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Combination of high resolution melting (HRM) analysis and SSR molecular markers speeds up plum genotyping: case study genotyping the Greek plum GeneBank collection

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Abstract

In a germplasm bank collection the conservation and characterization of genetic resources is a prerequisite in order to use the material in breeding projects aiming the creation of new cultivars. In the present study, 54 *Prunus salicina domestica* and *Prunus domestica* genotypes (including seven Greek cultivars), maintained in the *ex situ* National Genebank Collection of Greece, were classified using microsatellite (simple sequence repeat, SSR) markers on high resolution melting (HRM) analysis. The SSR primer pairs were chosen from the published literature as originally designed on *Prunus* species. This combined approach was used to genotype all plum accessions of the collection highlighting the benefits of either method (HRM and SSRs) for cultivar identification. Dendrograms for *P. domestica* and *P. salicina* and a combined one with all the genotypes assayed were produced. A total of 15 from the 19 *P. domestica* accessions analysed, including all the Greek accessions but 'Avgati Skopelou', were grouped into the same clade in the combined dendrogram, whereas the remaining four were dispersed into the *P. salinica* clades. Bayesian structure analysis confirmed that 'Avgati Skopelou' differs from the rest of the Greek plum cultivars since it was not grouped into the same cluster. The combination of HRM and SSRs, provided a considerably faster, cost-effective, closed-tube microsatellite genotyping method for molecular characterization of plum cultivars.

Keywords: Local germplasm, microsatellites, *Prunus domestica*, *Prunus salicina*

Introduction

Plums are classified into the genus *Prunus* of the Rosaceae family (subfamily Amygdaloideae – also called Prunoideae) alongside other economically important stone fruit trees, such as peach, almond, apricot and

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sweet cherry. The phylogenetic classification of the plums, however, is still controversial so that depending on the taxonomist the genus *Prunus* contains from 19 to 40 plum species (Topp *et al.*, 2012 and references therein).

The knowledge of the available genetic diversity is important in plant breeding programs (Goulão *et al.*, 2001); in the past, this diversity was accessed by morphological, pomological and phenological characters, whereas nowadays molecular methods have been introduced. It is also important to evaluate the challenges during growth conditions of cultivars, such as their response to biotic and abiotic factors, as well as consumers' preferences, in order to address effectively the issue of awareness to the genetic resources and provide a useful germplasm for breeders who intent to produce new cultivars (Ganopoulos *et al.*, 2015). Many methods have been developed to assess genetic diversity; traditionally, distinction of cultivars was based on phenotypical observation and morphological description. Although guidelines have been created for thorough and comprehensive description (UPOV, 1982), it is a process that requires personal experience while it is influenced by environmental factors (This *et al.*, 2004). To overcome such limitations biochemical or molecular analysis methods have been introduced. However, not a single method is superior to all others; instead the available methods could be used complementary to each other as was shown recently when phenotypic observations together with biochemical and molecular analysis were all incorporated to distinguish grapevine accessions (Merkouropoulos *et al.*, 2015). Regarding the genetic diversity of plums, morphological descriptors (Hend *et al.*, 2009; Gharbi *et al.*, 2014; Kazija *et al.*, 2014), biochemical profiles (Mowrey and Werner, 1990) and molecular methods such as random amplified polymorphic DNA (RAPDs) (Shimada *et al.*, 1999; Boonprakob *et al.*, 2001), amplified fragment length polymorphisms (AFLPs) (Goulão *et al.*, 2001; Ilgin *et al.*, 2009), simple sequence repeats (SSRs) (Ahmad *et al.*, 2004; Gharbi *et al.*, 2014; Kazija *et al.*, 2014; Klabunde *et al.*, 2014), chloroplast DNA markers (Horvath *et al.*, 2011) and inter simple sequence repeats (ISSRs) (Goulão *et al.*, 2001; Carrasco *et al.*, 2012; Athanasiadis *et al.*, 2013) have all been employed. Recently, a further analytical technique has been introduced: high resolution melting (HRM) analysis (Wittwer *et al.*, 2003) and has been extensively applied in the fields of cultivar identification (Madesis *et al.*, 2014 and references therein). Identification of cultivars using SSRs and/or single nucleotide polymorphisms (SNPs) through HRM analysis has successfully been performed before in other species (Mackay *et al.*, 2008; Ganopoulos *et al.*, 2011; Bosmali *et al.*, 2012; Hwang *et al.*, 2012; Ricci *et al.*, 2012; Xanthopoulou *et al.*, 2013). The aim of the current research was dual: firstly, to molecularly genotype and discriminate the Greek GeneBank Collection of plum cultivars and secondly, to evaluate HRM on a set of microsatellite molecular markers as a tool for the molecular genotyping of plums. The information obtained here may be useful for the conservation, management and application of plum breeding programs.

