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ORIGINAL PAPER

Is the genetic diversity of small scattered forest tree populations at the southern limits of their range more prone to stochastic events? A wild cherry case study by microsatellite-based markers

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Abstract Wild cherry (*Prunus avium* L.) is a widespread, partially asexual, noble hardwood European species characterized by a scattered distribution, small population sizes, and human exploitation for its valuable wood. These characteristics, especially at the southern limits of the species natural distribution where additional varying stresses may occur, render *P. avium* populations prone to potential stochastic, genetic, and demographic events. In this study, we used dominant inter simple sequence repeat (ISSR) and codominant simple sequence repeat (SSR) markers to infer the genetic structure of *P. avium*. Five populations from northern Greece were evaluated based on 46 ISSR and 11 SSR loci. Populations presented a relatively high level of genetic variation, with a mean genetic diversity of H_e=0.166 and H_e=0.740 regarding ISSR and SSR analysis, respectively. We observed moderate population differentiation for ISSR (G_{ST}=0.113) and SSR (F_{ST}=0.097) markers. AMOVA also detected significant differentiation among populations for ISSRs (Φ_{ST}=0.338) and SRRs (Φ_{ST}=0.162). According to linkage disequilibrium analysis, estimates of effective population size were generally sufficient for maintaining extant genetic variability and evolutionary potential. A possible bottleneck was detected for only one population. In general, it appears that despite the particular characteristics of the *P. avium* populations studied, genetic stochasticity events were not apparent. The studied populations, located at the rear edge of the species European distribution, reveal a wealth of genetic variation that is very valuable for the genetic conservation of local adaptive gene complexes, especially under contemporary climatic change scenarios.

Keywords *Prunus avium* · Genetic structure · ISSR · SSR · Conservation

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Introduction

Plant species that are partially asexual and present small, isolated populations of scattered individuals are more prone

to genetic, demographic, and environmental stochasticity (Ellstrand and Elam 1993), especially in southern areas of their distribution (rear-edge populations; Hampe and Petit 2005) where climatic change is expected to be more manifested. They are therefore at a risk of losing genetic diversity and future adaptive potential as a result of events such as decreased gene flow among populations, reduction of recombination as an effect of partially asexual reproduction, elevated levels of inbreeding, fixation of deleterious alleles, and higher occurrence of genetic drift. Demography is the main factor influencing genetic histories; nevertheless, neutral markers may present stochasticity in a given demographic context that is modulated by non-stochastic forces such as selection. The influence of the above factors in shaping extant population genetic diversity and structure is of paramount importance in order to secure the adaptive potential of populations and to formulate appropriate management and conservation strategies for sustainability (Tessier du Cros et al. 1999; Boshier and Amaral 2004; Belletti et al. 2008).

Wild cherry (*Prunus avium* L.) is a perennial woody angiosperm with a scattered distribution featuring small scattered populations at differential densities in nearly all regions of Europe (Beaver et al. 1995). *P. avium* is a strictly allogamous diploid ($2n=2x=16$; Crane and Brown 1937) reproduced asexually via suckering and sexually by insect pollination and subsequent seed dispersal by birds and mammals (Mohanty et al. 2001). It is an economically important noble hardwood species with valuable timber and edible fruits (Russell 2003). The trans-European distribution of wild cherry, and its cultivated form sweet cherry, is the result of a complex interplay between postglacial colonization and anthropogenic expansion. Chloroplast DNA studies in *P. avium* (even though partial and geographically incomplete) suggest the presence of different postglacial colonization routes (Russell 2003). Early domestication of *Prunus* has been indicated in Asia Minor since 7,000 BP (Behre 1978), and *P. avium* was apparently first cultivated in Greece since at least 2,300 BP according to Theophrastos (Hedrick 1915; Marshall 1954). It was introduced to western Europe by the Romans (Zohary and Hopf 2000), and there is accumulating evidence of multiple domestication events (Mariette et al. 2010). The current species distribution presents its southeastern limits in Greece, forming small isolated populations.

Initial investigations of the wild cherry genetic diversity were based on morphological traits (Weiser 1996; Meyer-Dingel et al. 1997), followed by analyses of isoenzyme variation (Santi et al. 1990; Frascaria et al. 1993; Mariette et al. 1997), AFLP markers (Tavaud et al. 2001), and the maternally inherited chloroplast DNA (Mohanty et al. 2001). The development of specific simple sequence repeat (SSR) markers (Clarke and Tobutt 2003; Vaughan and Russell 2004) allowed more detailed investigations of genetic diversity in *P. avium* (Schueler et al. 2006; Aravanopoulos et al. 2008; Guarino et al. 2009). Nevertheless, genetic information especially at the southern limits of the species natural distribution remains as scattered, as the species itself.

SSRs, as highly polymorphic codominant markers that evolve faster than cytoplasmic or nuclear genes, are particularly useful for resolving population structure at a finer geographic and evolutionary scale. Another microsatellite-based method, inter simple sequence repeats (ISSRs), permits the detection of polymorphisms in inter-microsatellite loci (Zietkiewicz et al. 1994). As different markers have different properties and reflect different aspects of genetic diversity (Karp et al. 1996), ISSRs may present an additional view of geographic diversity at the genetic level. ISSR analysis has been used for the assessment of genetic variation in numerous plant species, including *Castanea sativa* (Mattioni et al. 2008), *Morus* spp. (Kar et al. 2008), and cultivated *P. avium* (Ganopoulos et al. 2011). To our knowledge, this is the first time that ISSR analysis is applied to wild cherries.

The importance of rear-edge populations has been a matter of intense debate focusing on the validity of the center-periphery hypothesis that distinguishes between the abundant populations at the core of a species distribution and the small fragmented populations of its periphery (Lesica and Allendorf 1992; Hampe and Petit 2005; Eckert et al. 2008). Do rear-edge populations behave as predicted by this hypothesis and accordingly both effective population size and rate of gene flow should be highest at the "abundant" center and lowest at the edges of the species distribution range (Sagarin and Gaines 2002; Eckert et al. 2008)? Or, range-wide patterns of population genetic diversity are usually shaped by past climate-driven range dynamics rather than by demo-genetic stochasticity per se, and therefore, marginal rather than central populations commonly harbor the bulk of species genetic diversity (Hewitt 2000, 2004; Hampe and Petit 2005)?

According to the "center-periphery" model, rear-edge populations are of limited and questionable conservation (Lesica and Allendorf 1992) and potentially breeding value: they may face more rapid cycles of extinction, recolonization, and associated founder events or strong population bottlenecks that lead to a stochastic reduction of within-population genetic diversity and evidently of their evolutionary potential (Hoffman and Parsons 1996; Eckert et al. 2008). Their anticipated lower genetic diversity and higher genetic differentiation are further aggravated by their exposure to notable environmental pressure in contrast to central populations (Eckert et al. 2008). On the other hand, if recolonization history has been the main driver in shaping extant genetic diversity compared to demography and local conditions, then rear populations may constitute an invaluable reservoir of within-species genetic diversity (Petit et al. 2003; Hewitt 2004). Rear-edge populations may preserve

rare alleles and gene combinations important for adaptation to extreme environmental conditions (Lesica and Allendorf 1992; Hampe and Petit 2005) being often regarded extremely important for the survival and evolution of the biota (Hampe and Petit 2005). Their long-term persistence may have shaped current biogeographical patterns through its mitigating effect on extinctions. Rear-edge populations have likely played key roles in the maintenance of biodiversity throughout the Quaternary (Hampe and Petit 2005) and can therefore be considered as evolutionary key populations of utmost importance for conservation.

The objectives of this study were: (1) to assess and compare population genetic structure and diversity of natural *P. avium* rear-edge populations present in northern Greek forests, by employing dominant (ISSR) and codominant (SSR) molecular markers, (2) to investigate genetic divergence among natural populations, (3) to determine how demographic history and underlying stochasticity have determined extant population genetic structure, effective population size, and gene flow, and (4) to assist in establishing management and conservation practices for *P. avium* natural populations.

Materials and methods

Study site and sampling design

Five natural populations, covering most of the northern Greek range of *P. avium*, were sampled: three from the Region of Macedonia (Vorras, Cholomontas, Katafito) and two from the Region of Thrace (Hayntou, Nymphaea; Fig. 1). Levels of sample size per population were influenced by census population numbers, the noncontinuous distribution of wild cherry in nature, and the need to avoid sampling clonal or filial structures. According to field observations, populations consisted of a minimum of 80–400 trees present as isolated individuals or in small groups in mountainous terrain.

We collected young leaves from individual trees spaced at least 150 m apart in order to avoid sampling-related individuals. The sample size of each population ranged from 15 to 20 individuals (mean, 18.6); 93 individuals were sampled in total (Table 1). Collected leaves of each individual were stored at −80°C prior to DNA extraction.

