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Genetic diversity of Italian populations of *Abies alba*

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Abstract: Silver fir (*Abies alba*) is a common tree species in the mountainous areas in Europe. A number of natural stands in the hilly regions of northern Europe represent relic populations. The aim of the research was to evaluate the diversity present in Italian populations of the species. Genetic diversity was assessed in 45 silver fir populations covering the species' distribution range in Italy, based on the allelic variation present at seven microsatellite loci (SSRs). A consistent level of intra-population variability was present. Several of the populations displayed signs of ongoing genetic erosion, and evidence for a recent bottleneck in some was identified. Populations from the eastern Alps and the Apennines were more variable than those sampled from the western Alps. About 8% of the overall genetic variance was found between populations, with the remainder representing variation present within the populations. The data suggested that the southern Apennines acted as a refugium during the most recent Ice Age, and that many of the populations from this area have remained isolated over a prolonged period. Smaller and more isolated populations have experienced genetic drift, whereas the larger ones have preserved a high level of diversity. Identification of genetically homogeneous regions could be informative for the management of genetic resources.

Keywords: Silver fir, genetic differentiation, glacial refugia, regions of provenance, SSR markers

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Introduction

The *Abies* genus (Pinaceae) is represented by over 50 species, eight of which are endemic to the Mediterranean region and have a very limited distribution, namely *Abies bornmülleriana* Mattf., *A. cephalonica* Loudon, *A. cilicica* (Antoine & Kotschy) Carrière, *A. equi-trojani* (Asch. & Sint. ex Boiss.) Mattf., *A. nebrodensis* Lojac. (Mattei), *A. nordmanniana* Steven (Spach), *A. numidica* de Lannoy ex Carrière and *A. pinsapo* Boiss, divided in the two subspecies *tazaotana* and *marocana* (Dering et al., 2014). *A. borisii-regis* Mattf. is thought to be a natural hybrid between *A. alba* and *A. cephalonica* (Vidaković, 1991).

Fig. 1. Geographical distribution of 45 Italian populations of silver fir. WAl (western Alps), CEAl (central and eastern Alps), NAp (northern Apennines), GAl (Graian Alps), SCAp (central and southern Apennines). The European distribution of the species (according to EUFORGEN, http://www.euforgen.org/species/abies-alba/) is shown in the top right corner

Silver fir (*A. alba* Mill.) is common in the European mountainous areas, with several relic populations occupying certain hilly regions of the northern Europe. Three effective refugia during the Holocene have been identified: one lies in the Apennines and the other two in the Balkans (Liepelt et al., 2009; Mosca et al., 2012; Cheddadi et al., 2014). According to Muller et al. (2007), the Apennine refugium should be considered as two sub-areas, one at their northern and the other at their southern end, with a further area taking in the Pyrenees and adjacent parts of north-eastern Spain. However, fir did not expand from refugia in the Pyrenees and Southern Italy (Cheddadi et al., 2014). A further refuge in southern France, speculated in the past, has been then excluded (Muller et al., 2007).

In Italy, the silver fir covers an area of about 68,000 ha, of which about two thirds represent pure stands (http://www.sian.it/inventarioforestale/jsp/risultati_introb.jsp). The tree is relatively common in the central and eastern parts of the Alps, while its populations in the Apennines tend to be small and somewhat fragmented (Piovani et al., 2010); however, large stands are still be found in a few parts of the Apennines (Ducci et al., 1998). The cultivation of silver fir forests has a long tradition in Italy: the records show that both Franciscan and Benedictine monks were managing stands already 1,000 years ago as far apart as Tuscany in the northern part of the country and Calabria in the south. Its timber was used for ship construction by the Venetians and for supporting church roofs in both Florence and Rome (Ducci et al., 1998). In more modern times, its distribution has been strongly influenced by deforestation and/or replacement by European larch (*Larix decidua*).

Both anthropogenic activity and climate change exert a downwards pressure on the genetic diversity of natural forests. The pressure is particularly severe for marginal and peripheral populations (Jump & Peñuelas, 2006; Reich & Oleksyn, 2008; Leonardi et al., 2012). The long-term survival of such forests is heavily dependent on the preservation of intraspecific genetic diversity (Tessier du Cros et al., 1999; Kramer & Havens, 2009).

The characterization of genetic diversity pattern within species and among populations is a fundamental condition to establish any program aimed at biodiversity preservation. Genetic markers are basic to supply information on the genetic structure of populations, to analyse the pattern of within-species variability distribution (Pautasso, 2009), and to support the management of seed supply, taking into account the limits to be respected for seed collection (Escudero et al., 2003). In particular, knowledge of genetic variation should be the basis for ecological restoration actions (Väli et al., 2008). This is even more important where the preservation of local, marginal and well adapted and/or differentiated gene pools must be achieved through the implementation of such small and probably inbreeded populations (Rice & Emery, 2003; Frankham, 2005; Lefèvre et al., 2012). As a consequence, the objective is focal not only for scattered and rare species, but also for those characterised by wide distribution and that could seem less sensitive to genetic erosion (Eckert et al., 2008; Montoya, 2008).

The purpose of the present study was to assess the genetic variation retained in a number of silver fir populations sampled from various locations across Italy, based on genotyping at a set of nuclear microsatellite loci. Results are also discussed in the light of the European Directive 1999/105/CE, which deals with marketing of forest reproductive material and defines principles and criteria on which the forest seed chain should be based.