Materials and methods

A total of 44 plum cultivars were used in this study, all growing in the *ex situ* GeneBank Collection of the Institute of Plant Breeding and Genetic Resources, Department of Deciduous Fruit Trees in Naoussa (Supplementary Table S1), representing the richest plum Germplasm Bank in Greece, which is the result of multiyear national survey. Isolation of DNA from leaves was performed using the 'NucleoSpin Plant II' kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop-1000 (Thermo Scientific, Wilmington, DE, USA) spectrophotometer at 260 and 280 nm ultraviolet lengths, whereas the integrity of the DNAs was estimated by gel electrophoresis in a 0.8% agarose gel. Samples were then diluted to 20 ng/µl work concentration.

For microsatellite analysis, PCR amplification, DNA melting and end point fluorescence level acquiring were performed in a total volume of 20 µl in a 72-well carousel of the Rotor-Gene 6000 real-time 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia) according to Ganopoulos *et al.* (2011). PCR reaction mixture consisted of 20 ng genomic DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 300 nM forward and reverse primers, 1.5 mM Syto®9 green fluorescent nucleic acid stain and 1 U KapaTaq DNA polymerase (Kapa Biosystems, USA). The PCR protocol used was as follows: an initial denaturing step at 95°C for 3 min followed by 35 cycles at 95°C for 20 s, 52°C for 30 s, and 72°C for 20 s, then a final extension step at 72°C for 2 min (Table 1). The fluorescent data were acquired at the end of each extension step during PCR cycles. In order to perform the HRM analysis, the products were initially denatured at 95°C for 5 s, and then annealed at 50°C for 30 s to randomly form DNA duplexes.

HRM protocol steps: pre-melt at the first appropriate temperature for 90 s, and melt at a ramp of 10°C in an appropriate temperature range at 0.1°C increments every 2 s. The fluorescent data were acquired at the end of each increment step. End point fluorescence level was acquired following the melting process by holding at 60°C for 5 min. The sequences of the microsatellite primers used in the study were chosen from the available published literature (Cipriani *et al.*, 1999; Downey and Iezzoni, 2000; Dirlewanger *et al.*, 2002; Struss *et al.*, 2003; Messina *et al.*, 2004), whereas HRM was performed as described previously (Ganopoulos *et al.*, 2011). For microsatellite genotyping by HRM analysis, the genotype of each DNA sample

Table 1. Details of microsatellite primers used and number of HRM genotypes obtained from 54 plum cultivars

Microsatellite loci	Forward (5′–3′)	Reverse (5′–3′)	Amplicon size	Motif[a]	Tm range	PIC	I	HRM genotypes	Species of origin	Reference
BPPCT034	CTACCTGAAATAAGCAGAGCCAT	CAATGGAGAATGGGGTGC	228	$(GA)_{19}$	77.4–79.1	0.064	0.137	18	*P. persica*	1
BPPCT037	CATGGAAGAGGATCAAGTGC	CTTGAAGGTAGTGCCAAAGC	155	$(GA)_{25}$	76.7–78.92	0.121	0.130	16	*P. persica*	1
PceGA34	GAACATGTGGTGTGCTGGTT	TCCACTAGGAGGTGCAAATG	155	NA	81.38–81.70	0.111	0.271	5	*P. cerasus*	2
PS12A02	GCCACCAATGGTTCTTCC	AGCACCAGATGCACCTGA	200	NA	77.73–79.62	0.152	0.257	9	*P. cerasus*	2
UDP96–001	AGTTTGATTTTCTGATGCATCC	TGCCATAAGGACCGGTATGT	120	$(CA)_{17}$	76.5–78.73	0.120	0.235	13	*P. persica*	3
UDAp-404	CATGAACAGGGTCAAAAGCA	TATATCCTTACGCGGCCTCA	181	$(GA)_{21}$	79.75–82.4	0.091	0.175	16	*P. armeniaca*	4
UCD-CH17	TGGACTTCACTCATTTCAGAGA	ACTGCAGAGAATTTCCACAACCA	188	$(CT)_{11}$	75.4–77.42	0.122	0.218	12	*P. avium*	5
Mean	–	–	–	–	–	0.111	0.203	12.71	–	–