DNA extraction and microsatellite-based analysis

Total genomic DNA was isolated using the procedure described by Doyle and Doyle (1987). The DNA amount was quantified by a UV spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany). Samples were then diluted to a 20-ng/μL working concentration.

For ISSR analysis, amplification was performed in a total volume of 25 μl including 30 ng of total cellular DNA, 200 mM of each dNTPs, 2 mM MgCl2, 40 pmol of primers, 2.5 μl of 10× Taq DNA polymerase buffer, and 1 U DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). PCR amplifications were performed in a PTC 200 (MJ Research) 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 1.5% agarose gel and stained with ethidium bromide. A 100-bp or 1-Kb DNA ladder (Invitrogen, USA) was used as a size marker. The selected ISSR primers (University of British Columbia, Canada; Table 2) were used for PCR amplification. Data points correspond to 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 the UVIDoc software (UVItec, Cambridge, UK) to quantify signal intensity. To ensure reproducibility, three control samples previously amplified using the same primers were included in each gel. Reproducibility was also tested by reamplification of a subset of 10% of the samples chosen at random. Two researchers performed DNA bands scoring independently; all ISSR reproducible fragments were recorded irrespective of their intensity.

For SSR analysis, PCR reactions were conducted in an 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′ labeled with IR700/IR800 dyes. Temperature profiles including annealing temperatures were those from Clarke and Tobutt (2003), Downey and Iezzoni (2000), Testolin et al. (2000), and Vaughan and Russell (2004). PCR reactions were electrophoresed in 25 cm long denaturing 6% polyacrylamide (SequaGel XR, Polymed) gels in 1× TBE buffer. PCR products were electrophoresed and visualized on 6.5% polyacrylamide gels using a LI-COR 4,300 DNA analyzer. Results were analyzed using LI-COR SAGA^{GT} software. Furthermore, the MICRO-CHECKER software was used to test the genotyping of microsatellite (SSR) data and to identify various genotyping and typographic errors (Van Oosterhout et al. 2004). The MICRO-CHECKER analysis suggested there were no indications for scoring error due to stuttering or due to large allele dropout.

Data analysis

Microsatellite-based (ISSR and SSR) variation and genetic diversity within populations

The percentage of polymorphic loci (*P*), effective numbers of alleles (n_e), gene diversity (expected heterozygosity, H_e),

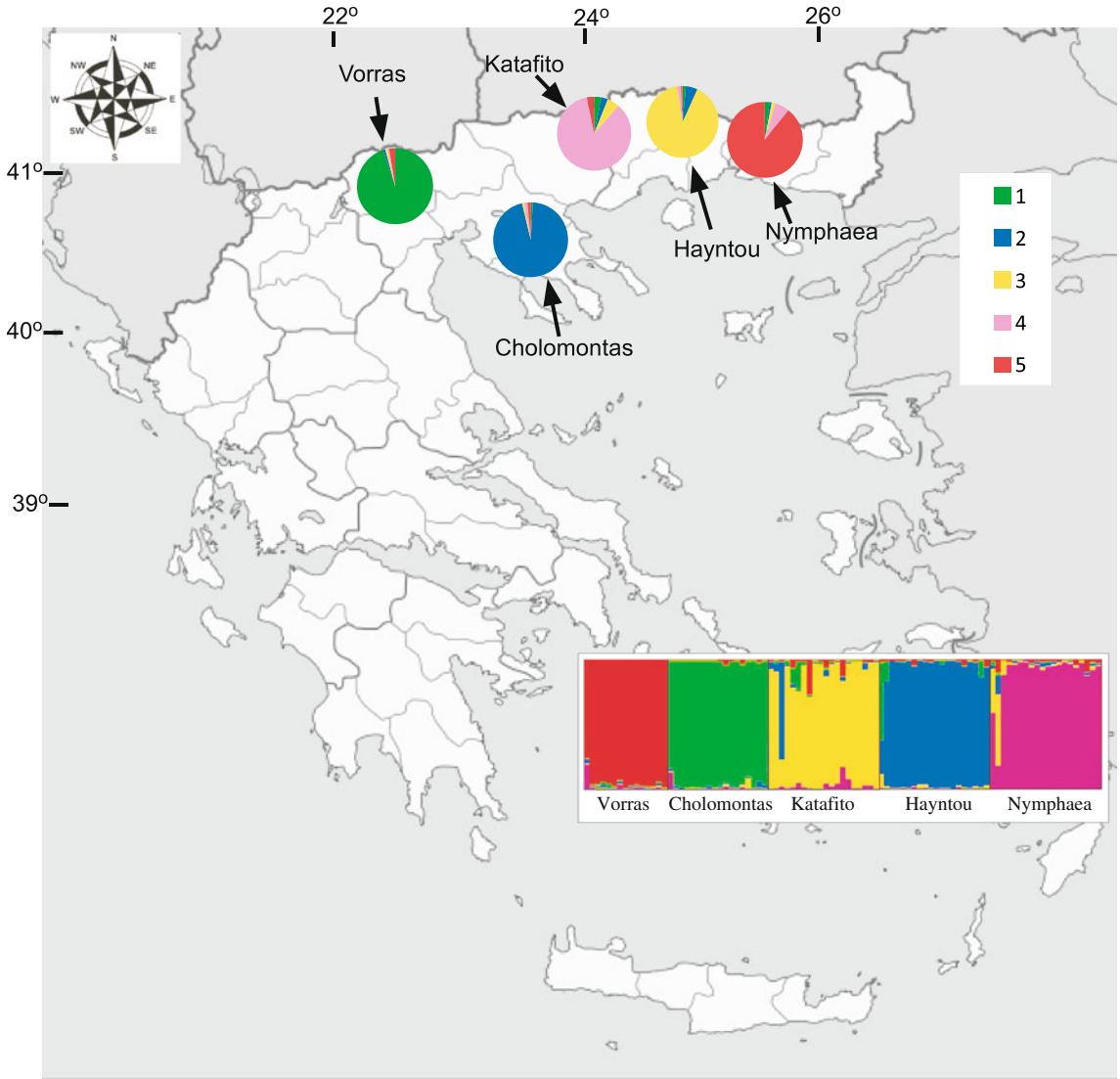

Fig. 1 Distribution of cluster memberships at individual and population levels in the five P. *avium* populations, obtained using the STRUCTURE method (Pritchard et al. 2000). Number of clusters, $K=5$. Each *vertical bar* in the histograms (*lower panels*) represents the proportion of cluster memberships in each individual. The *pie charts* superimposed on the maps (*upper panels*) show the average proportions of cluster memberships among the individuals sampled in each population

and Shannon's diversity index (I) were calculated using PopGene32 (Yeh et al. 1999), for both markers. ISSRs are dominant markers, and each band represents the phenotype at a single biallelic locus. Only bands that could be unambiguously scored were used in the analysis. ISSR amplified bands were scored for band presence (1) or absence (0), and a binary qualitative data matrix was formed. For SSR data, we used the hierarchical rarefaction method available in HP-RARE (Kalinowski 2005) to calculate, in addition to the above parameters, allelic

Table 1 Geographic locality of *Prunus avium* natural populations in this study

Population code	Geographic localities	Latitude	Longitude	Altitude (m)	Estimated census population size	Sample size
VOR	Vorras, Imathia	21° 95′	40° 93′	1,200	80	15
HOL	Cholomontas, Chalkidiki	23° 52′	40° 42′	800	100	18
KAT	Katafito, Drama	23° 69′	41° 35′	1,000	900	20
HAI	Hayntou, Xanthi	24° 66	41° 30′	1,200	200	20
NIM	Nimphaea, Rodopi	25° 45′	41° 22′	700	400	20

Table 2 Characteristics and genetic diversity parameters of ISSR and SSR primers used in this study

Primer (UBC)	Annealing Temperature (°C)	Fragment size range	Fraction polymorphic fragments	Percentage of polymorphism (%)	Locus	References	n_a	Allele size (bp)	H_e	F_{ST}	R_{ST}	F_{IS}	f_n
811	52	400–2,100	6/8	75	EMPA004	Clarke and Tobutt 2003	12	178–206	0.756	0.068	0.141	0.039	0.030
827	55	800/3,100	7/9	77.7	EMPA005	Clarke and Tobutt 2003	13	231–255	0.771	0.119	0.216	0.064	0.031
834	55	560–2,600	14/14	100	EMPAS002	Vaughan and Russell (2004)	15	128–162	0.752	0.133	0.012	0.134	0.045
841	55	500–2,000	7/10	70	EMPAS006	Vaughan and Russell (2004)	12	203–229	0.798	0.082	0.098	0.148*	0.066
881	60	600–3,000	10/13	77	EMPAS010	Vaughan and Russell (2004)	14	148–188	0.723	0.099	0.061	−0.007*	0.000
					EMPAS011	Vaughan and Russell (2004)	15	66–110	0.772	0.118	0.027	0.133	0.047
					EMPAS012	Vaughan and Russell (2004)	13	118–148	0.742	0.093	0.027	0.036	0.015
					EMPAS014	Vaughan and Russell (2004)	8	194–212	0.560	0.078	0.068	0.291*	0.111
					PS12A	Sosinski et al. (2000)	12	147–186	0.706	0.107	0.056	0.040	0.028
					UDP98–412	Testolin et al. (2000)	11	98–128	0.778	0.028	0.018	0.049	0.052
					PCEGA34	Downey and Iezzoni (2000)	15	142–174	0.784	0.126	0.233	0.287*	0.117
Mean	–	–		79.9			12.7	–	0.740	0.095	0.114	0.110	0.049

n_a number of alleles, H_e gene diversity, F_{ST} gene differentiation coefficient, R_{ST} unbiased gene differentiation coefficient, F_{IS} inbreeding coefficient, f_n null alleles frequency

