Limited genetic variability and phenotypic plasticity detected for cavitation resistance in a Mediterranean pine

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Summary

• Resistance to cavitation is a major determinant of plant survival under severe drought and can be used to quantify species adaptive potential. Interspecific variation in this key trait is well defined in woody species, but intraspecific variation (level and structure) resulting from standing genetic variation and phenotypic plasticity has never been determined.

• Combining for the first time *in situ* characterization of natural populations and two reciprocal common gardens in dry and wet sites, we estimated variance components (phenotypic, genetic, environmental, and genetic × environmental) of cavitation resistance based on 513 genotypes of a Mediterranean pine, *Pinus pinaster*.

• Despite the selected populations being climatically contrasted, phenotypic plasticity in resistance to cavitation remained low and was essentially attributed to family level. Between-population variation in cavitation resistance for both phenotypic and genetic variation was limited.

• These results strongly suggest that cavitation resistance is buffered against genetic and to a lesser extent environmental variation (canalization) in maritime pine. Consequently, in a drier world, the increasing drought tolerance of *Pinus* species might be severely constrained by the low level of cavitation resistance variation, resulting in a large-scale loss of productivity.

Introduction

Given the magnitude of the expected increase in world average temperatures and in the frequency of extreme climatic events (Beniston *et al.*, 2007; Della-Marta & Beniston, 2008; Sterl *et al.*, 2008; van Oldenborgh *et al.*, 2009; Wigley, 2009), the rate of adaptation driven by natural selection and migration may no longer keep pace with climate change (Davis & Shaw, 2001; Davis *et al.*, 2005; Corlett & Westcott, 2013; S. Delzon *et al.*, unpublished). This is particularly true for long-lived organisms such as forest tree species forced to cope with these drastic rapid climate changes – possibly within a single generation (Breda *et al.*, 2006; Bréda & Badeau, 2008; Lindner *et al.*, 2010). Indeed, several authors have reported that recent forest die-backs could be linked to severe drought events, which are manifestations of climate change (Allen & Breshears, 1998; Breshears *et al.*, 2005; Breda *et al.*, 2006; Granier *et al.*, 2007; Allen, 2009; Allen *et al.*, 2010). Whether organisms can pass through such abiotic filters depends on their fitness, that is, the probability to both survive and reproduce. The reproduction component is often considered the main driver of fitness, but in a changing world with stochastic extreme droughts, survival could become a

bigger challenge than reproduction. Quantifying the extent and relative amount of genetic and environmental variations in relevant fitness-related traits during extreme drought is therefore a prerequisite to understanding the evolutionary processes that lead these organisms to cope with such climatic events and predicting their adaptive potential in response to climate change (Lindner *et al.*, 2008, 2010).

One of the most relevant traits for tracking tree survival during extreme droughts is cavitation resistance, that is, the ability to conduct water though the xylem even during drought events (Cochard *et al.*, 2008). This is grounded by several lines of evidence. Meta-analyses have shown that, on average, species from drier climates are more cavitation-resistant than species from wetter climates (Maherali *et al.*, 2004; Choat *et al.*, 2012), while experimental drought and recovery monitoring experiments have established a causal link between resistance to cavitation and lethal water potential (Brodribb & Cochard, 2009; Brodribb *et al.*, 2010). For instance, in conifers, a 50% loss of hydraulic conductance in the seedling stem leads to death by dehydration, showing that more cavitation-resistant species survive stronger drought (Brodribb & Cochard, 2009; Brodribb *et al.*, 2010). Overall, resistance to cavitation (estimated by the pressure

corresponding to 50% loss of hydraulic conductance (P_{50})) varies widely among species (Maherali *et al.*, 2004), especially conifers (P_{50} ranged between -2 and -16 MPa; see Delzon *et al.*, 2010). A recent study showed that most of the variability in this trait was attributable to genera within a botanical family, whereas species explained < 10% of the variance (S. Delzon *et al.*, unpublished).

To cope with increasingly severe drought events, the adaptation of sessile organisms will rely on the level of standing genetic variation and phenotypic plasticity (Aitken *et al.*, 2008). Genetic diversity can be seen as a pool of variants among which natural selection, at a given time and in a given point in space, keeps the fittest. This diversity is naturally renewed over time by the interplay of evolutionary forces (migration, genetic drift, natural selection, recombination and mutation). Only three recent studies have assessed genetic variation in resistance to cavitation (Corcuera *et al.*, 2011; Lamy *et al.*, 2011; Wortemann *et al.*, 2011). It was found that phenotypic variation is low (coefficient of variation < 10%) and that variation between populations is limited, as most genetic variation resides within population. Lamy *et al.* (2011) proposed that cavitation resistance is a genetically canalized trait in *Pinus pinaster*, that is, the average value of this trait is similar for populations of dry and wet origins, making it robust to genetic perturbations (i.e. mutation, recombination, etc). Phenotypic plasticity is a second component of adaptation. It is classically split into two components: a reaction norm corresponding to a (linear, quadratic or sigmoid) function that links phenotypic variation to environmental changes; and the genetic variability of the reaction norm, that is, the genotype-by-environment (G × E) interaction (Debat & David, 2001; Pigliucci, 2005). G × E is of major concern in the attempt to develop plants adapted to wide geographical ranges. It is of considerable concern for forest trees species, as it provides clues to understand mechanisms that have shaped local adaptation. The term 'phenotypic plasticity' can be found in most discussion sections of papers dealing with ecological implications of variation in cavitation resistance (Kolb & Sperry, 1999; Maherali & DeLucia, 2000; Maherali *et al.*, 2002; Jacobsen *et al.*, 2007; Beikircher & Mayr, 2009; Martinez-Vilalta *et al.*, 2009), yet it has rarely been quantified by appropriate experiments and robust estimators. This has prompted some authors to postulate that cavitation resistance is a highly plastic trait (Jacobsen *et al.*, 2007; Beikircher & Mayr, 2009), whereas other authors claim that cavitation resistance is not a plastic trait at all (Maherali *et al.*, 2002; Martinez-Vilalta *et al.*, 2004, 2009). These conclusions arose from the comparison between phenotypic variation, as assessed *in situ*, and genetic variation, as measured in provenance trials. However, until recently, working with a large sample size (to properly estimate variance components in well-designed experiments) was inconceivable given the technology available. This phenotyping barrier was removed thanks to technical advances allowing high-throughput phenotyping (Cochard, 2002; Cochard *et al.*, 2005). This high-throughput method has reduced the cost of experimentation, allowing researchers on a fixed budget to obtain much more precise estimates of variances and variance components, opening up new perspectives for quantifying the relative amount of genetic variation and phenotypic plasticity in resistance to cavitation.

