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1 **High rate of adaptive evolution in two widespread European pines**

2

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26 evolution

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33 Running title (45 char including spaces): Adaptive evolution in two European pines

34 **Abstract** (≤ 250 words),

35

36 Comparing related organisms with differing ecological requirements and evolutionary
37 histories can shed light on the mechanisms and drivers underlying genetic adaptation. Here,
38 by examining a common set of hundreds of loci, we compare patterns of nucleotide
39 diversity and molecular adaptation of two European conifers (Scots pine and maritime pine)
40 living in contrasted environments and characterized by distinct population genetic structure
41 (low and clinal in Scots pine, high and ecotypic in maritime pine) and demographic
42 histories. We found higher nucleotide diversity in Scots pine than in maritime pine, whereas
43 rates of new adaptive substitutions (ω_a), as estimated from the Distribution of Fitness
44 Effects (DFE), were similar across species, and among the highest found in plants. Sample
45 size and population genetic structure did not appear to have resulted in any significant bias
46 in ω_a . Moreover, the species-specific population contraction-expansion dynamics did not
47 seem to have affected differentially the rate of adaptive substitution in these two pines.
48 Several methodological and biological factors may underlie the unusually high rate of
49 adaptive evolution of Scots pine and maritime pine. By providing two new case studies with
50 contrasting evolutionary history, we contribute to disentangling the multiple factors
51 potentially affecting adaptive evolution in natural plant populations.

52 **Introduction**

53

54 Understanding of the mechanisms of plant adaptation has been advanced through
55 comparative studies of species differing in demographic history (Slotte *et al.* 2010),
56 effective population size (Gossmann *et al.* 2012; Strasburg *et al.* 2011), genetic structure
57 among populations (Wright & Andolfatto 2008), ecological conditions (Tellier *et al.* 2011),
58 or phylogenetic relationships (Eckert *et al.* 2013a; Grivet *et al.* 2013; Palmé *et al.* 2009).
59 For long-lived species such as most forest trees, unraveling adaptive processes is
60 challenging, as during their long life-cycle, individuals experience different selective
61 pressures that accumulate over time. Genomic research in forest trees, moreover, has been
62 hindered because of large genome sizes and the lack of model species. Nevertheless, recent
63 advances in tree genomics and reanalysis of common garden experiments have fostered a
64 body of literature that provides insights into the mechanisms underlying forest tree
65 adaptation in space and time. Emerging from these studies are the ideas that forest trees are
66 locally adapted to different environmental conditions, especially to temperature,
67 photoperiod, drought, or biotic stress (see examples in Alberto *et al.* 2013a; Savolainen *et al.*
68 *et al.* 2007), and that they respond to contrasted selection pressure across life stages (Alía *et al.*
69 *et al.* 2014). Based on this knowledge, it is of prime interest to identify major environmental
70 drivers of adaptation, as well as the genes involved in the process, as they can help forecast
71 the future distribution of these ecologically important species in the face of climate change.

72 Identifying footprints of natural selection within genomes is complex, as the
73 observed patterns of polymorphism may result from many distinct yet interacting
74 evolutionary forces, including neutral processes such as migration or genetic drift, all acting
75 on variation generated by mutation. Because demography and selection can leave similar
76 patterns within genomes (Biswas & Akey 2006; Excoffier *et al.* 2009; Nielsen 2005), tests

77 aiming at attributing these patterns to the action of natural selection must take into account
78 the demographic history of the species, as well as the complex interactions between these
79 two processes (Li *et al.* 2012; Schrider & Kern 2016). Tests robust to demography often
80 contrast polymorphism within species with divergence between species. For example, the
81 popular McDonald-Kreitman (MK) test (McDonald & Kreitman 1991) compares the
82 amount of polymorphism to divergence for categories of sites that are expected to evolve
83 differently (e.g., synonymous/silent vs. non-synonymous). From the original MK test,
84 several extensions have been developed. Two of the most popular are the MKPRF test
85 (Bustamante *et al.* 2002, 2003, 2005), which is more powerful but is based on specification
86 of a population genetic model, and the more recent SnIPRE test (Eilertson *et al.* 2012),
87 which is a nonparametric method that does not require estimation of population genetic
88 parameters.

89 Other methods based on the MK test provide overall estimates of adaptive evolution
90 that can be correlated with population parameters (e.g., effective population size; Gossmann
91 *et al.* 2012) or environmental characteristics (e.g., Lourenço *et al.* 2013) in comparative
92 studies. First, the Distribution of Fitness Effects (DFE) of new mutations at functional sites
93 is estimated from polymorphism data and under a specific demographic scenario. Second,
94 the number of substitutions originating from neutral and slightly deleterious mutations is
95 predicted from the estimated DFE (e.g., the method II of Eyre-Walker & Keightley 2009).
96 Any excess of substitutions (with respect to the neutral expectation) can then be attributed
97 to directional selection, as measured by α (the proportion of adaptive nucleotide
98 substitutions) or ω_a (the relative rate of adaptive substitutions scaled by the rate of neutral
99 substitution) (Bierne & Eyre-Walker 2004; Eyre-Walker & Keightley 2009; Smith & Eyre-
100 Walker 2002). Although α has been extensively used (e.g., (Eckert *et al.* 2013a; Gossmann
101 *et al.* 2010; Slotte *et al.* 2010; Strasburg *et al.* 2011), this estimate is of limited value for

102 estimating the efficiency of the adaptive process, because α is also influenced by the rate of
103 non-adaptive substitutions (Gossmann *et al.* 2012). The parameter ω_a , which is roughly
104 equivalent to K_a (the number of non-synonymous substitutions per non-synonymous site),
105 is thus more appropriate for comparing adaptive evolution across genomic regions or
106 species (Gossmann *et al.* 2012; Lourenço *et al.* 2013).

107 In the present study, we assess the effect of distinct demographic histories and
108 selection regimes on inferences of adaptive evolution for two widespread conifer species.
109 Scots pine (*Pinus sylvestris* L.) is widely and continuously distributed in Eurasia, occupying
110 regions that differ greatly in climate (Krakau *et al.* 2013). Its demographic history is
111 characterized by an ancient bottleneck (Kujala & Savolainen 2012; Pyhäjärvi *et al.* 2007)
112 and limited population genetic structure, which is only found along the margins of its wide
113 range (Gullberg 1985; Kujala & Savolainen 2012; Pyhäjärvi *et al.* 2007). The absence of
114 genetic structure shown by molecular markers across much of its range, however, is not
115 indicative of a lack of quantitative trait variation across populations (Andersson &
116 Fedorkov 2004; García-Gil *et al.* 2003; Notivol *et al.* 2007; Oleksyn *et al.* 1998). In
117 particular, strong clines in photoperiod-related traits are often observed across latitudinal
118 gradients in the species (e.g., for timing of growth cessation and budset; see Beck *et al.*
119 2004; Hurme *et al.* 1997; Mikola 1982; Oleksyn *et al.* 1998).

120 In contrast to Scots pine, maritime pine (*Pinus pinaster* Ait.) is patchily distributed
121 across the western Mediterranean Basin and the Atlantic regions of Portugal, Spain and
122 France. This conifer grows in warm temperate regions with an oceanic influence on climate
123 (Abad Viñas *et al.* 2016), and is particularly well adapted to dry and fire-prone
124 environments. Its demographic history is characterized by a more recent bottleneck relative
125 to Scots pine (Naydenov *et al.* 2014), and it has a strong genetic structure among
126 populations across its range (e.g., Burban and Petit 2003; Bucci *et al.* 2007; Jaramillo-

127 Correa et al. 2015) that is accompanied by morphological and physiological differences
128 (Kremer and Roussel 1986; Alía et al. 1995; Santos del Blanco et al. 2010; Lamy et al.
129 2011; Corcuera et al. 2012), defining various subspecies and eco-types (Richardson 1998).
130 This genetic structure likely results from post-Pleistocene events (Burban and Petit 2003;
131 Bucci et al. 2007; Naydenov et al. 2014), including adaptation to local climate (Serra-
132 Varela et al. 2015).

133 Given the large differences between Scots pine and maritime pine with respect to
134 their climatic niches, we focused on genes for general responses to different type of abiotic
135 stress. This is also consistent with previous studies ranking the importance of different
136 fitness-related traits for local adaptation in the species (for Scots pine see Castro *et al.* 2002;
137 Galiano *et al.* 2010; Ryyppö *et al.* 1998; and for maritime pine see Corcuera *et al.* 2011;
138 Gaspar *et al.* 2013; Lamy *et al.* 2014; Ramírez-Valiente & Robledo-Arnuncio 2014). Then,
139 we used complementary methods to get insights into the action of selective forces, both at
140 specific genes and genome-wide, and considered the specific demographic, ecological and
141 historical settings of each species to discuss the possible factors (both methodological and
142 biological) that may explain our results. Our hypotheses are: i) Scots pine has high
143 nucleotide diversity resulting from its large distribution and expected large effective
144 population size; ii) Large effective population size in Scots pine would have also resulted in
145 higher efficiency of selection and thus a higher number of fixed adaptive substitutions (see
146 also Gossmann *et al.* 2012); and iii) the highly fragmented distribution in maritime pine
147 would have resulted in lower levels of overall adaptive evolution. Scots pine and maritime
148 pine, with their contrasting characteristics, allow for exploring how different biological
149 factors may interact with natural selection and adaptive evolution in plants.

150

151

152 **Materials and methods**

153

154 *Sampling*

155 Scots pine was sampled across 8-10 populations from its western range (Figure 1 and Table
156 S1) for eight loci from six candidate genes for abiotic stress response and phenology (3-20
157 individuals per population, and a total of 36-115 individuals per locus), and across a smaller
158 number of populations (7) for the much larger CRSP (Comparative Re-Sequencing in
159 Pinaceae initiative; <http://dendrome.ucdavis.edu/NealeLab/crsp/>; see Wegrzyn *et al.* 2008)
160 set of 364 loci (1-2 individuals per population, and a total of 6-12 individuals per locus). In
161 a first phase, maritime pine was sampled across 10-12 populations from its full range
162 (Figure 1 and Table S1) for the same eight abiotic stress response and phenology loci as for
163 Scots pine (1-19 individuals per population for a total of 49-100 individuals per locus), and
164 across 11 populations for the CRSP gene dataset (1-3 individuals per population for a total
165 of 6-14 individuals per locus). More details on the gene datasets are presented in the
166 *Sequence data* section. In a second phase, another dataset produced in maritime pine
167 comprising 128 loci common with the CRSP dataset (2-4 individuals per population for a
168 total of 28-36 individuals per locus) was obtained within the Conifer Re-sequencing
169 Initiative for European Conifers (CRIEC, www.evoltree.eu; Figure 1 and Table S1). This
170 dataset with more individuals per population was examined in order to study the effect of
171 sample size and population structure on the DFE-based estimates. Finally, the CRSP and the
172 CRIEC datasets were combined for the 128 loci in common (2-6 individuals per population
173 for a total of 34-49 individuals per locus) to reach a bigger sample size for the statistical
174 analyses (“extended CRSP” dataset). Sequence datasets used for the different analyses are
175 detailed in Figure S1.