*$P<0.05$, significant deviations from zero

richness (AR), private allelic richness (pAR; alleles which are unique to a particular population), and the inbreeding coefficient (F_{IS}). As null alleles were suspected in some loci, allelic frequency estimation and comparison between observed and Hardy–Weinberg expected heterozygote frequencies were computed by the maximum likelihood method described in Kalinowski and Taper (2006). Following null allele detection, F_{IS} was also estimated after removing three loci (EMPAS006, EMPAS014, and PCEGA34) where null allele frequency exceeded 6% (Dakin and Avise 2004; Chapuis and Estoup 2007). Conformance to Hardy–Weinberg equilibrium was determined by assessing the significance of the F_{IS} values by means of Fisher's exact tests implemented in GENEPOP with specified Markov chain parameters of 5,000 dememorization steps, followed by 1,000 batches of 5,000 iterations per batch. The sequential Bonferroni correction was applied to obtain critical confidence limits for multiple comparisons, with an initial probability $p=0.05$.

Genetic divergence between populations

Gene differentiation coefficients (G_{ST}) for ISSR markers were calculated using PopGene32 (Yeh et al. 1999). For SSRs, the program FSTAT was used to calculate F_{ST} (Weir and Cockerham 1984) and R_{ST} (Slatkin 1995) across all populations, while pairwise population differentiation was evaluated using FSTAT (Goudet 1995) and RSTCALC (Goodman 1997), respectively. A total of 1,000 randomizations were used to determine the statistical significance of the estimates; means and significant values over loci, populations, and subdivisions were obtained by jackknifing. The GENEPOP (Raymond and Rousset 1995) software (http://genepop.curtin.edu.au/) was used to test for linkage disequilibrium between all pairs of loci after Bonferroni correction.

The hierarchical distribution of genetic variation among and within populations for both markers was also characterized by an analysis of molecular variance (AMOVA; Excoffier et al. 1992; Michalakis and Excoffier 1996). We conducted a hierarchical AMOVA using the GENALEX 6 software (Peakall and Smouse 2006). The tests were implemented using estimates of Φ_{ST} based on distances calculated from allelic data. Tests of significance were performed using 9,999 permutations within the total dataset.

Unbiased genetic distances among populations were estimated according to (Nei 1978) by using PopGene32 (Yeh et al. 1999). The relationships among populations were initially investigated by an unweighted pair group method using arithmetic means (UPGMA) dendrograms based on Nei's (1978) genetic distances which were constructed for both ISSR and SSR markers. The reliability of the trees was tested using bootstrap estimates (Felsenstein

1985). High bootstrap scores (>90%) suggest strong support for a particular cluster. The DISPAN computer program was used for the UPGMA tree constructions and bootstrap analysis (Ota 1993). Principal coordinate analysis (PCoA) was also used to visualize the genetic structure based on the ISSR data set. The analysis was executed in GENALEX (Peakall and Smouse 2006). The nature of the SSR data set permitted a more elaborate ordination analysis, in particular, the factorial correspondence analysis (FCA) using the GENETIX 4.05 software (Belkhir 1999). A model-based Bayesian clustering method was applied to ISSR and SSR data to infer genetic structure and define the number of clusters (gene pools) in the dataset using the software STRUCTURE version 2.3.2 (Pritchard et al. 2000). We ran the program using the admixture model and the correlated allele frequencies option, which are considered most appropriate for detecting structure among populations that are likely to be similar due to migration or shared ancestry (Falush et al. 2003). The STRUCTURE software 2.2 uses a Markov chain–Monte Carlo procedure to infer unstructured subpopulations, without considering prior classification within the sample. The proportional membership of each cluster was estimated based on its genotype. The same Bayesian analysis was applied to ISSRs where each class of genotypes was treated as being a haploid allele.

Assessment of effective population size, genetic drift, gene flow, and population bottlenecks

We quantified drift by estimating effective sizes of the studied populations (N_E) by employing two different methodologies: a point estimation method, based on linkage disequilibrium (Hill 1981), was applied to determine the effective size of each population using the calculation suggested by Bartley et al. (1992) and implemented by the N_EEstimator software (Peel et al. 2004). The second method uses more than one-locus genetic data to estimate the population effective size and is expected to give reliable evaluations also for small populations with a limited number of microsatellite loci (Vitalis and Couvet 2001). The estimation was based on a Wright–Fisher island model, which assumes that all migrants originate from a random sample of all populations. The ESTIM 1.2. software (Vitalis and Couvet 2001) was used. Results of both methods were compared. Historical gene flow (N_m) among populations was estimated indirectly based on the G_{ST} (ISSR) and R_{ST} (SSR) statistics. In addition, we estimated gene flow using Slatkin's (1985) private allele method, which is based on the average frequency of unique alleles found in local populations.

Genetic effects of demography in populations were examined based on SSR markers following two approaches. Initially, recent population bottlenecks were investigated using the empirical approach of Luikart and Cornuet (1998), which compares gene diversity with the expected equilibrium gene diversity calculated from the observed number of alleles under mutation-drift equilibrium. If a significant number of loci show excessive gene diversity, then the population is likely to have undergone a recent bottleneck. The probability distribution was estimated using a Wilcoxon test with 10,000 simulations. A two-phase model (TPM) was advocated as an appropriate mutation model after an allele permutation test was applied to assess whether stepwise mutation had contributed to population differentiation ($R_{ST}>F_{ST}$, Hardy et al. 2003). The second approach involved testing a mode shift away from an L-shaped distribution of allelic frequencies (Luikart and Cornuet 1998). Both analyses were conducted using BOTTLENECK ver. 1.2.02 (Cornuet and Luikart 1996; Piry et al. 1999).

Results

Genetic diversity within populations

A total of 46 ISSR bands were obtained from the five selected primers that corresponded to an average of 9.2 bands per primer. The size of the ISSR bands ranged from 400 to 3,100 bp (Table 2). The percentage of polymorphic loci (P) at the population level ranged from 50% (population Vorras) to 69.57% (population Hayntou), with an average value of 59.13%. The effective number of alleles per locus (n_e) ranged from 1.238 (population Vorras) to 1.306 (population Hayntou) with an average value of 1.270 (Table 3). The value of Shannon's I showed similar trends and ranged from 0.225 to 0.295 in populations Vorras and Hayntou, respectively (mean I=0.258). Nei's (1978) gene diversity (H_e) at the population level ranged from 0.145 (population Vorras) to 0.189 (population Hayntou) with an average value of 0.166. Results showed that the genetic diversity of *P. avium* from the Hayntou population was the richest among the five populations. The Vorras population presented the poorest genetic diversity. The genetic diversity of the total sample was relatively high (P=100%, n_e=1.329, I=0.338, H_e=0.209; Table 3).

