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Assessing inter- and intra-cultivar variation in Greek *Prunus avium* by SSR markers

Ioannis V. Ganopoulos[1,2], Evagellia Avramidou[1], Dionisia A. Fasoula[2†], Grigorios Diamantidis[3] and Filippos A. Aravanopoulos[1]*

[1]*Laboratory of Forest Genetics and Forest Tree Breeding, Faculty of Forestry and Natural Environment, Aristotle University of Thessaloniki, PO Box 238, Thessaloniki 54124, Greece,* [2]*Laboratory of Genetics and Plant Breeding, Faculty of Agriculture, Aristotle University of Thessaloniki, PO Box 261, Thessaloniki 54124, Greece and* [3]*Laboratory of Agricultural Chemistry, Faculty of Agriculture, Aristotle University of Thessaloniki, PO Box 261, Thessaloniki 54124, Greece*

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Abstract

Prunus avium cultivars widely used in northern Greece were investigated in terms of inter- and intra-cultivar genetic variation and DNA fingerprinting. Based on 11 simple sequence repeats loci, the average number of alleles per locus ($N_a = 2.82$), probability of identity ($P_{ID} = 0.327$), polymorphic information content (0.451) and expected heterozygosity ($H_e = 0.494$) were within the range reported in similar studies. The most informative markers were BPPCT039 and EMPa018. The cultivars were clearly separated in both an unweighted pair group method with arithmetic mean dendrogram and a multivariate space ordination. Any two cultivars differed on the average at 6.30 loci. The null hypothesis of zero intra-cultivar variability was tested and could not be rejected. Two cultivars (Tragana Edessis and Tragana Sarakinon) were genetically similar, but not identical. This study, the first of its kind for sweet cherry in Greece, presents a useful molecular tool for resolving issues of intra-cultivar variability and synonimity and provides a warranty of genetic identity in the handling and management of local traditional germplasm.

Keywords: DNA fingerprinting; microsatellites; probability of identity; sweet cherry

Introduction

Plant germplasm utilization and conservation rely on the understanding of the wealth of genetic variation in the genome. The knowledge of allelic variation is indispensable to breeders and conservationists as it reflects the raw material upon which selection acts. The advent of molecular genetic markers increased the resolution of genetic variation to levels not attainable in the past, especially regarding perennial woody species with complex genomes that are at relatively early stages of domestication.

Prunus avium L. (sweet cherry trees and wild cherries used for their excellent wood) is such an example. Cherries are thought to have originated in the Caucasus areas and are currently found across mainland Europe and western Asia (Webster, 1996). *P. avium* was apparently first cultivated in Greece (Marshall, 1954) and is currently one of the most popular tree crops in the country (Koukouroyiannis, 1996). Almost 73% of the sweet cherry fresh market production is concentrated in northern Greece, particularly in the region of Central Macedonia, Edessa district (50% of the total). Suitable environmental conditions and a demanding export market have resulted in the rapid increase of sweet

* Corresponding author. E-mail: aravanop@for.auth.gr
†Present address: Agricultural Research Institute, PO Box 22016, 1516 Nicosia, Cyprus.

cherry yield. Cherries are appreciated in local and international markets because of the fruit and the traditional agricultural practices used. However, confusion still exists concerning local cultivars used in production and their trueness-to-type.