To explore, in concert, the phenotypic variability, phenotypic plasticity and standing genetic variation of this key fitness trait, we carried out a unique case study on maritime pine (*Pinus pinaster*), a forest tree species with a fragmented distribution in the western Mediterranean region. The scattered distribution of this species may have prevented or limited gene flow between different groups of populations, promoting high genetic divergence between ecotypes as a result of genetic drift (Ribeiro *et al.*, 2002a, b; Bucci *et al.*, 2007) and/or natural selection (Quezel & Barbero 1998, in Richardson, 1998). In this study, we analysed growth and resistance to cavitation data of six populations planted in France (Lamy *et al.*, 2011) and in Spain using a provenance-progeny design, and data from individuals of these populations were sampled *in situ*. We characterized the phenotypic variance *in situ*, the genotypic variance in two constrasted trials and the phenotypic plasticity. In total, we measured 513 genotypes for resistance to cavitation, which to the best of our knowledge makes the results of this study the largest data set produced for cavitation resistance.

The aims of this study were three-fold: to quantify the magnitude of phenotypic variability in resistance to cavitation *in situ*; to study the degree of environmental or genetic determinism for this trait by quantifying both genetic variation and phenotypic plasticity using common garden experiments; and to estimate the correlations between resistance to cavitation and climate variables. This knowledge leads us to elaborate hypotheses on both micro- and macro-evolution of resistance to cavitation in this pine species.

Materials and Methods

Genetic material and trait measurements

Provenance-progeny trials We established a replicated provenance-progeny trial (i.e. same families of the same populations; Fig. 1) in Calcena (Aragon, Spain; 41°3′N, 01°43′W; mean annual precipitation ≈ 400 mm), hereafter called the 'dry' trial, and in Cestas (Aquitaine, France; 44°44′N, 00°46′W; mean annual precipitation ≈ 800 mm), hereafter called the 'wet' trial (Table 1, Fig. 1, Supporting Information Fig. S1). Seedlings were grown in a nursery from open-pollinated seeds collected from 24 natural populations (or ecotypes) in France, Italy, Morocco, Spain and Tunisia (Table S2), to cover the fragmented distribution of *Pinus pinaster* Aiton. Each population was represented by 20–30 half-sib families (*c*. 15 offspring per family). Each trial was planted using an incomplete randomized block design (a multi-tree plot design in the dry site and a single-tree plot design in the wet site). The wet and dry trials were planted in winter 2003 and winter 2004 (Eveno, 2008), respectively.

In both trials, total height at 4 yr old (*h*; m) was measured in 2008 (dry trial) and 2007 (wet trial) for each individual of the 24 populations (i.e. > 7000 measurements for both trials). For resistance to cavitation, we subsampled six populations out of the 24 populations (see Table 1). In each population, eight families were randomly sampled with at least one half-sib in each block (i.e. 6 populations × 8 families × 5–4 blocks; Table 1).
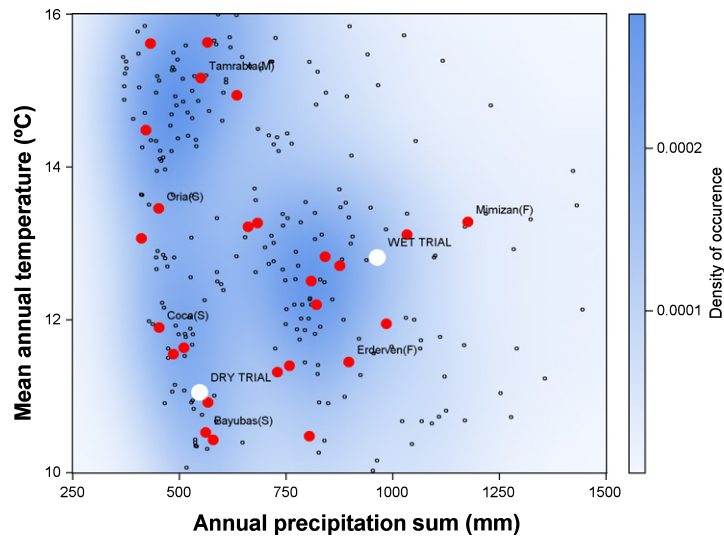
Fig. 1 Distribution of provenance-progeny trials (white circles) and populations (red dots) in bioclimatic parameter space (some dots are climatically very similar and can barely be distinguished on the map). The labelled red dots are the selected populations (see the main text). The small black dots are 769 population locations used to represent the climatic variability of *Pinus pinaster*. The contour plot represents the probability of the presence (kernel density estimate) of *P. pinaster* populations within the bioclimatic envelope. The probabilities of occurrence of *P. pinaster* populations within the bioclimatic envelope are indicated in white (low probability) and blue (high probability), respectively. A principal component analysis is also provided (Supporting Information Fig. S1) on the same data. The letter in parenthesis after the provenance names indicate the country (F, France; S, Spain; M, Morocco; I, Italy).

Table 1 Climate data, location, elevation, soil and genetic information for each selected population of *Pinus pinaster* and each provenance-progeny trial

Population	Longitude (°)	Latitude (°)	n	Altitude (m)	P_i (mm)	T_m (°C)	VPD_{max} (hPa)	ETP (mm)	Mitotype	Chlorotype
Bayubas de Abajo (Spain)	−2.87	41.52	37–182	955	561	10.5	11.42	882.9	W	a
Coca (Spain)	−4.08	41.37	37–122	788	452	11.9	14.23	718.7	W	a
Mimizan (France)	−1.30	44.13	38–154.5	37	1176	13.2	7.26	751.59	W	a
Oria (Spain)	−2.62	37.87	32–155.5	1232	451	13.4	14.29	922.59	W	j
San Cipriano (Spain)	−8.70	42.13	35–114	310	1625	13.8	8.54	721.91	W	g
Tamrabta (Morocco)	−5.02	33.66	38–113	1760	550	15.1	18.56	976.54	M	k
Erdeven (France)[a]	−2.86	47.65	11–11	20	898	11.4	4.42	640.33	na	na

Provenance-progeny trial	Longitude (°)	Latitude (°)	n	Elevation (m)	P_i (mm)	T_m (°C)	VPD_{max} (hPa)	ETP (mm)	Soil
Dry, Calcena (Spain)	−1.72	41.62	196–1857	997	452	11.1	11.1	778.2	Shaly sandstone
Wet, Cestas (France)	−0.78	44.74	240–5569	61	800	12.7	6.70	743.8	Sandy podzol

n, number of individuals for cavitation resistance and total height, respectively; P_i, mean annual precipitation; T_m, mean annual air temperature; VPD_{max}, maximum water vapour pressure deficit (in July for all provenances); ETP, annual sum of potential evapotranspiration. Mitotype and chlorotype correspond to the most frequent chloroplastic and mitochondrial haplotypes present in the populations (Burban & Petit, 2003).
[a]This population was not sampled in the provenance-progeny trials because there is some doubt about its origin.
The grey highlight corresponds to the provenance-progeny trial only.