176 Trees were sampled within populations following standard protocols to avoid
177 sampling related trees (i.e., leaving a minimum distance of 50 m between sampled
178 individuals) and without any phenotypic selection.

179

180 *Sequence data*

181 Sequence alignment and editing

182 Eight loci from previously studied candidate genes, including two full-length genes (*coLI*
183 and *gia*), were amplified with available primers from different sources (see [Table S2](#)).
184 Another 1,600 gene amplicons (hereafter referred as the CRSP gene set) were obtained by
185 resequencing in seven conifers (including Scots pine and maritime pine) some loci
186 originally developed in loblolly pine, *Pinus taeda* (Eckert *et al.* 2013b). For both gene sets,
187 DNA sequences were obtained by direct sequencing from haploid seed megagametophytes.
188 In this way, (i) phase is directly known and does not need to be estimated and (ii) co-
189 amplification of paralogs, a common problem in plant species with large genomes such as
190 conifers, is more easily detected. For the CRSP gene set, loci with at least six successfully
191 sequenced individuals in both Scots pine and maritime pine were accepted for further
192 analyses. This led to 491 loci that were subsequently checked manually and edited with
193 Codon Code Aligner (CodonCode Corporation, Centerville, MA, USA) or Sequencher 4.7
194 (Gene Codes Corporation, Ann Arbor, MI, USA). Low quality sequences were removed, as
195 well as putative paralogous genes based on phylogenies including the three pines (the two
196 target species together with loblolly pine), as detected by PRANK runs with default
197 parameters (Löytynoja & Goldman 2005). This last quality-filtering step led to a set of 389
198 loci common to the two target species.

199

200 Annotation

201 Gene annotation for all loci was obtained from homology with loblolly pine EST contigs
202 and the NCBI reference protein database using Geneious version 6.1 (Biomatters,
203 Auckland, New Zealand). All 389 genes had high sequence similarity with loblolly pine
204 genes, suggesting low copy-number genes and orthology across the different species. The
205 biological function of the genes was determined based on their homology with the
206 *Arabidopsis thaliana* and/or *Pinus taeda* protein database with an E-value threshold of 10^{-10}
207 (see [Table S3](#)) and the Protein Knowledgebase – UniProtKB
208 (<http://www.uniprot.org/uniprot/>). Of the 389 genes, 364 could be annotated, leading to a
209 total of 372 annotated loci in the two species when adding the eight previously-studied
210 candidate gene loci. Loblolly pine was used as outgroup when needed.

211

212 Target and reference genes

213 The 372 loci under study, as well as their annotations, are publicly available (NCBI
214 GenBank: MF385275-MF385581 and MF385585-MF397901). From these 372 loci, 64
215 were selected as target genes, including the eight previously-studied candidate gene loci
216 (see [Table S2](#) for details and references) and 56 other annotated loci selected from the
217 CRSP dataset (see above). All 64 target genes had highly confident annotation associated to
218 adaptive traits: 48 loci related to abiotic stress responses (mostly cold, heat, drought, salt
219 and other oxidative stresses); and 16 loci related to photosynthesis and photosystem (9 loci),
220 and phenology (7 loci). Only target genes were tested for footprints of selection.

221 The reference genes consisted of all other loci (308) from the CRSP dataset,
222 including loci with unknown function or loci for which there is no evidence of involvement
223 in forest tree adaptation. Because of implementation limitations and statistical power
224 requirements (i.e., the minimum number of required segregating sites) in the MKPRF test
225 (see below), reference genes were combined for this analysis into compound loci. All loci

226 with a known position on a genetic map (loblolly pine, Eckert *et al.* 2010; maritime pine,
227 Chancerel *et al.* 2011; and our own unpublished genetic map of Scots pine) were grouped
228 according to the linkage groups forming 28 compound loci (see [Table S4](#)). To balance the
229 number of loci used in the MKPRF test (64 target versus 28 compound reference loci), we
230 used also 36 other reference loci selected at random among those reference genes not
231 included in the compound loci to also reach 64 loci for the reference group in which the
232 statistical power was mainly driven by the compound loci (higher number of segregating
233 sites).

234

235 *Nucleotide diversity, genetic divergence and overall patterns of polymorphism*

236 Nucleotide diversity π (Nei & Li 1979) and Watterson's θ_w (Watterson 1975) were
237 calculated for all sites, as well as separately for silent, synonymous and non-synonymous
238 sites, in Scots pine and maritime pine. For each species, the divergence from loblolly pine
239 was characterized by the number of synonymous substitutions per synonymous site (K_s) and
240 of non-synonymous substitutions per non-synonymous site (K_a), both with Jukes-Cantor
241 correction, and their ratio (K_a/K_s), as well as the statistics of shared and fixed segregating
242 sites requested by some of the neutrality tests (see below).

243 To identify overall differences in patterns of polymorphism across species, different
244 statistics were computed based on the observed Site Frequency Spectrum (SFS): Tajima's D
245 (Tajima 1989), which evaluates the difference between low- and intermediate-frequency
246 variants; Zeng *et al.*'s E (Zeng *et al.* 2006), which evaluates the difference between low-
247 and high-frequency variants; and the normalized Fay and Wu's H (H_n ; Zeng *et al.* 2006),
248 which evaluates the difference between intermediate- and high-frequency variants. These
249 statistics were computed, separately, for the 64 target loci and the 308 reference loci, and
250 compared with those under a standard neutral equilibrium model (constant population size).

251 All nucleotide diversity statistics were computed using MANVa and mstatspop
252 (<https://bioinformatics.cragenomica.es/numgenomics/people/sebas/software/software.html>),
253 and, when needed, the SDMTools package in R was used to correct for sample size
254 variation across loci, by weighting means and standard deviations.

255

256 *Neutrality tests robust to demography*

257 To identify potential genes under selection, we first used the SnIPRE approach (Eilertson *et*
258 *al.* 2012), which considers polymorphism and divergence data from synonymous/silent and
259 non-synonymous sites under a Poisson Random Effect model. This method is based on the
260 McDonald-Kreitman test, and it can reliably identify genes under weak and strong negative
261 as well as positive selection, without requiring the specification of a population genetic
262 model. We considered both the selection (specific selection effect of a gene relative to
263 neutrality) and constraint (proportion of non-synonymous mutations that are non-lethal, thus
264 having effects on counts) effects provided by the program. The selection effect is useful to
265 identify selection on mildly deleterious and advantageous mutations, while the constraint
266 effect is useful to identify strong negative or purifying selection (Eilertson *et al.* 2012).
267 Thus, significant effects on sequence data are classified as being neutral, negative or
268 positive.

269 Second, we used the McDonald-Kreitman Poisson Random Fields (MKPRF) test
270 (Bustamante *et al.* 2002, 2005), which implements a Markov chain Monte Carlo (MCMC)
271 Bayesian approach to estimate parameters of the Poisson Random Field (PRF) model
272 (Sawyer & Hartl 1992), allowing to compare groups of genes for selection signatures.
273 MKPRF not only identifies non-neutrally evolving loci but it also estimates the associated
274 strength of selection ($\gamma=2N_e s$). The MKPRF analysis was run using the *mkprf* program
275 (kindly provided by Carlos D. Bustamante and Adam Boyko), with the following

276 parameters: 10 independent MCMC chains per run and 10,000 samples from each chain
277 drawn on every 10 steps, after burn-in of 1,000 steps. Default values were used for prior
278 distributions and other MCMC parameters. Convergence of the MCMC runs was checked
279 in the MCMC output files generated by the program.

280

281 *Distribution of fitness effects (DFE) and adaptive evolution*

282 We used a method based on the DFE to estimate the overall rates of adaptive substitutions
283 (ω_a): the method II of Eyre-Walker and Keightley (2009) as implemented in the DoFE 3.1
284 program. This method accounts for demographic changes that can affect the shape of the
285 SFS by comparing the observed folded SFS at neutral sites to the folded SFS expected from
286 neutral mutations in a stationary population at equilibrium and assumes that demography
287 has the same proportional effect on the SFS of selected sites.

288 Input files were created using Python and Perl scripts kindly provided by Jaakko
289 Tyrmi and Adrian Schneider, respectively (available upon request). To build the input files
290 for the method II of Eyre-Walker and Keightley (2009), we computed the statistics for non-
291 synonymous sites and synonymous sites, at the intraspecific level (for Scots pine and
292 maritime pine, respectively) and at the interspecific level (using loblolly pine as outgroup
293 for each target pine) using the same number of alleles per species and dataset: 305 loci with
294 10 alleles each for Scots pine and 291 loci with 11 alleles for maritime pine for the CRSP
295 datasets; 126 loci with 23 alleles each for the CRIEC dataset. The other input statistics were
296 then computed with MANVa (see above) using the same reduced datasets.

297 Loblolly pine was used as outgroup for both Scots pine and maritime pine and, thus,
298 DFE-based estimates of adaptive evolution for these two pine species were done along a
299 partially shared branch. In phylogenetically close species, shared divergence with the
300 outgroup may result in differences in ω_a mainly due to differences in within-species

301 polymorphism, i.e., estimates can be biased if the nucleotide divergence between the
302 species is low relative to within species variation (Keightley & Eyre-Walker 2012). To
303 exclude this potential source of bias, shared divergence between loblolly pine and Scots
304 pine/maritime pine was estimated using two approaches. First, one, three or five sequences
305 from each locus and species were randomly sampled and concatenated, leading to three
306 aligned fasta files. Aligned fasta files were subsequently used to build unrooted
307 phylogenetic trees using PhyML (Guindon & Gascuel 2003). The analyses were run with
308 default parameters, using the General Time Reversible (GTR) model (Tavaré 1986), and for
309 all sites, and both 0-fold and 4-fold degenerate sites. The proportion of shared divergence
310 was estimated then as the fraction of the sum of branch lengths due to the common branch
311 leading to loblolly pine (i.e., the common branch leading to loblolly pine divided by the
312 total sum of branch lengths). Second, fixed differences between loblolly pine and both Scots
313 pine and maritime pine were counted, and shared fixed differences were directly obtained
314 for all sites as well as for 0-fold and 4-fold degenerate sites.

