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Unexpected scenarios from Mediterranean refugial areas: disentangling complex demographic dynamics along the Apennine distribution of silver fir / Piotti, Andrea; Leonarduzzi, Cristina; Postolache, Dragos; Bagnoli, Francesca; Spanu, Ilaria; Brousseau, Louise; Urbinati, Carlo; Leonardi, Stefano; Vendramin, Giovanni Giuseppe. - In: JOURNAL OF BIOGEOGRAPHY. - ISSN 0305-0270. - ELETTRONICO. - 44:7(2017), pp. 1547-1558. [10.1111/jbi.13011]

Published
DOI:10.1111/jbi.13011

Article type: Original Article

Word count: Abstract (328), main body of the text (6985)

Title: Unexpected scenarios from Mediterranean refugial areas: disentangling complex demographic dynamics along the Apennine distribution of silver fir

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Running head: Demographic history of *Abies alba* in the Apennines

ABSTRACT

Aim Mediterranean refugial areas are generally underrepresented in large-scale genetic surveys of forest trees. In the case of silver fir (*Abies alba* Mill.), this has led to divergent hypotheses about the exact location of glacial refugia and the trajectory of recolonization routes. Based on comprehensive sampling of Apennine populations, we aimed to reconcile discrepancies about the number and location of refugia for silver fir in the Apennines and test alternative demographic scenarios developed from palaeobotanical and genetic data.

Location Mediterranean Basin; the Apennines and surrounding areas.

Methods 1167 individuals from 16 Apennine populations, extensively covering the species' distribution along the Italian Peninsula, and eight populations from the Alps and Eastern Europe were genotyped at 16 nuclear and three chloroplast microsatellite markers. The geographical distribution of genetic variation was explored using Bayesian clustering and multivariate methods. Based on inferred genetic structure, the demographic history of *A. alba* was assessed by approximate Bayesian computation (ABC) analysis.

Results Two unexpected characteristics of genetic structure emerged: a sharp genetic boundary in the central Apennines and a tight genetic connection between southern Apennine and Eastern European gene pools. Two Apennine areas, corresponding precisely with refugial areas hypothesized in most recent palaeobotanical syntheses, have high genetic diversity on a par with Eastern European populations. ABC analysis showed an ancient separation between Apennine and Eastern European gene pools followed by an admixture event that, mainly through directional gene flow via pollen, might have established the genetic similarity between southern Apennine and Eastern European populations. In addition, there was evidence that the central Apennines acted as a small-scale, isolated refugium during the Last Glacial Maximum.

Main conclusions Silver fir rear edge populations have experienced a complex demographic history across several glacial-interglacial cycles, leading to unexpected genetic structure. Our study provides new insights into forest tree dynamics in the Mediterranean, showing the putative presence of multiple refugia for silver fir in the Apennines and a trans-Adriatic connection between silver fir populations in southern Italy and the Balkans.

Keywords *Abies alba*; approximate Bayesian computation; glacial refugia; Mediterranean basin; phylogeography; Pleistocene; post-glacial recolonization; nuclear and chloroplast microsatellites; rear edge populations; trans-Adriatic gene flow

INTRODUCTION

There is continuing debate about the location of glacial refugia. Such interest is justified by the importance of correctly placing glacial refugia and recolonization routes when interpreting current species' distributions, estimating species' migration potential and foreseeing possible range shifts (Petit *et al.*, 2008). Coupling genetic and palaeobotanical data is considered the most effective approach to infer past retraction-colonization dynamics in plants (Hu *et al.*, 2009). For a very small number of tree species, genetic and palaeobotanical records covering both the distribution core and edges are available, and well-grounded hypotheses on the topography of glacial refugia and post-glacial recolonization routes have been developed. Inevitably, such inference on past demography has also been tried for species with much less complete data available. In particular, biogeographic literature highlights that northern Mediterranean refugial areas, such as the Italian and Balkan Peninsulas, are generally underrepresented in genetic and palaeobotanical large-scale surveys on forest trees (Hampe & Petit, 2005; Liepelt *et al.*, 2009; Linares, 2011). Trees, and plants in general, have experienced complex dynamics due to environmental heterogeneity and palaeoclimatic events in these areas, leading to high phylogeographic complexity and idiosyncratic patterns (Nieto Feliner, 2014). The increasing availability of molecular data and refined statistical approaches are providing unprecedented power to unravel complex demographic histories, but to be effective these tools must be applied to appropriate sampling of the focal species.

Silver fir (*Abies alba* Mill.) is one of the most important forest tree species in Europe and results from available palaeobotanical and genetic studies (e.g. Liepelt *et al.*, 2002, 2009; Linares, 2011; Cheddadi *et al.*, 2014) have generated contrasting hypotheses about its Quaternary history. In particular, important but unsolved points are the location of isolated refugia (i.e. refugial populations that did not expand after the Ice Ages) and effective refugia (i.e. refugial populations that contributed to recolonization) and the phylogenetic relationships among populations from refugial areas. There is general agreement about the existence of at least two effective refugia in the Apennines and southern

Balkans, but so far only inconsistent speculations on their exact location and on recolonization routes have been proposed (Liepelt *et al.*, 2009; Linares, 2011; Cheddadi *et al.*, 2014). An attempt to synthesise different interpretations of genetic and palaeobotanical data is difficult since both disciplines suffer low availability of data for refugial areas (Liepelt *et al.*, 2009; Linares, 2011; Tinner *et al.*, 2013).

