Establishing a baseline to monitor future climate-change-effects on peripheral populations of *Abies alba* in central Apennines

Fulvio Ducci¹, Anna De Rogatis², Roberta Proietti¹, Alexandru Lucian Curtu³, Maurizio Marchi⁴, Piero Belletti⁵


**Abstract** Understanding tree species responses to climate change is crucial for preserving biodiversity especially in Southern Europe hot spots where *Abies alba* is widely spread. Three Apennine silver fir populations, Pigelleto (PIG), La Verna (LV) and Bocca Trabaria (BT), ensured gene flows in interglacial periods between the two phylogenetically different groups of northern and southern Apennines. These stands were analysed (nuclear and chloroplast SSRs) with the aim to establish a baseline for their future management in view of the expected changes. The three forests were tested for the Centre-Periphery Hypothesis (CPH) compared to forty-five Italian populations. At the same time, permanent areas were surveyed within LV and PIG on dominant (a) and dominated or natural regeneration (r) tree layers, and on age classes. In two consecutive years, spring cambial phenology activity was also weekly monitored on microcores, and critical phenology dates recorded. The stands matched CPH only partially, showing different phylogenetic history and their bridging between northern and southern groups of silver fir populations was confirmed. LV was distinct from PIG and BT. The within-population variance component was significantly high, and no narrow relatedness was observed between dominant and dominated/regeneration spatially closer trees, and genetic parameters were comparable in both layers at LV and PIG. In both stands, older age classes ensured natural regeneration. Cambium phenology was highly variable within populations, consistently to other Mediterranean conifers, and highly sensitive to local and year’s conditions and monitoring will improve population’s adaptive capacity detection. Shelterwood-system silvicultural treatments are suggested on small areas to drive the demographic and panmictic balance towards an uneven-aged more resilient structure, and iterated monitoring will help to adapt the forest management to the isotherm shift.

**Keywords:** *Abies alba*, silver fir, genetic diversity, cambial phenology, marginal/peripheral populations, Apennines, isotherm shift

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Introduction

Southern Europe is considered a hot spot of genetic diversity, encompassing forest tree populations that served as sources of variation for the post-glacial northward re-colonisation (Petit et al. 2003), including silver fir (Abies alba Mill. 1759; Linares 2011, Ducci 2015, Piotti et al. 2017). The silver fir natural distribution range has been shaped by both climate oscillations (Pfister & Brazdil 1999, Magri et al. 2006) and anthropogenic activities (Lefèvre 2004). This species is highly sensitive to climate fluctuations, and potential scenarios for its future distribution range were recently formulated (Piotti et al. 2017). In response to the predicted rise in global temperature effects (IPCC 2014), silver fir is expected to retreat northwards and into higher altitude environments, primarily to be replaced by broad-leaved tree species, such as beech (Miozzo et al. 2014, Marchi & Ducci 2018). Several surveys and forest inventories (Marchi & Ducci 2018, Frejaville et al. 2019) have shown the risks of increasing marginalization of peripheral silver fir stands (Fig. 1).

Several Apennine silver fir populations can be classified both marginal and peripheral (MaP; Lesica & Allendorf 1995, Eckert et al. 2008, Pironon et al. 2014) and have long been found to harbor high levels of variation and sometimes unique adaptive traits (Bergmann & Gregorius 1992, Ducci et al. 1998, Thuiller et al. 2008, Steen & Barrett 2015).

**Figure 1** Current environmental suitability map (a) and predicted scenarios for 2050s RCP 8.5 (b) for silver fir in the Italian peninsula after Marchi & Ducci (2018). Suitable lands are shown in green. Y and X axes in plots are respectively latitude and longitude.
MaP populations grow at the edges of a species’ ecological niche space and can be poorly connected, or not connected at all, by gene flows to other populations of the same species (Fady et al. 2016). According to Eckert et al. (2008), peripheral populations would have reduced genetic diversity and increased genetic differentiation compared to the species core, due to supposedly lower effective population size, higher levels of genetic drift, founder effect and isolation, or a combination of these factors. On the other side, according to Hampe & Petit (2005), most of the marginal isolated populations could have preserved high genetic distinctiveness. In these populations, selection for local adaptation may be expected resulting in the development of distinct ecotypes. Indeed, they may have remained sufficiently isolated to retain alleles responsible for their unique traits (Thuiller et al. 2008, Steen & Barrett 2015) and may represent an important genetic resource for adaptation (Lindner et al. 2010, Fady et al. 2016).

MaP should be monitored and potentially preserved, especially in view of climate-change-associated effects (Piovani et al. 2010, Fady et al. 2016). Monitoring is among the priority areas put forth by the Global Plan of Action for Forest Genetic Resources (FGR, FAO 2014) and it is also stressed in the Convention on Biological Diversity (Laikre et al. 2009). The concept of “genetic monitoring” defines the systematic study of within-population adaptive and neutral genetic variation with the aim of detecting changes over time and to improve the sustainability of applied forest management practices (Aravanopoulos 2016, Fady et al. 2016, Fussi et al. 2016). Most gene-ecological approaches in monitoring consider several genetic indicators and verifiers of neutral and adaptive genetic variation, including phenological parameters (Aravanopoulos 2011, 2016, Konnert et al. 2011, Kavaliauskas et al. 2018). Indeed, phenological traits are of focal interest as a response to the environmental factors, influencing and shaping the fitness of populations at local level (Chuine & Beaubien 2001, Denny et al. 2014).

Two main groups of A. alba populations can be genetically distinguished on Apennines, one northern and one southern (Konnert & Bergmann 1995), whose border was roughly identified among the populations of Bocca Trabaria (BT, province of Pesaro-Urbino), La Verna (LV, province of Arezzo) and Pigelleto (PIG, province of Siena) (Belletti et al. 2017, Piotti et al. 2017). According to Konnert & Bergmann (1995) and confirmed by Santini et al. (2018), this area may also represent an ancient point of contact/introgression with Balkan populations (Longauer et al. 2003, Liepelt et al. 2009, Piotti et al. 2017), as also observed in other silver fir populations found in central-eastern Europe (Gömöry et al. 2012, Bosela et al. 2016, Popović et al. 2017). Bocca Trabaria, La Verna and Pigelleto stands grow at the edges of both the northern and the southern Apennine phylogenetic groups and can be therefore considered transitional metapopulations between them (Piovani et al. 2010, Belletti et al. 2017, Piotti et al. 2017). Their monitoring in real-time may help to assess their response to environmental change.

For this reason, between 2010 and 2012, long-term monitoring began in the framework of the Life Project ReSilFor (REstoring SILver-fir FORest, LIFE08 NAT /IT/000371) on Bocca Trabaria, La Verna and Pigelleto populations.

At this stage, phylogeographic relationships among populations based on data in Belletti et al. (2017) were examined to provide their historical context.

A baseline of their status was therefore required to assess their genetic variation and relationships among adult/dominant regeneration/dominated demographic layers and age classes.

In this baseline framework, cambial phenology behavior is an important functional trait (Housset et al. 2016) related to the
population adaptive potential and its fitness. Dormancy breaking is conditioned by species population chilling requirements and seasonal variation of cambial activity is a way of woody plants to acclimatize to new environmental conditions as a trait conditioned by phenotypic plasticity (Heide 1974, Martinez-Meier et al. 2009, Fonti & Jansen 2012). At the geographical edge of a natural range, the length of the growing season can be very often influenced by climatic instability, i.e. with frequent extreme climatic events variations (Camarero et al. 2010, Aravanopoulos 2016, Antonucci et al. 2019, Cuny et al. 2019). Since there is a close correlation between dormancy and winter temperatures, even minimal variations in winter temperatures could determine a noticeable effect on the whatever budburst timing (Myking & Heide 1995).

This study was aimed 1) to form a high informative baseline for 1a) adopting suitable silvicultural systems for the conservation of the MaP populations in the current context, which are by themselves important for the Apennine biodiversity, 1b) to allow their future monitoring and to adapt progressively their silviculture to environmental changes, 2) to highlight their relevance as past and possibly future bridges to be preserved for the gene flow between the southern and northern Apennine sub-groups, 3) to test the “Centre-Periphery Hypothesis” (Eckert et al. 2008, Pironon et al. 2014) in case of the Apennine bridge populations.

Materials and Methods

Site description

The three studied sites are described in Figure 1 and Table 1. Site 1, Bocca Trabaria (BT), is near Fonte Abeti on the eastern slope of the Apennines (region of Marche, Pesaro, and Urbino province); site 2, La Verna (LV), lies within the Casentino Forest National Park on the western slope of the Apennines in eastern Tuscany (Arezzo province); site 3, Pigelleto (PIG) is located on Mt. Amiata (western Tuscany, Siena province).