Cherry cultivar identification has traditionally been based on morphological and phenological traits (UPOV, 1976; IPGRI, 1985). Nevertheless, phenotypic characteristics are influenced by environmental factors which may cause elevated diversity in scoring desirable traits, thus lowering their reliability (Aravanopoulos, 1999). DNA markers permit a far more precise identification of individuals and present a great potential for the characterization of economically important germplasm. Isoenzymes (Beaver *et al*., 1995; Granger, 2004) and random amplified polymorphic DNAs (RAPD) (Gerlach and Stosser, 1997) were initially used to identify unique genotype profiles in *P. avium*. Recently, simple sequence repeats (SSRs or microsatellites) designed for peach (*Prunus persica*) have been used to amplify loci in other *Prunus* species, such as *P. dulcis* (almond), *P. armeniaca* (apricot), *P. domestic*a (plum) and *P. avium*, and have been recommended for use in comparative mapping within Rosaceae (Cipriani *et al*., 1999; Sosinski *et al*., 2000; Clarke *et al*., 2009). Because SSR-flanking sequences are conserved between *P. persica* and *P. avium* (Wunsch, 2009), SSR primers developed for *P. persica* were an obvious choice for use in *P. avium* (Cipriani *et al*., 1999; Dirlewanger *et al*., 2002; Schueler *et al*., 2003). More recently, SSR markers isolated in sweet cherry (Clarke and Tobutt, 2003) and wild cherry (Vaughan and Russell, 2004) have been used in sweet cherry genetic diversity studies (Marchese *et al*., 2007; Lacis *et al*., 2009).

This study reports the use of SSR markers for the identification of traditional *P. avium* cultivars present in the region of Central Macedonia, Greece. In addition, the extent of genetic variability including intra-cultivar variation was investigated. The null hypothesis of zero intra-cultivar variability was tested. The overall objective was to produce a molecular standard which could provide assurance of genetic identity in the handling and management of the genetic material, especially during the multiplication and distribution stages.

Materials and methods

Plant material

The most representative sweet cherry cultivars of the region of Central Macedonia, Greece were included in this study. The three most frequently planted cultivars, as well as two less widespread but well-known traditional cultivars, were included (Table 1). The main morphological characteristics of the cultivars upon which earlier cultivar identification was largely based are also given. Plant material was collected from the reference collection of the Pomology Institute of Naousa. Since it was not known with any degree of confidence if the widely planted cultivars represent a multiclonal assembly or a single genotype, the potential of intra-cultivar variability was assessed by sampling different individuals of the same cultivar name across the region of Central Macedonia, Greece (Table 1). Total genomic DNA was isolated according to Doyle and Doyle (1987).

Polymerase chain reaction (PCR) amplification and electrophoresis

Seventeen SSR markers originating from *P. avium* and *P. persica* (Table 2) were PCR-amplified as follows: PCR buffer (20 mM Tris–HCl, 50 mM KCl), 1.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer, 0.7 U of Taq DNA polymerase (Gibco BRL) and 20 ng of *P. avium* genomic DNA in a 15 μl final volume. PCR reactions were performed on a GeneAmp 9600 thermal cycler (Perkin-Elmer Cetus), which was programmed to follow the conditions reported by Dirlewanger *et al*. (2002), Clarke and Tobutt (2003) and Vaughan and Russell (2004). PCR products were separated by electrophoresis using 3% 'Metaphor' high-resolution agarose gels (FMC Bioproducts) in 1 × Tris–Borate–ethylenediamine tetraacetic acid buffer at 5 V/cm (Pinto *et al*., 2003), stained with ethidium bromide and visualized under ultraviolet light. Fragment sizes were estimated with 10 base pair ladder DNA-sizing markers (Gibco BRL).

Table 1. *Prunus avium* cultivars, their code designation, sample sizes and main morphological characteristics

Cultivar	Prevalence	Sample size (*N*)	Fruit size[a]	Flesh colour	Fruit shape
Lemonidi	Widespread	5	–	–	–
Mpakirtzeika	Widespread	15	Very large	Red	Cordiform
Tragana Edessis	Widespread	15	Large	Mahogany complexion	Nephroid
Tragana Sarakinon	Rare	5	Very large	Mahogany complexion	Nephroid
Vasiliadi	Rare	2	–	–	–

–, No data.
[a] Fruit size: large, 7 g; very large, >9 g (Chatzicharisis *et al*., 2000).