315

316

317 **Results**

318

319 *Nucleotide diversity, genetic divergence and overall patterns of polymorphism*

320 The two pines showed a significant difference (non-overlapping 95% confidence intervals)
321 in nucleotide diversity, with Scots pine ($\theta_{w-syn}=0.00687$, 95% CI: 0.00594-0.00781)
322 displaying 1.64 times the nucleotide diversity of maritime pine ($\theta_{w-syn}=0.00419$, 95% CI:
323 0.00362-0.00476) (Table 1). In both species, the overall K_a , K_s , and K_a/K_s ratios (relative to
324 loblolly pine) were similar, with overlapping 95% confidence intervals (data not shown),
325 indicating equal divergence from loblolly pine (K_s of 0.0323 for Scots pine and of 0.0375

326 for maritime pine) and similar rates of evolutionary constraint (Table 1). Scots pine and
327 maritime pine only shared about 2-4% of their synonymous polymorphic sites (depending
328 on the species used as outgroup).

329 Statistics based on the SFS (Fay & Wu 2000; Tajima 1989; Zeng *et al.* 2006)
330 revealed different polymorphism patterns in the two pines (Table 2). Scots pine displayed
331 an excess of low-frequency variants compared to intermediate-frequency (Tajima's D) and
332 high-frequency (Zeng *et al.*'s E) variants, but no differences between intermediate- and
333 high-frequency (Fay and Wu's H_n) variants. In contrast, maritime pine displayed no
334 difference of intermediate- with respect to low-frequency variants (Tajima's D), and an
335 excess of high-frequency variants compared to low-frequency (Zeng *et al.*'s E) and
336 intermediate-frequency (Fay and Wu's H_n) variants. Thus, overall, Scots pine displayed an
337 excess of low-frequency variants, while maritime pine showed an excess of high-frequency
338 variants. This pattern is present in both target and reference loci suggesting that
339 demographic processes (and not selective processes) underlie the observed differences
340 between the two pines. The impact of population structure on the statistics is reflected
341 especially by Fay and Wu's H_n , which is more sensitive to population subdivision (Zeng *et al.*
342 *al.* 2006), and that deviates more strongly from the standard neutral model in the species
343 with stronger population structure (i.e., maritime pine).

344

345 *Neutrality tests robust to demography*

346 The robust-to-demography SnIPRE method (Empirical Bayes and Bayesian SnIPRE tests)
347 suggested four and three target genes as being under selection in Scots pine (3 positive and
348 1 negative selection events) and maritime pine (2 positive and 1 negative selection events),
349 respectively (Table 3 and Table S5). Overall, the 64 target loci had a positive average
350 selection effect in Scots pine, while in maritime pine such effect was not observed (Figure

351 S2). All genes found under selection with the MKPRF test but one for Scots pine (five and
352 two for Scots pine and maritime pine, respectively) were among those revealed by the
353 SnIPRE method (Table 3). As expected, a higher proportion of loci from the previously-
354 studied candidate gene dataset (see *Material and Methods*) was found under selection (50%
355 and 25% based on the two methods in Scots pine and maritime pine, respectively) compared
356 to the CRSP dataset (0.27% and 0.55%, respectively). Two of these genes were common to
357 both pines using the two methods, with the same pattern of negative (*dhn1*) and positive
358 (*coLI*) selection.

359

360 *Distribution of fitness effects (DFE) and adaptive evolution*

361 The inferred DFE using DoFE was similar for the two pines, with most of the mutations
362 being strongly deleterious and subject to purifying selection (Figure 2). However, there
363 were also some differences in the DFE spectrum. Scots pine had a significantly higher
364 proportion of deleterious mutations for the three classes with the least deleterious mutations,
365 while the opposite pattern was observed at the other extreme of the DFE spectrum with
366 about 77% of mutation being highly deleterious for Scots pine and about 85% for maritime
367 pine. Increasing the sample size for maritime pine (the “extended CRSP” dataset, see
368 *Sampling* section) resulted in higher proportion of the three classes with the least deleterious
369 mutations but still in lower proportion compared to Scots pine, while the pattern was the
370 opposite for highly deleterious mutations (Figure 2). In terms of adaptive substitutions, both
371 species displayed a relative rate significantly different from zero (Scots pine $\omega_a = 0.1156$
372 vs. maritime pine $\omega_a = 0.1535$), with no significant differences between them (overlapping
373 95% CIs, see Figure 2). Increasing the sampling size for maritime pine (“extended CRSP”
374 dataset) led to a slightly smaller ω_a (0.1447), not different from that estimated with the
375 CRSP datasets for Scots pine and maritime pine (Figure 2). Estimating ω_a with a simple

376 and non-parametric method $\omega_a = \frac{d_N - d_S(P_N/P_S)}{d_S}$ (Gossmann *et al.* 2010; Kousathanas *et al.*
377 2014) gave $\omega_a = -0.0740$ with 95% CIs (-0.2430; 0.0871) for Scots pine and $\omega_a = 0.0976$
378 with 95% CIs (-0.0778; 0.2867) for maritime pine. These estimates confirmed the trend of a
379 higher ω_a in maritime pine than in Scots pine, albeit differences were still not significant
380 (i.e., overlapping CIs).

381 Shared divergence between loblolly pine and Scots pine/maritime pine was
382 estimated using two approaches. First, a three-species unrooted phylogenetic tree was built
383 using 168,534 bp of concatenated sequences, which showed only 43% of shared divergence
384 due to the common branch from loblolly pine to Scots pine/maritime pine (Figure S3). A
385 similar estimate was obtained for 0-fold and 4-fold degenerate sites (43 to 44% of shared
386 divergence). Furthermore, we obtained the same estimates with one, three or five sequences
387 randomly-sampled from each locus and species. Second, counts of fixed differences
388 between loblolly pine and Scots pine/maritime pine showed 629 (all sites), 152 (0-fold
389 degenerate sites) and 106 (4-fold degenerate sites) mutations specific to the Scots pine
390 lineage, while these numbers were 960, 196 and 185 mutations, respectively, for maritime
391 pine (Table S6).

392

393

394 Discussion

395

396 The comparison of the same sets of loci between Scots pine and maritime pine, two related
397 species that occupy different ecological niches and are characterized by different
398 evolutionary histories, allows for exploring how different biological factors may interact
399 with natural selection and adaptive evolution in plants. We discuss how our results are in
400 agreement with each species evolutionary history, as well as how the comparison between

401 species helps to better understand the evolutionary forces responsible for the observed
402 molecular pattern.

403

404 *Nucleotide diversity*

405 This study presents the first large set of loci sequenced in a range-wide sample of two
406 important European conifers. The primers used in this study were transferred from a related
407 New World species, loblolly pine, and thus may suffer from ascertainment bias due to
408 enrichment with low-diversity conserved genes. To test this hypothesis, we compared a set
409 of 364 loblolly pine loci orthologous to those used in this study with 1,000 sets of 364 loci
410 randomly selected with replacement from a larger set of ca. 6,000 loci available in this
411 species (see Eckert *et al.* 2013b). This comparison indicated a significant bias towards
412 lower nucleotide diversity in our gene set (i.e., non-overlapping 95% confidence intervals),
413 but only when considering statistics for all sites (see [Table S7](#)). This suggests that
414 comparisons based on synonymous/silent sites or even non-synonymous sites in our study
415 are subject to only limited bias. Moreover, because the ascertainment bias would affect both
416 species in the same way, it would not prevent a comparative analysis between the two pines,
417 which is the main focus of this study.

418 The sequencing of the same 372 loci in both pines revealed significantly higher
419 nucleotide diversity in the widespread and continuously distributed Scots pine than in the
420 narrower and more patchily distributed maritime pine. Only a limited number of genes has
421 been sequenced for each pine (and rarely the same genes across species) in previous studies,
422 making interspecific comparisons difficult. Watterson's nucleotide diversity for silent sites
423 (compare with [Table S8](#)) in Scots pine was 0.00525 for 16 loci (Pyhäjärvi *et al.* 2007), and
424 0.00620 for 11 loci (Kujala & Savolainen 2012); while that of maritime pine was 0.00824
425 for 11 loci (Eveno *et al.* 2008), but was only 0.00280 for six other loci (Grivet *et al.* 2011)

426 To test whether using a smaller gene set could lead to biased nucleotide diversity estimates,
427 we subsampled different number of loci from the total 372 in our study, and compared
428 nucleotide diversity for these subsamples. Subsampling for 10, 50 or 100 loci did not affect
429 average nucleotide diversity estimates (see confidence intervals in [Table S9](#)), suggesting
430 that the number of genes used did not necessarily lead to bias. Nevertheless, subsets of
431 specific genes may indeed have substantially different levels of nucleotide diversity. For
432 example, the set of six previously-studied candidate genes (see *Material and methods*)
433 showed higher nucleotide diversity (Scots pine $\theta_{w-silent}=0.00895$; maritime pine θ_{w-}
434 $silent=0.00664$) than the 372-locus average ([Table S8](#)). These results show that nucleotide
435 diversity can only be properly compared across species when using a large set of
436 (preferably) common genes, as this estimate can be very variable across small specific gene
437 sets.

438

439 *Insights into demographic history*

440 Patterns within the SFS for each species (as summarized by statistics such as Tajima's D ,
441 Zeng et al.'s E , and Fay and Wu's H_n) can provide insights into demographic history (see
442 references in Gravel *et al.* 2011). In Scots pine and maritime pine, they reflected relatively
443 well what is known for each pine species. Scots pine has likely been through a very ancient
444 (~1-2 Ma) and severe (shrinking populations to about 1% of present time population size)
445 bottleneck (Pyhäjärvi *et al.* 2007). In more recent times, the species would have recolonized
446 a vast territory in northern, central and Eastern Europe, reaching northern Fennoscandia
447 some 10,000 to 7,000 years ago (see Pyhäjärvi *et al.* 2007, 2008; Savolainen *et al.* 2011;
448 Kujala *et al.* 2012; Cheddadi *et al.* 2006; Naydenov *et al.* 2007). This wide-range
449 colonization process would have led to a weak population genetic structure in Scots pine
450 (except especially at the southern margins) (Cheddadi *et al.* 2006; Dvornyk *et al.* 2002;

451 García-Gil *et al.* 2003; Karhu *et al.* 1996; Pyhäjärvi *et al.* 2007). The excess of low-
452 frequency variants in this species could have originated during repeated long-range
453 expansions and would correspond (mostly) to relatively new mutations (but likely before
454 the most recent ice age). In contrast, maritime pine has likely survived in multiple glacial
455 refugia (Bucci *et al.* 2007; Burban & Petit 2003), from which it would have recolonized its
456 current range around the Last Glacial Maximum (~20,000 years ago; Naydenov *et al.* 2014).
457 Its spatially limited expansion, combined with population fragmentation, would have led to
458 distinct and regionally restricted gene pools (Bucci *et al.* 2007; Burban & Petit 2003;
459 Jaramillo-Correa *et al.* 2015), which would have been relatively stable across time, leading
460 to the accumulation of high-frequency variants when considering full-range SFS patterns.
461 Because of the very distinct demographic histories of the two pines, we used methods
462 integrating demography to characterize their pattern of adaptive evolution at the molecular
463 level and to test loci for signatures of positive and negative selection.