Historically, BT has been used for timber production, starting during the Roman age and through the beginning of the 20th century, while in LV timber has been occasionally harvested until a few decades ago. PIG was intensively managed to produce props and beams for local cinnabar mines between 1897 and 1972.

In BT, two permanent plots (BT1 and BT2 respectively) were initially established to sample different aspects. Anyway, according to local forester’s information an artificial origin of the stand was hypotized in BT1, while BT2 was indicated of natural origin. Each silver fir stands showed an even-aged structure with an evident lack of natural regeneration.

In LV, two permanent plots (LV1 and LV2) were also established, on the NW and NE slopes of the mountain, respectively. This stand is an unevenly aged beech and silver fir mixed forest, along with maple and ash. Natural regeneration consists of groups, filling the gaps created by silvicultural treatments or caused by relatively frequent (20-50 years) natural disturbances (catastrophic storms).

In PIG, a single plot was established, due to the smaller extension of the surely native core of the forest. In this stand the demographic structure is evenly aged and natural regeneration also occurs in patches.

At all sites, the size of the permanent plots was adjusted to the local ground topography, varying between 7,700 m² and 9,800 m² (Table 1). These plots were established as reference areas for future regular monitoring according to a two-decade schedule, to detect possible variations compared to the initial baseline.

Meteorological and climatic data

Climatic data for the general site description were obtained from the nearest Italian Climatic Service meteorological stations. Monthly data for the reference period from 1981–2010 were averaged for each study site to obtain mean values for the climatic normal period (Table 1). BT’s site climatic data were obtained from
Table 1 Main ecological characteristics and descriptors of the investigated silver fir populations (modified after Manetti et al. 2014).

<table>
<thead>
<tr>
<th>Stand name</th>
<th>Bocca Trabaria</th>
<th>La Verna</th>
<th>Pigelleto</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID Plot</td>
<td>BT1</td>
<td>BT2</td>
<td>LV1</td>
</tr>
<tr>
<td>Geografic coordinates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silvicultural structure and dominant species</td>
<td>Monoplane structure by clusters. Silver fir, several broad-leaved trees and <em>Picea abies</em> (planted).</td>
<td>Uneven aged high forest, silver fir mixed with beech, maple and ash.</td>
<td>Uneven aged high forest. Silver fir mixed with sporadic broad-leaved trees,</td>
</tr>
<tr>
<td>Mean altitude (m)</td>
<td>930-1080</td>
<td>1166</td>
<td>1188</td>
</tr>
<tr>
<td>Aspect</td>
<td>NW</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Mean annual temperature (°C)</td>
<td>12.5°</td>
<td>12.5°</td>
<td>9.2°</td>
</tr>
<tr>
<td>Mean annual precipitation (mm)</td>
<td>1011</td>
<td>1200</td>
<td>1250</td>
</tr>
<tr>
<td>Slope %</td>
<td>30-80</td>
<td>60</td>
<td>40-60</td>
</tr>
<tr>
<td>Permanent plot area (m²)</td>
<td>9800</td>
<td>9800</td>
<td>9200</td>
</tr>
<tr>
<td>Maximum age of adult/ dominant layer trees (years)</td>
<td>119</td>
<td>130</td>
<td>258</td>
</tr>
<tr>
<td>Mean age of adult/ dominant layer trees (years)</td>
<td>78</td>
<td>100</td>
<td>157</td>
</tr>
<tr>
<td>Minimum natural regeneration/ dominated layer age (years)</td>
<td>-</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>Dominant height (m)</td>
<td>33.8</td>
<td>32.5</td>
<td>26.5</td>
</tr>
<tr>
<td>No. tree per hectare (n ha⁻¹)</td>
<td>326</td>
<td>332</td>
<td>674</td>
</tr>
<tr>
<td>Basal area per hectare (m² ha⁻¹)</td>
<td>61.25</td>
<td>63.5</td>
<td>44.14</td>
</tr>
<tr>
<td>No. of species occurring in the plot</td>
<td>6</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Shannon index</td>
<td>1.59</td>
<td>1.59</td>
<td>1.71</td>
</tr>
</tbody>
</table>
Gallucci & Urbinati (2009). According to Blasi et al. (2018), BT is included in the 1C2a Umbria and Marche Apennine Subsection of the Apennine Ecoregion of Italy. The site of LV experiences cold winters (average mean minimum temperature: 1.3°C) and mild summers, with a mean annual temperature and average precipitation of 9.5°C and 1225 mm, respectively. Blasi et al. (2018) included this site in the 1C1a Toscana and Emilia-Romagna Apennine Subsection. PIG enjoys partially a temperate oceanic (sub-Mediterranean) climate included in the 1C1b Tuscan Basin Subsection of the Apennine Ecoregion. There, in July, the temperature ranges from 20 to 23°C, and the annual rainfall is more than 1270 mm, peaking in November (185 mm), with the lowest levels in July (49 mm).

Concerning the evaluation of responses of cambial activity to environmental pressures, the daily minimum and maximum air temperatures and precipitation were acquired from the nearest meteorological stations of the Tuscan Regional Hydrological Service over three years (2010-2012) for the LV and PIG sites. For the LV site, the weather station was located at Chiusi della Verna (TOS01000639: 43.707°N, 11.931°E, altitude 1125 m), whereas for PIG, the weather station was located at Abbadia San Salvatore (TOS07000001: 42.984°N, 11.669°E, altitude 855 m). Air temperature is the primary factor that determines the onset of cambial activity (Rossi et al. 2008b). Therefore, the statistical software R (R Development Core Team 2019) was used to assess the chilling and heating requirements for each year, starting with 2010, using the temperature thresholds of ≤ 5°C for chilling and > 5°C for heating before the start of the growing season (Rossi et al. 2008b).

Methods used to forecast scenarios related to possible shifts in the silver fir range in Italy and for the above populations were proposed by Marchi and Ducci (2018) (Figure 1).

![Figure 2](image-url) Spatial distribution of (a) and (r) layers at LV and PIG.
Plant material and sample collection

In *Abies* spp., saplings can remain dominated by the crown canopy for several decades and trees are able to resume their growth when conditions become favorable. For this reason, trees were classified in agreement with the structural patterns established by dendrochronological studies for these populations (Manetti et al. 2014, Mazza et al. 2014) as an adult/dominant (a) layer, for trees with diameter at breast height (DBH) > 30 cm, and as a natural regeneration/dominated (r) layer, for trees with diameters between 2 and 30 cm. Dendrometric data collected in (a) and (r) layers will be compared in future monitoring to detect possible changes. For each sampled tree GPS coordinates were recorded and in Fig. 2, the spatial distribution of (a) and (r) layers in LV and PIG was shown.

The age of the (a) trees was estimated by core-ring analysis, taking samples approximately 50 cm from ground level (Manetti et al. 2014), whereas the age of the (r) trees was estimated both by core-ring analysis in bigger trees and estimating the age by counting whorls in the smaller ones. In the (a) layer, tree age classes ranged between 40 and 175 years at LV1, between 60 and 171 years at LV2 and between 30 to 101 years at PIG. In the (r) layer, tree age classes ranged respectively between 20 and 80 years at LV1, between 30 and 111 years at LV2, and between 25 and 68 years at PIG. At BT1 and BT2, due to lack of natural regeneration only class (a) occurred with age classes up to 119 years.

In LV and PIG all silver fir trees growing in the permanent plots were analyzed using nuSSRs and cpSSRs to investigate 1) the within-population genetic variability and structures, 2) the relatedness among demographic layers (a) and (r), and 3) among age classes always separating the (a) and the (r) classes given the appearance of the dominated plants, and 4) the possible filial structures. Instead, in BT plots, where only (a) trees existed, nuSSRs were used. Indeed, in this study, cpSSR markers were mainly investigated to understand paternal relationships of natural regeneration (r) with the (a) trees, to assess gene flow.

The “Centre-Periphery Hypothesis” (CPH) was tested by comparing the three studied populations to other Apennine and the Alpine populations, using data by Belletti et al. (2017) (Table S1).

At LV and PIG, the (a) silver fir trees were also monitored using cambial phenology (Bigler et al. 2004). The two sites were used due to their geographical position in relation to the distance-from-the-sea and the availability of relevant climate time series. BT was excluded from this monitoring because of logistics and of access-to-the-site reasons impeded the required frequent surveys.

Table 2 lists the numbers of individuals sampled respectively for genetic and phenological analyses. Parameters selected for this genetic monitoring were chosen from the main indicators and their verifiers reported in the literature (Koskela et al. 2013, Aravanopoulos et al. 2015, Aravanopoulos 2016).