For SSR data, a total of 140 alleles were observed across the 11 loci studied. The number of alleles per locus ranged from 8 (EMPAS014) to 15 (EMPAS002; EMPAS011; PCEGA34), with a mean value of A=12.7. The frequency of null alleles (f_n) was generally low (mean value $f_n \leq 0.05$) and was more pronounced ($0.10 \leq f_n \leq 0.12$) in two loci only (EMPAS014, PCEGA34), while the expected heterozygosity (H_e) ranged from 0.560 (EMPAS014) to 0.798 (EMPAS006). Null alleles, which may result from an accumulation of mutations in the primer binding sites, were

Table 3 Genetic diversity at population and species levels in five natural *Prunus avium* populations

Populations	ISSR					SSR							
	n_e	I	H_e	P (%)	G_{ST}	n_e	H_e	AR	pAR	F_{IS}	F_{IS} after null allele correction	F_{ST}	R_{ST}
Vorras	1.238	0.225	0.145	50.00		4.120	0.734	6.833	0.860	0.004	−0,016		
Cholomontas	1.304	0.285	0.185	63.04		3.952	0.730	6.939	1.050	0.083*	0.072		
Katafito	1.254	0.241	0.155	54.35		4.520	0.765	6.500	0.320	0.117*	0.117*		
Hayntou	1.306	0.295	0.189	69.57		4.315	0.720	7.519	0.500	0.093	0.053		
Nimphaea	1.249	0.241	0.154	58.70		4.360	0.752	6.455	0.380	0.102	0.142		
Mean	1.270	0.258	0.166	59.13			0.740	6.849	0.622	0.079	0.073		
Species level	1.329	0.338	0.209	100	0.113		0.824					0.097	0.123

P percentage of polymorphic loci, H_e gene diversity (expected heterozygosity), *I* Shannon's index, F_{ST} gene differentiation coefficient (SSR), G_{ST} gene differentiation coefficient (ISSR), F_{IS} inbreeding coefficient, n_e effective number of alleles; *AR* allelic richness, *pAR* private allelic richness, R_{ST} unbiased gene differentiation coefficient

*$P<0.05$, significant deviations from zero

detected. Three loci (EMPAS006, EMPAS014, and PCEGA34) where null allele frequency exceeded 6% were excluded during F_{IS} calculations (Chapuis and Estoup 2007). The inbreeding coefficient (F_{IS} without null alleles) ranged from −0.007 (EMPAS010) to 0.291 (EMPAS014). At four loci (EMPAS006, EMPAS010, EMPAS014, and PCEGA34) the F_{IS} value deviated significantly from zero (Table 2). The number of alleles per locus independent of sample size AR (the allelic richness) ranged from 6.455 (Nimphaea) to 6.939 (Cholomontas). Private alleles were also noticed, and on the average, private allelic richness (pAR) amounted to about 10% of total AR. The average heterozygosity was highest in Katafito (0.765) and lowest in Hayntou (0.720). At the population level, SSR indicated high levels of genetic variation in Katafito and Nimphaea and relatively high levels in the three remaining populations of Cholomontas, Hayntou, and Vorras (Table 3). Two out of the five populations showed significant positive F_{IS} values, indicating a heterozygote deficit in *P. avium* populations, especially manifested in the Katafito population (F_{IS}=0.117, Table 3).

Genetic divergence between populations

Genetic differentiation is an important index in order to evaluate the population genetic structure of a species. For ISSR markers, the inter-population coefficient (G_{ST}) of 0.113 indicated genetic differentiation among populations. With regard to linkage disequilibrium, there was one population (Hayntou) that presented a high number of loci combinations (>20 out of a total of 65) with significant values ($p<0.05$). After Bonferroni correction, there was significant (at $p<0.05$) linkage disequilibrium between 23 pairs of loci in the Hayntou population and between 15 pairs in the Katafito population. The lowest number of significant combinations was detected in the Vorras population, where only five locus pairs had significant ($p<0.05$) linkage disequilibrium values.

According to AMOVA of the ISSR data set, there were highly significant ($p<0.001$) genetic differences among the five *P. avium* populations. Thirty-six percent of the total genetic diversity was attributed to among-population differentiation. Thus, AMOVA (Φ_{ST}=0.338) also supports the results of Nei's (1978) gene diversity statistics and Shannon's *I*, indicating genetic differentiation among populations (Table 4). To elucidate further gene differentiation among populations, Nei's (1978) unbiased genetic distances were evaluated (Table S1). Nei's distances ranged from 0.028 to 0.232 based on the ISSR analysis (below the diagonal) with an average 0.109. The largest genetic difference (0.232) occurred between Vorras and Hayntou populations and the least (0.028) between Katafito and

Table 4 Analysis of molecular variance results for five natural *Prunus avium* populations based on ISSR and SSR markers

	Source of variation	*df*	SSD	Variance component	Total variance (%)	Φ statistics	*P* value
ISSR	Among populations	4	185.983	2.267	34		
	Within populations	88	391.372	4.447	66	Φ_{ST}=0.338	<0.001
	Total	92	577.355	6.714	100		
SSR	Among populations	4	170.754	1,800	16		
	Within populations	88	818.622	9,303	84	Φ_{ST}=0.162	<0.001
	Total	93	989.376	11,102	100		

Hayntou populations. Relatedness among populations was illustrated by a dendrogram using the UPGMA algorithm based on Nei's (1978) distance. An ISSR data-based dendrogram grouped the five populations into two main clusters (Fig. 2). The Vorras population formed a separate cluster showing less similarity to the other four populations studied. The latter cluster was further divided into two sub-clusters consisting of Cholomontas, Hayntou, and Katafito (first subgroup) and Nimphaea (second subgroup). Furthermore, PCoA results illustrated three major clusters, which was congruent to the UPGMA dendrogram (Fig. 2) especially regarding the clear separation of Vorras and the formation of a large cluster corresponding to the remaining populations. The first two principal coordinates accounted for 56.36% of the total variation (Fig. 3).

F_{ST} parameters varied among SSR loci, ranging from 0.028 (UDP98−412) to 0.126 (PCEGA34; Table 2). Global genetic differentiation across all populations for SSR markers was estimated as F_{ST} and R_{ST} (0.097 and 0.123, respectively). All pairwise F_{ST} (0.760–0128) and R_{ST} (0.823–0.147) values were highly significant ($p<0.001$; Table S2). Gene differentiation coefficients (F_{ST}, R_{ST}) suggested significant population differentiation in *P. avium* at the species level (moderately high F_{ST} values, Table 3). The AMOVA of the SSR data set produced congruent results (significant Φ statistics, Table 4). AMOVA also revealed a high percentage of variation due to population subdivision (88%). Nei's (1978) unbiased genetic distances ranged from 0.383 (Katafito vs. Nimphaea) to 0.674 (Cholomontas vs. Nimphaea; Table S1). After UPGMA, the *P. avium* populations were clustered into two groups (Fig. 2). The FCA of the SSR data set presented a clear separation of the populations (Fig. 4). Overall, a high amount of the total genetic variation (79%) was explained in three-dimensional

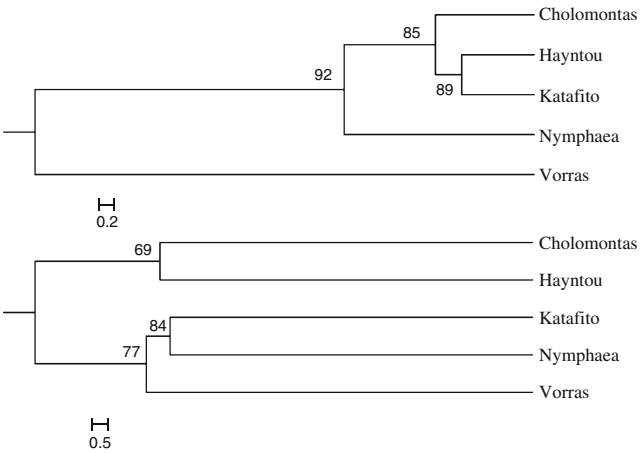

Fig. 2 UPGMA dendrogram, derived from Nei's (1978) unbiased genetic distances, showing the relationships among the five examined populations of wild cherry using ISSR (**a**) and SSR (**b**) datasets. Branch lengths are proportional to genetic distances. The *bootstrap values* (1,000 replicates) are given as a percentage at nodes

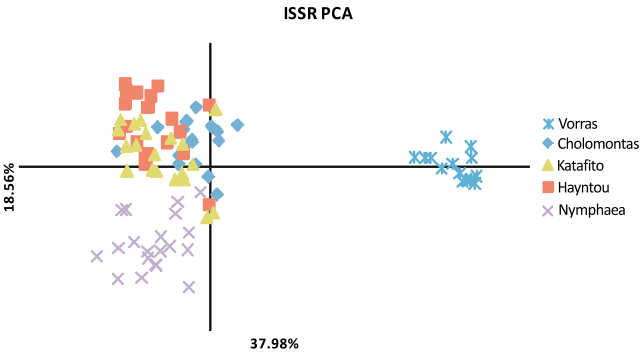

Fig. 3 Scatter plot of the first and second principal coordinates based on the molecular variation of ISSR markers for five natural *P. avium* populations

multivariate space. In Axis 1 individual populations were separated. More interesting was the partition at Axis 2 that revealed the formation of two groups: the first (Hayntou, Katafito, Nimphaea) corresponds to the north-eastern Greek *P. avium* populations and the second (Cholomontas, Vorras) to the north-central Greek populations.

STRUCTURE analysis with SSR markers was performed without prior information on the geographic origin of samples, and the highest likelihood of the data was obtained for $K=5$. The data set was partitioned into clusters or gene pools corresponding roughly to geography (Fig. 1): individuals from Cholomontas, Hayntou, Katafito, Nimphaea, and Vorras tended to be classified in separate clusters. Low levels of admixture were observed in four populations. A moderate level of admixture was detected in the Katafito population. The same Bayesian analysis was applied on ISSR data, resulting in very similar clustering of genotypes (results not shown). Some concordance of population clustering to geography was also evident in previous analyses, particularly in the FCA (Fig. 4), although not at such a refined level.

