



**HAL**  
open science

## Evolutionary rate and genetic load in an emblematic Mediterranean tree following an ancient and prolonged population collapse

Juan Jaramillo-correa, Francesca Bagnoli, Delphine Grivet, Bruno Fady, Filippos Aravanopoulos, Giovanni Vendramin, Santiago C. Gonzalez-Martinez

### ► To cite this version:

Juan Jaramillo-correa, Francesca Bagnoli, Delphine Grivet, Bruno Fady, Filippos Aravanopoulos, et al.. Evolutionary rate and genetic load in an emblematic Mediterranean tree following an ancient and prolonged population collapse. *Molecular Ecology*, 2020, 29, pp.4797-4811. 10.1111/mec.15684 . hal-03025269

**HAL Id: hal-03025269**

**<https://hal.inrae.fr/hal-03025269>**

Submitted on 21 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



23 **Abstract**

24 Severe bottlenecks significantly diminish the amount of genetic diversity and the speed  
25 at which it accumulates (i.e. evolutionary rate). They further compromise the efficiency  
26 of natural selection to eliminate deleterious variants, which may reach fixation in the  
27 surviving populations. Consequently, expanding and adapting to new environments may  
28 pose a significant challenge when strong bottlenecks result in genetic pauperization.  
29 Herein, we surveyed the patterns of nucleotide diversity, molecular adaptation and  
30 genetic load across 177 gene-loci in a circum-Mediterranean conifer (*Pinus pinea* L.) that  
31 represents one of the most extreme cases of genetic pauperization in widespread  
32 outbreeding taxa. We found very little genetic variation in both hypervariable nuclear  
33 microsatellites (SSRs) and gene-loci, which translated into genetic diversity estimates  
34 one order of magnitude lower than those previously reported for pines. Such values  
35 were consistent with a strong population decline that began some ~1Ma. Comparisons  
36 with the related and parapatric maritime pine (*Pinus pinaster* Ait.) revealed reduced  
37 rates of adaptive evolution ( $\alpha$  and  $\omega_a$ ) and a significant accumulation of genetic load. It is  
38 unlikely that these are the result from differences in mutation rate or linkage  
39 disequilibrium between the two species; instead they are the presumable outcome of  
40 contrasting demographic histories affecting both the speed at which these taxa  
41 accumulate genetic diversity, and the global efficacy of selection. Future studies, and  
42 programs for conservation and management, should thus start testing for the effects of  
43 genetic load on fitness, and integrating such effects into predictive models.

44

45 **Introduction**

46 Evolutionary rate, the speed at which genetic diversity accumulates within a species'  
47 genome, depends on both the rate at which mutations appear, and the likelihood that  
48 they reach fixation (Charlesworth et al. 1995; Eyre-Walker and Keightley 2007;  
49 Charlesworth 2009). This likelihood is conditioned by each mutation's effect on fitness,  
50 and stochastic processes that may eliminate them. Consequently, evolution (and  
51 adaptation) indirectly relies on species' biological features like population size (or its  
52 change over time), migration rate and mating system, which condition both the efficacy  
53 of selection and the extent of genetic drift (e.g. Charlesworth 2009; Chen et al. 2017). For  
54 instance, deleterious mutations accumulate at very low frequencies in large populations  
55 (often in heterozygous states), while they can more easily reach fixation in smaller  
56 populations, because of a diminished efficiency of purifying selection (Charlesworth et  
57 al. 1993; Eyre-Walker and Keightley 2007). Similarly, advantageous variants may  
58 increase in frequency at faster rates in large populations or under elevated gene flow  
59 regimes, than in smaller or isolated stands, where they can disappear through the effect  
60 of drift (Charlesworth and Eyre-Walker 2007; Eyre-Walker and Keightley 2009).

61 The consequences of drastic population size reductions on the efficacy of selection  
62 and the accumulation of genetic load (the reduction of mean fitness in a population  
63 caused by deleterious variation relative to a mutation-free population) are thus  
64 fundamental aspects of population genetics (e.g. Kimura et al. 1968; Ohta 1992; Gillespie  
65 2001). They are also of high interest in current conservation, domestication,  
66 improvement and clinical genetics projects (e.g. Marsden et al. 2016; Robinson et al.  
67 2016; Pedersen et al. 2017). Theory predicts that neutral variation accumulates  
68 proportionally to mutation rate, and independently of population size (Lanfear et al.  
69 2014). As a result, the (neutral) evolutionary rate of population/species should not be

70 affected after a bottleneck if mutation rate remains unchanged. However, a negative  
71 relationship is expected between the accumulation of deleterious mutations and  
72 population size, with larger populations/species purging more efficiently  
73 disadvantageous variants than smaller ones (e.g. Chen et al. 2017). Consequently,  
74 comparing how different sorts of mutations (neutral vs. putatively deleterious)  
75 accumulate in populations/species with contrasting demography may be used as a  
76 proxy for inferring adaptive evolutionary rates (e.g. Böndel et al. 2015; González-  
77 Martínez et al. 2017; Grivet et al. 2017).

78 Some studies (e.g. Balloux and Lehmann 2012; Wei et al. 2015) have nevertheless  
79 suggested that mutation rate can also be affected by demographic fluctuations,  
80 particularly when generations overlap, as in most long-lived taxa, including mammals  
81 and forest trees. While such a possibility may flaw the comparisons above, other authors  
82 have claimed that it is the generation time that is affected by demographic changes,  
83 rather than mutation rate itself (Lanfear et al. 2014). A recent empirical survey on two  
84 pines with contrasting demography, and similar generation times and life-history traits,  
85 (Grivet et al. 2017) pointed in that direction, as it reports no differences in the rate of  
86 evolution between these taxa.

87 Forest trees are reputed for their resilience to stochastic processes, particularly those  
88 derived from population size changes (Petit and Hampe 2006). They have long  
89 generation times, extensive gene flow and large reproductive output over the years,  
90 which, combined with strong selective pressures at early life stages, reduce the impact  
91 of inbreeding depression and maintains (ancestral) genetic diversity (e.g. Ledig et al.  
92 2000). On the other hand, such features also result in a lowered accumulation of  
93 mutations per unit of time, which suggests a slow recovering of molecular genetic  
94 variation after a strong and prolonged bottleneck (Lanfear et al. 2014; Jaramillo-Correa

95 et al. 2015a). Interestingly, these features can also make them more impermeable to the  
96 accumulation of genetic load and favour the spread of new advantageous mutations  
97 (Petit and Hampe 2006; Lanfear et al. 2014).

98 Stone pine (*Pinus pinea* L.) is an emblematic Mediterranean tree that has been  
99 associated to humans for thousands of years. For instance, fatty acids related to stone  
100 pine nuts have been identified in calculus from hominid teeth remnants more than 300  
101 ka old (Hardy et al. 2015), and charcoal fragments assigned to stone pine have been  
102 reported in several human settlements dating back to the last ~50 ka (Prada et al. 1997;  
103 Badal 1998; Carrión et al. 2008). Archaeological evidence indicates that this species has  
104 been cultivated for over 6,000 years (Prada et al. 1997; Pérez-Jordà et al. 2017), and its  
105 nuts have been traded since the Phoenician civilization (i.e. 3,500 BP; Wilcox 1977;  
106 Popova and Hristova 2017). Stone pine is thus considered an archaeophyte (i.e. a species  
107 distinguished by unrecorded early man introductions) currently distributed around the  
108 whole Mediterranean basin, where it forms small populations growing on different  
109 types of soil, from the sea level up to 1,000 meters (Richardson 1998). Despite this  
110 distribution, stone pine is one of the most genetically depauperate outbreeding plants in  
111 the world, with virtually no genetic variation at allozyme or chloroplast microsatellite  
112 loci (Fallour et al. 1997; Vendramin et al. 2008). These features make stone pine an ideal  
113 model for testing hypotheses related to evolutionary rate changes and the accumulation  
114 of genetic load after strong and prolonged bottlenecks.

115 Herein, we collected nuclear microsatellite and DNA sequence data to estimate more  
116 accurately the genomic diversity of stone pine and infer its demographic history. For  
117 comparison, we used data available for the parapatric sister taxon maritime pine (*Pinus*  
118 *pinaster* Ait.), which inhabits similar environments but has much higher amounts of  
119 genetic diversity (Bucci et al. 2007; Jaramillo-Correa et al. 2015b; Grivet et al. 2017). We

120 then determined the accumulation of putatively neutral and deleterious genetic  
121 variation since its divergence from maritime pine, by using the distantly related (and  
122 native to North America) loblolly pine (*Pinus taeda* L.) as outgroup. We inferred various  
123 evolutionary parameters by taking into account both the shared and exclusive portion of  
124 genetic variation to each taxon. We hypothesized that the low effective population size  
125 of stone pine resulted in a diminished evolutionary rate and in higher genetic load when  
126 compared to maritime pine. However, such values should be different from those  
127 expected under a simple bottleneck model with no selection. That is, we expected that  
128 purifying selection have managed to purge most of the deleterious part of the genetic  
129 load and allowed for the retention of some advantageous variants in stone pine (i.e. the  
130 number of fixed adaptive mutations should be different from zero).

131

## 132 **Materials and Methods**

### 133 *Sampling, genotyping and sequencing*

134 Two datasets were used to infer demographic and population genetic patterns in stone  
135 pine. First, nuclear microsatellite (nuSSR) data were obtained for 735 individuals from  
136 33 populations distributed along the species range (Fig. 1A; Table S1). Sample size per  
137 population varied between 16 and 30 individuals; all of which were at least 50 m apart.  
138 DNA was extracted from foliage using DNeasy Plant Mini Kits (QIAGEN, Valencia, CA,  
139 USA) and amplified using stone pine specific primers for 13 nuSSRs. Primer sequences  
140 and PCR conditions are described in Pinzauti et al. (2012). PCR amplification was carried  
141 out in a GeneAmp PCR 9700 thermal-cycler (Applied Biosystems, Foster City, California,  
142 USA), and PCR products were separated in an ABI 3130xl automatic sequencer (Applied  
143 Biosystems). Electrophoregrams were scored using GeneMapper version 4.0 (Applied  
144 Biosystems).