<table>
<thead>
<tr>
<th>Site/Forest</th>
<th>Adult/dominant layer (a)</th>
<th>Natural regeneration/dominated layer (r)</th>
<th>Tree sampled for growth and cambial phenology</th>
<th>Trees sampled for genetic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bocca Trabaria 1</td>
<td>17</td>
<td>*</td>
<td>**</td>
<td>17</td>
</tr>
<tr>
<td>Bocca Trabaria 2</td>
<td>30</td>
<td>*</td>
<td>**</td>
<td>30</td>
</tr>
<tr>
<td>La Verna 1</td>
<td>26</td>
<td>56</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>La Verna 2</td>
<td>35</td>
<td>19</td>
<td>35</td>
<td>36</td>
</tr>
<tr>
<td>Pigelleto</td>
<td>30</td>
<td>46</td>
<td>23</td>
<td>26</td>
</tr>
</tbody>
</table>

Note: * No natural regeneration; ** No phenological survey
DNA extraction and SSR analyses

From the (a) and (r) trees the DNA was extracted from the needles frozen and stored at -80°C. From some too high (a) trees, DNA was extracted from cambium obtained from microcores. The DNA extraction was carried out using the Invisorb Spin Plant Mini Kit (Stratec Molecular GmbH, Berlin, Germany) and the PCRs (Eppendorf® MasterCycler® Pro) were performed as described by Belletti et al. (2017). Amplicons were separated using an ABI Prism 3730xl DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Data were read with GeneMapper 4.1 software (Life Technology). Seven nuSSR loci, SF1, SFb4, SFb5, SFg6, SF78, SF324, SF333 (Cremer et al. 2006) and two cpSSR loci, Pt30204 and Pt71936 were analysed (Vendramin et al. 1996).

Genetic data analysis

Genetic parameters

Regarding the formation of the baseline for future monitoring, for each permanent plot keeping distinguished the subpopulations a) and r) for both demographic and age classes analyses, the following genetic parameters were calculated based on the seven nuSSR loci using the GenAlEx software v.6.5b4 (Peakall & Smouse 2012): observed heterozygosity (H₀), expected heterozygosity (Hₑ) and inbreeding coefficient (Fᵢₛ). The allelic richness (A_r) was evaluated using Fstat v.2.9.3.2 software (Goudet 2002).

Haplotype diversity (h) and haplotype richness (h_r) were calculated from the cpSSR data, using Contrib 1.4 (Petit et al. 1998).

F statistics were calculated according to Wright (1965) and Weir and Cockerham (1984). The molecular variance analysis (AMOVA; Excoffier et al. 1992) was performed using Arlequin v.3.5.2.2 (Excoffier & Lischer 2010). When F_is values were positive, the presence of null alleles was investigated, using Micro-Checker software (van Oosterhout et al. 2004). The estimation of the inbreeding coefficients, which accounted for these null allele frequencies, was performed using INEst software, selecting the individual inbreeding model with a Gibbs sampler of 105 iterations (Chybicki & Burczyk 2009).

For both nuSSR and cpSSR markers, a Bayesian cluster analysis was performed, using STRUCTURE v.2.3.4 software (Pritchard et al. 2010), for each locality separately and then combined. Even if a higher number of loci for cpSSR should be required, a first screening was anyway decided with these two loci only. The K parameter varied from 1 to 10, and n admixture-ancestry model and correlated allele frequency were used to perform a Markov chain Monte Carlo simulation algorithm (MCMC), with a 100,000 burn-in length and a run length of 100,000. The optimum K value was determined based on ΔK, which was calculated within the STRUCTURE Harvester v.0.6.94 program (Evanno et al. 2005, Earl & von Holdt 2012). Graphical displays of the clusters were produced using the plotting programs Clump 1.1.2 (Jakobsson & Rosenberg 2007) and Distrut (Rosenberg 2004).

Concerning nuSSRs, the Mantel test (Diniz-Filho et al. 2013) was performed to test possible correlation between (a) and (r) layer underlying trees, among geographical distance (Euclidean, coordinate GPS geolocation) and Nei’s genetic distance matrix using Past 3.2 software (Hammer et al. 2001, Hammer & Harper 2006).

Testing the Centre-Periphery Hypothesis(CPH)

To check the CPH, for the evaluation of the effect of the distance of each of the three populations from the other Italian stands, the Pearson r was tested to evaluate the correlation among genetic parameters (A_r, Hₑ, F_is on F_st max, G_st on G_stmax) and geographic distance (km), Past software and Multivariate Correlation Matrix (v 1.0.11) in Free Statistics Software v.1.2.1 (Wessa 2020) were used.

In addition, the Welch modified t test
was conducted to verify any differences in mean values between genetic parameters ($A_r$, $H_e$, $F_{st}$, and $G_{st}$ on $G_{st \, \text{max}}$) of Apennine versus western (WA\textit{lp}), central-western (CWA\textit{lp}) and central-eastern (CEA\textit{lp}) Alpine populations using the Paired and Unpaired Two Samples Tests about the Mean (v 1.0.7) in Free Statistics Software (Wessa 2020).

The graphic linear trends of those parameters in relation to the kilometric distances were implemented by Excel software, comparing each of the three populations to the Apennine populations and to the Alpine ones.

The T test with Welch modification was assessed to test for significant difference in mean values of the above genetic parameters into the age classes using the Paired and Unpaired Two Samples Tests about the Mean (v 1.0.7) in Free Statistics Software (v 1.2.1). It was performed to identify the best ways to manage genetic diversity in age classes and to monitor changes over time within them.

**Cambial phenology**

Sample collection and preparation for monitoring cambial phenology

Eighty-six (a) layer trees (29 in LV1, 34 in LV2, and 23 in PIG), with straight stems, similar diameters at breast height, and without apparent damage, were monitored for their cambial phenology. This sampling method was adopted to keep uniform tree characteristics (Rathgeber et al. 2011b). For each sampled tree mean age, average height and diameter at breast height were recorded (Tables 2 and 3). In 2011 and 2012, cambial phenology and the intra-annual dynamics of wood formation were monitored from April to November. Wood microcores (total number = 2,064), 4 mm in diameter and 10-15 mm length, were collected from 30 cm below to 30 cm above breast height (1.3 m), using a Trephor® tool (Rossi et al. 2006a).

In the first part of the growing season, from mid-April to mid-May, samples were processed weekly, while through the end of June they were processed biweekly. On this way, highlighting the onset of cambial activity and precocity was possible as well as to assess the onsets of the radial-enlargement and wall-thickening phases (Güney et al. 2015, Cocozza et al. 2016, Fernández-de-Uña et al. 2018). Between July and November, cambial phenology was monitored at monthly intervals, due to both the remoteness of the monitored sites and the slower rates of tracheid formation during the summer in the Mediterranean area (Camarero et al. 2010, Vieira et al. 2014 and 2017). Although less frequently, microcore sampling was updated to determine the duration of the secondary growth season.

Microcores were stored in Eppendorf microtubes containing a solution of 50% ethanol and stored at 5°C, to avoid tissue deterioration.

<table>
<thead>
<tr>
<th>Trait</th>
<th>La Verna 1</th>
<th>La Verna 2</th>
<th>Pigelleto</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dbh (cm)</td>
<td>58.0 ± 29.0</td>
<td>72.0 ± 25.0</td>
<td>44.0 ± 15.0</td>
</tr>
<tr>
<td>H (m)</td>
<td>30.6 ± 8.7</td>
<td>39.0 ± 8.6</td>
<td>26.9 ± 4.2</td>
</tr>
<tr>
<td>CPA (m²)</td>
<td>37.6 ± 18.7</td>
<td>35.0 ± 15.2</td>
<td>24.7 ± 11.0</td>
</tr>
<tr>
<td>Age (years)</td>
<td>94.07 ± 53.63</td>
<td>131.67 ± 33.94</td>
<td>75.91 ± 19.45</td>
</tr>
</tbody>
</table>

Note: The measured traits (diameter at breast height - Dbh, height - H, crown projection area - CPA and age) averaged ± standard deviation within the three examined populations is reported.
Figure 3 Cambial zone and phases of xylem differentiation. A - The beginning of xylem differentiation with dividing cambium and cells in enlargement phase. B - All differentiation phases. PZ: phloem; CZ: cambial zone; EZ: enlarging cells; cell wall thickening (WZ) and mature cells (MZ).

