDNA haplotypes grouped together with *P. cerasifera* haplotypes. Therefore Reales *et al*. (2010) and Horvath *et al*. (2011) argued that *P. domestica* originated from *P. cerasifera*, at least in its maternal lineage.

In the current study *P. domestica* cultivars were grouped together, in some cases showing weak relationships. Bootstrap support values were rather low, fluctuating from 14% to 95%. This is also an effect of the complex relationships among the polyploid prunes. The only cultivars showing high affinity were 'Stanley' and 'Bluefree' (95% bootstrap support) followed by 'Tuleu dulce' and 'Tuleu gras' bistro (95% bootstrap support). 'Stanley' is acknowledged as the standard plum variety, released by the Cornell-Geneva

station in 1926 (Andersen *et al*., 2006). 'Bluefree' on the other hand is a more recent cultivar that has been released in order to extend the harvest plum season after 'Stanley'; therefore a common origin cannot be excluded.

Moderate affinity (35% bootstrap support) was recorded among two of the prune landraces 'Feher besztercei' and 'Mirabelle de Nancy'. Mirabelle plum is believed to have originated in Asia Minor and specifically in the district lying amongst Bosporus and the Black Sea, introduced in Europe through the crusades (the Lorraine region of France). Till present, this region produces almost 70% of the worlds production of Mirabelle plums. On the other hand, 'Feher besztercei' has a Hungarian origin; it has been reported, that before the 18th century only one outstanding and universal Besztercei plum was cultivated (Surányi, 2006). 'Anna Späth' Pitesti was clustered together with 'President', while 'Anna Späth' Oradea had affinity to the 'Feher besztercei'/'Mirabelle de Nancy' group. 'Anna Späth' is a Hungarian plum of unknown parentage, first raised near Kadoszbeg and discovered by Späth in 1874. Finally, 'President', a very large blue-black dual purpose plum, was developed at the start of the 20th century by the Rivers Nursery, in Hertfordshire, England.

The two Greek plum varieties, 'Goulina' and 'Asvestochoriou', were highly affiliated and somewhat diverged from the foreign cultivars. Especially in the Bayesian analysis, where no admixture was calculated, they were considered as a distinctive population. Finally, two plum accessions, 'Scoldus' SS (broadly used as a rootstock) and 'Kisnanai' (a traditional Hungarian cultivar/landrace also used as a rootstock) had little similarity to other *P. domestica* genotypes and remained as single entities.

Genetic relationship among the diploid Japanese plumes (*P. salicina*) seems to be less complicated compared to their hexaploid equivalents, since a possible common origin between cultivars is indicating and corroborates with the hypothesis of ploidy level. *Prunus salicina*, originated in China, was introduced into Japan no earlier than 1500 AD. Later on, many cultivars were introduced into California (around 1870) and afterwards to Europe (Liu *et al*., 2007). The foundations of the Japanese plum cultivation were set by Luther Burbank who introduced several Japanese plum accessions and hybridized them with American native species, resulting in a series of improved cultivars (Ryugo, 1988). This is documented by the limited diversity detected among the plum cultivars in relation to other tree fruit species. The major Japanese plum cultivars decent from a few genotypes produced by hybrization between *P. salicina*, *P. simonii* Carrière and native North American species. Todays breeding programs are utilizing a fraction of improved cultivars, thus narrowing even further the genetic base (Ilgin *et al*., 2009).

In the present study, *P. salicina* accessions were organized in three subclusters. The first was composed by 'Black Gold' and 'Kelsey', sharing high affinity, and clearly separated by the other two sublusters. 'Kelsey' is one of the oldest cultivars introduced from Japan, more than 100 years ago, while 'Black Gold' was bred and patented in 1980 by Superior Farms (Okie and Ramming, 1999). The close genetic affinity among them and the Bayesian analysis (at k=2 and k=5) indicates that the genetic composition of the latter has a proportion of the 'Kelsey' genotype. The second subgroup is composed by cultivars 'Angeleno', 'Black Beauty' and 'Friar' (the predominant plum in the industry, since it is very productive with large fruit and its black skin colour does not show bruises). 'Angeleno' and 'Black Beauty' have been both produced by a hybridization of 'Gariota' x 'Eldorado' and share common lineage, while 'Friar' has a partial 'Santa Rosa' genotype (Okie and Ramming, 1999). The last subgroup contains 'Santa Rosa', 'Black Diamond' and 'Calita'. 'Santa Rosa' is a complex hybrid produced by Luther Burbank, 'Black Diamond' is also produced by a 'Gariota' x 'Eldorado' crossing and 'Calita' was released as a seedling (Okie and Ramming, 1999). Therefore, nowadays *P. salicina* cultivars have a narrow genetic base and almost 10 cultivars producing about 75% of the total world production in recent years. The rest 25% is produced by other secondary cultivars, which are essentially derived from the same germplasm, all trace back to just five parents and released by Luther Burbank: 'Santa Rosa', 'Eldorado, 'Gaviota', 'Formosa' and 'Burbank' (Okie and Ramming, 1999). Hence, it is clear from the parentages that many introductions are mutations or chance seedlings, rather than the result of planned hybridizations.

In conclusion, ISSR and RAPD markers permitted the distinction between plum species and established the identity of the Greek National plums collection. The correlation among the molecular techniques suggests their utility for identification and characterization of germplasm and could provide a profound understanding of plum germplasm diversity.

Conclusions

In conclusion, the current work revealed the equivalent effectiveness of RAPD and ISSR markers for the investigation of the Greek plum germplasm diversity and its relationships to introduced accessions. High inter- and intra- genetic variability was recorded among *Prunus* spp. and related genera; clustering reflected association among most of the accessions according to species genotype/ploidy. Greek cultivars 'Goulina' and 'Asvestochoriou' revealed a distinctive genetic composition that differentiated them from the other *P. domestica* accessions. Possibly the estimation of diversity among these genotypes can be significant for implementation in future breeding programs.

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