In the Apennines, the presence of *A. alba* during the late-glacial period has been demonstrated by palynological surveys, mainly from the northern Apennines and the Tyrrhenian side of the central Apennines (e.g. Follieri *et al.*, 1998; Vescovi *et al.*, 2010; Magri *et al.*, 2015). Previous genetic work, based on a limited number of populations located mostly in Calabria and the northern Apennines, inferred the presence of at least two genetic clusters (Konnert & Bergmann, 1995; Liepelt *et al.*, 2009). This led to the hypothesis of an isolated refugium in the southern Apennines and an effective refugium, located either in the central (e.g. Konnert & Bergmann, 1995) or in the north-western Apennines (e.g. Cheddadi *et al.*, 2014), from which the recolonization of the Alps and central Europe may have started. In contrast, following early hypotheses about southern Italy as the starting point for the recolonization of Central Europe, a possible genetic continuity along the entire Apennine chain has been hypothesized several times (Scaltsoyiannes *et al.*, 1999; Linares, 2011; Camerano *et al.*, 2012). Some genetic studies even considered central Italy as a possible melting pot of recolonization routes from the Balkans and southern Apennines (Parducci *et al.*, 1996) or from the northern and southern Apennines (Larsen & Mekic, 1991). However, all of these previous studies have been based on limited sampling and critical questions remain regarding the genetic relationship between populations from the northern and southern Apennines and the origin of populations from the central Apennines. Establishing the evolutionary history of these fragmented Apennine silver fir populations is particularly urgent because they have high evolutionary and conservation value due to their unique genetic and eco-physiological features (Hansen & Larsen, 2004; Carrer *et al.*, 2010; Cheddadi *et al.*, 2014; Brousseau *et al.*, 2016). An intensive genetic survey covering the entire Italian Peninsula will allow robust testing of different phylogeographic hypotheses developed from palaeobotanical and genetic data and resolution of these questions.

The main objective of this work was to investigate the genetic structure of Apennine silver fir populations and reconstruct past demographic and recolonization dynamics affecting Apennine genetic clusters. We intensively sampled 16 populations along the entire Apennine range, with a particular focus on previously unsampled areas and adding several populations from surrounding regions (i.e. the Alps and Eastern Europe). Overall, 1167 individuals were genotyped with biparentally inherited nuclear and paternally inherited chloroplast microsatellite markers (hereafter nSSRs and cpSSRs, respectively). The intensive sampling strategy and the large marker set used allowed us to investigate: *i*) the genetic relationship between populations from the northern and southern Apennines and the origin of central Apennine populations, *ii*) the possible presence of genetic discontinuities and/or contact zones between different genetic clusters along the Apennines, and *iii*) the genetic relationship between Apennine gene pools and those from surrounding areas. Based on the genetic structure emerging from analyses of our data, we assessed the support for alternative hypotheses about Pleistocene dynamics of silver fir populations in the Apennines developed from palaeobotanical and genetic data through approximate Bayesian computation (ABC) analyses.

MATERIALS AND METHODS

Sample collection and genotyping

Sixteen putatively autochthonous populations were sampled along the Apennine chain (five in the northern, five in the central, six in the southern Apennines, Fig. 1 and Table S1.1) according to two general criteria: *i*) extensively covering the species' distribution in this area, and *ii*) increasing the sampling effort in terms of number of populations in areas sparsely covered by previous studies (i.e. the central Apennines). We included five populations from the Alps and three populations from

Eastern Europe to investigate the genetic relationship of Apennine populations with those from surrounding areas. From each population, needle tissues were collected from c. 50 adult individuals at least 20 metres apart in order to adequately cover a large area (c. 3 ha) within each stand.

All sampled individuals were genotyped at 16 unlinked nSSRs (Aag01, Aat01, Aat02, Aat03, Aat04, Aat05, Aat06, Aat08, Aat09, Aat10, Aat11, Aat13, Aat14, Aat15 and Aat16, Postolache *et al.*, 2014; NFF7, Hansen *et al.*, 2005) and three cpSSRs (Pt71936, Vendramin *et al.*, 1996; Pt30141 and Pt30249, Liepelt *et al.*, 2001). The multiplexing and amplification procedures for nSSRs are reported in Postolache *et al.* (2014). CpSSRs were multiplexed using the Type-it Microsatellite PCR kit (Qiagen, Germany) with primer concentrations 0.1µM (Pt71936), 1µM (Pt30141) and 1.5µM (Pt30249). PCR products were run on AB 3500 (Applied Biosystems, USA), with LIZ-500 as the internal size standard. The resulting profiles were sized using GeneMarker (SoftGenetics).

Genetic diversity and population structure

Standard genetic parameters describing within-population genetic variation and genetic differentiation were estimated by GENALEX (Peakall & Smouse, 2012) and HP-RARE (Kalinowski, 2005) for nSSRs, and CONTRIB (Petit *et al.*, 1998) for cpSSRs.

The presence of a genetic structure among sampled populations and the putative number of different genetic clusters were evaluated using the model-based Bayesian clustering algorithm implemented in STRUCTURE 2.3 (Pritchard *et al.*, 2000) and the empirical statistic ΔK (Evanno *et al.*, 2005; Earl & von Holdt, 2012) on nSSR data. STRUCTURE was run using default settings and parameter values, and varying K from one to 10. Each run consisted of 1×10^5 burn-in iterations and 5×10^5 data collection iterations, and was replicated 10 times. After checking for convergence of diagnostic statistics, different runs for the same K were averaged using the software CLUMPP (Jakobsson & Rosenberg, 2007). Bayesian analysis of population structure was run on the cpSSR dataset using BAPS (Corander *et al.*, 2008) based on a non-spatial genetic mixture analysis for linked loci, with K varying from one to 10. The best partition of populations into K clusters with the highest marginal log-likelihood after 10 replicates was chosen as the most representative one. Principal component analysis (PCA) was also performed on the arcsine square root transformed population allele and haplotype frequencies in R 3.2.4 (R Core Team, 2015) to evaluate the main features of the genetic structure obtained through Bayesian clustering with an independent approach.

The hierarchical partitioning of total molecular variance due to the genetic structure emerging from Bayesian clustering analyses was estimated through AMOVA (Excoffier *et al.*, 1992). Statistical significance of each hierarchical level (among K genetic clusters, among populations within genetic clusters, and within populations) was evaluated by 1×10^4 nonparametric permutations using GENALEX.

Approximate Bayesian computation to infer demographic history

To trace the demographic history of *A. alba*, the ABC procedure (Beaumont *et al.*, 2002) implemented in DIYABC 2.1 (Cornuet *et al.*, 2014) was performed based on the nSSR dataset. To simplify the analysis and limit the number of scenarios tested, we relied on the results of Bayesian and multivariate clustering analyses on our datasets (see Results) and on previously available palaeobotanical and genetic information. Therefore, ABC analyses were carried out on four groups of populations, hereafter referred to as NAPP (northern Apennines and the Alps), CAPP (central Apennines), SAPP (southern Apennines), and EAST (Eastern Europe), and scenarios were designed to cover plausible phylogenetic relationships among such genetic clusters.

