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The genetic structure of silver birch (*Betula pendula* Roth) in Campania (southern Italy)

D. DE LUCA[1], L. PAINO[1], E. DEL GUACCHIO[2]

[1]Dipartimento di Biologia, Università degli Studi di Napoli Federico II, Via Foria 223, 80139 Napoli, Italy. [2]Dipartimento di Scienze Formative, Psicologiche e della Comunicazione, Università degli Studi Suor Orsola Benincasa di Napoli, Via Santa Caterina da Siena 37, 80132 Napoli, Italy.
daniele.deluca088@gmail.com

Abstract. We investigated genetic diversity and population structure of four silver birch (*Betula pendula* Roth) stands occurring on the Mt. Cerasuolo, Mt. Cervati, Mt. Polveracchio and Mt. Somma (Campania Region, southern Italy) using nuclear and chloroplast microsatellite markers and the intergenic spacer *trnH-psbA*. Our results support the occurrence of four distinct genetic groups.

Riassunto. Abbiamo analizzato la diversità e la struttura genetica di quattro popolazioni di betulla (*Betula pendula* Roth) del Monte Cerasuolo, del Monte Cervati, del Monte Polveracchio e del Monte Somma (Regione Campania, sud Italia) utilizzando microsatelliti plastidiali e nucleari e lo spaziatore intergenico *trnH-psbA*. I nostri risultati indicano la presenza di quattro gruppi genetici distinti.

Key words: *Betula pendula*, Conservation, Microsatellites, Population structure, Relic populations, Southern Italy.

INTRODUCTION

The silver birch (*Betula pendula* Roth) is a typical tree of the Euro-Siberian landscape, which reaches its southernmost distribution in the Iberian Peninsula, South Italy, and Greece (MEUSEL & JÄGER 1998), where it occurs in relic populations from the last glacial event. In Italy, it is common throughout the Alpine and Prealpine regions and is particularly widespread in Piedmont and Lombardy, where it finds favourable environmental conditions for its survival, as low summer temperatures and high humidity (PLINI & TONDI 1989). Along the Apennine ridge, however, due to the Mediterranean-mountain climate, with high temperatures and prolonged summer drought, its distribution is more scattered and relegated to isolated stations in Abruzzo, Campania (predominantly) and Calabria (PLINI & TONDI 1989). However, since the most recent bibliography for the Southern Apennines dates back to AGOSTINI (1981) and various other authors mentioned in the publication of PLINI & TONDI (1989), the species might have disappeared from some of such sites as consequence of climatic variations and human pressure.

Campania is the Region in which the silver birch, despite its relic origin, is widely distributed and often forms monospecific

stands (PLINI & TONDI 1989, and references therein). Since these stands can be either relatively small and isolated or large and geographically continuous, the question arises whether scattered populations are to be considered as distinct genetic groups or not. To date, no genetic analyses to assess population structure in this area have been conducted, but CENNAMO *et al.* (2002) found high levels of genetic diversity in a population sample from the Cilento, Vallo di Diano and Alburni National Park area (Campania Region, southern Italy) using ISSR markers.

The occurrence of discontinuous and isolated populations, especially at the edge of distribution areas, can harm genetic diversity, and very little is known about the amount and organisation of genetic variation in the southern marginal areas of this species (VAKKARI 2009). According to genetic theory, marginal populations should be less genetically variable but more differentiated in respect to central ones; however, the interplay of different evolutionary processes and reproductive strategies of the species can alter such predictions.

Here we analysed the genetic variability and population structure of four silver birch stands in Campania Region (southern Italy) using nuclear and chloroplast microsatellite markers and the chloroplast intergenic spacer *trnH-psbA*, with the aim of assessing whether these stands constitute single or multiple genetic groups. Such information can be used as preliminary guideline for conservation purposes.

MATERIALS AND METHODS

Sampling plan and DNA extraction. Silver birch specimens have been collected from four stands within Campania (Fig. 1). Twenty-six samples were taken from a stand located at about 700 m a.s.l. on the Mount Cervati, in the municipality of Sassano (Cilento area, Salerno Province), specifically in the areas of Valtasso, Campolongo, and Nicola Bedda. No young trees were observed in such areas, probably due to grazing; therefore, leaves were only taken from adult individuals.