HRM, high resolution melting; PIC, polymorphic information content.
1 = Dirlewanger *et al.* (2002), 2 = Downey and Iezzoni (2000), 3 = Cipriani *et al.* (1999), 4 = Messina *et al.* (2004), 5 = Struss *et al.* (2003).
[a]The repeat motif of each marker is given based on information provided, or not, by the original reference that published primers for the marker.

was determined based on the shape of curves depicted by temperature-shifted melting curves or difference plots, and was scored for the binary data matrix (e.g. '1' denoted the presence of a melting curve, whereas '0' denoted the absence of a melting curve). Briefly for each marker we assign 1 if the HRM curve exists and 0 if it does not by comparing horizontally one genotype with all the others. Thus, the HRM curves become markers, allowing the performance of genotyping via the HRM analysis. The matrices were then analysed by FreeTree v. 0.9.1.50 software (Hampl *et al.*, 2001). Similarity of qualitative data was calculated using the Nei and Li/Dice similarity index (Nei and Li, 1979), and similarity estimates were analysed using UPGMA (Unweighted Pair Group Method using Arithmetic Averages). The matrices of mutual coefficients of similarity calculated by FreeTree were converted to MEGA 5 software (Tamura *et al.*, 2011) and the resulting clusters were expressed as dendrograms.

Possible population structure was investigated using a model based on the Bayesian procedure implemented in the software STRUCTURE v2.3.2 (Pritchard *et al.*, 2000). The analysis was carried out using a burning period of 10,000 iterations and a run length of 200,000 MCMC replications. We tested a continuous series of K, from 1 to 8, in 10 independent runs. We did not introduce prior knowledge about the population of origin, and assumed correlated allele frequencies and admixture (Falush *et al.*, 2003). For selecting the optimal value of K, ΔK values (Evanno *et al.*, 2005) were calculated using STRUCTURE HARVESTER (Earl, 2012).

For each SSR-HRM locus, we calculated the Shannon's information index $(-1 \times \text{Sum}\ (p_i \times \ln(p_i))$ and the polymorphic information content $(1 - \text{Sum}\ pi^2)$, where p_i the frequency of the *i*th HRM curve for the population.

Results

In order to genotype and distinguish the 54 plum cultivars maintained in the National GeneBank Collection of Greece, we evaluated seven microsatellite markers, revealing a total of 89 HRM profiles. For all the primer pairs, detection sensitivity and reproducibility tests have been confirmed by replicated DNA samples. Polymorphisms among the 54 plum cultivars were detected based on the pattern of temperature-shifted curves and difference plot (Fig. 1(a) and (b)). Analysis of conventional melting curves does not allow the sufficient discrimination of the different cultivars, as it uses only the Tm values, whereas the resolving power of HRM is much greater than the conventional melting curve analysis.

The normalized HRM melting curves of nine representative plum genotypes (using the microsatellite marker PS12A02) are depicted in Fig. 1(a); where only the unique

