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High-density SNP assay development for genetic analysis in maritime pine (*Pinus pinaster*)

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2

3

High-density SNP assay development

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for genetic analysis in maritime pine (*Pinus pinaster*)

5

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24

25

26 **Running title:** SNP discovery and validation in maritime pine

27

28

For Review Only

29 **Abstract**

30

31 Maritime pine provides essential ecosystem services in the south-western Mediterranean
32 basin, where it covers around 4 million ha. Its scattered distribution over a range of
33 environmental conditions makes it an ideal forest tree species for studies of local adaptation
34 and adaptive responses to climatic change. Highly-multiplexed single-nucleotide
35 polymorphism (SNP) genotyping arrays are increasingly used to study genetic variation in
36 living organisms and for practical applications in plant and animal breeding and genetic
37 resource conservation. We developed a 9k Illumina Infinium SNP array and genotyped
38 maritime pine trees from i) a three-generation inbred (F2) pedigree, ii) the French breeding
39 population, and iii) natural populations from Portugal and the French Atlantic coast. A large
40 proportion of the exploitable SNPs (2,052 / 8,410, i.e. 24.4%) segregated in the mapping
41 population and could be mapped, providing the densest ever gene-based linkage map for this
42 species. Population genetics and structure analyses based on 3,981 SNP markers common to
43 the Portuguese and French gene pools revealed high levels of differentiation, leading to the
44 identification of a set of highly differentiated SNPs that could be used for seed provenance
45 certification. Finally, we discuss how the validated SNPs could facilitate the identification of
46 ecologically and economically relevant genetic factors in this species, improving our
47 understanding of the demography and selective forces shaping its natural genetic diversity,
48 and providing support for new breeding strategies.

49

50

51 **Introduction**

52

53 Maritime pine (*Pinus pinaster* Aiton, Pinaceae) is a long-lived wind-pollinated forest tree
54 species native to the western part of the Mediterranean Basin. Its natural range extends from
55 northern Morocco in the south to French Brittany in the north, and from Portugal in the west
56 to Italy in the east (<http://www.euforgen.org/distribution-maps/>). It is found in various
57 ecological situations, from sea level to an altitude of 2,100 m in the High Atlas (Morocco),
58 from regions characterised by heavy annual rainfall in a maritime climate to dry regions in the
59 semi-arid Mediterranean climate, and from calcareous to acidic soils (Alía and Martín, 2003).
60 Its scattered distribution has resulted in local adaptations and high levels of genetic
61 differentiation for adaptive traits across its natural distribution range (González-Martínez *et*
62 *al.* 2002, Santos-del-Blanco *et al.* 2012, Lamy *et al.* 2011, 2014). Considerable genetic
63 differentiation between ecotypes has been reported for various neutral molecular markers,
64 providing clear evidence for a geographic structure of genetic diversity (Bucci *et al.* 2007,
65 Santos-del-Blanco *et al.* 2012, Jaramillo-Correa *et al.* 2010, 2015).

66

67 This fast-growing tree has been widely planted in systematic reforestation programs since the
68 19th century, to secure coastal (along the Atlantic) and inland (Castilian plateau) sand dune
69 areas, to drain marshes and to create new forests for resin production. Over the last 50 years,
70 this species has been commercially exploited as a timber resource for the forestry industry
71 (sawmills, wood panels, pulp and paper). In recent years, it has also been used as a source of
72 chemicals for the bio-industry (Rohdewald 2002, Touriño *et al.* 2005, Jorge *et al.* 2002), as
73 bioactive phenolic compounds can be extracted from its bark. There are now 4.2 million ha
74 under maritime pine within its natural range and 200,000 ha outside this range (mostly in
75 Australia) (Bouffier *et al.* 2013). The breeding of maritime pine began in the 1960s in

76 southwestern France, after several species and provenance trials had shown that the local
77 ecotype was the best adapted and fastest growing tree in the Aquitaine soil and climatic
78 conditions (Illy 1966, Harfouche 1995). This programme has now reached its third generation,
79 and is one of the most advanced conifer breeding programmes in the world (Mullin *et al.*
80 2011).

81

82 The maritime pine has also been adopted by the forest tree genetics research community as a
83 key model species for investigations of the genetic variation or linked mutations underlying
84 phenotypic variability, particularly those selected by the environment and involved in local
85 adaptation (reviewed by González-Martínez *et al.* 2011). It is now hoped that the discovery of
86 polymorphisms causing changes in gene expression and/or amino-acid sequences will lead to
87 innovations in genetic resource management, for both breeding (Isik 2014) and conservation
88 strategies (Ouborg *et al.* 2010). Such discoveries should also lead to changes in silviculture
89 practices to take into account the evolutionary processes inferred from neutral and selected
90 markers (Lefèvre *et al.* 2014). Major efforts have been devoted to the sequencing and
91 assembly of the maritime pine transcriptome (Canales *et al.* 2014), for studies of the
92 molecular basis of the phenotypic response to biotic and abiotic constraints (e.g. Le Provost *et*
93 *al.* 2013). Moreover, since the pioneering work of Lepoittevin *et al.* (2010), describing the
94 design of the first multiplex single-nucleotide polymorphism (SNP) genotyping assay in
95 maritime pine, medium-scale SNP-arrays have been developed (Supplemental Table 1). These
96 assays have made it possible to characterise hundreds of trees, at hundreds of loci, for various
97 applications: nucleotide diversity analysis (Plomion *et al.* 2014), QTL detection (de Miguel *et*
98 *al.* 2014), association mapping (Lepoittevin *et al.* 2012, Budde *et al.* 2014), analyses of the
99 correlations between genotype frequencies and environmental gradients (Jaramillo-Correa *et*
100 *al.* 2015) and linkage map construction (Chancerel *et al.* 2013).

101

102 Given the high throughput and reliability of the Infinium platform from Illumina (e.g.
103 Bartholomé *et al.* 2015a for eucalyptus, Pavy *et al.* 2013 for spruce), we used this platform to
104 design a customised genotyping array for maritime pine, including about 5,000 SNPs from
105 the studies cited above and about 4,000 SNPs newly identified from RNA-seq data and new
106 amplicon resequencing. The resulting 9k SNP array is the largest genotyping chip ever
107 produced for this species. We assessed the suitability of this array for genetic linkage mapping
108 and genetic differentiation analysis between two gene pools (Portugal and French Atlantic
109 coast). Finally, we discuss the potential utility of this SNP array for exploring genetic
110 diversity and its contribution to phenotypic variation, genetic inferences about historical
111 demographic events, the past action of natural selection and adaptive evolution, and the
112 implementation of novel tree breeding strategies.

113

114 **Materials and methods**

115

116 *Design of an Illumina Infinium array for maritime pine*

117

118 We designed a 9k Illumina Infinium SNP array for maritime pine, including the two subsets
119 of SNPs described below.

120 *Previously available SNPs*

121 Over the past five years, several studies have reported the development of SNP markers in
122 maritime pine (see Supplemental Table 1). We selected a total of 4,997 SNPs from assays
123 based on VeraCode, GoldenGate or Infinium Illumina technologies (Illumina, San Diego, CA,
124 USA) and incorporated them into the same Infinium genotyping array. Some SNPs had been
125 used on a number of occasions (270 SNPs were common to at least two studies), whereas

126 others were developed specifically for one study, as shown in Figure 1. SNP redundancy was
127 checked by aligning flanking sequences with the maritime pine UniGene (Canales *et al.* 2014:
128 http://www.scbi.uma.es/sustainpinedb/home_page). Alignments were performed by carrying
129 out BlastN searches in the Blastall V2.2.26 suite (e-value = 10^{-5}). We retained the best aligned
130 contig for each sequence and the position of the SNP was retrieved from that contig. When a
131 redundant SNP was associated with different dbSNP accessions, we retained the accession
132 with the longest flanking sequence. Overall, we retained 4,712 different SNPs in this first
133 subset.