Population selection for resistance to cavitation measurements The procedure to select the six populations representing the range of the climatic envelope of maritime pine was as follows. Working with a total of 754 grid points covering the entire natural range of the species (Bucci *et al.*, 2007), we first extracted climate data from the CRU CL 2.0 10' global data set for the period 1961–1990 (New *et al.*, 1999, 2000, 2002). The climate data included monthly precipitation, mean, minimum and maximum temperature, diurnal temperature range, water vapour pressure, cloud cover, wet day frequency, ground frost frequency, mean global radiation, mean wind speed, de Martonne's index,

Turc's potential evapotranspiration, and soil water deficit. These parameters were also used to derive air vapour pressure deficit (Jones, 1992). Principal component analysis (PCA) on the correlation matrix was performed to reduce the number of dimensions (14 climatic variables × 12 months) over the whole set of climate variables (Figs 1, S1). All data were centred and scaled before PCA. The 24 populations available in the provenance-progeny trials were finally placed on the main plane of the PCA (first two axes accounting for 70% of variation) and six were selected to cover the climate envelope of *P. pinaster* (Table 1, Fig. 1). We also checked that climatic data from the CRU CL 2.0 10' global

data set for the period 1961–1990 are correlated with the climatic data during the studied period (see Fig. S3). The historical temperature and precipitation followed the same pattern as the trend observed in the world climatic database in both provenance-progeny tests (dry test: $r_s = 0.96$ and $P < 0.0001$ for mean temperature; $r_s = 0.49$ and $P = 0.0004$ for precipitation; wet test: $r_s = 0.93$ and $P < 0.0001$ for mean temperature; $r_s = 0.33$ and $P = 0.002$ for precipitation). These populations also displayed different mitotypes and chlorotypes and therefore different evolutionary histories (Vendramin *et al.*, 1998; Burban *et al.*, 1999; Bucci *et al.*, 2007). We finally checked that this sampling procedure did not bias population genetic differentiation towards high values, as the estimated F_{ST} (Wright's fixation index, quantifying population differentiation due to genetic structure) from the selected population (0.098; Lamy *et al.*, 2011) was close to the species average (F_{ST} 0.10–0.14 from Vendramin *et al.*, 1998; Burban & Petit, 2003; Bucci *et al.*, 2007)).

***In situ* natural populations** To assess phenotypic variation for *in situ* natural populations, we phenotyped seven populations, including the six populations selected in the provenance-progeny trials. The additional population was from Erdeven (Bretagne, France; see Table 1), one of the northernmost populations of *P. pinaster*. We used a simplified sampling scheme of 11 individuals per populations (77 genotypes in total) set at least 50 m apart to avoid genetic autocorrelations as a result of possible relatedness between individuals (Gonzalez-Martinez *et al.*, 2003). We measured collar diameter, tree height and age (by counting stem whorls, which is considered a valid method for monocyclic conifers) and harvested two branches per individual on the two last growth units for resistance to cavitation measurements. In order to plot and characterize the within-population variability of growth performances between the provenance-progeny trials and *in situ* natural populations, we roughly estimated tree height at 4 yr old by dividing total height by the actual age (we obtained an average annual increment) and then multiplying by 4.

Assessment of resistance to cavitation

We established the whole vulnerability curve for each of the 513 genotypes (see Fig. 2). Vulnerability curves were based on the Cavitron technique, as previously described in Cochard (2002) and Cochard *et al.* (2005). Measurements were performed at the high-throughput phenotyping platform for hydraulic traits (Cavit_Place, University of Bordeaux, Talence, France) using a custom-built honeycomb rotor (SamPrecis 2000, Bordeaux, France) mounted on a Sorvall RC5 superspeed centrifuge (Thermo Fisher Scientific, Munich, Germany). Samples were kept refrigerated, and vulnerability to cavitation was determined within 2 wk of collection. All samples were debarked to avoid resin exudation and then re-cut under water to a standard length of 27 cm, and both ends were trimmed with a fresh razor blade to obtain perfectly smooth surfaces with open tracheids. A solution of ultrapure and degassed water including 10 mM KCl and 1 mM $CaCl_2$ was used as the reference solution for hydraulic

measurements. After measuring maximum hydraulic conductance under high (i.e. close to zero) xylem pressure (P), centrifuge rotation speed was gradually increased from -0.5 to -7 MPa by 0.5 MPa to determine percentage loss of hydraulic conductance (PLC). Rotor velocity was monitored with a 10 rpm-resolution electronic tachymeter (A2108-LSR232; 202 Compact Inst, Bolton, UK) and xylem pressure was adjusted to $c. \pm 0.02$ MPa. CAVI_SOFT software (version 1.5; University of Bordeaux) was used to measure conductances and compute all the vulnerability curves, which were adjusted according to Pammenter & Vander Willigen (1998). P_{50} (MPa) was defined as the pressure corresponding to 50% PLC. S_{50}, which corresponds to the speed of cavitation spread, was defined as the slope (% MPa^{-1}) of a tangent at the inflexion point (P_{50}).

## Statistical analysis and estimation of genetic parameters

Single trial analysis We used the following mixed model to estimate variance components used to derive all the genetics quantitative parameters:

$$y_l(b(pf(p))) = \beta_b + \gamma_p + \gamma_{f(p)} + \varepsilon_l(b(pf(p))) \qquad \text{Eqn 1}$$

(y, resistance to cavitation (or height at 4 yr old) for the lth observation in the bth block and pth population and the fth family nested within the pth population; β, a fixed block effect; γ, random effect; ε, the residual.) A variance component was estimated from each random effect: σ_{pop}^2 is genetic variance between populations, and $\sigma_{f(pop)}^2$ is genetic variance between mother trees (i.e. families) nested within a population. Usually, residuals (ε) are assumed to be distributed as $\sim N(0, \boldsymbol{R})$, where $\boldsymbol{R}$ is a covariance matrix as ($\boldsymbol{R} = \sigma_e^2 I$). However, spatial autocorrelations in the field trial between individuals as a result of soil and microclimatic effects violated the normality assumption of residuals. To avoid a biased estimation of variance components, we modelled an $\boldsymbol{R}$ covariance structure that assumes separable first-order autoregressive processes in rows and columns with spatially dependent and independent error variances (Dutkowski *et al.*, 2002, 2006). Spatially explicit models were only performed for h because the P_{50} data were spatially too sparse (see Table 2).

Variance and/or covariance components were estimated using the restricted maximum likelihood method, assuming a normal distribution of the random effects. The significance of variance components was tested using log-likelihood ratio tests. We included population as a random effect to draw inference at the species level and to obtain an unbiased estimate of heritability and genetic population differentiation (Wilson, 2008). The normality, identity and independence of residuals of each trait were graphically checked by plotting Studentized marginal and conditional residuals, which confirmed that the data matched the assumption of the mixed model.