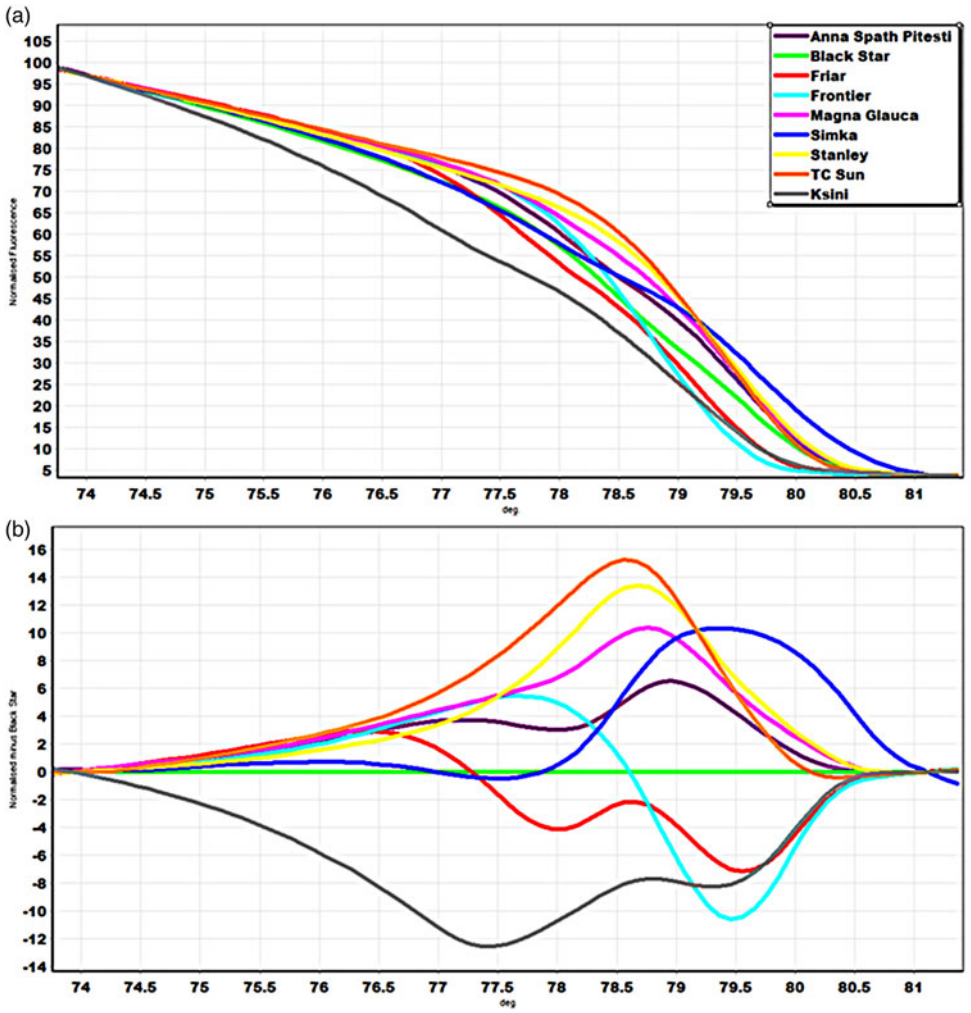

Fig. 1. HRM analysis of representative plum genotypes with microsatellite marker PS12A02. (a) Normalized HRM melting curves for nine unique plum genotypes that are using HRM analysis, (b) representative profiles of the melting curves (difference plot curves) of PS12A02 amplicons for plum genotypes. Difference graph of nine unique genotypes using the cultivar 'Black Star' as reference genotype.

HRM genotypes are shown. The shape of the melting curves could reveal the differences between the cultivars under investigation and show that all cultivars used could be easily distinguished visually by their melting curves, as for example in cultivars 'Stanley' and 'Frontier'. The results with the rest of the microsatellite markers were similar, showing a clear discrimination of most of the genotypes used (data not shown).

Fig. 1(b) depicts the difference graph produced by the PS12A02 marker on a representative set of 9 unique plum cultivars as compared with cultivar 'Black Star', which was used as the baseline. As shown in Fig. 1, the confidence value of similarity between 'Black Star' and the rest cultivars was estimated, and showed that PS12A02 was a sufficient molecular marker to differentiate most of the 54 plum cultivars. PS12A02 marker combined with HRM analysis represents a polymorphic microsatellite marker, which identified nine different HRM profiles while all other 45 genotypes followed one of these nine distinct curves.

The detected polymorphic HRM curves originating from the seven microsatellite markers were used to construct three similarity dendrograms using the UPGMA cluster algorithm: two dendrograms, which consist of either the *P. domestica* or *P. salicina* cultivars (Figs 2 and 3, respectively) and a third dendrogram, the combined dendrogram, with all the cultivars analysed (Fig. 4). In the *P. domestica* dendrogram, four distinct clades of 8, 3, 5, and 2 cultivars, respectively, have been formed. Similarly, four clades of 9, 6, 14 and 5 cultivars, respectively, were formed in the *P. salicina* dendrogram. In the combined similarity dendrogram, the 54 *P. domestica* and *P. salicina* cultivars were grouped