Material and methods

Silver fir population sampling

A set of 45 populations of silver fir was selected, covering the natural range of the species in Italy (Fig. 1, Table 1). Most of these populations are thought to

Table 1. Site characteristics of the 45 sampled Italian silver fir populations. The rainfall and temperature records are means calculated from at least five years of data

Code	Population	Geographical region	Latitude N	Longitude E	Sampling area elevation (m s.l.m.)	Annual (summer) rainfall (mm)	Annual average temperature (°C)
GOU	Gouta	Western Alps	43°56’	7°36’	1000–1300	1510 (286)	11.8
NAV	Navette	Western Alps	44°07’	7°43’	1400–1600	1320 (230)	8.2
GOR	Prel-Buscaiè	Western Alps	44°12’	7°39’	1000–1200	1290 (269)	10.6
VAL	Terme di Valdieri	Western Alps	44°12’	7°16’	1400–1600	1260 (220)	8.1
BAL	Pietraporzio	Western Alps	44°20’	7°02’	1300–1400	1000 (193)	5.4
CHI	Chiotspars, Prazzo	Western Alps	44°29’	7°05’	900–1100	980 (140)	5.9
SAL	Salza di Pinerolo	Western Alps	44°56’	7°03’	1200–1400	1130 (129)	5.4
GRB	Gran Bosco Salbertrand	Western Alps	45°03’	6°54’	1600–1800	760 (180)	6.2
FON	Fondo	Western Alps	45°31’	7°41’	1100–1200	1210 (320)	4.8
SES	Campello	Western Alps	45°41’	8°03’	1400–1600	1580 (450)	7.1

Code	Population	Geographical region	Latitude N	Longitude E	Sampling area elevation (m s.l.m.)	Annual (summer) rainfall (mm)	Annual average temperature (°C)
CER	Cervatto	Western Alps	45°53’	8°09’	1000–1100	1320 (260)	7.3
ANZ	Ceppo Morelli	Western Alps	45°58’	8°04’	800–1000	1240 (300)	5.2
BOG	S. Bernardo	Western Alps	46°09’	8°12’	1500–1700	1250 (346)	6.0
TOC	Bosco della Colma	Western Alps	46°09’	8°28’	1100–1300	1610 (420)	7.2
VAR	Vargno	Western Alps	45°39’	7°54’	1400–1700	780 (120)	5.4
CRX	Croix de la Fana	Western Alps	45°47’	7°25’	1700–2000	670 (100)	5.8
BON	Bondinaz	Western Alps	45°43’	7°23’	1100–1500	690 (110)	6.1
PEN	Pendine	Western Alps	45°42’	7°17’	950–1450	700 (120)	6.7
CHA	Chabodey	Western Alps	45°44’	7°03’	1000–1400	890 (170)	6.2
BEL	Bellouque	Western Alps	45°49’	7°21’	1000–1700	970 (190)	5.6
GRE	Gressoney	Western Alps	45°50’	7°49’	1400–1900	810 (210)	6.1
BAG	Camaluf	Central Alps	45°51’	10°26’	1200–1400	1340 (370)	6.8
ALB	Albaredo	Central Alps	46°05’	9°37’	1000–1300	1230 (220)	6.9
AND	Paganella	Central Alps	46°09’	11°00’	1000–1200	1080 (270)	4.8
VPT	Fleres	Eastern Alps	46°58’	11°21’	1300–1600	670 (220)	5.1
LAU	Laudes	Eastern Alps	46°41’	10°32’	1100–1400	690 (210)	5.3
TRO	Trodena	Eastern Alps	46°19’	11°22’	1200–1500	1100 (180)	6.9
FAV	Favogna	Eastern Alps	46°17’	11°11’	1200–1500	730 (220)	7.1
SCA	S. Candido	Eastern Alps	46°43’	12°16’	1300–1600	770 (260)	4.7
TES	Tesimo	Eastern Alps	46°32’	11°10’	1000–1300	720 (240)	7.8
VIS	Val Visdende	Eastern Alps	46°37’	12°39’	1400–1600	920 (260)	5.2
DOS	Dosso	Eastern Alps	45°55’	11°30’	1300–1500	1430 (320)	5.9
PAU	Paularo	Eastern Alps	46°32’	13°07’	900–1100	1850 (450)	7.0
MNR	Monte Nero	Northern Apennines	44°34’	9°30’	1400–1600	1320 (218)	8.8
VEN	Ventasso	Northern Apennines	44°23’	10°16’	1100–1300	1400 (250)	8.4
APU	Alpi Apuane	Northern Apennines	44°09’	10°26’	900–1200	1790 (370)	10.3
ABE	Abetone	Northern Apennines	44°09’	10°40’	1200–1400	2540 (470)	7.2
VER	Foreste Casentinesi	Northern Apennines	43°42’	11°56’	1100–1300	1620 (320)	8.6
RTR	Bocca Trabaria	Central Apennines	43°34’	12°11’	800–1100	1320 (260)	10.7
PIG	Monte Amiata	Central Apennines	42°53’	11°38’	1500–1700	1330 (320)	8.7
MAC	Macera della Morte	Central Apennines	42°44’	13°24’	900–1200	1480 (320)	9.8
FVP	Fonte Volpona	Central Apennines	41°53’	14°21’	800–1000	1230 (210)	10.4
VAC	Vaccarizzo	Southern Apennines	40°09’	16°05’	700–1000	1030 (320)	10.2
GAR	Gariglione	Southern Apennines	39°08’	16°39’	1500–1600	2140 (410)	10.3
ARC	Archiforo	Southern Apennines	38°33’	16°21’	1000–1200	2020 (390)	10.7

be native, although some silver fir planting has been carried out in the past, especially in the northern Apennines. In particular, the populations sampled in MNR (Piovani et al., 2010), APU (Amorfini et al., 2004) and VER (Piovani et al., 2010; Ducci & Proietti, 1997) have been shown to have evolved naturally; in any case, only trees old enough (> 80 years) to have been in place prior to any recent afforestation activity were sampled. The populations from GOU, GOR, CHI, SAL, GBR, VIS, DOS, PAU, VER, ABE, FVP, ARC and GAR have been registered in the Italian National Book of Seed Stands: they were selected on basis of their phenotype and their health status (Morandini & Magini, 1975; Ducci & De Rogatis, 2010). The BAL, TOC, ALB and TRO populations are also “registered seed stands”, although only at the regional level (Camerano et al., 2012). Most of the populations occur as mixed stands, accompanied by Norway spruce (*Picea abies*) and European larch (*Larix decidua*) in the western and central Alps, Norway spruce and black pine (*Pinus nigra*) in the eastern Alps, and the European beech (*Fagus sylvatica*) in the Apennines. With one exception, each populations was represented by 24 adult, non-contiguous trees, selected at random. The exception was the APU population, where the samples comprised only 17 trees. The overall number of trees subjected to genotyping was 1,073.