For the single-site analysis, narrow-sense heritability was estimated as: $h_{ns}^2 = 4\sigma_{f(pop)}^2 / (\sigma_\varepsilon^2 + \sigma_{f(pop)}^2)$. We estimated σ_A^2 by $4\sigma_{f(pop)}^2$, as trees from the same family were presumed to be half-sibs (open-pollinated seeds). We did not include population
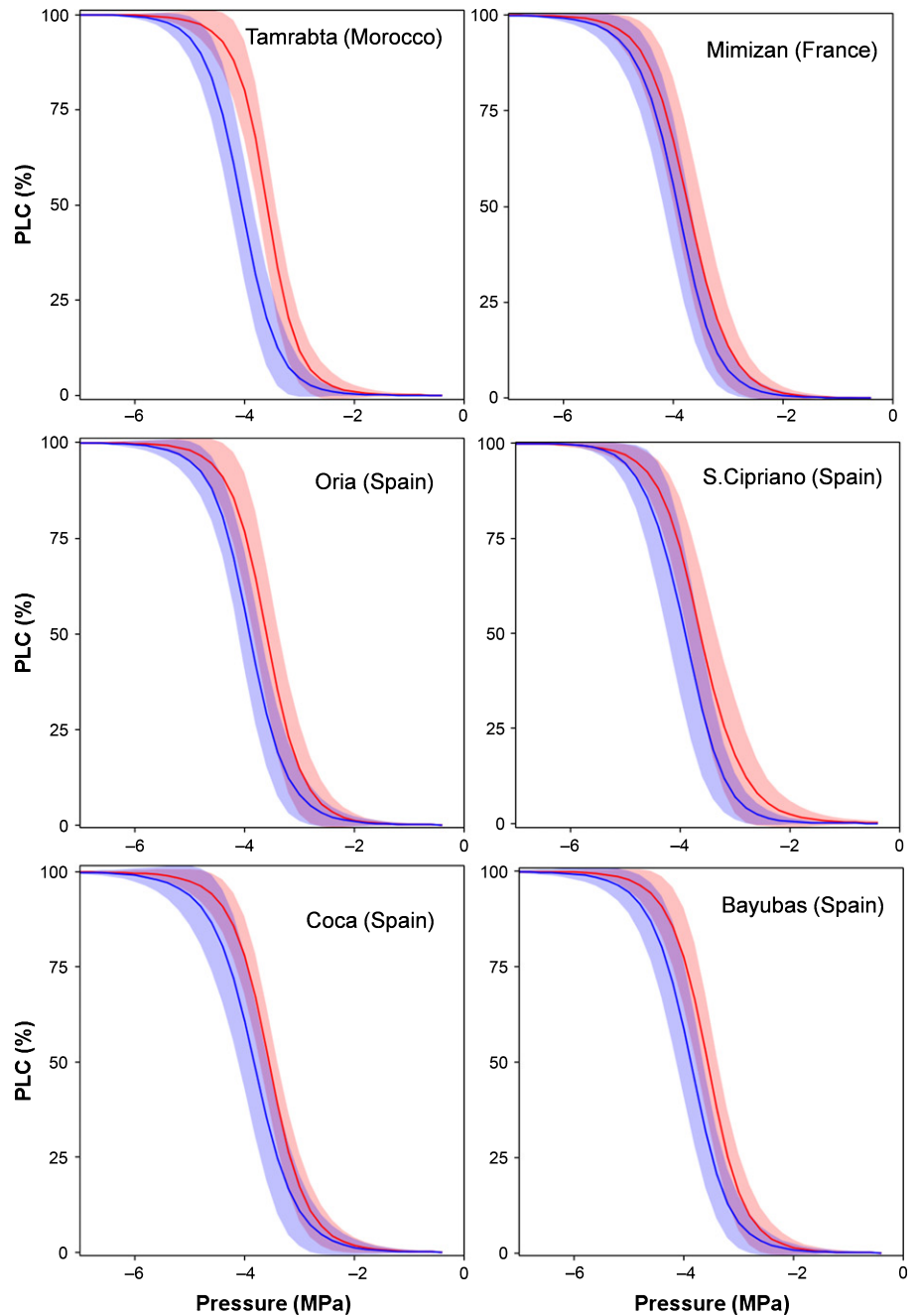
Fig. 2 Mean vulnerability curves for each of the six populations of *Pinus pinaster* in each trial (wet and dry; $n = 36 \pm 1$). The shaded band represents the standard deviation. The mean vulnerability curves for the dry and wet trials are shown in red and blue, respectively. PLC, percentage loss of hydraulic conductance.

effect in the heritability estimation, as natural selection appeared to occur within each population (Visscher *et al.*, 2008). Standard deviation of heritability was calculated with delta method equations of Lynch & Walsh (1998). CV is the coefficient of variation (or $\overline{CV}$ when averaged across trials), that is, the variance component is standardized by the trait mean (Houle, 1992) as follows: $CV = \frac{\sqrt{\sigma^2_{trait}}}{X_{trait}} 100$. For each variance component, we also calculated the corresponding CV (CV_A: additive coefficient of variation; CV_{pop}: between-population coefficient of variation; CV_P: phenotypic coefficient of variation; CV_R: residual coefficient of variation).

The estimate of between-population phenotypic differentiation, Q_{ST} (Spitze, 1993), was calculated as $Q_{ST} = \sigma^2_{pop}/(\sigma^2_{pop} + 2\sigma^2_A)$. Details of the methodology used for Q_{ST} and F_{ST} comparisons are reported in Lamy *et al.* (2011) (or see Notes S1).

Multi-trial analysis We used the following mixed model to estimate phenotypic plasticity for both h and P_{50}:

$$y_l\big(sbp(s)f(p)f(p(s))\big) = \beta_s + \gamma_b + \gamma_{sp} + \gamma_{f(p)} + \gamma_{sf(p)} \\ + \varepsilon_l\big(sbp(s)f(p)f(p(s))\big) \qquad \text{Eqn 2}$$

(y, resistance to cavitation (or height at 4 yr old) for the lth observation in the sth site and the bth block and pth

Table 2 Variance component estimates for the (a) multi-trial and (b) single-trial models (*Pinus pinaster*)

(a)

Effects	Overall			
	h		P_{50}	
	Variances	P-value	Variances	P-value
Trial	3424	<0.001	0.054	<0.001
Block (site)	37.00		0.001	
Population	438.7	<0.001	0.001	0.08
Site*population	76.84	<0.001	0.002	0.23
Family (Population)	10.85	<0.001	7×10^{-9}	0.003
Site*family (population)	28.27	<0.001	0.008	0.03
Residuals	374.7		0.052	

(b)