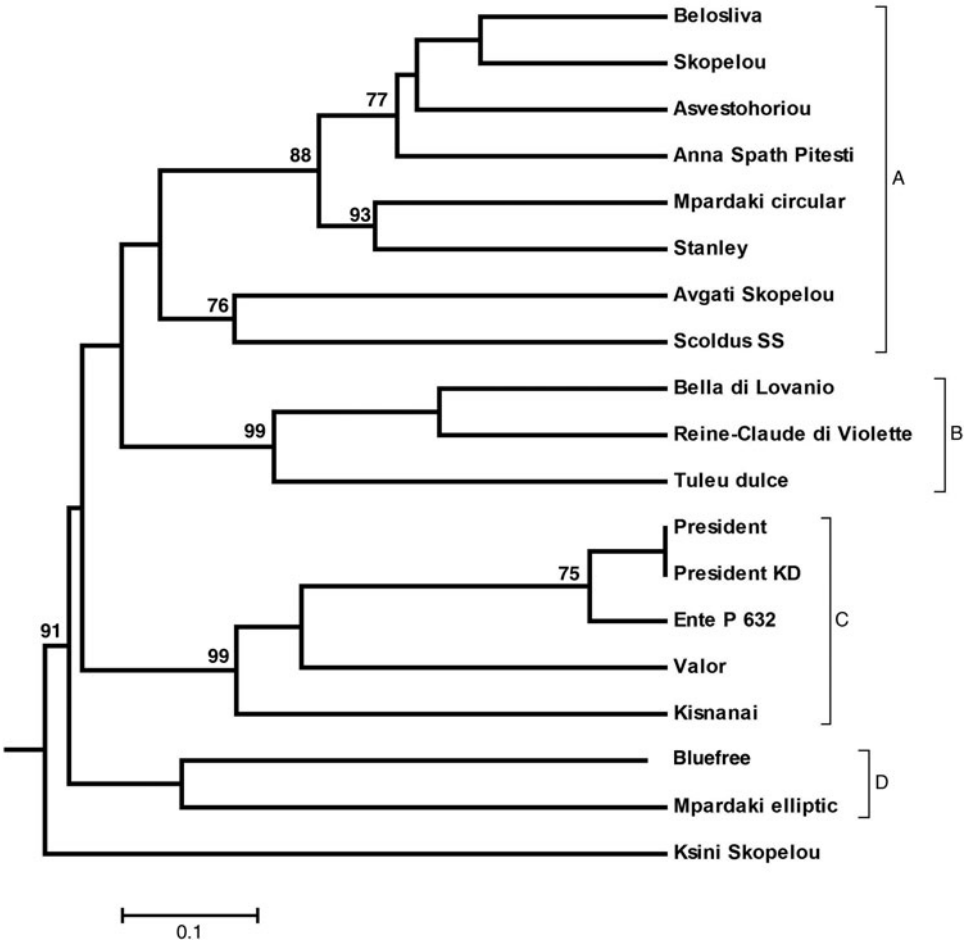

Fig. 2. Dendrogram showing the genetic relationships among 19 *P. domestica* cultivars as revealed by the microsatellite genotyping with HRM analysis. Numbers above the lines indicate bootstrap values (percentage of 1000 replicates). Bootstrap values greater than 50% are shown.

into five clades (A–E) of 15, 7, 11, 10 and 11 cultivars, respectively (Fig. 4), with each of the clades divided in smaller clades. Clade A consists purely of *P. domestica* cultivars, whereas clades B and E consist purely of *P. salicina* cultivars; this confirms the distinct genetic differences among the two species. There are, however, four *P. domestica* cultivars, namely 'Avgati Skopelou', 'Scoldus SS', 'Bella di Lovanio' and 'Tuleu dulce' that are dispersed among *P. salicina* cultivars in clades C and D suggesting the occurrence of a level of genetic similarities between *P. salicina* and the four *P. domestica* cultivars. The successful discrimination of *P. domestica* from *P. salicina* cultivars was achieved using SSR primers that had originally been designed on a variety of *Prunus* species, such as *P. persica, P. armeniana* and *P. cerasus*. This heterologous amplification is particularly effective in evolutionary relative species (Downey and Iezzoni, 2000) and has been extensively used in the case of plum genotyping where SSRs primers originating from other *Prunus* species have been used. The current microsatellite analysis identified cultivars 'Bluefree' and 'Belosliva' sharing high level of genetic relationship as revealed by their position on the dendrogram. Further, two cultivars were identified as synonyms within the set of the foreign plum cultivars: 'President' and 'President KD' confirming their morphological identity; most probably 'President KD' is a clone of 'President'.

To get a clearer picture of the genetic relationships between the analysed groups of cultivars, principal coordinate analysis (PCoA) was performed on the molecular data. In the PCoA scatter plot (Fig. 5), a slight separation is present between the traditional Greek cultivars and the foreign plum cultivars supporting the combined dendrogram (Fig. 4) results in a robust way, considering the high percentage of the total genetic diversity in the first two axes (23.74%).