464

465 *Genes under selection*

466 Three dehydrins were found to be under selection in the two pines. Previous studies looking
467 at various members of this family, and using different approaches (neutrality tests, F_{ST} -
468 outlier detection, allele frequency-environment correlations), also showed the action of
469 natural selection on dehydrin genes: *dhn1*, *dhn3* and *dhn9* in Scots pine (Palmé *et al.* 2009;
470 Wachowiak *et al.* 2009); *dhn1* (Eveno *et al.* 2008), *dhn2* and *dhn5* (Grivet *et al.* 2011) in
471 maritime pine. In this study, two new dehydrin genes (*dhn2* and *dhn5*) were identified as
472 possible targets of positive selection in Scots pine, while our results confirmed the adaptive
473 role of *dhn1* in maritime pine. Gene expression of dehydrins in water stress experiments
474 pointed to their involvement in drought resistance in maritime pine (Perdiguero *et al.* 2012;
475 Velasco-Conde *et al.* 2012). They have also been shown to be involved in wounding, cold,

476 and drought stress response in white spruce and loblolly pine (Lorenz *et al.* 2011; Richard
477 *et al.* 2000; Watkinson *et al.* 2003). Altogether these studies point to the pivotal role of
478 dehydrins in conifer adaptive response to abiotic stress, over the short- (F_{ST}) and long-term
479 (SnIPRE) timescale, emphasizing their importance.

480 An interesting case is that of locus *O_4042_01* which showed a signal of positive
481 selection in maritime pine (16 fixed non-synonymous mutations compared to only three in
482 Scots pine). Locus *O_4042_01* shows high similarity (E-value of 2×10^{-21}) with *glutathione*
483 *S-transferase* (*gst*) in *Arabidopsis thaliana*, an enzyme involved in secondary metabolism
484 response to the processes of detoxification and stress response to cold (Goulas *et al.* 2006),
485 salt (Jiang *et al.* 2007), and pathogens (Jones *et al.* 2006). In the Chinese pine, *Pinus*
486 *tabuliformis*, five residues within *gst* were found under positive selection, four of them
487 involved in the enzyme activity and specificity (Lan *et al.* 2013). Interestingly, one of these
488 residues codes for a different amino acid in maritime pine (a proline) compared to loblolly
489 pine (alanine), while in Scots pine most individuals maintain the putative ancestral form.
490 This amino acid (positions 31-33) is located very close to the catalytically active G-site in
491 the spatial conformation of the protein, and the substitution of this residue could cause
492 structural changes in the GSH binding pocket (where the conjugation of intracellular
493 glutathione to a wide variety of molecules occurs; Lan *et al.* 2013).

494 Finally, *constans-like 1* (*coL1*), which was found under positive selection in both
495 pines, codes for a putative transcription factor suggested to affect flower development in
496 *Arabidopsis* (Ledger *et al.* 2001). In trees, homologues to *constans* are involved in bud
497 development (Alberto *et al.* 2013b; Ruttink *et al.* 2007), as well as in photoperiodic control
498 of shoot elongation (Holefors *et al.* 2009). Evidence of selection on *constans*-like genes,
499 moreover, has been found in poplar (Chen *et al.* 2014; Ma *et al.* 2010; Ruttink *et al.* 2007;

500 Smith *et al.* 2004; Wei *et al.* 2013), spruce (Holliday *et al.* 2010), oak (Lind-Riehl *et al.*
501 2014), and the perennial *Arabidopsis lyrata* (Mattila *et al.* 2016).

502

503 *Distribution of fitness effects (DFE) and adaptive evolution*

504 Scots pine and maritime pine case studies provided new insights on conifer adaptive
505 evolution and the evolutionary forces shaping conifer genomes. The DFE was similar
506 between the two pines and to other plants (e.g., Eckert *et al.* 2013a; Gossmann *et al.* 2010),
507 as well as to various other organisms (see references in Eyre-Walker & Keightley 2007),
508 with most of the mutations being strongly deleterious and subject to purifying selection.
509 Compared to other conifers (and plants) however (see Eckert *et al.* 2013a, and Hodgins *et*
510 *al.* 2016), both targeted pines presented an atypical pattern with a lower proportion of
511 slightly deleterious mutations and a higher proportion of highly deleterious mutations. The
512 relative rates of new adaptive substitutions (ω_a) for Scots pine (0.1156) and maritime pine
513 (0.1535) were within the range found in other species (between -0.14 and 0.31; Gossmann
514 *et al.* 2012), although at the upper range limit for plants (Gossmann *et al.* 2010, 2012) as
515 well as higher by a factor over two than those estimated with the same methodology in
516 other pines (ω_a = from -0.0477 to 0.0325 for 11 species of soft pines in Eckert *et al.* 2013a;
517 ω_a = 0.0592 for lodgepole pine in Hodgins *et al.* 2016).

518 Several outcomes emerge from these results. First, sampling intensity may bias the
519 estimate of DFE and ω_a , as illustrated by the “extended CRSP dataset” that led to weaker
520 differences between the two pines. Second, albeit very distinct in terms of their evolutionary
521 histories (i.e., demographic history, population structure, and effective population size), the
522 two pines present similar rates of adaptive evolution. This suggests that other factors may
523 also govern the efficacy of selection across these taxa (see below). Third, it is noteworthy to
524 highlight the high efficiency of natural selection at purging highly deleterious mutations as

525 well as the high rate of positive selection in both pines, in regards to plants in general and
526 more specifically to other conifers studied so far. Plants tend to have low rates of adaptive
527 evolution, linked to contracting populations and a high level of population structure
528 (Gossmann *et al.* 2010). Since pines present in general large effective population size, being
529 also relatively undomesticated species, it is expected that they display also high rates of
530 adaptive evolution. However, this trend was not found in previous studies including pines,
531 suggesting that other factors, still largely unknown, may also be relevant (Chen *et al.* 2017).

532 The reliability of our estimates may depend on methodological factors, among them
533 the choice of the loci under study, the outgroup species, or the analytical method, as well as
534 the sampling intensity and distribution (Eckert *et al.* 2013a; Phifer-Rixey *et al.* 2012;
535 Städler *et al.* 2009). More specifically, (i) because we used mostly primers transferred from
536 another species (loblolly pine), gene sets in our study may be more conserved and thus
537 undergo less adaptive evolution (Bachtrog 2008; Eckert *et al.* 2013a; Gossmann *et al.*
538 2010); notice that this would make even more remarkable the high ω_a found in both pines.
539 (ii) Different genic regions may be under different evolutionary constraints (e.g., Hodgins *et*
540 *al.* 2016), and therefore lead to different estimates of adaptive evolution. We computed
541 DFE and ω_a for silent sites (intron + untranslated regions, UTR) and found lower rates of
542 positive selection (data not shown) although not significantly different from those estimated
543 with synonymous sites. (iii) Recombination and mutation rates may differ among selected
544 loci and thus can directly affect the proportion of segregating sites (Bachtrog 2008). (iv)
545 The level of divergence of the species of interest with the outgroup may reveal different
546 proportion of segregating sites, i.e., the closer the outgroup the fewer differences will be
547 detected (Gossmann *et al.* 2010; Strasburg *et al.* 2011). In our study, low phylogenetic
548 distance and partial sharing by the targeted species of the branch conducting to the outgroup
549 could have overestimated ω_a (Keightley & Eyre-Walker 2012). However, phylogenetic

550 analyses indicated less than 50% of shared divergence between loblolly pine and each of the
551 targeted species suggesting that observed patterns were not only due to within-species
552 polymorphism. Furthermore, counts of fixed differences between the outgroup and each of
553 the targeted species pointed to sufficient level of lineage specific mutations in both Scots
554 pine and maritime pine as to correctly estimate ω_a . (v) The choice of the methodology used
555 to infer adaptive evolution (Eckert *et al.* 2013a; Welch 2006) may give different output. By
556 selecting the same loci and outgroup, as well as the same methodology for the two pines
557 and the main studies in other organisms, we attempted to control for these factors and
558 ensured that the estimates (although probably conservative, see point (i)) were comparable
559 across species. Finally, both sampling intensity and distribution may have affected our
560 estimate of adaptive evolution rate. (vi) To test effects of sampling intensity, in terms of
561 both individuals and loci, first, the CRSP dataset (less samples per population but more
562 loci) was compared with the “extended CRSP” dataset (more samples per population but
563 fewer loci) in maritime pine and, second, simulated datasets with different number of loci
564 were compared. Neither approach suggested any effect of sampling intensity on our
565 estimates. (vii) Sampling scheme influences what aspects of the history are emphasized (see
566 Städler *et al.* 2009). Scots pine populations were sampled from both the main range
567 (Finland, Sweden and Poland) and the margins (Spain, Italy and UK), representing fairly
568 well the species evolutionary history overall. Nevertheless, there may still be a bias in Scots
569 pine estimates, as the CRSP loci were not sampled across the full species range (in
570 particular the eastern-northern range was poorly sampled). Thus, some apparently fixed
571 non-synonymous mutations may show polymorphism in the unsampled range, resulting in
572 an upward bias. In maritime pine, however, this bias should be minimal (if any) as
573 populations were sampled across its full range, considering all gene pools known in the
574 species. In addition, population genetic structure does not seem to have affected the

575 proportion of adaptive substitutions, with estimates being similar between the weakly
576 structured Scots pine and the highly structured maritime pine, and still higher than
577 published estimates for other species. Finally, other factors may constrain selective forces,
578 as suggested by comparative studies and theoretical work, and may have influenced the
579 estimates of adaptive evolution in the two pines (Galtier 2016; Lanfear *et al.* 2014): genetic
580 draft and background selection (Messer & Petrov 2013; Peischl *et al.* 2013), environmental
581 heterogeneity (Tellier *et al.* 2011), phenotypic space dimensionality (i.e., fitness landscape)
582 and rate of environmental change (Gillespie 2001; Lourenço *et al.* 2013). These factors are
583 challenging to tease apart.

584

585 *Conclusion*

586 By analyzing a common set of 372 gene loci, we detected specific patterns of molecular
587 evolution and adaptation in two widespread European conifers. First, as expected,
588 nucleotide diversity was higher in the continuously distributed Scots pine than in the
589 patchily distributed maritime pine. Second, by using methods that incorporate demographic
590 effects, we detected an unexpected high relative rate of adaptive substitutions in both pines,
591 and in particular in maritime pine. Although we cannot fully discard methodological
592 caveats, these high rates of adaptive evolution do not seem to be correlated with population
593 genetic structure nor demographic histories that differ between the two pines. Altogether,
594 our results suggest that more than one factor may be responsible of the high rate of adaptive
595 evolution found in these two emblematic pine species, with several factors being difficult to
596 tease apart.