145 Second, DNA sequences for 264 gene loci originally identified on loblolly pine were  
146 obtained for 12 individuals from as many populations dispersed across the full species'  
147 range (Fig. 1A; Table S1). Sanger resequencing was performed on haploid seed  
148 megagametophytes, which allows for direct phase inference and identification of co-  
149 amplified gene paralogs. DNA sequences were visually checked and manually edited  
150 with SEQUENCHER 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). Low-quality  
151 sequences and those exhibiting double peaks (indicative of paralog co-amplification)  
152 were removed, which resulted in a final dataset of 177 gene loci scattered over most  
153 pine chromosomes (see Westbrook et al. 2015; Plomion et al. 2016), and covering up to  
154 56 Kbp. The retained sequences were aligned with their maritime pine and loblolly pine  
155 orthologs, and annotated from homology with EST contigs from the last species and the  
156 NCBI reference protein database using GENEIOUS version 6.1 (Biomatters, Auckland, New  
157 Zealand).

158

#### 159 *Genetic diversity and population structure*

160 For nuSSR data, standard genetic diversity statistics were estimated for each population  
161 using GENALEX version 6.503+ (Peakall and Smouse 2006). These included the mean ( $A$ )  
162 and effective ( $A_e$ ) number of alleles, the number of private alleles, and the observed ( $H_0$ )  
163 and expected ( $H_E$ ) heterozygosity, from which  $F$ -statistics were computed with FSTAT  
164 (Goudet 2002). Deviations from Hardy-Weinberg (H-W) expectations and linkage  
165 disequilibrium between loci were also evaluated with GENALEX. Only one locus  
166 ( $pEST2669$ ) showed deviations from H-W in over 70% of the populations and was  
167 removed from all analyses. No linkage between pairs of loci was systematically  
168 observed. Population clustering and differentiation were evaluated using a principal  
169 coordinates analysis (PCoA, performed with GENALEX; Peakall and Smouse 2006), and

170 with Bayesian software STRUCTURE (Pritchard et al. 2000). For the latter analysis, ten  
171 runs of 1,000,000 iterations of an admixture model with correlated allele frequencies  
172 and no priors on population locations were performed for  $K$ -values ranging from 1 to 13,  
173 after a burn-in period of 500,000 steps. Convergence among runs with the same  $K$ -value  
174 was verified using CLUMPP (Jakobsson and Rosenberg 2007), and the most plausible  
175 number of clusters was determined in STRUCTURE HARVESTER (Earl and von Holdt 2011)  
176 following Evanno et al. (2005) and Janes et al. (2017).

177 For the DNA sequence dataset, standard diversity estimates, such as the number of  
178 segregating sites ( $S$ ) and haplotypes ( $h$ ),  $\pi$  (Nei and Li 1979) and Watterson's  $\theta_w$   
179 (Watterson 1975) were calculated using DnaSP v.6 (Rozas et al. 2017) for all sites, and  
180 separately for synonymous and non-synonymous sites. In addition, the pairwise  
181 divergence ( $D_{xy}$ ) from loblolly pine was computed together with the number of  
182 synonymous substitutions per synonymous site ( $K_s$ ) and of non-synonymous  
183 substitutions per non-synonymous site ( $K_a$ ), both using the Jukes-Cantor correction. To  
184 ease some of the demographic and genetic-load inferences below, the two last  
185 parameters ( $K_s$  and  $K_a$ ) were also determined between maritime and loblolly pines.  
186 Sequence alignments were also used to separate divergence counts into those that were  
187 exclusive to each maritime and stone pines (i.e. that arose after their divergence), and  
188 those that were shared between these two taxa (i.e. that appeared before their split but  
189 after the separation from their common ancestor with loblolly pine).

#### 190 191 *Phylogeny and demographic inferences*

192 The phylogenetic relationships and divergence times between stone, maritime and  
193 loblolly pines were co-inferred from a random subset of 50 gene-sequence alignments.  
194 This subset was selected to reduce computing time and facilitate convergence among

195 partitions and runs. First, the best-fit gene partition, with the corresponding molecular  
196 evolution models, was selected based on a Bayesian Information Criterion with  
197 PARTITIONFINDER 1.1 (Lanfear et al. 2012) using linked branch lengths and a greedy  
198 algorithm. Then, phylogenies were inferred using the Bayesian framework implemented  
199 in BEAST v1.7 (Drummond et al. 2013) by assuming a *log*-normal relaxed molecular clock  
200 and specifying *log*-normal-distributed priors for the parameters, and a Calibrated Yule  
201 model for the tree. Following previous phylogenetic work on pines (e.g. Gernandt et al.  
202 2005; Saladin et al. 2017), the divergence between North American loblolly pine  
203 (subsect. *Australes*) and the two Mediterranean pines (stone and maritime pines; subsect.  
204 *Pinaster*) was calibrated using a *log*-normal distribution centred at 35 million years ago  
205 (Ma) and a 95% probability interval of  $\pm 10$  Ma; such a calibration covers the subgenus  
206 *Pinus* split, which was dated back to 45Ma based on *P. bailey* fossils (Saladin et al. 2017).  
207 Analyses consisted of ten runs of 100 million iterations each; samples were collected  
208 every 1,000 steps after removing the first 25% of trees as burn-in. Chain mixing,  
209 likelihood stability, and convergence across runs were surveyed using TRACER v1.6  
210 (Drummond et al. 2012). Subsampled trees were finally summarized by Maximum Clade  
211 Credibility with common ancestor heights as node heights on TREEANNOTATOR 1.8.0  
212 (Drummond et al. 2012).

213 The demographic history of stone pine was initially surveyed using STAIRWAYPLOT (Liu  
214 and Fu 2015), an approach that uses the site frequency spectrum (SFS) to fit a  
215 demographic model consisting of population size changes over various periods of time.  
216 This analysis was performed from the unfolded SFS derived from all DNA sequences  
217 (55,833 sites) and using the median mutation rate inferred on the phylogenetic analyses  
218 above (i.e.  $4.18 \times 10^{-8}$  per site per generation); as for other pines (Brown et al. 2004;  
219 Willyard et al. 2006), a generation time of 25-40 years was assumed. Median values and

220 confidence intervals for population size estimates were derived from 200 replicates of  
221 the input file using the built-in bootstrap function. Stairway-plots were generated for the  
222 whole sample and for each genetic cluster separately (see below).

223 In addition, and to gain a more detailed insight of the most recent demographic  
224 history of stone pine, we tested several potential evolutionary scenarios based on  
225 present-day population structure (involving two differentiated genetic clusters for  
226 eastern and western Mediterranean populations, see Results), using the approximate  
227 Bayesian procedure implemented in DIYABC (Cornuet et al. 2010).

228 The tested scenarios were as follows (see also Fig. S1, provided as Supplementary  
229 Information):

230 *Scenario 1 (null hypothesis)*: no population genetic structure and no population size  
231 changes;

232 *Scenario 2 (ancient bottleneck)*: no population genetic structure and ancient and  
233 severe bottleneck;

234 *Scenario 3 (ancient split + bottleneck 1)*: ancient divergence followed by collapse of  
235 both eastern and western Mediterranean genetic clusters;

236 *Scenario 4 (ancient split + bottleneck 2)*: ancient divergence followed by collapse of  
237 the eastern genetic cluster;

238 *Scenario 5 (ancient bottleneck + split)*: ancient and severe bottleneck predating the  
239 divergence of the two genetic clusters;

240 *Scenario 6 (ancient bottleneck + split + recent bottleneck)*: identical to *Scenario 5* but  
241 including a collapse for the eastern genetic cluster after divergence.

242 After fine-tuning prior parameters (all of which had uniform distributions, except for  
243 mutation rate, which had a *log*-uniform distribution), a reference table with one million  
244 simulated datasets per scenario was built. Then, the 1% of these datasets that were the

245 closest to the observed data were used to infer the posterior probability of each  
246 scenario. Goodness-of-fit was assessed for each scenario by model checking using the  
247 Principal Component Analysis (PCA) implemented in DIYABC, which measures the  
248 discrepancy between simulated and real data. Confidence in scenario choice (i.e. Type I  
249 and II errors) was estimated by simulating 500 datasets under each scenario to  
250 determine the probability of not choosing each scenario when it was the true one, or to  
251 choosing it when it was not the true scenario. The most likely scenario was finally  
252 employed to estimate demographic parameters for the two genetic clusters: time in  
253 number of generations, and effective sizes for the current and ancestral populations (see  
254 Figs. 2 and S1).

255

#### 256 *Adaptive evolution and accumulation of genetic load*

257 The proportion of nonsynonymous substitutions fixed by adaptive evolution ( $\alpha$ ) and the  
258 rate of adaptive substitutions scaled by the rate of neutral substitutions ( $\omega_a$ ) were  
259 inferred from DNA sequence data using methods available in DOFE 4.0  
260 ([http://www.sussex.ac.uk/lifesci/eyre-walkerlab/documents/dofe-31-for-](http://www.sussex.ac.uk/lifesci/eyre-walkerlab/documents/dofe-31-for-windows.zip)  
261 [windows.zip](http://www.sussex.ac.uk/lifesci/eyre-walkerlab/documents/dofe-31-for-windows.zip)). To ease the results interpretation, statistics were estimated and  
262 compared between stone and maritime pines by using loblolly pine as an outgroup; only  
263 the non-shared part of the divergence (i.e. the fixed exclusive differences for each taxon  
264 determined above) from loblolly pine was included in these calculations. Briefly,  $\alpha$  was  
265 estimated based on Smith and Eyre-Walker (2002), and  $\omega_a$  was determined using the  
266 non-parametric method of Gossman et al. (2010) and method II of Eyre-Walker and  
267 Keightley (2009). This last statistic incorporates putative demographic changes that  
268 may affect the patterns of nucleotide polymorphism by comparing the observed SFS at  
269 neutral sites, with the one expected in a stationary population at equilibrium. This

270 method was also used to infer the distribution of fitness effects (DFE) for 0-fold  
271 degenerate mutations exclusive to each species. For stone pine, DFE was estimated for  
272 the whole species and for each genetic cluster, and summarized in three bins of  
273 increasing purifying selection ( $0 < N_e s < 1$ ;  $1 < N_e s < 10$ ;  $N_e s > 10$ ).