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Table 2. Simple sequence repeat markers assayed and polymorphism obtained in the *Prunus avium* cultivars studied

Locus	Origin	Size range (bp)	Remarks	N_a
BPPCT002[a]	*P. persica*	175–185	Polymorphic	3
BPPCT005[a]	*P. persica*	155–199	Polymorphic	2
BPPCT012[a]	*P. persica*	154–165	Polymorphic	2
BPPCT026[a]	*P. persica*	140–190	Polymorphic	3
BPPCT028	*P. persica*	179–185	Monomorphic	2
BPPCT034[a]	*P. persica*	225–240	Polymorphic	3
BPPCT037	*P. persica*	–	Complex pattern	–
BPPCT038[a]	*P. persica*	100–125	Polymorphic	4
BPPCT039[a]	*P. persica*	134–150	Polymorphic	5
BPPCT040[a]	*P. persica*	122–140	Polymorphic	3
UDP96-001	*P. persica*	125–140	Monomorphic	2
UDP98-021	*P. persica*	100–110	Monomorphic	2
UDP98-410	*P. persica*	130–160	Monomorphic	2
EMPA015[a]	*P. avium*	215–240	Polymorphic	3
EMPA018[a]	*P. avium*	95–106	Polymorphic	4
EMPAS10	*P. avium*	185–195	Monomorphic	2
EMPAS12[a]	*P. avium*	122–140	Polymorphic	2

bp, base pair; N_a, number of alleles per locus.
[a] Data employed in DNA fingerprinting.

The sizes of the bands were estimated using the UVIDocMw software (version 99.04 for Windows).

Data analysis

Probabilities of identity (P_{ID}) and polymorphic information content (PIC) were calculated as follows:

$$\mathrm{PIC} = 1 - \left(\sum_{i=1}^{n} p_i^2\right) - \sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2p_i^2 p_j^2$$

$$P_{ID} = \sum_{i=1}^{n} p_i^4 + \sum_{i=1}^{n-1}\sum_{j=i+1}^{n} (2p_i p_j)^2,$$

where p_i and p_j are the frequencies of the ith and jth alleles and n is the total number of such alleles (Botstein *et al.*, 1980; Paetkau *et al.*, 1995). These indices provide an estimate of the discrimination ability of each microsatellite by taking into account not only the number of revealed alleles but also their relative frequencies. Hence, different values of P_{ID} and PIC for microsatellites detecting the same number of alleles are due to their different allelic distribution (Pasqualone *et al.*, 1999). P_{ID} measures the probability that two randomly drawn diploid genotypes will be identical assuming observed allele frequencies and random assortment (Paetkau *et al.*, 1995).

Match probability (Jeffreys *et al.*, 1991) based on allele frequencies of a natural northern Greek wild cherry population (Avramidou *et al.*, 2007) was also calculated. A set of five loci common to this study was employed. Match probabilities for multiple loci indicate the probability that one unknown sample will match a sample drawn at random from the population. Independence within and among loci has been assumed. In addition, match probability ratio (MPR) was calculated as the square frequency of the most common allele to provide the most conservative estimate of match rate within the multicultivar assembly. Cumulative MPR was estimated as the sequential product of MPR at each SSR locus (DeNise *et al.*, 2004). The percent polymorphic loci (P), number of alleles per locus (N_a) and the observed (H_o) and expected (H_e) heterozygosities (assuming Hardy–Weinberg equilibrium), were calculated using the CERVUS software (Marshall *et al.*, 1998). The number of effective alleles was calculated as $N_e = 1/(1 - H_e)$.

The SSR-reproducible fragments were classified as present (1) or absent (0) and analyzed by the FreeTree v. 0.9.1.50 software (Hampl *et al.*, 2001). The similarity of qualitative data was calculated using the Nei and Li/Dice similarity index (Nei, 1973) and the similarity estimates were analyzed using unweighted pair group method with arithmetic mean (UPGMA). The matrices of mutual coefficients of similarity calculated by FreeTree were converted to MEGA 4 v. 4.1 software (Tamura *et al.*, 2007), and the resulting clusters were expressed as dendrograms. The robustness of the dendrogram was assessed by bootstrap analysis running 2000 iterations, also performed by FreeTree. Principal coordinate analysis (PCoA) was executed with the GenAlEx software (Peakall and Smouse, 2006). For the identification of unique genotypes, all possible pairwise comparisons between two cultivars were attempted.