The sample preparation method was modified from that described by Rossi et al. (2006a). A simpler method was developed for slice preparation, without the preventive embedding of microcores in paraffin (Pâques et al. 2016). Microcores were directly glued onto small wooden supports, and transverse sections of 10-12-µm thickness (Fernández-de-Uña et al. 2018, Ziacò et al. 2018) were cut with a GSL1 microtome (MICROT L; Gärtner & Schweingruber 2013, Gärtner et al. 2014). This method minimized the necessary time, costs and chemical consumption, while still preparing good-quality slices.

Sections were stained with aqueous 0.16% w/v cresyl violet acetate (Rossi et al. 2006b) and examined under visible and polarised light, at 400× magnification, to distinguish between the developing and mature xylem cells. An Axiocam ICc 1 digital microscope camera (www.zeiss.com), supported by proprietary AxioVision software, was used.

Microscopic observations

For each slice, along three radial files, the radial number of cells in the cambial zone (nC), cells in the radial-enlargement phase (nE), cells in the wall-thickening phase (nW), and mature cells (nM) were counted, according to Rossi et al. (2006b). The widths of the tree rings formed during the previous growing season (PR) were also measured (Rathgeber et al. 2016). Discrimination between cells in the enlargement, wall-thickening, and lignification phases was determined according to both the reaction between cresyl violet acetate and lignin and to observations under polarized light. Cells in the cambial and radial-enlargement phases showed thin, pink-stained cell walls. Enlarging cells were larger than cambial cells and showed thin and non-birefringent walls under polarized light (Kutscha et al. 1975 in Rathgeber et al. 2011a). During the subsequent stages of thickening and lignification, the cell walls become light purple at the beginning of the process and blue when the tracheids reached maturity (Fig. 3). Furthermore, tracheids in the radial-enlarging phase were not visible under polarised light because of the lack of a secondary wall (Ren et al. 2018), whereas when the lignification process began, the cell walls were birefringent (Deslauriers et al. 2003, Rathgeber et al. 2011a). Indistinct and identical xylem and phloem cells were originated from cambial cell division and were later subjected to differentiation processes (Rathgeber et al. 2011b).

In winter, the dormant cambium consisted of four to five cell layers while in spring, when the number of new xylem cells increased in the cambial zone, the onset of cell division was considered begun (Wilson 1970). When at least one radial file of enlarging cells was
observed, the formation of new tracheids was considered to have begun (Rossi et al. 2008a) (Fig. 3). In late summer, when no further cells were observed in the wall-thickening or lignification phases, xylem formation was considered completed and radial tree growth ended (Rathgeber et al. 2011a).

Data analysis of xylem phenology and timing of wood formation

The phenology of the intra-annual dynamics of wood formation (Rossi et al. 2006b) was analysed using the CAVIAR package (Rathgeber et al. 2011a, 2018), built for R statistical software. Logistic regression was applied to the cell-counting dataset to calculate five critical dates for which there was a 50% probability of a specific phase having started or ended, while the associated confidence interval (95%) was the time expressed in Days of the Year (DOY), spent between the dates defined by the 2.5% and 97.5% probabilities (Rathgeber et al. 2018). These dates reflected the following: (1) the onset of the enlargement period (bE); (2) the onset of the wall-thickening period (bW); (3) the appearance of the first mature tracheids (bM); (4) the cessation of the enlargement period (cE); and (5) the cessation of wall-thickening and the hardening period (cW). These dates were then used to define the durations of three wood-formation phenophases, namely enlargement (dE = cE - bE), wall-thickening (dW = cW - bW) and wood formation/xylogenesis (dX = cW - bE).

A bootstrap permutation test (Rathgeber et al. 2011a, 2018) was applied to compare the critical dates and the duration of wood formation among groups of trees. As the cell numbers in the microcores can vary by sample, sampling date, and tree, the cell numbers each year were standardized against the width of the ring for the previous year, on three radial files per sample (Rossi et al. 2003, Cuny et al. 2012, Rathgeber et al. 2011a, 2018). The Gompertz function was used to assess the cell number increment during the growing season and to describe and analyse the intra-annual dynamics of xylem formation, both between and within monitoring areas, across different years (Deslauriers et al. 2003, Rossi et al. 2003, 2006b).

Results

Climatic data

The mean annual temperature difference between the PIG and LV sites was approximately 2.3 ± 1.06°C. The smallest difference (0.95°C) occurred during 2010, and the largest difference (5.6°C) occurred during the winter of 2011/2012. A linear regression of temperature against the yearly time scale, revealed an increase of approximately 1.5°C per year (Fig. 4). This result matched the overall data at the national level for 2012. Indeed, 2012 in Italy was the 4th warmest year since 1961, but with a record-breaking February for cold and snow (Desiato et al. 2013). The pattern of rainfall was irregular, but overall, the PIG site was wetter than the LV site. The mean annual temperature differences recorded at the PIG and LV sites influenced the thermal sum; however, for both years, at the beginning of the microcore sampling period, the thermal sum of days with temperatures ≥ 5°C was greater for the PIG compared with the LV.

Genetic analyses

Genetic variation among populations

The extent of the genetic variability differed among the three sites (Table 4). \( H_o \) was generally lower than \( H_e \), and deviations from the Hardy-Weinberg equilibrium were statistically significant (\( P \leq 0.01-0.001 \)). The within-population component of the variation accounted for 74% of the variance, as suggested by AMOVA results, and F coefficients were always positive (\( F_{is} = 0.117, F_{st} = 0.164, F_{it} = 0.262; \) all \( P \leq 0.001 \)), still showing a \( F_{st} \) higher than \( F_{is} \) and a slight loss of heterozygosity. The STRUCTURE analysis (\( K = 2 \)) confirmed that
LV was distinct from PIG and BT, which were grouped together in the same cluster. However, when $K = 3$ was tested, BT2 was admixed into a third cluster including LV2 (a) material (Fig. 5).

**Figure 4** Temperature and precipitation distribution (upper panel: mean daily temperature, lower panel mean daily rainfall) at PIG and LV over the period 2010-2012.

**K=2**

<table>
<thead>
<tr>
<th>LV1 a</th>
<th>LV2 a</th>
<th>LV1 r</th>
<th>LV2 r</th>
<th>PIG a</th>
<th>PIG r</th>
<th>BT 1</th>
<th>BT 2</th>
</tr>
</thead>
</table>

**K=3**

<table>
<thead>
<tr>
<th>LV1 a</th>
<th>LV2 a</th>
<th>LV1 r</th>
<th>LV2 r</th>
<th>PIG a</th>
<th>PIG r</th>
<th>BT 1</th>
<th>BT 2</th>
</tr>
</thead>
</table>

**Figure 5** STRUCTURE clusters obtained for (a) and (r) layers of all three sites with $K=2$ and $K=3$ based on nuSSR data.
Table 4 Genetic parameters derived from nuclear and chloroplast SSR data in Bocca Trabaria, La Verna and Pigelleto permanent areas (Plots).

<table>
<thead>
<tr>
<th>Bocca Trabaria</th>
<th>nuSSR</th>
<th>cpSSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plots</td>
<td>N</td>
<td>(A_r)</td>
</tr>
<tr>
<td>BT 1</td>
<td>17</td>
<td>5.400</td>
</tr>
<tr>
<td>BT 2</td>
<td>30</td>
<td>7.800</td>
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<tr>
<td>mean</td>
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<td>6.600</td>
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</table>

<table>
<thead>
<tr>
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<th>nuSSR</th>
<th>cpSSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plots</td>
<td>N</td>
<td>(A_r)</td>
</tr>
<tr>
<td>LV 1 a</td>
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</tr>
<tr>
<td>LV 2 a</td>
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<td>6.448</td>
</tr>
<tr>
<td>LV 1 r</td>
<td>56</td>
<td>7.007</td>
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<tr>
<td>LV 2 r</td>
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<td>7.286</td>
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<tr>
<td>mean</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pigelleto</th>
<th>nuSSR</th>
<th>cpSSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plots</td>
<td>N</td>
<td>(A_r)</td>
</tr>
<tr>
<td>PIG a</td>
<td>26</td>
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</tr>
<tr>
<td>PIG r</td>
<td>46</td>
<td>6.669</td>
</tr>
<tr>
<td>mean</td>
<td></td>
<td>7.049</td>
</tr>
</tbody>
</table>

Legend: \(N\) = number of trees; \(A_r\) = allelic richness; \(H_o\) = observed heterozygosity; \(H_e\) = expected heterozygosity; \(h\) = haplotype diversity; \(h_r\) = haplotype richness; \(F_{rec}\) = inbreeding coefficient, considering null alleles; \(a\) = layer (a); \(r\) = layer (r).