The analysis of different demographic models (see Appendix S2) was performed following a two-step approach. In the first step, we compared six scenarios in which, for simplicity, all populations were assumed to have an identical prior distribution (Uniform distribution: 10-500000, Table S2.5) for the effective population size (*Ne*). In the second step, the whole dataset was used to compare the two scenarios that showed comparably high posterior probability in the previous analysis (Fig. 2). In this final analysis, the prior distributions of effective population size were set according to results from the first step analysis (Table S2.6). To underline this main difference with respect to scenarios from the first step analysis, the two retained scenarios were then referred to as scenario A and B (Fig. 2).

The main characteristics and peculiarities of the two scenarios compared in the second step of the DIYABC analysis were as follows:

Scenario A is a hierarchical split scenario directly following STRUCTURE results (see Results), in which NAPP separated from EAST before generating, respectively, CAPP and SAPP.

Scenario B takes into account *i*) that silver fir populations from the Italian Peninsula and Eastern Europe are characterized by different and almost fixed mitochondrial variants (Liepelt *et al.*, 2002), and *ii*) the genetic structure emerging from STRUCTURE results. Therefore, considering that SAPP shares the same mitotype with NAPP and that SAPP and EAST belong to the same STRUCTURE cluster, the scenario was designed hypothesizing that, after an initial split between NAPP and EAST, SAPP was generated by admixture of EAST and an unsampled ghost population, which merged with NAPP. CAPP merged relatively recently with NAPP considering the likely post-glacial connection between these two groups of populations (Magri *et al.*, 2015). The admixture rates *ra* and 1-*ra* are the genetic contribution of each of the source populations to the origin of SAPP.

In both scenarios, $t_\#$ represents the time of occurrence of an event (expressed in number of generations) and $N_\#$ refers to the effective population size of the corresponding populations (N_{NAPP}, N_{CAPP}, N_{SAPP}, and N_{EAST} for the four clusters described above, N_{GHOST} for an unsampled ghost population, and Na for the ancestral population ‘a’) during each time period (e.g. $0 – t_1$ or $t_2 – t_3$) (Fig. 2).

Details of each competing scenario in the first step analysis, mutation rate, summary statistics, and model checking are provided in Appendix S2, Fig. S2.5 and Table S2.5.

RESULTS

Genetic diversity and population structure

The 16 nSSRs showed a total and mean number of alleles of 151 and 9.43 (from two alleles at Aat16 to 34 at NFF7), respectively. No evidence was found for the presence of null alleles or significant genotypic disequilibrium among locus pairs. In addition, no evidence for selection was found by outlier detection tests (FDIST and BAYESCAN, see Appendix S3 and Fig. S3.6), indicating that the analyzed nSSRs were likely to be neutral, as suggested by Postolache *et al.* (2014). The allelic richness (Ar_{84}) and expected heterozygosity (H_E) were above average in southern Italy, Eastern Europe and the NER population in the northern Apennines (Fig. 3a, Table S1.1). Differentiation indices showed a globally moderate differentiation among populations, with F_{ST}=0.097 and Hedrick's G'_{ST}=0.155.

CpSSRs displayed 12 (Pt71936), 17 (Pt30141), and 6 (Pt30249) size variants, which combined into 164 haplotypes and a mean haplotypic diversity over populations of h=0.935. The haplotype richness per population (Hr_{37}) ranged from 10 (COR) to 28 (ROM) (Fig. 3b, Table S1.1). Genetic differentiation was in line with that found at nSSRs (G_{ST}=0.045).

STRUCTURE analysis revealed an optimal grouping at K=2 (Fig. S1.1) clearly distinguishing, along the Apennines, populations located north and south of the Gran Sasso and Majella massifs (blue and red, respectively, in Fig. 3c). Populations from the southern Apennines clustered with those from Eastern Europe. AMOVA analysis showed that the proportion of total genetic variation explained by differences between these two main genetic clusters was 7% (P<0.001). The next strongest level of structuring was K=4, which grouped populations from the central Apennines separately from those in the northern Apennines and the Alps, and populations from the southern Apennines were separated from those in Eastern Europe (Fig. 3d). The main characteristics of the genetic structure emerging from STRUCTURE analysis were confirmed by BAPS analysis on cpSSRs (Fig. 3e), PCA on both marker types (Fig. S1.2), and pair-wise differentiation indices (Tables S1.2 and S1.3). In particular, PCA analyses of both marker types showed a main separation resembling STRUCTURE results at K=2 along the first principal component (i.e. southern Apennine and Eastern European populations differentiated from northern Apennine and Alpine populations), whereas the second principal component highlighted the differentiation between southern Apennine and Eastern European populations.

Approximate Bayesian computation to infer demographic history

In the first step of DIYABC analysis, it was not possible to distinguish the most-likely scenario because similarly high posterior probabilities were found for scenarios 3 and 4 (respective probabilities 0.49 and 0.42, with largely overlapping 95% CIs; Table 1). Therefore, we decided to compare the two scenarios in a final analysis, in which scenario B showed a posterior probability significantly higher than scenario A (Table 1). The observed summary statistics and PCA results (Table S1.4 and Fig. S1.3) confirmed the good fit of scenario B to the data. The type I error rate was 0.314, and the average type II error rate was 0.257.

Under scenario B, SAPP originated at t_2 by an admixture event between EAST and a ghost population. The ghost population merged with NAPP at t_3 and gave the largest contribution (ra=0.780) to the formation of SAPP. The median values of the effective population sizes were 41800, 15500, 97900, 93500, 51300, and 2870 for N_{NAPP}, N_{CAPP}, N_{SAPP}, N_{EAST}, N_{GHOST}, and Na, respectively (Table 2 and Fig. S1.4). The posterior parameters showed that the effective population size of the ancestral population was estimated to be 14.5 and 32.5 times lower than those of NAPP and EAST, respectively, suggesting an expansion event at t_4. The results also indicated that the demographic expansion continued in the following events, which led to the formation of SAPP at t_2. More recently, a bottleneck is likely to have given rise to the formation of CAPP at t_1.

