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Genetic diversity, structure and fruit trait associations in Greek sweet cherry cultivars using microsatellite based (SSR/ISSR) and morpho-physiological markers

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Abstract It is important to couple phenotypic analysis with genetic diversity for germplasm conservation in gene bank collections. The use of molecular markers supports the study of genetic marker-trait associations of biological and agronomic interest on diverse genetic material. In this report, 19 Greek traditional sweet cherry cultivars and two international cultivars, which were used as controls, were grown in Greece and characterized for 17 morpho-physiological traits, 15 simple sequence repeat (SSR) loci and 10 inter simple sequence repeat (ISSR) markers. To our knowledge, this is the first report on molecular genetic diversity studies in sweet cherry in Greece. Principal component analysis (PCA) of nine qualitative and eight quantitative morphological parameters explain over 77.33% of total variability in the first five axes. The SSR markers yielded a combined matching probability ratio (MPR) of $9.569 \times e{-}12$. The 15 SSR loci produced a total of 92 alleles. Ten ISSR primers generated 91 bands, with an average of 9.1 bands per primer. Expected heterozygosity (gene diversity) values of 15 SSR loci and 10 ISSR markers averaged at 0.683 and 0.369, respectively. Based on stepwise multiple regression analysis (MRA), SSR alleles were found associated with harvest time and fruit polar diameter. Furthermore, three ISSR markers were correlated with fruit harvest and soluble solids and four ISSR markers were correlated with fruit skin color. Stepwise MRA identified six SSR alleles associated with harvest time with a high correlation ($P < 0.001$), with linear associations with high F values. Hence, data analyzed by the use of MRA could be useful in marker-assisted breeding programs when no other genetic information is available.

Keywords Sweet cherry · SSR/ISSR · MRA · Fruit association markers · Morpho-physiological traits

Abbreviations

SSR	Simple sequence repeat
ISSR	Inter simple sequence repeat
AFLP	Amplified fragment length polymorphism

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RAPD	Random amplified polymorphic DNA
MRA	Multiple regression analysis
MPR	Matching probability ratio
PCA	Principal component analysis
PCR	Polymerase chain reaction
QTL	Quantitative trait locus

Introduction

Cherries (*Prunus avium* L.) include sweet cherry trees cultivated for human consumption and wild cherry trees, also called mazzards, cultivated for their wood. Cherries are thought to have originated in the Caucasus area, whereas at present they are found across mainland Europe and western Asia (Webster 1996). Undoubtedly, cherries were an early food source for primitive inhabitants of Europe, as pits have been recovered from cave dwellings that date back to 4000–5000 B.C. Sweet cherries were probably first cultivated in Greece (Hedrick 1915; Marshall 1954). Today, cherry cultivation is one of the most popular of fruit tree crops in Greece (Koukourojiannis 1996). Commercial demand for sweet cherry resulted in the contemporary increased agricultural production.

To identify and analyze the genetic diversity of the various sweet cherry cultivars used, one can rely solely on the phenotypic traits (IPGRI 1985; UPOV 1976). Morphological analysis is quick and commonly used method to identify and characterize the germplasm through phenotyping. Phenotyping characteristics that are influenced by environmental factors, however, may cause elevated diversity in the desirable agronomic traits (Marinoni et al. 2003), thus lowering the reliability of the method.

To overcome these difficulties, the DNA-based molecular methods of RAPDs (Gerlach and Stosser 1997), AFLPs (Struss et al. 2002, 2003) and SSRs (Schueler et al. 2003; Struss et al. 2002, 2003; Wunsch and Hormaza 2002, 2004) are widely used for sweet cherry germplasm characterization and the assessment of genetic diversity. The SSRs or microsatellites are routinely used for germplasm identification studies because of their ease of use and reproducibility, and also for their co-dominant inheritance and high polymorphism. A more recent microsatellite-based method, inter simple sequence repeats (ISSRs), has been developed, which involves PCR amplification of DNA by a single, 16–18 bp long primer composed of a repeated sequence. This approach is highly reproducible and of relatively low cost (Powell et al. 1996). ISSR analysis has been used for cultivar identification in numerous plant species, including rice (Joshi et al. 2000), apple (Goulao and Oliveira 2001), strawberry (Arnau et al. 2003) and mulberry (Kar et al. 2008). To our knowledge, this is the first time that ISSR analysis has been applied to sweet cherries.

As association analysis has not been implemented in sweet cherry to date, the present study aimed to develop this procedure in sweet cherry by using markers and plant materials that provide the best probability of high inter-cultivar diversity. Therefore, through morpho-physiological markers and microsatellite (SSR/ISSR) based methods we characterized a gene bank collection of Greek sweet cherry cultivars and reference genotypes. Molecular data of SSRs were used to infer the existence of a genetic structure in the collection studied. The objective is to produce a molecular standard which could provide a warranty of genetic identity in the handling and management of the genetic material, especially during the propagation and distribution stages of plant propagation material. Furthermore, the development of F_2 generations after crosses, inbred lines and back cross progenies in trees like sweet cherry is an elaborate process, taking into consideration the high degree of heterozygosity and the long juvenile period. The development of the marker-based technology offers a fast, reliable, and easy way to perform MRA and comprise an alternative approach to breeding in diverse species of animals and plants (Chatterjee et al. 2004; Kar et al. 2008; Ruan et al. 2009; Virk et al. 1996). Towards this end, we studied the association of molecular markers (SSR/ISSRs) with fruit characteristics, such as fruit weight, fruit polar, skin color, soluble solids and harvest time by applying MRA.