134

135 *Newly developed SNPs*

136 This second subset comprised SNPs obtained by the random screening of EST data or
137 specifically detected in candidate gene sequences.

138

139 *Newly developed SNPs from 454 sequence reads*

140 A flowchart describing the steps involved in the identification of SNPs from 454 data is
141 shown in Figure 2. Three genotypes involved as progenitors of interprovenance hybrids in the
142 framework of the maritime pine breeding programme (accessions 110-4019-1 from Corsica
143 (C), 0284-2 from Landes (L) and 112-4-1 (M) from Morocco) were used. A composite cDNA
144 library was constructed with the SMART PCR cDNA synthesis kit (Clontech, Laboratories
145 Inc., Mountain View, CA, USA) for each tree. The C and M libraries contained equal
146 proportions of cDNAs from differentiating xylem, swelling buds and young needles, whereas
147 the L library consisted of equal proportions of cDNAs from differentiating xylem, swelling
148 and quiescent buds, and young and mature needles. Pyrosequencing (454 Titanium, Roche,
149 Branford, CT, USA) was performed with the Roche-454 Genome Sequencing platform (FLX
150 Titanium technology). Sequences (available under accession numbers SRX031589,

151 SRX208012 and SRX031592 from the NCBI short-read archive) were cleaned with the
152 Pyrocleaner tool (Mariette *et al.* 2011), which removes particularly short (<150 bp) and long
153 reads (>600 bp), reads with a percentage of Ns (ambiguous base calls) greater than 2%, low-
154 complexity regions, and duplicated reads. For each library, the SNPs were identified by
155 aligning each set of sequences against the 198,425 contigs of the second maritime pine
156 UniGene established by Chancerel *et al.* (2013). Alignments were performed with the CLC
157 Genomics Workbench Reference Mapping function of CLC Genomics Workbench V6.0
158 (CLC Bio, Aarhus, Denmark), with the default parameters. SNPs were then detected with the
159 neighbourhood quality standard algorithm (NQS) and the following parameters: minimum
160 coverage = 6, minimum central base quality = 20, minimum neighbourhood quality over a
161 window length of 11 nucleotides = 15, maximum gap and mismatch count = 2 and minimum
162 allele frequency = 20%.

163

164 Newly developed SNPs from candidate genes

165 Grivet *et al.* (2015) obtained 105 *in vitro* SNPs from two full-length candidate genes with
166 functions relating to phenology (*coll* and *gia*) and 64 amplicons sequenced as part of the
167 CRIEC (Comparative Re-sequencing in European Conifers) project, an EvolTree
168 (<http://www.evoltree.eu>) initiative. An additional set of *in silico* SNPs was then obtained from
169 281 candidate genes with functions relating to abiotic and biotic (plant defence) stress
170 responses in forest trees selected from the following sources: i) 66 genes from published
171 (Perdiguero *et al.* 2013) and unpublished maritime pine sequences available from GenBank,
172 ii) 149 genes from sequencing studies in other conifers (Wachowiak *et al.* 2009, Kujala and
173 Savolainen 2012), iii) 53 transcripts displaying differential expression in the presence and
174 absence of pine wood nematode infection (Santos *et al.* 2013), iv) 10 genes associated with
175 adventitious shoot induction and plant development in pines (Alonso *et al.* 2007, Ordás *et al.*

176 unpublished for *knox* genes), and v) 3 genes potentially involved in cavitation resistance in
177 beech (Lalagüe *et al.* 2014). These sequences were blasted (BlastN, e-value = 10^{-5}) against the
178 maritime pine UniGene to retrieve the best matching contig and the sequences surrounding
179 the SNP from a catalogue of 55,607 available SNPs (Figure 3). Redundant SNPs were
180 removed.

181

182 *Annotation of synonymous and non-synonymous substitutions*

183

184 The respective positions of SNPs were defined in the contigs of the maritime pine UniGene
185 from Canales *et al.* (2014). Within the coding sequences (when characterised), non-
186 synonymous and synonymous SNPs were annotated by comparing the amino acids translated
187 from the reference codon to the codon containing the SNP. The functional annotation was
188 retrieved from the study by Canales *et al.* (2014). All these items of information are available
189 in Supplemental Table 3.

190

191 *The populations studied and genetic analysis*

192

193 The SNP assays were tested and validated on the basis of Mendelian segregation in mapping
194 pedigrees and genetic diversity analysis in an elite breeding population from France and
195 natural populations of different origins (French Atlantic coast and Portugal).

196

197 *Mapping population and linkage analysis*

198

199 The mapping population consisted of a three-generation inbred pedigree (F2) obtained by the
200 self-pollination of an inter-provenance “Landes x Corsica” hybrid (accession H12 resulting

201 from the control cross between L146 and C10 genotypes). In total, 638 F2 seeds were planted
202 in a nursery in June 1998 and 626 saplings were transplanted into the field in March 1999 (4
203 m × 2 m, 0.51 ha, Lacanau de Mios, France). After 15 years, 565 F2 plants were still available
204 for genetic analysis. We used 92 F2 plants to test the Mendelian segregation of the SNP
205 markers and to associate them with a particular genetic position. The F2 plants with the most
206 recombinant genotypes were selected with MapPop software
207 (<http://www.bio.unc.edu/faculty/vision/lab/mappop/>, Vision *et al.* 2000) and a linkage map
208 was established by Lavigne *et al.* (manuscript in preparation), by genotyping 477 F2 plants
209 for 248 SNPs distributed over 12 linkage groups (LG), the haploid chromosome number in
210 pines.

211

212 The *R* package *onemap* v2.0-3 (Margarido *et al.* 2007, Mollinari *et al.* 2009) was used for
213 linkage mapping. Only SNPs and individuals for which there was less than 5% and 1%
214 missing data, respectively, were considered in the analysis. SNPs were clustered into LGs on
215 the basis of a LOD score greater than 10. The LG names were defined on the basis of
216 previously mapped loci (Chancerel *et al.* 2013). The RECORD algorithm (Os *et al.* 2005) was
217 used to order markers within LGs, with the following parameters: LOD=3 and max.rf=0.4.
218 Recombination rates were converted into genetic distances (cM) with the Kosambi mapping
219 function (Kosambi 1943). The goodness-of-fit of SNP segregations to the expected Mendelian
220 segregation ratio (i.e. 1:2:1 for an F2 population) was assessed in chi-squared tests, with
221 adjustment of the significance threshold for simultaneous multiple tests (Benjamini and
222 Yekutieli 2001) within each LG. The same procedure was also applied to a previous SNP
223 dataset genotyped in the same F2 family, but with different genotypes and mapping software
224 (Chancerel *et al.* 2013). The two genetic maps were then combined into a composite linkage
225 map with the *R* package *LPmerge* (Endelman and Plomion, 2014).