On Mount Somma (Vesuvius volcanic complex, Naples Metropolitan Area), despite bibliographic data supported the presence of very large birch nuclei in the past (e.g., PASQUALE 1869), only repeated field trips allowed us to detect few specimens, sparsely located in poorly accessible areas. Only eight specimens were found, in isolated nuclei of 1-2 individuals and mixed to chestnut trees (*Castanea sativa* Mill.) at about 600 m a.s.l.

On the other hand, on Mt. Cerasuolo (Naples Metropolitan Area) and Mt. Polveracchio (Salerno Province) we observed pure and dense stands of silver birch, with young and adult trees, the latter harbouring mature seeds. This is probably due to ecological and climatic factors (plants were found at greater altitude than that of Mt. Somma, at about 1.300 m a.s.l. on Mt. Polveracchio) and to the larger number of remaining individuals. Twenty trees were sampled in both sites.

We collected 2-3 leaves per plant in each sampled site and stored them at -80 °C until DNA extraction, which was performed following the protocol of DOYLE & DOYLE (1987). We also used polyvinylpyrrolidone (PVP) during DNA extraction to handle the high content in polyphenols of birch leaves, which can hamper the process (HOWLAND *et al.* 1991). Quality and concentration of DNA were verified using a size standard (MII, Boehringer Mannheim).

Amplification of nuclear microsatellites. Four nuclear microsatellite loci, L1.10, L3.1, L5.4 and L7.1a, (KULJU *et al.*

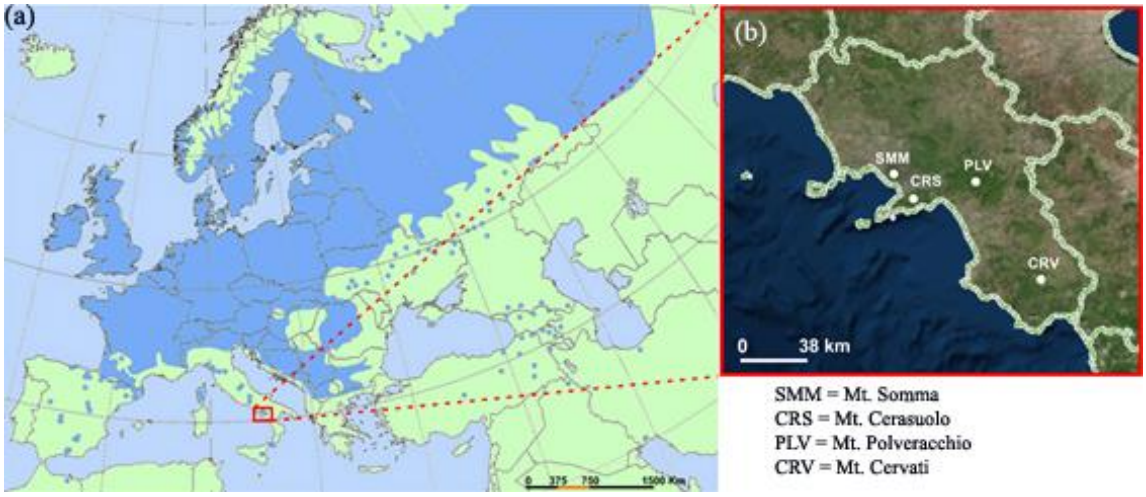

Fig. 1 - (a) Distribution range of *Betula pendula* Roth (modified from European Forest Genetic Resources (EUFORGEN) database (http://www.euforgen.org/distribution-maps/), and (b) sampling localities within Campania Region.

Table 1 - Genetic diversity indices per locus.