Results

Eleven of the seventeen (64.7%) microsatellite primer pairs used produced stable and repeatable polymorphic amplification fragments (Fig. 1). Six primer pairs were excluded from the analysis. One (BPPCT037) presented complex banding patterns, a finding probably implying locus duplication. Five primer pairs produced monomorphic patterns ($P = 0.71$). The number of alleles per

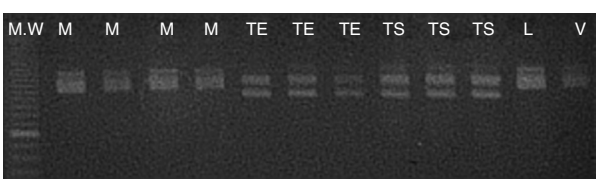

Fig. 1. Metaphor agarose gel showing the allelic profile of nuclear SSR marker BPPCT012 in the sweet cherry cultivars assayed (MW: 10 bp DNA ladder; M, Mpakirtzeika; TE, Tragana Edessis; TS, Tragana Sarakinon; L, Lemonidi; V, Vasiliadi).

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Table 3. Genetic diversity of simple sequence repeat loci used on *Prunus avium* cultivars

Locus	N_e	P_{ID}	PIC	H_e	H_o
BPPCT02	2.38	0.256	0.499	0.580	0.465
BPPCT005	1.20	0.576	0.230	0.171	0.186
BPPCT012	1.15	0.639	0.192	0.131	0.140
BPPCT026	2.38	0.364	0.386	0.408	0.535
BPPCT034	2.02	0.375	0.375	0.505	0.953
BPPCT038	3.12	0.160	0.622	0.680	1.000
BPPCT039	4.13	0.111	0.702	0.758	1.000
BPPCT040	2.57	0.225	0.537	0.611	0.953
EMPa018	4.00	0.115	0.693	0.750	1.000
EMPa015	2.11	0.342	0.408	0.528	1.000
EMPaS12	1.47	0.435	0.323	0.321	0.395
Mean	2.41	0.327	0.451	0.494	0.693

N_e, effective number of alleles; P_{ID}, probability of identity; PIC, polymorphic information content; H_e, expected heterozygosity; H_o, observed heterozygosity.

locus ranged from 2.00 to 5.00, with a mean of 2.82 ($N_e = 2.41$; Table 3). PIC values ranged from 0.192 to 0.693 (average of 0.451) and P_{ID} values ranged from 0.111 to 0.639 (average of 0.327). The most informative markers were BPPCT039 and EMPa018. The match probabilities on a per-cultivar basis were very small ranging from 2.4×10^{-6} (cultivar Vasiliadi) to 2.0×10^{-8} (cultivar Lemonidi). Cultivars Mpakirtzeika, Tragana Edessis and Tragana Sarakinon presented intermediate values of 1.0×10^{-7}, 2.0×10^{-7} and 2.0×10^{-7}, respectively. The MPR was 6.1×10^{-4} across cultivars. All individual trees sampled from different areas and bearing the same cultivar name presented the same genotype.

Expected heterozygosity ranged from 0.171 to 0.758 with an average of $H_e = 0.494$ (Table 3). In this case, H_e was equivalent to marker index based on the assumption that in microsatellites each assay reveals a single locus (Powell *et al.*, 1996; Milbourne *et al.*, 1997). The observed heterozygosity ranged from 0.140 to 1.000 with a mean value of $H_o = 0.693$ (Table 3). Pairwise comparisons showed genetic differences among cultivar pairs in a range of one to nine loci. The minimum value was observed between the Tragana Edessis and Tragana Sarakinon cultivars and the maximum between the Tragana Sarakinon and Vasiliadi cultivars (data not shown). Any two cultivars differed on average at 6.30 loci. A key for the identification of cultivars by classifying individuals according to an ascending systematic arrangement of the different SSR genotype designations was developed (data not shown).

