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

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6

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

25

26 **ABSTRACT**

27

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

34

35 **Location** Mediterranean Basin; the Apennines and surrounding areas.

36

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

43

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

52 refugium during the Last Glacial Maximum.

53

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

59

60

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

64

65 INTRODUCTION

66

67 There is continuing debate about the location of glacial refugia. Such interest is justified by the
68 importance of correctly placing glacial refugia and recolonization routes when interpreting current
69 species' distributions, estimating species' migration potential and foreseeing possible range shifts
70 (Petit *et al.*, 2008). Coupling genetic and palaeobotanical data is considered the most effective
71 approach to infer past retraction-colonization dynamics in plants (Hu *et al.*, 2009). For a very small
72 number of tree species, genetic and palaeobotanical records covering both the distribution core and
73 edges are available, and well-grounded hypotheses on the topography of glacial refugia and post-
74 glacial recolonization routes have been developed. Inevitably, such inference on past demography has
75 also been tried for species with much less complete data available. In particular, biogeographic
76 literature highlights that northern Mediterranean refugial areas, such as the Italian and Balkan
77 Peninsulas, are generally underrepresented in genetic and palaeobotanical large-scale surveys on
78 forest trees (Hampe & Petit, 2005; Liepelt *et al.*, 2009; Linares, 2011). Trees, and plants in general,
79 have experienced complex dynamics due to environmental heterogeneity and palaeoclimatic events
80 in these areas, leading to high phylogeographic complexity and idiosyncratic patterns (Nieto Feliner,
81 2014). The increasing availability of molecular data and refined statistical approaches are providing
82 unprecedented power to unravel complex demographic histories, but to be effective these tools must
83 be applied to appropriate sampling of the focal species.

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

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

96 In the Apennines, the presence of *A. alba* during the late-glacial period has been demonstrated by
97 palynological surveys, mainly from the northern Apennines and the Tyrrhenian side of the central
98 Apennines (e.g. Follieri *et al.*, 1998; Vescovi *et al.*, 2010; Magri *et al.*, 2015). Previous genetic work,
99 based on a limited number of populations located mostly in Calabria and the northern Apennines,
100 inferred the presence of at least two genetic clusters (Konnert & Bergmann, 1995; Liepelt *et al.*,
101 2009). This led to the hypothesis of an isolated refugium in the southern Apennines and an effective
102 refugium, located either in the central (e.g. Konnert & Bergmann, 1995) or in the north-western
103 Apennines (e.g. Cheddadi *et al.*, 2014), from which the recolonization of the Alps and central Europe
104 may have started. In contrast, following early hypotheses about southern Italy as the starting point for
105 the recolonization of Central Europe, a possible genetic continuity along the entire Apennine chain
106 has been hypothesized several times (Scaltsoyiannes *et al.*, 1999; Linares, 2011; Camerano *et al.*,
107 2012). Some genetic studies even considered central Italy as a possible melting pot of recolonization
108 routes from the Balkans and southern Apennines (Parducci *et al.*, 1996) or from the northern and
109 southern Apennines (Larsen & Mekic, 1991). However, all of these previous studies have been based
110 on limited sampling and critical questions remain regarding the genetic relationship between
111 populations from the northern and southern Apennines and the origin of populations from the central
112 Apennines. Establishing the evolutionary history of these fragmented Apennine silver fir populations
113 is particularly urgent because they have high evolutionary and conservation value due to their unique
114 genetic and eco-physiological features (Hansen & Larsen, 2004; Carrer *et al.*, 2010; Cheddadi *et al.*,
115 2014; Brousseau *et al.*, 2016). An intensive genetic survey covering the entire Italian Peninsula will
116 allow robust testing of different phylogeographic hypotheses developed from palaeobotanical and

117 genetic data and resolution of these questions.