597

598

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600

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952
 953

954 **Data Accessibility**

955

956 DNA sequences are deposited in GenBank under accessions MF385275-MF385581 and
 957 MF385585-MF397901.

958

959

960 **Author Contribution**

961

962 DG, OS and SCGM conceived the study. AJE, DBN and SCGM designed and produced
963 the sequence data sets. DG and KA analyzed the data and drafted the manuscript. AV
964 and SCGM contributed to analyze the data. All the authors contributed to editing and
965 revising the manuscript.

966 **Tables**

967

968 **Table 1.** Summary statistics for 372 common loci sequenced in Scots pine and maritime pine.

969

	Scots pine	maritime pine
Number of loci	372	372
Total length (bp)	165,048	166,641
Segregating sites	1543	983
Average length (bp)	444	448
$\theta_{w\text{-syn}}$ (stdev)	0.0069 (0.0090)	0.0042 (0.0054)
π_{syn} (stdev)	0.0063 (0.0098)	0.0044 (0.0069)
$\theta_{w\text{-nsyn}}$ (stdev)	0.0015 (0.0027)	0.0008 (0.0014)
π_{nsyn} (stdev)	0.0013 (0.0025)	0.0009 (0.0018)
K_s (stdev)	0.0323 (0.0253)	0.0375 (0.0313)
K_a (stdev)	0.0070 (0.0081)	0.0070 (0.0080)
K_a / K_s (stdev)	0.3988 (1.0366)	0.3335 (0.8254)

970

971 bp=base pair

972 stdev=standard deviation

973 syn=synonymous

974 nsyn=nonsynonymous

975 θ_w : average Watterson's nucleotide diversity (Watterson 1975) per site

976 π : average Tajima's nucleotide diversity (Tajima 1989) per site

977 K_s : number of synonymous substitutions per synonymous site with Jukes-Cantor correction, using *P. taeda* as outgroup

978 K_a : number of non-synonymous substitutions per non-synonymous site with Jukes-Cantor correction, using *P. taeda* as outgroup

979 **Table 2.** SFS-based statistics for 64 target and 308 reference loci in Scots pine and maritime pine, with their 95% Confidence Interval
 980 in square brackets.
 981

982

Locus set	Statistic	Scots pine	maritime pine
Target	Tajima's D	-0.5230 [-0.8559,-0.1901]	0.1195 [-0.2020,0.4410]
Target	Zeng et al.'s E	-0.3596 [-0.6483,-0.0709]	0.5309 [0.1473,0.9145]
Target	Fay and Wu's H_n	-0.0844 [-0.2788,0.1100]	-0.5269 [-0.9309,-0.1229]
Reference	Tajima's D	-0.4776 [-0.5831,-0.3720]	0.0106 [-0.1122,0.1335]
Reference	Zeng et al.'s E	-0.3376 [-0.4517,-0.2235]	0.2898 [0.1566,0.4230]
Reference	Fay and Wu's H_n	0.0610 [-0.0483,0.1703]	-0.3077 [-0.4587,-0.1567]

983 **Table 3.** Neutrality tests and type of selection for target genes in Scots pine and maritime
 984 pine. Symbols in parenthesis represent genes related to biotic stress (S) and
 985 phenology/photosystem (P).
 986

Locus	Scots pine		maritime pine	
	SnIPRE*	MKPRF	SnIPRE*	MKPRF
<i>dhn1</i> (S)	negative	negative		negative
<i>dhn2</i> (S)	positive	positive		
<i>dhn5</i> (S)	positive	positive		
<i>coL1</i> (P)	positive	positive	positive	
<i>0_4042_01</i> (S)			positive	positive
<i>2_9480_01</i> (S)			negative	
<i>0_12156_02</i> (P)		positive		

987
 988 *Only genes in common between the Empirical Bayes and the Bayesian SnIPRE tests are
 989 reported. Outputs for each method are presented in full in [Table S5](#).
 990

991 **Figure Legends**

992

993 **Figure1.** Species distribution and sampling for Scots pine (blue, black dots) and maritime
994 pine (green, red dots).

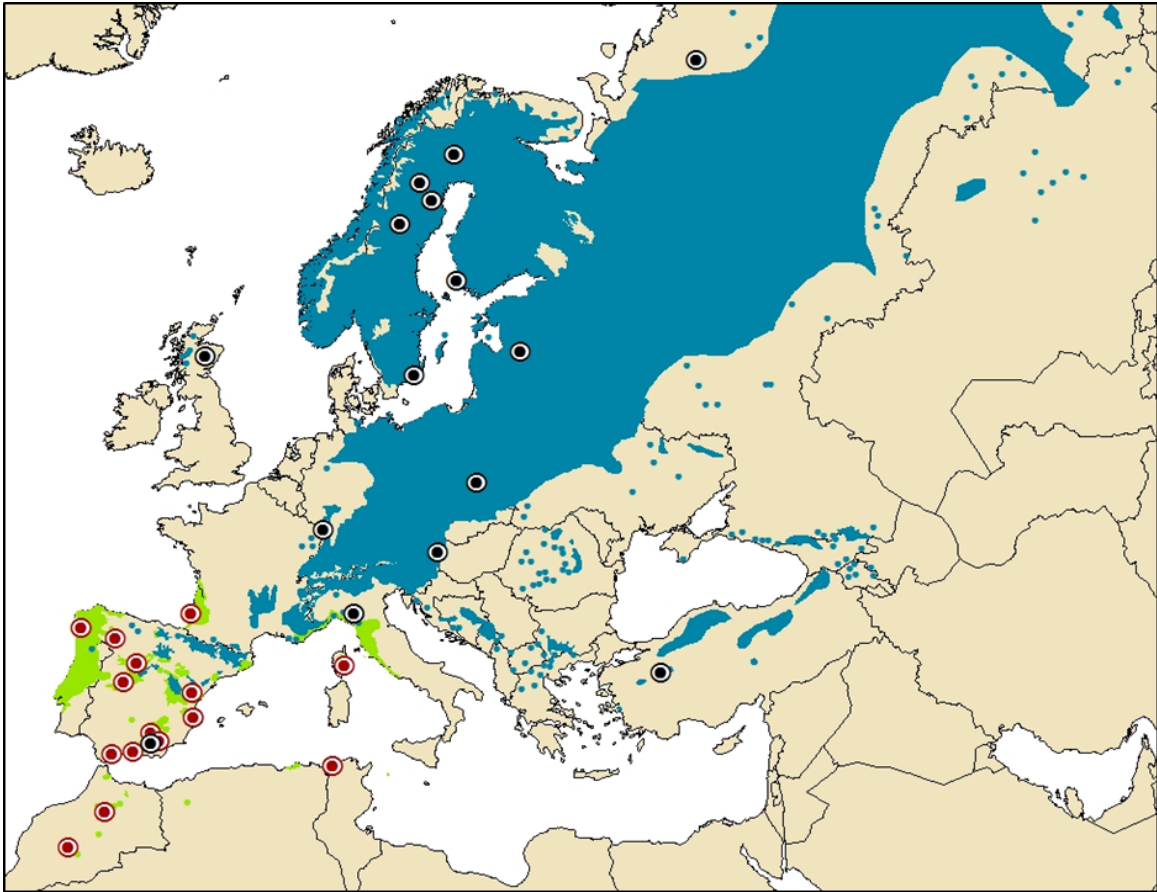
995

996 **Figure 2.** Distribution of Fitness Effects of new mutations (DFE) (a), and relative rate of
997 adaptive substitutions ω_a (b) for the CRSP dataset in Scots pine and maritime pine, and for
998 the “extended CRSP” dataset (only maritime pine), using the method II of Eyre-Walker and
999 Keightley (2009), as implemented in DoFE. $N_e s$ denotes the product of the effective
1000 population size N_e and the strength of selection s , with $N_e s < 1$ corresponding to slightly
1001 deleterious mutations and $N_e s > 100$ corresponding to highly deleterious mutations. Bars in
1002 (a) represent standard errors (with different letters indicating significant differences), while
1003 bars in (b) represent 95% Confidence Intervals.

1004

1005 **Figure1.**

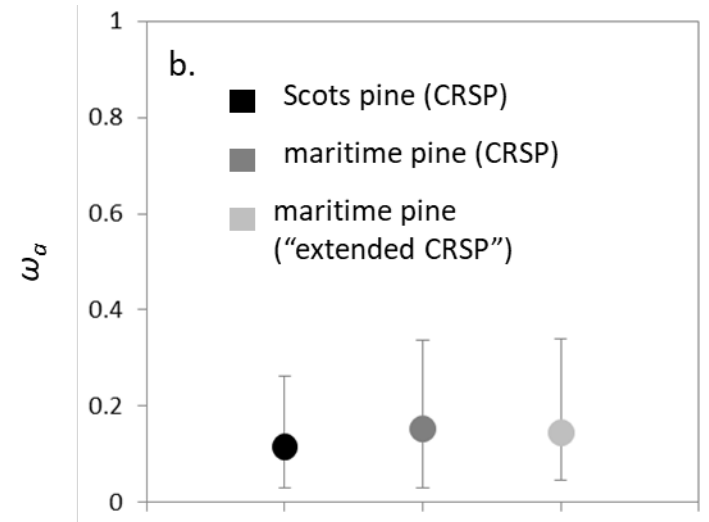
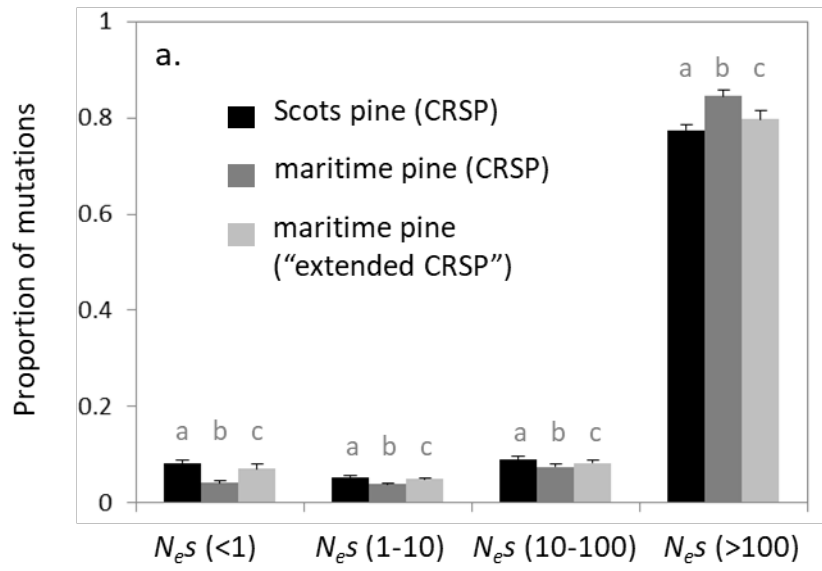
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1008 **Figure 2.**