The median values of the divergence times t_1 (for CAPP and NAPP), t_2 (for the appearance of SAPP from the admixture between EAST and a ghost population), t_3 (for the ghost population and NAPP), and t_4 (for EAST and NAPP) were 1320 (95%CI: 300-3110), 4750 (95%CI: 1800-6850), 7790 (95%CI: 4230-9860), 12000 (95%CI: 6950-14800) generations ago, respectively (Table 2 and Fig. S1.4). Assuming a generation time of 50 years (Liepelt *et al.*, 2002; Dering *et al.*, 2014; Ruosch *et al.*, 2016), these values can be translated into 66000 (95% CI: 15500-155500), 237500 (95% CI: 90000-342500), 389500 (95% CI: 211500-493000), 600000 yrs BP (95% CI: 347500-740000) for t_1, t_2, t_3, and t_4 respectively.

DISCUSSION

Spatial distribution of genetic diversity

Along the Apennine chain there are only two areas where genetic diversity is large and comparable with populations from Eastern Europe. They correspond strictly to refugial areas hypothesized according to the most recent and detailed palaeobotanical syntheses (Magri *et al.*, 2015).

The first area is located in the southern Apennines (Fig. 1), from the southernmost population (GAM) to the latitude of CIL and LAU, with the highest haplotype and allelic richness recorded for the TDP population. The only Apennine silver fir populations regularly included in genetic surveys at the biogeographical scale were those from Calabria, which often showed higher genetic variation than northern ones (Bergmann *et al.*, 1990; Vicario *et al.*, 1995; Liepelt *et al.*, 2002; Longauer *et al.*, 2003; Liepelt *et al.*, 2009). Such high diversity, together with the high vitality and growth vigour seen in provenance trials (e.g. Larsen & Mekic 1991; Kerr *et al.*, 2015), has led many authors to consider Calabrian populations as part of a long-lasting but isolated refugial area (Liepelt *et al.*, 2009; Cheddadi *et al.*, 2014). Our results strongly support the hypothesis of an isolated refugium in the southern Apennines and indicate that the upper latitudinal limit of this refugial area was at ~40.5° N, i.e. the latitude of CIL and LAU. These two populations have retained high genetic diversity during the postglacial period despite erosion to their current small, highly-isolated state. In particular, CIL is composed of few hundred individuals and located ~40 km from the nearest stand (Rovelli, 1995; Di Pietro & Fascetti, 2005). Nevertheless, CIL still maintains levels of genetic diversity comparable to much larger stands (e.g. TDP, the largest stand in southern Italy; Schettino & Travaglio, 2015).

The second area is located in northern Apennines, a region for which several palaeobotanical surveys are available but whose silver fir populations have rarely been included in large-scale genetic studies. Among the three populations sampled, it is surprising that NER is the only one showing high genetic diversity because it is the northernmost population along the Apennines, and highly isolated and extremely small (c. 500 individuals, Piovani *et al.*, 2010). From NER to the central Apennines, genetic diversity gradually decreases to populations north of the Gran Sasso massif which, along with Alpine populations, are the least genetically diverse in our survey. NER has an allelic and haplotype richness 16% and 54% higher, on average, than much larger and less isolated Alpine populations. This suggests that the northernmost part of the Apennine distribution is the most likely refugial area or, at least, the area where silver fir was most abundant during the Last Glacial Maximum (LGM): a hypothesis that fits well with palaeobotanical data (Vescovi *et al.*, 2010; Magri *et al.*, 2015). Besides its conservation relevance, the correct location of refugial populations can help solve the long-lasting problem of accurately calculating historical migration rates (Cheddadi *et al.*, 2014). This might be particularly important for silver fir, whose northern Apennine effective refugium has often been hypothesised in different locations, from the north-western (e.g. Cheddadi *et al.*, 2014) to central Apennines (e.g. Konnert & Bergmann, 1995). The high genetic similarity between populations from the northern Apennines and eastern Alps, with the former being much more diverse despite the much lower census size, seems to confirm the hypothesised post-glacial migration route from the northern Apennines to central Europe (e.g. Konnert & Bergmann 1995; Liepelt *et al.*, 2009; Cheddadi *et al.*, 2014).

A third area might also have acted as a refugium in the Italian Peninsula. Bayesian clustering results, genetic differentiation from surrounding populations, and DIYABC estimate that CAPP diverged from NAPP ~66,000 yrs BP, all point towards the local persistence of silver fir during the last glacial period in the central Apennines. The existence of a glacial refugium in this area has been postulated several times, but always as an alternative to the northern Apennine one (e.g. Konnert & Bergmann, 1995). On the contrary, our analyses suggest the existence of two separate genetic clusters during the LGM north of the Gran Sasso massif, one in the northern and one in the central Apennines. The latter was characterized by a lower effective population size than other Apennine refugia, in accordance with palaeobotanical records showing a marked demographic reduction of silver fir since 70,000 yrs BP in central Italy (Follieri *et al.*, 1998). Although northern and central Apennine populations are generally small and fragmented, and considered as highly impacted by human intervention in the last millennia (Piovani *et al.*, 2010; Urbinati & Romano, 2012; Tinner *et al.* 2013; Leonarduzzi *et al.*, 2016), no clear signals of translocation and/or mixing of different provenances were found, contrary to what was observed in other European conifers (e.g. Wagner *et al.*, 2015), and the spatial distribution of genetic diversity seems mainly related to events dating to before the Neolithic age.

Genetic structure and demographic history

Two unexpected characteristics of the genetic structure of silver fir in the Apennines emerged from our analyses: the existence of a sharp genetic boundary separating populations north and south of the Gran Sasso and Majella massifs in the central Apennines, and the tight genetic connection between southern Apennine and Eastern European gene pools.