Testing the Centre-Periphery Hypothesis (CPH)

The Pearson’s \(r\) correlation performed on the Italian silver fir stands examined by Belletti et al. (2017), showed for LV a significant positive correlation of \(G_{st}\) (\(r = 0.36, P \leq 0.0147\)) with the geographic (km) distances from the other populations. PIG has a very significant positive correlation for \(F_{st}\) and \(G_{st}\) (\(r = 0.44, P \leq 0.003\); \(r = 0.41, P \leq 0.0061\)) and a significant negative correlation of \(A_r\) (\(r = -0.36, P = 0.028\)). BT has a very significant correlation for \(F_{st}\) and \(G_{st}\) (\(r = 0.45, P \leq 0.0018\); \(r = 0.46, P \leq 0.0016\)) (Table S2).

The Welch’s T test (Table S3) showed for \(A_r\) significant differences among the mean values of Apennine populations and the three sectors of Alpine populations. The same for \(H_e\), except for the Central Eastern Alpine (CEAlp) populations. Moreover, concerning the \(F_{st}\) on \(F_{st_{max}}\) and \(G_{st}\) on \(G_{st_{max}}\) the LV and PIG populations showed significant and highly significant differences among their mean values and those of the Alpine stands. While BT for \(F_{st}\) on \(F_{st_{max}}\) showed only a low difference vs CEAlp and for \(G_{st}\) on \(G_{st_{max}}\) vs Western Alpine (WAAlp) populations and vs Western-Central Alpine populations (WCAIp).

Graphics in Figures S1, S2 and S3, represent the linear regressions of genetic parameters with geographic distances for each studied population, separately for the Alpine and for Apennine populations. BT, LV and PIG showed \(A_r\) and \(H_e\) trend decreasing for the Alpine populations, as well as LV’s \(F_{st}\). At the same time, the linear regressions of \(G_{st}\) of BT, LV and PIG increased towards Alps. The same trend was also observed for \(F_{st}\) of PIG and BT. The Alpine population parameter values were narrowly grouped around a mean distance, while the Apennine ones were scattered in...
relation to geographic distances.

Genetic diversity within populations and structural layers

In BT, the genetic analysis on (a) trees showed differences between BT1 and BT2 (Table 4). Indeed, allelic richness A_r and expected heterozygosity H_e were higher in BT2. The inbreeding coefficient F recalculated (F_{rec}), lower in BT1, was positive, indicating a deficiency of heterozygotes and some divergence from the panmictic equilibrium. The F_{is} (0.108, P ≤ 0.001), F_{st} (0.180, P ≤ 0.001) and F_{it} (0.269, P ≤ 0.001) coefficients (Wright 1965) underlying the differences between BT1 and BT2.

That results were confirmed by AMOVA, revealing a proportion of assigned genetic variance to the within population component of 72%, and by STRUCTURE analysis according to a best K value of 3, where these two study areas were in separated clusters. A small group only of BT2 plants and few BT1 trees were admixed in a third cluster (Fig. S4). In LV, trees belonging to LV1 and LV2 and in addition to (a) and (r) tree layers were surveyed. Values of A_r and H_e detected in layer (a) were relatively lower than in layer (r) (Table 4) and H_o was generally lower than H_e, except for layer (a) at LV2. Concerning the cpSSRs, comparable genetic diversity was present in the (a) and (r) tree layers, the h and h_r values were similar, except in LV1 that showed in (r) layers higher values than in (a).

The inbreeding coefficient F_{rec} was positive in both the sub-populations, higher in LV1 (a) and in LV2 (r), diverging from the Hardy-Weinberg equilibrium (P ≤ 0.01 – 0.001).

Both allele and genotype frequencies were comparable in (a) and (r) groups and (r) layer did not show variability. The AMOVA showed that the within-population variance component was 92% for nuSSRs and 100% for cpSSRs. This was confirmed by F_{is}, F_{st} and F_{it} coefficients 0.128, 0.047, and 0.169, respectively (all P ≤ 0.001). No genetic correlation between (a) and (r) spatially closer trees (nuSSRs: r = 0.08, P ≤ 0.005; cpSSRs: r = -0.06, P ≤ 0.990) was detected by the Mantel test.

The STRUCTURE analysis (K = 2), based on nuSSRs, showed the majority of LV2 (a) trees clustered together, while the majority of LV1 and LV2 (r) individuals were admixed in a second cluster with LV1 (a) trees (Fig. 5). When cpSSRs were analysed, a lack of genetic structure was detected (K = 2), and the clusters did not identify specific groups (Fig. S4). At PIG, both (a) and (r) tree layers were also recorded. There, parameters as H_e, h and h_r were comparable between these layers except A_r, with a higher value in (r). The genetic diversity among the (r) trees was slightly lower than among the (a) trees (Table 4). The inbreeding coefficients F_{rec} was positive but lower than in LV and BT and tended to increase in (r). The divergence from the Hardy-Weinberg equilibrium (P ≤ 0.01) was therefore relatively low.

The AMOVA revealed that most of variation observed resided within-populations (93% for nuSSRs and 90% for cpSSRs). The F coefficients (F_{is} = 0.101; F_{st} = 0.038; and F_{it} = 0.135, all P ≤ 0.001) confirmed the high variation among trees, but not between (a) and (r) layers.

According to the Mantel test, no spatial genetic correlation between (a) and (r) trees was detected (nuSSRs: r = 0.03, P = 0.173; cpSSRs: r = 0.02, P = 0.737) and this finding was also confirmed by the STRUCTURE analysis (K = 2), in which one of clusters was mainly composed by (r) trees admixed with only a few (a) trees. The other cluster was composed predominantly of (a) trees. When cpSSRs were used, a lack of genetic structure was noted (K = 2), and clusters did not identify specific groups (Fig. S4).

Genetic diversity analysis based on tree age

The Table 5 reports results of genetic analyses performed on age classes. In LV several trees were attributed to the (r) layer because of their
small size and their dominated appearance even if the ring analysis revealed some of these trees aged as dominant (a) trees.

Concerning nuSSRs, \( A_r \) values ranged from 4.724 (71-110- a) to 5.719 (41-50- r), \( H_o \) values from 0.683 (41-50-r) to 0.760 (111->131- a) and \( H_e \) values ranged from 0.760 (71-110-a) to 0.735 (111->131-a).

The \( H_o \) value was generally lower than \( H_e \), determining a significant divergence from the Hardy-Weinberg equilibrium (\( P \leq 0.01-0.001 \)) and \( F_{\text{rec}} \) coefficients, always positive, were in general low, except for the age class (41-50-r) where \( F_{\text{rec}} \) reached 0.139. The Wright’s coefficients (\( F_{st} = 0.139; F_{is} = 0.024; \) and \( F_{it} = 0.159, \) all \( P \leq 0.001 \)) confirmed high variation among trees, but not among age classes.

Concerning cpSSRs, the \( h \) value ranged between 0.927 (71-110-a) and 0.989 (41-50-r), and the \( h_r \) value ranged from 4.685 (age 71-110-a) among dominant trees to 5.770 (41-50-r) among dominated/natural regeneration plants.

The STRUCTURE analysis (Fig. S5), based on nuSSR data, demonstrated that most of the regeneration (r) trees were clustered together with adult (a) age class 40-70-a, along with a few trees that belonged to the adult class >111- a. Therefore, both 40-70-a and 41-50-r classes appear genetically closer to the oldest trees than to the younger trees. Two clusters were also detected using cpSSRs that showed trees widely distributed among classes, without clear relationships among them.

In PIG, also, some (a) trees were found of similar age to some (r) trees. When examining nuSSRs, \( A_r \) values ranged from 4.244 (71->100-a) to 4.367 (31-70-a), \( H_o \) values ranged from 0.646 (31-70-a, 41-70-r) to 0.702 (71->100-a), and \( H_e \) values ranged from 0.695 (71->100-a) to 0.719 (31-70-a).

\( H_o \) values were generally lower than \( H_e \), showing divergence from the Hardy-Weinberg equilibrium, except for the 71->100-a class, which had a higher \( H_e \) value.

The \( F_{\text{rec}} \) coefficients were in general low and positive, except for a higher value associated to age class 31-70-a (0.106) and a negative one.