Marker analysis

Needles were stored at –20°C until processed for DNA extraction. A 100 mg batch of needles of each entry was treated with an E.Z.N.A. HP Plant DNA Mini kit (Omega Bio-tek Inc., Norcross, GA, USA), following the manufacturer’s instructions. The resulting DNA templates were amplified using seven pairs of primers, each targeting a variable

Table 2. Experimental details of the seven microsatellite loci assayed. A: mean number of alleles per population; N_e: mean effective number of alleles per populations; I = Shannon's information index; H_o: mean observed heterozygosity; H_e: mean expected heterozygosity

Locus	Repeat motif	Forward and reverse primer sequences (5' → 3')	Fluorescent label	Total number of alleles	Molecular weight range (bp)	A	N_e	I	H_o	H_e
SF1	$(CCG)_9$	TTGACGTGATTAACAATCCA AAGAACGACACCATTCTCAC	IRD-700	10	202–229	3.6	2.2	0.930	0.583	0.543
SFb4	$(GT)_{16}$	GCCTTTGCAACATAATTGG TCACAATTGTTATGTGTGTGG	IRD-800	26	144–200	11.8	6.5	2.094	0.695	0.833
SFb5	$(CT)_{15}$	AAAAAGCATCACTTTTCTCG AAGAGGAGGGGAGTTACAAG	IRD-700	12	135–159	7.5	4.1	1.594	0.560	0.728
SFg6	$(AC)_9$	GTAACAATAAAAGGAAGCTACG TGTGACACATTGGACACC	IRD-800	7	102–116	4.7	3.2	1.271	0.214	0.665
SF78	$(CGCA)_8(CA)_{15}G(CA)_8$	CATTGTTGTCTTTGTTTCACA TGCACCGTTTTGTTTTTCC	IRD-700	51	145–263	14.5	8.8	2.352	0.828	0.872
SF324	$(CCG)_8$	TTTGAACGGAAATCAAATTCC AAGAACGACACCATTCTCAC	IRD-800	12	94–154	3.6	2.3	0.934	0.521	0.550
SF333	$(CA)_{12}(TA)_4$	ATTTGTTCATTTTGGTCCTG ACACAGGAAAAAGTCGGTAA	IRD-800	13	150–180	6.4	4.1	1.551	0.528	0.743

microsatellite locus (Cremer et al., 2006): SF1, SFb4, SFb5, SFg6, SF78, SF324, SF333 (Table 2).

For six of the seven microsatellites (the exception was SF333), each 13 μL PCR, made up in 1x Promega (Madison, WI, USA) buffer, contained 2.5 mM $MgCl_2$, 0.65 U *Taq* polymerase (Promega), 0.5 μM of each primer, 0.2 mM dNTP and ~20 ng template DNA. For the SF333 assay, the $MgCl_2$ concentration was reduced to 0.5 mM and the primer concentration to 0.4 μM. For the six assays excluding SF333, a touchdown amplification protocol was applied, which comprised an initial denaturation of 94°C/5 min, followed by ten cycles (94°C/30 s, 65°C/30 s reducing by 1°C per cycle, 72°C/40 s), a further 23 cycles of 94°C/30 s, 55°C/50 s, 72°C/40 s and a final extension of 72°C/7 min. For the SF333 assay, the initial denaturation was 94°C/3 min, and this was followed by 30 cycles of 94°C/30 s, 50°C/30 s, 65°C/90 s, and a final extension of 65°C/15 min. The forward primers were fluorescently 5' labelled with either IRD-700 or IRD-800. The resulting amplicons were electrophoresed through a denaturing 6% polyacrylamide gel and detected automatically using a DNA 4200 Sequencer (LI-COR Biotechnology, Lincoln, NE, USA). The gels were run for 2 h at a constant 2,000 V in 1x TBE buffer. IRD 700- and IRD 800-labelled ladders were included to enable the estimation of fragment sizes.

Data analysis

Genetic variation within populations averaged over the seven loci was estimated using GenAlEx v.6 software (Peakall & Smouse, 2006, 2012), which also allowed for the calculation of allele frequencies, the mean number of alleles per locus, the mean number of private and effective alleles per locus, along with the observed and expected heterozygosity level (H_o and H_e respectively). Allelic richness was derived using FStat software (Goudet, 1995). Genotypic disequilibrium between pairs of loci was tested at the single population level and across all populations, with Fisher's exact test using Arlequin software (Excoffier et al., 2005). Fisher's exact test based on the Markov Chain algorithm (Guo & Thompson, 1992) was used to detect deviations from Hardy-Weinberg equilibrium for each population and each locus. Where a significant deficiency of heterozygotes was detected, the presence of a null alleles was inferred (Pemberton et al., 1995). Loci showing a high frequencies of null alleles were identified using Micro-Checker software (Van Oosterhout et al., 2004). The estimation of the inbreeding coefficients took into account the frequency of null alleles; the necessary calculations were performed with INEst software (Chybicki & Burczyk, 2009), selecting the individual inbreeding model (IIM) with a Gibbs sampler of 10^5 iterations (Chybicki & Burczyk, 2009). The Bottleneck v.1.2.02 program (Piry et al., 1999) was employed to predict recent population bottlenecks. A Wilcoxon's sign rank test compared the level of heterozygosity expected assuming a Hardy-Weinberg equilibrium with predicted heterozygosity at mutation-drift equilibrium, on the basis of the observed allele number (Piry et al., 1999). The program was run both assuming a two-phase model (TPM) of mutation, and a pure stepwise or infinite allele models (SMM, Di Rienzo et al., 1994). A thousand simulations were performed for each sample, consisting of 90% single mutations and 10% multistep alterations. FreeNA (Chapuis & Estoup, 2007) was used to compute the Weir (1996) F-statistics; F_{ST} was taken as an estimate of the proportion of the overall genetic variance contributed