226

227 *Populations of unrelated individuals and genetic diversity estimation*

228 The French Atlantic coast gene pool was represented by two subsets of individuals: i) 50 trees
229 from two natural populations, Hourtin and Petrocq, sampled from a clonal collection
230 (CLONAPIN) established directly from the source populations (as described by Rodríguez-
231 Quilón *et al.* 2015). Hourtin (45°11'N, 1°09'E) and Petrocq (44°04'N, 1°18'E) are coastal
232 populations growing at low altitude (<30 m a.s.l.) and under typical maritime climate (annual
233 precipitation of 980-1248 mm and mean annual temperature of 12-13°C); and ii) 50 elite trees
234 from a larger set of about 600 genotypes mass-selected in natural forests of the Aquitaine
235 region in the early 1960s to constitute the first generation of the maritime pine breeding
236 program (Illy 1966). These 50 genotypes constitute the founders of a three-generation
237 pedigreed population used to develop proof-of-concept for genomic selection in maritime
238 pine.

239

240 The Portuguese population was also represented by two subsets of genotypes: i) 19 trees
241 sampled from two provenances in a provenance trial carried out at Mimizan (France:
242 44°20'N, 1°28'W). These provenances were described by Illy (1966): 6 trees were from seeds
243 collected at “Pinhal de Leiria” (an 11 ha coastal forest located at 39°79'N, 8°98'W; 0-50 m
244 a.s.l.; annual precipitation of 700-900 mm) and 13 trees were from seeds collected at “Trás-
245 os-Montes” (a mountain forest located at 41°57'N, 7°50'W; 1150 m a.s.l., annual
246 precipitation of 1200-1400 mm); and ii) 23 additional trees from “Pinhal de Leiria” collected
247 from the CLONAPIN maritime pine collection.

248

249 We first tested departure from Hardy-Weinberg equilibrium, in standard chi-squared tests
250 with a nominal significance threshold of $1.05-1.15 \times 10^{-5}$, corresponding to an experiment-

251 wise type I error of 5 % (Bonferroni correction to account for multiple testing). We then
252 estimated three genetic diversity parameters for each SNP: minor allele frequency (MAF),
253 observed (H_o) and expected heterozygosity (H_e , Nei's index of genetic diversity corrected for
254 sample size, Nei 1987). These three parameters were highly correlated (>0.95). We therefore
255 present data for MAF and H_e only. Genetic differentiation (F_{st}) was assessed for pairs of
256 populations, with a set of 3,981 polymorphic SNPs common to both populations, with GDA
257 software (Lewis and Zaykin 2001). Finally, Bayesian clustering analysis (Structure software,
258 Pritchard *et al.* 2000, Falush *et al.* 2003) was performed to identify different gene pools in
259 maritime pine, as described by Jaramillo-Correa *et al.* (2015).

260

261 *Folded site frequency spectrum (SFS) for the French and Portuguese groups*

262

263 Sites with missing data were excluded from the samples, to ensure general consistency across
264 loci in terms of the number of sequences analysed for each site. Furthermore, we retained the
265 same SNPs for the French and Portuguese groups, 3,513 SNPs in total, corresponding to 88%
266 of the original dataset (3,981 SNPs). Finally, two individuals were discarded from the French
267 group as they presented 19% (INIA_PET11) and 10% (INIA_PET12) missing data across the
268 3,981 SNPs. The estimate of folded SFS was therefore based on 3,513 common SNPs with no
269 missing data, genotyped in 94 French and 42 Portuguese individuals.

270

271 SNPs were analysed as unpolarized (i.e. no ancestral state inferred), with the least frequent
272 nucleotide at a given SNP site considered to correspond to the minor allele and thus used for
273 the calculation of minor allele frequency (MAF). MAF was calculated for the French and
274 Portuguese groups separately. The folded SFS was then plotted from the observed MAF and
275 from the expected SFS. We used equation 6 from the paper by Ganapathy and Uyenoyama

276 (2009) to determine the expected SFS. This equation is widely used to describe the expected
277 SFS for a genomic sample of SNPs, each of which is assumed to correspond to a mutation on
278 an independent gene genealogy.

279

280 *DNA extraction and genotyping*

281

282 Developing needles were collected after bud burst for each genotype of the F2, natural and
283 breeding populations. They were stored at -80°C or dried with silica gel before DNA
284 extraction. About 30 - 40 mg of frozen/dried needles were crushed with a mixer mill (Retsch
285 MM300, Haan, Germany). Genomic DNA was isolated with the Invisorb DNA plants 96 kit
286 from Invitex (GmbH, Berlin, Germany), according to the manufacturer's instructions. All
287 concentrations were determined with a NanoDrop spectrophotometer (NanoDrop
288 Technologies, Wilmington, DE, USA) and a fluorescence assay (Quant-IT kit, Invitrogen,
289 Carlsbad, CA, USA). Samples with DNA concentrations greater than 50 ng/μl (based on
290 fluorescence measurements) were used for the Infinium assay. Genotyping was carried out at
291 the Genes Diffusion Facility (Douai, France).

292

293 **Results**

294

295 *SNP detection*

296

297 The SNP genotyping array included 8,949 SNPs, 4,712 of which (i.e 52.65%) were already
298 available (166 from Lepoittevin *et al.* 2010, 835 SNPs from Chancerel *et al.* 2011, 3,378
299 SNPs from Chancerel *et al.* 2013, 434 SNPs from de Miguel *et al.* 2014, and 184 SNPs from

300 Lavigne *et al.* (manuscript in preparation) (Figure 1). The other 4,237 (i.e 47.35%)
301 corresponded to newly developed SNPs.

302

303 *Newly developed SNPs*

304

305 *In silico* SNPs from 454 sequence reads (Figure 2)

306 Pyrosequencing of the C (Corsica), M (Morocco) and L (Landes) libraries provided 188,494,
307 611,673 and 458,882 reads, respectively. After cleaning, we retained 107,089, 484,348 and
308 225,842 reads for C, L and M, respectively. We identified 2,039 SNPs in the C dataset, 4,937
309 in the L dataset and 4,752 in the M dataset. We then selected SNPs in a test-cross
310 configuration (1:1 segregation), i.e. heterozygous in one parent and homozygous in the other,
311 because, in a full-sib family with biallelic codominant markers, pairs of markers presenting
312 this configuration provide the best estimate of recombination rates, particularly if compared
313 with pairs having a test-cross and intercross (1:2:1) configuration (Plomion *et al.* 1997). Thus,
314 we selected 2,830, 1,695 and 2,957 SNPs for the C × M, L × C and L × M crosses,
315 respectively. In total 784, 2,046 and 911 SNPs were informative for the C, M and L parents,
316 respectively. This procedure resulted in the retention of 3,741 unique SNPs as particularly
317 suitable for linkage mapping.

318

319 *In vitro* and *in silico* SNPs from candidate genes (Figure 3)

320 Of the 281 candidate genes, 166 were associated with at least one contig of the maritime pine
321 UniGene. We retrieved 888 SNPs from these 166 contigs. Finally, after removing duplicated
322 sequences and SNPs located less than 100 bp apart, we retained 391 SNPs for SNP array
323 development. In addition, 105 *in vitro* SNPs from 66 resequenced candidate genes were
324 identified and incorporated into the SNP array.

325

326 *Annotation of synonymous and non-synonymous substitutions*

327

328 In total, 4,257 (47.6%) SNPs were found in contigs for which a full-length protein was
329 predicted, with 1,557 (36.6%) of these SNPs located in non-coding regions. SNPs in coding
330 sequences (2,700 *i.e.* 63.4%) were characterised in terms of their synonymous (S) or non-
331 synonymous (NS) nature and codon responsible for the NS change (Figure 4A and
332 Supplemental Table 3). We identified 567 (21%) synonymous SNPs, and found that the point
333 mutation affected the third position in the codon in 87.5% of cases. There were 2,133 (79%)
334 non-synonymous SNPs with the mutation affecting the first codon position in 21.5% and the
335 second codon position in 70.6%. For NS mutations, the change in the protein is indicated in
336 Supplemental Table 3. Most of the SNPs were transitions (59.3%). The most common
337 transition was C↔T. The most frequent transversion was G↔T (Figure 4B).