In the central Apennines, the four populations within the Gran Sasso e Monti della Laga National Park (VDC, CEP, COR and TOS) and ABS are separated by only 90 km, an area comprising the Gran Sasso and Majella massifs where no natural populations of silver fir are present. Despite their geographic proximity, they showed pair-wise genetic differentiation values among the highest detected ($G'_{ST} = 0.22$, 0.21, 0.25 and 0.15, respectively, Table S1.3). According to DIYABC estimates, the Apennine populations from the two main gene pools detected by STRUCTURE analysis have had separate dynamics during the last 400,000 yrs. Therefore, hypotheses based on a recent genetic continuity along the entire Apennine chain and about the central Apennines as a melting pot between different recolonization routes (Larsen & Mekic, 1991; Scaltsoyiannes *et al.*, 1999; Linares, 2011; Camerano *et al.*, 2012) seem highly unlikely. The processes underlying the origin and persistence of a long-lasting genetic boundary in central Italy are not clear and any hypotheses will require a multidisciplinary approach considering a longer period, from the Neogene Apennine orogeny to climatic dynamics during several Pleistocene glacial cycles. However, it is worth noting that this area represents a steep discontinuity at different time scales. For example, it is considered as the tectonic separation between the northern Apennines Arc and the southern Apennines-Calabrian Arc (Satolli & Calamita, 2008), and an ecotone between different precipitation patterns that have had a large, long-lasting influence on vegetation dynamics in the Italian Peninsula (Comborieu-Nebout *et al.*, 2015). Populations along the rear edge of silver fir distribution are indeed separated by neat genetic boundaries, as previously hypothesized, but the present study demonstrates their location is not where they were previously thought. In particular, we found a genetic similarity between populations from Eastern Europe and southern Italy that are almost fixed for different mitotypes (Liepelt *et al.*, 2002). A genetic similarity at isozymes and chloroplast markers between these two areas was reported by Liepelt *et al.* (2002) and Longauer *et al.* (2003). The former hypothesized a possibly extensive trans-Adriatic gene flow via pollen homogenizing chloroplast haplotype frequencies over long distance. Our data support this hypothesis as the most likely scenario, considering that SAPP was generated by the admixture of EAST and a population originating from NAPP. These ancient gene pools would have diverged long before the last glacial period, corroborating the hypothesis of a separation lasting for several Quaternary glacial cycles (Liepelt *et al.*, 2009). The median admixture rate estimated (78% from NAPP *vs*. 22% from EAST) is compatible with an introgression via-pollen from the Balkan into the northern Apennine gene pool having shaped, together with the isolation between populations at the two extremes of the Apennine chain, the genetic layout of southern Apennine populations between 340,000 and 90,000 yrs BP. Geological studies demonstrated that the Apulian platform connected several times to the Balkans by a trans-Adriatic land bridge (Patacca *et al.*, 2008), and the presence of shared haplotypes in the Balkan and Italian Peninsulas for other tree species (e.g. Bagnoli *et al.*,

2016) supports the idea of effective gene flow being not rare across what is nowadays considered as a geographic barrier.

The scenario depicted by our intensive genetic survey of Apennine populations provides new insights into the Quaternary history of silver fir. Among them, two seem particularly relevant for studying adaptive responses of rear edge silver fir populations. First, a solid knowledge about past demographic patterns can improve the study of local adaptation. So far, it has been based on considering a main separation between Alpine and Apennine populations in silver fir (Mosca *et al.*, 2014), which seems quite unlikely from our results. Incorrectly considering demographic scenarios can hinder the study of local adaptation by introducing biases in selection tests (Nielsen, 2005). In addition, silver fir tree-ring growth series in Italy show three differentiated groups of populations: 1) the western Alps, 2) the northern Apennines and the eastern Alps, and 3) the central and southern Apennines (Carrer *et al.*, 2010). This subdivision partially corresponds to the genetic structure found in our survey, with some exceptions. In fact, central Apennine populations are genetically similar to northern populations but show growth responses comparable to the ones from southern populations, raising several questions about the relative strength of past migration dynamics *vs.* adaptation to climate in highly heterogeneous regions.

Second, the existence of a sharp genetic boundary in the central Apennines poses the basis for studying possible south-to-north adaptive gene flow in this area. With ongoing climate warming, genotypes able to perform well in warmer and drier conditions are expected to be selectively advantaged both in local persistence and during latitudinal and altitudinal migrations, in particular at Mediterranean latitudes (Fady *et al.*, 2016). In this context, the genetic boundary found in central Italy, corresponding to an area where ecological conditions markedly change (Brunetti *et al.*, 2004; Comborieu-Nebout *et al.*, 2015), might represent an ideal location for studying the role of gene flow in promoting adaptive responses to climate change (Kremer *et al.*, 2012). In such a study, it should be noted that, although Mediterranean silver fir provenances are often considered less sensitive to drought (Aussenac, 2002; Carrer *et al.*, 2010, but see Gazol *et al.*, 2015), fossil data from the last few millennia, which were characterized by increasing aridity, show that silver fir populations have declined similarly throughout the Italian Peninsula (Magri *et al.*, 2015).

Our study has revealed that unexpected processes have shaped the spatial distribution of silver fir genetic diversity in the environmentally heterogeneous Apennine area through several Quaternary glacial cycles. These findings, besides helping to resolve questions about forest tree dynamics at the distributional edge and raising new testable hypotheses, emphasise the importance of robust sampling within refugial areas to quantitatively describe genetic structure for the conservation of extant diversity. The availability of suitable genetic data is essential for effective protection of forest genetic resources (de Vries *et al.*, 2015). Although rear edge silver fir populations have been heavily impacted by human activities for centuries, recently they have also displayed a marked growth decline with changing environmental conditions (Gazol *et al.*, 2015) and their adaptive potential might be constrained by their small size and high geographic marginality. A conservation plan is urgently needed and our data can play a key role in getting it established.

