

Capturing neutral and adaptive genetic diversity for conservation in a highly structured tree species

ISABEL RODRÍGUEZ-QUILÓN,^{1,6} LUIS SANTOS-DEL-BLANCO,^{2,3} MARÍA JESÚS SERRA-VARELA,^{1,3} JARKKO KOSKELA,⁴
SANTIAGO C. GONZÁLEZ-MARTÍNEZ,^{1,3,5} AND RICARDO ALÍA^{1,3}

¹Department of Forest Ecology and Genetics, Forest Research Centre, INIA-CIFOR, Carretera A Coruña km 7.5, Madrid, 28040 Spain

²Department of Ecology and Evolution, University of Lausanne, Biophore Building, Lausanne, 1015 Switzerland

³Sustainable Forest Management Research Institute, University of Valladolid-INIA, Palencia, 34071 Spain

⁴Biodiversity International, Via dei Tre Denari 472/a, Maccaresse, 00057 Italy

⁵BIOGECO, INRA, University of Bordeaux, Cestas, 33610 France

Abstract. Preserving intraspecific genetic diversity is essential for long-term forest sustainability in a climate change scenario. Despite that, genetic information is largely neglected in conservation planning, and how conservation units should be defined is still heatedly debated. Here, we use maritime pine (*Pinus pinaster* Ait.), an outcrossing long-lived tree with a highly fragmented distribution in the Mediterranean biodiversity hotspot, to prove the importance of accounting for genetic variation, of both neutral molecular markers and quantitative traits, to define useful conservation units. Six gene pools associated to distinct evolutionary histories were identified within the species using 12 microsatellites and 266 single nucleotide polymorphisms (SNPs). In addition, height and survival standing variation, their genetic control, and plasticity were assessed in a multisite clonal common garden experiment (16544 trees). We found high levels of quantitative genetic differentiation within previously defined neutral gene pools. Subsequent cluster analysis and post hoc trait distribution comparisons allowed us to define 10 genetically homogeneous population groups with high evolutionary potential. They constitute the minimum number of units to be represented in a maritime pine dynamic conservation program. Our results uphold that the identification of conservation units below the species level should account for key neutral and adaptive components of genetic diversity, especially in species with strong population structure and complex evolutionary histories. The environmental zonation approach currently used by the pan-European genetic conservation strategy for forest trees would be largely improved by gradually integrating molecular and quantitative trait information, as data become available.

Key words: conservation genetics; dynamic conservation; microsatellites; phenotypic plasticity; *Pinus pinaster*; quantitative genetic differentiation; single nucleotide polymorphisms (SNP).

INTRODUCTION

Human-driven environmental change is a broadly recognized threat to global biodiversity (Bellard et al. 2012) and how to ensure species persistence under rapid climate change remains a challenge in conservation biology (Sgrò et al. 2011). Mediterranean forest ecosystems are particularly sensitive to such rapid changes, especially trees, their keystone species (Fady et al. 2015). In fact, the latest predictions forecast a shift in their distribution due to climate change (Aitken et al. 2008, Benito et al. 2011). However, the exact patterns they will follow and their microevolutionary responses remain highly unpredictable, given the multiple factors involved in the process, the uncertainty of future climate scenarios, their long generation times, and the spatial and temporal environmental heterogeneity of their distribution ranges

(Alía et al. 2014, Fady et al. 2015). Recurrent wildfires, changes in land use, and new biotic risks add to threats directly derived from global warming, such as drought stress and increased temperatures (Leone and Lovreglio 2004). In response, trees can adapt, migrate, or become extinct (Aitken et al. 2008). But we still lack data integrating approaches to assess the evolutionary consequences of global environmental changes and their impact on genetic variation, phenotypic plasticity, and the adaptability of forest tree populations (Chevin et al. 2010, Alberto et al. 2013), which are essential information to design and implement conservation strategies.

Maintaining species diversity and habitats has been the main objective of conservation policies so far, while intraspecific genetic diversity has been commonly overlooked (Laikre et al. 2010). At the same time, conservation of particular populations within species has been a central task in the European Forest Genetic Resources Program (EUFORGEN), as most widely distributed

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⁶E-mail: rodriguez.isabel@inia.es

forest trees in Europe are not endangered at the species level, but often include threatened valuable populations. The reduced level of domestication and widespread local adaptation in forest trees (Wang et al. 2010, Alberto et al. 2013) have promoted specific in situ dynamic conservation strategies worldwide (Koskela et al. 2013, Lefèvre et al. 2013). Dynamic conservation aims at maintaining evolutionary processes and adaptive potential in natural populations and manmade stands, to ensure their long-term sustainability (Namkoong 1997). Since first applied to forest trees (Ledig 1986), it has been further implemented and translated into conservation management (e.g., Koskela et al. 2013, Lefèvre et al. 2013). However, despite significant improvements in recent decades, there are still several questions that need to be addressed, such as how to select target units for conservation and how many, how to assess their viability, and how to manage them (Graudal et al. 2014).

The importance of forest genetic diversity for forest sustainability is widely recognized, but nevertheless usually ignored when developing indicators and guidelines for forest management under climate change (Sgrò et al. 2011, Graudal et al. 2014, Fady et al. 2015). A reasonable starting point is to consider that a conservation network should span a species' entire standing genetic variation (Crandall et al. 2000). Then, two main approaches have been used to define tree conservation units: (1) methods based on the contribution of particular populations to the total neutral genetic diversity or differentiation of the species (Petit et al. 1998), and (2) methods based on ecological and geographical information (e.g. Hamann et al. 2005, Lefèvre et al. 2013). The existing European network of dynamic conservation units is largely based on this second approach (Lefèvre et al. 2013), assuming that main climate types in Europe are closely linked with genetic differentiation, indirectly including adaptive processes under the hypothesis of local adaptation and clinal or ecotypic variation in tree species (see Alberto et al. [2013] for a review). Neutral molecular markers are considered an appropriate first step to designate conservation units (Fraser and Bernatchez 2001, Sagnard et al. 2002), as they provide information about historical evolutionary divergence and demographic processes, although they are often unsuitable to quantify genetic changes responding to particular selection pressures (Reed and Frankham 2001, Ouborg et al. 2006). Patterns identified in quantitative adaptive traits normally differ from those observed using neutral or weakly selected loci (Fraser and Bernatchez 2001, Sagnard et al. 2002, Frankham 2010). However, difficulty (real and perceived) of measuring adaptive genetic diversity has prevented its integration in conservation planning and management. It is urgent to overcome this situation, particularly for fragmented, structured, and locally adapted tree species facing climate change, where intraspecific diversity becomes of paramount importance (Kapeller et al. 2012). Moreover, phenotypic plasticity needs to be considered too, as it

can buffer selection toward adaptation, or make populations overcome stressful conditions via plastic changes (Chevin et al. 2010, Fady et al. 2015). Phenotypic traits may thus be more useful than previously thought in selecting populations for conservation.