274 In addition, the relative proportion of new deleterious mutations that had  
275 accumulated since the divergence of each taxa was approximated in two ways. First, the  
276 ratio  $\pi_a/\pi_s$  (a *proxy* of the efficacy of selection) was estimated, using loblolly pine as  
277 outgroup, from the diversity of 0-fold and 4-fold positions of all polymorphic coding  
278 sequences with only two variants per polymorphic site. Second, the functional effects of  
279 all variants (fixed and polymorphic) exclusive to each stone or maritime pines were  
280 inferred using PROVEAN (Choi and Chan 2015). This tool uses alignments from protein  
281 blasts against public databases to determine a score for any given site change, by  
282 assuming that the rarer a mutation is, the most likely it is to be deleterious. It gives  
283 particularly low scores to nonsense replacements and to those resulting in substantial  
284 biochemical changes, especially in conserved protein regions (Choi and Chan 2015).  
285 Following a similar survey in spruce (Conte et al. 2017), variants with scores below -  
286 2.282 were retained as deleterious candidates (see also Zhang et al. 2016 for poplar).  
287 Candidate counts per species, per gene and per individual were finally compared with  
288 non-parametric tests.

289

## 290 **Results**

### 291 *Microsatellite variation and genetic structure*

292 Genetic diversity estimated with nuSSRs was extremely low in stone pine. Four out of  
293 the 13 loci surveyed were monomorphic; the nine polymorphic nuSSRs carried between  
294 two and five alleles, totalling only 29 size variants (mean = 2.23 alleles per locus). The

295 mean number of alleles per population ranged between 1.00 and 1.69 (mean = 1.38),  
296 and the average number of effective alleles per population between 1.00 and 1.41 (mean  
297 = 1.19; Table S1). In addition, allele frequencies were highly homogeneous across  
298 populations. Most loci only displayed one frequent allele common to all populations  
299 (frequency > 0.8) and a few rare variants scattered across isolated populations with no  
300 apparent geographic trend. Ten out of the 29 alleles observed (34.5%) were private;  
301 four of these were observed in populations from the Iberian Peninsula, although some  
302 were also found in France, Tunisia, Lebanon and Turkey (Fig. 1A); two of these private  
303 alleles were in homozygous state. Heterozygosity was also very low, ranging from 0.014  
304 and 0.176 for  $H_0$  (mean = 0.083) and between 0 and 0.221 for  $H_E$  (mean = 0.114; Fig. 1A;  
305 Table S1). Mean population divergence was high ( $F_{ST} = 0.242$ ), and the most plausible  
306 number of genetic clusters was two (Fig. 2). These clusters were mostly located in the  
307 eastern or western parts of the Mediterranean Basin. Increasing  $K$ -value to three  
308 revealed an additional subdivision for the eastern genetic cluster, separating  
309 populations from Israel/Lebanon from those of Greece/Turkey; stands from Cyprus  
310 were genetically intermixed.  $K$ -values 4 and 5 revealed no further subdivisions (Fig. S2).  
311 For all examined  $K$ -values, assignment of the Portuguese population and of a Spanish  
312 marginal population (Garrovillas, see Fig. 2B) was dubious; they both had a large genetic  
313 component from the eastern genetic cluster, suggesting a recent anthropogenic  
314 introduction. These populations were removed from further analyses. Similarly weak  
315 population genetic structure was observed with the first two coordinates of the PCoA,  
316 which explained 44% and 23.7% of the data variance, respectively (Fig. S2).

317

318 *Species divergence and demography*

319 Only one tree topology was supported by the phylogenetic analyses (posterior  
320 probability = 1.0 for all nodes), indicating (as expected) that stone pine is more closely  
321 related to maritime pine than to loblolly pine. Divergence between stone pine and its  
322 common ancestor with maritime pine dated back to ~29 Ma (95%CI: 35-12Ma), while  
323 its separation from the common ancestor with loblolly pine was inferred in ~40Ma  
324 (95%CI: 25-52Ma; Fig. 3A).

325 The demographic history of stone pine, as inferred with STAIRWAYPLOT (on DNA  
326 sequence variation), was marked by a deep trend of effective population size decrease  
327 starting ~1Ma. This trend was observed for the whole species (Fig. 3C), and for each  
328 independent genetic cluster (Fig. S3). For the whole species, a first initial collapse  
329 reduced the stone pine population size by about 80%, while two more recent episodes  
330 narrowed it down to 1-5% of its ancestral size during the last ~200Ka (Fig. 3C).  
331 Population collapse seemed stronger for the eastern genetic cluster than for the western  
332 one (Fig. S3). Interestingly, maritime pine suffered a population size decline of a similar  
333 magnitude during this period, from which it was able to recover to approximately  
334 ancestral population sizes before the decline (Fig. 3B). No population size recovery was  
335 inferred for stone pine.

336 A more detailed exploration of the recent demographic dynamics of stone pine was  
337 performed by testing several hypotheses by approximate Bayesian inference on the  
338 nuSSR data. The most likely scenario (*scenario 5*, see Materials and Methods; Posterior  
339 Probability = 0.8) involved a severe bottleneck in the ancestral population, and a more  
340 recent population subdivision between the western and eastern genetic clusters, which  
341 currently have highly dissimilar population sizes (much larger in the west; Fig. 3D).  
342 These episodes were respectively inferred 5,510 and 238 generations into the past. If we  
343 consider a generation time of 25-40 years, as for other pines, the ancestral population

344 collapse would date back to between 220Ka and 135 Ka, which roughly coincides with  
345 the previous interglacial and the second to last glacial period (i.e. the Mindel-Riss  
346 interglacial and Riss Glaciation in the Alps), respectively, and with the second population  
347 decline inferred from the DNA-sequence data (Fig. 3C). Moreover, population divergence  
348 would have occurred during the Holocene (9.5 – 6Ka), roughly coinciding with the  
349 earliest fossil evidence of stone pine cultivation (see Discussion).

350

### 351 *Nucleotide diversity, adaptive evolution and genetic load*

352 DNA sequence variation was also extremely low for stone pine (even lower than for the  
353 nuSSRs, in comparative terms); only 15 of the 177 gene loci were polymorphic (8.5%),  
354 and contained 21 variable sites, which represents one SNP every ~2.5 Kbp. For a  
355 comparison, 171 of the same gene loci were polymorphic in maritime pine, and  
356 harbored 548 SNPs (one SNP every ~102 bp). This translated into a mean nucleotide  
357 diversity that was one order of magnitude lower in stone pine ( $\pi = 0.000212$  [95%CI:  
358 0.000184–0.000246];  $\theta_w = 0.000149$  [95%CI: 0.000126–0.000178]) than in maritime  
359 pine ( $\pi = 0.002423$  [95%CI: 0.002065–0.002778];  $\theta_w = 0.002305$  [95%CI:  
360 0.001993–0.002621]; Table 1). Both  $\pi$  and  $\theta_w$  estimates were equally low and not  
361 significantly different between the two stone pine genetic clusters (Table S2).

362 Despite this very low nucleotide diversity, the mean rate of total ( $D_{xy}$ ), synonymous  
363 ( $K_s$ ) and non-synonymous substitutions ( $K_a$ ) accumulated in stone pine since its  
364 divergence from loblolly pine (0.0168 [95%CI: 0.0147–0.0185], 0.0382 [95%CI:  
365 0.0338–0.0426], 0.0099 [95%CI: 0.0073–0.0116]; respectively) were virtually identical  
366 to those observed for maritime pine ( $D_{xy} = 0.0164$  [95%CI: 0.0146–0.0182];  $K_s = 0.0384$   
367 [95%CI: 0.0336–0.0432];  $K_a = 0.0101$  [95%CI: 0.00836–0.0118]). Decomposing these  
368 figures revealed that they were mostly due to the number of shared substitutions

369 between species (Fig. 4). The non-shared portion was significantly smaller in stone pine  
370 than in maritime pine, and it was composed of a relatively similar number of  
371 synonymous and non-synonymous substitutions (152 vs. 113). In maritime pine, the  
372 non-shared variants contained almost twice as many silent than replacement  
373 substitutions (325 vs. 188; Fig. 4). Such values translated in a larger  $K_a/K_s$  ratio  
374 (estimated per gene) for stone pine than for maritime pine (0.46 [95%CI: 0.41–0.52] vs.  
375 0.33 [95%CI: 0.24–0.40]), suggesting relaxed purifying selection in stone pine (Table 1).  
376 The efficacy of selection, as determined with the  $\pi_a/\pi_s$  ratio, pointed in the same  
377 direction, as estimates were again higher in stone pine (0.448 [95%CI: 0.371–0.526])  
378 than in maritime pine (0.256 [95%CI: 0.186–0.326]; Table 1). As for nucleotide  
379 diversity, no differences were observed for  $K_a/K_s$  and  $\pi_a/\pi_s$  ratios between stone pine  
380 genetic clusters (Table S2).

381 In terms of adaptive evolution, stone pine also seemed hampered when compared to  
382 maritime pine, not only because the proportion of (non-shared) non-synonymous  
383 substitutions fixed by adaptive evolution ( $\alpha$ ) and the relative rate of (non-shared)  
384 adaptive substitutions ( $\omega_a$ ) were both significantly lower, but because they were also  
385 not significantly different from zero; estimates of  $\alpha$  and  $\omega_a$  were respectively 0.171  
386 (95%CI: -0.027–0.309) and 0.062 (95%CI: -0.001–0.119) for stone pine, while they were  
387 0.494 (95%CI: 0.346–0.609) and 0.304 (95%CI: 0.219–0.391) for maritime pine (Fig. 4).  
388 Estimates of  $\alpha$  and  $\omega_a$  were virtually identical for both stone pine genetic clusters (Table  
389 S2). On the other hand, the general shape of the distribution of fitness effects (DFE) was  
390 similar for both stone and maritime pines. However, the first species had a significantly  
391 higher proportion of least deleterious mutations (Fig. 4), while maritime pine had a  
392 higher proportion of highly deleterious mutations. Again, no differences were observed  
393 between stone pine eastern and western genetic clusters. It must be reminded that all

394 calculations above for stone and maritime pines (i.e. DFE,  $\alpha$ ,  $\omega_a$ ,  $K_a/K_s$  and  $\pi_a/\pi_s$ ) were  
395 performed using only exclusive polymorphisms and replacements accumulated after the  
396 divergence from loblolly pine.