Locus	**Allele size (bp)**	**A**	**H_e**	**uH_e**	**H_o**
L1.10	172-289	24	0.899	0.905	0.606
L3.1	213-235	8	0.773	0.778	0.778
L5.4	238-266	14	0.863	0.869	0.851
L7.1a	135-154	14	0.859	0.864	0.795

A = number of alleles; H_e = expected heterozygosity; uH_e = unbiased expected heterozygosity according to NEI (1978); H_o = observed heterozygosity.

Table 2 - Genetic diversity indices for population sample.

Population Sample	**Pop Code**	**N**	**A_p**	**$R_{(8)}$**	**$R_{(20)}$**	**H_e**	**uH_e**	**H_o**	***F_{IS}***
Mt. Cerasuolo	CRS	20	3	4.118	5.929	0.720	0.739	0.702	0.014^{ns}
Mt. Cervati	CRV	26	8	4.662	7.017	0.797	0.813	0.817	-0.005^{ns}
Mt. Polveracchio	PLV	20	10	4.958	7.657	0.821	0.843	0.811	0.026^{ns}
Mt. Somma	SMM	8	4	3.480	-	0.594	0.636	0.531	0.081^{ns}

N = number of sampled individuals; A_p = number of private alleles; $R_{(8)}$ = allelic richness corrected for the sample size of 8; $R_{(20)}$ = allelic richness corrected for the sample size of 20; H_e = expected heterozygosity; uH_e = unbiased expected heterozygosity according to NEI (1978); H_o = observed heterozygosity; F_{IS} = inbreeding coefficient. ^{ns} = not significant.

Table 3 - p values of Hardy-Weinberg equilibrium related to differences between Ho and He.

	CRS	**CRV**	**PLV**	**SMM**
L1.10	0.011	0.004	0.530	0.010
L3.1	0.688	0.615	0.640	0.552
L5.4	0.268	0.025	0.044	0.163
L7.1a	0.151	0.000	0.015	0.485

CRS = Mt. Cerasuolo; CRV = Mt. Cervati; PLV = Mt. Polveracchio; SMM = Mt. Somma.

Table 4 - Linkage disequilibrium between loci per population sample and relative p-value.

Pop	Locus_1	Locus_2	p-value
CRS	L1.10	L5.4	0.336937
CRS	L1.10	L3.1	0.598099
CRS	L5.4	L3.1	0.560034
CRS	L1.10	L7.1a	0.566721
CRS	L5.4	L7.1a	1.000000
CRS	L3.1	L7.1a	0.780870
CRV	L1.10	L5.4	0.000000
CRV	L1.10	L3.1	0.000000
CRV	L5.4	L3.1	0.000009
CRV	L1.10	L7.1a	0.081427
CRV	L5.4	L7.1a	0.134152
CRV	L3.1	L7.1a	0.002038
PLV	L1.10	L5.4	1.000000
PLV	L1.10	L3.1	1.000000
PLV	L5.4	L3.1	0.424447
PLV	L1.10	L7.1a	1.000000
PLV	L5.4	L7.1a	0.238809
PLV	L3.1	L7.1a	0.177126
SMM	L1.10	L5.4	0.399304
SMM	L1.10	L3.1	0.199284
SMM	L5.4	L3.1	0.130858
SMM	L1.10	L7.1a	0.800130
SMM	L5.4	L7.1a	0.055761
SMM	L3.1	L7.1a	0.130732

CRS = Mt. Cerasuolo; CRV = Mt. Cervati; PLV = Mt. Polveracchio; SMM = Mt. Somma.

Table 5 - Population structure estimates using pair-wise Weir and Cockerham's FST (above the diagonal) and Jost's D (below the diagonal) indices.

	CRS	CRV	SMM	PLV
CRS	-	0.129^{***}	0.140^{***}	0.099^{***}
CRV	0.535^{***}	-	0.118^{***}	0.069^{***}
SMM	0.381^{***}	0.324^{***}	-	0.427^{***}
PLV	0.377^{***}	0.491^{***}	0.105^{***}	-

*** $p < 0.001$ (after 1000 permutations). CRS = Mt. Cerasuolo; CRV = Mt. Cervati; PLV = Mt. Polveracchio; SMM = Mt. Somma.