338

339 *Linkage mapping*

340

341 Of the 8,410 SNPs used to genotype the 92 trees of the F2 mapping population, 4,634 (55.1%)
342 were monomorphic, 2,052 (from 1,672 different contigs) were polymorphic (24.4%) and the
343 remaining 1,724 (20.5%) corresponding to failed assays. All polymorphic SNPs passed the
344 quality threshold and were therefore used in linkage analysis. The total map length was 1,993
345 cM, spread over 12 LGs corresponding to the haploid number of chromosomes for the
346 maritime pine genome. LG length ranged from 129.2 (LG11) to 198.2 cM (LG5) and the
347 mean number of SNPs per LG was 171 cM (F2_N in Table 1). The mean SNP density was
348 0.98, but more than half of the markers were grouped into clusters (*i.e.* groups of markers
349 displaying no recombination). This resulted in 901 unique positions, separated by a mean

350 distance of 2.25 cM. Two factors could account for the clustering of SNPs: i) the small size of
351 the mapping population (92 genotypes) and ii) the presence of more than one SNP in 260 of
352 the 1,672 mapped contigs. Distorted SNPs were retained in the linkage analysis and accounted
353 for 3.6% of the mapped markers. They were grouped together in five regions located on three
354 LGs (segregation distortion regions, SDRs): two linked SDRs on LG#3 with 10 and 5
355 markers, one SDR on LG#6 (26 markers) and three SDRs on LG#10 (14, 12 and 2 markers)
356 (Figure 5). The order of SNPs was similar (Spearman $\rho=0.98$) to that obtained from the
357 linkage map reconstructed with the dataset of Chancerel *et al.* (2013) based on different
358 genotypes of the same progeny (F2_O in Table 1). Only 56 of the 1,180 markers common to
359 the two genetic maps differed in order between the two maps (Figure 5). These differences in
360 marker order occurred between tightly linked markers (<5 cM), mostly on LG#1 and LG#10,
361 for which 10 and 15 markers were involved, respectively. Finally, based on the highly
362 conserved marker order between the two maps, a composite map was established (F2_C in
363 Table 1) with LPmerge software. It contained 2,353 SNPs (including 1,121 SNPs in the same
364 contigs). This composite map included 1,661 different loci, and is therefore the densest yet
365 gene-based linkage map for this species. The total map length was 1,711.7 cM. There were
366 955 unique positions, separated by a mean distance of 1.82 cM.

367

368 *Genetic diversity and population structure analysis*

369

370 Only a few markers displayed significant departure from Hardy-Weinberg equilibrium in each
371 population: 6 (0.10%) for the French Atlantic-based breeding population, 16 (0.26%) for the
372 natural French Atlantic population and 18 (0.35%) for the Portuguese population. These
373 markers, together with those for which >10% of the data were missing in any of the three
374 populations, were removed from further analyses, resulting in a set of 5,016 loci (including

375 24% monomorphic loci) successfully scored in all populations. We found no differences in
376 genetic diversity, assessed with this common SNP set, between the natural and breeding
377 populations in the French Atlantic region (H_e of 0.336 vs. 0.332, based on polymorphic loci
378 only; Table 2), suggesting that the mass selection in natural forests for constitution of the base
379 breeding population for this species was broad enough to collect most of the standing genetic
380 variation of this ecotype. This is further supported by the absence of significant genetic
381 differentiation between the natural and mass-selected populations ($F_{st} = 0.0005$, 95% CI: -
382 0.00002, 0.00107). Genetic diversity was slightly lower for the Portuguese population (H_e of
383 0.319) than for both the breeding and natural populations in France. High levels of genetic
384 differentiation were observed between the French Atlantic and Portuguese populations ($F_{st} =$
385 0.0847, 95% CI: 0.08087, 0.08864), with 263 SNPs having F_{st} values above 0.25. These SNP
386 markers are good candidates to replace the biochemical assay (based on terpene content
387 analysis, Baradat and Marpeau-Bezard, 1988) currently used to determine the putative origin
388 of adult forest stands in Aquitaine before the collection of seeds and their distribution for
389 commercial purposes in France. Indeed, seed-lots from Portugal were introduced into
390 Aquitaine in the 1950s and the stands they formed suffered frost damage after the
391 exceptionally cold winter of 1985 (Ribeiro *et al.* 2002). Therefore, from 1986 onwards,
392 candidate stands for seed collection in Aquitaine had to be certified as of French origin. The
393 Bayesian clustering pattern observed, with the identification of only two gene pools ($K=2$),
394 with the French natural and breeding populations grouped together into a single gene pool
395 significantly separated from the Portuguese population (Figure 6), is an strong asset in this
396 respect. This result will guide the development of highly informative genotyping multiplexes
397 based on the Sequenom MassARRAY technology that has already been developed for this
398 species (Chancerel *et al.* 2013).

399

400 As expected, the SFS of both the French and Portuguese groups showed a deficit of the
401 observed low-frequency variants (Supplemental Figure 1). This deficit is probably due to a
402 combined effect of the approach developed to select most of the SNPs (i.e. *in silico* analysis
403 of ESTs obtained from small numbers of genotypes, precluding the capture of rare variants of
404 the SFS), and the sampling scheme (a small sample size for each group), hindering the
405 detection of rare variants even if present on the SNP array.

406

407 **Discussion**

408

409 In this section, we discuss how the substantial number of polymorphisms detected and
410 validated in this study will help researchers working on maritime pine to address scientific
411 questions in population genomics and quantitative genetics with large samples of individuals
412 from natural and pedigreed populations that can now be surveyed for genetic variation at a
413 few thousand expressed genes across the genome.

414

415 *Linkage and QTL mapping*

416

417 High-throughput SNP genotyping has been successfully implemented in several conifers
418 (Pavy *et al.* 2008; Eckert *et al.* 2009a; Chancerel *et al.* 2011, 2013). This approach has
419 generated thousands of markers for genetic mapping, significantly improving our
420 understanding of large, complex conifer genomes (Jermstad *et al.* 2011, Pavy *et al.* 2012,
421 Martínez-García *et al.* 2013, Neves *et al.* 2014). We show here that a combination of highly
422 multiplexed SNP genotyping and a selective mapping strategy is useful for the mapping of
423 hundreds of genes at low cost. The genetic map obtained with this strategy was highly reliable
424 and accurate, as revealed by comparison with a previously developed map based on different

425 genotypes from the same progeny (Chancerel *et al.* 2013). The use of markers common to the
426 two sets of offspring made it possible to construct a composite genetic linkage map
427 confirming the positions of most of the previously mapped markers. Highly saturated gene-
428 based maps are very useful for the identification of orthologous genes, facilitating the study of
429 genome structure and evolution in conifers (Mackay *et al.* 2012, Ritland *et al.* 2011). Conifers
430 display a high degree of genome macrostructure conservation (Chagné *et al.* 2003, Pavy *et al.*
431 2012, Krutovsky *et al.* 2004, Jermstad *et al.* 2011). Comparative mapping is therefore a useful
432 tool to transfer information between species. SNP-based genetic maps have been used to
433 anchor large genome scaffolds to support conifer sequencing projects (Martínez-García *et al.*
434 2013, Neale *et al.* 2014). In addition, high-density gene-based maps are essential for the
435 identification of genes controlling targeted traits through their colocalization with QTLs
436 (Monclus *et al.* 2012, de Miguel *et al.* 2014, Lind *et al.* 2014, Li *et al.* 2014, Bartholomé *et al.*
437 2015b). Thus, the development and application of high-throughput genotyping technologies to
438 non-model species without a reference genome sequence, such as maritime pine, represents a
439 major advance towards the identification of genes controlling traits of interests.