Effects	Dry trial, Calcena (Spain)				Wet trial, Cestas (France)			
	h		P_{50}		h		P_{50}	
	Variances	P-value	Variances	P-value	Variances	P-value	Variances	P-value
Block	1058	<0.001	12.87	<0.001	13 830	<0.001	15.30	<0.001
Population	8.03	<0.001	0.0003	0.173	204.14	<0.001	0.0028	0.06
Family (population)	6.79	<0.001	0.0083	0.009	47.51	<0.001	0.0088	0.003
Spatial residuals	102.14	<0.001	na	na	307.10	<0.001	na	na
Non spatial residual	42.25	<0.001	0.0456	na	216.23	<0.001	0.059	na
Spatial autocorrelation (row)	0.92	<0.001	na	na	0.87	<0.001	na	na
Spatial autocorrelation (columns)	0.92	<0.001	na	na	0.91	<0.001	na	na

(a) Variance estimators are from a multi-trial model (no. 2). The trial effect (in grey) was not declared as a random effect for statistical reasons (see the Materials and Methods section) but we calculated a variance from the values estimated for each trial (fixed effect) and the *P*-value is from the full model. This model assumes the same error variance for both trials. Results from a full model assuming two different error variances for each trial are provided in Supporting Information Table S1. (b) Variance estimators are from the mixed model for single-trial analysis (no. 1). Spatial residuals and spatial autocorrelation coefficients are given for the growth trait only, as the data for cavitation resistance were too spatially sparse. na, not available. In both models, *P*-values are from a log-likelihood ratio test, which is why block effects do not have *P*-values.

population, and the *s*th site and the *f*th family nested within the *p*th population, and the *f*th family nested within the *p*th population nested within the *s*th site; β, a fixed effect; γ, random effect; ε, the residual.) A variance component was estimated from each random effect: σ^2_{pop} is genetic variance between populations, $\sigma^2_{\text{site.pop}}$ is the variance of the interaction term between population and site, $\sigma^2_{f(\text{pop})}$ is genetic variance between mother trees nested within a population, $\sigma^2_{\text{site}.f(\text{pop})}$ is the variance of the interaction term between mother trees nested within population and site, and σ^2_{e} is residual variance. Other model formulations were tested to take into account the heteroscedasticity of the error variance but did not change the results of the analysis (see Notes S2 and Table S1). Mixed models were run using SAS (SAS, 2008) and ASREML (Gilmour *et al.*, 1995, 2006; Gilmour, 1997).

Given the difference in terms of sample size between P_{50} and *h*, we also ran all the analyses on a reduced data set for *h*, that is, the same sample size as P_{50}. Considering that the absolute values of variances were, as expected, different, but the patterns of significance were similar between the reduced data set and full data set analyses, it was decided to present the most accurate estimates using the whole data set for *h*.

Assessment of phenotypic plasticity According to Falconer & Mackay (1996), the total phenotypic variance of a population can be modelled as:

$$\sigma^2_{\text{P}} = \sigma^2_{\text{G}} + \sigma^2_{\text{E}} + 2\text{cov}_{\text{GE}} + \sigma^2_{\text{GE}} + \sigma^2_{\varepsilon}$$

where σ^2_{P} is the phenotypic variance of a population, σ^2_{G} is the genetic variance of a population, and σ^2_{E} is the general environmental effect (macro-environmental effect). In others terms, it is the difference between sites. 2cov_{GE} corresponds to genotype–environment covariance, that is, it corresponds to the covariance between the estimations of the two main effects. From an experimental point of view, it measures the physical association of particular genotypes with particular environmental effects. In our experimental design, if individuals are randomly distributed with respect to macro-environments then $2\text{cov}_{\text{GE}} = 0$. σ^2_{GE} is the genotype–environment interaction, corresponding to variation in the phenotypic response of specific genotypes to specific environments. Assuming that $2\text{cov}_{\text{GE}} = 0$ in our provenance-progeny trials, then the variance associated with phenotypic plasticity (σ^2_{PP}) can be written as: $\sigma^2_{\text{PP}} = \sigma^2_{\text{E}} + \sigma^2_{\text{GE}}$.

In this study, two phenotypic estimators were used. (1) Scheiner's phenotypic plasticity index: $S = (\sigma^2_{\text{E}} + \sigma^2_{\text{GE}})/\left(\sigma^2_{\text{GE}} + \sigma^2_{\text{G}} + \sigma^2_{\text{E}} + \sigma^2_{\varepsilon}\right)$ adapted from Scheiner & Lyman (1989), which is based on the estimation of the interaction term of the mixed-model analysis (see model 2). This index, which includes both terms of phenotypic plasticity, is not bounded and always positive. (2) The Relative Distance Plasticity Index (RDPI), defined by Valladares *et al.* (2006) as the absolute phenotypic distance between individuals of the same genotype (in this case family) placed in different environments, divided by the highest of the two phenotypic values. This index relies less on the implicit assumption made about the data distribution.

Results

Phenotypic variability

For resistance to cavitation, CV_P for *in situ* natural populations ($CV_P^{in\ situ}$) and CV_P estimated from the provenance-prog-enytrial ($\overline{CV_P^{ppt}}$) were 6.97 and 6.5, respectively, meaning that the phenotypic variability of *in situ* natural populations was close to the variation found in the provenance-progeny trials. Population effect was significant for *in situ* natural populations ($P = 0.03$) but no differences were found after population clustering between wet-trial and dry-trial populations ($P = 0.63$). In fact, this trend was driven by a population from Erdeven (France), and there are some doubts over the 'naturalness' of the sampled individuals (genetically calibrated material from seed orchards).

Phenotypic plasticity

Overall trend At the population level, h displayed much higher phenotypic plasticity than P_{50} (S for P_{50} and h: 0.51 and 0.90, respectively). At the family level, the two traits displayed similar levels of phenotypic plasticity (S for P_{50} and h: 0.90 and 0.98, respectively). The most robust estimator (RDPI and Fig. 3) of phenotypic plasticity clearly showed that P_{50} was less plastic than h (0.08 versus 0.65, respectively). Note that these results were not biased by the unbalanced data sets between h and P_{50}, as the same trend was found on the reduced sample size for h, and also using another model formulation (see Notes S1).

Causal sources of phenotypic plasticity Phenotypic plasticity attributable to environment ('site effect'; see model 2) was significant for both cavitation resistance (P_{50}) and height growth (h), but the relative contribution of environmental variance (σ_E^2) to total variance was stronger for h than P_{50} (based on the total variance from both fixed and random effects, 79% for h and 46% for P_{50}). Genotype $\times$ environment interaction at the population level (σ_{GE}^2 for population) was only significant for h (1.7%), whereas at the family level (σ_{GE}^2 for family) it was significant for both traits (0.6% for P_{50} and 6% for h). Therefore, h and, to a lesser extent, P_{50} showed phenotypic plasticity mainly driven by σ_E^2.