Bayesian analyses performed with the STRUCTURE software were based on seven microsatellite loci and included all 54 plum cultivars. Subsequent ΔK analyses (Evanno

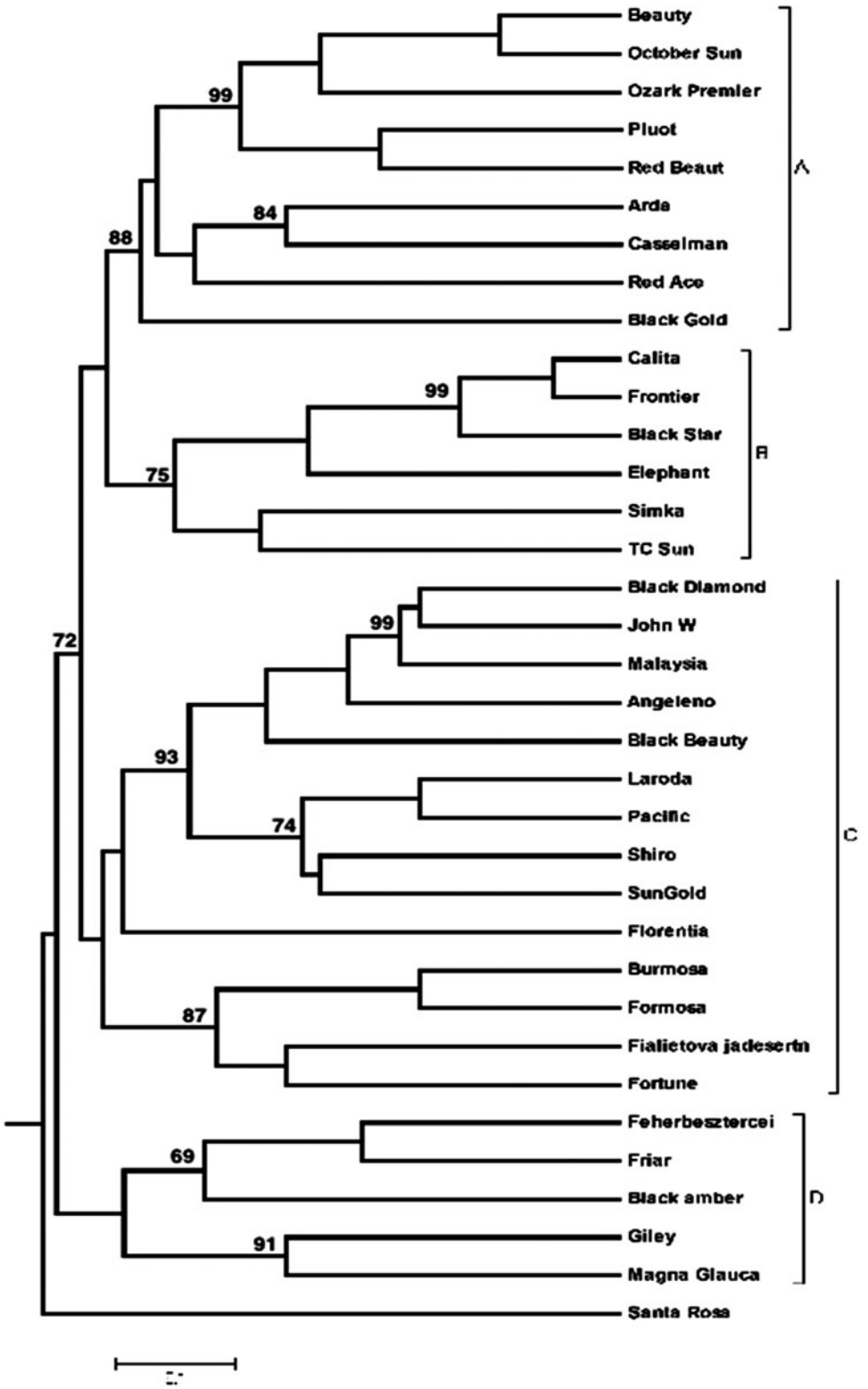

Fig. 3. Dendrogram showing the genetic relationships among 35 *P. salicina* cultivars as revealed by the microsatellite genotyping with HRM analysis. Numbers above the lines indicate bootstrap values (percentage of 1000 replicates). Bootstrap values greater than 50% are shown.