A combined approach including molecular, quantitative, and ecological data, although rarely employed, is fundamental and would allow the integration of evolutionary processes in conservation planning (Sagnard et al. 2002, Ouborg et al. 2006, Wang et al. 2010, Sgrò et al. 2011). Here, we use maritime pine (*Pinus pinaster* Ait.) as a case study to show the usefulness of considering both neutral and quantitative genetic variation to identify dynamic conservation units. Maritime pine is an outcrossing, wind-pollinated long-lived tree of great ecological and economic importance that grows along a wide range of elevations, substrates, and climate regimes. It survived previous glaciations in multiple refugia (Burban and Petit 2003, Bucci et al. 2007), maintaining large amounts of genetic diversity despite its fragmented distribution, notably in the Mediterranean area (Fig. 1). As a result of this complex evolutionary history, it has a strong population genetic structure (González-Martínez et al. 2004, Santos-del-Blanco et al. 2012, Jaramillo-Correa et al. 2015) and shows signals of local adaptation (e.g., genetic differences among populations for adaptive traits [Alía et al. 1997, Santos-del-Blanco et al. 2012], or correlations between allele frequencies of polymorphisms in candidate genes for climate adaptation and climate variables [Jaramillo-Correa et al. 2015]).

We used previously developed molecular markers to classify populations into gene pools with distinct evolutionary histories. We considered these gene pools inferred from neutral genetic variation as a basis to identify a minimum set of potential conservation units. Based on a multisite clonal test, we then assessed levels of standing genetic variation, differentiation among populations, and phenotypic plasticity for two adaptive traits. We used this information to identify genetically differentiated population groups and evaluated their viability for dynamic conservation. Finally, based on our results, we assessed the suitability of the climatic zonation currently used by the European Forest Genetic Resources Program for maritime pine conservation. From this case study, we introduce a cascade decision approach to identify candidate populations for dynamic conservation and suggest key genetic parameters to account for in future forest tree conservation, based on both molecular markers and quantitative traits.

MATERIALS AND METHODS

Plant material

We collected open-pollinated seeds from 35 natural populations covering the main climates across the maritime pine natural range (Fig. 1). About 30–40 trees,

separated at least 50 m from each other, were sampled, and one single seed from each tree was planted in nursery. Then, each sapling (the “mother plants”) was propagated by cuttings to obtain clones. These clones (average of 15 clones per population, for details see Appendix S1: Table S1) were used to establish a common garden experiment of 16544 trees, in 2010. Each of the four replicated trials comprised 4136 trees, with 517 clones (i.e., genotypes) and eight replicates per genotype, in a randomized complete block design. The four trial sites provided contrasting environmental conditions (Fig. 1). Trials in Asturias (Spain) and Portugal were located in the Atlantic region, with high annual rainfall (1160 and 985 mm, respectively) and mild temperatures (11.3°C and 14.2°C, respectively), although Portugal shows less than half the summer rainfall than Asturias (63 and 152 mm, respectively). Trials in Madrid and Cáceres (Spain) are located in continental areas under Mediterranean influence (annual rainfall of 443 and 898 mm, and mean annual temperature of 13.7°C and 15.5°C, respectively), with large seasonal temperature oscillations and a marked summer drought.

Molecular markers

Needles from all the 517 genotypes were collected from mother plants in nursery and dried in silica gel for subsequent DNA extraction. Samples were genotyped for 12 nuclear microsatellites (nuSSRs and 266 single nucleotide polymorphisms (SNPs; data deposited in Dryad with doi: 10.5061/dryad.c289v). Details for DNA extraction, marker typing, and scoring can be found in Santos-del-Blanco et al. (2012) and Jaramillo-Correa et al. (2015), and references therein. Both sets of molecular markers have proved to be mostly neutral in previous studies (7% of markers associated with environmental variables and a fire-related trait; see Budde et al. 2014, Jaramillo-Correa et al. 2015). Here we used both sets of molecular markers to assess population structure using the Bayesian clustering method implemented in STRUCTURE 2.3.4, running an admixture model with correlated allele frequencies between clusters. Ten runs were performed for each number of clusters from $K = 1$ to $K = 10$, with a burn-in length of 1,00,000 and a run length of 10,00,000 iterations. The optimal number of population clusters,