Genetic variation

Within-population Microenvironmental sensitivity (through the analysis of CV_R) and evolvability (through the analysis of CV_A) were lower for P_{50} ($\overline{CV_A} = 4.9$ and $\overline{CV_R} = 6.0$; CVs averaged across trials) than h ($\overline{CV_A} = 13.65$ and $\overline{CV_R} = 29.7$), that is, P_{50} was less influenced by microenvironmental variations and showed a limited amount of additive genetic variation compared with h (Table 3).

Between-population variation and Q_{ST} and F_{ST} comparisons The same pattern was observed in both trials. No significant population effect was detected for P_{50}, whereas a significant population effect was found for h (Table 3). On average, across trials, the between-population coefficient of variation ($\overline{CV_{pop}}$) was 0.7% for P_{50}, that is, much lower than the value measured for h (10.5%; see Table 3). For P_{50}, the Q_{ST} distribution was lower than the F_{ST} distribution (Fig. S2), that is, the observed between-population variation was lower than the variation expected under genetic drift alone, suggesting a mechanism (uniform selection and/or genetic constraints; see the Discussion section) favouring the same phenotypic mean in the studied populations from contrasted climates. For h, we found the opposite trend (Fig. S2). The Q_{ST} distribution was higher than the F_{ST} distribution, indicating that the studied populations displayed more differentiation than would be expected from drift alone (Fig. S2).
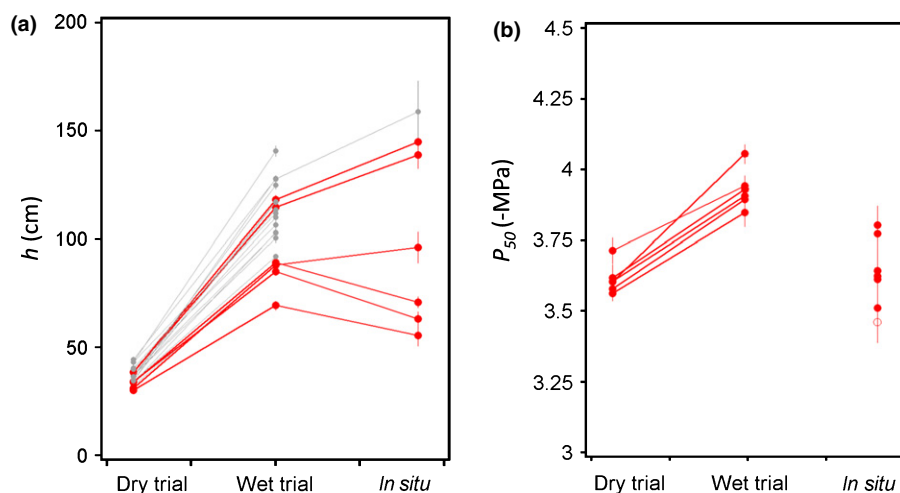
Fig. 3 Chart showing population means of *Pinus pinaster* in each provenance-progeny trial (dry and wet) connected by solid lines, and means for natural populations collected *in situ* (dots). (a) In both progeny-provenance trials, total height (h) was assessed at 4 yr old; $n = 163 \pm 14.56$ individuals per population. For *in situ* natural populations, h was estimated at 4 yr old from, on average, 11-yr-old trees; $n = 11$ individuals per natural population. (b) In both progeny-provenance trials, cavitation resistance (P_{50}) was assessed using $n = 36 \pm 1$ individuals per population. For *in situ* natural populations, cavitation resistance was assessed using $n = 11$ individuals per natural population. Bars, $\pm$ SE. Red dots are used for the six selected populations, while grey dots are used for the other populations present in the data set.

Table 3 Variance components (σ_P^2, σ_{pop}^2, σ_A^2, σ_R^2), narrow-sense heritability (h_{ns}^2), coefficients of variation (CV$_P$, CV$_A$, CV$_{pop}$ and CV$_R$) and population differentiation (Q$_{ST}$) of *Pinus pinaster* populations

Trial	Traits	σ_P^2	σ_{pop}^2	σ_A^2	σ_R^2	h_{ns}^2 (SE)	CV$_P$	CV$_A$	CV$_{pop}$	CV$_R$	Q$_{ST}$[a]
Dry trial	*h*	151.2	8.043	27.13	144.4	0.17 ± 0.07	34.96	14.8	8.0	34.1	0.12
Dry trial	P_{50}	0.053	0.0004	0.034	0.045	0.61 ± 0.27	6.43	5.06	0.4	5.9	0.005
Wet trial	*h*	570.8	204.1	190.0	523.3	0.33 ± 0.03	21.7	12.5	13	20.8	0.34
Wet trial	P_{50}	0.067	0.0027	0.035	0.058	0.51 ± 0.23	6.60	4.75	1	6.1	0.04
In situ	*h*	2514	1789[b]	na	724.9	na	47.2	na	39.8[b]	25.3	na
In situ	P_{50}	0.064	0.010[b]	na	0.053	na	6.97	na	2.83[b]	9.64	na

Variance components are from single-trial mixed model analysis (no. 1). h_{ns}^2, narrow-sense heritability; SE, standard error of heritability; σ_P^2, phenotypic genetic variance; σ_A^2, additive genetic variance; σ_{pop}^2, between-population variance; σ_R^2, residual variance; CV$_A$, the variation coefficient of additive variance after adjustment for block effect; CV$_P$, variation coefficient of phenotypic variance after adjustment for block effect; CV$_R$, residual coefficient of variation; CV$_{pop}$, between-population coefficient of variation. Q$_{ST}$ is the genetic quantitative variation between populations (Spitze, 1993).
[a]For the comparison with F_{ST}, see Fig. S2. *In situ* variance components are from a mixed model with one random parameter (population).
[b]This variance component (or the associated coefficient of variation) is not equal to the same variance estimated in the provenance-progeny trial because it includes variation from the family level. P_{50}, cavitation resistance; *h*, tree height; na, not available.
The grey area indicates results for the analysis done for *in situ* data; for further explanation see main text.

**Between-population variation estimated from *in situ* natural populations and provenance-progeny trials** We were unable to compare the coefficients of variation for cavitation resistance between *in situ* natural populations (CV$_{pop}^{in\,situ}$: 2.83) and between populations in the progeny-provenance trials ($\overline{CV_{pop}^{ppt}}$: 0.7) because family variation ($\overline{CV_{f(pop)}^{ppt}}$; see Table 2) is not estimated *in situ*. However, it is possible to compare CV$_{pop}^{in\,situ}$ with the sum of $\overline{CV_{pop}^{ppt}}$ and $\overline{CV_{f(pop)}^{ppt}}$. The estimation of CV$_{pop}^{in\,situ}$ (= 2.83) turned out to be of the same order of magnitude as estimated in the provenance-progeny test ($\overline{CV_{pop}^{ppt}} + \overline{CV_{f(pop)}^{ppt}} = 1.9$), with the small difference between the two estimators being probably caused by a sample-size effect.