ACKNOWLEDGEMENTS

This project was funded by the Italian MIUR project “Biodiversitalia” (RBAP10A2T4), the COST Action FP1202, and the Gran Sasso e Monti della Laga National Park project “Caratterizzazione genetica, ecologico-strutturale e dendrocronologica dei popolamenti di abete bianco del Parco Nazionale del Gran Sasso e dei Monti della Laga”. We wish to thank all the Parks and Reserves where sampling sites are located for permissions and logistic support. Special thanks are due to the Pollino National Park, the Gran Sasso e Monti della Laga National Park, the Tara National Park, the National Forest Service of Tarvisio and Abetone, and the Comunità Montana Vallo di Diano for particular support during sampling and/or financial contribution, and to P.Piovani for his inspiring work showing the conservation value of relic silver fir populations in the northern Apennines. Valuable contributions to the choice and/or sampling of study sites came from J. Aleksic, R. Di Pietro, R.

Motta, R. Berretti, F. Meloni, P. Belletti, L. Ceccarelli, F. Bini, D. DiSanto, V. Gallucci, A. Piermattei, E. Santini, M. Garbarino, E. Bianchi, E. Vajana, A. Saracino, A. Basile, N. Guarino, M. Borghetti, F. Ripullone, C. Menguzzato, P. Zhelev, F. Popescu, D. Pitar, O. Iordan, D. Suciu. We also would like to thank S. Di Fazio, S. Oddou-Muratorio, S. Cavers, D. Magri, two anonymous Reviewers and the Editor for valuable feedbacks on an earlier version of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary tables and figures

Appendix S2 DIYABC materials and methods details

Appendix S3 Selection tests

DATA ACCESSIBILITY

Microsatellite data with geographic coordinates are available on Figshare at https://doi.org/10.6084/m9.figshare.4704748.v1

BIOSKETCH

The authors belong to a research consortium dealing with population genetics of forest tree species. Their research is particularly focused on the analysis of the distribution of extant genetic variation of forest tree species using genetic markers to dissect the role of demography and selection in shaping genetic diversity at different temporal and spatial scales. They have been involved in projects studying the range-wide phylogeography of several forest tree species in Europe and the Mediterranean Basin.

Editor: Lyn Cook

Author contributions: A.P., C.L. and G.G.V. designed the study; A.P., C.L., D.P., C.U. and S.L. organized and performed the sampling; C.L., I.S., D.P. and G.G.V. arranged the molecular laboratory analyses; A.P., C.L., F.B., L.B., S.L. carried out the data analysis; A.P. and C.L. led the writing with contributions from all authors, who revised and approved the manuscript.

TABLES

Table 1 Posterior probability of each tested demographic scenario and its 95% confidence interval based on the logistic estimate according to DIYABC. In Step 1 analysis, scenarios 1, 2, and 6 are based on an ancient separation between populations from the Apennines and Eastern Europe, scenario 3 strictly represents STRUCTURE results (Fig. 3c,d), and scenarios 4 and 5 consider an admixed origin of southern Apennine populations (see details in the Appendix S2). Scenarios from Step 2 analysis are summarized in Fig. 2.

Scenario	**Posterior probability**	**95% CI (lower - upper)**
Step 1 - Preliminary test with 100 individuals per population		
1	0.0666	0.0266-0.1066
2	0.0021	0.0000-0.0478
3	0.4923	0.4315-0.5531
4	0.4200	0.3582-0.4818
5	0.0172	0.0000-0.0611
6	0.0018	0.0000-0.0476
Step 2 - Analysis with the whole dataset		
A	0.2680	0.2252-0.3109
B	0.7320	0.6891-0.7748

Table 2 Parameter estimates for the best demographic scenario based on approximate Bayesian computation.

				Quantiles			
Parameter	Mean	Median	Mode	2.5%	5%	95%	97.5%
N_{NAPP}	4.25×10^{4}	4.18×10^{4}	4.29×10^{4}	2.00×10^{4}	2.31×10^{4}	6.57×10^{4}	7.13×10^{4}
N_{CAPP}	1.94×10^{4}	1.55×10^{4}	1.19×10^{4}	3.90×10^{3}	5.02×10^{3}	4.98×10^{4}	6.11×10^{4}
N_{SAPP}	1.02×10^{5}	9.79×10^{4}	8.56×10^{4}	4.25×10^{4}	5.03×10^{4}	1.72×10^{5}	1.84×10^{5}
N_{EAST}	9.65×10^{4}	9.35×10^{4}	8.40×10^{4}	4.55×10^{4}	5.29×10^{4}	1.53×10^{5}	1.70×10^{5}
N_{GHOST}	6.72×10^{4}	5.13×10^{4}	1.11×10^{4}	2.90×10^{3}	4.96×10^{3}	1.76×10^{5}	1.89×10^{5}
Na	3.50×10^{3}	2.87×10^{3}	9.10×10^{2}	1.88×10^{2}	3.44×10^{2}	8.57×10^{3}	9.22×10^{3}
t_1	1.41×10^{3}	1.32×10^{3}	1.16×10^{3}	3.00×10^{2}	4.26×10^{2}	2.68×10^{3}	3.11×10^{3}
t_2	4.65×10^{3}	4.75×10^{3}	4.78×10^{3}	1.80×10^{3}	2.23×10^{3}	6.69×10^{3}	6.85×10^{3}
t_3	7.61×10^{3}	7.79×10^{3}	8.06×10^{3}	4.23×10^{3}	4.73×10^{3}	9.72×10^{3}	9.86×10^{3}
t_4	1.17×10^{4}	1.20×10^{4}	1.25×10^{4}	6.95×10^{3}	7.78×10^{3}	1.46×10^{4}	1.48×10^{4}
ra	7.23×10^{-1}	7.80×10^{-1}	9.05×10^{-1}	1.48×10^{-1}	2.64×10^{-1}	9.71×10^{-1}	9.84×10^{-1}
µmic	2.64×10^{-5}	2.46×10^{-5}	2.12×10^{-5}	1.03×10^{-5}	1.22×10^{-5}	4.64×10^{-5}	5.32×10^{-5}
pmic	2.28×10^{-1}	2.37×10^{-1}	3.00×10^{-1}	1.19×10^{-1}	1.30×10^{-1}	2.97×10^{-1}	3.00×10^{-1}
snimic	1.62×10^{-6}	6.89×10^{-7}	1.78×10^{-8}	1.72×10^{-8}	2.50×10^{-8}	7.05×10^{-6}	8.63×10^{-6}

N_{NAPP} = NAPP effective population size; N_{CAPP} = CAPP effective population size; N_{SAPP} = SAPP effective population size; N_{EAST} = EAST effective population size; N_{GHOST} = unsampled ghost population effective population size; Na = effective population size of the ancestral population; times are considered from present (0) backwards in time, t_1=divergence of CAPP from NAPP; t_2= generation of SAPP by admixture of EAST and an unsampled ghost population; t_3 = divergence of the ghost population from NAPP; t_4 = divergence of NAPP and EAST; *ra* = admixture rate; µmic = mean mutation rate of microsatellites; pmic = mean parameter of geometric distribution (GSM, Generalized Stepwise Mutation Model); snimic = individual locus SNI (Single Nucleotide Insertion/deletion) rate.