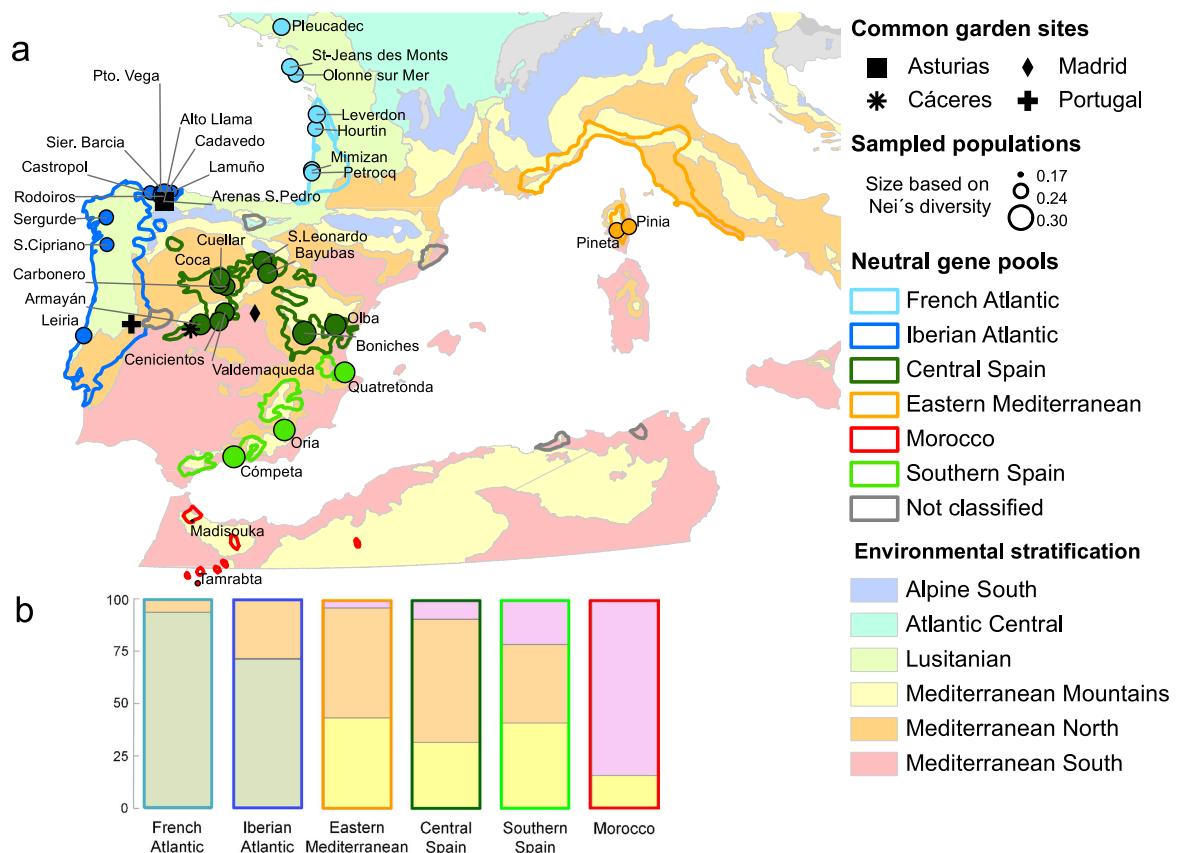


FIG. 1. (A) Climatic stratification of Europe (Metzger et al. 2005) and maritime pine (*Pinus pinaster* Ait.) distribution, with location of the 35 source populations (circles) and clonal common gardens (other symbols). Size of population symbols is proportional to Nei's diversity (H_e) for 266 single nucleotide polymorphisms (SNP). Different symbol and contour colors indicate the six different neutral gene pools derived from 12 nuclear microsatellites (nuSSRs) and 266 SNP markers. (B) Proportion of each neutral gene pool area covered by different climatic regions.

K , was calculated following Evanno et al. (2005). Additionally, we tested for neutral genetic differentiation within each neutral gene pool following the same procedure. Allelic richness and unbiased Nei's index of genetic diversity (H_c ; Nei 1978) for both marker types were computed using SPAGeDi v1.4 (Hardy and Vekemans 2002). F_{ST} was also estimated for both markers at different hierarchical levels (i.e. species, among and within gene pools). F_{ST} distributions were later computed following the parametric bootstrap procedure proposed by Lamy et al. (2011), to be compared to Q_{ST} ones (see following section).

Quantitative traits

Survival and height were measured three years after establishment in the field. Clonal common gardens allowed multiple phenotypic measurements of each genotype. Recorded phenotypic variables were analyzed with Bayesian mixed models in R (R Development Core Team 2015; see Appendix S2: Bayesian mixed models for details). Quantitative genetic parameters (clonal variance and population variance), and best linear unbiased predictors (BLUPs) for genotypes and populations were derived from Markov chain Monte Carlo (MCMC) models. Then, we computed pairwise cross-environment correlations among trait estimates for clone and population mean data to estimate genotype \times environment ($G \times E$) interactions. Within-population broad-sense heritability (H^2 ; Falconer and Mackay 1996) for survival and height was estimated as

$$H^2 = (\sigma_{cwp}^2) / (\sigma_{cwp}^2 + \sigma_e^2)$$

where σ_{cwp}^2 is the variance among clones within populations and σ_e^2 , the error variance. For the estimation of broad-sense heritability of survival, we included an extra term in the denominator ($\pi^2/3$) to account for implicit logit link function variance (Nakagawa and Schielzeth 2010).

Phenotypic plasticity for survival and height was assessed by computing the ratio of the mean trait estimate at the most favorable site minus the mean trait estimate at the least favorable site, divided by the mean for the same trait in the most favorable site (Valladares et al. 2006). We estimated the heritability of plasticity as the ratio of the $G \times E$ interaction variance to the total phenotypic variance (Scheiner and Lyman 1989). To estimate the variance component of the interaction we used a multi-site two-step analysis (see Appendix S2: Heritability of plasticity for details).

Genetic differentiation for quantitative traits, Q_{ST} (Spitze 1993), was estimated as

$$Q_{ST} = \frac{\sigma_{Bpop}^2}{\sigma_{Bpop}^2 + 2\sigma_{Wpop}^2}$$

where σ_{Bpop}^2 and σ_{Wpop}^2 are the genetic variance between populations and within populations, respectively. Even

though our experimental design did not include families, we were able to compute a broad sense Q_{ST} proxy (Q_{ST}^*), approaching genetic variance from clonal variance estimates. Genetic variance is mainly additive in complex quantitative traits (Hill et al. 2008), as reported in maritime pine (Cotterill et al. 1987), and thus we expect negligible biases with our approach. Moreover, Q_{ST}^* was computed from common garden data with low environmental heterogeneity, and independently in each location, thus minimizing potential biases due to $G \times E$ interactions. MCMC models provided 1000 independent Q_{ST}^* estimates. To disentangle the effects of genetic drift from those of selection, Q_{ST}^* probability distributions were compared to those of neutral F_{ST} , using a nonparametric and free distribution two-sample test for equality of the 2.5% and the 97.5% quantiles with Bonferroni correction for multiple comparisons (Lamy et al. 2011). Both Q_{ST}^* and F_{ST} were computed at the species level and within neutral gene pools.

Identification of conservation groups

We considered gene pools revealed with molecular markers (Fig. 1) as a baseline for conservation needs because they reflect the demographic history of the species. However, we found high quantitative genetic differentiation (Q_{ST}^*) for adaptive traits within those neutral gene pools (see *Results*). Thus, to identify adaptive population groups that are relevant for genetic conservation within neutral gene pools, we conducted a hierarchical clustering analysis considering nine variables: survival and height at the four common gardens and the plasticity index for height. For this analysis, we excluded populations with five clones or less (Madisouka, Carbonero, and Cómpeeta) and Rodoiros population, which proved to be nonnative, judging by cluster analyses (see Appendix S1: Figure S1) and historical records. Uncertainty of hierarchical clusters was assessed via multiscale bootstrap resampling, using pvclust package (Suzuki and Shimodaira 2006) in R (R Development Core Team 2015). We run 10000 bootstraps using the average agglomerative method and correlation as distance measure. The most significant factors for population clustering were identified with the Random Forest package (Liaw and Wiener 2002) attending to mean decrease accuracy and mean decrease in Gini index. These estimates indicate, respectively, how important are individual variables for the correct estimation of the tree, and how much they contribute to the homogeneity of the nodes and leaves in the resulting random forest. We considered all variables randomly sampled as candidates at each split, and grew 10^6 trees. Height in Asturias (i.e., the site with the highest survivorship and best growth rates) was by far the most influential variable (Appendix S2: Figure S1) providing the highest among-population differentiation. Then, we also carried out subgroup identification using just height data from the Asturias site. These subgroup definitions within neutral gene pools

were based on pairwise post hoc population comparisons at the $\alpha = 0.1$ level, as phenotypic variation within populations is typically large.