440

441 *Association mapping*

442

443 In long-lived outbreeding organisms, such as forest trees, association mapping is an attractive
444 alternative to QTL mapping, as it can be used to identify molecular variation underlying
445 phenotypes from multiple genetic backgrounds without the need to produce segregating
446 progenies (Neale and Savolainen 2004). Under certain circumstances (e.g. moderate-high
447 heritability), association mapping can also be applied to natural populations. This approach is
448 useful for studies of adaptive traits in the precise environments in which they evolved (see
449 Parchman *et al.* 2012; Budde *et al.* 2014 for forest trees). Recent years have seen an explosion

450 in the number of association mapping studies in model organisms, such as humans and
451 *Arabidopsis* (see reviews in Stranger *et al.* 2011; Weigel 2012). However, only a few studies,
452 mostly focusing on technological characters associated with wood quality, have been carried
453 out on forest trees (e.g. González-Martínez *et al.* 2011 and references therein; Cappa *et al.*
454 2013; Guerra *et al.* 2013). Progress has been particularly slow in maritime pine, partly due to
455 the lack of a reliable and cost-efficient genotyping platform capable of dealing with the large
456 sample sizes required to detect small- and medium-sized allelic effects. However, promising
457 results were obtained in the first genetic association studies in maritime pine, based on only a
458 few hundred markers. Lepoittevin *et al.* (2012) found two polymorphisms significantly
459 associated with growth (in a HD-Zip III transcription factor) and wood cellulose content (in a
460 fasciclin-like arabinogalactan protein), respectively. Cabezas *et al.* (2015) discovered a strong
461 association between several (linked) polymorphisms in *korrigan*, a gene encoding a
462 membrane-bound endo-1,4- β -d-glucanase involved in cellulose biosynthesis, and various
463 growth traits, including height and polycyclism. Our SNP array includes all SNPs reported to
464 display significant genotype:phenotype associations in previous studies. It is also enriched in
465 SNPs from candidate genes, based on all the available information (published or unpublished)
466 concerning genes displaying signatures of natural selection (e.g. Eveno *et al.* 2008; Grivet *et al.*
467 2011), involved in environmental associations (e.g. Jaramillo-Correa *et al.* 2015) or
468 displaying differential expression (e.g. for pine nematode resistance, Santos *et al.* 2012; or
469 drought response, Perdiguero *et al.* 2013) in this species. We therefore expected the use of
470 this SNP genotyping array to generate highly informative data, increasing our understanding
471 of the ecological drivers of adaptation and of use for breeding applications, including genomic
472 selection (see below). For example, Westbrook *et al.* (2013) found that a small number of
473 significantly associated SNPs (~20-30) had the same predictive power as the full dataset
474 (4,854 SNPs) in SNP-based models for oleoresin flow in *Pinus taeda* L. Finally, this SNP

475 array will allow unprecedented explorations of the molecular basis of polygenic quantitative
476 traits, through the implementation of multilocus association models (e.g. piMASS, Guan and
477 Stephen 2011), for which high-density genotyping is required. Most of the traits relevant for
478 adaptation or breeding are polygenic. The development of high-density SNP genotyping
479 assays for forest trees thus constitutes a first step towards the use of molecular information in
480 operational forestry.

481

482 *Genomic selection*

483

484 High-throughput genotyping platforms, such as the new SNP array developed here, can guide
485 breeding and selection decisions (Eggen 2011). Genomic selection (GS) is a paradigm shift
486 first introduced into animal breeding in 2008 for the selection of superior individuals in many
487 countries (Goddard 2009). The major difference between GS and marker-assisted selection
488 (MAS) is the number of markers used. GS makes use of a much larger number of markers to
489 trace all the QTLs with small or large effect (Hayes and Goddard 2010). SNP-based
490 genotyping platforms are reliable and repeatable for the genotyping of large numbers of
491 individuals. There are few missing genotypes, and these genotypes can be handled (dropped
492 or imputed) without markedly decreasing data quality. Thus, SNP arrays have become the
493 choice genotyping platform for animal breeding and human genetics studies, despite advances
494 in the efficiency of DNA sequencing technologies. Whole-genome sequences have been
495 obtained for several major forest trees subjected to breeding programmes in recent years, and
496 draft assemblies of whole genomes are now available even for conifers, which have
497 particularly large genomes (Birol *et al.* 2013, Nystedt *et al.* 2013, Neale *et al.* 2014). These
498 resources should facilitate the development of efficient genotyping by sequencing platforms
499 in the near future. GS is expected to revolutionise forest tree breeding, by decreasing the need

500 for expensive and time-consuming progeny-testing practices. If successful, GS could halve
501 the long breeding cycles (>15 years) of forest trees and double the genetic gain per unit time
502 (Isik 2014). One probable application of GS application in forest trees would involve the use
503 of data from different sources, such as progeny tests and genotyping centres, in a single-step
504 approach to predicting the genetic merit of individuals (Legarra *et al.* 2009). The predicted
505 model for one cycle can then be refined as new data become available. As breeding cycles
506 progress and genotyping/sequencing costs fall, progeny testing will have a lesser effect on
507 selection decisions. Nevertheless, the lack of a reliable and cost-efficient genotyping platform
508 remains the major bottleneck for the routine application of GS in forest trees. The new high-
509 density SNP array presented here constitutes a first step towards the implementation of GS in
510 maritime pine.

511

512 *Population genomics*

513

514 The high-density SNP array developed in this study provides a powerful tool for the genome-
515 wide genotyping of a large number of populations across the full distribution range of the
516 species. The genotyping of hundreds of individuals across the entire range of maritime pine,
517 with SNPs located both in coding and noncoding regions, would increase our understanding
518 of the role of the evolutionary, demographic and adaptive mechanisms acting on natural
519 populations. The available molecular markers have shown that maritime pine populations are
520 spatially structured into regional gene pools connected by gene flow, particularly in the
521 Iberian Peninsula (Burban and Petit 2003, Bucci *et al.* 2007, Jaramillo-Correa *et al.* 2015).
522 However, the timing of the historical events leading to this spatial separation, and the degree
523 of connectivity between gene pools, remain to be determined. In addition, maritime pine
524 grows in diverse environmental conditions, resulting in the local adaptation of populations

525 over the range of this species (González-Martínez *et al.* 2002, Jaramillo-Correa *et al.* 2015,
526 Serra-Varela *et al.* 2015), but the molecular mechanisms underlying this adaptation are poorly
527 understood. Most ecologically and economically important traits are quantitative in nature and
528 controlled by many loci (Rockman 2012). Extensive genome coverage is therefore required to
529 disentangle selection from other factors that have shaped genomic variation across
530 populations (e.g. genetic drift). This SNP array provides a means of identifying functional
531 variation and the molecular bases of adaptation, through various methods based on the
532 differentiation of allele frequencies between populations (F_{ST} -based methods; see e.g. Prunier
533 *et al.* 2011, Chen *et al.* 2012), site-frequency spectrum statistics (e.g., Eckert *et al.* 2009b),
534 correlations with environmental variables (e.g., Eckert *et al.* 2010, Jaramillo-Correa *et al.*
535 2015), or combinations of these approaches. ‘Reverse’ ecology approaches connecting
536 genomic data with environmental parameters have also proved useful for identifying the
537 major ecological drivers of adaptation (Levy and Borenstein 2012). This SNP array is
538 therefore highly promising for studies aiming to interpret the signatures left in the genome by
539 different evolutionary forces, making inferences about complex population processes, or
540 investigating geographic patterns of genome-wide genetic variation.