Experimental effects and spatial analysis Block effect was significant for all traits and models, which means that the experimental design captured environmental variation. Spatial analysis for *h* showed a high autocorrelation (0.87) between closely spatially related individuals (Table 2). However, changes in terms of variance estimation between a spatial analysis and a classical analysis were low (*c.* 2–5%; data not shown). The P_{50} data were spatially too sparse to allow the same spatial analysis.

Relationships between climate and traits

The first principal component axis (PC1) was interpreted as an aridity index, as it was positively correlated with atmospheric water demand and soil water deficit and negatively correlated with number of wet days (Fig. S1b). In addition, Fig. S1(c) shows that the values of climatic variables during June, July, August and September are best correlated with PC1. Mean population *h* values showed a negative correlation with the composite aridity index (Fig. 4a). Populations from mesic provenances showed better growth than populations from xeric provenances in both trial stands and *in situ* natural stands. The same trend was observed with the reduced data set (*P*-values = 0.051, 0.009 and 0.001 in
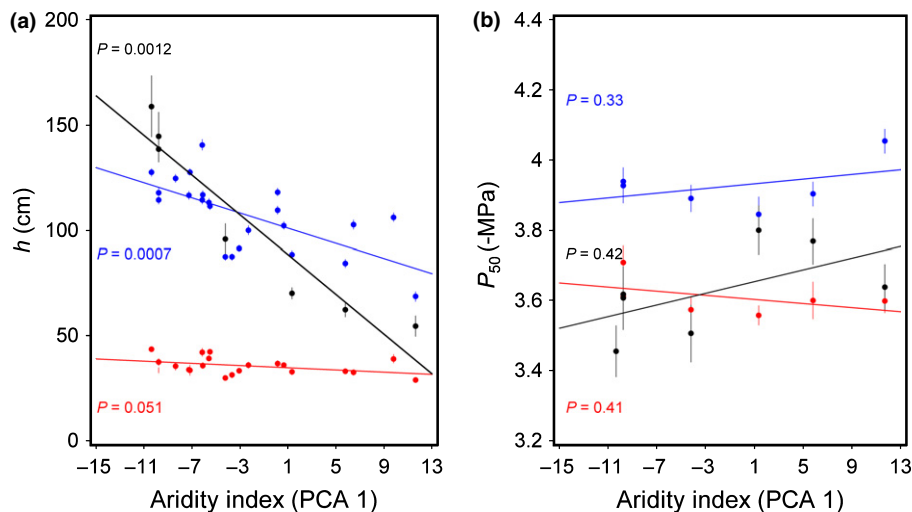
Fig. 4 The population mean of *Pinus pinaster* for (a) tree height (*h*) and (b) cavitation resistance (P_{50}) according to aridity index. The aridity index corresponds to the projection on the first principal component axis (PC1) of population position within the main plane space described by the principal component analysis (PCA). Red, blue and black colours correspond to regression in the dry and wet provenance-progeny trials and *in situ* natural stands, respectively. The *P*-value was from a linear model. Bars, ± SE.

dry trials, wet trials, and *in situ*, respectively; data not shown). For P_{50}, we did not detect any significant relationship between aridity index (Fig. 4b) and other climatic variables (data not shown). Furthermore, the mean value for resistance to cavitation was higher in the dry trial (-3.6 ± 0.02; $P < 0.05$; see Table 2) than the wet trial (-3.9 ± 0.02), which is the opposite to what we expected (see Fig. 3). The lower growth in the dry trial matched our expectation based on the site's dryness as estimated from climatic variables (Fig. 3).

Discussion

We have carried out the first comprehensive study of the determinism of resistance to cavitation variation by simultaneously quantifying phenotypic variability, genetic variation and phenotypic plasticity in a pine species. The three most significant findings are: phenotypic variation in resistance to cavitation (P_{50}) is low (below 7%); additive genetic variation within and between populations is low for P_{50} (below 2%) compared with growth; P_{50} shows low phenotypic plasticity, mainly driven by family-level plasticity, and its magnitude is eight-fold lower than for growth (based on RDPI). Taken together, these findings suggest that resistance to cavitation is buffered against genetic variation and, to a lesser extent, against environmental variation.

Cavitation resistance displays low phenotypic variation in *Pinus pinaster*

The naturally occurring variability of fitness-related ecophysiological traits is rarely monitored, despite its crucial importance for predicting the impacts of climate change on distribution range (Arntz & Delph, 2001; Leinonen *et al.*, 2008; Dawson *et al.*, 2011). Focusing on resistance to cavitation as a proxy for survival under extreme drought, only two studies (Martinez-Vilalta *et al.*, 2009; Corcuera *et al.*, 2011) have provided robust estimates of within-species variability, but both these studies failed to make inferences on the micro-evolutionary processes shaping this variability. In two provenance trials conducted for *P. pinaster*, Corcuera *et al.* (2011) measured a $\overline{CV}_P^{ppt} \approx 5\%$ which is similar to our estimate of 6.4%. In *Pinus sylvestris*, Martinez-Vilalta *et al.* (2009) obtained a $CV_P^{in\ situ} \approx 8\%$, again similar to the value obtained here (6.9%) for *P. pinaster*. Therefore, our results agree well with those of previous experiments on *in situ* natural populations as well as populations grown in provenance-progeny trials, and strongly suggest that cavitation resistance shows low phenotypic variability for these two pine species. Whether this result would extend to other pines or beyond to other members of the Pinaceae calls for further investigations.

Phenotypic plasticity of cavitation resistance is low

While many authors studying natural populations have discussed the magnitude of phenotypic plasticity of resistance to cavitation (Kolb & Sperry, 1999; Maherali & DeLucia, 2000; Maherali *et al.*, 2002; Martinez-Vilalta & Pínol, 2002; Jacobsen *et al.*, 2007), only two quantitative estimates of phenotypic plasticity

have been reported (Corcuera *et al.*, 2011; Wortemann *et al.*, 2011). The diversity of the species studied (broad phylogenetic and ecological sampling) and the low number of replicates means that no consensus has been able to emerge from the above-cited studies. Our estimate of phenotypic plasticity of resistance to cavitation is more robust than those of previously mentioned studies, as we measured a large number of genotypes and used a statistical framework (mixed model) that allowed broad inference, and we provided a reference trait (height growth) for comparison purposes, considering that absolute numbers have no biological meaning *per se*. Indeed, growth-related traits are known to show a high level of phenotypic plasticity in *P. pinaster* (Alia *et al.*, 1997; Gonzalez Martinez *et al.*, 2005). A strict comparison between resistance to cavitation and growth traits shows that resistance to cavitation is much less plastic (see the RDPI values). However, this pattern should be confirmed in larger experiments including more sites.