Characterization of conservation groups

For each of the population groups identified, we computed heritability and evolvability (the clonal variance to mean ratio for each trait), two key parameters related to short-term evolutionary potential. Genetic variance components for each trait and site were obtained from MCMC models, where population groups were included as a random factor. We also computed Q_{ST}^* , F_{ST} , and height plasticity for each conservation-relevant population group as described previously. The effective population size (N_e) of each group was assessed for both nuSSRs and SNPs using the heterozygote-excess method of Pudovkin (Pudovkin et al. 2009), as implemented in NeEstimator V2 (Do et al. 2014). To investigate levels of inbreeding, we also computed a multilocus estimate of selfing for each group using RMES software (David et al. 2007).

Climatic zonation

The pan-European genetic conservation strategy for forest trees relies mainly on the climatic classification of Europe (Metzger et al. 2005, 2013). We wanted to test the usefulness of broadscale climatic boundaries as a proxy for genetic differentiation in maritime pine. To do so, we overlapped the climatic stratification of Europe (as defined in Lefèvre et al. 2014, modified from the original map in Metzger et al. 2005), the distribution of maritime pine neutral gene pools, the location of the existing 42 EUGIS conservation units and the conservation-relevant population groups proposed here. Additionally, we tested for correlations among height and continuous environmental variables. We retrieved summary climate data for the period 1950–2000 from Worldclim (Hijmans et al. 2005) and a regional climatic model (Gonzalo Jiménez 2010) for the 11 non-Spanish and the 24 Spanish maritime pine populations, respectively. Pearson

correlation coefficients were estimated between height estimates at each site and nine geographic and environmental variables related to temperature and rainfall (summarized in Appendix S3: Table S1). We also ran a principal component analysis (PCA) for those geographic and environmental variables, based upon the correlation matrix, with varimax rotation. The analysis was implemented by the psych package (Revelle 2011) on the R platform (R Development Core Team 2015). PCA loadings of the three most important PCs were later used for correlation analysis with height.

RESULTS

Molecular markers

Both nuSSRs and SNPs revealed similar genetic differentiation to previous markers (e.g., chloroplast SSRs; Bucci et al. 2007), leading to the definition of six extant gene pools (optimal $K = 6$): French Atlantic coast, Iberian Atlantic coast, central Spain, southern Spain, Eastern Mediterranean (including Corsica), and Morocco (Appendix S1: Figure S1). We found no population structure (optimal $K = 1$) within each specific gene pool (data not shown). Maritime pine's H_e for nuSSRs and SNPs was 0.65 ± 0.01 and 0.30 ± 0.00 , respectively. H_e estimates were consistently above 0.23 for nuSSRs and 0.5 for SNP markers, for all tested populations (Appendix S1: Table S1 and Figure S2) and for the six distinct neutral gene pools (Table 1). Allelic richness ranged from 2.20 to 3.19 for nuSSRs and from 1.39 to 1.63 for SNP markers in the studied populations (for details see Appendix S1: Table S1), with no significant differences among gene pools (Table 1). F_{ST} estimates at the species level were moderate for both nuSSRs (0.099, CI of 0.085–0.117) and SNP markers (0.130, CI of 0.118–0.142). Among gene pools, F_{ST} explained most of the neutral population differentiation (0.070 for nuSSRs and 0.116 for SNP markers). As expected, we detected a reduced level of molecular marker genetic differentiation among populations within all neutral gene pools, except for Morocco (Table 1).

TABLE 1. Genetic diversity (allelic richness and Nei's expected heterozygosity, H_e ; Nei 1978) and population genetic differentiation (F_{ST}) based on 12 nuclear microsatellites (nuSSRs) and 266 single nucleotide polymorphisms (SNPs), for the six extant maritime pine (*Pinus pinaster* Ait.) gene pools; standard error (SE) is shown in parentheses.

Gene pool	Allelic richness		Nei's H_e		F_{ST}	
	nuSSRs	SNPs	nuSSRs	SNPs	nuSSRs	SNPs
French Atlantic	2.55	1.53	0.588	0.262	0.008 (0.004)	0.006 (0.001)
Iberian Atlantic	2.49	1.52	0.581	0.257	0.023 (0.090)	0.025 (0.002)
Central Spain	2.63	1.59	0.603	0.290	0.024 (0.005)	0.022 (0.002)
Southern Spain	2.83	1.60	0.677	0.301	0.045 (0.015)	0.045 (0.006)
Eastern Europe	2.28	1.51	0.520	0.258	0.002 (0.012)	0.016 (0.007)
Morocco	2.36	1.40	0.568	0.192	0.161 (0.094)	0.155 (0.036)
Species	2.52	1.53	0.647	0.303	0.099 (0.000)	0.130 (0.006)

Quantitative traits

Survival ranged from 3% to 98% across the four common gardens (98% CI, 0.97–0.99 in Asturias, 66% CI, 0.60–0.70 in Portugal, 21% CI, 0.18–0.26 in Madrid, and 3% CI, 0.20–0.40 in Cáceres). Mean height per site ranged from 27.7 cm (CI, 25.9–28.8) in the most favorable environment (Asturias) to 17.0 (CI, 14.8–18.9) in the harshest (Cáceres; for details see Appendix S2: Table S1). Both traits evidenced highly contrasted levels of environmental stress among trial sites. Phenotypic variance for survival was almost entirely driven by environmental variability as suggested by low differentiation across clones. Height thus turned out to be a more suitable adaptive trait than survival to study population differentiation in our study. We identified highly significant site-to-site correlations for both traits among sites with similar environmental conditions (e.g. correlation for height in sites under Atlantic climate: $r = 0.49$, $P < 10^{-10}$, and under Mediterranean climate: $r = 0.24$, $P < 10^{-3}$). Overall, cross-environment correlations were stronger for height, with all correlations being significant ($P < 0.01$; Appendix S2: Table S2).