Materials and methods

Plant material

Morpho-physiological and molecular genetic diversity was assessed in 21 sweet cherry (*Prunus avium*

Table 1 Cultivars, code designation, prevalence and geographic origin of the sweet cherry material studied

No	Cultivar	Registration code	Prevalence	Geographic region
1	Proimo Kolindrou	PK	Rare	North Greece
2	Tragana Edessis	TE	Widespread	North Greece
3	Mpakirtzeika	MP	Widespread	North Greece
4	Basiliadi	BA	Rare	North Greece
5	Tragana Edessis-Naousis	TEN	Widespread	North Greece
6	Hybrid Tragana Edessis × unknown	HTEU	Rare	North Greece
7	Mavro Anastasias	MA	Rare	North Greece
8	Kokkino Anastasias	KA	Rare	North Greece
9	Hybrid Tragana Edessis × Germersdorfer	HTEG	Rare	North Greece
10	Lemonidi	LE	Rare	North Greece
11	Agiorgitiko Lilantiou	AL	Rare	South Greece
12	Kifisias	KI	Rare	South Greece
13	Proimo Axaias	PA	Rare	South Greece
14	Mavro Proimo Vitalou	MPV	Rare	South Greece
15	Kapsiotika	KAP	Rare	South Greece
16	Napoleon Karamela	NK	Rare	South Greece
17	Petrokeraso Axaias	PEA	Rare	South Greece
18	Fraoula Volou	FV	Widespread	South Greece
19	Samou	SA	Rare	South Greece
20	Burlat	BU	Widespread	International
21	Van	VA	Widespread	International

L.) cultivars cultivated in Greece. All these cultivars exist in the Greek Fruit Gene Bank collection in Naousa (Table 1) and represent the total diversity of Greek sweet cherry cultivars. Nineteen of these 21 cultivars are traditional Greek cultivars, whereas the remaining two ('Burlat' and 'Van') have been used as controls for quantitative traits and for molecular analysis. The cultivars were arranged in a randomized complete block design with three replicates and four plants per elementary experimental unit for 3 years. Trees were grown in open fields with standard agronomic practices.

Analysis of morpho-physiological traits

Leaves were collected at adult stage, at approximately the end of July. From each of the four trees studied per cultivar, seven leaves were sampled per year, and the following parameters measured using a digital calliper with a sensitivity of ±0.01 mm. Flowers were collected at full bloom 10 flowers were taken from each of the four trees studied per cultivar and year. Cherry fruit was collected at maturity. Maturity was determined on the basis of the color characteristics of each cultivar, taking into account information provided by growers and from personal experience and observation. A sample of a total of 106 cherry fruits was taken from each of the five trees studied per cultivar and year. Of these, 100 were used to determine the mean fruit weight. The remaining six cherries were used to study a series of quantitative and qualitative descriptors.

Seventeen morphological and biochemical traits of leaves, flowers and fruits were used to group each cultivar: yield (kg/tree), leaf type (score), number of floriferous buds/spur (score), lamella shape (score), lamella length (cm), flowering time (days), fruit shape (score), weight of harvested fruits (g), fruit size (score), fruit polar diameter (mm, stem to blossom end, on a representative fruit), equatorial diameter (mm), fruit skin color (score), fruit crispness (score), harvest time (days), soluble solids (%),titratable acidity (%) and ratio soluble solids to titratable acidity (score). These traits were selected from the International Union for the Protection of New Cultivars of Plants descriptors proposed for sweet

cherry (UPOV 1976; IPGRI 1985). The scoring system is the same as used by UPOV and IPGRI. When possible, all the measurements of a trait were made on the same date to avoid differences in the environment or developmental stages of the tree.

DNA extraction and PCR amplification conditions

Total genomic DNA was isolated using the procedure described by (Doyle and Doyle 1987). The DNA amount was estimated by a fluorimeter (Dyna Quant 200, Hoefer Pharmacia Biotech) using Hoechst H 33258 and a human standard at 144 ng/µl. Samples were then diluted to 20 ng/µl working concentration.

PCR reactions for SSR analysis were conducted in a 8 µl final volume using primers at 0.3 µM, 20 ng of total genomic DNA and 2× Qiagen Multiple PCR master mix buffer (Qiagen, Crawley, UK). Forward primers were 5′ labelled with FAM/HEX dyes. Temperature profiles including annealing temperatures were those from the literature (Clarke and Tobutt 2003; Downey and Iezzoni 2000; Testolin et al. 2000; Vaughan and Russell 2004). Electrophoresis (EP) was performed of DiSA (Department of Agriculture and Environmental Sciences, Udine, Italy) with the MegaBace 500 (Amersham Biosciences, UK) automatic sequencer, and using a Et-ROX internal standard (Et400-R Size Standard, Amersham Biosciences, UK) for all SSR loci. All samples were analysed two to four times with independent PCRs and EP runs.

Amplifications for ISSR analysis were performed in a total volume of 25 µl including 30 ng of total cellular DNA, 200 mM of each dNTPs, 2 mM $MgCl_2$, 40 pmol of primers, 2.5 µl of 10× *Taq* DNA polymerase buffer and 1 U of 1 U DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). PCR amplifications were performed in a PTC 200 (MJ Research Inc., Watertown, MA) as follows: an initial step of 5 min at 94°C, followed by 35 cycles, each one including 30 s at 94°C for denaturation, 90 s at 45–60°C (depending on the used primer) for annealing and 90 s at 72°C for elongation. A 5 min step at 72°C was programmed as a final extension. Amplification products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. A 100 bp or 1 Kb DNA ladder (Invitrogen, USA) was used as size markers. The selected ISSR primers (University of British Columbia, Canada; Table 4) were used for PCR amplification and data points are the presence/absence of each distinguishable band across all samples for the same primer, in both replicate sets of amplifications. Gels and images were analyzed using UVIDoc software (UVItec, Cambridge, UK) to quantify signal intensity.

Data analysis

The mean values regarding the quantitative traits of each cultivar were calculated (Table S1). PCA was performed with the XLSTAT software version 2008.4.02 (Addinsoft USA, New York, NY). In the PCA, the data used to generate eigenvalues and percentage of the variation were accumulated by the PCA and the load coefficient values between the original characters and respective PC. The first two principal components which accounted for the highest variation were used to plot the scatter diagram of the cultivars. The significance of diversity among the genotypes for each of the attributing traits was tested through one-way ANOVA (Table 2).

Electrophoretic runs were analysed with Fragment Profiler (v.1.2, Amersham Biosciences), that automatically sizes and bins the alleles. The SSR and ISSR reproducible fragments were classified as present (1) or absent (0), and were typed into a computer file as a binary matrix, one for each molecular marker. The matrices were then analyzed 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 analyzed using Unweighted Pair Group Method using Arithmetic Averages (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 resulting clusters were expressed as dendrograms. The robustness of the dendrogram was assessed by bootstrap analysis running 1,000 iterations, also performed by FreeTree.