541

542 **Conclusion**

543

544 In this study we report the discovery of new SNP markers in maritime pine, from RNA-seq
545 and amplicon resequencing data, and the establishment of an Infinium genotyping array
546 including SNPs that have already been validated. The SNP array presented here represents a
547 major step forward for population and conservation genetics and for breeding in maritime
548 pine. However, this array is subject to a number of limitations. Ascertainment biases, resulting
549 from the small panel of individuals from which SNPs were obtained (ascertainment width)

550 and from the stringent criteria in terms of minimum allele frequency and read coverage used
551 to retain the SNPs (ascertainment depth), must be taken into account when interpreting the
552 results of future studies of natural populations. A systematic bias would be expected for
553 estimates of nucleotide diversity, inferences about population structure, evolutionary
554 processes based on natural selection and/or historical demographic models, particularly if
555 based on the site frequency spectrum (reviewed by Helyar *et al.* 2011). Furthermore, most of
556 the SNPs were obtained from independent contigs of the UniGene, precluding the use of
557 linkage disequilibrium and haplotype diversity as metrics for population genetic inferences. In
558 the near future, we will develop genotyping-by-sequencing approaches, which should make it
559 possible to decrease the problem of ascertainment bias, because the individuals of interest are
560 sequenced directly. Considering allele drop out of genotyping-by-sequencing approaches
561 which reduces genome complexity through restriction digest (e.g. restriction-site-associated
562 DNA sequencing, Baird *et al.* 2008) as a major drawbacks for genetic inferences in highly
563 heterozygous species such as maritime pine (Davey *et al.* 2013, Gautier *et al.* 2013, Puritz *et*
564 *al.* 2014, Mastretta-Yanes *et al.* 2015), future development of marker technology in this
565 species will be based on sequence capture and direct sequencing (Gnirke *et al.* 2009).

566

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568

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583

584 **Data accessibility**

585

586 Roche 454 raw data have been deposited in the short-read archive of NCBI (accessions:
587 SRX031589 for genotype 110-4019-1, SRX208012 for genotype 0284-2 and SRX031592 for
588 genotype 112-4-1). The assembled contigs of the second maritime pine UniGene are available
589 from:

590 http://genotoul-contigbrowser.toulouse.inra.fr:9092/Pinus_pinaster2/index.html.

591

592 **Authors' contributions**

593

594 IL and HL: *in silico* SNP detection in mapping populations; IL: SNP array design and SNP
595 annotation; CB, LB, JMG, FB, GGV, DG, IRQ, and SCGM: logistical aspects of genotyping;
596 CB, JB IRQ, and DG: SNP scoring; CP, IL, MdM, MTC, NdM and FH: database compilation
597 from published and unpublished data; JB: linkage mapping; SCGM and DG: population
598 genetics analysis; CP: design of the study and overall coordination. All the authors
599 participated in the writing of the paper, and have read and approved the manuscript submitted.

600

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966

967 **Figure and table legends**

968

969 **Figure 1** Number of SNPs developed over the last five years for maritime pine and
970 interdependence of studies (for more details see Supplemental Table 2). Studies from de
971 Miguel *et al.* (2014) in orange, Chancerel *et al.* (2013) in blue, Chancerel *et al.* (2011) in
972 green, Lepoittevin *et al.* (2010) in red, and Lavigne *et al.* (manuscript in preparation) in black.
973

974 **Figure 2** Strategy for developing SNPs segregating in three full-sib progenies (CxM, LxC,
975 LxM), from high-throughput sequencing of the parental lines (C, L and M).
976

977 **Figure 3** Strategy for developing new SNPs in candidate genes.
978

979 **Figure 4 Synonymous and non-synonymous substitutions.** (A) Synonymous (S) and non-
980 synonymous (NS) mutations at the three codon positions. (B) Proportion of transitions and
981 transversions.
982

983 **Figure 5** Comparison between the two F2 genetic linkage maps (F2_N in blue and F2_O in
984 white). Segregation distortion regions are indicated in orange.
985

986 **Figure 6** Bayesian clustering analysis for $K=2$, showing the French Atlantic (red) and
987 Portuguese (green) maritime pine gene pools. The red and green portions of each bar
988 (individual tree) indicate the probability of genetic ancestry (y-axis) for each cluster.
989

990 **Table 1** Characteristics of the F2 genetic linkage maps. F2_N: map obtained from the 9 k-
991 array (this study), F2_O: map constructed with data from Chancerel *et al.* (2013) and F2_C:
992 composite map generated from the F2_N and F2_O maps.
993

994 **Table 2** Genetic diversity estimates for French Atlantic and Portuguese maritime pine
995 populations.

996

997 **Supporting Information**

998

999 **Supplemental Table 1** List of SNP arrays already developed for maritime pine and
1000 associated research questions.

1001

1002 **Supplemental Table 2** List of SNP markers already developed for maritime pine

1003

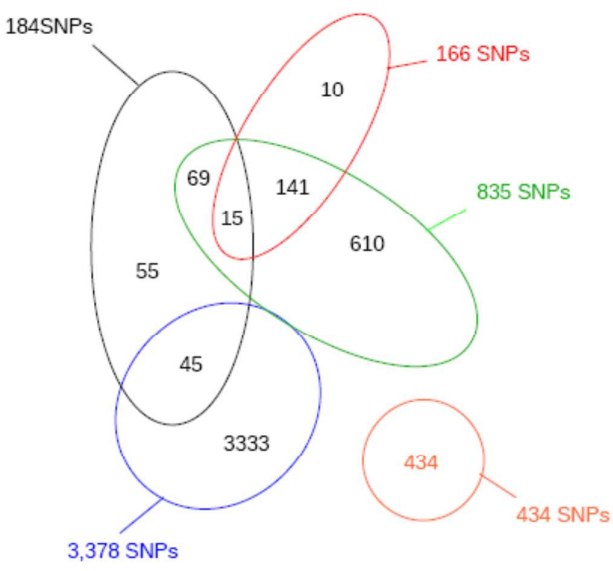
1004 **Supplemental Table 3** List of SNP markers with sequence, dbSNP accession, type of SNP,
1005 corresponding contig ID in PineV3 and functional annotation, and linkage group assignment
1006 on the F2, CL, ML, CM linkage maps.

1007

1008 **Supplemental Figure 1** Distribution of minor allele frequency (MAF from France [A] and
1009 Portugal [B]) and folded site frequency spectrum (SFS from France [C] and Portugal [D]).

1010 The expected distribution of minor allele variants is skewed towards an excess of rare
1011 variants. According to Hartl and Clark (1998), more than one third of all polymorphisms
1012 would be expected to have a frequency below 5%, reflecting purifying selection.