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

133

134 **MATERIALS AND METHODS**

135

136 **Sample collection and genotyping**

137

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

143 Eastern Europe to investigate the genetic relationship of Apennine populations with those from
144 surrounding areas. From each population, needle tissues were collected from c. 50 adult individuals
145 at least 20 metres apart in order to adequately cover a large area (c. 3 ha) within each stand.
146 All sampled individuals were genotyped at 16 unlinked nSSRs (Aag01, Aat01, Aat02, Aat03, Aat04,
147 Aat05, Aat06, Aat08, Aat09, Aat10, Aat11, Aat13, Aat14, Aat15 and Aat16, Postolache *et al.*, 2014;
148 NFF7, Hansen *et al.*, 2005) and three cpSSRs (Pt71936, Vendramin *et al.*, 1996; Pt30141 and
149 Pt30249, Liepelt *et al.*, 2001). The multiplexing and amplification procedures for nSSRs are reported
150 in Postolache *et al.* (2014). CpSSRs were multiplexed using the Type-it Microsatellite PCR kit
151 (Qiagen, Germany) with primer concentrations 0.1 μ M (Pt71936), 1 μ M (Pt30141) and 1.5 μ M
152 (Pt30249). PCR products were run on AB 3500 (Applied Biosystems, USA), with LIZ-500 as the
153 internal size standard. The resulting profiles were sized using GeneMarker (SoftGenetics).

154

155 **Genetic diversity and population structure**

156

157 Standard genetic parameters describing within-population genetic variation and genetic
158 differentiation were estimated by GENALEX (Peakall & Smouse, 2012) and HP-RARE (Kalinowski,
159 2005) for nSSRs, and CONTRIB (Petit *et al.*, 1998) for cpSSRs.

160 The presence of a genetic structure among sampled populations and the putative number of different
161 genetic clusters were evaluated using the model-based Bayesian clustering algorithm implemented in
162 STRUCTURE 2.3 (Pritchard *et al.*, 2000) and the empirical statistic ΔK (Evanno *et al.*, 2005; Earl &
163 von Holdt, 2012) on nSSR data. STRUCTURE was run using default settings and parameter values, and
164 varying K from one to 10. Each run consisted of 1×10^5 burn-in iterations and 5×10^5 data collection
165 iterations, and was replicated 10 times. After checking for convergence of diagnostic statistics,
166 different runs for the same K were averaged using the software CLUMPP (Jakobsson & Rosenberg,
167 2007). Bayesian analysis of population structure was run on the cpSSR dataset using BAPS (Corander
168 *et al.*, 2008) based on a non-spatial genetic mixture analysis for linked loci, with K varying from one

169 to 10. The best partition of populations into K clusters with the highest marginal log-likelihood after
170 10 replicates was chosen as the most representative one. Principal component analysis (PCA) was
171 also performed on the arcsine square root transformed population allele and haplotype frequencies in
172 R 3.2.4 (R Core Team, 2015) to evaluate the main features of the genetic structure obtained through
173 Bayesian clustering with an independent approach.

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

179

180 **Approximate Bayesian computation to infer demographic history**

181

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

190 The analysis of different demographic models (see Appendix S2) was performed following a two-
191 step approach. In the first step, we compared six scenarios in which, for simplicity, all populations
192 were assumed to have an identical prior distribution (Uniform distribution: 10-500000, Table S2.5)
193 for the effective population size (N_e). In the second step, the whole dataset was used to compare the
194 two scenarios that showed comparably high posterior probability in the previous analysis (Fig. 2). In

195 this final analysis, the prior distributions of effective population size were set according to results
196 from the first step analysis (Table S2.6). To underline this main difference with respect to scenarios
197 from the first step analysis, the two retained scenarios were then referred to as scenario A and B (Fig.
198 2).

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

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

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

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

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

219

220 **RESULTS**

221

222 **Genetic diversity and population structure**

223

224 The 16 nSSRs showed a total and mean number of alleles of 151 and 9.43 (from two alleles at Aat16
225 to 34 at NFF7), respectively. No evidence was found for the presence of null alleles or significant
226 genotypic disequilibrium among locus pairs. In addition, no evidence for selection was found by
227 outlier detection tests (FDIST and BAYESCAN, see Appendix S3 and Fig. S3.6), indicating that the
228 analyzed nSSRs were likely to be neutral, as suggested by Postolache *et al.* (2014). The allelic
229 richness (Ar_{84}) and expected heterozygosity (H_E) were above average in southern Italy, Eastern
230 Europe and the NER population in the northern Apennines (Fig. 3a, Table S1.1). Differentiation
231 indices showed a globally moderate differentiation among populations, with $F_{ST}=0.097$ and Hedrick's
232 $G'_{ST}=0.155$.