For SSR analysis, polymorphic information content (PIC) and probability of identity (P_{ID}) were calculated as follows (Botstein et al. 1980; Paetkau et al. 1995):

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

$$P_{\mathrm{ID}} = \sum_{i=1}^{n} p_i^4 + \sum_{i=1}^{n-1}\sum_{j=i+1}^{n} (2p_i p_j)^2$$ where p_i and p_j are the frequencies of the *i*th and *j*th alleles and *n* is the total

Table 2 Eigenvalue, eigenvector and scores of the five first factors retained from the PCA of 17 morpho-physiological traits performed on a Greek sweet cherry Gene Bank collection

Trait	PC-1	PC-2	PC-3	PC-4	PC-5
Yield	**0.332**	0.245	0.008	0.075	−0.239
Leaf type	−0.054	0.056	0.041	**0.468**	−0.359
Number of floriferous bud/spur	0.071	0.001	**0.344**	−0.415	0.044
Lamella shape	−0.168	−0.165	**0.310**	0.269	−0.140
Lamella length	0.259	−0.022	−0.085	−0.150	−0.496
Fruit shape	−0.131	−0.286	−0.251	−0.408	−0.140
Fruit polar diameter	0.236	−0.315	−0.360	0.100	−0.082
Equatorial diameter	**0.399**	0.043	−0.048	0.002	−0.077
Fruit size	**0.383**	0.011	−0.108	0.197	−0.065
Gristly of fruit	**0.360**	−0.086	0.228	0.029	0.209
Fruit weight	**0.393**	−0.011	−0.140	−0.045	0.070
Soluble solids	0.174	−0.179	−0.022	**0.304**	**0.586**
Titratable acidity	0.175	−0.449	**0.349**	−0.009	0.084
Proportion of soluble solids with titratable acidity	0.084	−0.419	**0.387**	−0.162	−0.224
Eigenvalue	5.357	2.651	2.072	1.816	1.251
Total variance (%)	31.512	15.592	12.189	10.680	7.357
Cumulative (%)	31.512	47.104	59.293	69.973	77.330

Values in bold indicate the most relevant characters (>0.3) that contributed most to the variation of the particular component

number of such alleles. The number of alleles per locus, the observed (Ho) and expected (He) heterozygosity, were calculated using the CERVUS software (Marshall et al. 1998). Wright's fixation index was estimated according to the formula ($F = 1 - \text{Ho/He}$) (Wright 1951). The number of effective alleles was calculated as $\text{Ne} = 1/(1 - \text{He})$. In addition, 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).

For ISSR analysis, Nei's gene diversity (He) (Nei 1973) and Shannon's information index (I) (Lewontin 1972) were estimated via the POPGEN 1.32 software (Yeh and Boyle 1997). Resolving power (Rp) of a primer is: $\text{Rp} = \Sigma\ IB$ where IB (band informativeness) takes the value of: $1 - [2 \times (0.5 - p)]$, p being the proportion of the 21 sweet cherry cultivars analyzed containing the band (Prevost and Wilkinson 1999). The similarity between matrices based on different marker system (SSR and ISSR) was calculated using the standardized Mantel coefficient (Mantel 1967).

Gene bank collection structure

We designed a clustering approach in order to study how geographic-based groups (Table 1) represent the inter-cultivar similarity in genetically homogeneous populations. For that purpose we used the STRUCTURE v.2.2 software (Baldoni et al. 2006; Parker et al. 2004; Pritchard et al. 2000; Rosenberg et al. 2002). This software places cultivars in K clusters that have distinct marker frequencies, where K is chosen a priory and can be varied across different runs. Cultivars can have memberships in several clusters, with membership coefficients equaling 1 across clusters. Cultivars were divided into genetic clusters using the STRUCTURE software package. SSR data were analyzed by treating each class of cultivars as being, effectively, diploid alleles, according to the software documentation. A no-admixture ancestry model was used and allele frequencies were correlated, with a burn-in length of 30,000 followed by 100,000 runs at each K. Five STRUCTURE runs produced nearly identical membership coefficients at each K (data not shown).

Traits association with molecular markers

Association between SSR/ISSR markers and the quantitative traits was estimated through stepwise MRA. In this method each quantitative or qualitative trait was treated as a dependent variable, while the SSR/ISSR markers were treated as independent variables. The analysis was based on the model $Y = a + b_1m_1 + b_2m_2 + \cdots + b_jm_j + \cdots + b_nm_n + d + e$, which related the variation in the dependent variable (Y = cultivar means for a quantitative trait) to a linear function of the set of independent variables m_j, representing SSR and ISSR markers (as first described by Virk et al. 1996). The b_j terms are the partial regression coefficients that specify the empirical relationships between Y and m_j, d represents between accession residuals, which is left after regression, and e is the random error of Y that includes environmental variation (Virk et al. 1996). To select independent variables for the regression equation, F values with 0.045 and 0.099 probabilities were used to enter and remove, respectively (Affifi and Clark 1984; Roy and Bargmann 1957). R^2 denotes the square of R, the multiple correlation coefficient. Selected markers were further tested independently with linear curve fitting using linear models for confirming the significance of β-statistics for each band identified by MRA. Beta can be defined as the standardized regression coefficient $= BS_x/S_y$, where B is the regression coefficient or slope and S_x and S_y are the standard deviations of independent (x) and dependent (y) variables (Affifi and Clark 1984; Kar et al. 2008). Student's t-test was performed to test significance between mean trait estimates of cultivars where specific markers were present and absent.