397 Finally, genetic load, evaluated as the predicted number and frequency of non-shared  
398 replacements with putatively deleterious effects according to PROVEAN (Choi and Chan  
399 2015), was higher in stone pine than in maritime pine. The first species accumulated  
400 relatively less replacements (116) and in less genes (20) than maritime pine (166  
401 replacements in 38 genes; Mann-Whitney U-test;  $P = 0.0394$  and  $P = 0.0142$  for the  
402 number of replacements and genes, respectively). However, 34 of the replacements  
403 observed in stone pine (29.3% of the total) were putatively deleterious, and most of  
404 them (27 out of the 34; or 23.3% of the total substitutions) were fixed in the individuals  
405 analysed (Fig. 4). On the other hand, 48 (28.9%) putatively harmful replacements were  
406 inferred for maritime pine, while only 15 of them (9% of the total substitutions) were  
407 fixed (Fig. 4). Both the total number of putatively deleterious mutations and the number  
408 of fixed deleterious mutations were significantly different between the two pines  
409 (Mann-Whitney U-test;  $P = 0.0271$  and  $P = 0.0128$ , respectively).

410 Differences in genetic load were more evident when evaluated at the individual level.  
411 In stone pine, individuals had on average 0.22 putatively deleterious mutations per  
412 gene, while they were significantly less (mean = 0.15) in maritime pine (Mann-Whitney  
413 U-test;  $P = 0.0184$ ). After scaling by the number of synonymous mutations per species,  
414 the mean number of putatively deleterious mutations per gene per individual was still  
415 significantly higher for stone pine (0.042) than for maritime pine (0.019; Mann-Whitney  
416 U-test;  $P = 0.0036$ ), indicating relaxed purifying selection in the first species.

417 Interestingly, deleterious variants were not equally distributed across polymorphic  
418 and monomorphic genes in stone pine (Table S3), particularly those that were fixed for

419 significantly more replacement substitutions than the average gene (i.e. five or more  
420 replacements;  $n=39$ ). More than half of the polymorphic genes (53%) contained  
421 putatively deleterious variants, while only a fifth of the monomorphic genes bearing five  
422 or more replacements exhibited such harmful variants (Mann-Whitney U-test;  $P =$   
423 0.0083). These monomorphic genes with reduced fixation of deleterious mutations were  
424 further biased towards regulation and stress response processes and have often been  
425 reported as candidate genes for adaptation in other pines (see Discussion), while the  
426 polymorphic genes showed no particular function enrichment (Table S3).

427

## 428 **Discussion**

429 In this study, we demonstrate that the widespread and outbreeding stone pine has  
430 unprecedentedly low genome-wide levels of genetic diversity. This near absence of  
431 genomic variation is the product of a deep and prolonged demographic collapse, rather  
432 than changes in the speed at which it accumulates genetic diversity. This demographic  
433 framework further resulted in a lowered efficacy of purifying selection and the fixation  
434 of deleterious variants.

435

### 436 *Low levels of genome-wide genetic diversity and phylogeographic inferences*

437 In line with previous studies on allozymes (Fallour et al. 1997) and chloroplast  
438 microsatellites (cpSSRs) (Vendramin et al. 2008), we found very little genetic variation  
439 in both hypervariable nuclear microsatellites (29 alleles in 13 nuSSRs) and gene loci (21  
440 SNPs in 177 gene loci spanning more than 55 Kbp). While estimation accuracy of  
441 individual parameters might be at stake because of our relatively small dataset (both in  
442 terms of markers and sample size), it is unlikely that such low diversity values are the  
443 result of limited population or genome sampling. Indeed, both population genetics and

444 coalescence theory indicates that much of the genetic variability within a species can be  
445 captured with a limited number of individuals, while strong demographic changes  
446 affecting the whole genome can be detected even when surveying a modest part of the  
447 genome (Tajima 1983; Nielsen and Slatkin 2013).

448 In plants, low diversity values are usually observed in self-fertilizing taxa or in species  
449 with very restricted distributions, but are unusual in widespread outbreeding taxa  
450 (Table 2). Within the genus *Pinus*, and forest trees in general, estimates of genomic  
451 diversity are usually much higher than those obtained herein for stone pine ( $\pi =$   
452 0.0002), even for endemic species, such as *P. balfouriana* ( $\pi = 0.0028$ ) or *P. longaeva* ( $\pi =$   
453 0.0021; Eckert et al. 2013). Indeed, to our knowledge, the only conifer for which such an  
454 equally low nucleotide diversity has been reported is the tertiary relict *Picea breweriana*  
455 ( $\pi = 0.0001$ ; Chen et al. 2010), which is distributed in small scattered stands at the  
456 border between California and Oregon.

457 Previous works have suggested an ancient and prolonged bottleneck to account for  
458 the low genetic variation in stone pine, but without providing further insights on times  
459 or population sizes (Fallour et al. 1997; Vendramin et al. 2008). Our demographic  
460 framework indicated a general and strong trend of population collapse that could have  
461 started as early as  $\sim 1$ Ma, reduced the ancestral stone pine population to 1-10% of its  
462 original effective size, and continued well within the Holocene (Fig. 3). The number and  
463 timing of decline pulses vary according to the type of marker (SSRs vs. gene-loci) and  
464 method used (ABC vs. SFS-based), but they all coincide with glacial periods of the  
465 Pleistocene (e.g. Günz, Riss, and Würm; Cohen and Gibbard 2011; Fig. 3). While such  
466 differences might be the result of our limited sample size, variation in demographic  
467 parameters is rather common when using different markers and methods (e.g. Patton et

468 al. 2019). This deserves a more detailed study, likely including simulations, which is out  
469 of the scope of the present work.

470 In addition to demographic history, some biological features of stone pine, such as its  
471 inability to disperse its seeds by wind (contrarily to other Mediterranean pines) or the  
472 3-year cone maturation (all other Mediterranean pines take only two years), may have  
473 also contributed to its unusually low genetic diversity. For example, because of  
474 restricted gene flow, all mutations that appeared after or during the population decline  
475 would have been only locally dispersed, increasing their chances to be lost by genetic  
476 drift. Ancient alleles would have suffered a similar fate, being differentially fixed or lost,  
477 but never dispersed among distant populations. Indeed, such a feature can also explain  
478 the genetic structure of stone pine, which is mostly driven by private nuSSR alleles,  
479 whose spatial distribution (together with that of polymorphic SNPs and rare cpDNA  
480 haplotypes; see Vendramin et al. 2008) could be mostly the result of restricted gene flow  
481 among populations.

482 According to our Bayesian simulations, the main genetic clusters of stone pine did not  
483 diverge until the Holocene (9 – 6.5 Ka; Fig. 3), while none of the main genetic clusters  
484 showed signs of expansion. This implies that stone pine should have colonized its  
485 current distribution very quickly. However, its low seed dispersal ability hints that a  
486 circum-Mediterranean colonization could not have occurred without the support of  
487 idiosyncratic dispersers (Vendramin et al. 2008). Evidence indicates that stone pine  
488 seeds have been consumed by humans since at least ~50 Ka BP, that it has been  
489 cultivated for over 6,000 years, and its nuts traded during no less than three millennia  
490 (Prada et al. 1997; Badal 1998; Carrión et al. 2008; Pérez-Jordà et al. 2017). Estimated  
491 divergence times between genetic clusters roughly coincide with the beginning of stone  
492 pine cultivation, which points at anthropogenic factors as the main drivers of recent

493 expansion, divergence and ecological success (Barbero et al. 1998; Richardson, 1998;  
494 Vendramin et al. 2008).

495 Archeological remnants and analyses of ancient trade routes further suggest that the  
496 ancestral stone pine population (from where colonization took place) was likely located  
497 within the Iberian Peninsula (Carrión et al. 2008; Mutke et al. 2019; Fig. 3.4 from  
498 Rubiales et al. 2011); a view supported by the distribution of private nuSSR alleles (Fig.  
499 1) and rare cpDNA types (Vendramin et al. 2008), and by the higher effective population  
500 sizes estimated herein for the western genetic cluster (Fig. 3D). Unfortunately, the low  
501 genetic diversity of this species hampers for the time being more detailed  
502 phylogeographic and genomic analyses (including the putative effects of incipient  
503 domestication for nut taste and size).

504

#### 505 *Evolutionary rates and adaptive variation*

506 Our estimates of adaptive evolution ( $\alpha$ ,  $\omega_a$ , and DFE) pointed to a diminished (adaptive)  
507 evolutionary rate in stone pine when compared to maritime pine, as well as a relaxation  
508 of purifying selection (Table 1; Fig. 4). These results agree with theoretical expectations,  
509 implying less potential for adaptive evolution in species with small effective population  
510 size (i.e. stone pine). Although limited by our rather modest genome sampling,  $\pi_a/\pi_s$ ,  $\alpha$   
511 and  $\omega_a$  values, and the DFE, obtained herein for stone pine are not unusual for pines or  
512 plants in general (Gossman et al. 2010; Chen et al. 2017; Grivet et al. 2017). The nearly  
513 neutral theory of molecular evolution predicts a simple relationship between adaptive  
514 evolution and effective population size, which can be derived into a *log-log* linear  
515 correlation between  $\pi_s$  and  $\pi_a/\pi_s$  (Kimura 1968; Welch et al. 2008). Such a relationship  
516 was indeed reported for a wide variety of organisms (Chen et al. 2017), and the values  
517 obtained herein for stone pine fit well within this regression line (Fig. S4). Thus, current

518 effective population size resulting from the peculiar demography of this species seems  
519 to account for both the rate at which it is accumulating genetic diversity, and the global  
520 efficacy of selection. Because, the sequenced gene-loci were widespread within the stone  
521 pine genome, it is unlikely that sequencing additional genes would reverse the patterns  
522 observed herein.

523 It could be argued that other elements, such as variation in mutation rate ( $\mu$ ) and  
524 linkage disequilibrium (LD), or spatiotemporal changes of fitness effects, may underlie  
525 observed differences between stone and maritime pines. For instance, it has been  
526 suggested that demographic fluctuations can affect  $\mu$ , particularly in species with  
527 overlapping generations (Balloux and Lehmann 2012; Wei et al. 2015). Although we did  
528 not formally estimate this parameter, no significant differences were observed between  
529 the two pines for  $D_{xy}$ ,  $K_a$  or  $K_s$ , which suggest that their mutation rates should be at least  
530 in the same order of magnitude. Indeed, to our knowledge, no differences in mutation  
531 rates have been reported so far among pine taxa, including those from different  
532 subsections (Willyard et al. 2009).