<table>
<thead>
<tr>
<th>LV</th>
<th>nuSSR</th>
<th>cpSSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pop</td>
<td>N</td>
<td>( A_r )</td>
</tr>
<tr>
<td>40-70-a</td>
<td>14</td>
<td>5.229</td>
</tr>
<tr>
<td>71-110-a</td>
<td>9</td>
<td>4.724</td>
</tr>
<tr>
<td>111-&gt;131-a</td>
<td>41</td>
<td>5.590</td>
</tr>
<tr>
<td>20-40-r</td>
<td>15</td>
<td>5.547</td>
</tr>
<tr>
<td>41-50-r</td>
<td>44</td>
<td>5.719</td>
</tr>
<tr>
<td>51-111-r</td>
<td>16</td>
<td>5.631</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PIG</th>
<th>nuSSR</th>
<th>cpSSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pop</td>
<td>N</td>
<td>( A_r )</td>
</tr>
<tr>
<td>31-70-a</td>
<td>7</td>
<td>4.367</td>
</tr>
<tr>
<td>71-&gt;100-a</td>
<td>15</td>
<td>4.244</td>
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<td>20-40-r</td>
<td>25</td>
<td>4.285</td>
</tr>
<tr>
<td>41-70-r</td>
<td>21</td>
<td>4.252</td>
</tr>
</tbody>
</table>

Legend: N=tree number, \( A_r \) = allelic richness; \( H_o \) = observed heterozygosity; \( H_e \) = expected heterozygosity; \( h \) = haplotype diversity; \( h_r \) = haplotype richness; \( F_{\text{rec}} \) = inbreeding coefficient, considering null alleles; a=Layer (a); r=Layer (r).
associated to class 71 - >100-a (-0.026). The $F$ coefficients ($F_{is} = 0.098; F_{it} = 0.121, F_{st} = 0.026; \text{all } P \leq 0.001$) confirmed low variation among trees and age classes.

When examining cpSSRs, the $h$ value ranged from 0.738 (41-70-r) to 0.999 (31-70-a) and the $h_r$ value ranged from 2.369 (41-70-r) to 4.000 (31-70-a). The nuSSR STRUCTURE analysis (Fig. S6) showed most (r) trees clustered together with trees of age class 71->100-a. CpSSRs divided again trees into two clusters, one of which clustered (r) trees together with oldest (a) trees. Comparing the significance between the average values of the genetic parameters on age classes (Table S4) using the Welch’s test, the T values, ranged from 2.89 for LV-$h_r$ to 4.32 for PIG-$F_{is}$, and showed significant genetic diversity among age classes ($P <0.05$) for all parameters.

**Phenological behavior of xylem formation in (a) layer trees at LV and PIG**

The phenological phases of xylem growth were presented in Fig. S7. Over the two study years, the timing (Fig. S7) of bE in both regions (end of April/early May) was very consistent, with the sole exception of PIG in 2012, when bE coincided with the first half of April (DOY 102.8 ± 4.95), some two weeks earlier than the bE for LV. In 2011, at both LV and PIG, bW occurred approximately one week after bE. In 2012, this stage was reached approximately six days later in LV2 (DOY 133.4 ± 12.82) than in LV1 (DOY 127.6 ± 11.2); however, this difference was not significant.

The timing of bW in PIG (DOY 115.8 ± 9.19) occurred somewhat earlier (approximately the end of April).

The bM stage was reached in LV1 during the third week of May for both years, as was the case in LV2 in 2011; however, in 2012, bM was delayed by approximately six days. In PIG, the bM stage occurred significantly later than in LV for the year 2011 compared with 2012.

In 2011 and 2012, differences in the mean values of cumulative temperature (days with temperature ≤ 5°C) were observed between sites with respect to the observation of the first E cells (mean value of 319.8°C in LV1 and 202.2°C in PIG in 2011, at DOY 119.9 ± 6.89 and DOY 118.7 ± 6.91, respectively). The thermal sum increased progressively during the growing season in both sites, but in PIG, at the onset of bW and bM, the mean value of cumulative temperature (days with temperature ≥ 5°C) was higher (year 2011: bW = DOY 126.6 ± 9.4 and mean value of cumulative temperature = 1374.7°C in PIG; bW = DOY 127.3 ± 9.63 and mean value of cumulative temperature = 929.6°C in LV1). After June, the monthly samplings continued both to follow the growth of the annual ring and to assess the length of the vegetative season. The duration of the three phenophases (dE, dW and dX) is illustrated in Fig. S8. The longest lasting dX phase occurred in 2012 for the three stands.

**Discussion**

**Genetic variability**

The inter-population analysis

Genetic parameters of the focused populations were consistent with other fir populations in Italy (Cremer et al. 2006, Piovani et al. 2010, Belletti et al. 2017, Piotti et al. 2017, Popović et al. 2017, Santini et al. 2018).

The climatic scenarios for Italy (Fig. 1; Marchi & Ducci 2018) highlighted the need to establish monitoring networks on the isotherm shift effects in this specific area of central Apennines, where silver fir metapopulations for millennia act as a bridge for the periodic genetic flows between southern and northern groups. This area is crucial for adaptation to progressively harder environmental conditions (Arnaud-Haond et al. 2006). Therefore, establishing a baseline for future surveys at BT, LV and PIG to preserve their transitional
Establishing a baseline to monitor future...
(Aravanopoulos 2011, Alberto et al. 2013, Piotti et al. 2017), which could be achieved through a combination of both in situ and ex situ interventions.

Among results, PIG and BT2 were detected genetically quite distant, but not BT1 which instead resulted closer. This similarity is compatible with the information about the artificial origin of BT1, which was indeed planted in the 19th century. The origin of BT1 is not proven, but based on historical information and genetic analysis, it was clear that BT1 trees did not originate from natural regeneration and probably derive from silver fir populations belonging to the central-northern group. BT2 was instead declared to be of natural origin and based on $H_e$ and $A_r$ values, exhibited higher genetic diversity.

It also should be highlighted that BT2 clustered for K=3 together LV2 (a) trees representing a probable transitional status between the northern and southern groups of Apennine silver fir. That can explain the apparent discrepancy between Belletti et al. (2017), who included the BT population within the southern group, while Piotti et al. (2017) instead included it within the northern one. The present work confirmed the inclusion in the northern group. Most likely, both studies sampled the local silver fir population, without distinguishing between planted and naturally regenerated individual trees areas. Because of its non-native, unknown origin, BT1 should be therefore excluded from future monitoring reiterations.

The greater genetic richness of BT2 can favor adaptability and plasticity and there an adaptive management to open artificial gaps in the crown canopy should be considered. At the same time, BT1 should be progressively harvested, and the area naturally regenerated or planted with BT2 materials.

The intra population analyses

In this framework, a dynamic management strategy (Shaw & Etterson 2012, Lefèvre et al. 2013, Schueler et al. 2014) should consider the within-population demography (Aitken et al. 2008). In these populations, inbreeding was common but occurred with lower intensity than is generally expected in isolated populations. Comparing the demographic layers in general, the (r) saplings showed inbreeding ratios like (a) trees. These findings were close to Leonarduzzi et al. (2016) for the nearest Abruzzi silver fir populations and suggested a still-active population dynamism (Bagnoli et al. 2011). This is a reason why long-term monitoring is required to identify incoming stressors for these populations and therefore adapting silvicultural interventions to changing situations.

Silvicultural treatments should be focused on promoting natural regeneration by opening gaps and reducing the population of older dominated (r) trees. The eldest age classes of the (a) layers generally hosted a large proportion of the stand genetic diversity and their fructification should be favored before they are harvested. Generally, a higher within-population component of the total variance, was evidenced by the AMOVA results confirming Bagnoli et al (2011). However, relatively high differentiation rates revealed the existence of genetic diversity between the three stands.

The distinction of both (a) and (r) layers was possible either in LV or PIG. There, their similar level of genetic diversity suggested the ability of the (r) gene pool to contribute to the maintenance of genetic variability. Consequently, the gene pool could support the efficacy of silvicultural interventions aimed at creating optimal microhabitats for the installation of natural regeneration thanks to the capacity of dominated saplings to re-start their growth when gaps are open in the crown canopy. Patches of (r) trees were generally better identifiable at LV than in PIG, likely due to variations in the microclimate and favored by gaps in the crown canopy (Bottalico et al. 2014).
Among other characteristics, these populations showed a strong capacity for within gene flow, not genetic correlation between neighboring plant was highlighted, indeed many of the trees (r) were not genetically related to the adult plants growing within the permanent plots. The cpSSR genotype analyses suggested that some male parents may be located outside of the permanent plots, and pollen can have travelled long distances, as recorded in similar studies (Kremer et al. 2012, Leonarduzzi et al. 2016, Santini et al. 2018).

Concerning age class relationships, the highest gene variability was observed for the oldest tree age class (111- >131 a), even though with low inbreeding rates. Genetic relatedness was detected among the younger age classes 40-70-a, 20-40-r, 41-50-r, and a group of the mature trees > 111-a. Instead, the others > 111-a mature trees showed proximity to the trees >70 years. The patched distribution of natural regeneration in the forest was likely determined by gaps caused by fallen large trees.