Broad-sense heritability (H^2) varied depending on the trait and trial site. Survival H^2 was very low, ranging from 0.02 (CI, 0.01–0.02) in Portugal to 0.05 (CI, 0.04–0.06) in Asturias. Height H^2 was substantially higher (range of 0.15–0.28), particularly in more stressful environments (e.g., 0.28 CI of 0.13–0.41 in Cáceres). Mediterranean neutral gene pools showed higher H^2 compared to Atlantic ones (see Appendix S2: Table S3).

Quantitative genetic differentiation (Q_{ST}^*) ranged from 0.11 (CI, 0.08–0.16) in Asturias to 0.39 (CI, 0.30–0.48) in Madrid for survival, and from 0.31 (CI, 0.23–0.50) in Portugal to 0.45 (CI, 0.31–0.58) in Asturias for height. In the four sites, overall Q_{ST}^* estimates were significantly higher than F_{ST} estimates at the species level (Fig. 2). Quantitative population differentiation was still high within neutral gene pools, suggesting the existence of distinct phenotypic groups ($Q_{ST}^* = 0.10$ to 0.87 for height, Fig. 2; and $Q_{ST}^* = 0.01$ to 0.35 for survival; Appendix S2: Table S3).

Maritime pine showed high levels of phenotypic plasticity, although variable among neutral gene pools. Phenotypic plasticity for height varied between Atlantic and Mediterranean populations, decreasing from northern to southern gene pools, the French Atlantic and Iberian Atlantic being those with the highest plasticity index (0.96 and 0.93, respectively) and the Moroccan populations those with the least (0.75). Heritability of height plasticity was low (estimate for the combined analysis of 0.06), albeit higher in more stressful environments (in decreasing order of harshness: 0.11 ± 0.04 in Cáceres, 0.08 ± 0.03 in Madrid, 0.07 ± 0.02 in Portugal and 0.05 ± 0.02 in Asturias).

Identification and characterization of conservation groups

Hierarchical cluster analysis within neutral gene pools allowed their further subdivision based on quantitative traits. Among the nine variables used for clustering, height measured in Asturias was the most

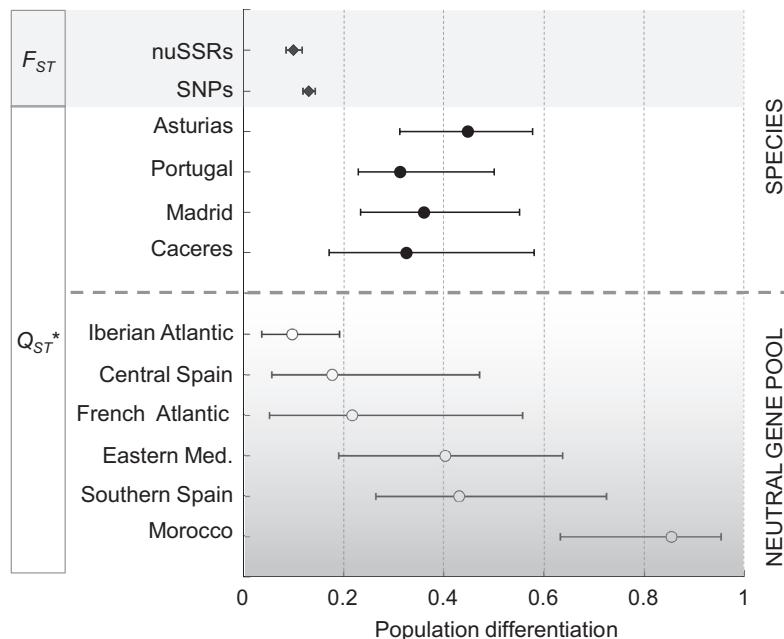


FIG. 2. Molecular (F_{ST}) and quantitative (Q_{ST}^*) genetic differentiation among range-wide maritime pine populations for height. Results are given at the species level for the four trial sites (common gardens; see Fig. 1) and within neutral gene pools in the Asturias trial site.

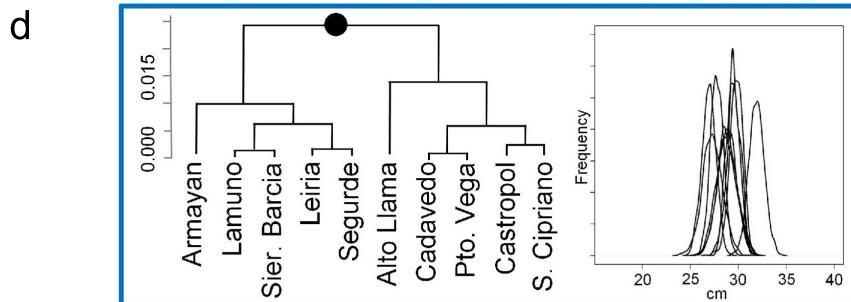
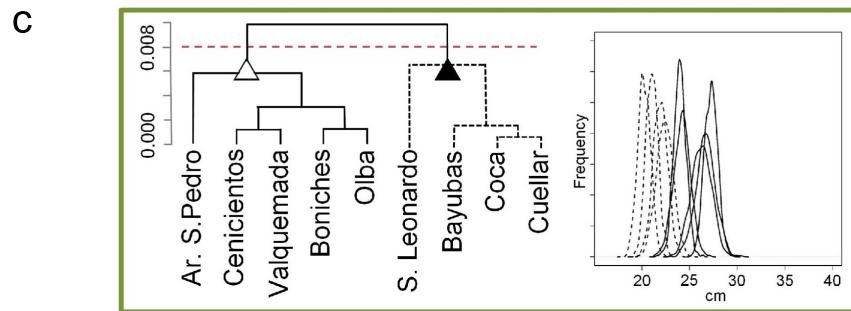
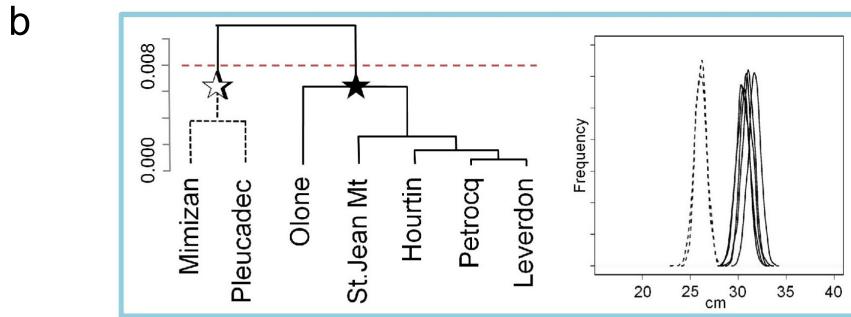
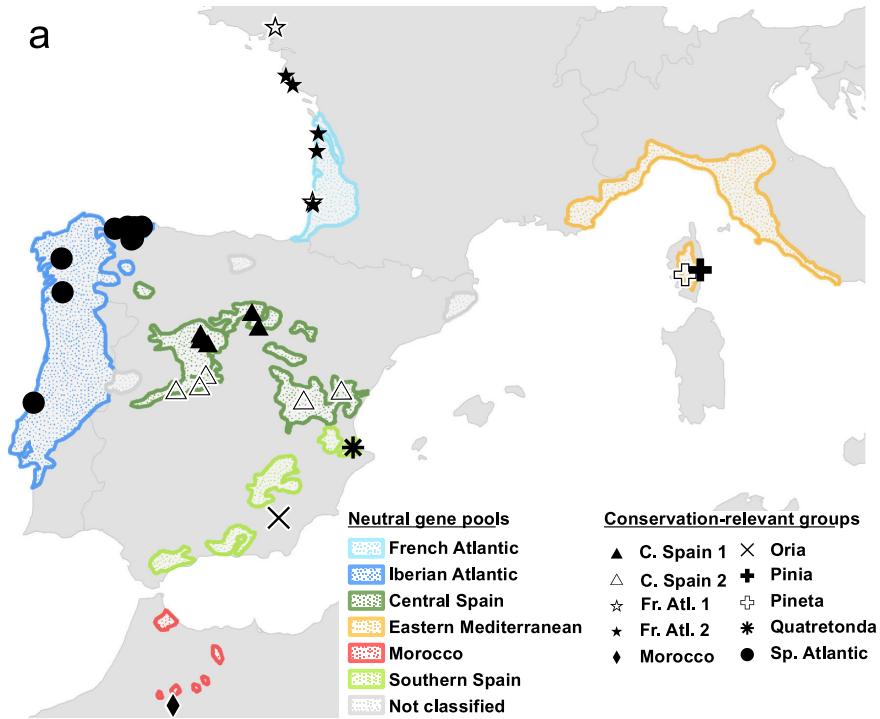