233 CpSSRs displayed 12 (Pt71936), 17 (Pt30141), and 6 (Pt30249) size variants, which combined into
234 164 haplotypes and a mean haplotypic diversity over populations of $h=0.935$. The haplotype richness
235 per population (Hr_{37}) ranged from 10 (COR) to 28 (ROM) (Fig. 3b, Table S1.1). Genetic
236 differentiation was in line with that found at nSSRs ($G_{ST}=0.045$).

237 STRUCTURE analysis revealed an optimal grouping at $K=2$ (Fig. S1.1) clearly distinguishing, along
238 the Apennines, populations located north and south of the Gran Sasso and Majella massifs (blue and
239 red, respectively, in Fig. 3c). Populations from the southern Apennines clustered with those from
240 Eastern Europe. AMOVA analysis showed that the proportion of total genetic variation explained by
241 differences between these two main genetic clusters was 7% ($P<0.001$). The next strongest level of
242 structuring was $K=4$, which grouped populations from the central Apennines separately from those
243 in the northern Apennines and the Alps, and populations from the southern Apennines were separated
244 from those in Eastern Europe (Fig. 3d). The main characteristics of the genetic structure emerging
245 from STRUCTURE analysis were confirmed by BAPS analysis on cpSSRs (Fig. 3e), PCA on both
246 marker types (Fig. S1.2), and pair-wise differentiation indices (Tables S1.2 and S1.3). In particular,

247 PCA analyses of both marker types showed a main separation resembling STRUCTURE results at $K=2$
 248 along the first principal component (i.e. southern Apennine and Eastern European populations
 249 differentiated from northern Apennine and Alpine populations), whereas the second principal
 250 component highlighted the differentiation between southern Apennine and Eastern European
 251 populations.

252

253 **Approximate Bayesian computation to infer demographic history**

254

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

262 Under scenario B, SAPP originated at t_2 by an admixture event between EAST and a ghost population.
 263 The ghost population merged with NAPP at t_3 and gave the largest contribution ($ra=0.780$) to the
 264 formation of SAPP. The median values of the effective population sizes were 41800, 15500, 97900,
 265 93500, 51300, and 2870 for N_{NAPP} , N_{CAPP} , N_{SAPP} , N_{EAST} , N_{GHOST} , and N_a , respectively (Table 2 and
 266 Fig. S1.4). The posterior parameters showed that the effective population size of the ancestral
 267 population was estimated to be 14.5 and 32.5 times lower than those of NAPP and EAST,
 268 respectively, suggesting an expansion event at t_4 . The results also indicated that the demographic
 269 expansion continued in the following events, which led to the formation of SAPP at t_2 . More recently,
 270 a bottleneck is likely to have given rise to the formation of CAPP at t_1 .

271 The median values of the divergence times t_1 (for CAPP and NAPP), t_2 (for the appearance of SAPP
 272 from the admixture between EAST and a ghost population), t_3 (for the ghost population and NAPP),

273 and t_4 (for EAST and NAPP) were 1320 (95%CI: 300-3110), 4750 (95%CI: 1800-6850), 7790
274 (95%CI: 4230-9860), 12000 (95%CI: 6950-14800) generations ago, respectively (Table 2 and Fig.
275 S1.4). Assuming a generation time of 50 years (Liepelt *et al.*, 2002; Dering *et al.*, 2014; Ruosch *et*
276 *al.*, 2016), these values can be translated into 66000 (95% CI: 15500-155500), 237500 (95% CI:
277 90000-342500), 389500 (95% CI: 211500-493000), 600000 yrs BP (95% CI: 347500-740000) for t_1 ,
278 t_2 , t_3 , and t_4 respectively.