In PIG, the highest genetic variability was observed within the younger age class (31–70-a) characterized by a notable inbreeding. Genetic proximity among the (r) age classes and only some trees of the adult class > 71-a was detected there. Indeed, as for nuSSR and cpSSR, this group of mature trees likely contributed to the gene pool of the younger classes, probably reflecting the effects of past intensive selective and/or occasional management (Perrin 1954, Bernetti 1995) for the mine timber supply.

The evolution of genetic variability between and within the different age classes and their contribution to address the panmictic equilibrium in each population should be closely monitored.

The significant genetic differences among age classes at LV and PIG should be considered in the management of these populations to keep genetic variability and maintaining allelic richness. In the same way, the consistency and genetic variability among age classes should be redistributed by future silvicultural interventions to balance the population’s structure.

Genetic monitoring is therefore necessary to assess any significant variations in genetic diversity within the population and in its different layers and age classes to allow adequate silvicultural management (Aravanopoulos 2018). Indeed, according to the future climatic scenarios in the Mediterranean regions (Marchi & Ducci 2018), genetic erosion and fitness reduction can be likely expected due to the isotherm shift effects (Fady et al. 2016).

Aravanopoulos (2018) reviewed studies on the effects of silvicultural treatments on population genetic diversity as well as on population’s resilience. He found that silviculture can determine long-term impacts on the genetic diversity, structure and resilience of forest tree populations and should be based on indicators and parameters suitable to detect perturbations and bottlenecks.

In view of the adaptation to environmental changes as well as to assess the effect of silvicultural treatments, a monitoring approach is required to provide insight and potential solutions for future silviculture and management regimes (Aravanopoulos 2016). Treatments should be therefore focused on reducing inbreeding by promoting natural regeneration after good fructification years and reducing the amount of aged but dominated trees in (r) layer. At the same time, the extension of populations must be increased where possible to rise the effective size and to improve the demographic structure by various age classes.

**Phenology of xylem formation**

A progressive loss of fitness can be also connected to an adaptive disorder determined by the changing site conditions due to isotherm shift and "the empirical evidence often suggests a lack of decrease in abundance or
fitness at the limits of the range" (Sexton et al. 2009 in Csilléry et al. 2020a and 2020b). When analyzing the phenology of silver fir offspring from France southern populations, it was reported adaptive divergences between these populations also for bud break and growth traits in turn related to water stress. Generally, less growth allows for better resistance to stress and this decoupling between growth and phenology could be advantageous from an evolutionary point of view. This aspect therefore makes important the evolutionary and adaptive significance of phenology, including phenology of xylem formation and highlights the need to know its behavior over time through monitoring.

In temperate and boreal environments, spring phenology is generally driven by the interaction of key biological traits and climatic factors, such as chilling requirements, rising temperatures and, in broader geographical terms, the photoperiod at the latitude of origin of the populations (Basler & Körner 2012, Friedl et al. 2014, Primack et al. 2015, Zohner et al. 2016). This last cue is particularly important in late-successional species, such as silver fir and beech (Basler & Körner 2012, Garate-Escamilla et al. 2020). However, in our case, because the very narrow spatial distribution of the three populations, the photoperiod must be considered as a constant in the phenological study, while the effects of the other two components and the individual factors prevail over the populations under consideration. Constituting a parallel and contemporary phenological baseline will therefore serve either to local monitoring to identify behavioral anomalies with possible / probable effects on the populations fitness and to bring them into a wider silver fir monitoring network, which allows to have a more global vision of the species and its reactions to climate shift. In Italy, Antonucci et al. (2019) monitored xylem phenology formation on Italian silver fir, studying variability in a North-South transect and comparing 3 origins, 1 from Dolomites, 1 from central-southern Apennines and 1 from southern Apennines. Our study focused on meaningful metapopulations growing at the borders between the northern and southern groups of Apennine firs and aimed to establish a reference baseline to highlight any future anomalies in the phenological behavior of these populations that could alter their fitness and thus increase their genetic erosion.

The start timing of xylem enlargement has an important evolutionary meaning since it is a trade-off between the decreasing risk of frost damage and the optimization of the growing season length (Housset et al. 2016). In our study, timing of the first enlarging cells (end of April) was consistent with Antonucci et al. (2019) in Italy and Cuny et al. (2012) in France.

During the two years of cambium phenology monitoring, the primary differences between LV and PIG were found in 2012, which was warmer at the global level (Desiato et al. 2013, Friedl et al. 2014). In PIG, where the mean annual temperature is higher than in LV due to the lower altitude and the shorter distance from the sea, the effect of warmer 2012 spring was stronger at the onset of the enlarging phase (bE), approximately two weeks earlier compared with 2011. Deslauriers et al. (2008) observed similar behavior in Pinus leucodermis Ant. when monitored during two contrasting years.

The interruption of the dormant phase in both conifers and broad-leaved trees is linked to the increasing temperatures after winter, both in the Mediterranean and boreal regions (Oribe et al. 2001, Nielsen & Jørgensen 2003, Deslauriers et al. 2008, Rossi et al. 2008b, Cocozza et al. 2016, Delpierre et al. 2019), but the role of the photoperiod is crucial in recognizing the new growing season. In fact, thanks to the interaction between temperature and photoperiod, in early springs, when the risk of late frosts is high, a too early start is delayed (Pletsers et al. 2015). In conifers, Rossi et al. (2006b) observed as the maximum growth ring rates occurred between the end of
spring and the start of summer at the maximum day length.

The earlier onset of the bE phase in PIG compared with LV could also be attributed to the different altitudes between the two sites, as changes in altitude can determine significant variations in temperature and precipitation (Antonucci et al. 2015, Cocozza et al. 2016, Kraus et al. 2016). In Picea abies, Kraus et al. (2016) observed at higher altitude with decreasing temperatures the time needed to cell maturation increases progressively.

In PIG, where the mean age was lower than that in LV1 and LV2, cells reached every maturation stage sooner, during both monitored years. At La Verna, a later onset of the cambium activity and cell differentiation was more evident in LV2 probably favored by the different aspect. Xylem formation is a complex process, associated also with physiological characteristics of single trees, including age and size. In timberline conifers, where microenvironmental conditions can be more difficult, Rossi et al. (2008a) found a shorter tree-ring-formation period in mature trees, due to the cambium division onset occurring later than in younger trees, with reduced cell production.

Anyway, the longer dX observed for 2012 in all 3 populations is likely related to the high spring temperatures observed for that year. Low temperatures are the primary limiting factor for the radial growth of trees at high latitudes or altitudes (Rossi et al. 2007, Hänninen & Tanino 2011); therefore, the expected rise in temperatures, together with increased atmospheric CO$_2$ levels, may favor tree growth and lengthen the growing season. This effect is also expected to occur in the natural core range of A. alba (Gazol et al. 2015). Furthermore, in the Mediterranean region, where hot and dry summers are common, another important factor to consider in xylem dynamics is the availability of water (Kramer et al. 2000, Cherubini et al. 2003, Camarero et al. 2010, Lebourgeois et al. 2012, Vieira et al. 2014). The amount of seasonal precipitation will affect not only the length of phenological phases but also the length of the growing season (Giagli et al. 2016, Housset et al. 2016, Ren et al. 2018, Antonucci et al. 2019). Indeed, water availability influences the processes of cell division and expansion (Fonti et al. 2010), and among the Mediterranean firs, A. alba seems one of the most sensitive species to climatic fluctuations and water stress because of its lower water-use efficiency (Battipaglia et al. 2009). Combined with climatic fluctuations, also soil characteristics can influence the individual tree behavior (Gewehr et al. 2014). Therefore, high spring and summer temperatures (especially in July and August), combined with reduced precipitations, can influence the cambial activity, decreasing the annual ring growth. In addition, annual growth is also influenced by the previous year climate, particularly the summer rain distribution, this influence is stronger in southern slopes (Battipaglia et al. 2009, Carrer et al. 2010, Mazza et al. 2014, George et al. 2015). In particular, the water balance of the previous growing season influences the spring wood production, while the current summer rainfall can influence the late wood thickness (Battipaglia et al. 2009). In Thuja occidentalis L., intra- and among population variability in cambial phenology could be considered plastic responses to different stand environmental conditions (Housset et al. 2016). Differences in behavior were observed between and within populations for bE, bW and bM in relation to the thermal sums recorded in the two years of monitoring. The differences for those traits suggested an adaptive potential which may be particularly meaningful in southern Europe, where according to the RCP8.5 scenario, the range of A. alba could be significantly reduced (Ruosch et al. 2016). Similar levels of variability of cambial phenology traits were observed for the Mediterranean climate in Pinus pinaster (Vieira et al. 2014).