Results and discussion

Analysis of morpho-physiological traits

As shown in Table S1, significant variation was recorded for different morpho-physiological traits of the cultivars examined. For instance the mean weight of harvested fruit was ranged from 4.9 g in KI to 12.1 g for MP. The fruit yield ranged from 25 kg/tree in NK to 120 kg/tree in V. The distribution of cultivars based on the PC-1 and PC-2 shows the phenotypic variation among the cultivars and how widely dispersed they are along both axes (Fig. 1). Scores on the first principal component (PC-1) which accounted for 31.51% of the total variation were highly correlated (correlation coefficient >0.3) to characters related to yield, equatorial diameter of fruit, fruit weight and crispness (Table S1). The second principal component (PC-2) explained 15.59% of the total variation and was highly associated with the fruit skin color. The third component (PC-3) which explained 12.18% of the variation was mainly correlated to characters related to the number of floriferous bud/spur, lamella shape, titratable acidity, proportion of soluble solids to titratable acidity, and blooming time. The fourth component (PC-4) explained 10.68% of the variation and was determined by leaf type and soluble solids. The fifth component (PC-5) was related to the soluble solids

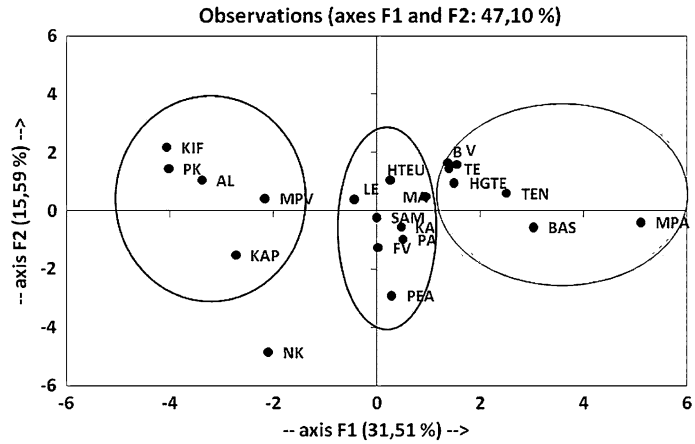

Fig. 1 PCA with phenotypic data from the 21 sweet cherry cultivars

and accounted for 7.35% of the total variability. PCA showed that 17 phenotypic traits were important in explaining multivariate polymorphism.

Multivariate analysis assumes inclusion of genotypes with maximum genetic divergence (Amurrio et al. 1995; Bhatt 1970). Variability was observed in all the 17 measured characteristics suggesting presence of a high degree of phenotypic polymorphism among the cultivated cultivars of sweet cherry in Greece. This indicates the presence of diverse morphotypes at the individual genotype level, pointing to ample possibilities for obtaining desirable trait combinations in specific cultivars. This would be crucial in a breeding program for meeting the diverse demands of farmers, researchers and consumers of this tree crop.

Molecular characterization and assessment of genetic diversity with microsatellite based (SSR/ISSR) markers

In total 92 alleles were detected for the 15 microsatellites evaluated (Table 3). The number of alleles per locus in the 21 sweet cherry cultivars ranged from 3.00 to 8.00, with a mean of 5.80 (3.48 for the effective number of alleles, Ne) for the 15 polymorphic microsatellites scored. The average number of alleles per locus identified in this study is bigger compared to the number identified in other studies of sweet cherry. For example, Clarke and Tobutt (2003) genotyped fourteen sweet cherry cultivars using 19 SSR primer pairs and detected an average of 3.3 alleles per locus, whereas Dirlewanger et al. (2002) genotyped 21 sweet cherry cultivars from all over the world using 33 SSR primer pairs and identified an average of 2.80 alleles per locus.

The expected heterozygosity in the present study ranged from 0.337 to 0.804 with an average of 0.683; in the three earlier studies on sweet cherry, the expected heterozygosity averaged 0.600 (Dirlewanger et al. 2002), 0.460 (Clarke and Tobutt 2003) and 0.600 (Vaughan and Russell 2004). The observed heterozygosity ranged from 0.238 to 0.952 (mean 0.711). Expected and observed heterozygosity values were compared using the fixation index (F), which ranged between −0.386 and 0.363 and had an average over all loci of −0.032 (Table 3). The fixation index value indicates an overall excess of

Table 3 Genetic diversity among the 21 sweet cherry cultivars as revealed by SSR analysis

Locus	Linkage group[a]	Position (cM)	Na	Ne	Ho	He	F	PIC	P_{ID}
EMPA004	G6	48.7	6.000	4.388	0.952	0.772	−0.233	0.736	0.160
EMPA005	G1	32.7	3.000	2.549	0.619	0.608	−0.019	0.537	0.365
EMPA015	G4	1.7	7.000	4.410	0.857	0.773	−0.109	0.742	0.146
EMPA018	G8	33.5	8.000	3.196	0.667	0.687	0.030	0.665	0.164
EMPAS01	G6	28.0	5.000	3.500	0.857	0.714	−0.200	0.666	0.227
EMPAS02	G3	77.0	7.000	4.455	0.905	0.776	−0.167	0.748	0.133
EMPAS06	G4	25.9	8.000	4.570	0.857	0.781	−0.097	0.751	0.138
EMPAS10	G4	50.9	6.000	1.508	0.238	0.337	0.293	0.326	0.471
EMPAS11	G5	46.4	5.000	3.279	0.714	0.695	−0.028	0.656	0.211
EMPAS12	G3	38.5	6.000	3.955	0.476	0.747	0.363	0.704	0.193
EMPAS14	G5	45.9	4.000	3.328	0.857	0.700	−0.225	0.649	0.243
UDP96-005	G1	48.5	3.000	2.222	0.762	0.550	−0.386	0.480	0.413
UDP98-412	G6	76.9	7.000	2.665	0.476	0.625	0.238	0.592	0.239
UDP98-022	G1	124.8	5.000	3.139	0.714	0.681	−0.048	0.626	0.266
PceGA34	G2	86.8	7.000	5.098	0.714	0.804	0.111	0.779	0.111
Mean	–	–	5.800	3.484	0.711	0.683	−0.032	0.643	0.232
Total									8.265175 × e−11

[a] Indicated linkage groups and map positions according to Clarke et al. (2009)

Table 4 Details of ISSR primers used, number of markers obtained, polymorphism and genetic diversity indices of ISSR markers from 21 sweet cherry cultivars