279

280 **DISCUSSION**

281

282 **Spatial distribution of genetic diversity**

283

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

287 The first area is located in the southern Apennines (Fig. 1), from the southernmost population (GAM)
288 to the latitude of CIL and LAU, with the highest haplotype and allelic richness recorded for the TDP
289 population. The only Apennine silver fir populations regularly included in genetic surveys at the
290 biogeographical scale were those from Calabria, which often showed higher genetic variation than
291 northern ones (Bergmann *et al.*, 1990; Vicario *et al.*, 1995; Liepelt *et al.*, 2002; Longauer *et al.*, 2003;
292 Liepelt *et al.*, 2009). Such high diversity, together with the high vitality and growth vigour seen in
293 provenance trials (e.g. Larsen & Mekic 1991; Kerr *et al.*, 2015), has led many authors to consider
294 Calabrian populations as part of a long-lasting but isolated refugial area (Liepelt *et al.*, 2009;
295 Cheddadi *et al.*, 2014). Our results strongly support the hypothesis of an isolated refugium in the
296 southern Apennines and indicate that the upper latitudinal limit of this refugial area was at $\sim 40.5^\circ$ N,
297 i.e. the latitude of CIL and LAU. These two populations have retained high genetic diversity during
298 the postglacial period despite erosion to their current small, highly-isolated state. In particular, CIL is

299 composed of few hundred individuals and located ~40 km from the nearest stand (Rovelli, 1995; Di
300 Pietro & Fascetti, 2005). Nevertheless, CIL still maintains levels of genetic diversity comparable to
301 much larger stands (e.g. TDP, the largest stand in southern Italy; Schettino & Travaglio, 2015).

302 The second area is located in northern Apennines, a region for which several palaeobotanical surveys
303 are available but whose silver fir populations have rarely been included in large-scale genetic studies.
304 Among the three populations sampled, it is surprising that NER is the only one showing high genetic
305 diversity because it is the northernmost population along the Apennines, and highly isolated and
306 extremely small (c. 500 individuals, Piovani *et al.*, 2010). From NER to the central Apennines, genetic
307 diversity gradually decreases to populations north of the Gran Sasso massif which, along with Alpine
308 populations, are the least genetically diverse in our survey. NER has an allelic and haplotype richness
309 16% and 54% higher, on average, than much larger and less isolated Alpine populations. This suggests
310 that the northernmost part of the Apennine distribution is the most likely refugial area or, at least, the
311 area where silver fir was most abundant during the Last Glacial Maximum (LGM): a hypothesis that
312 fits well with palaeobotanical data (Vescovi *et al.*, 2010; Magri *et al.*, 2015). Besides its conservation
313 relevance, the correct location of refugial populations can help solve the long-lasting problem of
314 accurately calculating historical migration rates (Cheddadi *et al.*, 2014). This might be particularly
315 important for silver fir, whose northern Apennine effective refugium has often been hypothesised in
316 different locations, from the north-western (e.g. Cheddadi *et al.*, 2014) to central Apennines (e.g.
317 Konnert & Bergmann, 1995). The high genetic similarity between populations from the northern
318 Apennines and eastern Alps, with the former being much more diverse despite the much lower census
319 size, seems to confirm the hypothesised post-glacial migration route from the northern Apennines to
320 central Europe (e.g. Konnert & Bergmann 1995; Liepelt *et al.*, 2009; Cheddadi *et al.*, 2014).

321 A third area might also have acted as a refugium in the Italian Peninsula. Bayesian clustering results,
322 genetic differentiation from surrounding populations, and DIYABC estimate that CAPP diverged from
323 NAPP ~66,000 yrs BP, all point towards the local persistence of silver fir during the last glacial period
324 in the central Apennines. The existence of a glacial refugium in this area has been postulated several

325 times, but always as an alternative to the northern Apennine one (e.g. Konnert & Bergmann, 1995).
326 On the contrary, our analyses suggest the existence of two separate genetic clusters during the LGM
327 north of the Gran Sasso massif, one in the northern and one in the central Apennines. The latter was
328 characterized by a lower effective population size than other Apennine refugia, in accordance with
329 palaeobotanical records showing a marked demographic reduction of silver fir since 70,000 yrs BP
330 in central Italy (Follieri *et al.*, 1998). Although northern and central Apennine populations are
331 generally small and fragmented, and considered as highly impacted by human intervention in the last
332 millennia (Piovani *et al.*, 2010; Urbinati & Romano, 2012; Tinner *et al.* 2013; Leonarduzzi *et al.*,
333 2016), no clear signals of translocation and/or mixing of different provenances were found, contrary
334 to what was observed in other European conifers (e.g. Wagner *et al.*, 2015), and the spatial distribution
335 of genetic diversity seems mainly related to events dating to before the Neolithic age.