Assessment of effective population size, genetic drift, gene flow, and population bottlenecks

The populations with the highest effective population sizes calculated with N_E Estimator were Nimphaea and Katafito (N_E ranging from 82.3 to 84.1; Table 5). The population with the smallest effective size was Vorras (N_E=44.3. 95% confidence interval (CI), 27.7–98.5). The linkage disequilibrium method (Hill 1981) also provided low to moderate N_E estimates (mean across populations N_E=64.54), but considerably wider confidence intervals (95% CI ranging from 27.7 to 269.9). Estimated effective population sizes based on the ESTIM software for all *P. avium* populations ranged from N_E=49.93 (Cholomontas; 95% CI=19.05–∞) to infinity (Nimphaea; 95% CI=49.93–∞). Notably, the lower 95% CIs were about 40 for all north-eastern populations (Table 5).

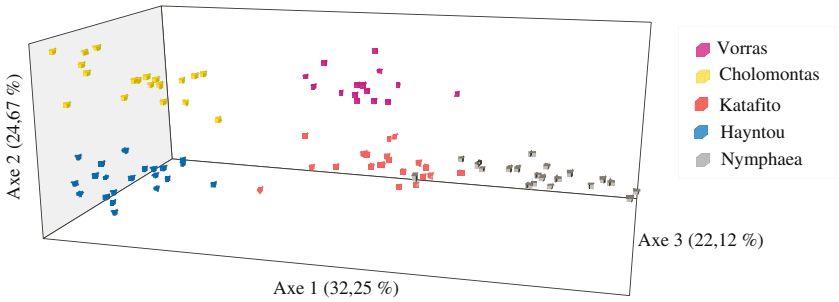

Fig. 4 Ordination of five natural *P. avium* populations depicted after a factorial correspondence analysis (FCA) based on SSR data

The indirect estimates of historical gene flow (N_m), inferred from G_{ST} (ISSR) and R_{ST} (SSR) were 1.962 and 1.782, respectively. The respective N_m calculated from the private allele method (Slatkin 1985) was 1.190. Apparently, all these estimates consistently exhibit a low level of historical gene flow between the studied *P. avium* populations. This result was further reinforced by an assignment analysis (using the GENECLASS2 software; results not shown), which detected only a single first-generation migrant between the Hayntou and Katafito populations, suggesting almost no contemporary gene flow taking place among natural populations of the species.

Population bottlenecks were investigated with the TPM model after an allele size permutation test (Hardy et al. 2003) indicated that mutations do not appear to contribute to genetic differentiation at this scale (data not shown). When the TPM model was employed, the Wilcoxon signed-rank tests used for detecting bottlenecks in the populations indicated a rather weak bottleneck ($p<0.05$) in the Nimphaea population only (Table 5).

Discussion

Genetic diversity within populations

Microsatellite-based markers in population genetics are highly powerful for the detection of differentiation due to their high levels of polymorphism. Using ISSR and SSR markers, we estimated population genetic diversity and investigated genetic structure in five natural *P. avium* populations. The mean genetic diversity within populations (H_e=0.740) was high and in agreement with the elevated microsatellite heterozygosity found in most tree species (Sanou et al. 2005). In fact, it was higher than corresponding values from other studies in managed and unmanaged *P. avium* populations that originated from the central and northern extant wild cherry distribution range (France, Central Europe, UK; Mariette et al. 2010; Stoeckel et al. 2006; Vaughan et al. 2007). Average allelic richness across loci was also high (AR=6.849), but lower than the mean values reported from SSR *P. avium* studies (Stoeckel et al. 2006; Vaughan et al. 2007; Mariette et al. 2010). The same trend was found after comparing average H_e and AR values from our study to that of Vaughan et al. (2007) who used populations from the northern part of the species contemporary range in UK. An evaluation of the same ten common SSR loci showed that H_e and AR were 0.744 and 6.590 in our study and 0.692 and 7.524 in the Vaughan et al. (2007) study. Private allelic richness could not be compared with earlier *P. avium* studies as it has not been reported before for wild cherry. Allelic richness is potentially more informative than expected heterozygosity in assessing genetic erosion in natural populations (Spencer et al. 2000), being more sensitive to the effects of demographic bottlenecks and their immediate consequences. High levels of variation among populations were also observed based on Shannon's diversity index (I=0.338 for ISSR markers). The above estimate was higher than those previously obtained in *Prunus mahaleb* and *Prunus*

Table 5 Estimation of effective population size, tests for mutation-drift equilibrium, probabilities from Wilcoxon signed-rank tests for heterozygosity excess and mode shift using BOTTLENECK for five natural *Prunus avium* populations (values in parentheses indicate 95% confidence intervals)

Population	N_E Estimator	N_E ESTIM	TPM	Mode shift
Cholomontas	44.5 (30.3–78.6)	49.93 (19.05–∞)	0.5170 (ns)	Normal
Hayntou	67.5 (42.7–146.3)	183.75 (39.14–∞)	0.5170 (ns)	Normal
Katafito	84.1 (51.1–211.5)	202.47 (40.28–∞)	0.6499 (ns)	Normal
Nimphaea	82.3 (46.9–269.9)	∞ (53.45–∞)	0.0268*	Normal
Vorras	44.3 (27.7–98.5)	237.87 (22.51–∞)	0.1030 (ns)	Normal

N_E effective population size, *TPM* two-phase model, *ns* nonsignificant test

*$p<0.05$

africana based on RAPD markers (Dawson and Powell 1999; Jordano and Godoy 2000). It appears that our populations generally present adequate, if not high, amounts of genetic diversity, which attest to their future stability. This result differentiates from what might have been expected for glacial refugial populations which have been considered as sanctuaries for allelic richness, but not for gene diversity (Widmer and Lexer 2001). In this case, it is clear that our peripheral populations, compared to core populations from France and the UK, present lower allelic richness and higher gene diversity. As such, our results do not appear to conform to the center-periphery model (Lesica and Allendorf 1992), and these populations present considerable amounts of genetic diversity.

An excess of homozygotes was found for ten out of 11 microsatellite loci under study. For all populations, a positive inbreeding coefficient was observed (mean F_{IS}= 0.079), but the F_{IS} value was significant in two out of the five populations studied. Mariette et al. (2010) also reported a heterozygote deficiency in natural wild cherry populations (F_{IS}=0.04). In general, the overall F_{IS} value suggests a weak departure from Hardy–Weinberg equilibrium due to heterozygote deficiency. This result is to some extent differing to the majority of earlier results from natural *P. avium* populations where heterozygote excess has been reported in populations originating from the core of the species natural distribution (France, Italy; Ducci and Santi 1997; Frascaria et al. 1993; Stoeckel et al. 2006). Heterozygote excess is likely the result of the extensive clonality of *P. avium* natural populations (Stoeckel et al. 2006; Mariette et al. 2010). This suggestion could provide an explanation to the discrepancy between our results and the earlier ones, as in our case individuals were sampled at least 150 m apart. When sampling of clonal or filial structures is avoided, the population at large may not be characterized by heterozygosity excess. Finally, the increased levels of homozygosity and the departure from Hardy–Weinberg equilibrium could also be attributable to mating between related individuals, small population size, or the scattered distribution of *P. avium*, which may not form always random mating populations (Durel et al. 1996; Boys et al. 2005; Degen et al. 2006).

Genetic divergence between populations

SSRs provided higher within-population genetic diversity and lower genetic differentiation among populations than ISSRs, probably due to the higher polymorphism of the former (Gaudeul et al. 2004) and to the potential underestimation of ISSR heterozygosity (Tang et al. 2008). However, similar estimates of the relative differentiation among populations for ISSRs and SSRs were observed (G_{ST}=0.113; F_{ST}=0.097; Table 3). As expected, AMOVA results indicated that most of the variation was partitioned within populations. Among-population variation was highly significant and reached 34% in the ISSR analysis, but only 16% in the SSR analysis (Table 5). The lower differentiation observed in the SSRs analysis may reflect higher SSR mutation rates (Hedrick 1999; Schlötterer 2000; Queney et al. 2001; Tang et al. 2008) or homoplasy (Gaudeul et al. 2004). The above estimates were higher than those obtained in previous investigations of *P. avium*, based on different types of genetic markers. Santi (1988), Frascaria et al. (1993) and Mariette et al. (1997) found a relatively low genetic differentiation among samples of four (F_{ST}=0.012), four (F_{ST}=0.049), and five populations (F_{ST}=0.052) using isoenzyme markers in populations originating from the central range of the species in France. Stoeckel et al. (2006) found an F_{ST}=0.066 using SSR and S-allele markers studying *P. avium* populations in France, while Vaughan et al. (2007) reported an F_{ST}=0.022 in the UK. According to Mariette et al. (2010), the F_{ST} between wild cherry and landraces in France has been estimated to be F_{ST}=0.060. Indeed, tree species with outcrossing breeding systems tend to harbor high within-population genetic diversity and low to moderate population differentiation (Hamrick et al. 1992) as a consequence of high gene flow. The significant proportion of among-population differentiation of this study probably reflects a higher fragmentation and reproductive isolation of the Greek populations compared to those of Western Europe. Concerning linkage disequilibrium, the pairs of loci with significant disequilibrium were different in the five populations, suggesting that disequilibrium was probably due to different demographic histories of these populations. Overall, there is accumulating evidence that in these rear-edge populations, the potential action of historical genetic drift and isolation favors genetic structure and differentiation (Schaal and Leverich 1996; Vucetich and Waite 2003; Hampe and Petit 2005; Eckert et al. 2008).