Primer (UBC)	Annealing temperature (°C)[a]	Fragment size range	Fraction polymorphic fragments	Percentage polymorphism (%)	Gene diversity (GD)	Shannon index (I)	Resolving power (R_P)
811	52	820–2,700	2/8	25	0.404	0.591	1.99
816	52	800–2,100	3/4	75	0.375	0.562	5.60
821	48	1,700–2,200	2/3	66.6	0.485	0.678	3.24
823	54	640–2,540	5/11	45.5	0.389	0.568	5.29
827	55	1,180–3,100	5/10	50	0.353	0.521	5.26
834	55	560–2,600	10/16	62.5	0.354	0.525	11.05
841	55	530–1,940	6/12	50	0.354	0.527	5.43
860	52	530–2,000	4/6	66.6	0.344	0.524	4.76
881	60	600–3,000	9/14	64.2	0.295	0.460	5.21
891	58	560–2,000	5/7	71.4	0.339	0.513	6.20
Mean	–	–	5.1/9.1	57.7	0.369	0.546	5.24

[a] Determined empirically

heterozygosity in the material studied. The mean values for He (0.680) and Ho (0.711) were high and very similar because self-incompatibility in sweet cherry prevents selfing and reduces inbreeding.

The PIC values ranged from 0.326 to 0.779 with an average of 0.643. This particular PIC value (0.643) was similar to that (0.650) identified in another survey (Marchese et al. 2007). The P_{ID} values ranged from 0.111 to 0.471 with an average of 0.232. According to the values of PIC and P_{ID}, the most informative markers are the PceGA34 and EMPA004 (Table 3). In addition, MPR was calculated based on the most likely homozygote probability for each locus. MPR was $9.569 \times e{-}12$ across cultivars indicating that the probability of another unrelated cherry tree having the same genotype is very low.

The set of 10 ISSR primers showed multiband patterns in each cultivar (Table 4). This primer set amplified a total of 91 reliable and reproducible bands from the DNA of 21 sweet cherry cultivars tested. The total number of bands scored ranged from three to sixteen and the number of polymorphic bands from 2 to 10 per primer. Primer UBC821 resulted in the smallest number of bands (three) and primer UBC834 generated the largest number of bands (sixteen). The average number of bands per primer was 9.1. Band size ranged from 530 bp (UBC841 and UBC860) to 3.1 kb (UBC827). Among analyzed cultivars, 51 (57.95%) of the ISSR bands were polymorphic. The diversity of the Gene Bank collection was represented with Nei's gene diversity (GD), as well as Shannon's information index (I). Data for GD and I for all the 21 cultivars were analyzed using 10 ISSR markers and their corresponding mean values were found as 0.369 and 0.546 (Table 4). The R_P provided a modest indication of the ability of ISSR primers to distinguish between cultivars. The resolving power (R_P) of each primer was estimated in order to determinate the most informative ones for the discrimination between sweet cherry cultivars. The R_P of the 10 primers ranged from 1.99 for primer UBC811 to 11.05 for primer UBC834 (Table 4) with a mean value of 5.24. Three of the ISSR primers (UBC834, UBC891 and UBC816) possessed high R_P values (11.05, 6.20 and 5.60, respectively) and are the most efficient for surveying genetic diversity in the 21 cultivars of the Gene Bank collection.

The results provide guidance for future efficient use of these molecular methods in the genetic analysis of sweet cherry. The collection of primers used in this study gave a reasonable number of amplification products for the genetic diversity analysis. Based on the results, the genetic diversity among 21 cultivars is discussed. This study reveals the great importance of ensuring the differentiation of sweet cherry cultivars and their application for certification purposes. A comparison of genetic similarity matrices revealed that estimates based on SSRs and ISSRs

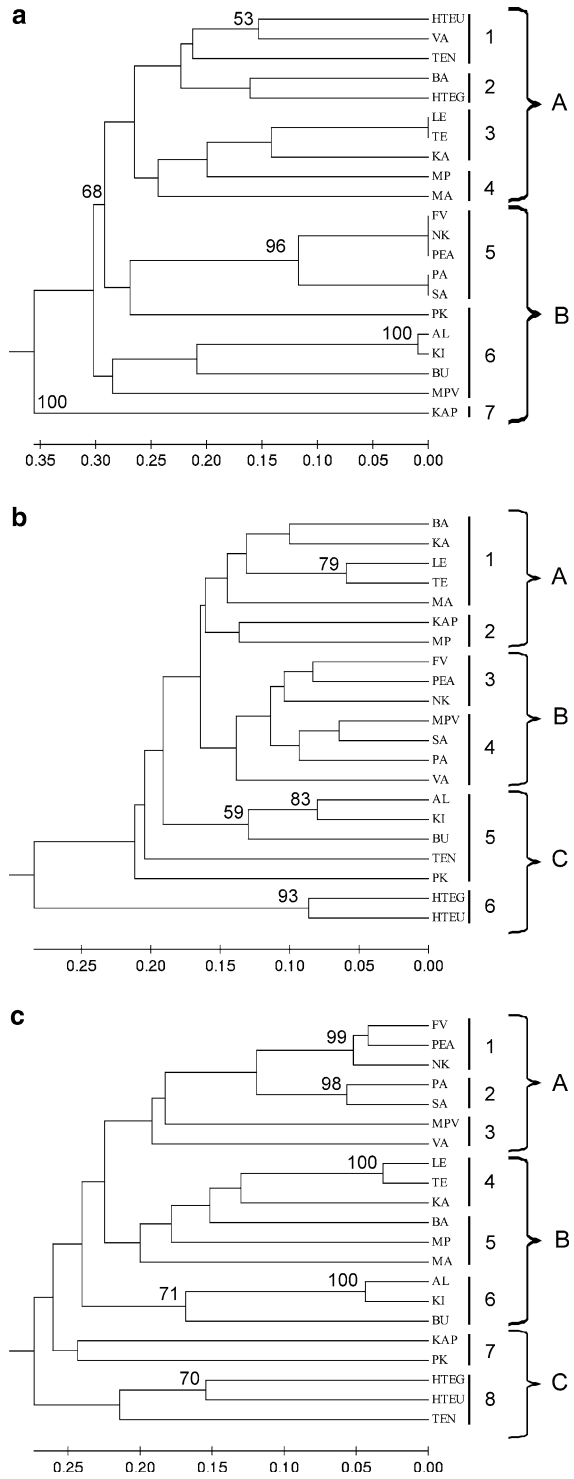

Fig. 2 Dendrograms of 21 sweet cherry cultivars based on UPGMA analysis of SSR (**a**), ISSR (**b**) and SSR/ISSR (**c**) polymorphisms (Table 1). The sweet cherry cultivars clustered into groups (*A*, *B* and *C*) and subgroups (numbers on *left*). Numbers below the lines indicate bootstrap values (percentage of 1,000 replicates). Bootstrap values greater than 50% are shown

are highly correlated, indicating congruence between these assays (data not shown).