533 Linkage disequilibrium can cause selection at one locus to affect the fixation of  
534 mutations in neighbouring genomic regions (i.e. interference; Lanfear et al. 2014).  
535 Conifers are among the plants with the lowest genome-wide recombination rates  
536 (Jaramillo-Correa et al. 2010b), which indicates that interference should be indeed taken  
537 into account. Unfortunately, because of its low diversity, no reliable recombination  
538 inference could be performed herein for stone pine. However, given that fixations  
539 between loci (including both nuSSRs and gene-sequences) appeared independent from  
540 each other, and that even modest amounts of recombination can be sufficient to alleviate  
541 interference (Presgraves 2005; Weissman and Barton 2012), the contribution of LD  
542 variation to explain observed differences across stone and maritime pines should be

543 negligible. Future studies with more complete genome data are however necessary to  
544 test this hypothesis more formally.

545 Selective pressures may also vary across a species range and over time, having  
546 heterogeneous effects on substitutions rates, and thus on evolutionary rate (Bell 2010).  
547 However, if our demographic and phylogeographic inferences are correct, such changes  
548 should be affecting more maritime pine than stone pine; given that the first species is  
549 composed by six ancient genetic clusters distributed across contrasting environments  
550 (Bucci et al. 2007; Jaramillo-Correa et al. 2015b), while population structure in stone  
551 pine is less marked and more recent (Fig. 3). While there is still little information on how  
552 spatiotemporal variation of fitness affects evolutionary rate (Lanfear et al. 2014), no  
553 differences in the proportion of adaptive substitutions were observed across the more  
554 ancient maritime pine gene pools (Grivet et al. 2017). Thus, suggesting again that  
555 differences in demography appear as the most likely explanation for the contrasting  
556 rates at which these two parapatric pines are accumulating genetic diversity.

557 Genomes of heavily bottlenecked species usually exhibit overall genetic depletion,  
558 with isolated and randomly distributed peaks of diversity (Robinson et al. 2016). Stone  
559 pine seems to fit such expectations. The few SNPs detected were distributed in only 15  
560 genes that were not particularly biased towards specific biological processes. They  
561 participated in functions as varied as transport, ATP binding and secondary metabolism,  
562 and are scattered in various pine linkage groups (Westbrock et al. 2015; Plomion et al.  
563 2016). More than half of these genes (53%) contained putatively deleterious variants  
564 (either fixed or polymorphic; Table S3).

565 Interestingly, among the monomorphic genes, those that were fixed for significantly  
566 more replacement substitutions than the average gene (i.e. five or more replacements;  
567  $n=39$ ) tended to have fewer deleterious variants. These genes were further enriched

568 towards regulation and stress response processes, and most of them (60%) have been  
569 reported as candidate genes for local adaptation in other pines and/or are upregulated  
570 under drought stress in stone pine (Perdiguero et al. 2013; Table S3). Although these  
571 observations are preliminary, given the modest gene sampling of our study and the lack  
572 of current knowledge on gene function in conifers, they do provide an interesting  
573 starting point to understand how adaptive variation arises and reaches fixation in these  
574 taxa.

575

#### 576 *Long-term persistence in the absence of genetic variation*

577 An interesting question that arises from our study is how a species genetically depleted  
578 has been able to survive, thrive, and successfully compete with taxa that occupy a  
579 similar ecological niche. Extrapolating from the limited number of genes surveyed,  
580 maritime pine bore significantly more silent and replacement substitutions and more  
581 putatively harmful mutations than stone pine. The large majority of these changes  
582 (91%) were in a heterozygous state in maritime pine, whereas most of the non-  
583 synonymous substitutions detected for stone pine, including many evaluated as  
584 deleterious, were homozygous (Fig. 4). These results fit the expectations of a differential  
585 accumulation of neutral and putatively deleterious mutations within a species after a  
586 bottleneck (Gillespie 2001; Lanfear et al. 2014; Chen et al. 2017), and it is unlikely that  
587 this trend could be reversed when additional gene-regions are sequenced.

588 The accumulation of partially deleterious mutations in stone pine also points to  
589 reduced inbreeding depression. In forest trees, this phenomenon generally results in the  
590 elimination of virtually all inbred progeny at early life-stages (Petit and Hampe 2006),  
591 which is believed to be homozygous for recessive deleterious mutations. When  
592 populations are large (as for maritime pine), deleterious mutations tend to accumulate

593 in heterozygous states. However, after a bottleneck (as for stone pine), these mutations  
594 are exposed and can be purged more efficiently, which leads to inbreeding tolerance (i.e.  
595 inbreds are no longer selected against), and to a more accelerated diversity loss by  
596 genetic drift (Lanfear et al. 2014). Early work in stone pine and other genetically  
597 depleted conifers seem to point in that direction (Ammannati 1989; Ledig et al. 2000).

598 It must be noted, however, that genetic load in stone pine is likely underestimated  
599 because of our limited genome sampling, and because virtually all mutations evaluated  
600 as deleterious came from bioinformatics predictions. That is, they were either very rare  
601 within conifer databases or produced biochemical changes in the proteins. No strongly  
602 harmful mutations, like premature stop codons or insertions changing the protein  
603 reading frames, were observed in stone pine. While such types of mutations might be  
604 detected with a more in-depth genome survey, they are also expected to be more  
605 common in species with lower inbreeding depression than forest trees, such as annual  
606 plants or vertebrates, including humans (Robinson et al. 2016; González-Martínez et al.  
607 2017; Pedersen et al. 2017; Laenen et al. 2018). Should this trend be confirmed after a  
608 more extensive exome re-sequencing, it will indicate that purifying selection is still  
609 operating for highly-deleterious variants in stone pine, as could be inferred from the  
610 DFE (Fig. 4).

611 Another mechanism fostering stone pine long-term persistence despite depleted  
612 genetic variation might be the presence of islands of genetic diversity within its genome,  
613 and which may be associated to relevant adaptive quantitative genetic variation and/or  
614 phenotypic plasticity (Carrasquinho and Gonçalves 2013; Mutke et al. 2013; Sánchez-  
615 Gómez et al. 2011). Typically, this type of variation is produced by small allele frequency  
616 changes in multiple loci across the genome (Pritchard et al. 2010; Boyle et al. 2017;  
617 Csilléry et al. 2018); variation that is better preserved during bottlenecks by balancing

618 or frequency-dependent selection than newly arisen mutations (Lynch 1996). If this is  
619 the case for stone pine, a more detailed genomic survey (including full genome or  
620 transcriptome sequencing) should reveal further diversity-rich regions upon which  
621 selection may operate. Another possibility is methylation diversity, which has already  
622 been shown to be substantial in stone pine (Sáez-Laguna et al. 2014). Such variability is  
623 often inheritable, and can rapidly accumulate after bottlenecks and contribute to  
624 phenotypic plasticity in adaptive traits (Chinnusamy and Zhu 2009; Johanness et al. 2009;  
625 Balao et al. 2018).

626 Finally, future studies should formally survey the effects on fitness (e.g. via  
627 association with adaptive phenotypes, as in Zhang et al. 2016) of the putatively  
628 deleterious variants inferred in this study; given that these are merely bioinformatics  
629 predictions (Choi and Chan 2015; Conte et al. 2017). Integrating such effects, and genetic  
630 load, into predictive models should become a priority for the improvement of  
631 commercially important traits in stone pine, such as those related to nut production (see  
632 Wallace et al. 2018). Considering deleterious variants is also important for conservation  
633 and management programs in forest trees (Holliday et al. 2017). Indeed, avoiding  
634 deleterious alleles, especially in homozygosity, might account for a larger part of the  
635 phenotypic variance of species than the avidly-searched adaptive genes usually reported  
636 in most population genomic studies.

637

### 638 **Acknowledgements**

639 We are grateful to Diana Barba and Denis Vauthier for sampling, and Carmen García-  
640 Barriga and Mario Zabal-Aguirre for laboratory assistance and DNA sequence edition.  
641 We further thank Yoshiaki Tsuda for help with ABC analyses and Michele Bozzano for  
642 stimulating and passionate discussions on stone pine demographic history. Samples

643 were collected from common gardens in France and Spain, installed within the FAO-  
644 Silva Mediterranea collaborative framework. French common gardens belong to the  
645 GEN4X network ([http://www.efpa.inra.fr/Outils-et-Ressources/Systemes-d-  
647 experimentation-et-d-observation/Reseau-GEN4X/](http://www.efpa.inra.fr/Outils-et-Ressources/Systemes-d-<br/>646 experimentation-et-d-observation/Reseau-GEN4X/)). This research was supported by  
648 funding from EVOLTREE ([www.evoltree.eu](http://www.evoltree.eu)), a former EU Network of Excellence, and  
649 currently a European Research Group facilitated by the European Forest Institute (EFI),  
650 and B4EST, a project funded by the European Union's Horizon 2020 research and  
651 innovation programme under grant agreement No 773383. JPJ-C was supported by the  
652 "Dirección General de Asuntos del Personal Académico" (DGAPA-UNAM) through a  
653 PASPA fellowship, for a sabbatical leave during which this study was elaborated.

653

#### 654 **Data accessibility**

655 Nuclear microsatellite (nuSSR) data, DNA sequence alignments, gene annotations, and  
656 site type counts are available in Dryad, with doi: 10.5061/dryad.59zw3r23r.

657

#### 658 **Author contributions**

659 JPJC, GGV and SCGM conceived the study. GGV, BF, SCGM, DG and FAA produced the data.  
660 DG and FB contributed to data analyses. JPJC and SCGM analysed the data and drafted  
661 the manuscript. All authors revised and edited the text, and produced the final  
662 manuscript.

663

#### 664 **References**

665 Ammannati, R. (1988). Effeti dell'autoimpollinazione sulla crescita in altezza in *Pinus*  
666 *pineae* L. *Monti e Boschi*, 3, 50–52.

667 Badal, E. (1998). *Interés económico del pino piñonero para los habitantes de la Cueva de*  
668 *Nerja*. In J.L. Sanchidrián, & M.D. Simón (Eds.), *Las culturas del Pleistoceno Superior en*  
669 *Andalucía* (pp. 287-300). Málaga: Patronato de la Cueva de Nerja.

670 Balao, F., Paun, O., & Alonso, C. (2018). Uncovering the contribution of epigenetics to  
671 plant phenotypic variation in Mediterranean ecosystems. *Plant Biology*, 20, 38-49.

672 Balloux, F., & Lehmann, L. (2012). Substitution rates at neutral genes depend on  
673 population size under fluctuating demography and overlapping generations.  
674 *Evolution*, 66, 605–611.

675 Barbéro, M., Loisel, R., Quézel, P., Richardson, D.M., & Romane, F. (1998). *Pines of the*  
676 *Mediterranean basin*. In D.M. Richardson (Ed.), *Ecology and biogeography of Pinus* (pp.  
677 153–170). Cambridge: Cambridge University Press.