by the between population component (Slatkin, 1995). The Phylip package (Felsenstein, 2013) used the resulting genetic distance matrix to construct a UPGMA-based dendrogram. A cophenetic value matrix was then calculated by the Coph routine within Ntsys-pc software (Rholf, 2006); this was used by the Mxcomp routine to evaluate the goodness-of-fit of the predicted clusters. An analysis of molecular variance (AMOVA; Excoffier et al., 1992) was performed using the Arlequin package (Excoffier et al., 2005). The genetic structure of the populations was obtained by deploying the Pritchard et al. (2000) Structure program, setting a burn-in period of 10^5 followed by $5x10^5$ iterations, and using both the admixture ancestry and the correlated allele frequency model. K values were calculated from a mean of 20 runs for each value of K between 1 and 10. The optimum value of K was determined following Rosenberg et al. (2001) and Evanno et al. (2005).

Results

Allelic diversity and within population genetic variation

A summary of the within population genetic diversity statistics is shown as Table 3. A total of 131 alleles (size range 94–263 bp) were revealed at the seven microsatellite loci, representing a mean of about 19 per locus. The most variable locus was SF78 (51 alleles) and the least variable SFg6 (seven alleles). In total, 16 of the alleles were "private" (i.e., present in only a single population); on a per locus basis, their number ranged from zero (SFb5) to six (SF78). The FVP and GAR populations each harboured two, and the 12 populations NAV, GOR, GRB, BOG, TOC, VPT, SCA, TES, DOS, APU, MAC and ARC each harboured one private allele. On average, at least seven alleles per locus were present in all 45 populations; the lowest number occurred in the APU population, but even here, allele richness and the levels of heterozygosity lay close to the global means. Populations collected from the Alps were the least variable (Fig. 2, Table 3): this was particularly evident for populations derived from the north-western sector. The global H_e was 0.724, while H_o was only 0.563: this difference likely reflected a combination of non-random mating and the presence of null alleles. The latter were present at significant frequency with respect to both SFg6 (average frequency 0.14) and SFb5 (0.05). Re-calculating the inbreeding coefficients following adjustment for the frequency of null alleles considerably reduced the extent of the deviation from the Hardy-Weinberg equilibrium (F_{IS} ranged from 0.013 in ABE to 0.080 in

Table 3. Within population measures of genetic variation. A: mean number of alleles per locus; Ne: effective number of alleles per locus; Ar10: allele richness; Pa: frequency of private alleles per locus; H_o: mean observed heterozygosity; H_e: mean expected heterozygosity; F_{IS}: mean inbreeding coefficient, taking into account null alleles. None of the F_{IS} values are significantly different from zero. Values in parenthesis for overall means are standard errors. BTPM and BSMM: Wilcoxon test parameters regarding evidence for recent bottlenecks; the former is derived from the two-phase model of mutation (TPM), and the latter from the pure stepwise or infinite allele model (SMM). ns: not significant, *, **: significant at p <0.05, <0.01

Population	A	N_e	A_{r10}	P_a	H_o	H_e	F_{IS}	B_{TPM}	B_{SMM}
GOU	7.4	4.4	4.336	0	0.565	0.688	0.031	ns	ns
NAV	7.6	4.0	4.371	0.143	0.621	0.722	0.015	ns	ns
GOR	7.7	4.5	4.527	0.143	0.637	0.735	0.024	ns	ns
VAL	7.3	4.0	4.418	0	0.581	0.734	0.073	ns	ns
BAL	6.7	3.8	4.135	0	0.556	0.690	0.019	ns	ns
CHI	7.1	3.9	4.406	0	0.593	0.708	0.014	ns	ns
SAL	7.6	4.1	4.342	0	0.554	0.686	0.016	ns	ns
GRB	6.4	3.6	3.884	0.143	0.513	0.657	0.020	ns	ns
FON	6.6	4.0	4.342	0	0.497	0.727	0.024	ns	ns
SES	6.6	3.8	4.161	0	0.509	0.698	0.020	ns	ns
CER	6.6	3.7	4.084	0	0.560	0.700	0.021	ns	ns
ANZ	7.4	3.8	4.203	0	0.520	0.716	0.023	ns	ns
BOG	6.9	3.4	3.973	0.143	0.523	0.679	0.030	ns	ns
TOC	7.0	3.5	4.076	0.143	0.604	0.706	0.017	ns	ns
VAR	6.9	3.7	4.207	0	0.461	0.689	0.080	ns	ns
CRX	7.1	4.2	4.474	0	0.488	0.722	0.046	ns	ns
BON	6.3	4.0	4.275	0	0.488	0.732	0.051	ns	ns
PEN	7.0	3.9	4.234	0	0.462	0.683	0.037	ns	ns
CHA	7.7	4.1	4.524	0	0.502	0.722	0.055	ns	ns
BEL	5.9	3.0	3.772	0	0.450	0.649	0.065	ns	ns
GRE	6.6	3.9	4.132	0	0.492	0.680	0.032	ns	ns