Phylogenetic comparisons of SSR and ISSR markers

SSR markers distinguished 12 Greek sweet cherry cultivars with unique fingerprints, while 7 cultivars (LE–TE, FV–NK–PEA and PA–SA) fell into three non-distinguishable pairs indicating possible homonymy (Fig. 2a). The dendrogram displaying the molecular relationships among the 21 sweet cherry cultivars tested separates them into two main groups and several subgroups (A1–4 and B5–7; Fig. 2a). In Group A, we can distinguish clearly two sets of subgroups: Subgroups A-1 + A-2, having the cultivars BA, HTEG, HTEU, TEN and the reference cultivar 'Van' (VA). The cultivars AL and KI were found closely related and were grouped together (Subgroup B-6). The close genetic relationship of these cultivars has been confirmed in this study; however, one is not a clone of the other in the strict genetic sense. These cultivars differ in one of the 15 polymorphic loci tested.

For ISSR markers, the dendrogram generated from the UPGMA cluster analysis (Fig. 2b) based on the Nei and Li (1979) similarity index, classified the 21 sweet cherry cultivars in three main groups with two subgroups in each main group (A1–2, B3–4 and C5–6; Fig. 2b). Some sweet cherry cultivars originating from the same geographic location were found to be clustered closely. Two cultivars, namely MPV and SA, showed the closest relationship. The two hybrids, HTEG and HTE that classify in group C consist a distinct subgroup (C6) and they show the highest genetic distance compared to the remaining cultivars. Using the ISSR marker technology all the cultivars were distinguished, in contrast to the previously applied SSR method. Therefore the use of ISSRs may reflect a greater degree of genetic resolution than the SSR method, as was shown in the case of cultivars 'Lemonidi' (LE) and 'Tragana Edessis' (TE). These two cultivars were indistinguishable by the SSRs, whereas the application of ISSRs showed that these cultivars, although very

closely related, were indeed different. Similar results also obtained for the FV, NK, PEA and for the MPV and SA sweet cherry cultivars. ISSR markers, which were originally devised for discriminating among closely related plant cultivars (Wolfe 2005), were proven effective in our study with sweet cherries in distinguishing closely related cultivars that otherwise remain indistinguishable with the conventional SSR method. Another crop with a low level of variation is lemon (*Citrus limon*); new cultivars originate mainly from somatic mutation and nuclear variation. Although they are highly heterozygous, the majority of 57 lemon cultivars remained indistinguishable when analyzed with microsatellite markers whereas better discrimination was achieved by analyzing the same material using ISSRs (Gulsen and Roose 2001).

From the combined data analysis, we constructed a dendrogram in order to evaluate the resolving power of both techniques (Fig. 2c). The dendrogram produced by combined (SSR/ISSR) analysis data presents a higher resolution in separating the different sweet cherry cultivars. All sweet cherry cultivars were distinguished very well and clustered into three main groups which were subdivided into smaller groups (Fig. 2c). Group A is consisted of cultivars from Southern Greece and also the commercial cultivar Van (VA). Group B is consisted of three subgroups; in subgroup B6 are clustered Agiorgitika Lilantiou (AL) and Kifisias (KI) which are of unknown pedigree, suggesting a possible genetic relatedness between them. Another interesting feature is that cultivars Lemonidi (LE) and Tragana Edessis (TE), which were indistinguishable in SSR analysis and marginally separated by ISSR markers, were clearly distinguished by the combined analysis. Finally, Group C is consisted of two subgroups (C7, 8). Noteworthy, hybrids HTEG and HTEU are clustered together with their common parent TEN.

For the 21 sweet cherry cultivars analyzed in the present study, there exists a theoretical expectation for the clustering of cultivars within the dendrogram, based on their botanical pedigree. Genetic similarity dendrograms were constructed either using the results taken by SSR or ISSR analysis and by their combination. The best correlation with theoretical expectations is clearly shown by the dendrogram developed on the basis of ISSR results. The more convenient shape of the ISSR-based dendrogram can be explained by comparing the principles of each method. For example, every ISSR fragment represents an individual locus with two possible alleles (0—absent, 1—present). On this basis, the results presented in this report correspond to a total of 91 loci. The same interpretation was used for the evaluation of SSR results; although 92 fragments were evaluated, they in fact represented only 15 loci. After reading the above, the most important impression you should come away with is that there is no single method for all questions. For studies of cultivar identification, SSR experiments have a significant advantage over ISSR in that they are free of the poor agarose resolving power. On the other hand, ISSR experiments identify higher band sharing and a greater number of loci per assay. In these regards, ISSRs, even with their limitations are better suited to detect these types of individuals.

The prevailing opinion over the past few years is that microsatellite based approaches, either SSRs or ISSRs, are complementary methods to provide the most complete coverage of the genome. Hence, in the pedigree analysis, the combined SSR + ISSR analysis was found to be the most accurate system for the identification of cultivars from the same parental source.

Gene bank collection structure with SSR and ISSR markers

STRUCTURE analysis with SSRs (Fig. 3) showed that at $K = 2$ sweet cherry cultivars from Northern Greece were grouped together with the two international cultivars included in this study and four cultivars from Southern Greece (AL, KI, MPV and KAP), while the remaining five from Southern Greece were grouped separately. In contrast to what was expected for sweet cherries, not all cultivars clustered according to geographical origin. At $K = 3$, the Northern group was separated well enough from the Southern Greece sweet cherry cultivars. Cultivar 'Burlat' perhaps indicated a commonality in origin with the two cultivars from Southern Greece. At higher K values, the new groups were composed of individuals belonging to different clusters, making it difficult to identify the underlying classification criterion.