The UPGMA dendrograms presented generally similar results. The major finding concerns mainly the Vorras population, the westernmost population studied (Fig. 1). Vorras appears well differentiated especially in the ISSR analysis (Fig. 2). A difference between the two graphs involves Katafito a central population among the ones studied in the east–west axis (Fig. 1). This population appeared belonging to different groups in the ISSR and SSR analyses (Fig. 2). The PCoA results based on ISSR markers indicated three well-separated clusters. In concordance to the respective UPGMA dendrogram, the differentiation of the Vorras population, and to a lesser extent of the Nimphaea, was prominent. More interesting as well as more informative based on the nature of the marker data and the high proportion of the total variation explained in low multivariate space was the FCA based on SSRs. Results were overall congruent to the previous analyses of

both markers, but the apparent distinction of the two groups formed (north-central and north-eastern populations; Fig. 4) was more clearly elucidated by this approach.

Possible admixture in these populations was assessed by STRUCTURE which revealed that each population studied corresponded to a unique gene cluster. STRUCTURE results further verified PCoA and FCA results and showed that most individuals clustered into distinct population-specific groups (Figs. 3 and 4). The presence of private alleles in each population (Table 3) also implies at least some degree of population independence. Overall, STRUCTURE suggested a low level of population admixture in these *P. avium* populations. Population differentiation, as portrayed by the above analyses, calls for a detailed definition of population structure in *P. avium* which presents a twofold importance: it allows for informed forest management and facilitates the establishment of a proper conservation program of natural populations.

Effective population size, genetic drift, gene flow, and population bottlenecks

N_E estimates provide a measure of the effects causing, or being analogous to, drift in natural populations. Effective population size (N_E) estimates were computed by the linkage disequilibrium and the Wright–Fisher island model methods. *P. avium* as indicated by STRUCTURE seems to present a restricted dispersion which may violate the assumptions of the second approach; however, when the total migration rate is low, the differentiation even between adjacent populations is close to that expected in an island model (Kimura and Maruyama 1971; Rousset 2000). The estimation is also relatively robust with respect to finite deme number and to the assumption of migration–drift equilibrium (Vitalis and Couvet 2001). Both methods are therefore reliable for the present study, having indicated rather sufficient levels of effective sizes for all populations that will probably be enough for their stability and adaptive evolution in the future. Interestingly, the north-eastern populations present fairly high N_E levels compared to the north-central ones. Especially population Cholomontas appears to have a comparatively restricted effective population size.

The occurrence of scattered stands may lead to high genetic divergence among populations and regions mainly due to exposure to genetic drift (Belletti et al. 2008). Furthermore, insect-pollinated forest tree species with a scattered distribution, such as wild cherry, may experience genetic drift due to nonrandom mating (Demesure et al. 2000), which affects the pattern of genetic diversity both among and within populations, even in outcrossing species (Belletti et al. 2008). Moreover, frequent size reductions of scattered wild cherry populations may occur causing population size reductions and bottlenecks (Belletti et al. 2008) that may further increase genetic drift and differentiation (Demesure et al. 2000). However, these processes were not that obvious in the present study. Some drift may for example explain the significant heterozygote deficiency in two populations, but overall, most effective population sizes were fairly large, indicating general population stability despite a significant and rather strong population differentiation (among-population variation averages 20% of the total variation considering both marker analyses).

The values of historical gene flow estimated from private alleles (Slatkin 1985) and population differentiation (G_{ST} and R_{ST}) methods are concordant (N_m=1.190–1.962) and indicate moderate to low gene flow among the *P. avium* populations. The N_m value of *P. avium* is near the average reported for outcrossed animal-pollinated species (N_m= 1.154; Hamrick and Godt 1990). Furthermore, only a single first-generation migrant was detected between the populations Katafito and Hayntou (GENECLASS2; results not shown). In agreement to STRUCTURE results, such a low migration level, even if it materializes into realized gene flow, is in theory not sufficient to prevent genetic differentiation between populations due to drift (Slatkin 1987).

Bottleneck detection used the Wilcoxon signed-rank test for heterozygosity excess and was based on the TPM model which, being a combination of SMM and IAM models, gives probably the most reliable results (Di Rienzo et al. 1994; Spencer et al. 2000). Significant deviations for mutation-drift equilibrium under the TPM model may suggest a recent population size decline, or population fluctuations (Kang et al. 2008) in the Nimphaea population only (Table 5). The second approach used for historical bottleneck detection based on a mode shift of allele frequency distribution did not support any sign of recent bottlenecks; all five populations displayed a normal distribution of allele frequencies. As effective population size does not appear to be considerably low in these rear-edge populations, it may be safe to assume that any transitory reductions in size in these populations have not resulted in significant bottlenecks. Bottleneck comparisons between rear-edge refuge and recolonized regions are not available for *P. avium*. However, in *Prunus serotina*, potential bottlenecks were investigated in a comparison of native North American and introduced European populations; no bottlenecks were detected in the native range, while some populations exhibited bottlenecks in the introduced range (Pairon et al. 2010). In species with similar life history traits, refugial and recolonized populations have not been subjected to bottleneck analysis within the same study that would assure the use of the same SSR loci and analytical approaches. Therefore, results cannot be directly comparable; however, pertinent literature reflects that bottlenecks detected via SSR analysis are rather more common in recolonized than in refugial populations (Tsuda

and Ide 2005; Tsuda et al. 2009; Hu et al. 2010; Kato et al. 2010; Omondi et al. 2010; Pairon et al. 2010).

General discussion and conclusions

Results revealed high amounts of genetic diversity and significant population differentiation as about 80% of the total variation resides within the rear-edge *P. avium* populations studied. These values were higher than those reported for population from the center of the species distribution, while allelic richness was lower. Populations were rather isolated genetically, characterized by fairly low levels of gene flow; however, significant population bottlenecks were not detected, while the lowest estimated effective population size was N_E>44 (Table 5). Overall, it appears that even at the southern distribution limits of the species, demo-genetic stochasticity is not clearly apparent. The center-periphery hypothesis does not apply very well in these data, and the notion of rear-edge populations as advanced by Hampe and Petit (2005) describes much better the observed results. However, the importance of past climate-driven range dynamics as the major, or the only factor, in shaping extant genetic diversity and structure in these rear-edge populations is not clear. Besides a general scarcity of relevant genetic data for the Balkans (Hewitt 2000), *P. avium* as an entomophilous species with a low pollen production (Faegri and Iversen 1989) presents scarce pollen profile records which do not allow for a definite detection of glacial refugia and a reconstruction of the species European natural migration/colonization routes. The geographical location of this study, in addition to being considered a glacial refugium for many species of the present European flora, has been under constant and strong anthropogenic influence at least over the past 4,000 years, resulting in a multifaceted interaction of nature and humans regarding population establishment, expansion, contraction, and gene flow. Natural migration routes could have been influenced by artificial plantings of early selected *P. avium* varieties that may progressively become naturalized. The result can be a complex mosaic of extant genetic diversity and differentiation where admixture of divergent lineages during recolonization might yield complex distribution patterns of diversity (Petit et al. 2003; Arana et al. 2010). Mohanty et al. (2001), by studying cpDNA in 23 European *P. avium* populations, indicated the absence of a strong phylogenetic structure which they attributed to dispersal mechanisms including anthropogenic activities. Nevertheless, this study, as well as a study of selected wild cherry plus trees (Avramidou et al. 2010), has shown fairly strong genetic structure in rear-edge northern Greek populations. In the latter case, two distinct gene pools were identified even within a locally restricted population (Vathytopos, NE Greece; Avramidou et al. 2010).