Population	A	N_e	A_{r10}	P_a	H_o	H_e	F_{IS}	B_{TPM}	B_{SMM}
BAG	7.0	3.9	4.290	0	0.578	0.712	0.040	ns	ns
ALB	7.9	4.2	4.405	0	0.569	0.715	0.022	ns	ns
AND	7.7	4.6	4.405	0	0.574	0.729	0.014	ns	ns
VPT	7.9	4.6	4.600	0.143	0.554	0.735	0.020	ns	ns
LAU	8.3	5.5	4.907	0	0.553	0.786	0.030	ns	ns
TRO	8.4	4.5	4.492	0	0.515	0.719	0.040	ns	ns
FAV	7.9	4.4	4.354	0	0.605	0.713	0.030	ns	ns
SCA	8.1	5.3	4.688	0.143	0.546	0.763	0.036	ns	ns
TES	8.6	4.5	4.592	0.143	0.517	0.731	0.033	ns	ns
VIS	8.3	4.7	4.481	0	0.499	0.732	0.029	ns	ns
DOS	7.9	4.5	4.461	0.143	0.555	0.720	0.041	ns	ns
PAU	7.9	4.7	4.396	0	0.568	0.709	0.017	ns	ns
MNR	6.9	4.6	4.602	0	0.593	0.760	0.051	*	ns
VEN	6.3	4.6	4.520	0	0.529	0.737	0.040	**	ns
APU	5.1	3.8	4.333	0.143	0.539	0.707	0.035	ns	ns
ABE	8.1	6.0	5.011	0	0.673	0.774	0.013	**	**
VER	8.0	5.7	4.934	0	0.692	0.779	0.022	**	*
RTR	8.3	4.6	4.495	0	0.645	0.725	0.016	ns	ns
PIG	8.4	6.5	5.139	0	0.636	0.778	0.022	**	**
MAC	8.4	5.8	4.984	0.143	0.615	0.762	0.021	*	ns
FVP	7.7	4.8	4.698	0.286	0.649	0.760	0.037	ns	ns
VAC	8.7	6.4	5.197	0	0.697	0.791	0.014	**	*
GAR	8.3	5.8	4.942	0.286	0.532	0.753	0.029	ns	ns
ARC	9.0	5.4	4.990	0.143	0.709	0.768	0.014	ns	ns
Overall mean	7.45 (0.24)	4.47 (0.15)	4.453	0.046	0.563 (0.012)	0.724 (0.008)	0.030 (0.016)		

VAR, with a mean of 0.030). Since the confidence intervals calculated by INEst software overlapped zero in every population, it was concluded that none of the F_{IS} values were significantly different from zero. Although for most of the populations the H_e value and the gene diversity at mutation-drift equilibrium

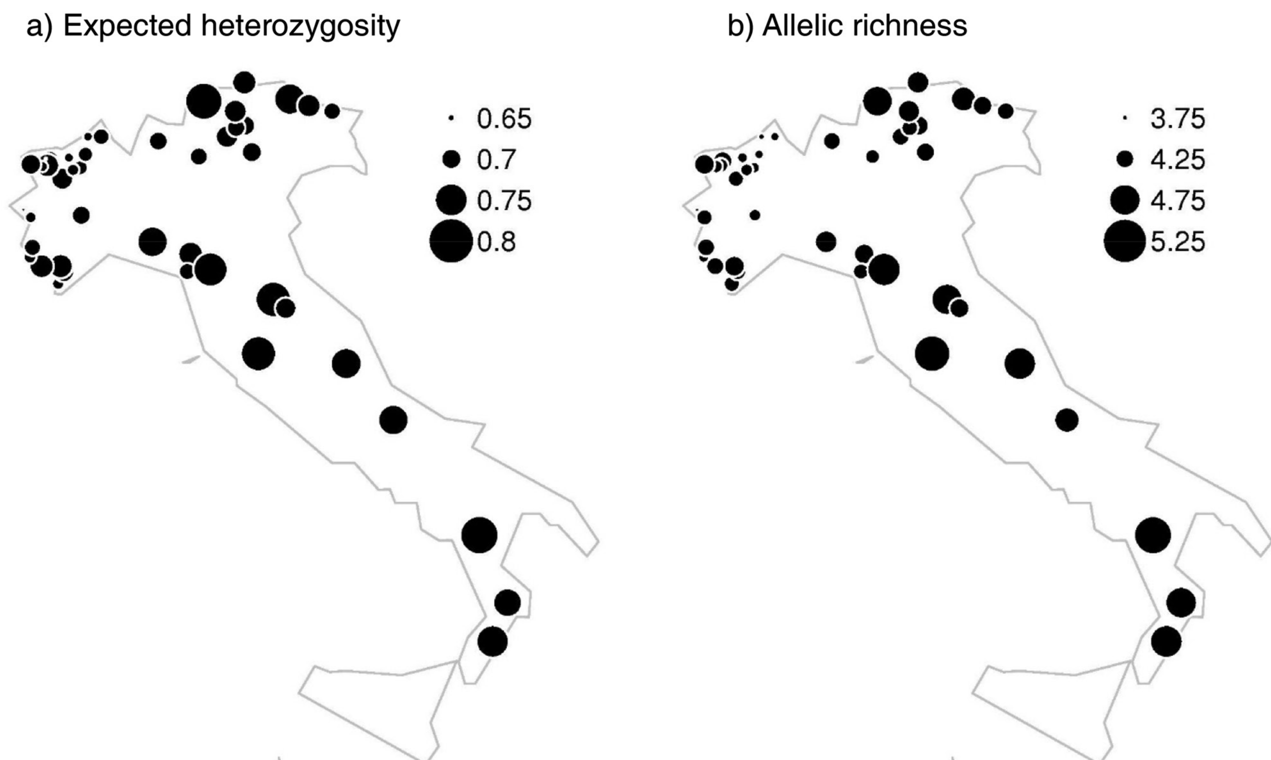

Fig. 2. Genetic variation in Italian silver fir populations. (a) Expected heterozygosity (H_e), (b) allele richness

did not differ significantly from one another, some evidence for recent bottlenecks was revealed in populations sampled from the central and southern parts of the Apennines (MNR, VEN, ABE, VER, PIG, MAC and VAC: see Table 3).