The results of the STRUCTURE analysis with ISSR markers are reported in Fig. 3. At $K = 2$

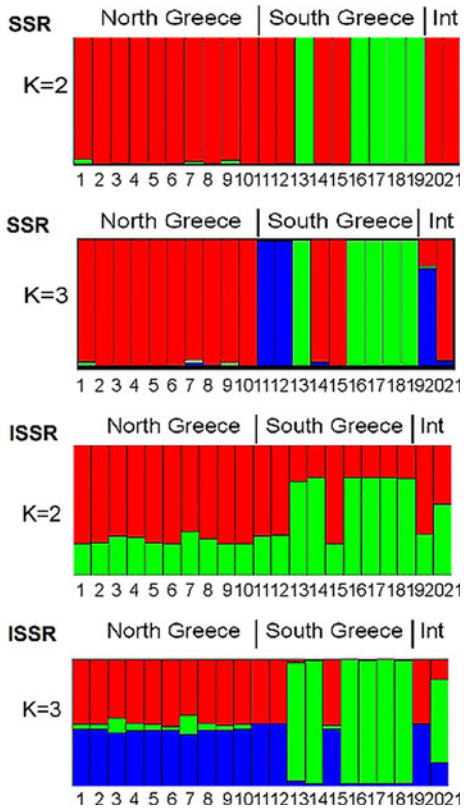

Fig. 3 Estimated geographic group structure for 21 sweet cherry cultivars with SSR and ISSR markers (Table 1). Each individual is represented by a *vertical line*, which is partitioned into K segments that represent the individual's estimated membership fractions in K clusters. *Different greyscale colors* indicate different geographic groups (North Greece, South Greece and International = INT). *Long black lines* indicate the separation among a priori assigned groups

cultivars were clustered similarly to STRUCTURE analysis with SSR markers, with the Northern Greek cultivars being in the same cluster with the two International cultivars 'Van' and 'Burlat'. At $K = 3$, the Southern Greek trees differentiated into a new population, together with one international cultivar ('Van'). With ISSR markers, the degree of relationship to southern Greece cultivars is evident.

Only a part of the cultivars clearly clustered according to the geographic origin, while others showed a partial or even predominant membership of populations of other regions, for example 'Van'. This finding is probably due to humans moving cultivars to different sites during the past several thousand years of sweet cherry cultivation. These results suggest that traditional cultivars of Greece which correspond to pre-defined regional groups partly belong to genetically distinct groups. The STRUCTURE analysis methodology has been previously used in wild and cultivated olives from the Mediterranean region by Baldoni et al. (2006) in order to investigate their genetic structure. It is interesting to note that most of the North Greece cultivars resolved in the same way obtained by STRUCTURE analyses both with SSR and ISSR markers.

SSR and ISSR markers associated with fruit traits through MRA

Trees and other crop types are routinely investigated using QTL technology, markers developed for traits with significant agronomic importance, using planned crosses. The developing markers could then be used in studies with a variety of other organisms, such as cereal species (rice: Virk et al. 1996; barley: Pakniyat et al. 1997; wheat: Breseghello and Sorrells 2006; Maccaferri et al. 2005), as well as tree crops such as mulberry (Kar et al. 2008; Vijayan et al. 2006). There are, however, two major limitations in this approach. Firstly, this technology is currently under development, therefore there is no established QTL information specifically on sweet cherry; moreover, tree planned crosses need years before fruit traits can be evaluated. To avoid such limitations, germplasm collections could be used (Virk et al. 1996). We therefore used a collection of 21 cultivars in order to find out the putative associations between a combination of established SSRs (Clarke and Tobutt 2003; Downey and Iezzoni 2000; Testolin et al. 2000; Vaughan and Russell 2004), ISSRs and morpho-physiological traits.

The SSR marker analysis revealed a number of markers showing statistically significant correlation with different phenotypic traits, as identified through MRA. Six markers were correlated with harvest time and four with fruit polar. The proportion of phenotypic variation accounted for by each primer pair (R^2) was calculated (Table 5). Marker $EMPAS02_{147}$ in particular was found to be associated with harvest time. MRA identified 14 SSR markers (alleles) in association with different phenotypic traits. These markers were correlated negatively or positively with the studied phenotypic characters. A combination of six markers accounted for up to 94.6% of the variation in fruit harvest time. The location on the

Table 5 Markers (SSR/ISSR) associated with different morpho-physiological traits in sweet cherry as revealed by MRA and the coefficients

	SSR markers (alleles)	*r*	R^2	R^2 change	*F* change	Standard error	Standardized beta coefficients	*t* value	*P* value
Harvest time	$EMPAS02_{147}$	0.624	0.390	0.390	12.125	0.778	0.587	6.770	0.002
	$+PceGA34_{160}$	0.764	0.584	0.195	8.442	0.311	−0.652	−8.093	0.000
	$+EMPA015_{241}$	0.854	0.729	0.145	9.085	0.299	0.633	8.175	0.000
	$+PceGA34_{143}$	0.906	0.822	0.092	8.295	0.314	−0.293	−3.786	0.000
	$+EMPAS06_{207}$	0.956	0.914	0.093	16.266	0.310	−0.443	−5.521	0.000
	$+EMPAS12_{142}$	0.973	0.946	0.032	8.359	0.387	0.284	2.891	0.000
Fruit polar diameter	$EMPA005_{248}$	0.578	0.334	0.334	9.535	0.355	−0.950	−7.841	0.000
	$+EMPAS14_{199}$	0.775	0.600	0.266	11.969	0.381	0.574	4.881	0.000
	$+EMPAS11_{110}$	0.859	0.739	0.138	9.000	0.486	0.408	3.515	0.000
	$+EMPA004_{185}$	0.907	0.823	0.085	7.680	0.349	−0.320	−2.771	0.000
Fruit weight	$+EMPAS02_{147}$	0.669	0.447	0.447	15.368	1.341	0.669	3.920	0.001
	ISSR markers (bands)	*r*	R^2	R^2 change	*F* change	Standard error	Standardized beta coefficients	*t* value	*P* value
Harvest time	$ISSR881_{600}$	0.600	0.359	0.359	8.977	1.697	0.970	4.620	0.000
	$+ISSR823_{1160}$	0.726	0.527	0.167	5.295	1.507	−0.590	−2.605	0.021
	$+ISSR816_{800}$	0.809	0.655	0.129	5.218	1.331	0.363	2.284	0.038
Skin color	$ISSR860_{680}$	0.640	0.409	0.409	11.090	1.009	−0.863	−7.574	0.000
	$+ISSR834_{1860}$	0.768	0.590	0.181	6.633	0.868	−0.439	−3.690	0.003
	$+ISSR821_{3000}$	0.849	0.661	0.130	6.515	0.742	−0.414	−3.811	0.002
	$+ISSR834_{2600}$	0.924	0.808	0.132	11.693	0.559	−0.378	−3.419	0.005
Soluble solids	$ISSR891_{1200}$	0.557	0.310	0.310	7.188	2.853	0.553	4.161	0.000
	$+ISSR881_{2200}$	0.796	0.634	0.324	13.252	2.147	−0.701	−5.009	0.001
	$+ISSR841_{1500}$	0.872	0.760	0.126	7.352	1.800	−0.384	−2.712	0.000