336

337 **Genetic structure and demographic history**

338

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

343 In the central Apennines, the four populations within the Gran Sasso e Monti della Laga National
344 Park (VDC, CEP, COR and TOS) and ABS are separated by only 90 km, an area comprising the Gran
345 Sasso and Majella massifs where no natural populations of silver fir are present. Despite their
346 geographic proximity, they showed pair-wise genetic differentiation values among the highest
347 detected ($G'_{ST} = 0.22, 0.21, 0.25$ and 0.15 , respectively, Table S1.3). According to DIYABC estimates,
348 the Apennine populations from the two main gene pools detected by STRUCTURE analysis have had
349 separate dynamics during the last 400,000 yrs. Therefore, hypotheses based on a recent genetic
350 continuity along the entire Apennine chain and about the central Apennines as a melting pot between

351 different recolonization routes (Larsen & Mekic, 1991; Scaltsoyiannes *et al.*, 1999; Linares, 2011;
352 Camerano *et al.*, 2012) seem highly unlikely. The processes underlying the origin and persistence of
353 a long-lasting genetic boundary in central Italy are not clear and any hypotheses will require a
354 multidisciplinary approach considering a longer period, from the Neogene Apennine orogeny to
355 climatic dynamics during several Pleistocene glacial cycles. However, it is worth noting that this area
356 represents a steep discontinuity at different time scales. For example, it is considered as the tectonic
357 separation between the northern Apennines Arc and the southern Apennines-Calabrian Arc (Satolli &
358 Calamita, 2008), and an ecotone between different precipitation patterns that have had a large, long-
359 lasting influence on vegetation dynamics in the Italian Peninsula (Comborieu-Nebout *et al.*, 2015).
360 Populations along the rear edge of silver fir distribution are indeed separated by neat genetic
361 boundaries, as previously hypothesized, but the present study demonstrates their location is not where
362 they were previously thought. In particular, we found a genetic similarity between populations from
363 Eastern Europe and southern Italy that are almost fixed for different mitotypes (Liepelt *et al.*, 2002).
364 A genetic similarity at isozymes and chloroplast markers between these two areas was reported by
365 Liepelt *et al.* (2002) and Longauer *et al.* (2003). The former hypothesized a possibly extensive trans-
366 Adriatic gene flow via pollen homogenizing chloroplast haplotype frequencies over long distance.
367 Our data support this hypothesis as the most likely scenario, considering that SAPP was generated by
368 the admixture of EAST and a population originating from NAPP. These ancient gene pools would
369 have diverged long before the last glacial period, corroborating the hypothesis of a separation lasting
370 for several Quaternary glacial cycles (Liepelt *et al.*, 2009). The median admixture rate estimated (78%
371 from NAPP vs. 22% from EAST) is compatible with an introgression via-pollen from the Balkan into
372 the northern Apennine gene pool having shaped, together with the isolation between populations at
373 the two extremes of the Apennine chain, the genetic layout of southern Apennine populations between
374 340,000 and 90,000 yrs BP. Geological studies demonstrated that the Apulian platform connected
375 several times to the Balkans by a trans-Adriatic land bridge (Patacca *et al.*, 2008), and the presence
376 of shared haplotypes in the Balkan and Italian Peninsulas for other tree species (e.g. Bagnoli *et al.*,

377 2016) supports the idea of effective gene flow being not rare across what is nowadays considered as
378 a geographic barrier.