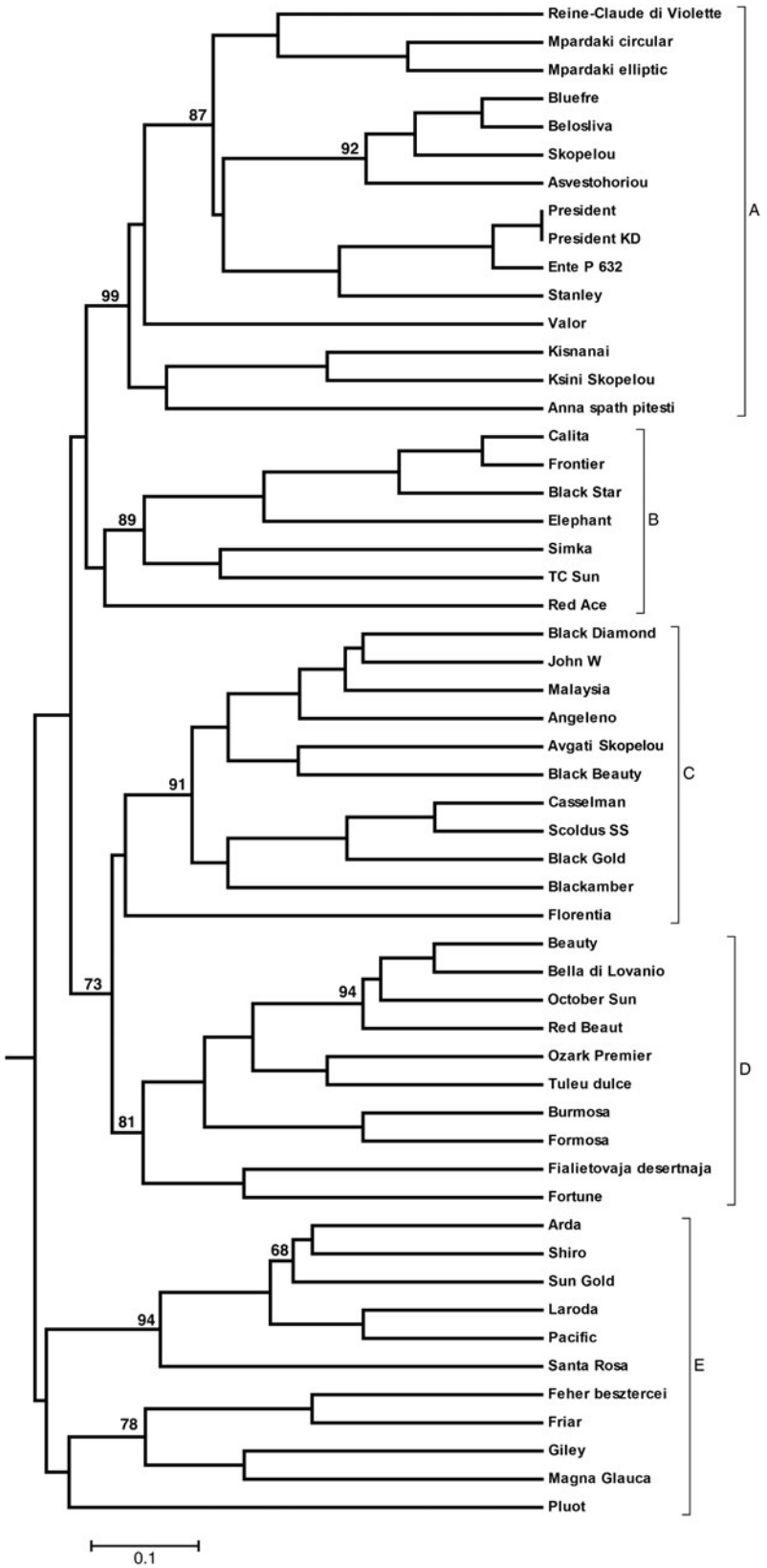

Fig. 4. Dendrogram showing the genetic relationships among 54 plum (*P. domestica* and *P. salicina*) cultivars as revealed by the microsatellite genotyping with HRM analysis. Numbers above the lines indicate bootstrap values (percentage of 1000 replicates). Bootstrap values greater than 50% are shown.