+ Denotes the inclusion of marker(s) in the preceding step(s) in the stepwise MRA

molecular linkage map of sweet cherry of the SSR markers used is known (Table 3) (Clarke et al. 2009). A total of six alleles ($EMPAS02_{147}$, $PceGA34_{160}$, $EMPA015_{241}$, $PceGA34_{143}$, $EMPAS06_{207}$, $EMPAS12_{142}$) showed strong correlation with harvest time. One of them, marker $EMPA015_{241}$ has a value beta coefficient (0.633) that showed a strongly positive correlation and it was statistically significant ($P < 0.001$, $t = 8.175$) with harvest time among the selected sweet cherry cultivars. The association of the marker $EMPAS02_{147}$ with the harvest time of sweet cherry cultivars is presented in Fig. 4. Similarly, the marker $EMPA005_{248}$ showed maximum (−0.950) and highly significant ($P < 0.001$, $t = -7.41$) negative correlation with fruit polar (Table 5). Finally, the marker $EMPAS02_{147}$ showed very high positive correlation with fruit weight. The significance of this marker based on beta coefficient (β) and *t*-value revealed that this is highly significant ($P < 0.001$, $t = 3.920$). The standardized beta coefficient was also very high (0.669) (Table 5).

Following the ISSR marker analysis the results revealed statistically significant association with different fruit traits (quantitative and qualitative), as identified through MRA. The markers identified varied from 4 for skin color and 3 for fruit harvest and for soluble solids. The proportion of phenotypic variation accounted for by each primer pair (R^2) was computed (Table 5). MRA identified 10 ISSR markers (bands) in association with different phenotypic traits. The statistical analysis of these markers based on beta coefficients and *t*-values revealed that they are all highly significant. Particularly, a combination of four ISSR markers ($UBC860_{680}$, $UBC8341_{860}$, $UBC821_{3000}$ and $UBC834_{2600}$) exhibited significant correlation with skin color. Out of them, the marker

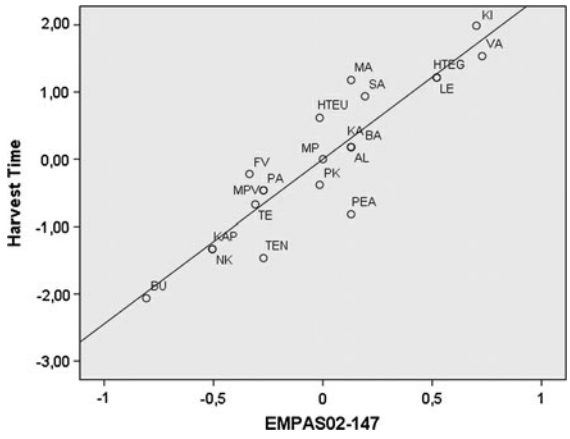

Fig. 4 Regression plot for harvest time with SSR marker $EMPAS02_{147}$. The *x*-axis variable is residuals from regressing $EMPAS02_{147}$ against the remaining independent variables

$UBC860_{680}$ has a beta coefficient value of (-0.863, $P < 0.001$, $t = -7.574$) and showing a strong and significant negative correlation with the skin color (Table 5). Lastly, the marker $UBC881_{600}$ showed very strong positive correlation ($\beta = 0.970$) with harvest time and highly significant ($P < 0.001$, $t = 4.620$) (Table 5). In conclusion we identified six SSR and three ISSR molecular markers showing very strong association with harvest time of sweet cherry fruits.

The methods described in this study provide a reliable and easy analysis for identification of promising cultivars at the early stages of breeding programs. Previously, an association analysis approach using MRA was adopted by Virk et al. (1996) to establish two qualitative traits, and by Kar et al. (2008) to identify a suite of markers linked to biochemical traits in mulberry tree. The MRA approach is a convenient tool for tree crops and a quick method for establishing marker–trait association avoiding the need for mapping populations. The markers identified in this study can be used for MAS breeding programs.

The obvious advantages of the stepwise multiple regression analysis are (a) that this could allow the detection of quantitative trait locus (QTL) that varies across a wide spectrum of biodiversity rather than just between two planned parental genotypes; (b) that QTL for any quantitative trait can be studied in the same investigation and (c) that this requires less inputs of time, labor and financial resources, compared to the linkage-based QTL identification (Ruan 2010). Our data reveal a high level of molecular diversity in sweet cherry cultivars. Such diversity has allowed the inference of the existence of a genetic structure that was factored into the association analysis.

Conclusion

The importance of the utilization of morpho-physiological and DNA markers in the management of the sweet cherry Gene Bank collection was established in this study improving the conservation and management of the relevant genetic resources. The genetic diversity data obtained is helpful in the identification of duplicate cultivars, verification of synonyms and homonyms and determination of misidentified cultivars. SSR and ISSR markers can be used in order to specify the gaps in the gene pools and organize the future additions. The application of the SSR/ISSR approach enables us to predict positive correlation between data generated by molecular markers and morpho-physiological traits.

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