678 Bazile-Robert, E. (1981). Le pin pignon (*Pinus pinea* L.) dans le Würm récent de  
679 Provence. *Géobios*, 14, 395–397.

680 Bell, G. (2010). Fluctuating selection: the perpetual renewal of adaptation in variable  
681 environments. *Philosophical Transactions of the Royal Society of London, Series B:*  
682 *Biological Sciences*, 365, 87–97.

683 Böndel, K.B., Lainer, H., Nosenko, T., Mboup, M., Tellier A., & Stephan, W. (2015). North-  
684 South colonization associated with local adaptation of the wild tomato species  
685 *Solanum chilense*. *Molecular Biology and Evolution*, 32, 2932-2943.

686 Boraks, A., & Broders, K.D. (2016). Population genetic diversity of the rare hardwood  
687 butternut (*Juglans cinerea*) in the northeastern USA. *Tree Genetics & Genomes*, 12, 43.

688 Boyle, E.A., Li, Y.I., & Pritchard, J.K. (2017) An expanded view of complex traits: from  
689 polygenic to omnigenic. *Cell*, 169, 1177-1186.

690 Boys, J., Cherry, M., & Dayanandan, S. (2005). Microsatellite analyses reveals genetically  
691 distinct populations of red pine (*Pinus resinosa*, Pinaceae). *American Journal of*  
692 *Botany*, 92, 833-841.

693 Bromham, L., Hua, X., Lanfear, R., & Cowman, P.F. (2015). Exploring the relationships  
694 between mutation rates, life history, genome size, environment, and species richness  
695 in flowering plants. *The American Naturalist*, 185, 507-524.

696 Brown, G. R., Gill, G. P., Kuntz, R. J., Langley, C. H., & Neale, D.B. (2004). Nucleotide  
697 diversity and linkage disequilibrium in loblolly pine. *Proceeding of the National*  
698 *Academy of Sciences of USA*, 101, 15255–15260.

699 Bucci, G., González-Martínez, S.C., Le Provost, G., Plomion, C., Ribeiro, M.M., Sebastiani, F.,  
700 Alia, R., & Vendramin, G.G. (2007). Range-wide phylogeography and gene zones in  
701 *Pinus pinaster* Ait. revealed by chloroplast microsatellite markers. *Molecular Ecology*,  
702 16, 2137–2153.

703 Carrasquinho, I., & Gonçalves, E. (2013). Genetic variability among *Pinus pinea* L.  
704 provenances for survival and growth traits in Portugal. *Tree Genetics & Genomes*, 9,  
705 855–866.

706 Carrión, J.S., Finlayson, C., Fernández, S., Finlayson, G., Allué, E., López Sáez, J.A., López  
707 García, P., Gil-Romera, G., Bailey G., & González-Sampériz, P. (2008). A coastal  
708 reservoir of biodiversity for Upper Pleistocene human populations: palaeoecological  
709 investigations in Gorham's Cave (Gibraltar) in the context of the Iberian Peninsula.  
710 *Quaternary Sciences Reviews*, 27, 2118-2135.

711 Charlesworth, B. (2009). Fundamental concepts in genetics: effective population size  
712 and patterns of molecular evolution and variation. *Nature Reviews, Genetics*, 10, 195–  
713 205.

714 Charlesworth, B., Morgan, M.T., & Charlesworth, D. (1993). The effect of deleterious  
715 mutations on neutral molecular variation. *Genetics*, 134, 1289-1303.

716 Charlesworth, D., Charlesworth, B., & Morgan, M.T. (1995). The pattern of neutral  
717 molecular variation under the background selection model. *Genetics*, 141, 1619–1632.

718 Charlesworth, J., & Eyre-Walker, A. (2007). The other side of the nearly neutral theory,  
719 evidence of slightly advantageous backmutations. *Proceedings of the National*  
720 *Academy of Sciences of USA*, 104, 16992–16997.

721 Chen, J., Källman, T., Gyllenstrand, N., & Lascoux, M. (2010). New insights on the  
722 speciation history and nucleotide diversity of three boreal spruce species and a  
723 Tertiary relict. *Heredity*, 104, 3–14.

724 Chen, J., Glémin, S., & Lascoux, M. (2017). Genetic diversity and the efficacy of purifying  
725 selection across plant and animal species. *Molecular Biology and Evolution*, 34, 1417–  
726 1428.

727 Chinnusamy, V., & Zhu, J.K. (2009). Epigenetic regulation of stress responses in plants.  
728 *Current Opinion in Plant Biology*, 12, 133–139.

729 Choi, Y., & Chan, A.P. (2015). PROVEAN web server: a tool to predict the functional effect  
730 of amino acid substitutions and indels. *Bioinformatics*, 31, 2745-2747.

731 Cohen, K.M., & Gibbard, P. (2011). *Global chronostratigraphical correlation table for the*  
732 *last 2.7 million years*. Cambridge: Subcommission on Quaternary Stratigraphy  
733 (International Commission on Stratigraphy). Retrieved from  
734 <http://quaternary.stratigraphy.org/charts/>

735 Conte, G.L., Hodgins, K.A., Yeaman, S., Degner, J.C., Aitken, S.N., Rieseberg, L.H., &  
736 Whitlock, M.C. (2017). Bioinformatically predicted deleterious mutations reveal  
737 complementation in the interior spruce hybrid complex. *BMC Genomics*, *18*, 970.

738 Cornuet, J. M., Pudlo, P., Veyssier, J., Dehne-Garcia, A., Gautier, M., Leblois, R., ... Estoup, A.  
739 (2014). DIYABC v2.0: A software to make approximate Bayesian computation  
740 inferences about population history using single nucleotide polymorphism, DNA  
741 sequence and microsatellite data. *Bioinformatics*, *30*, 1187– 1189.

742 Csilléry, K., Rodríguez-Verdugo, A., Rellstab, C., & Guillaume F. (2018). Detecting the  
743 genomic signal of polygenic adaptation and the role of epistasis in evolution.  
744 *Molecular Ecology*, *27*, 606-612.

745 Cuenca, A. (2003) *Evidencia de dos linajes genéticos en Pinus cembroides revelada por*  
746 *microsatélites de cloroplasto* (Unpublished MSc thesis). Universidad Nacional  
747 Autónoma de México.

748 Drummond, A.J., Suchard, M.A., Xie, D., & Rambaut, A. (2012). Bayesian Phylogenetics  
749 with BEAUti and the BEAST 1.7, *Molecular Biology and Evolution*, *29*, 1969–1973.

750 Earl, D.A., & vonHoldt, B.M. (2012). STRUCTURE HARVESTER: a website and program  
751 for visualizing STRUCTURE output and implementing the Evanno method.  
752 *Conservation Genetics Resources*, *4*, 359-361.

753 Echt, C.S, De Verno, L.L., Anzidei, M., & Vendramin, G. G. (1998). Chloroplast  
754 microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait.  
755 *Molecular Ecology*, *7*, 307-316.

756 Eckert, A. J., Bower, A. D., Jermstad, K. D., Wegrzyn, J. L., Knaus, B. J., Syring, J. V., & Neale,  
757 D. B. (2013). Multilocus analyses reveal little evidence for lineage-wide adaptive  
758 evolution within major clades of soft pines (*Pinus subgenus* *Strobus*). *Molecular*  
759 *Ecology*, *22*, 5635–5650.

760 Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of  
761 individuals using the software structure: a simulation study. *Molecular Ecology*, *14*,  
762 2611–2620

763 Eyre-Walker, A., & Keightley, P.D. (2007). The distribution of fitness effects of new  
764 mutations. *Nature Reviews, Genetics*, 8, 610-618.

765 Eyre-Walker, A., & Keightley, P.D. (2009). Estimating the rate of adaptive molecular  
766 evolution in the presence of slightly deleterious mutations and population size  
767 change. *Molecular Biology and Evolution*, 26, 2097–2108.

768 Fallour, D., Fady, B., & Lefèvre, F. (1997). Study on isozyme variation in *Pinus pinea* L.:  
769 evidence for low polymorphism. *Silvae Genetica*, 46, 201–207.

770 Gaut, B.S., Díez, C.M., & Morrell, P.L. (2015). Genomics and the contrasting dynamics of  
771 annual and perennial domestication. *Trends in Genetics*, 31, 709-719.

772 Gernandt, D.S., López, G.G., García, S.O. & Liston, A. (2005). Phylogeny and classification  
773 of *Pinus*. *Taxon*, 54, 29-42.

774 Gillespie, J. H. (2001). Is the population size of a species relevant to its evolution?  
775 *Evolution*, 55, 2161–2169.

776 González-Martínez, S.C., Ridout, K., & Pannell, J.R. (2017). Range expansion compromises  
777 adaptive evolution in an outcrossing plant. *Current Biology*, 27, 2544-2551.

778 Gossmann, T.I., Song, B.-H., Windsor, A.J., Mitchell-Olds, T., Dixon, C.J., Kapralov, M.V., ...  
779 Eyre-Walker, A. (2010). Genome wide analyses reveal little evidence for adaptive  
780 evolution in many plant species. *Molecular Biology and Evolution*, 27, 1822–1832.

781 Goudet J. (2002). FSTAT, A program to estimate and test gene diversities and fixation  
782 indices. Retrieved from <http://www2.unil.ch/popgen/softwares/fstat.htm>

783 Grivet, D., Avia, K., Vaattovaara, A., Eckert, A.J., Neale, D. B., Savolainen, O., & González-  
784 Martínez, S.C. (2017). High rate of adaptive evolution in two widespread European  
785 pines. *Molecular Ecology*, 26, 6857–6870.

786 Hardy, K., Radini, A., Buckley, S., Sarig, R., Copeland, L., Gopher, A., & Barkai, R. (2016).  
787 Dental calculus reveals potential respiratory irritants and ingestion of essential plant-  
788 based nutrients at Lower Palaeolithic Qesem Cave Israel. *Quaternary International*,  
789 398, 129-135.

790 Holliday, J.A., Aitken, S.N., Cooke, J.E.K., Fady, B., González-Martínez, S.C., Heuertz, M.,  
791 Jaramillo-Correa, J.P., Lexer, C., Staton, M., Whetten, R.W., & Plomion, C. (2017).  
792 Advances in ecological genomics in forest trees and applications to genetic resources  
793 conservation and breeding. *Molecular Ecology*, 26, 706-717.