The results of this study can be useful in the protection of genetic resources which aims at securing the long-term adaptive evolutionary potential of a species. The populations under study can be regarded as high priority for gene conservation purposes which can be achieved by maintaining gene conservation units among natural populations that are characterized by high genetic diversity and unique gene pools. Population clustering and ordination provide an indication of population groups that should be represented in a *P. avium* gene conservation scheme. Evidently, all studied *P. avium* populations can be used in a breeding program. Only population Cholomontas, due to a fairly restricted effective population size, may be in need of some future protection through forest management. It is unlikely that this population will be currently endangered due to genetic factors, but a limited effective population size will make it vulnerable to future losses of genetic diversity. A possible exclusion from harvesting for a decade may provide an adequate means of facilitating future regeneration and increase in population size. The other populations, especially the north-eastern ones, do not appear to need any particular immediate forest management intervention and could be employed in gene conservation. Overall, the north-eastern group that was depicted especially by FCA could be represented by population Hayntou as a gene conservation unit. From the north-central group, population Vorras could also be included, in the absence of a better alternative, as this geographical area has not been extensively studied; the only concern is the small value of the N_E lower confidence limit (N_E≤30). In conclusion, northern Greek *P. avium* populations present a wealth of genetic variation and are potentially very valuable as well as important in preserving genetic diversity and local adaptive complexes, especially under contemporary climatic change scenarios.

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References

Arana MV, Gallo LA, Vendramin GG, Pastorino MJ, Sebastiani F, Marchelli P (2010) High genetic variation in marginal fragmented populations at extreme climatic conditions of the Patagonian Cypress *Austrocedrus chilensis*. Mol Phyl Evol 54:941–949

Aravanopoulos AF, Avramidou E, Ganopoulos I (2008) Genetics of wild cherry (*Prunus avium* L.) in Greece. Growing valuable broadleaved tree species, 6–8 October, Freiburg, Germany

Avramidou E, Ganopoulos IV, Aravanopoulos FA (2010) DNA fingerprinting of elite Greek wild cherry (*Prunus avium* L.) genotypes using microsatellite markers. Forestry 83:527–533

Bartley D, Bagley M, Gall G, Bentley B (1992) Use of linkage disequilibrium data to estimate effective size of hatchery and natural fish populations. Conserv Biol 6:365–375

Beaver JA, Iezzoni AF, Ramm CW (1995) Isozyme diversity in sour, sweet and ground cherry. Theor Appl Genet 90:847–852

Behre K (1978) Formenkreise von *Prunus domestica* L. von der Wikingerzeit bis in die fru¨ he Neuzeit nach Fruchtsteinen aus Haithabu und Alt-Schleswig. Berichte der Deutschen Botanischen Gesellschaft 91:161–179

Belkhir K (1999) GENETIX (v4.04): a Windows program for population genetic analysis. Laboratorie Genome, Populations Interactions, UPR 5000 du CNRS, Universite Montpellier II, Montpellier

Belletti P, Monteleone I, Ferrazzini D (2008) A population genetic study in a scattered forest species, wild service tree [*Sorbus torminalis* (L.) Crantz], using RAPD markers. Eur J Forest Res 127:103–114

Boshier D, Amaral W (2004) Threats to forest ecosystems and challenges for the conservation and sustainable use of forest genetic resources. In: Vinceti B, Amaral W, Meilleur B (eds) Challenges in managing forest genetic resources for livelihoods. IPGRI, Rome, pp 7–22

Boys J, Cherry M, Dayanandan S (2005) Microsatellite analysis reveals genetically distinct populations of red pine (*Pinus resinosa*, Pinaceae). Am J Bot 92:833–841

Chapuis M-P, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. Mol Biol Evol 24:621–631

Clarke JB, Tobutt KR (2003) Development and characterization of polymorphic microsatellites from *Prunus avium* 'Napoleon'. Molecular Ecology Notes 3:578–580

Cornuet J, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014

Crane M, Brown A (1937) Incompatibility and sterility in the sweet cherry, *Prunus avium* L. J Pomol Hort Sci 15:85–116

Dakin EE, Avise JC (2004) Microsatellite null alleles in parentage analysis. Heredity 93:504–509

Dawson IK, Powell W (1999) Genetic variation in the Afromontane tree *Prunus africana*, an endangered medicinal species. Mol Ecol 8:151–156

Degen B, Blanc L, Caron H, Maggia L, Kremer A, Gourlet-Fleury S (2006) Impact of selective logging on genetic composition and demographic structure of four tropical tree species. Biol Conserv 131:386–401

Demesure B, Guerrouι BL, Lucchi G, Prat D, Petit R-J (2000) Genetic variability of a scattered temperate forest tree: *Sorbus torminalis* L. (Crantz). Ann For Sci 57:63–71

Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB (1994) Mutational processes of simple-sequence repeat loci in human populations. Proc Natl Acad Sci USA 91:3166–3170

Downey SL, Iezzoni AF (2000) Polymorphic DNA markers in black cherry (*Prunus serotina*) are identified using sequences from sweet cherry, peach, and sour cherry. J Am Soc Hort Sci 125:76–80

Doyle J, Doyle J (1987) A rapid DNA isolation procedure for small amount of fresh leaf tissue. Phytochem Bull 19:11–15

Ducci F, Santi F (1997) The distribution of clones in managed and unmanaged populations of wild cherry (*Prunus avium*). Can J For Res 27:1998–2004

Durel CE, Bertin P, Kremer A (1996) Relationship between inbreeding depression and inbreeding coefficient in maritime pine (*Pinus pinaster*). Theor Appl Genet 92:347–356

Eckert CG, Samis KE, Lougheed SC (2008) Genetic variation across species' geographical ranges: the central–marginal hypothesis and beyond. Mol Ecol 17:1170–1188

Ellstrand NC, Elam DR (1993) Population genetic consequences of small population size: implications for plant conservation. Annu Rev Ecol Syst 24:217–242

Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Faegri K, Iversen J, Kaland PE, Krzywinski K (1989) Textbook of pollen analysis, 4th edn. Wiley, Chichester

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Frascaria N, Santi F, Guyon P (1993) Genetic differentiation within and among populations of chestnut (*Castanea sativa* Mill.) and wild cherry (*Prunus avium* L.). Heredity 70:634–641

Ganopoulos VI, Kazantzis K, Chatzicharisis I, Karayiannis I, Tsaftaris A (2011) Genetic diversity, structure and fruit trait associations in Greek sweet cherry cultivars using microsatellite based (SSR/ISSR) and morpho-physiological markers. Euphytica. doi:10.1007/s10681-011-0416-z

Gaudeul M, Till-Bottraud I, Barjon F, Manel S (2004) Genetic diversity and differentiation in *Eryngium alpinum* L. (Apiaceae): comparison of AFLP and microsatellite markers. Heredity 92:508–518

Goodman SJ (1997) R_{ST}Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. Mol Ecol 6:881–885

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. J Hered 86:485–486

Guarino C, Santoro S, De Simone L, Cipriani G (2009) *Prunus avium*: nuclear DNA study in wild populations and sweet cherry cultivars. Genome 52:320–337

Hampe A, Petit RJ (2005) Conserving biodiversity under climate change: the rear edge matters. Mol Ecol 8:461–467

Hamrick J, Godt M (1990) Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS (eds) Plant population genetics, breeding and germplasm resources. Sinauer, Sunderland, pp 43–63

Hamrick J, Godt M, Sherman-Broyles S (1992) Factors influencing levels of genetic diversity in woody plant species. New Forests 6:95–124

Hardy OJ, Charbonnel N, Freville H, Heuertz M (2003) Microsatellite allele sizes: a simple test to assess their significance on genetic differentiation. Genetics 163:1467–1482

Hedrick UP (1915) The cherries of New York. J B Lyon, Albany

Hedrick P (1999) Highly variable loci and their interpretation in evolution and conservation. Evolution 53:313–318

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. Philos Trans R Soc Lond B Biol Sci 359:183–195

Hill WG (1981) Estimation of effective population size from data on linkage disequilibrium. Genet Res 38:209–216

Hoffman A, Parsons P (1996) Extreme environmental change and evolution. Cambridge University Press, Cambridge

Hu L-J, Uchiyama K, Saito Y, Ide Y (2010) Contrasting patterns of nuclear microsatellite genetic structure of *Fraxinus mandshurica* var. *japonica* between northern and southern populations in Japan. J Biogeogr 37:1131–1143

Jordano P, Godoy JA (2000) RAPD variation and population genetic structure in *Prunus mahaleb* (Rosaceae), an animal-dispersed tree. Mol Ecol 9:1293–1305

Kalinowski ST (2005) HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. Mol Ecol Notes 5:187–189

Kalinowski S, Taper M (2006) Maximum likelihood estimation of the frequency of null alleles at microsatellite loci. Conserv Genet 7:991–995

Kang M, Wang J, Huang H (2008) Demographic bottlenecks and low gene flow in remnant populations of the critically endangered *Berchemiella wilsonii* var. *pubipetiolata* (Rhamnaceae) inferred from microsatellite markers. Conserv Genet 9:191–199

Kar P, Srivastava P, Awasthi A, Urs S (2008) Genetic variability and association of ISSR markers with some biochemical traits in mulberry (*Morus* spp.) genetic resources available in India. Tree Genet Genom 4:75–83