FIGURE LEGENDS

Fig. 1 Location of sampled populations, with the distribution of *Abies alba* in green (Source: EUFORGEN, http://www.euforgen.org/distribution-maps/).

Fig. 2 Demographic models tested in the second step of DIYABC analysis. In both scenarios, $t_{\#}$ represents the time of occurrence of an event (expressed in number of generations) and $N_{\#}$ is the effective population size of the corresponding populations during each time period (see the Materials and Methods for abbreviations). In scenario B, the admixture rates *ra* and 1-*ra* are the genetic contribution of each of the source populations to the origin of SAPP.

Fig. 3 Geographical distribution of allelic (a) and haplotype (b) richness, and STRUCTURE (c,d) and BAPS (e) results. Locations of populations (f) are shown for reference.

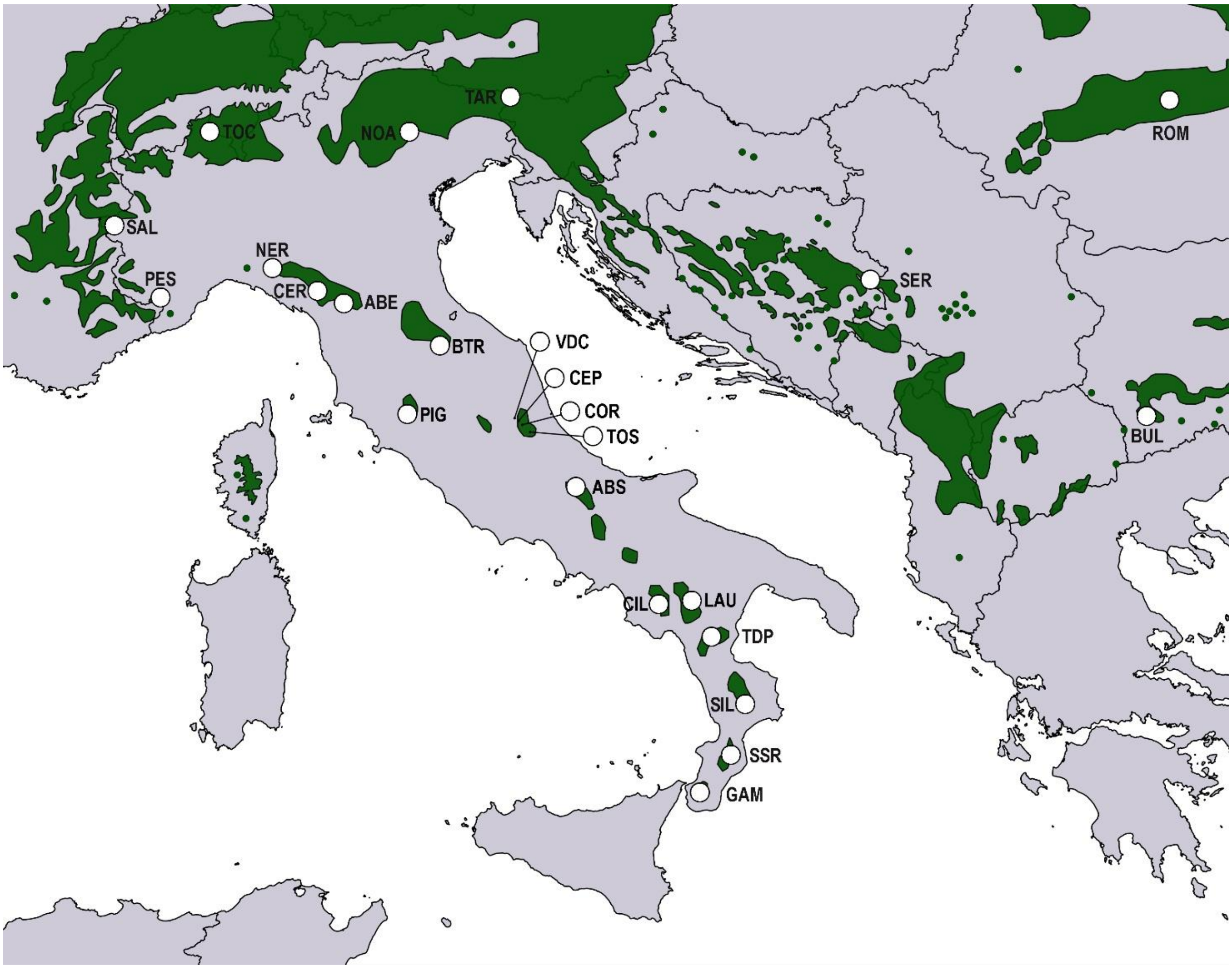
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Figure 1