794 Jakobsson, M., & Rosenberg, N.A. (2007). CLUMPP: a software matching and permutation  
795 program for dealing with label switching and multimodality in analysis of population  
796 structure. *Bioinformatics*, *23*, 1801–1806.

797 Janes, J.K., Miller, J.M., Dupuis, J.R., Malenfant, R.M., Gorell, J.C., Cullingham, C.I., &  
798 Andrew, R.L. (2017). The  $K=2$  conundrum. *Molecular Ecology*, *26*, 3594-3602.

799 Jaramillo-Correa, J.P., Gérardi, S., Beaulieu, J., Ledig, F.T., & Bousquet, J. (2015a). Inferring  
800 and outlining past population declines with linked microsatellites: a case study in two  
801 spruce species. *Tree Genetics & Genomes*, *11*, 9.

802 Jaramillo-Correa, J.P., Rodríguez-Quilón, I., Grivet, D., Lepoittevin, C., Sebastiani, F.,  
803 Heuertz, M., Garnier-Géré, P., Alía, R., Plomion, C., Vendramin, G.G., & González-  
804 Martínez, S.C. (2015b). Molecular proxies of climate maladaptation in a long-lived  
805 tree (*Pinus pinaster* Aiton, Pinaceae). *Genetics*, *199*, 793-807.

806 Jaramillo-Correa, J.P., Grivet, D., Terrab, A., Kurt, Y., de Lucas, A.I., Wahid, N., Vendramin,  
807 G.G., & González-Martínez, S.C. (2010a). The Strait of Gibraltar as a major  
808 biogeographic barrier in Mediterranean conifers: a comparative phylogeographic  
809 survey. *Molecular Ecology*, *19*, 5452-5468.

810 Jaramillo-Correa, J.P., Verdú, M., & González-Martínez, S.C. (2010b). The contribution of  
811 recombination to heterozygosity differs among plant evolutionary lineages and life-  
812 forms. *BMC Evolutionary Biology*, *10*, 22.

813 Johannes, F., Porcher, E., Teixeira, F.K., Saliba-Colombani, V., Simon M, Agier, N., ... Colot,  
814 V. (2009). Assessing the impact of transgenerational epigenetic variation on complex  
815 traits. *PLoS Genetics*, *5*, e1000530.

816 Kern, A.D., & Hahn M.W. (2018). The Neutral Theory in Light of Natural Selection.  
817 *Molecular Biology and Evolution*, *35*, 1366-1371.

818 Kimura, M. (1968) Evolutionary rate at the molecular level. *Nature*, *217*, 624-626.

819 Laenen, B., Tedder, A., Nowak, M.D., Toräng, P., Wunder, J., Wötsel, S., ... Slotte, T. (2018).  
820 Demography and mating system shape the genome-wide impact of purifying selection  
821 in *Arabis alpina*. *Proceedings of the National Academy of Sciences of USA*, *115*, 816-821.

822 Lanfear, R., Kokko, H., & Eyre-Walker, A. (2014). Population size and the rate of  
823 evolution. *Trends in Ecology and Evolution*, *29*, 33–41.

824 Ledig, F.T, Bermejo-Velázquez, B., Hodgskiss, P.D., Johnson, D.R., Flores-López, C., &  
825 Jacob-Cervantes, V. (2000). The mating system and genic diversity in Martínez spruce,  
826 an extremely rare endemic of México's Sierra Madre Oriental: an example of

827 facultative selfing and survival in interglacial refugia. *Canadian Journal of Forest*  
828 *Research*, 30, 1156-1164.

829 Lemieux, M.J., Beaulieu, J., & Bousquet, J. (2011). Chloroplast DNA polymorphisms in  
830 eastern hemlock: range-wide genogeographic analyses and implications for gene  
831 conservation. *Canadian Journal of Forest Research*, 41, 1047-1059.

832 Liu, X., & Fu, Y.-X. (2015). Exploring population size changes using SNP frequency  
833 spectra. *Nature Genetics*, 47, 555–559.

834 Lynch, M. (1996). *A quantitative-genetic perspective on conservation issues*. In J. C. Avise  
835 & J. L. Hamrick (Eds). *Conservation genetics: case histories from nature* (pp. 471–501).  
836 New York: Chapman and Hall.

837 Marsden, C.D., Ortega-Del Vecchyo, D., O'Brien, D.P., Taylor, J.F., Ramirez, O., Vilà, C., ...  
838 Lohmuller, K.E. (2016). Bottlenecks and selective sweeps during domestication have  
839 increased deleterious genetic variation in dogs. *Proceedings of the National Academy*  
840 *of Sciences of USA*, 133, 152–157.

841 Moyers, B.T., Morrell, P.L., & McKay, J.K. (2018). Genetic costs of domestication and  
842 improvement. *Journal of Heredity*, 109, 103–116.

843 Mutke, S., Gordo, J., Khouja, M., & Fady, B. (2013). Low genetic and high environmental  
844 diversity at adaptive traits in *Pinus pinea* from provenance tests in France and Spain.  
845 *Options Méditerranéennes*, 105, 73–79.

846 Mutke, S., Vendramin, G.G., Fady, B., Bagnoli, F., González-Martínez, S.C. 2019. *Molecular*  
847 *and quantitative genetics of stone pine (Pinus pinea L.)*. In D. Nandwani (Ed.). *Genetic*  
848 *diversity in horticultural plants* (pp. 61–84). Switzerland: Springer Nature.

849 Nei, M., & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of  
850 restriction endonucleases. *Proceedings of the National Academy of Sciences of USA*, 76,  
851 5269–5273.

852 Ohta, T. (1992). The nearly neutral theory of molecular evolution. *Annual Review of*  
853 *Ecology and Systematics*, 23, 263–286.

854 Peakall, R., & Smouse, P.E. (2006). Genalex 6: genetic analysis in Excel. Population  
855 genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288-295.

856 Pedersen, C.-E. T., Lohmueller, K.E., Grarup, N., Bjerregaard, P., Hansen, T., Siegismund,  
857 H.R., Moltke, I., & Albrechtsen, A. (2017). The effect of an extreme and prolonged  
858 population bottleneck on patterns of deleterious variation: insights from the  
859 Greenlandic Inuit. *Genetics*, 205, 787–801.

860 Perdiguero, P., Barbero M.C., Cervera, M.T., Collada, C., & Soto, A. (2013). Molecular  
861 response to water stress in two contrasting Mediterranean pines (*Pinus pinaster* and  
862 *Pinus pinea*). *Plant Physiology and Biochemistry*, 67, 199-208.

863 Pérez-Jordà, G., Peña-Chocarro, L., García Fernández, M., & Vera-Rodríguez, J.C. (2017).  
864 The beginnings of fruit tree cultivation in the Iberian Peninsula: plant remains from  
865 the city of Huelva (southern Spain). *Vegetation History and Archaeobotany*, 26, 527-  
866 538.

867 Petit, R.J., & Hampe A. (2006). Some evolutionary consequences of being a tree. *Annual*  
868 *Review of Ecology, Evolution, and Systematics*, 37, 187-214.

869 Pinzauti, F., Sebastiani, F., Budde, K.B., Fady, B., González-Martínez, S.C. & Vendramin,  
870 G.G. (2012). Nuclear microsatellites for *Pinus pinea* (Pinaceae), a genetically  
871 depauperate tree, and their transferability to *P. halepensis*. *American Journal of*  
872 *Botany*, 99, e362-e365.

873 Plomion, C., Bartholomé, J., Lesur, I., Boury, C., Rodríguez-Quilón, I., Lagravelle, H., ...  
874 González-Martínez, S.C. (2016). High-density SNP assay development for genetic  
875 analysis in maritime pine (*Pinus pinaster*). *Molecular Ecology Resources*, 16, 574-587.

876 Popova, T., & Hristova, H. (2018). Trees of eternity-*Pinus pinea* L. in daily life, rituals,  
877 religion and symbolism. Archaeobotanical evidence from the territory of Bulgaria.  
878 *Journal of Archaeological Science: Reports*, 19, 987-991.

879 Potter, K.M., Jetton, R.M., Dvorak, W.S., Hipkins, V.D., Rhea, R., & Whittier, W.A. (2012).  
880 Widespread inbreeding and unexpected geographic patterns of genetic variation in  
881 eastern hemlock (*Tsuga canadensis*), an imperiled North American conifer.  
882 *Conservation Genetics*, 13, 475-498.

883 Prada, M.A., Gordo, J., de Miguel, J., Mutke, S., Catalán-Bachiller, G., Iglesias, S., & Gil, L.  
884 (1997). *Las regiones de procedencia de Pinus pinea L. en España*. Madrid: Ministerio de  
885 Medio Ambiente, Organismo Autónomo Parques Nacionales.

886 Presgraves, D.C. (2005). Recombination enhances protein adaptation in *Drosophila*  
887 *melanogaster*. *Current Biology*, 15, 1651-1656.

888 Pritchard, J.K., Stephens, M., & Donnelly, P. (2000) Inference of population structure  
889 using multilocus genotype data. *Genetics*, 155, 945-959.

890 Pritchard, J.K., Pickrell, J.K., & Coop, G. (2010). The genetics of human adaptation: hard  
891 sweeps, soft sweeps, and polygenic adaptation. *Current Biology*, 20, R208-R215.

892 Richardson, D.M. (ed.). (1998). *Ecology and Biogeography of Pinus*. Cambridge:  
893 Cambridge University Press.

894 Robinson, J.A, Ortega-Del-Vecchio D., Fan, Z., Kim, B.Y., vonHoldt, B.M., Marsden, C.D.,  
895 Lohmuller, K.E., & Wayne, R.K. (2016). Genomic flatlining in the endangered island  
896 fox. *Current Biology*, 26, 1183-1189.

897 Ross-Davis, A., Ostry, M., & Woeste, K.E. (2008). Genetic diversity of butternut (*Juglans*  
898 *cinerea*) and implications for conservation. *Canadian Journal of Forest Research*, 38,  
899 899-907.

900 Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-  
901 Onsins, S.E, & Sánchez-Gracia, A. (2017). DnaSP v6: DNA sequence polymorphism  
902 analysis of large datasets. *Molecular Biology and Evolution*, 34, 3299-3302.

903 Rubiales, J.M., García-Álvarez, S., García-Amorena, I., Hernández, L., Morales-Molino, C.,  
904 Moreno, E., & Gómez-Manzaneque, F. (2011) Palaeobiogeographical perspectives on  
905 *Pinus pinea* L.: a controversial and enigmatic Mediterranean pine. 5<sup>th</sup> Biennial  
906 Conference of the International Biogeography Society, Irakleion, Greece.