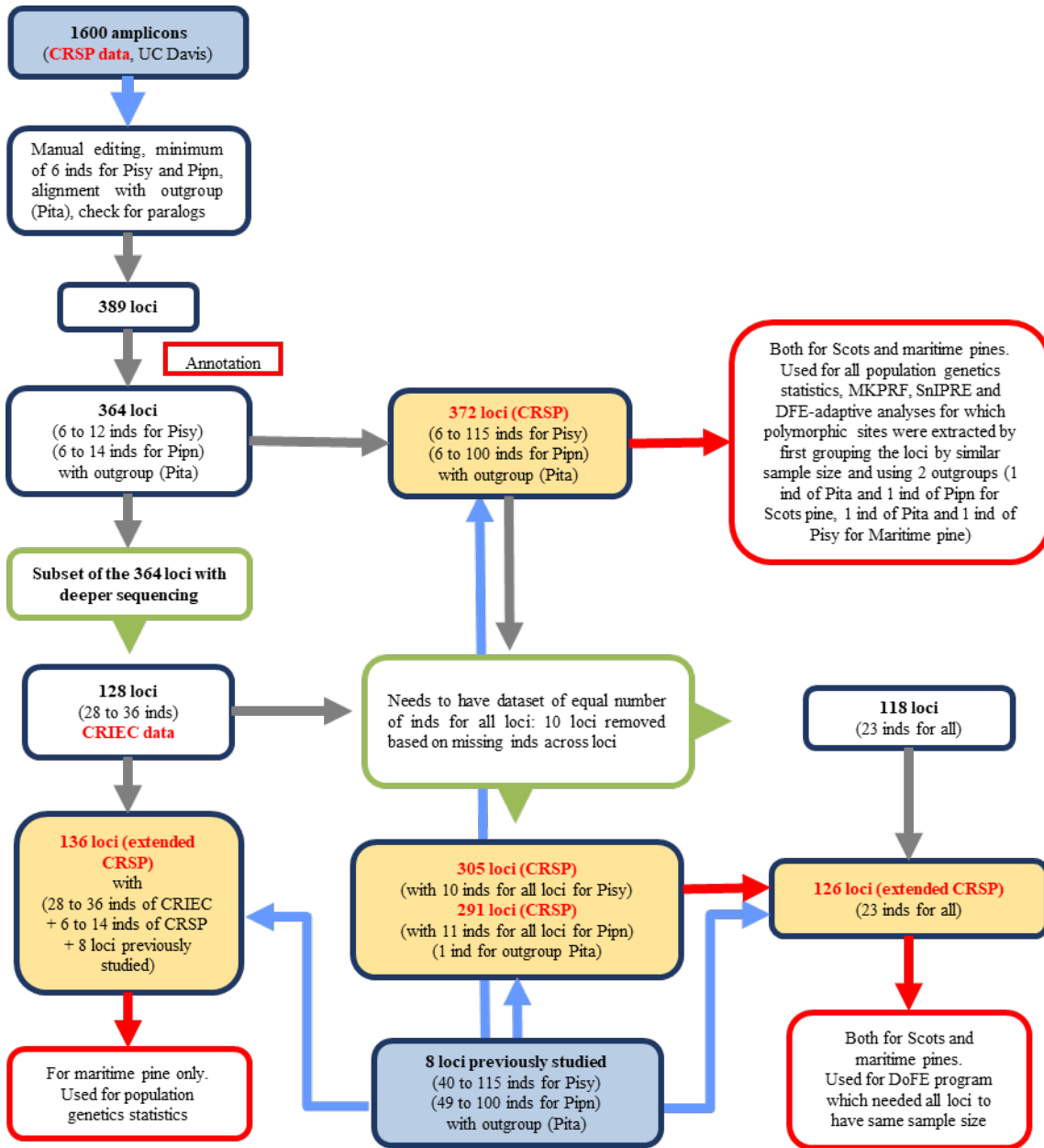
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1 **Figure S1.** Molecular datasets used for the different analyses. Species abbreviations are as
 2 follows: Loblolly pine (Pita), Scots pine (Pisy) and maritime pine (Pipn).

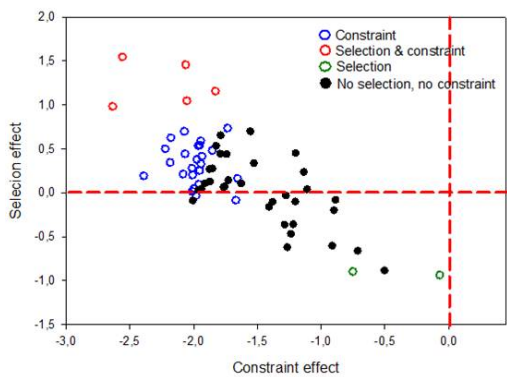
3



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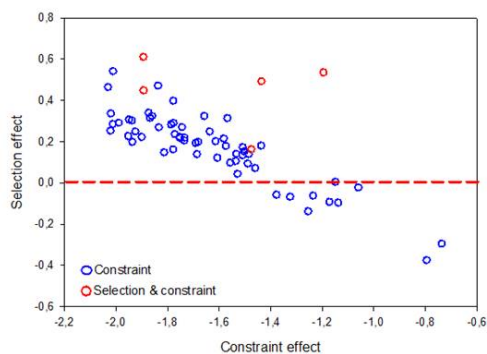
5

6 **Figure S2.** Selection events detected with the SnIPRE approach for the 64 target loci and
 7 silent sites in Scots pine and maritime pine. The graphs represent the selection effect
 8 (fixation rate of non-synonymous mutations) vs. constraint effect (deleterious mutations).
 9

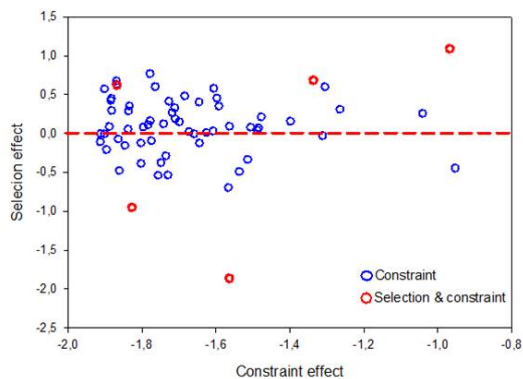


Empirical Bayes SnIPRE

Scots pine

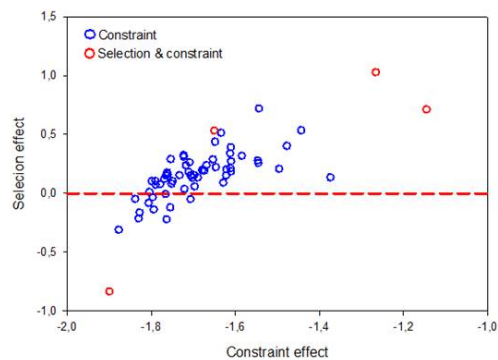


Bayesian SnIPRE



Empirical Bayes SnIPRE

maritime pine



Bayesian SnIPRE

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Figure S3. Maximum likelihood three-species unrooted phylogenetic tree to estimate shared divergence between loblolly pine and Scots pine/maritime pine (see main text).

Example of branch length estimates using 168,534 bp of concatenated sequence per species (Pipn_1: one sequence for maritime pine, Pisy_1: one sequence for Scots pine and Pita_1: one sequence for loblolly pine).

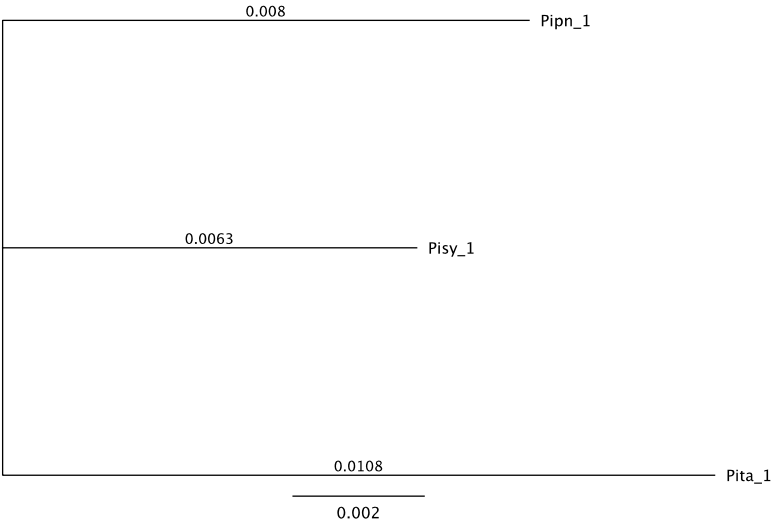


Table S1. Sampling for the two conifer species. Numbers under candidate genes' column correspond to the exact number of samples per population for each of the six previously studied candidate genes. For CRSP and "extended CRSP" datasets the numbers show the maximum sample size per population.

<i>Scots pine</i>										
Site	Country	Latitude	Longitude	8 loci (6 genes) from candidate genes						CRSP
				<i>coL1</i>	<i>dhn1</i>	<i>dhn2</i>	<i>dhn5</i>	<i>gia</i>	<i>4cl</i>	
Kolari	Finland	67.18	24.05	19	5	4	5	19	7	2
Usinsk	Russia	66.08	57.5	7				10	5	
Kaddekielas	Sweden	66.07	19.1	8				9	6	
Northern Sweden	Sweden	65.13	20.23							1
Northern Sweden	Sweden	64.15	16.07							1
Uusikaupunki	Finland	60.87	21.33	10	5	4	5	10	5	
Eastern Scotland	UK	57.05	-3.27	10				10	5	2
Kalsnava	Latvia	56.75	25.88	9				9	4	
Norra Gullabo	Sweden	56.47	15.92	10	5	5	5	10	6	
Radom	Poland	50.68	20.08	20	5	5	5	20	9	2
Haguenau	France	48.85	7.87	10	5	4	5	9	6	
Oberloisdorf	Austria	47.43	16.48		5	5	5			
Parma	Italy	44.62	10.15	10				9	3	2
Kalabak	Turkey	39.45	30.3		5	5	5			
Sierra de Baza	Spain	37.37	-2.83		5	4	5			2
Total (individuals)				113	40	36	40	115	56	12
Total (populations)				10	8	8	8	10	10	7

maritime pine

Site	Country	Latitude	Longitude	8 loci (6 genes) from candidate genes						CRSP	CRIEC
				<i>coL1</i>	<i>dhn1</i>	<i>dhn2</i>	<i>dhn5</i>	<i>gia</i>	<i>4cl</i>		
Landes	France	na	na							3	
Pleucadec	France	47.78	-2.34		5						
Mimizan (Landes)	France	44.13	-1.3		15	4	2		1		2
Galicia	Spain	na	na							1	
Unknown origin*	France	na	na							1	
Tabuyo del Monte	Spain	42.3	-6.22								2
San Cipriano de Ribarteme	Spain	42.12	-8.36		7						
Pinia (Corsica)	France	42.02	9.46	4	8	9	9	3	7		2
Pineta (Corsica)	France	41.96	9.04		6						
Coca	Spain	41.23	-4.5	13	17	15	13	10	16	2	4
Arenas de San Pedro	Spain	40.19	-5.12	12	19	15		10	13	1	4
Olba	Spain	40.17	-0.62	4		8	7	4	7	1	4
Quatretonda	Spain	38.97	-0.36	3		8	6	4	5	1	4
Cazorla	Spain	37.92	-2.92	9		7	7	9	9	1	4
Oria	Spain	37.52	-2.33	6	6	9	9	1	6	1	4
Tabarka	Tunisia	36.94	8.7	5	10	8	8	4	9	1	2
Cómpeta	Spain	36.85	-3.88			1	4		8		
Estepona	Spain	36.52	-5.12								2
Tamrabta	Morocco	33.6	-5.02	3	7	8	3	2	8	1	2
Sidi Meskour	Morocco	31.47	-6.83	1			1	2	3		
Total (individuals)				60	100	92	77	49	92	14	36
Total (populations)				10	10	11	12	10	12	11	12

* Parent of a QTL mapping progeny with only approximate known origin (France)

Table S2. Description of the six previously studied candidate genes (8 loci).