Genetic differentiation between populations

Most of the genetic diversity was partitioned to the within population component (F_{ST} of 0.082, SE 0.005). The AMOVA confirmed this conclusion, revealing that 8.25% of the variance was assignable to differences among populations, leaving the remaining 91.75% to the within population component (data not shown). The F_{ST} values on a per locus basis ranged from 0.060 (SF333) to 0.107 (SFg6). When the extent of genetic divergence between populations was quantified using the pairwise F_{ST} matrix, the F_{ST} values were found to lie between 0.009 (BOG and ANZ) and 0.108 (CHA and APU). Almost all of the pairwise F_{ST} values lay significantly above zero, suggesting the presence of a distinct population structure (data not shown). The UPGMA-based phylogenetic analysis (Fig. 3) confirmed a level of between population differentiation, as well as a consistent degree of structuring. The cophenetic correlation coefficient was 0.807 (P <0.001), implying a high goodness-of-fit for the cluster analysis. In particular, the populations sampled from the central and eastern Alps displayed a clear tendency to cluster (CEAl in Fig. 3), while the Apennine populations were split into two groups, one (NAp) including most of the

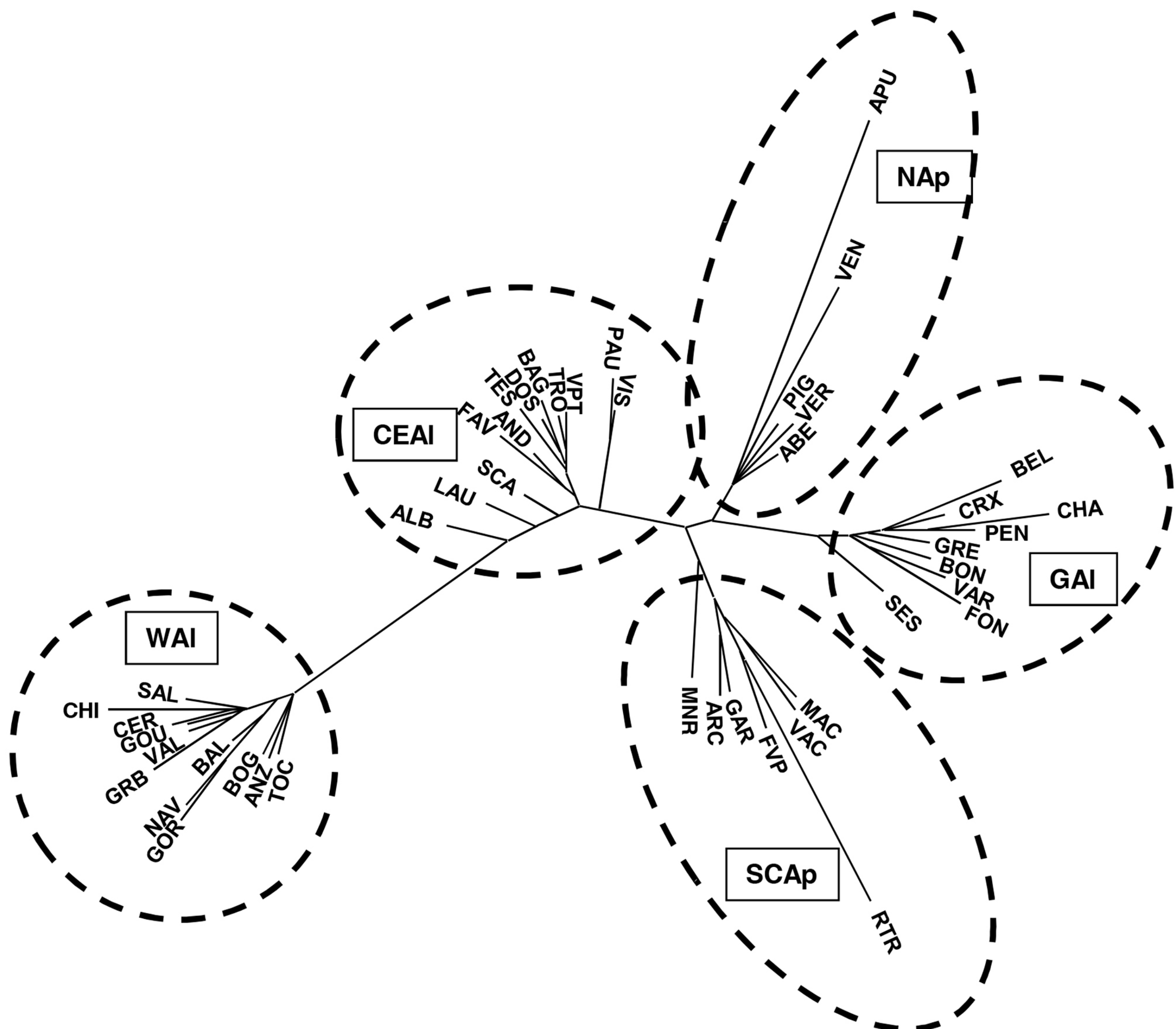

Fig. 3. Genetic structure of the Italian silver fir populations. Dotted lines link populations from a shared geographical region: WAl (western Alps), CEAl (central and eastern Alps), NAp (northern Apennines), GAl (Graian Alps), SCAp (central and southern Apennines)