The continued refinement of genotype/
phenotype association methodologies, which were not yet well-defined at the time of this initial survey (Heer et al. 2018), and the acquisition of multiple markers (especially functional ones) will likely simplify the characterization of genetic variation (Andersen & Luebberstedt 2003, Poczai et al. 2013, Bosela et al. 2016). Future monitoring periods should consider the development of genetic spatial-analysis methods to assist with silvicultural management (Verbylaitė et al. 2017) and to link cambial phenology and growth responses to climate and association genetics with stress responses associates with single-nucleotide polymorphism (SNP) genotypes (Pinosio et al. 2014, Heer et al. 2017). In addition, this framework can be relevant to deeper studies examining phenology, with particular focus on the winter chilling requirements and the effects of a prolonged growing season on xylogenesis (Gordo & Sanz 2010, Clark et al. 2014, Delpierre et al. 2019) at the southern edge of the silver fir range.

A baseline for future monitoring

The approach adopted in the framework of the multidisciplinary LIFE RESILFOR project (Miozzo et al. 2014) was used to establish a monitoring network for the considered silver fir populations and to provide a reference baseline to address their management towards the maintenance or the enhancement of genetic diversity. Thanks to the results obtained during this project, at the next monitoring it will be possible to verify if the selected parameters provide a sufficiently informative reference base to study the sensitivity of these South-Europe fir stands (Babst et al. 2013).

Among traits correlated to fitness, full seeds production and seed germination (Aravanopoulos et al. 2015) were impossible to be measured due to a lack of mast years during this study. Subsequent monitoring plans should consider the coverage of mast years, too. The relatively different forest and demographic structures (Manetti et al. 2014, Mazza et al. 2014) in the studied forest stands, were likely determined by former management and/or past disturbances (e.g. extreme climatic events). Anyway, major variability was detected among individuals and similar levels of genetic diversity were noted in both (a) and (r) layers, probably since in (r) layer also dominated aged trees were included. This structuring suggests the application of successive cuts followed by shelterwood cuts, when the removal of some (a) large trees will give to (r) trees the opportunity to recover their growth, favoring also natural regeneration and reducing possible losses of variability (Perrin 1954). With this silvicultural system, the silver fir dominated forests will result in more balanced uneven-aged stands. In silver fir, this management system, applied by groups or tree clusters will assume the definition of irregular shelterwood system. The baseline data will be useful as a reference for recording any changes occurring at the next monitoring cycles and will provide indications to fix silvicultural criteria and subsequently adjust management interventions to results from future monitoring. For this type of populations, the main purpose of silviculture should be focused on combating, by reducing or delaying them, the effects of genetic drift.

The adopted methodology

Fir management and interactions with other co-dominant species in mixed forests in relation to the effects of climate change appear to be common at these latitudes. It did not seem accidental that while the LIFE RESILFOR program was being implemented for the management of the relationships between beech and silver fir in the central Apennines, the LIFEGENMON program was also launched in central Europe and the Balkan peninsula. Both programs took into consideration the need to monitorsilverfirpopulations, which are currently more sensitive to competition with beech and habitat variations. The first program, mostly locally oriented towards an adaptive forestry
in the described transitional forests, intended to apply in those forest a permanent areas-based method for monitoring natural regeneration and adult strata within populations, and the habitat in general with the aim of establishing an initial baseline for future iterations and thus guide future silvicultural interventions (Miozzo et al. 2014). In this context, dendrological, genetic, and phenological parameters were examined, based on the literature and experience available at the time. The second one, more recently by a few years, intended to supply a significant contribution to the codification of shared methodological protocols for monitoring populations in the species range (Kavaliauskas et al. 2020).

Comparing the two project reports you can see that they are governed by two different philosophical and methodological approaches, as the first is aimed at supporting silvicultural choices to be applied over time in small isolated and at-risk populations, the latter aims at implementing a general method by providing shareable monitoring protocols. In our case study, given the small size of the populations, it was therefore decided to carry out a total analysis of all the material (adult trees and renewal) of fir present in permanent areas of about 1 hectare, in the latter it is proposed instead a random sampling of 50 plants at a minimum distance of 30 m, scattered over an area of at least 10 hectares; therefore, assuming much larger populations than those examined in central Italy. In fact, while a random positioning is also provided for the plots in the LIFGENMON protocols, it was not possible to adopt this criterion in our work, precisely because of the small size of our populations, in which all trees and seedlings with a diameter greater than 5 cm have been numbered and GIS mapped. In addition, LIFE RESILFOR does not provide for the replacement in subsequent iterations of the dead trees to be measured, but every 20 years the losses in the entire permanent plot are counted and the variations within the age and diameter classes were recorded.

The verifiers proposed by the two projects to characterize and monitor the populations and habitats are substantially the same, however differing in some respects concerning phenology and bloom synchronization which in central Italy, given the size of the mature tree layer, could not be detected with precision through the visual estimates of protocols proposed by Ducci et al. (2012) for fir nor by Kavaliauskas et al. (2020).

To obtain greater precision and allow the calculation of the average dates of passage from one phenological phase to another of the numbered and georeferenced adult plants, and genetically analyzed, we have preferred to resort to protocols providing for the analysis of cambium activity and xylogenesis (Rossi et al. 2006b, Rathgeber et al. 2011b). Likewise, being unable to accurately verify the blooming synchrony it was decided to record the progress of mast years. Carpet check genetic analyzes were carried in the Apennine populations on both the regeneration and adult layers, considering, in the latter, the subdivision into age and diameter classes. Future monitoring iterations will therefore make it possible to detect within population both changes in the forest structure and those relating to the genetic structuring, and at the same time to verify the progress of responses to the climate.

**Conclusions**

The temporal dynamics induced by historical climate and management changes might have a strong effect on the genetic structure of populations and an individual baseline should be established for each population, based on their own characteristics. A preliminary historical and phylogenetic investigation, based on anecdotal and scientific information is required in areas under long history of anthropic influence, as shown by the detection of the artificial origin of BT1 stand. Indeed, the artificial origin of several gene pools can influence the results of phylogenetic inference.
used to take decisions in managing silver fir genetic resources. The greater genetic richness of BT2 can favor adaptability and plasticity and there an adaptive management to open artificial gaps in the crown canopy should be considered. At the same time, BT1 should be progressively harvested, and the area naturally regenerated or planted with BT2 materials.

**Matching with the CPH**

The Centre-Periphery Hypothesis is not always valid for silver fir in the Italian peninsula but rather it should be considered as a relative principle and modular according to environmental variations in time and space as shown for the three studied forest stands.

**The genetic baseline**

Genetic markers used to form this initial baseline provided useful information to describe the studied populations at their present stage and surely the genetic parameters will be a reference for future reiterations. However, next surveys will have to consider advancements in single-nucleotide-polymorphisms (SNPs) applied to adaptive traits and their spatialization, will surely allow for a better management of studied silver fir populations.

The analysis of genetic parameters and genetic diversity by age classes can provide indications for the germplasm silvicultural management. Furthermore, these results allow to monitor over time the changes between age groups and social classes. This study also showed as at the time of monitoring, the intermediate age classes in PIG and the oldest in LV were those that most contributed to the dynamics of the studied populations.

**The cambial phenology baseline**

Cambial phenology in silver fir was characterised by within-population individual variability which is consistent to other Mediterranean conifers. Besides its inherited component, the annual radial growth can also be influenced by specific features of the forest stands, as underlined by dendroclimatic studies. The large size characterizing the fir trees of our populations could constitute a limit for their survival, due to a lower ability to adapt to more xeric conditions. Therefore, characterizing their average and individual phenological behavior can allow us to evaluate how much resilience they may have in the future. The high cambium phenological individual variability could support these populations for their fitness to cope the risks from the isotherm shift.

Considering adaptive traits, such as phenology, important for the population fitness, ecological factors *in situ* can interact and confound with genetic variability and a common garden experiment can help to distinguish the weight of these two phenotype’s components. In our case the insufficient seed production in the studied stands during the project period did not allow to start this phase.

**In situ conservation**

This baseline could be used to establish both silvicultural criteria and to define silvicultural treatments suitable for individual forest stands. If we consider the marginal populations investigated and their important bridging function between the southern and northern groups of silver fir, the main purpose of silviculture should be to reduce and / or delay the effects of isolation and genetic drift. Consequently, a silvicultural system applied on small areas (irregular shelterwood system) seems to be confirmed as the main management system to preserve the major demographic and genetic equilibrium favoring a more balanced uneven-aged forest structure.

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Author contribution statement

FD conceived the project. FD, ADR and RP conceived of the ideas and designed the methodology. ADR, RP, and MM collected and analyzed the data. FD and ADR wrote the original draft. FD, ADR, ALC and RP reviewed and edited the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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