The dendrogram generated from the UPGMA cluster analysis (Fig. 2) classified all individuals into three groups. The individuals of cultivars Tragana Edessis and Tragana Sarakinon were found closely related and were grouped together. The second group was composed of cultivars Vasiliadi and Lemonidi that appeared to have a common origin. The last group corresponded to individuals of cultivar Mpakirtzeika. The first two coordinates of the PCoA could interpret 87.38% of the total diversity observed and classified the cultivars into four groups (Fig. 3). The Tragana Edessis and Tragana Sarakinon cultivars appeared very closely associated and formed a well-defined group in this analysis as well. The Lemonidi and Vasiliadi cultivars were situated in the same quartile, while the Mpakirtzeika cultivar appeared distinctively differentiated from the other cultivars.

Discussion

The results showed a generally high degree of homology for the SSR loci between *P. persica* and *P. avium*, indicating marker transportability which agrees with other reports concerning the successful utilization of SSR markers in different *Prunus* species (Cipriani *et al.*, 1999; Wunsch and Hormaza, 2002; Dirlewanger *et al.*, 2002; Wunsch, 2009). The amount of polymorphism identified was rather low. Relatively low polymorphism was also detected by employing isoenzyme (Beaver *et al.*, 1995),

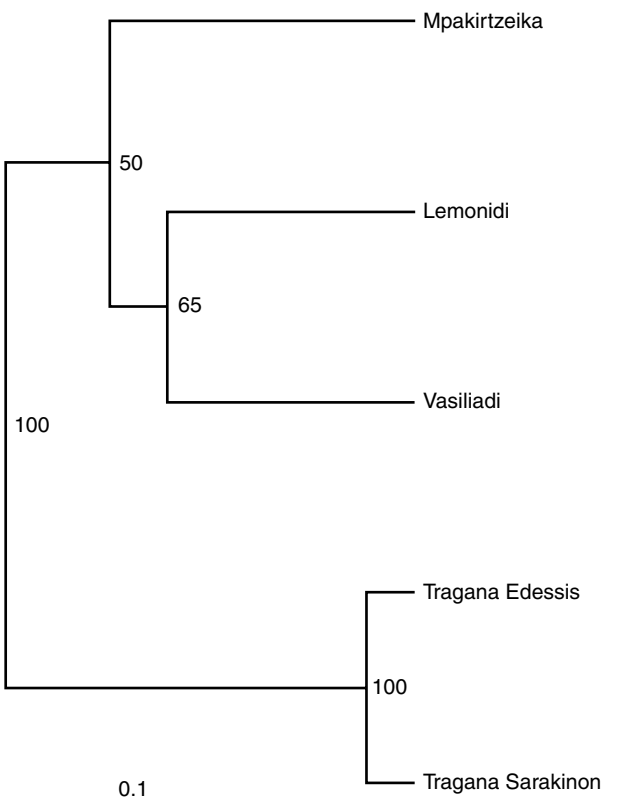

Fig. 2. Unweighted pair group method with arithmetic mean dendrogram showing similarities among five *Prunus avium* cultivars based on 11 simple sequence repeat loci. Numbers below branches represent bootstrapping values.

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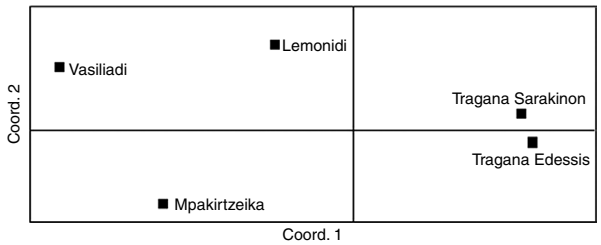

Fig. 3. Scatter plot of the first and second PCoA of the *Prunus avium* cultivars studied.