Furthermore, the same amount of phenotypic variability was observed in both provenance-progeny trials ($CV_P^{ppt} = 6.4$) and *in situ* natural populations ($CV_P^{in\ situ} = 6.9$), which provides sound evidence that phenotypic plasticity of cavitation resistance is low. Finally, a comprehensive literature survey using 'RDPI' as a keyword found that phenotypic plasticity for this trait is in the same range as the lowest values (0.19 ± 0.14; $n = 249$) found for photosynthetic traits (Baquedano *et al.*, 2008; Traveset *et al.*, 2008).

Cavitation resistance and aridity index do not covariate

Martinez-Vilalta *et al.* (2009), Herbette *et al.* (2010) and Corcuera *et al.* (2011) failed to detect phenotypic or genetic clinal (or counter-clinal) variation of resistance to cavitation with climatic variables. Likewise, here we were unable to find correlations between provenance climatic data and genetic (obtained from provenance-progeny trials) or phenotypic (*in situ* natural populations) variation (Fig. 4). Unexpectedly, resistance to cavitation was lower in the dry trial was than in the wet trial. Moreover, we did not detect any correlation between xylem-specific hydraulic conductivity and cavitation resistance ($r = -0.41$; $P = 0.1$). Both results provide sound evidence that P_{50} measurements in the dry trial were not biased by native embolism as a consequence of past cavitation events.

Several nonexclusive hypotheses can be formulated to explain this lack of correlation. First, we may not have targeted all the potential climatic variables driving resistance to cavitation. For instance, frost and soil compartments were not considered in this study. Frost is well known to induce cavitation but is unlikely to be involved because conifers are not prone to frost-induced cavitation (Pittermann & Sperry, 2003). As mentioned previously, the site effect for resistance to cavitation seems more affected by soil characteristics rather than 'air dryness'. Regarding soil compartments, no data were available, but future research should consider the fact that soil water shortage can buffer or even reverse our climatic ranking.

Secondly, we did not detect any genetic variability between populations despite purposively selecting contrasted populations. The lack of genetic differentiation for resistance to cavitation among populations could also explain why no significant trend

with climatic data was observed. Such results suggest that attempts to mitigate climate change impacts via strategies based on the transplantation of provenances from warmer origins (selected with climatic data only) may not be a robust approach. The capacity of a provenance to cope with warmer climate should be also experimentally assessed.

Is cavitation resistance a canalized trait in pines?

Based on a single provenance-progeny trial, Lamy *et al.* (2011) found that between-population genetic variance was lower than that expected under genetic drift ($Q_{ST} < F_{ST}$). The present study reinforces this finding over two climatically contrasted trials. The classical hypothesis to explain this pattern is uniform selection, a particular type of selection where stabilizing selection centred on the same selective optima acts in each population's environment. Here, the studied populations were selected on climatic bases in order to represent the full range of climatic variability, which means, implicitly, that the populations experienced different abiotic selective optima. Thus, the uniform selection hypothesis is unlikely in our case.

Alternatively, the $Q_{ST} < F_{ST}$ pattern can be explained by the complex genetic architecture of the trait. If the genes underlying the trait interact nonadditively (epistasis and/or dominance), simulation and/or analytical studies have shown that $Q_{ST} < F_{ST}$ without invoking a selection mechanism (Whitlock, 1999; Lopez-Fanjul *et al.*, 2003; Goudet & Buchi, 2006; Goudet & Martin, 2007). Traits showing low phenotypic and/or genetic variability under evolutionary stasis are called canalized traits (Flatt, 2005; Lamy *et al.*, 2012b). Given that resistance to cavitation showed low genetic variation between climatically contrasted populations, we suggest that cavitation resistance is a canalized trait.

What is the target of natural selection for drought tolerance in *Pinus pinaster*?

Pines are known to be isohydric, that is, during their short-term response to drought, they reduce their stomatal conductance as soil water potential decreases and atmospheric conditions become dry, thereby maintaining a relatively constant midday needle water potential regardless of drought conditions (Tardieu & Simonneau, 1998). However, there is no extensive data set for stomatal sensitivity to soil water potential (and/or VPD) from which to draw robust conclusions about the genetic variability and/or phenotypic plasticity of such a trait. To cope with long-term drought stress, isohydric species also adjust their carbon allocation as well as the ratio of transpiration on conducting surfaces (A_L/A_S), or 'hydraulic adjustment' (*Pinus ponderosa, Pinus palustris, P. sylvestris* and *Pinus halpensis*; Maherali & DeLucia, 2000; Addington *et al.*, 2006; Martinez-Vilalta *et al.*, 2009). If we take a step back and consider the genetic variability of traits involved in drought tolerance response in pines, most of the genetic variability is in morphological traits (A_L, collar diameter, and growth rate; McDowell *et al.*, 2002; Bouffier *et al.*, 2008; Lamy *et al.*, 2011, 2012a), in contrast to resistance to cavitation. This view is reinforced by the extent of phenotypic plasticity in

each of these traits, as morphological traits exhibit much more phenotypic plasticity than resistance to cavitation. Therefore, in *P. pinaster*, medium- and long-term adaptation to drought may rely more on changes in morphology (via phenotypic plasticity and standing genetic variation) than changes in xylem cavitation-related traits. In other terms, facing drought through xylem cavitation-related traits has limited adaptive potential (low genetic additive variance) compared with morphological and growth-related traits.

Assuming that resistance to cavitation is a central process in drought tolerance strategy but shows a low variability in *P. pinaster* and no difference between populations from contrasted climates, our hypothesis is that natural selection has shaped a robust underlying genetic architecture to avoid lethal deviation. This canalized trait seems not to be a current target of natural selection but was targeted a long time ago. These patterns are not expected for all conifers, particularly for more xeric species, that is, species highly resistant to cavitation such as *Juniperus, Cupressus* and *Callitris* (S. Delzon, pers. comm.). Indeed, further research is needed to explore genetic variability and phenotypic plasticity for this trait within the *Pinus* genus and the conifer taxa.

How vulnerable are the maritime pine forests in a drier world?

In a recent study, Choat *et al.* (2012) demonstrated the fundamental vulnerability of woody plant species to reduction in rainfall and increasing temperatures. The risky hydraulic strategy (weak safety margin ($P_{min} - P_{50}$)) exhibited by many species is probably the result of a trade-off that balances growth with protection against risk of mortality in a given environment. However, the limited plasticity and genetic variation of cavitation resistance found here suggest that it is unlikely that populations of *P. pinaster* will be able to hydraulically acclimate to accelerated climate change and evolve increased embolism resistance.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Another analysis of the climatic data to select population though a PCA.

Fig. S2 Comparison between Q_{ST} and F_{ST}.

Fig. S3 Correlation between climatic data from the world climate database and real climatic data from weather stations.

Table S1 Variance table from the model in Notes S2

Table S2 Geographical coordinates of populations growing in wet and dry trials

Notes S1 Exhaustive explanation of the comparison between Q_{ST} and F_{ST}.

Notes S2 Another model formulation for the multi-site analysis to take into account the heterogeneous error variances between sites.

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