907 Sáez-Laguna, E., Guevara, M.-Á., Díaz, L.-M., Sánchez-Gómez, D., Collada, C., Aranda, I., &  
908 Cervera, M.T. (2014). Epigenetic variability in the genetically uniform forest tree  
909 species *Pinus pinea* L. *PLoS ONE*, 9, e103145.

910 Saladin, B., Leslie, A.B., Wüest R.O., Litsios, G., Conti, E., Salamin, N. & Zimmermann, N.E.  
911 (2017). Fossils matter: improved estimates of divergence times in *Pinus* reveal older  
912 diversification. *BMC Evolutionary Biology*, 17, 95.

913 Sánchez-Gómez, D., Velasco-Conde, T., Cano-Martín, F.J., Ángeles-Guevara, M.A., Cervera,  
914 M.T., & Aranda, I. (2011). Inter-clonal variation in functional traits in response to  
915 drought for a genetically homogeneous Mediterranean conifer. *Environmental and*  
916 *Experimental Botany*, 70, 104–109.

917 Smith, N. G., & Eyre-Walker, A. (2002). Adaptive protein evolution in *Drosophila*. *Nature*,  
918 415, 1022–1024.

919 Tajima, F. (1983). 3. Evolutionary relationship of DNA sequences in finite populations.  
920 *Genetics*, 105, 437-460.

921 Vendramin, G.G., Fady, B., González-Martínez, S.C, Hu, F.-S., Scotti, I., Sebastiani, F., Soto,  
922 A., & Petit, R.J. (2008). Genetically depauperate but widespread: the case of an  
923 emblematic Mediterranean pine. *Evolution*, 62, 680–688.

- 924 Wallace, J.G., Rodgers-Melnick, E., & Buckler, E.S. (2018). On the road to Breeding 4.0:  
925 Unraveling the Good, the Bad, and the Boring of crop quantitative genomics. *Annual*  
926 *Review of Genetics*, 52, 421–444.
- 927 Walter, R., & Epperson, B.K. (2005). Geographic pattern of genetic diversity in *Pinus*  
928 *resinosa*: contact zone between descendants of glacial refugia. *American Journal of*  
929 *Botany*, 92, 92-100.
- 930 Watterson, G. A. (1975). On the number of segregating sites in genetical models without  
931 recombination. *Theoretical Population Biology*, 7, 256–276.
- 932 Wei, X., Zhao, L., Lascoux, M., & Waxman, D. (2015). Population structure and the rate of  
933 evolution. *Journal of Theoretical Biology*, 365, 486–495.
- 934 Weissman, D.B. & Barton, N.H. (2012). Limits to the rate of adaptive substitution in  
935 sexual populations. *PLoS Genetics*, 8, e1002740.
- 936 Welch, J.J., Eyre-Walker, A., & Waxman, D. (2008). Divergence and polymorphism under  
937 the nearly neutral theory of molecular evolution. *Journal of Molecular Evolution*, 67,  
938 418–426.
- 939 Westbrook, J.W., Chhatre, V.E., Wu, L.-S., Chamala, S., Gomide-Neves, L. Muñoz, P., ... Echt,  
940 C.S. (2015). A consensus genetic map for *Pinus taeda* and *Pinus elliottii* and extent of  
941 linkage disequilibrium in two genotype-phenotype discovery populations of *Pinus*  
942 *taeda*. *G3: Genes, Genomes, Genetics*, 5, 1685-1694.
- 943 Wilcox, G.H. (1977). Exotic plants from Roman waterlogged sites in London. *Journal of*  
944 *Archaeological Science*, 4, 269-282.
- 945 Willyard, A., Cronn, R., & Liston, A. (2009). Reticulate evolution and incomplete lineage  
946 sorting among the ponderosa pines. *Molecular Phylogenetics and Evolution*, 52, 498–  
947 511.
- 948 Zhang, M., Zhou, L., Bawa, R., Suren, H., & Holliday, J.A. (2016). Recombination rate  
949 variation, hitchhiking, and demographic history shape deleterious load in poplar.  
950 *Molecular Biology and Evolution*, 33, 2899–2910.

951

952 **Legends to Figures**

953 **FIGURE 1** Stone pine (*P. pinea*) distribution (green areas) and location of populations  
954 sampled for nuSSR (circles) and DNA sequence analyses (triangles). Circle color  
955 indicates the number of private alleles ( $A_{PT}$ ) found for nuSSRs.

956

957 **FIGURE 2 (A)** Geographic distribution of mean genetic-cluster membership for stone  
958 pine (*P. pinea*) populations, as obtained from STRUCTURE analysis of nuSSR variation  
959 when assuming two genetic clusters ( $K = 2$ ). **(B)** Individual bar-plot of genetic-cluster  
960 membership coefficients for the same analysis. **(C)** Distribution of  $\Delta K$  values for the  
961 various numbers of genetic clusters ( $K$ ) assumed in STRUCTURE analyses. See Table S1 for  
962 population codes and additional details.

963

964 **FIGURE 3** Genetic divergence and demographic history of stone pine (*P. pinea*). **(A)**  
965 Phylogenetic relationship and divergence times from maritime (*P. pinaster*) and loblolly  
966 (*P. taeda*) pines as determined from the Bayesian analyses of 50 nuclear gene-loci.  
967 Numbers and shaded rectangles over branches represent mean and 95% credible  
968 intervals (CI) for divergence time estimates (in Ma), respectively. All nodes had  
969 posterior probabilities of 1.0. **(B, C)** Changes of population size over time for maritime  
970 **(B)** and stone **(C)** pines inferred with a stairway-plot derived from the site frequency  
971 spectrum (SFS) of 55,833 DNA-sequence sites. Dark and light lines represent the median  
972 and 95% CI, respectively. Estimates based on the assumption of a mutation rate of  $1.64 \times$   
973  $10^{-9}$  per site per year, and a generation time of 25 years. Blue areas denote glacial  
974 periods; those mentioned in the text are indicated on top of **B**. **(D)** Schematic  
975 representation of the best demographic scenario for stone pine according to DIYABC

976 (based on the variation of 13 nuSSRs), and population parameters estimated from this  
977 scenario (see Fig. S1 for all scenarios).

978

979 **FIGURE 4 (A)** Shared and non-shared (exclusive) mean number of SNPs found in 177  
980 gene-loci in stone (*P. pinea*) and maritime (*P. pinaster*) pines after their divergence from  
981 loblolly pine (*P. taeda*). Exclusive SNPs are further subdivided in synonymous and non-  
982 synonymous (replacements); non-synonymous variants are additionally separated in  
983 putatively neutral and deleterious, according to functional effects inferred with PROVEAN  
984 (Choi and Chan 2015). The mean number of fixed deleterious SNPs per species is also  
985 indicated. **(B)** Proportion of (non-shared) non-synonymous substitutions fixed by  
986 adaptive evolution ( $\alpha$ ) and relative rate of (non-shared) adaptive substitutions ( $\omega_a$ ) per  
987 species. **(C)** Distribution of fitness effects (DFE) of exclusive mutations in each taxa.

988

989

990 **TABLE 1** Mean DNA sequence diversity and divergence from loblolly pine (*P. taeda*)  
 991 estimated from 177 common gene-loci (representing 55,833 bp) in stone (*P. pinea*) and  
 992 maritime (*P. pinaster*) pines

993

Parameter*	Stone pine	Maritime pine
$\theta_w$ ( <i>SD</i> )	0.00015 (0.00055)	0.00231 (0.00212)
$\pi$ ( <i>SD</i> )	0.00021 (0.00125)	0.00242 (0.00243)
$\pi_a$ ( <i>SD</i> )	0.00019 (0.00126)	0.00029 (0.00407)
$\pi_s$ ( <i>SD</i> )	0.00043 (0.00353)	0.00454 (0.00474)
$\pi_a/\pi_s$ ( <i>SD</i> )	0.4487 (0.5238)	0.2561 (0.4781)
$D_{xy}$ ( <i>SD</i> )	0.0168 (0.0115)	0.0164 (0.0117)
$K_a$ ( <i>SD</i> )	0.0099 (0.0114)	0.0101 (0.0115)
$K_s$ ( <i>SD</i> )	0.0382 (0.0289)	0.0384 (0.0317)
$K_a/K_s$ ( <i>SD</i> )	0.4598 (1.0666)	0.3315 (0.5126)

994 \* Abbreviations: *SD*, standard deviation;  $\theta_w$ , average Watterson's nucleotide diversity  
 995 per site;  $\pi$ , average Tajima's nucleotide diversity per site;  $\pi_a$ , average  $\pi$  for non-  
 996 synonymous sites;  $\pi_s$ , average  $\pi$  for synonymous sites;  $\pi_a/\pi_s$ , mean per gene ratio of  
 997 non-synonymous to synonymous nucleotide diversity;  $D_{xy}$ , average pairwise divergence;  
 998  $K_a$ , average number of non-synonymous substitutions per non-synonymous site;  $K_s$ ,  
 999 average number of synonymous substitutions per synonymous site;  $K_a/K_s$ , mean per  
 1000 gene ratio of non-synonymous to synonymous substitutions. The last three estimates  
 1001 were determined using the Jukes-Cantor correction.

**TABLE 2** Genetic diversity (cpDNA, cpSSR, and nuSSR) in genetically depauperate but widespread plants, including stone pine. Clonal and self-fertilizing plants were excluded.

Species	Markers	$H_0$	$H_E$	$H_S$	$H_T$	References
<i>Pinus resinosa</i>	9 cpSSR			0.543	0.618	Echt et al. 1998
	10 cpSSR		0.152			Walter and Epperson 2005
	5 nSSR	0.185	0.508			Boys et al. 2005
<i>Pinus pinea</i>	3 cpSSR				0.019	Vendramin et al. 2008
	9 nSSR	0.083	0.114			<i>This study</i>
<i>Juglans cinerea</i>	7 nSSR	0.760	0.750		0.762	Ross-Davis et al. 2008
	7 nSSR	0.659	0.691			Boraks and Broders 2016
<i>Tsuga canadensis</i>	4 cpDNA loci		0.727			Lemieux et al. 2011
	3 cpSSR		0.672			Lemieux et al. 2011
	13 nSSR	0.566	0.526			Potter et al. 2012