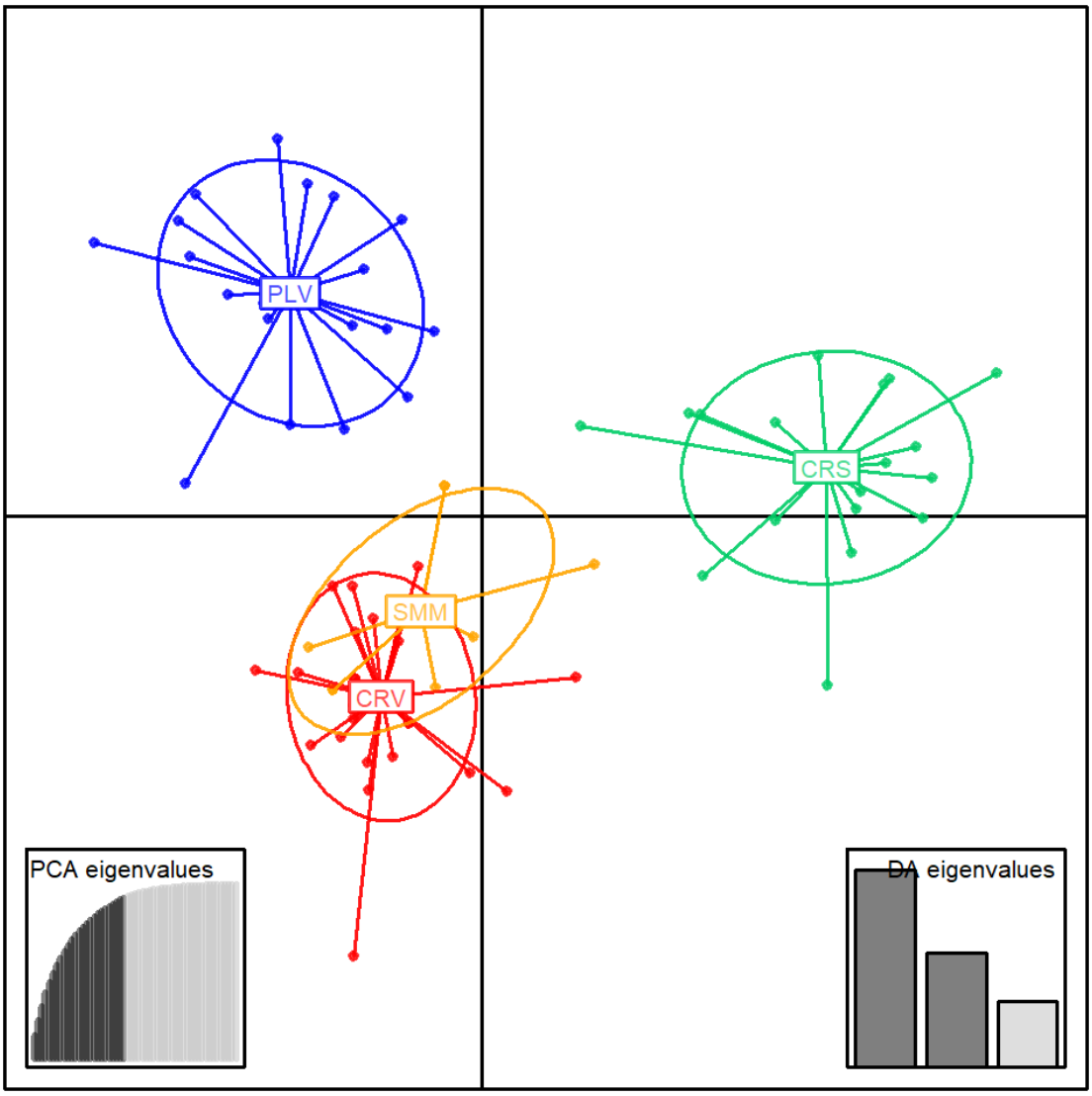

Fig. 2 - Discriminant analysis of principal components (DAPC).
CRS = Mt. Cerasuolo; CRV = Mt. Cervati; PLV = Mt. Polveracchio; SMM = Mt. Somma.