379 The scenario depicted by our intensive genetic survey of Apennine populations provides new insights
380 into the Quaternary history of silver fir. Among them, two seem particularly relevant for studying
381 adaptive responses of rear edge silver fir populations. First, a solid knowledge about past
382 demographic patterns can improve the study of local adaptation. So far, it has been based on
383 considering a main separation between Alpine and Apennine populations in silver fir (Mosca *et al.*,
384 2014), which seems quite unlikely from our results. Incorrectly considering demographic scenarios
385 can hinder the study of local adaptation by introducing biases in selection tests (Nielsen, 2005). In
386 addition, silver fir tree-ring growth series in Italy show three differentiated groups of populations: 1)
387 the western Alps, 2) the northern Apennines and the eastern Alps, and 3) the central and southern
388 Apennines (Carrer *et al.*, 2010). This subdivision partially corresponds to the genetic structure found
389 in our survey, with some exceptions. In fact, central Apennine populations are genetically similar to
390 northern populations but show growth responses comparable to the ones from southern populations,
391 raising several questions about the relative strength of past migration dynamics *vs.* adaptation to
392 climate in highly heterogeneous regions.

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

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

416

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418

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434

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597

598 **Supporting Information**

599

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

601 **Appendix S1** Supplementary tables and figures

602 **Appendix S2** DIYABC materials and methods details

603 **Appendix S3** Selection tests

604

605 **DATA ACCESSIBILITY**

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

608

609 **BIOSKETCH**

610

611 The authors belong to a research consortium dealing with population genetics of forest tree species.

612 Their research is particularly focused on the analysis of the distribution of extant genetic variation of

613 forest tree species using genetic markers to dissect the role of demography and selection in shaping

614 genetic diversity at different temporal and spatial scales. They have been involved in projects studying

615 the range-wide phylogeography of several forest tree species in Europe and the Mediterranean Basin.

616

617 Editor: Lyn Cook

618

619 Author contributions: A.P., C.L. and G.G.V. designed the study; A.P., C.L., D.P., C.U. and S.L.
620 organized and performed the sampling; C.L., I.S., D.P. and G.G.V. arranged the molecular laboratory
621 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
622 contributions from all authors, who revised and approved the manuscript.

623

624 **TABLES**

625

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

632

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

633

634

635

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

638

Parameter	Mean	Median	Mode	Quantiles			
				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
N_a	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}
μ_{mic}	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}