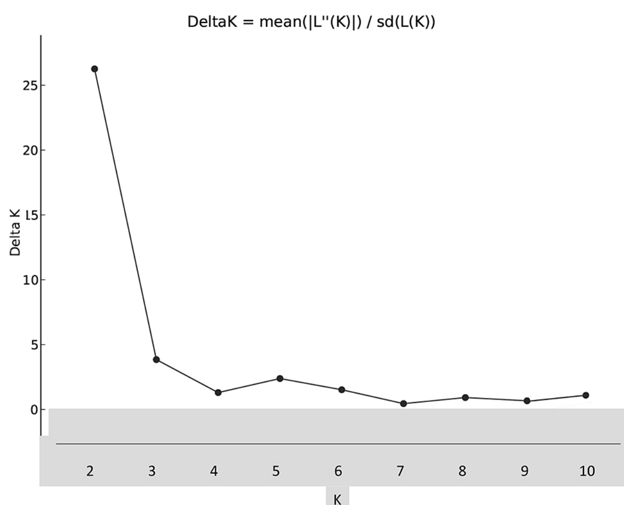

Fig. 4. Identification of the most likely number of clusters formed by 45 populations of silver fir in Italy

populations sampled from the northern part of the range together with PIG (central Apennines, but strongly isolated by others), while the other (SCAp) combined the populations sampled from the central and southern part with one sampled from the northern Apennines (MNR). The populations sampled from the western Alps also formed two clusters, one comprising the trees growing around the Aosta Valley (GAl) and the other those growing in the Ligurian, Maritime, Cottian and Pennine Alps (WAl). The Structure analysis implied that a K value of 2 was the most likely (Fig. 4). Most of the trees sampled from the north-western populations belonged to cluster 1, whereas those sampled from the eastern Alps and the Apennines were dominated by individuals belonging to cluster 2 (Fig. 5). Some admixed populations were detected among most of the geographical regions, although admixture was prominent in the GAl populations (FON, SES, VAR, CRX, BON, PEN, CHA, BEL and GRE).

Discussion

The objective of this research was to assess the level and the distribution of genetic variability of natural stands of silver fir in Italy, with a view to elaborate a rational strategy for conserving its genetic diversity. The genotyping of samples of the 45 populations, albeit at only a limited number of nuclear microsatellite loci, revealed an extensive bank of allelic variation: the mean number of alleles per locus was as high as 9.0 (mean 7.4), while H_e ranged from

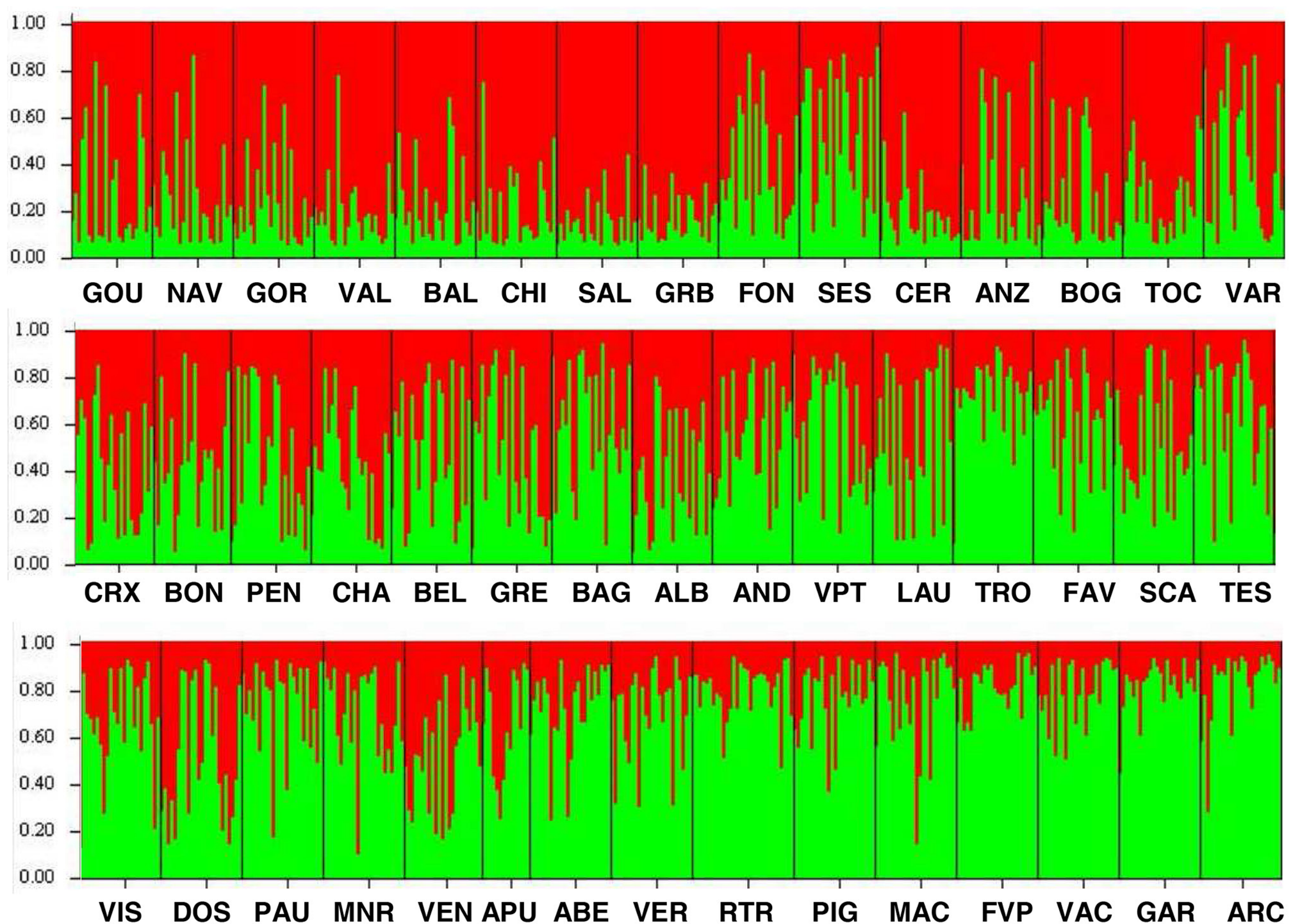

Fig. 5. Inference of population structure in silver fir using STRUCTURE software (K = 2)