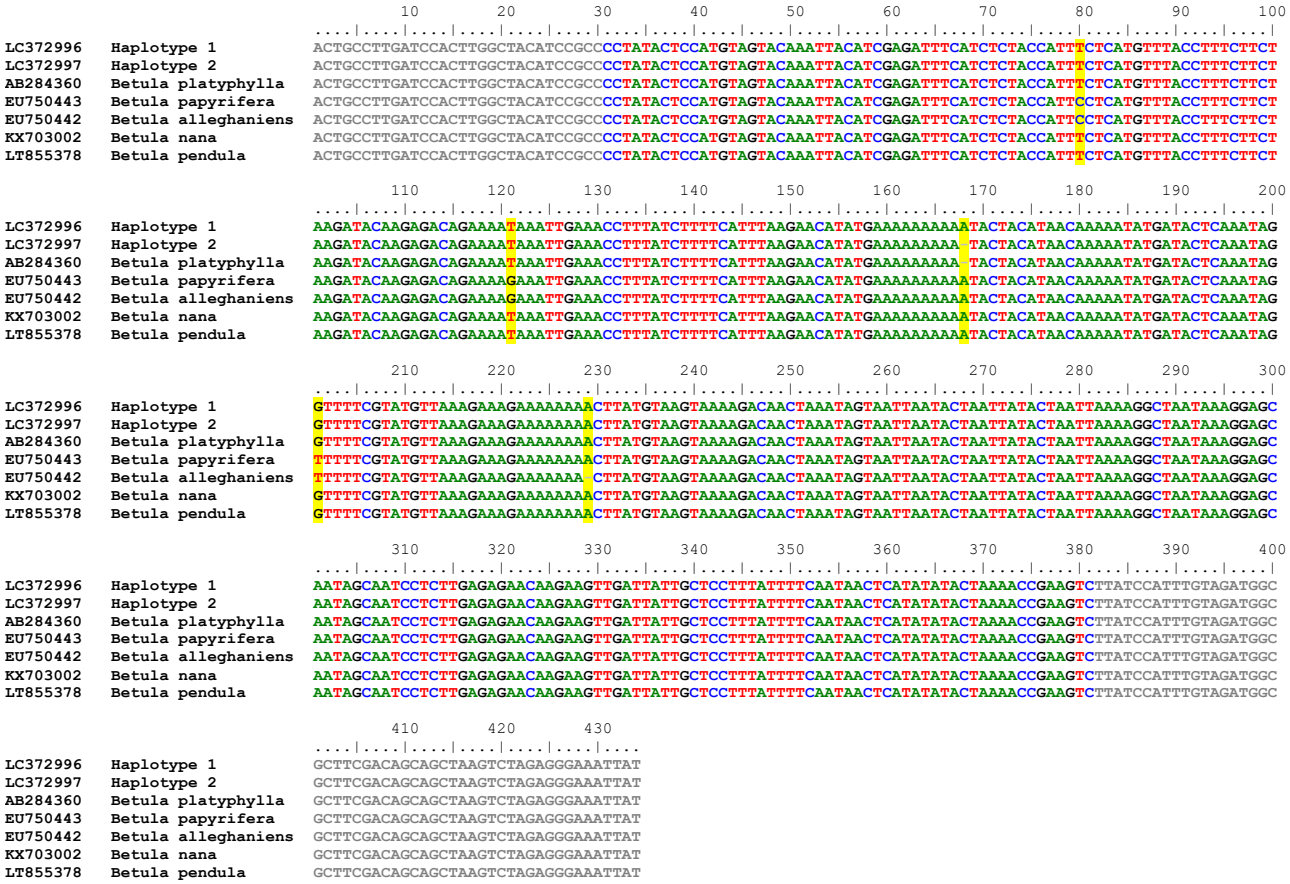

Fig. 3 - Alignment of *trnH-psbA* intergenic spacer among *Betula* species. In grey are the flanking gene regions (*trnH* and *psbA* respectively); variable sites are indicated in yellow.