RAPD (Gerlach and Stosser, 1997; Stockinger *et al.*, 1996) and SSR markers (Wunsch and Hormaza, 2002), probably reflecting a generally narrow genetic base in cultivated sweet cherry germplasm (Wunsch and Hormaza, 2002). The number of alleles per locus was similar to values reported in other sweet cherry studies. Clarke and Tobutt (2003) genotyped 14 sweet cherry varieties with 19 SSR primers and detected an average of 3.30 alleles per locus. Dirlewanger *et al.* (2002) genotyped 21 sweet cherry cultivars with 33 SSR primers and identified an average of 2.80 alleles per locus.

The absence of intra-cultivar variability was supported in cultivars Mpakirtzeika and Tragana Edessis, where the sample size was adequate for this assessment. Similar results were seen in the rest of the cultivars, although further verification is needed due to small sample sizes. In addition, the MPR indicated that the probability of another unrelated cherry tree having the same genotype was very low. Based on the values of the PIC and P_{ID} parameters, it was evident that the polymorphism detected is adequate for the unequivocal identification of all the cultivars studied. The PIC value found in this study was nevertheless lower than other values reported for *P. avium* (PIC = 0.65; Marchese *et al.*, 2007) and *P. persica* (PIC = 0.80; Yoon *et al.*, 2006). Higher PIC values may be attributed to the use of more informative markers and a larger sample size with relatively more diverse genotypes (Prasad *et al.*, 2000). The value of locus differences between any two cultivars was high, further supporting the sufficiency of the SSR marker set in cultivar identification that was indicated by PIC and P_{ID}. Average expected heterozygosity assuming Hardy–Weinberg equilibrium is presented to allow for comparisons with pertinent reports. It was within the range reported in earlier sweet cherry studies where expected heterozygosity averaged 0.600 (Dirlewanger *et al.*, 2002), 0.460 (Clarke and Tobutt, 2003), 0.600 (Vaughan and Russell, 2004) and 0.560 (Guarino *et al.*, 2009).

The UPGMA dendrogram (Fig. 2) depicts two important findings: (1) absence of intra-cultivar variation in all the cultivars studied and (2) clear separation of all the cultivars except Tragana Edessis and Tragana Sarakinon. The principal component analysis scatter plot further supported the dendrogram results in a robust way, considering the high percentage of the total genetic diversity (87.4%) that was accounted for in low multivariate space. In a comparable study of apple cultivars, only 40.43% could be accounted for by the first two coordinates (Song *et al.*, 2006).

The absence of intra-cultivar variability as indicated by 11 SSR loci, especially in widespread cultivars where a higher within-cultivar sample size was used, is particularly important. Furthermore, both the match probability on a per-cultivar basis and the MPR presented very small values. Previously, there was no information on whether the sweet cherry cultivars employed in the region represent single clones or multiclonal assemblies. Their classification was so far based on the general concordance of descriptors within each cultivar and on the traditional knowledge of local growers. Therefore, the null hypothesis of zero intra-cultivar variability cannot be rejected.

The relation of cultivars Tragana Edessis and Tragana Sarakinon has also been a standing issue that was resolved with DNA fingerprinting. Cultivar Tragana Sarakinon was thought to be synonymous with the Tragana Edessis cultivar (Chatzicharisis *et al.*, 2000). These cultivars were grouped together in this study, a sign of their genetic affinity; however, they are not identical. Their hypothesized synonymy has to be rejected. It is likely that these cultivars either arose from the same genetic background or one arose as a mutation from the other.

DNA fingerprinting proved by far more useful than phenotypic characterization in this particular cultivar set, as it was possible to unequivocally identify all the cultivars based on a subset of the SSR loci employed. Cultivar relationships have been identified and the null hypothesis of zero intra-cultivar variability could not be rejected, resolving a long-standing question among local scientists and practitioners. Therefore, with a relatively limited number of primers, molecular identification was possible in five of the most important sweet cherry cultivars of northern Greece. To the authors' knowledge, it is the first time that these cultivars have been DNA-fingerprinted and the absence of intra-cultivar variability was investigated. This SSR primer set could be a useful molecular tool for resolving issues of intra-cultivar variability and synonymy and for providing assurance of genetic identity in the handling and management of the local traditional genetic material.

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