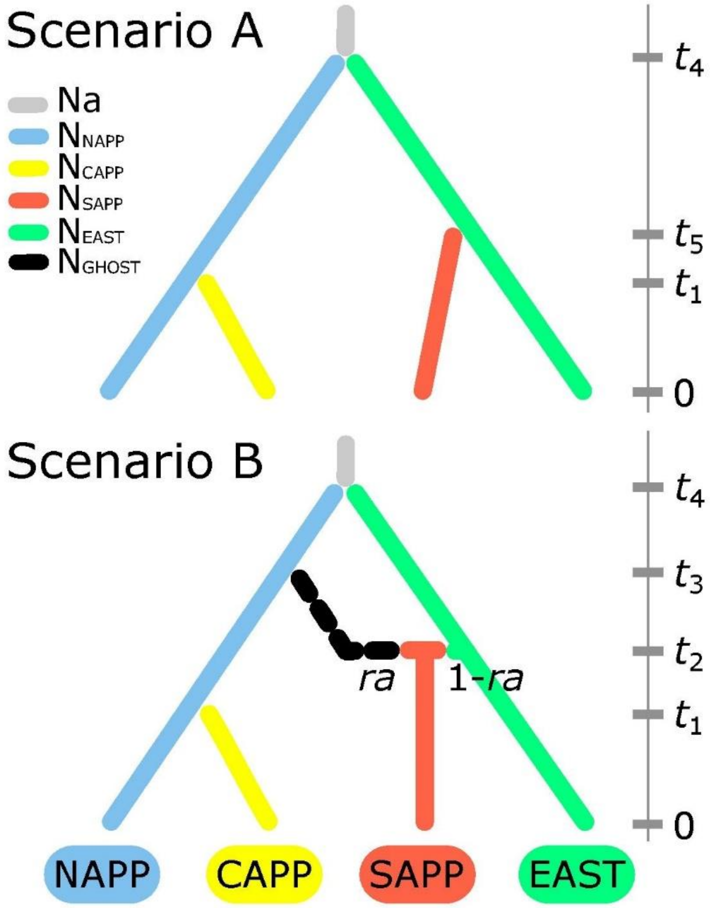
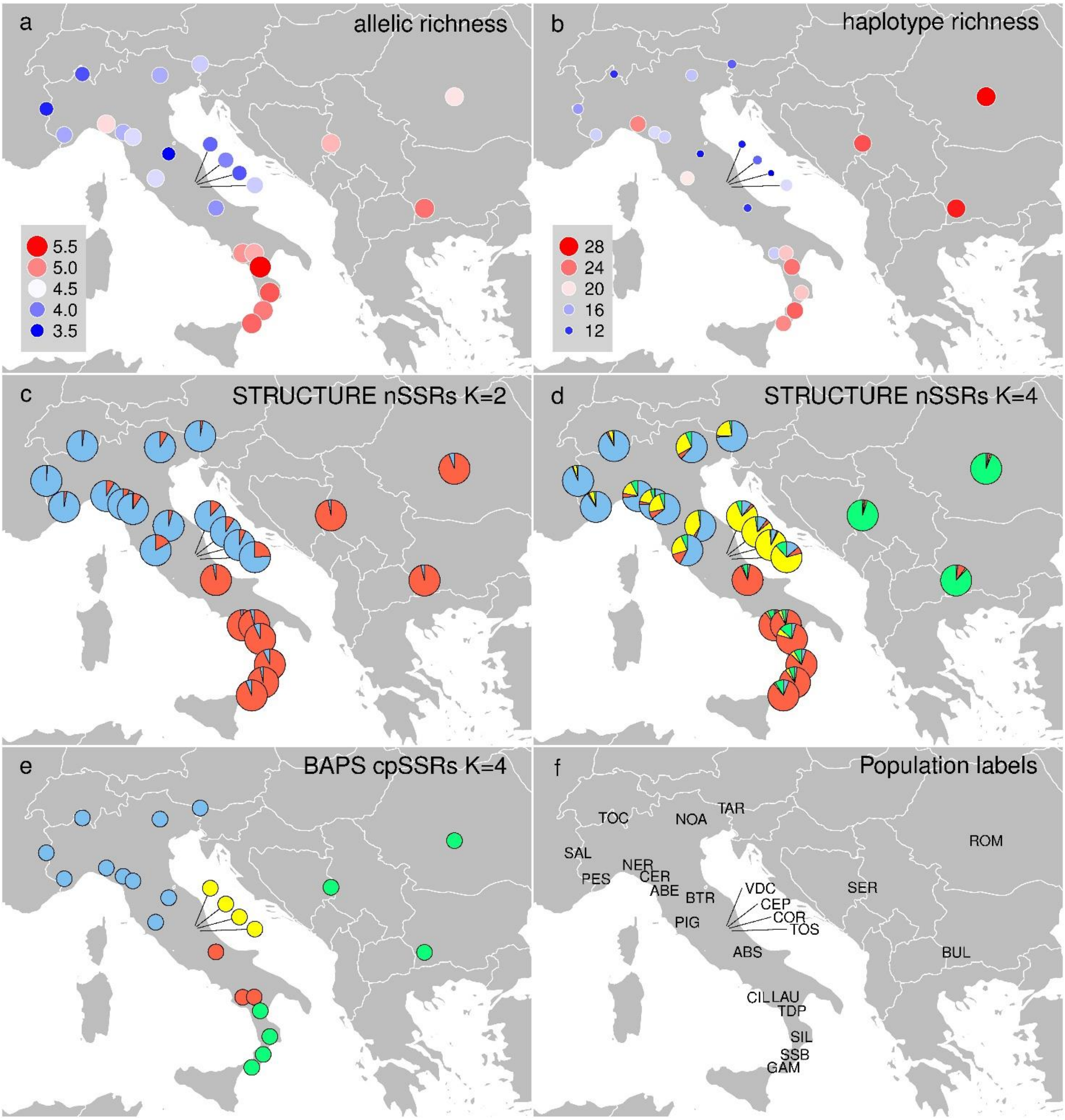

Scenario A
Na
N_{NAPP}
N_{CAPP}
N_{SAPP}
N_{EAST}
N_{GHOST}
t_4
t_5
t_1
0
Scenario B
t_4
t_3
t_2
ra
1-*ra*
t_1
0
NAPP
CAPP
SAPP
EAST

Figure 2


a
allelic richness
5.5
5.0
4.5
4.0
3.5
b
haplotype richness
28
24
20
16
12
c
STRUCTURE nSSRs K=2
d
STRUCTURE nSSRs K=4
e
BAPS cpSSRs K=4
f
Population labels
TOC
NOA
TAR
SAL
ROM
NER
CER
PES
ABE
BTR
VDC
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TOS
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Figure 3