Figure 1



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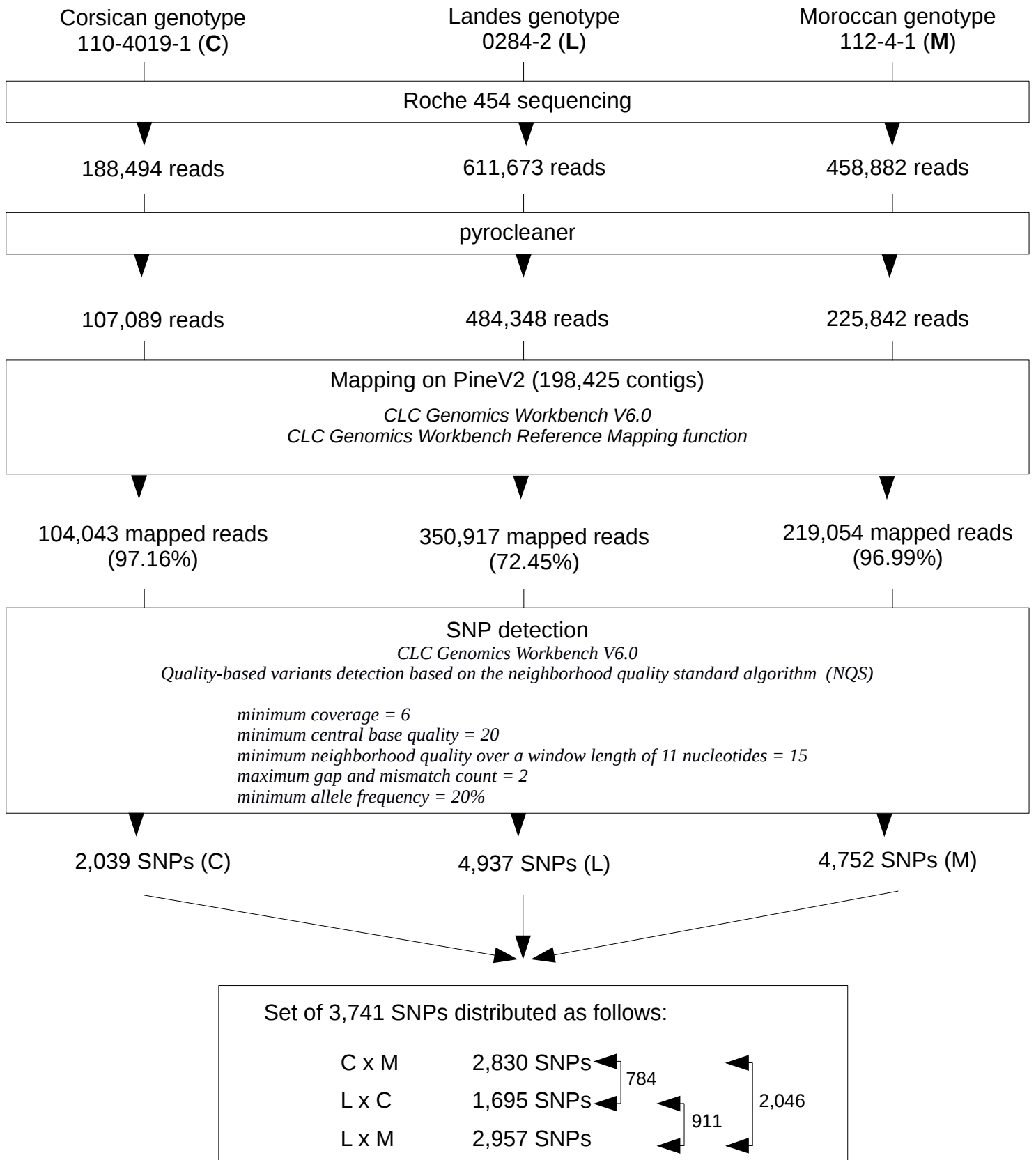
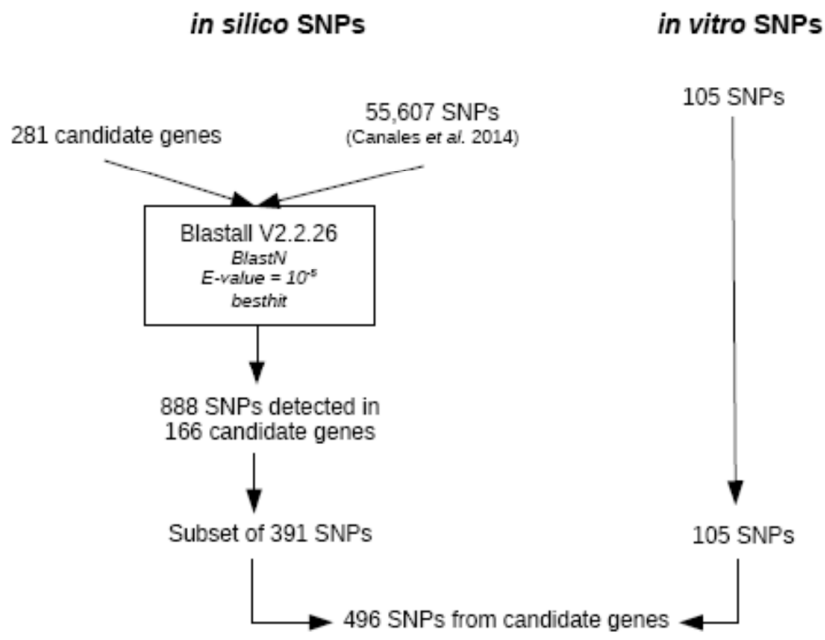
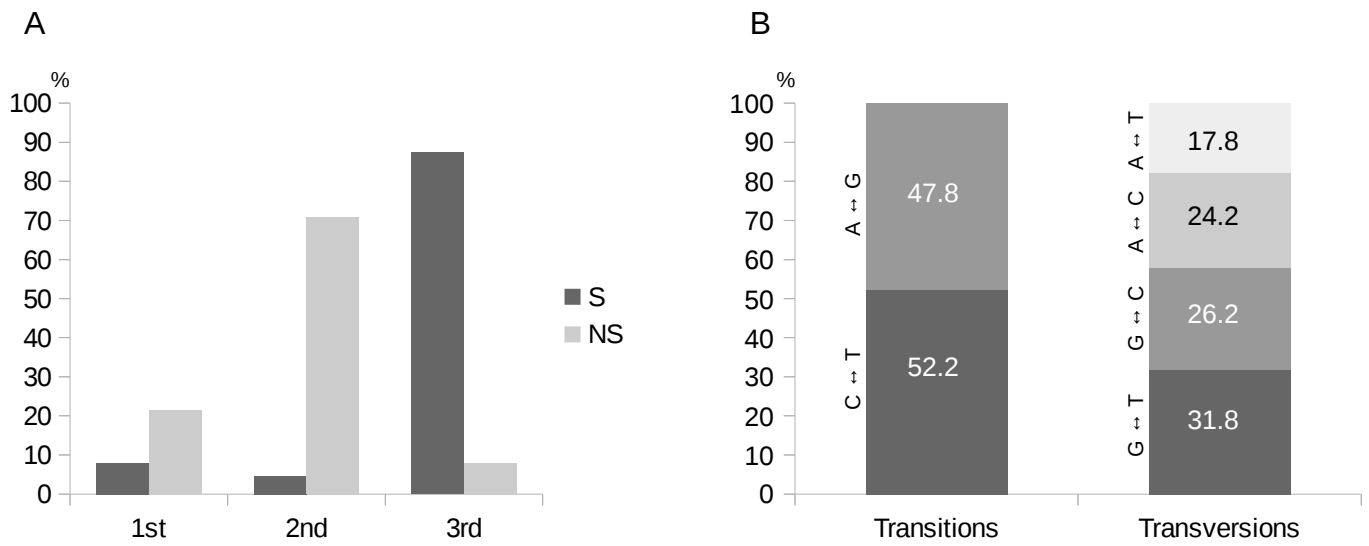