0.649 to 0.791 (mean 0.723) (Fig. 2 and Table 3). The presence of high diversity was not unexpected, since it is a feature of many woody plants, especially those which are not specialized to a narrow habitat (Hamrick et al., 1992). The genetic diversity present in the populations growing in the Apennine sites (especially in the central and southern part of the range) was higher than that of the Alpine ones; with respect to the latter, those sourced from the eastern part of the range tended to be more variable than those from the western part. An exception was found among populations sampled in the far south-western region (Ligurian and Maritime Alps: GOU, NAV, GOR and VAL), where genetic variability was close to the global mean. High levels of genetic variability in this area have been reported previously for a number of species (Minuto et al., 2006; Ferrazzini et al., 2007; Casazza et al., 2016), implying that this region has a rather unique ecology, reflecting the presence of very high mountains (up to 3,297 m a.s.l. of Argentera Mt) in close proximity to the Mediterranean Sea (maximum distance 50 km) (Casazza et al., 2008). The area is considered as an important biodiversity hot-spot (Medail & Quezel, 1999). A further indication of the greater variability of the Apennine populations was the high frequency of private alleles. Some evidence for recent population bottlenecks was associated with some of the sites along the Apennine chain (Table 3); the most probable cause of this was the widespread logging carried out in the area over the last 200 years. Genetic drift is expected to have been an issue only in populations experiencing fragmentation and isolation (e.g., MNR and VEN).

The overall level of between population diversity was comparatively high (F_{ST} 0.082), especially given the small size of the study area, and also confirmed by AMOVA. Conifer populations are typically associated with rather low levels of genetic differentiation, thanks to their allogamous mating system and their efficient dispersal systems for both pollen and seed (Petit & Hampe, 2006; Piotti et al., 2009; Williams, 2010). Still, a powerful enough environment-driven selection pressure can generate local adaptation even where gene flow is high, as exemplified in *Abies alba* by Mosca et al. (2012).

The Graian Alps, which include the Aosta Valley and neighbouring valleys, were highly differentiated from the other alpine populations, even though the physical distance involved are small (particularly for the Cottian and Pennine Alps). This could be due to the specific features of this area, which is bordered to the west and north by the highest mountains in the entire Alpine chain (Mont Blanc and Monte Rosa in particular); these represent a very effective barrier against gene flow. At the southern end of the Aosta Valley is a large area where silver fir is rare or even absent, which again likely constitutes a major obstacle to gene flow. The possibility is that the species entered Aosta Valley during postglacial period, but became isolated from the other lineages that diffused and evolved, by adaptation, at a faster rate. A similar scenario has been proposed for other species by Schönswetter et al. (2005), Bettin et al. (2007) and Thiel-Egenter et al. (2010).

For practical reasons, the number of trees analysed per population was limited to 24, which raises the possibility of sampling bias. Although the precision of any estimate of genetic variability can clearly be improved by increasing the sample size, F_{ST} is known to be associated with only a minor sampling variance at multi-allelic loci such as microsatellites (Kalinowski, 2005). Similarly, more precision can generally be obtained by assaying a greater number of loci than by increasing the number of individuals genotyped (Kalinowski, 2005). Both the genetic markers (highly polymorphic microsatellites) and the population genetic indices (F_{ST}) applied were directed at minimizing estimation biases caused by a small sample size (Miyamoto et al., 2008). With this proviso, the genetic architecture of the silver fir stands in the southern part of the Apennine range implied that the region represented a refugium during the last Pleistocene glaciation, leaving behind a number of well differentiated and isolated populations harbouring a considerable level of genetic variability. The occurrence of such high levels of genetic variability appears at odds with the norm for marginal populations, usually small and which tend to become highly adapted to a specific ecological niche and suffer from genetic erosion (Fady et al., 2016). However, this seems not the case of silver fir in southern Italy, probably because the large size of the populations and the presence of very favourable climatic conditions, as already observed in previous studies (Longauer et al., 2003)

The implication of the isolation of the populations growing in southern Apennine range is that the postglacial expansion of silver fir into central and northern Europe most probably originated from the northern Apennines, as suggested by Muller et al. (2007). Other species appear to have behaved in a similar way (Feliner, 2011). An important conclusion to be drawn is that silver fir genetic resources need to be preserved throughout the Apennines. The point of discontinuity between the non-migrating southern Apennine populations and the migrating northern ones remains undefined. The present data suggest that this demarcation lies between Tuscany/Emilia-Romagna and Umbria/Marche, but this conclusion is at best provisional. The PIG population is rather exceptional in this context: although it lies within the Northern Apennines area, it is very isolated, and represents a population in which the evidence for genetic bottleneck is strongest; nevertheless, its

level of diversity was higher than the global mean, in contrast to some of other small, isolated populations in the Northern Apennines zone, which showed clear symptoms of genetic erosion. The APU population is of particular conservation value, since despite its very small size (only a few tens of individuals), it harboured a number of private alleles and showed no evidence of any bottleneck. Valuable populations like this, as pointed out by Piovani et al. (2010), require careful management, which should include, for example, the cultivation of local seed. Overall, the diversity patterns revealed in the silver fir populations were consistent with the "rear edge versus leading edge" concept, which emphasizes the importance of peripheral populations for species survival and future adaptation to a changing environment (Hampe & Petit, 2005).

Apart from contributing to the understanding of silver fir diversity, the conclusions reached here can be used to set in place an effective programme of species diversity preservation. According to the European Council Directive 1999/105/CE, forest reproductive material falling into the "source-identified" and "selected" categories should be used only within the same region of provenance. The identification of these "regions of provenance" therefore represents both a priority for the *in situ* conservation of this species and an important management task for the authorities responsible for afforestation.

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