FIG. 3. (A) Proposed conservation-relevant population groups for maritime pine based on genetic distinctiveness and geographic boundaries of neutral gene pools. Clustering based on height and survival as measured in four common gardens with contrasting environments and mean height distribution assessed at Asturias site, for (B) French Atlantic, (C) Central Spain, and (D) Iberian Atlantic gene pools. Each inset shows a hierarchical clustering dendrogram on the left and a height distribution plot at the population level on the right, with populations represented with different symbols and line types (solid, dashed, etc.) corresponding to different conservation-relevant groups.

important (mean decrease accuracy = -113.61, mean decrease in Gini index = 1.29, Appendix S2: Figure S1). Pairwise post hoc population comparisons within gene pools for this variable led to very similar population clustering than with all nine variables. For the three neutral gene pools with more than three populations (French Atlantic, Iberian Atlantic, and Central Spain), clustering based on quantitative traits is shown in Fig. 3. The remaining neutral gene pools (Southern Spain, Eastern Mediterranean, and Morocco) exhibited significant differentiation among all populations within the gene pool. Accordingly, the six initial neutral gene pools led to the identification of 10 conservation-relevant population groups, subsequently considered as the minimum that should be depicted in a preliminary maritime pine genetic conservation program.

All conservation-relevant population groups showed high levels of neutral genetic diversity for both nuSSRs (minimum of 0.517 in *Pinia*) and SNPs (minimum of 0.187 in Tamrabta), reduced levels of inbreeding (Fig. 4) and high effective population sizes (NeEstimatorv2 gave infinite N_e for all groups, i.e., no evidence for variation caused by genetic drift due to a finite number of parents; Do et al. 2014). They also showed high evolvability for height and survival in the four trials, and high levels of phenotypic plasticity (Fig. 4), two key parameters determining their evolutionary potential. As expected, genetic differentiation among populations for neutral markers (F_{ST}) and quantitative traits (Q_{ST}^*) were markedly lower within the proposed conservation-relevant population groups than within the six initial neutral gene pools (Appendix S2: Table S4).

Usefulness of climatic zonation

The entire maritime pine natural range is comprised within six climatic zones (Fig. 1). The existing genetic conservation network (42 EUFGIS conservation units) comprises populations from the six climatic regions where the species can be found, but do not follow an even pattern within climatic regions (Appendix S3: Figure S1), showing gaps in the implementation of the pan-European strategy. The Mediterranean North climate is clearly overrepresented (52% of conservation units belong to this region), while the Atlantic Central, Lusitanian, and Mediterranean South climates are underrepresented (7%, 10%, and 10% of total, respectively). Moreover, it only includes populations from four out of the six neutral gene pools identified within the species, with the Eastern Mediterranean group clearly overrepresented, while populations from Iberian Atlantic or Moroccan gene

pools are lacking. Indeed, some populations within the same climatic zone belong to different neutral gene pools and, conversely, single neutral gene pools are distributed along distinct climatic zones (Fig. 1). Climatic boundaries were neither able to distinguish among population groups genetically distinct for adaptive traits within neutral gene pools (i.e. conservation-relevant population groups, as defined here). However, we found significant correlations between height and geographic and environmental variables in trials under Atlantic environments (e.g. $r = 0.55$, $P = 6.2 \times 10^{-4}$ with annual rainfall in Asturias; $r = -0.37$, $P = 2.9 \times 10^{-2}$, with mean temperature of the warmest month in Portugal; Appendix S3: Table S1). No significant correlations were found in trials under Mediterranean climates (Madrid and Cáceres), with lower survival. PCA showed that population mean height was highly correlated with PC1 (54% variance explained) in trials with higher trait variability ($r = 0.63$ in Asturias, $P = 6.5 \times 10^{-5}$; $r = 0.49$ in Portugal, $P = 3.3 \times 10^{-3}$; see details in Appendix S3: Tables S2 and S3). PC1 was mainly contributed by the continentality index and altitude, thus summarizing environmental harshness.

DISCUSSION

In this study, we used an integrated approach to identify a minimum set of genetically distinct population groups in maritime pine. We departed from gene pools defined using neutral molecular markers and, after incorporating information on genetic variation and adaptive potential in two key quantitative traits, we were able to distinguish 10 conservation-relevant population groups. Molecular marker and quantitative trait statistics suggested high levels of genetic diversity, plasticity, and evolutionary potential in these population groups. Their representation in a genetic conservation network would increase the chances for long-term maintenance of the species' genetic diversity. Our approach represents a step forward in setting priorities for upcoming conservation efforts, stressing the effectiveness of gradual integration of genetic information at different scales and time frames as it becomes available for the target species.