2004) were chosen to screen silver birch populations. Forward primers were labelled with 5' fluorescence tag (FAM, VIC, NED, Life Technologies, ThermoFisher Scientific Inc.) and amplified in simplex in a final volume of 25 µL using 1 U of DreamTaq™ DNA Polymerase (ThermoFisher Scientific Inc.) at the conditions specified in KULJU *et al.* (2004). PCR products were run on the Automated Sequencer 3130 Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific Inc.) after a 1:5 dilution and addition to 12 µL of formamide/ GeneScan™ 500 LIZ® size standard (Applied Biosystems, ThermoFisher Scientific Inc.).

Amplification of chloroplast microsatellites. Populations were also screened using three chloroplast microsatellite loci, ccmp4, ccmp5, and ccmp7 using the universal primers for chloroplast genome of dicotyledonous angiosperms developed by WEISING & GARDNER (1999). PCRs were carried out in a final volume of 25 µL containing: 20 ng of template DNA, 10X DreamTaq™ Buffer, 0.5 µM each of forward and reverse primers (forward primers were labelled using FAM fluorescence dye, Macrogen), 0.2 mM dNTP, 1 U of DreamTaq™ DNA Polymerase (ThermoFisher Scientific Inc.), and water to volume, following the protocol by WEISING & GARDNER (1999).

PCR fragments were run separately on the Automated Sequencer 3130 Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific Inc.) after a 1:50 dilution and addition to 12 µL of formamide/ GeneScan™ 500 LIZ® size standard (Applied Biosystems, ThermoFisher Scientific Inc.).

Analysis of microsatellite markers. Microsatellite peaks were analysed with Peak Scanner™ Software v1.0 (Life Technologies, ThermoFisher Scientific Inc.) and scored as PCR products. In case of null amplification, samples were run for a second time or PCR reactions were repeated; after these steps, lacking alleles were scored as missing. Input files for subsequent population genetic analyses were generated using GenAlEx v.6.501 (PEAKALL & SMOUSE 2006, 2012), PGDSpider (LISCHER & EXCOFFIER 2012) or manually.

Since chloroplast microsatellites revealed to be monomorphic in all the population samples under investigation, no further analyses were conducted on them; consequently, the following analyses were carried out on nuclear microsatellites only.

Observed heterozygosity (H_o), expected heterozigosity (H_e) and its unbiased form (uH_e) were calculated per locus and population in Genetix v4.05.2 (BELKHIR *et al.* 1996-2004).

Number of alleles (A), Hardy-Weinberg equilibrium (HWE) probabilities, linkage disequilibrium (LD) and inbreeding coefficient (F_{IS}) per population sample were calculated in Arlequin v3.5.2.2 (EXCOFFIER & LISCHER 2010). The Holm-Bonferroni correction was applied on HWE and LD values, whilst the statistical significance of F_{IS} values was determined using the p value statistics after 1000 replications.

Allelic richness (R) was calculated using ADZE v1.0 (SZPIECH *et al.* 2008) on the minimum number of samples (8 and 20), in order to compare results among samples.

Genetic differentiation and structure among population samples were inferred using: Weir and Cockerham's F_{ST} (WEIR & COCKERHAM 1984), Jost's *D* (JOST 2008), and Discriminant Analysis of Principal Components (DAPC). The three methods were chosen for the different approaches they use as follows: Weir and Cockerham's F_{ST} is based on heterozygosity, Jost's *D* on the effective number of alleles, whilst DAPC partitions the sample into a between- groups and within- group component to maximize discrimination between

groups. Calculations were computed in Arlequin v3.5.2.2 (EXCOFFIER & LISCHER 2010), and in the R (R CORE TEAM 2015) working packages DEMEtics (GERLACH *et al.* 2010) and ADEGENET v2.0.0 (JOMBART 2008) respectively. The statistical significance of pair-wise F_{ST} and *D* was determined after Bonferroni correction for multiple tests on 1000 bootstrap resamplings.