Karp A, Seberg O, Buiatti M (1996) Molecular Techniques in the Assessment of Botanical Diversity. Ann Bot 78(2):143–149

Kato S, Iwata H, Tsumura Y, Mukai Y (2010) Genetic structure of island populations of *Prunus lannesiana var. speciosa* revealed by chloroplast DNA, AFLP and nuclear SSR loci analyses. J Plant Res 124:11–23

Kimura M, Maruyama T (1971) Pattern of neutral polymorphism in a geographically structured population. Genet Res 18:125–131

Lesica P, Allendorf FW (1992) Are small populations of plants worth preserving. Conserv Biol 6:135–139

Luikart G, Cornuet J (1998) Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conserv Biol 12:228–237

Mariette S, Lefranc M, Legrand P, Taneyhill D, Frascaria-Lacoste N, Machon N (1997) Genetic variability in wild cherry populations in France. Effects of colonizing processes. Theor Appl Genet 94:904–908

Mariette S, Tavaud M, Arunyawat U, Capdeville G, Millan M, Salin F (2010) Population structure and genetic bottleneck in sweet cherry estimated with SSRs and the gametophytic self-incompatibility locus. BMC Genet 11:13

Marshall RE (1954) Cherries and cherry products. In: Economic crops, vol 5. Inter-Science, New York

Mattioni C, Cherubini M, Micheli E, Villani F, Bucci G (2008) Role of domestication in shaping *Castanea sativa* genetic variation in Europe. Tree Genet Genom 4:563–574

Meyer-Dingel A, Svolba J, Kleinschmit J (1997) Selektierte, mikrovermehrte Vogelkirschen-Klone. AIIg Forstz/Der Wald 52:963–964

Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. Genetics 142:1061–1064

Mohanty A, Martin JP, Aguinagalde I (2001) A population genetic analysis of chloroplast DNA in wild populations of *Prunus avium* L. in Europe. Heredity 87:421–427

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590

Omondi S, Kireger E, Dangasuk O, Chikamai B, Odee D, Cavers S, Khasa D (2010) Genetic diversity and population structure of *Acacia senegal* (L) Willd. in Kenya. Trop Plant Biol 3:59–70

Ota T (1993) DISPAN: genetic distance and phylogenetic analysis. Pennsylvania State University, University Park

Pairon M, Petitpierre B, Campbell M, Guisan A, Broennimann O, Baret PV, Jacquemart A-L, Besnard G (2010) Multiple introductions boosted genetic diversity in the invasive range of black cherry (*Prunus serotina*; Rosaceae). Ann Bot 105:881–890

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Peel D, Ovenden J, Peel S (2004) N_EEstimator: software for estimating effective population size, version 1.3

Petit RJ, Aguinagalde I, de Beaulieu JL, Bittkau C, Brewer S, Cheddadi R, Ennos R, Fineschi S, Grivet D, Lascoux M, Mohanty A, Muller-Starck GM, Demesure-Musch B, Palme A, Martin JP, Rendell S, Vendramin GG (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. Science 300:1563–1565

Piry S, Luikart G, Cornuet J (1999) Bottleneck: a computer program for detecting recent reductions in the effective population size using allele frequency data. J Hered 90:502–503

Pritchard J, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Queney G, Ferrand N, Weiss S, Mougel F, Monnerot M (2001) Stationary distributions of microsatellite loci between divergent population groups of the European rabbit (*Oryctolagus cuniculus*). Mol Biol Evol 18:2169–2178

Raymond M, Rousset F (1995) GENEPOP (version 1.2)—population genetics software for exact tests and ecumenicism. J Hered 86:248–249

Rousset F (2000) Genetic differentiation between individuals. J Evol Biol 13:58–62

Russell K (2003) EUFORGEN Technical Guidelines for genetic conservation and use for wild cherry (*Prunus avium*). International Plant Genetic Resources Institute, Rome

Sagarin RD, Gaines SD (2002) The 'abundant centre' distribution: to what extent is it a biogeographical rule? Ecol Lett 5:137–147

Sanou H, Lovett PN, Bouvet J-M (2005) Comparison of quantitative and molecular variation in agroforestry populations of the shea tree (*Vitellaria paradoxa* C.F. Gaertn) in Mali. Mol Ecol 14:2601–2610

Santi F (1988) Variabilité génétique intra et interpopulations chez le merisier (*Prunus avium* L.). Thèse de Doctorat de l'Institut National Agronomique de Paris Grignon

Santi F, Lemoine M, Bruant B (1990) Genetic markers for *Prunus avium* L: inheritance and linkage of isozyme loci. Ann For Sci 47:131–139

Schaal BA, Leverich WJ (1996) Molecular variation in isolated plant populations. Plant Species Biol 11:33–40

Schlötterer C (2000) Evolutionary dynamics of microsatellite DNA. Chromosoma 109:365–371

Schueler S, Tusch A, Scholz F (2006) Comparative analysis of the within-population genetic structure in wild cherry (*Prunus avium* L.) at the self-incompatibility locus and nuclear microsatellites. Mol Ecol 15:3231–3243

Slatkin M (1985) Rare alleles as indicators of gene flow. Evolution 39:53–65

Slatkin M (1987) Gene flow and the geographic structure of natural populations. Science 236:787–792

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462

Sosinski B, Gannavarapu M, Hager LD, Beck LE, King GJ, Ryder CD, Rajapakse S, Baird WV, Ballard RE, Abbott AG (2000) Characterization of microsatellite markers in peach [*Prunus persica* (L.) Batsch]. Theor Appl Genet 101 (3):421–428

Spencer CC, Neigel JE, Leberg PL (2000) Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks. Mol Ecol 9:1517–1528

Stoeckel S, Grange J, Fernandez-Manjarres J, Bilger I, Frascaria-Lacoste N, Mariette S (2006) Heterozygote excess in a self-incompatible and partially clonal forest tree species. Mol Ecol 15:2109–2118

Tang S, Dai W, Li M, Zhang Y, Geng Y, Wang L, Zhong Y (2008) Genetic diversity of relictual and endangered plant *Abies ziyuanensis* (Pinaceae) revealed by AFLP and SSR markers. Genetica 133:21–30

Tavaud M, Zanetto A, Santi F, Dirlewanger E (2001) Structuration of genetic diversity in cultivated and wild cherry trees using AFLP markers. International Society for Horticultural Science, Leuven

Tessier du Cros E, Matyas C, Kriebel H (1999) Contribution of genetics to the sustained management of global forest resources—conclusions and recommendations. In: Matyas C (ed) Forest genetics and sustainability, vol 63. Forestry Sciences, Netherlands, pp 281–287

Testolin R, Marrazzo T, Cipriani G, Quarta R, Verde I, Dettori MT, Pancaldi M, Sansavini S (2000) Microsatellite DNA in peach

(*Prunus persica* L. Batsch) and its use in fingerprinting and testing the genetic origin of cultivars. Genome 43:512–520

Tsuda Y, Ide Y (2005) Wide-range analysis of genetic structure of *Betula maximowicziana*, a long-lived pioneer tree species and noble hardwood in the cool temperate zone of Japan. Mol Ecol 14:3929–3941

Tsuda Y, Kimura M, Kato S, Katsuki T, Mukai Y, Tsumura Y (2009) Genetic structure of *Cerasus jamasakura*, a Japanese flowering cherry, revealed by nuclear SSRs: implications for conservation. J Plant Res 122:367–375

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

Vaughan SP, Russell K (2004) Characterization of novel microsatellites and development of multiplex PCR for large-scale population studies in wild cherry, *Prunus avium*. Mol Ecol Notes 4:429–431

Vaughan SP, Cottrell JE, Moodley DJ, Connolly T, Russell K (2007) Distribution and fine-scale spatial-genetic structure in British wild cherry (*Prunus avium* L.). Heredity 98:274–283

Vitalis R, Couvet D (2001) Estim 1.0: a computer program to infer population parameters from one-and two-locus gene identity probabilities. Mol Ecol Notes 1:354–356

Vucetich JA, Waite TA (2003) Spatial patterns of demography and genetic processes across the species' range: null hypotheses for landscape conservation genetics. Conserv Genet 4:639–645

Weir B, Cockerham C (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370

Weiser F (1996) Ergebnisse einer 33-jährigen Einzelbaum-Nachkommenschaftsprüfung nach freiem Abbliihen von Vogelkirsche. Prunus avium L. var. avium. Silvae Genet 45:260–266

Widmer A, Lexer C (2001) Glacial refugia: sanctuaries for allelic richness, but not for gene diversity. Trends Ecol Evol 16:267–269

Yeh F, Boyle T, Rongcai Y, Ye Z, Xian J (1999) Popgene version 1.31. Microsoft Window-based freeware for population genetic analysis. University of Alberta, Edmonton

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183

Zohary D, Hopf M (2000) Domestication of plants in the old world, 3rd edn. Oxford University Press, Oxford