Intraspecific conservation in forest trees

Most widespread European forest trees, including maritime pine, are included within the IUCN category of "least concern" or are still to be evaluated (Farjon 2013). However, the need for intra-specific diversity conservation in forest trees is widely recognized by the

research community (Ledig 1986, Kapeller et al. 2012 for Norway spruce, Leites et al. 2012 for Douglas-fir). Some particular maritime pine populations (e.g., Tramuntana in the Balearic Islands or those of subspecies *P. pinaster* ssp. *renouii* in North Africa) are endangered as a result of overexploitation and habitat degradation (Farjon 2013). Differential levels of forest decline have also recently been anticipated for distinct maritime pine gene pools (Jaramillo-Correa et al. 2015). In the same line, environmental niche modeling predicts that maritime pine will be able to withstand upcoming climate change, but with contrasted population responses (Benito et al. 2011). Population genetic structure analyses based on the studied molecular markers led to the definition of six extant gene pools (Fig. 1 and Appendix S1: Figure S1). This is consistent with the widely known phylogeographic history of the species (Santos-del-Blanco et al. 2012, Jaramillo-Correa et al. 2015). We found high levels of genetic variation and phenotypic plasticity at the species, gene pool, and conservation group levels (Fig. 4), suggesting potential for evolutionary change to cope with new selection pressures. However, our consistently higher Q_{ST}^* than F_{ST} estimates (Fig. 2), for both traits in the four common garden sites, point to widespread divergent selection too. Thus, contrasted evolutionary changes are expected in different parts of the maritime pine range, in particular under harsh environmental conditions. All these appeal for the need to further develop genetic conservation strategies at the intraspecific level for maritime pine, as for most other widespread forest trees (e.g., see CONFORGEN in Canada and EUFORGEN in Europe).

Usefulness of molecular markers and quantitative traits in conservation

Thirty years after the first definition of Evolutionary Significant Units by Ryder (1986), with scarce guidelines for operational application, it is still debated how conservation units should be defined and the relative importance that should be given to neutral and adaptive components of genetic variation (Crandall et al. 2000, Fraser and Bernatchez 2001). Our proposal is a cascade decision approach, using climatic boundaries when no other information is available, but gradually incorporating molecular marker and phenotypic data as they become available for the species. Extensive studies using molecular markers are now available, or could easily be in the near future, for many forest trees (e.g., *Eucalyptus*, *Pinus*, *Populus*, *Quercus*; Neale and Kremer 2011). Incorporating such information into conservation planning would already represent a qualitative leap forward from using traditional regions of provenance (Alía et al. 1996) or seed zones (Hamann et al. 2005). Molecular markers effectively define major cohesive groups with limited gene flow between them (Fraser and Bernatchez 2001). Nonetheless, they are often unsuitable to quantify adaptive variation or evolutionary potential

(Reed and Frankham 2001, Ouborg et al. 2006), as the ability of species to evolve is primarily determined by quantitative genetic variation (Frankham 2010). Thus, when data are available, phenotypic traits should be used in a second step to differentiate subgroups of populations under different selective pressures, improving previous proposals based on demographic observations or genetic differentiation estimated only with neutral molecular markers (e.g., Petit et al. 1998). Sagnard et al. (2002) provided one of the few examples combining quantitative traits and marker data in a conservation study in *Abies alba*. Extensive quantitative trait data from traditional common garden experiments (i.e., provenance trials) are already available for many economically and ecologically important tree species (see following section and Pâques [2013]). This information could be used in a third step to identify populations worth protecting, but we are unaware of any conservation program for forest trees using this information so far. The described methodology can be applied to any forest tree species, as data gradually become available. However, lack of genetic information could hinder a broad scale implementation of the described approach for species with less economic and ecological importance.

Once conservation-relevant population groups are identified, assessing to which extent they are able to maintain the species' evolutionary potential remains a complex issue. This can also be addressed by integrating molecular marker and quantitative trait data (Aravanopoulos 2011, Lefèvre et al. 2014). In maritime pine, the proposed conservation-relevant population groups display high levels of genetic diversity and effective population sizes, and negligible inbreeding (Fig. 4), suggesting high resilience to new environmental conditions. Low quantitative genetic differentiation among populations within these groups facilitates further selection of specific populations within them as conservation units, while maintaining genetic variation and evolutionary potential. The high phenotypic plasticity found in all conservation-relevant groups supports the available evidence on fast and strong plastic responses of Mediterranean plants to environmental change, with plasticity highly differing, as in our case study, among populations within a species (Matesanz and Valladares 2014). Maritime pine Atlantic populations are more plastic, and height plasticity decreases gradually as we move south within the species range (Fig. 4). For populations with higher plasticity, the demographic impact of strong selection would be smooth, allowing more time for evolutionary adaptations to take place and reducing the amount of evolutionary change necessary to track the moving environmental optimum (Chevin et al. 2010).

Implications for maritime pine conservation

The Mediterranean basin has been recognized as a biodiversity hotspot and a prime target for conservation efforts (Myers et al. 2000, Fady-Welterlen 2005).