Amplification and analysis of chloroplast trnH-psbA intergenic spacer. We also amplified the chloroplast intergenic spacer *trnH-psbA* in a subset of individuals (three) per population sample in order to detect possible differences among them. The region was amplified using the universal primers psbA3'f (SANG *et al.* 1997) and trnHf (TATE & SIMPSON 2003) at the following conditions: initial denaturation at 94 °C for 3 min, 32 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

PCRs were conducted in a volume of 25 µL containing: 30 ng of DNA, 1 U HotStarTaq Plus DNA Polymerase (QIAGEN), 10× PCR Buffer, 2.5 mM dNTPs (Promega), 0.2 mM forward and reverse primers (Macrogen), and water to reach the final volume. PCR products were sequenced with both forward and reverse primers using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, ThermoFisher Scientific Inc.).

RESULTS AND DISCUSSION

Nuclear microsatellites revealed to be polymorphic in all the samples analysed and therefore provided useful information for genetic diversity and population structure characterisation of the stands under investigation. On the contrary, chloroplast microsatellite loci were monomorphic (allele sizes: 115 bp for ccmp4, 104 bp for ccmp5, and 144 bp for ccmp7) and of no use for the purposes described above. The results discussed below, therefore, refer to nuclear microsatellite loci.

Genetic diversity estimations in terms of number of alleles and heterozygosity were quite similar across loci (Table 1). Locus L1.10 showed the highest variability in terms of number of alleles (24), whilst the others had comparable levels (8 alleles for locus L3.1 and 14 for L5.4 and L7.1a). This trend is similar to the one observed for a Finnish birch population of 30 individuals (KULJU *et al.* 2004), except for locus L7.1a that in our case showed three times more alleles. Expected heterozygosity ranged from 0.773 (L3.1) to 0.899 (L1.10), whilst observed one from 0.606 (L1.10) to 0.851 (L5.4).

When considering each population sample, genetic diversity was comparable among the groups with a similar number of individuals (Mt. Cerasuolo, Cervati and Polveracchio), with the samples from Mt. Cervati and Polveracchio exhibiting the highest levels of allelic richness, heterozygosity and private alleles (Table 2). The low values of allelic richness and heterozygosity observed in the Mt. Somma sample (Table 2) are more likely related to genetic drift than to the small sample size. Indeed, allelic richness corrected for SMM sample size is not much lower than the one of other samples (Table 2). Also the low values of expected heterozygosity (0.594, Table 2) cannot be related to the small sample size, since this genetic diversity estimate is generally insensitive to it (GORMAN & RENZI 1979); however, comparisons among populations are not valid unless many loci are examined (ALLENDORF *et al.* 2013).

Significant deviations from Hardy-Weinberg equilibrium were found in all the population samples and ascribed to differences between H_e and H_o (Table 3), whilst a significant linkage disequilibrium among

loci was only found in the sample CRV (Table 4). Inbreeding coefficient (F_{IS}) values indicated that individuals within the population samples under investigation are not significantly more related (CRS, PLV, SMM) or less related (CRV) than it would be expected under a model of random mating (Table 2).

The analyses of genetic structure revealed that silver birches in Campania Region do not form a single population but exhibit a local structure. According to F_{ST} and *D* indices, all the population samples are significantly different (Table 5), whilst DAPC analysis was able to clearly differentiate only the samples from Mt. Cerasuolo (CRS) and Mt. Polveracchio (PLV), with Mt. Cervati (CRV) and Somma (SMM) clustering together (Fig. 2). These contrasting results are mainly due to the different approaches on which the methods rely. Apart from the different information they use (see Material and Methods section), F_{ST} and *D* test differentiation on *a priori* groups (our population samples), whilst DAPC find clusters without any prior knowledge. Furthermore, unlike DAPC, F_{ST} and *D* estimates are affected by HWE deviations and linkage disequilibrium, which generally occur in every dataset (our included, Tables 3 and 4).