Locus	Length (bp) ^a	Scots pine	maritime pine
<i>4cl</i> (exon 1, 2-3, 5) ^b	516+360+251 = 1127	this study ³ (accessions MF385276-MF385442)	Grivet et al. (2011)
<i>dhn-1</i>	636	Wachowiak et al. (2009) ^d	Eveno et al. (2008)
<i>dhn-2</i>	713	Wachowiak et al. (2009)	Grivet et al. (2011) ^c
<i>dhn-5</i>	559	Wachowiak et al. (2009)	Grivet et al. (2011)
<i>coL1</i>	3829	Pyhäjärvi et al. (2007); Kujala and Savolainen (2012)	this study ¹ (accessions MF385443-MF385502)
<i>gia</i>	1376	Pyhäjärvi et al. (2007); Kujala and Savolainen (2012)	this study ² (accessions MF385510-MF385558)

^aLength based on the common alignment between Scots pine and maritime pine, including indels.

^bExon_1, exon_2-3, exon_5 correspond to exon_c, exon_a, exon_b in Grivet et al. (2011).

^cThe candidate gene corresponds to *dhn2-Ps*.

^d*dhn-1* corresponds to *dhn-9* in Wachowiak et al. (2009).

¹ PCR reaction: for primer pairs *Copr-promU2/ex1L1* and *Copr-ex2U2/3utrL2* the 20 µl mix of reaction contained 0.8 mM dNTP, 0.5 µM of each primer, 1x Phusion HF Buffer (Phusion, Finnzymes), 25 ng DNA and 0.4 unit Taq polymerase (Phusion, Finnzymes); PCR conditions: 1 min at 98 °C, 35 cycles of 10 sec at 98 °C, 30 sec at 66 °C, 30 sec 72 °C, followed by 10 min at 72 °C (see primer's specification in Kujala and Savolainen 2012). For primer pairs *Copr-109U/941L*, *Copr-718U/1919L* and *Copr1820U/1311L* the 20 µl mix of reaction contained 0.5 µM of each primer, 1x Phusion Flash PCR Master Mix (Phusion Flash, Finnzymes) and 25 ng DNA. PCR conditions: 1 min at 98 °C, 35 cycles of 10 sec at 98 °C, 30 sec at 67 °C (*Copr-109U/941L*) or 63 °C (*Copr-718U/1919L* and *Copr1820U/1311L*), 30 sec at 72 °C, followed by 10 min at 72 °C (see primer's specification in Kujala and Savolainen 2012).

² PCR reaction: the 20 µl mix of reaction contained 0.8 mM dNTP, 0.5 µM of each primer, 1x Phusion HF Buffer (Phusion, Finnzymes), 25 ng DNA and 0.8 unit Taq polymerase (Phusion, Finnzymes). PCR conditions: 1 min at 98 °C, 35 cycles of 10 sec at 98 °C, 30 sec at 65 °C (*ex11-U1/3utr-L1*) or 66 °C (*ex10-U1/ex11-L2*), 1 min at 72 °C, followed by 10 min at 72 °C (see primer's specification in Kujala and Savolainen 2012).

³ PCR conditions are the same as in Kujala and Savolainen (2012), and primers are identical to those described in Grivet et al. (2011).

Table S3. 64 target genes related to stress responses (S) and phenology/photosynthesis (P).

Locus	Function	E-value <i>Pinus</i> EST	E-value <i>A. thaliana</i>	Length (bp) ^a
<i>4cl (exon 1)</i> ^b	4-coumarate_CoA ligase (S)	0	7.00E-49	515
<i>4cl (exon 2-3)</i> ^b	4-coumarate_CoA ligase (S)	0	1.00E-24	359
<i>4cl (exon 5)</i> ^b	4-coumarate_CoA ligase (S)	0	1.00E-19	249
<i>dhn-1</i> ^c	Dehydrin (S)	0	5.00E-07	601
<i>dhn-2</i> ^d	Dehydrin (S)	0	5.00E-03	573
<i>dhn-5</i>	Dehydrin (S)	0	6.00E-04	386
<i>0_16976_02</i>	3-ketoacyl-CoA synthase 6 (S)	na	1.00E-41	402
<i>0_18745_02</i>	Mitogen-activated protein kinase 4 (S)	2.00E-089	8.00E-17	685
<i>0_2070_01</i>	Heat stress transcription factor B-2b (S)	na	7.00E-11	411
<i>0_3790_01</i>	Phospholipase D alpha 1 (S)	3.00E-120	5.00E-35	512
<i>0_4032_02</i>	ARM repeat superfamily protein (S)	na	3.00E-28	390
<i>0_4042_01</i>	Glutathione S-transferase TAU 8 (S)	1.00E-169	2.00E-21	476
<i>0_4285_01</i>	Amino acid permease 3 (S)	0	3.00E-27	495
<i>0_6683_01</i>	Salt-inducible zinc finger 1 (S)	na	6.00E-10	459
<i>0_6878_01</i>	F-box leucine-rich repeat family protein MAX2 (S)	7.00E-047	5.00E-62	435
<i>0_768_02</i>	Putative protein kinase (S)	1.00E-109	1.00E-38	481
<i>0_9082_01</i>	Putative beta-1,3-endoglucanase (S)	0	2.00E-32	421
<i>0_9524_02</i>	U-box domain-containing protein 41 (S)	na	3.00E-25	449
<i>0_990_01</i>	Putative calcium-binding protein CML25 (S)	na	7.00E-30	399
<i>2_1582_02</i>	DNAJ heat shock protein-like protein (S)	0	9.00E-56	457
<i>2_2931_01</i>	Ethylene-responsive transcription factor RAP2.4 (S)	0	7.00E-33	454
<i>2_3319_01</i>	Autophagy-related protein 18D (S)	na	2.00E-13	325
<i>2_3726_02</i>	DNAJ heat shock protein-like protein (S)	na	1.00E-28	450

<i>2_6731_01</i>	F-box protein GID2 (S)	na	4.00E-14	430
<i>CL1524Contig1_03</i>	Histidinol dehydrogenase (S)	5.00E -053	3.00E-17	439
<i>CL1536Contig1_03</i>	Mannose-1-phosphate guanylyltransferase (S)	5.00E -105	6.00E-37	207
<i>CL2332Contig1_01</i>	Calcium-dependent protein kinase 6 (S)	6.00E -057	3.00E-19	426
<i>CL263Contig2_03</i>	RNA-binding protein 47C' (S)	7.00E -047	6.00E-10	444
<i>CL3771Contig1_04</i>	Ubiquitin-conjugating enzyme E2 32 (S)	9.00E -091	3.00E-11	446
<i>UMN_2399_01</i>	U-box domain-containing protein 13 (S)	0	8.00E-27	437
<i>UMN_5272_01</i>	6-phosphogluconate dehydrogenase, decarboxylating 3 (S)	na	3.00E-83	444
<i>UMN_CL132Contig1_03</i>	Malate dehydrogenase (S)	7.00E -026	3.00E-62	319
<i>2_9480_01</i>	Malate dehydrogenase (S)	na	2.00E-63	420
<i>2_1014_01</i>	Heat stress transcription factor B-1 (S)	1.00E -059	2.00E-06	431
<i>0_1123_01</i>	Heat shock protein 70B (S)	0	1.00E-38	404
<i>0_11591_01</i>	Protein auxin RESPONSE 4 (S)	0	5.00E-33	419
<i>0_11649_01</i>	Tubulin beta-8 chain (S)	6.00E -128	4.00E-84	572
<i>0_11649_03</i>	Tubulin beta-2/beta-3 chain (S)	0	2.00E-72	344
<i>0_11684_01</i>	Coronatine-insensitive protein 1 (S)	0	7.00E-37	491
<i>0_12117_01</i>	Adenine nucleotide alpha hydrolase-like protein (S)	0	2.00E-15	414
<i>0_12896_01</i>	F-box protein SKIP2 (S)	2.00E -136	5.00E-55	434
<i>0_143_01</i>	Peroxidase 15 (S)	na	8.00E-22	434
<i>0_17010_02</i>	Putative UDP-glucose 6-dehydrogenase 1 (S)	0	1.00E-76	375
<i>0_4588_01</i>	Aldehyde dehydrogenase 2B4 (S)	na	1.00E-14	218
<i>CL1029Contig1_01</i>	Putative galactinol--sucrose galactosyltransferase 2 (S)	3.00E -085	3.00E-13	427
<i>CL305Contig1_05</i>	Dihydrolipoyl dehydrogenase 1 (S)	na	6.00E-44	302
<i>CL3795Contig1_01</i>	Amino acid dehydrogenase family protein (S)	na	1.00E-21	545
<i>0_7921_01</i>	Glucose and ribitol dehydrogenase homolog 1 (S)	na	2.00E-17	336
<i>0_15991_01</i>	E3 ubiquitin-protein ligase COP1 (P)	na	3.00E-17	317
<i>coL1</i>	Zinc finger protein Constans-like 3 (P)	0	8,00E-35	3797
<i>gia</i>	Gigantea protein (P)	0	1,00E-27	1287
<i>0_12156_01</i>	Inactive leucine-rich repeat receptor-like protein kinase	0	4.00E-47	431

	CORYNE (P)			
<i>0_12156_02</i>	inactive leucine-rich repeat receptor-like protein kinase CORYNE (P)	0	7.00E-37	450
<i>0_16400_01</i>	Protein UNUSUAL FLORAL ORGANS (P)	0	1.00E-49	437
<i>UMN_3408_01</i>	histone-binding protein RBBP4 (P)	0	2.00E-26	433
<i>0_3723_01</i>	STRUBBELIG-receptor family 3 (P)	2.00E -072	3.00E-16	572
<i>0_7454_01</i>	Probable serine/threonine-protein kinase (P)	na	9.00E-17	454
<i>0_8850_02</i>	Photosystem I P700 chlorophyll a apoprotein (P)	na	1.00E-52	320
<i>2_6995_01</i>	Phosphoenolpyruvate carboxylase 4 (P)	0	3.00E-61	407
<i>UMN_3561_02</i>	Photosystem II 47 kDa protein (P)	0	3.00E-41	352
<i>UMN_5101_03</i>	Cytochrome b6/f complex subunit V (P)	na	2.00E-13	402
<i>UMN_6852_02</i>	Cytochrome f (P)	0	1.00E-37	397
<i>UMN_6924_03</i>	Photosystem II 47 kDa protein (P)	na	2.00E-42	321
<i>CL1430Contig1_06</i>	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha 1 (P)	1.00E -056	4.00E-19	619

^aLength based on the common alignment between Scots pine and maritime pine, including indels.