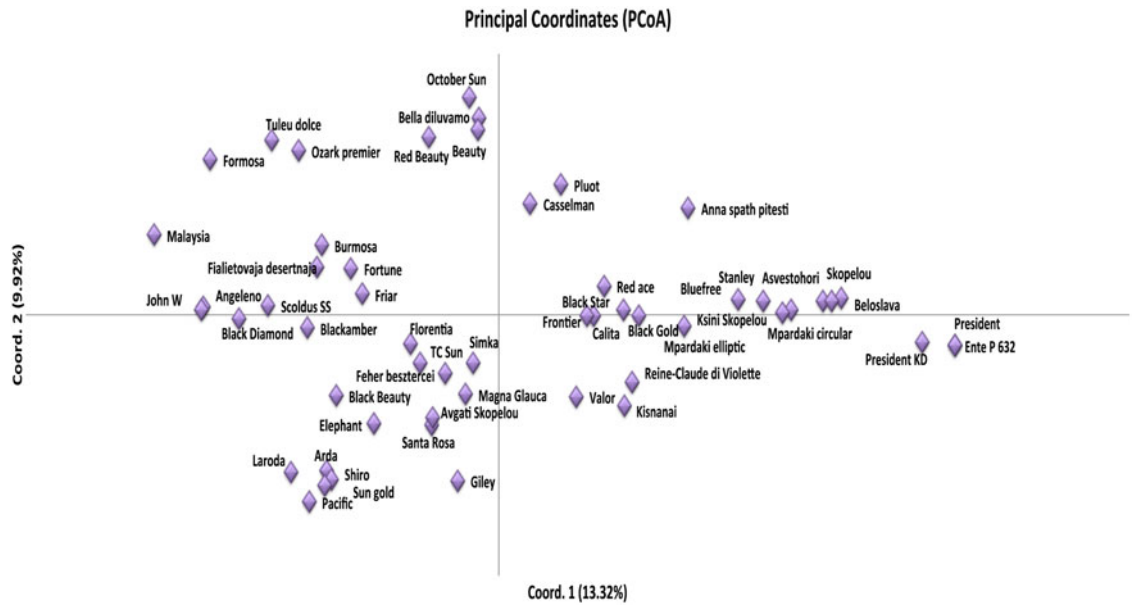

Fig. 5. Principal coordinate scatter plot of 54 plum cultivars based on microsatellite marker.

et al., 2005) revealed maximum values on $K = 3$. In the case where three clusters were defined ($K = 3$), the third cluster (blue) included the largest number of accessions (17), all with a probability of membership (qI) greater than 95% (Supplementary Table S1).

Discussion

Genotyping involving the use of 8 SSRs and 11 ISSRs markers on 29 plum cultivars (Carrasco *et al.*, 2012), and also 8 SSRs on 47 Japanese cultivars (Klabunde *et al.*, 2014) have been reported. Microsatellite genotyping with HRM analysis can be useful for differentiating cultivars which are phenotypically very similar (Ganopoulos *et al.*, 2011). In our case, microsatellite characterization has provided useful information for describing the genetic diversity and the relationships of plum cultivars established in the Greek plum GeneBank Collection. In the recent past, 26 cultivars of the same Collection were genotyped by RAPDs and ISSRs (Athanasiadis *et al.*, 2013). The current paper, however, is the first report to genotype the whole of the Greek plum GeneBank Collection (54 cultivars) using the combination of HRM and SSRs. Although some of the analysed genotypes are common in both studies, the relation between the genotypes detected by Athanasiadis *et al.* (2013) were not fully detected in the current study; this should not be of surprise since a different experimental approaches were performed focusing on different loci.

HRM represents a fast, simple, time efficient, high-throughput, very sensitive and with great analytical power approach for microsatellite genotyping, which involves lower risk of cross-contamination, since it is a simple closed-tube technique. HRM uses the whole amplicon sequence for detecting variations. As a result it has been shown that it could potentially reveal a greater amount of polymorphisms than other conventional gel-electrophoresis based methods. This can be attributed to the presence of SNPs in the flanking regions of the microsatellite repeats, which produce a higher number of alleles/genotypes (Distefano *et al.*, 2012). Thus, HRM has an advantage in revealing genetic information that cannot be detected by typical microsatellite analyses when allelic homoplasy is present, a phenomenon that has already been observed in *Prunus* (Ganopoulos *et al.*, 2011).

In the Bayesian analyses that have been performed with the STRUCTURE software, all of the Greek plum cultivars grouped inside the first cluster (red) except from the cultivar 'Avgati Skopelou' was recorded as admixture (yellow). Previous studies (Horvath *et al.*, 2011; Kazija *et al.*, 2014), which were performed on 80 plum varieties from the French National Collection and 55 Croatian and international plum cultivars, respectively, suggested the occurrence of complex genetic structure. With some exceptions, both the UPGMA clustering and the Bayesian approach, classification was according to species genotype and ploidy level; European and Japanese plums formed different clusters.

In conclusion, it is demonstrated herein that the identification of the genetically closely related plum cultivars could be achieved by combining the use of microsatellite markers and HRM. The use of only four microsatellite

markers in HRM was sufficient to discriminate 54 plum cultivars, whereas the use of a total of seven microsatellite markers led to the construction of an informative and discriminative dendrogram. Hence, we suggest the use of the HRM approach together with this set of seven microsatellites as a method for plum genotyping and/in breeding projects

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1479262116000022

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