The analysis of the intergenic spacer *trnH-psbA* showed no differences among individuals from different population samples (data not shown). In all the individuals analysed, a shift of 1 bp in position 168 (Fig. 3) was detected in the region of the intergenic spacer (position from 31 to 381), producing two sequences different for one bp (LC372996 and LC372997, 434 and 433 bp respectively). We are confident in excluding polymerase artefacts since this difference occurs in all the individuals, and also contamination of samples, since DNA extractions were conducted separately in time for each population sample. A BLAST analysis revealed that the 434 bp fragment (with an A more), is 100 % identical to both *B. pendula* and *B. nana*, and 99 % to *B. platyphylla, B. papyrifera* and *B. alleghaniensis* (Fig. 3). On the contrary, the 433 bp fragment (the shorter one) is 100 % identical to *B. platyphylla*, and 99 % to *B. pendula*, *B. nana*, *B. papyrifera* and *B. alleghaniensis* (Fig. 3). Such sequence similarity across species suggests that chloroplast markers are not useful for analyses neither at species nor population level in these taxa. Indeed, several studies conducted using chloroplast markers in European species of genus *Betula* revealed extensive haplotype sharing across species and lack of genetic differentiation (PALME *et al.* 2004; MALIOUCHENKO *et al.* 2007), supporting previous hypotheses of hybridisation and introgression among *Betula* species (HOWLAND *et al.* 1991; THÓRSSON *et al.* 2001). Furthermore, being birches wind-pollinated angiosperms and considering that intraspecific gene flow among populations is much higher for pollen dispersed biparentally inherited nuclear DNA than seed dispersed maternally inherited cpDNA (PETIT *et al.* 1993), we expect to detect higher levels of genetic structure using nuclear DNA markers than chloroplast DNA regions. Literature data support the latter hypothesis, proving nuclear microsatellite markers to be effective for detecting genetic structure within and among *Betula* species (TSUDA & IDE 2005; TSUDA *et al.* 2017).

Our results confirmed the findings discussed above: nuclear microsatellites were effective in finding genetic structure at local scale, whilst chloroplast data were not useful for such purposes. Birch stands of Mt. Cerasuolo, Mt. Polveracchio and Mt. Cervati constitute different genetic groups, with the latter encompassing also genotypes from Mt. Somma. Whilst the first tree birch stands appeared healthy and dense,

on Mt. Somma birches were sparse and not very lush, anticipating a likely disappearance; however, further and prompt investigations are needed to ascertain the status of such remnant trees on the volcano.

The role of marginal populations for gene conservation strategies is often debated. According to the distance to central populations, peripheral populations can be more or less interconnected. Generally, being geographically isolated (peripheral) means also being ecologically marginal (LESICA & ALLENDORF 1995). Ecologically marginal populations often experience different biotic and abiotic environments as compared to inner ones: they can be specifically adapted to stressful environmental conditions (i.e., length of growing season, frost, light, drought, etc.) which could promote genetic differentiation but, on the other hand, exhibit overall low genetic variability due to isolation or stochastic factors (LESICA & ALLENDORF 1995). However, empirical studies suggest that predictions cannot be easily made since many factors as life history, spatial distance, time and ecology can shape the genetic make-up of populations and species (LOVELESS & HAMRICK 1984; FOLL & GAGGIOTTI 2006; DUMINIL *et al.* 2007). In this context, marginal populations should be protected whenever possible.

The birch stands here investigated are found at different altitudes and grow on different substrates (volcanic, calcareous), so experience different ecological conditions that might promote local adaptation. Furthermore, the genetic structure here detected in some populations is a clue of isolation that can promote adaptation.

Campania region is home to another glacial relict, the silver fir (*Abies alba* Mill.) and a recent survey of the only population in this region revealed high genetic diversity respect to other populations despite small size and geographic isolation (DE LUCA *et al.* 2017). These studies, as well as others conduct at regional scale (e.g. CENNAMO *et al.* 2013; DE CASTRO *et al.* 2013), highlight the importance of characterising genetic diversity and population structure at small spatial scales, especially for management and conservation purposes.

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