^b*exon-1, exon-2-3, exon-5* correspond to *exon-c, exon-a, exon-b* respectively in Grivet et al. (2011).

^c*dhn-1* corresponds to *dhn-9* in Wachowiak et al. (2009).

^d*dhn-2* corresponds to *dhn2-Ps* in Grivet et al. (2011).

Table S4. 28 compound reference loci obtained using linkage map information.

Compound reference locus	Individual reference locus
LG1_a	0_18018_01, CL3054Contig1_01, UMN_1023_01
LG1_b	0_15329_01, 0_17206_01, 0_17607_02
LG2_a	2_9455_01, 0_8531_01, 0_9091_01, UMN_5867_01
LG2_b	0_13929_02, 0_16732_01, 0_2217_01, 2_9087_01
LG3_a	0_18261_01, 0_7001_01, UMN_3006_01, UMN_3444_01, UMN_927_01
LG3_b	0_17082_01, 0_5575_01, 0_9448_01, 2_1528_01
LG3_c	0_4756_01, 2_6618_01, 0_11270_01, 0_12683_01, 0_846_01, CL814Contig1_06
LG4_a	0_16068_01, 0_9444_01, CL4511Contig1_02
LG4_b	0_13383_01, 0_7171_01, 2_3591_03, CL1045Contig1_01, CL1238Contig1_01, UMN_1037_01
LG5_a	0_10453_01, 0_6465_01, CL3037Contig1_06, CL3758Contig1_05, 0_18350_01
LG5_b	0_10054_01, 2_3941_01, 2_5724_02, 2_9603_01, CL415Contig1_04
LG5_c	UMN_4904_01, 0_236_01, 2_2936_01, CL4342Contig1_01, UMN_801_01
LG6_a	0_9383_01, 2_7725_01, 2_8852_01, CL1004Contig1_08, CL4432Contig1_04
LG6_b	0_11980_01, 0_12929_02, 0_8359_01, 0_8844_01, 0_9329_02, 2_5064_01, CL544Contig1_03
LG7_a	0_10667_02, 2_5996_01, CL1848Contig1_01, CL572Contig1_02
LG7_b	0_1659_02, 0_18470_01, 0_2078_01, 2_5636_01, CL4470Contig1_01
LG7_c	0_14976_01, 0_4105_01, 0_4394_01, 2_6491_01, 2_9291_02
LG8_a	0_10267_01, 0_14221_01, 2_3947_01, CL1455Contig1_07
LG8_b	0_17127_01, 0_6999_01, 2_2960_02, CL1698Contig1_01, CL3539Contig1_01
LG9_a	0_17143_02, 2_10236_01, CL1694Contig1_04, UMN_6426_02
LG9_b	2_5099_01, 2_7852_01, 2_9930_01
LG9_c	0_13841_01, 0_16459_01, 2_684_01, 2_974_01
LG10_a	0_12021_01, 0_12978_02, 2_4724_01, 2_6130_01
LG10_b	0_13484_01, 0_16860_01, 2_6052_01, UMN_5833_01
LG11_a	0_12190_02, 0_16009_01, 0_17247_02, 2_7918_01, CL2472Contig1_01
LG11_b	0_16889_02, 0_2433_01, 0_5204_01, CL4023Contig1_01

LG12_a CL905Contig2_01, 0_16169_01, 0_2885_01, 0_3261_01
LG12_b 0_9922_01, 0_11090_01, 1_5675_01, 2_10212_01, UMN_2174_01

Table S5. Loci found under selection with the Empirical Bayes SnIPRE and the Bayesian SnIPRE methods (for silent sites).

Locus	Scots pine		maritime pine	
	Empirical Bayes SnIPRE	Bayesian SnIPRE	Empirical Bayes SnIPRE	Bayesian SnIPRE
<i>dhn1</i> (S)	negative	negative		positive
<i>dhn2</i> (S)	positive	positive		
<i>dhn5</i> (S)	positive	positive		
<i>coL1</i> (P)	positive	positive	positive	positive
<i>4cl_exon1</i> (S)	positive			
<i>0_4042_01</i> (S)			positive	positive
<i>2_9480_01</i> (S)			negative	negative
<i>0_143_01</i>		positive		
<i>0_3723_01</i>	negative			
<i>0_4032_02</i>			positive	
<i>0_4588_01</i>		positive		
<i>0_9082_01</i>	positive			
<i>0_11684_01</i>		positive		
<i>0_11649_01</i>			negative	
<i>CL1430Contig1_06</i>		positive		
Total positive	5	7	3	3
Total negative	3	2	2	1
Total	8	9	5	4

Table S6. Count of fixed differences between loblolly pine and Scots pine/maritime pine. “Originated in one species” refers to mutations that arose only in Scots pine or in maritime pine.

	Scots pine			maritime pine		
	All sites	0-fold sites	4-fold sites	All sites	0-fold sites	4-fold sites
Fixed sites	1868	402	333	2199	446	412
Shared sites	1239	250	227	1239	250	227
Originated in one species	629	152	106	960	196	185

Table S7. Bootstrapping procedure to compare nucleotide diversity estimates in loci transferred from loblolly pine to European pines (364 CRSP loci, $N=14$) with those randomly resampled from loblolly pine genome (about 6,000 loci available). Numbers in bold indicate significant departure from random set of loci.

	S total	π total	θ_w total	π syn	θ_w syn	π nsyn	θ_w nsyn
<i>Transferred loci set</i>	3.36	0.00213823	0.00253591	0.00480557	0.00560486	0.00093046	0.00112435
Low 95CI	3.02	0.00188983	0.00225759	0.00412203	0.00490509	0.00074009	0.00091097
High 95CI	3.70	0.00238664	0.00281423	0.00548911	0.00630463	0.00112082	0.00133772
<i>1,000 bootstraps (364 loci)</i>							
Low 95CI	3.38	0.00307339	0.00342669	0.00466651	0.00524553	0.00103738	0.00122625
High 95CI	4.48	0.00439749	0.00481324	0.00800163	0.00884918	0.00232990	0.00244981

S total: number of segregating sites per locus; π (total, syn, nsyn): Tajima's nucleotide diversity (Tajima 1989) for total sites, synonymous sites, and non-synonymous sites; θ_w (total, syn, nsyn): Watterson's nucleotide diversity (Watterson 1975) for total sites, synonymous sites, and non-synonymous sites; 95CI: 95% Confidence Intervals.

Table S8. Summary statistics for all sites, synonymous sites, non-synonymous sites and silent sites, for the two conifer species for all loci (target and reference genes) for the CRSP and for the “extended CRSP” datasets. Divergence estimates are given using loblolly pine as reference. Statistics were normalized for varying sample size across loci with the SDMTTools package in R.

	Scots pine (CRSP)			
	all	syn	nsyn	silent
θ_w (stdev)	0.003302 (0.003664)	0.006871 (0.008962)	0.001470 (0.002685)	0.005588 (0.006476)
π (stdev)	0.002926 (0.003886)	0.006346 (0.009822)	0.001254 (0.002494)	0.004978 (0.006712)
Divergence (JC) (stdev)	0.016598 (0.010255)	0.032276 (0.025316)	0.006973 (0.008064)	0.028613 (0.018187)

	maritime pine (CRSP)			
	all	syn	nsyn	silent
θ_w (stdev)	0.002141 (0.002162)	0.004189 (0.005440)	0.000823 (0.001393)	0.003749 (0.003783)
π (stdev)	0.002236 (0.002507)	0.004426 (0.006942)	0.000905 (0.001803)	0.003785 (0.004145)
Divergence (JC) (stdev)	0.019092 (0.014916)	0.037465 (0.031322)	0.007029 (0.007974)	0.033709 (0.025498)

	maritime pine (“extended CRSP”)			
	all	syn	nsyn	silent
θ_w (stdev)	0.002588 (0.001915)	0.00463 (0.005147)	0.001016 (0.00138)	0.004193 (0.003292)
π (stdev)	0.002912 (0.00252)	0.00543 (0.006992)	0.001083 (0.001996)	0.004768 (0.00428)
Divergence (JC) (stdev)	0.021141 (0.015417)	0.041243 (0.036966)	0.008142 (0.009089)	0.037145 (0.030381)

Table S9. Nucleotide diversity for maritime pine with CRSP (364 loci + 8 previously studied loci = 372 loci) and “extended CRSP” (128 + 8 previously studied loci = 136 loci) datasets using bootstrap resampling with replacement for different number of loci (indicated in brackets).

	CRSP		“extended CRSP”	
	π silent	θ_w silent	π silent	θ_w silent
Full dataset	0.003785	0.003749	0.004768	0.004193
Lower 95% CI	0.003282	0.003174	0.004076	0.003580
Higher 95% CI	0.004337	0.004358	0.005530	0.004770
Mean 1,000 bootstrats (10)				
Lower 95% CI	0.001220	0.001436	0.002448	0.002319
Higher 95% CI	0.007082	0.007389	0.007804	0.006756
Mean 1,000 bootstrats (50)				
Lower 95% CI	0.002380	0.002398	0.003612	0.003248
Higher 95% CI	0.005065	0.005451	0.005902	0.005192
Mean 1,000 bootstrats (100)				
Lower 95% CI	0.002783	0.002826	0.003939	0.003579
Higher 95% CI	0.004773	0.004946	0.005631	0.004904

Statistics were normalized for varying sample size across loci with the SDMTTools package in R.