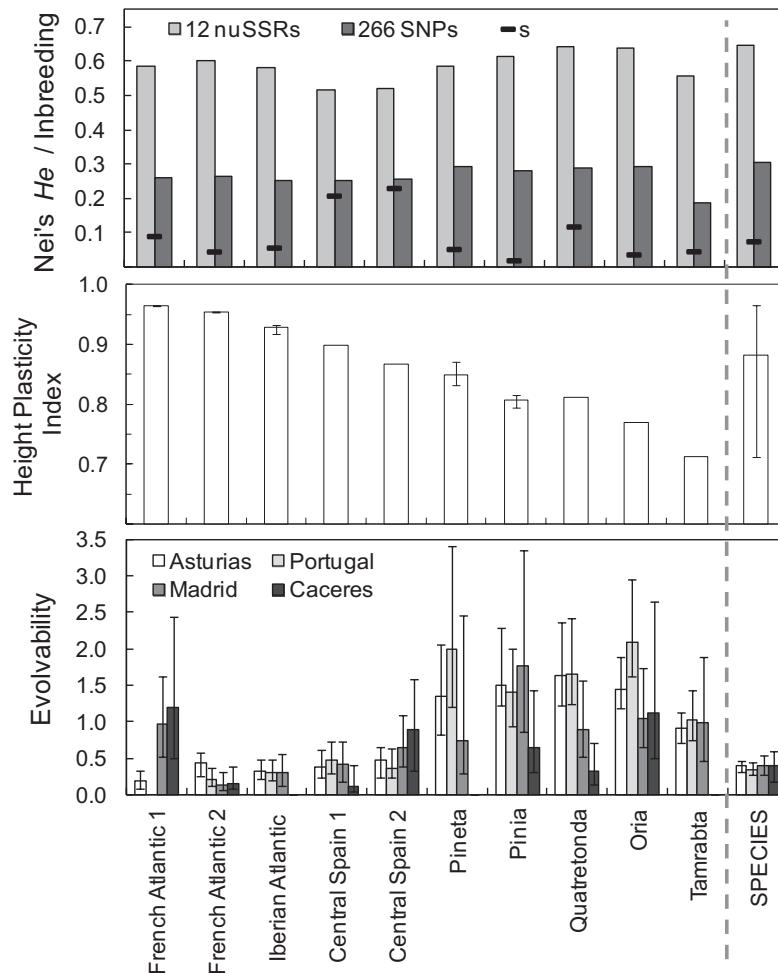


FIG. 4. Characterization of conservation-relevant population groups in maritime pine: Nei's genetic diversity (H_e) and inbreeding coefficient (s) based on 12 nuSSRs and 266 SNPs, plasticity index for height and evolvability in the four common garden sites (Asturias, Portugal, Cáceres, and Madrid). Bars show the range of height IP values within each group and 95% CI for evolvability.

However, Lefèvre et al. (2013) recently detected gaps in genetic conservation efforts for forest trees in the Mediterranean climatic zones. The pan-European genetic conservation strategy for forest trees uses climatic zones (Metzger et al. 2005, 2013) as a surrogate to account for adaptive processes. Still, the usefulness of broadscale climatic boundaries as a proxy for genetic differentiation to prioritize populations for genetic conservation remains questionable, even for species exhibiting phenotypic clines along environmental gradients. Our results corroborate the existence of a clinal variation along temperature and rainfall gradients in the species (Santos-del-Blanco et al. 2012, Jaramillo-Correa et al. 2015), and the important role of the environment in maritime pine local adaptation (Serra-Varela et al. 2015). However, neither neutral nor adaptive genetic variability are captured by the climatic zoning of Metzger et al. (2005), most likely because of its large spatial scale or inadequate selection of classification variables for maritime pine. Large-scale environmental classifications

constitute a good starting point, but should be considered with caution when addressing conservation priorities, especially when extensive genetic data is available (e.g., for some of the main forestry species in Europe, including Scots pine, Aleppo pine, common ash, European beech, wild cherry, and Norway spruce, among others; see Pâques 2013).

Once conservation-relevant population groups have been identified, as we did in this study, selection of particular populations within these groups should meet the minimum requirements for genetic conservation units developed by EUFORGEN (Koskela et al. 2013), as well as national and local use and socio-economic criteria. This highlights the difficulty of descending from conservation-relevant population groups at a regional scale (i.e., conservation planning) to practical conservation at the national or local level (i.e., choosing particular populations and protecting them). We present, nevertheless, a feasible approach to ease the challenge and improve practical conservation by using

available genetic information to produce candidate groups of populations to be represented in conservation networks.

The 10 conservation-relevant population groups presented here (Fig. 3) are the result of the integration of all genetic information currently available in maritime pine, but they still constitute the minimum to be considered. Collection of genetic data from unexplored regions and the inclusion of a wider range of adaptive and fitness-related traits, e.g., pest and disease resistance, germination and seedling establishment ability, and adult survival and reproduction traits, could increase the number of population groups to be considered for effective maritime pine conservation. Although it would be ideal to count on as many phenotypic traits as possible, in this species height has proved to be a highly integrative trait, closely related to resistance to biotic and abiotic factors (Santos-del-Blanco et al. 2012, Alía et al. 2014, Jaramillo-Correa et al. 2015). This made the classification based on height a reliable approximation.

Conservation efforts should additionally consider establishing genetic conservation units in marginal populations growing at distribution edges or under extreme ecological conditions (Fady et al. 2015), in particular those that have already shown genetic divergence from nearby core populations (e.g., Fuencaliente in Ciudad Real, Spain). The maritime pine range was traditionally divided into regions of provenance (e.g., Alía et al. 1996 for Spain, CEMAGREF 2003 for France) that correspond to areas with uniform ecological conditions and seed sources with putatively (i.e., not experimentally tested) similar phenotypes or genetic features (as evaluated by quantitative genetics, and terpene and isozyme studies) for forest management and afforestation purposes. These regions of provenance could be used to establish geographical boundaries for the defined conservation-relevant population groups. This strategy would work well, for example, in Spain, where a large number of disconnected provenance regions have been established (Alía et al. 1996, Appendix S3: Figure S2). However, our case study also shows that the Landes and Corsican provenance regions (CEMAGREF 2003) need to be subdivided for conservation purposes (Fig. 3). This highlights the need for standardization of criteria for selection of conservation units across countries.

CONCLUSIONS

To our knowledge, this is the first study considering jointly molecular marker, quantitative trait, and environmental variation to address forest tree conservation. We introduce key molecular and quantitative trait statistics to assess population differentiation and identify sound population groups for dynamic conservation purposes. Our results bear out that environmental variation is a good starting point for in situ gene conservation strategies, but neither a purely climatic nor a neutral molecular marker classification would capture all variability that

should be considered in conservation planning. Current conservation strategies fail to integrate available genetic information, despite its proving effective to identify populations worth protecting in tree species with complex evolutionary histories. Further work is needed to select particular populations for the establishment of genetic conservation units in maritime pine, but dissemination of first-step case studies, as the one presented here, represents a step forward in the urgent need to bridge the gap between basic research in ecology and evolution and the practical needs of conservation managers (Mace and Purvis 2008, Shafer et al. 2015). Incorporation of quantitative genetic information in conservation programs for long-lived organisms such as forest trees is challenging and complex, but precise and integrated demographic, neutral and adaptive genetic diversity data are essential to design effective tree dynamic conservation strategies to minimize biodiversity losses in the face of climate change.

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SUPPORTING INFORMATION

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DATA AVAILABILITY

Data associated with this paper have been deposited in Dryad: <http://dx.doi.org/10.5061/dryad.c289v>.