639

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

649

650 **FIGURE LEGENDS**

651

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

654

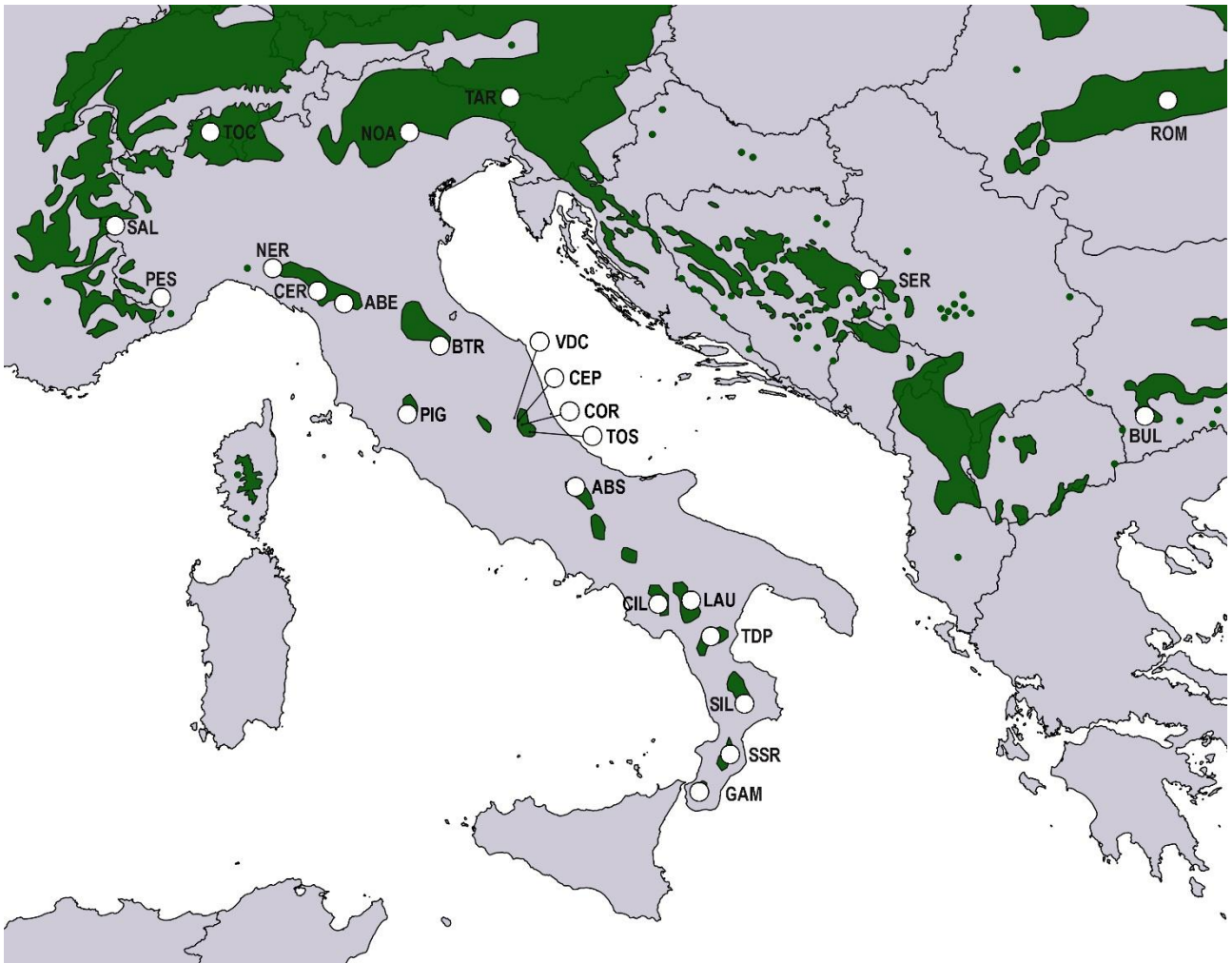
655 Fig. 2 Demographic models tested in the second step of DIYABC analysis. In both scenarios, $t_{\#}$
656 represents the time of occurrence of an event (expressed in number of generations) and $N_{\#}$ is the
657 effective population size of the corresponding populations during each time period (see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