Figure 3



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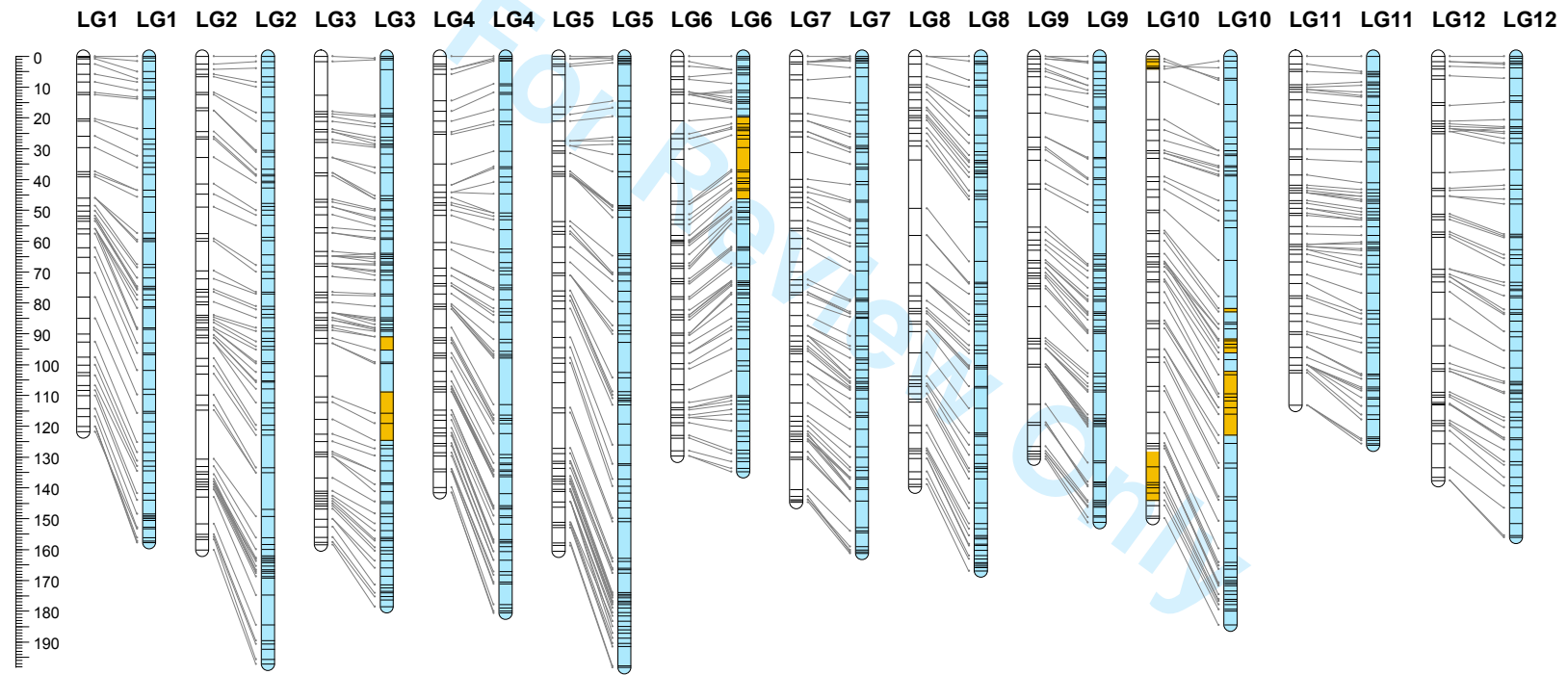


Synonymous and non-synonymous substitutions in the polymorphic SNPs.

(A) Synonymous (S) and non-synonymous (NS) mutations at the three codon positions.

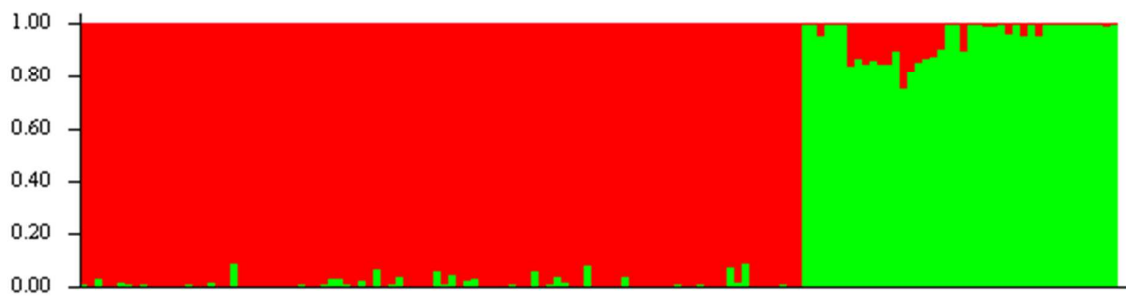
(B) Proportion of transitions and transversions.

Figure 5



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Figure 6



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Table 1

	LG	1	2	3	4	5	6	7	8	9	10	11	12	Total
F2_N	Size (cM)	157.7	197.2	178.5	180.6	198.2	134.8	161.2	166.9	151.17	184.4	126.2	156	1,992.9
	Number of SNPs	157	152	190	176	199	163	181	155	184	148	156	191	2,052
	Number of unique positions	65	81	88	77	80	80	76	79	74	66	68	67	901
	Distance between SNPs (cM)	1.01	1.31	0.94	1.03	1	0.83	0.9	1.08	0.83	1.25	0.81	0.82	0.98
	Distance between unique positions (cM)	2.46	2.47	2.05	2.38	2.51	1.71	2.15	2.14	2.07	2.84	1.88	2.36	2.25
	Distorted SNPs (%)	0	0	7.9	0	0	16	0	0	0	18.9	0	0	3.6
F2_O	Size (cM)	121.8	160.1	158.4	141.5	160.5	129.7	144.6	139.7	130.6	149.9	113.2	137.6	1,687.6
	Number of SNPs	106	117	147	138	126	113	124	93	134	107	130	146	1,481
	Number of unique positions	41	57	60	54	56	58	58	47	45	54	49	50	629
	Distance between SNPs (cM)	1.16	1.38	1.09	1.03	1.28	1.16	1.18	1.5	0.98	1.41	0.88	0.95	1.17
	Distance between unique positions (cM)	3.04	2.86	2.69	2.67	2.92	2.28	2.54	3.04	2.97	2.83	2.36	2.81	2.75
	Distorted SNPs (%)	0	0	0	0	0	0	0	0	0	33	0	0	2.75
F2_C	Size (cM)	124.1	160.1	159.3	142.2	160.5	131.3	144.6	139.7	132.2	155.4	123.7	138.8	1,711.7
	Number of SNPs	179	177	221	191	219	188	201	174	207	173	194	229	2,353
	Number of unique positions	64	81	95	75	88	88	83	81	75	76	74	75	955
	Distance between SNPs (cM)	0.7	0.91	0.72	0.75	0.74	0.7	0.72	0.81	0.64	0.9	0.64	0.61	0.74
	Distance between unique positions (cM)	1.97	2	1.69	1.92	1.84	1.51	1.76	1.75	1.79	2.07	1.69	1.88	1.82

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Table 2

Population		N	% mono	All loci			Common polymorphic loci		
				Loci #	MAF	<i>He</i>	Loci #	MAF	<i>He</i>
French Atlantic	Breeding population	46	0.242	5,016	0.201	0.266	3,981	0.254	0.336
	Natural population	50	0.241	5,016	0.198	0.263	3,981	0.250	0.332
Portugal		42	0.227	5,016	0.187	0.253	3,981	0.236	0.319

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