660

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

663

664



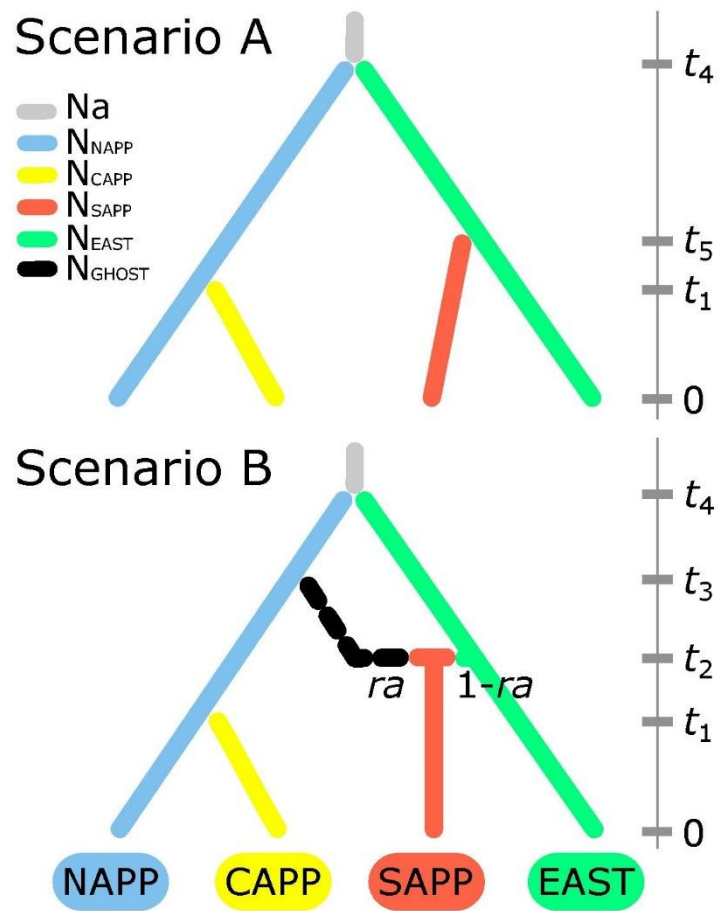
665

666 Figure 1

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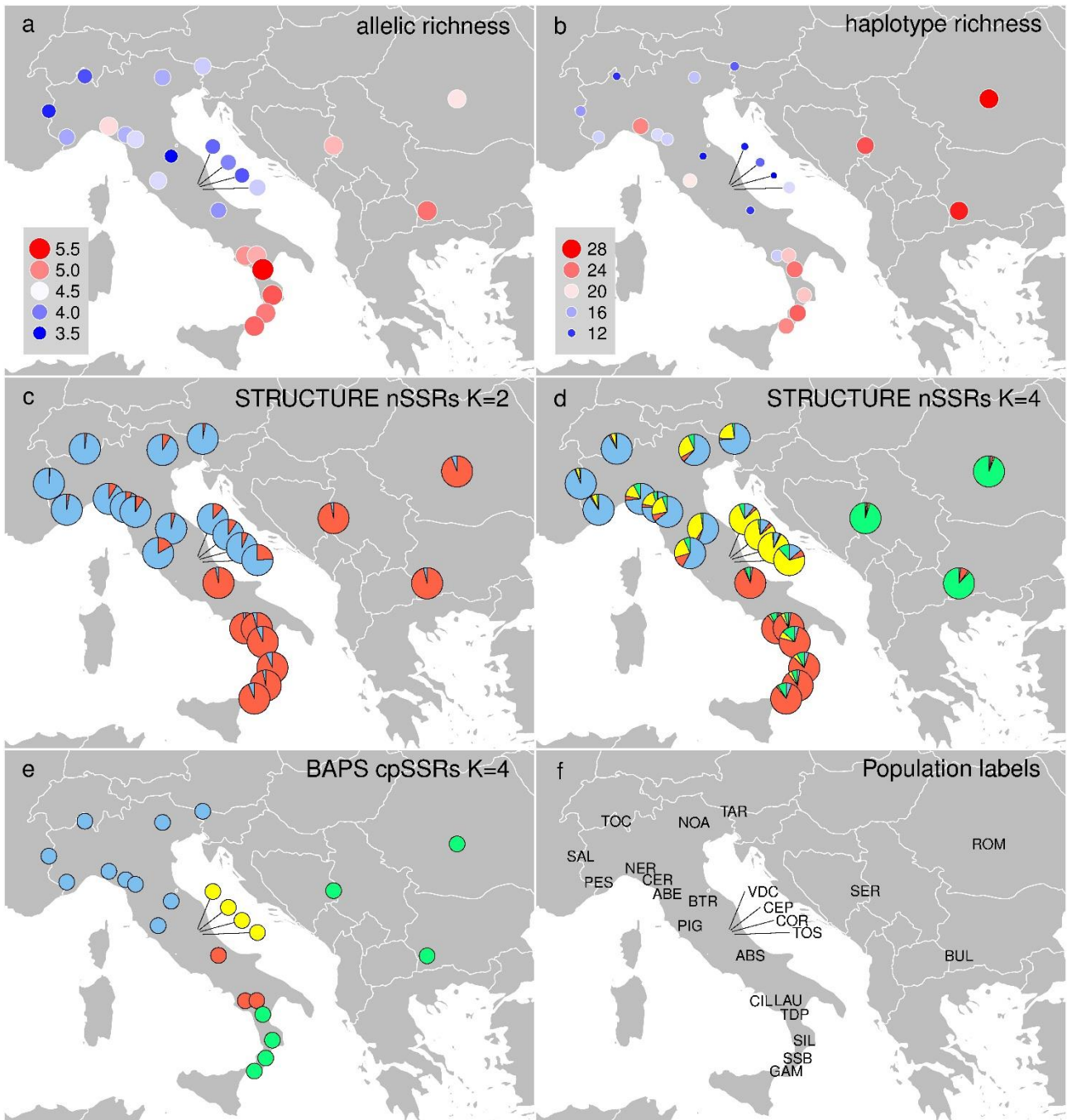


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671 Figure 2

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674

675 Figure 3