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Phenotypic plasticity is stronger than adaptative differentiation among Mediterranean stone pine provenances

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Abstract

The Mediterranean stone pine, *Pinus pinea* L., seems to be well adapted to the different climate zones of its distribution range that spans four thousand kilometres along the Northern shore of the Mediterranean Sea. But recent molecular studies revealed it to be extremely genetically depauperate for a widespread tree. In this context, a provenances trial should elucidate whether any differentiation in adaptative traits can be identified between 34 accessions covering its natural range. The presence of strong spatial autocorrelations throughout four test sites required iterative nearest-neighbours adjustments in their statistical analysis. No significant differences in survival or ontogeny were found between accessions, while height growth was slightly though significantly more vigorous in northern or inland provenances. But these differences were masked by a common, stable reaction norm in dependence on site and microsite. On the other hand, its strong developmental plasticity allows the stone pine to delay the heteroblastic phase change in order to survive in unfavourable conditions, a clear advantage in the limiting and unpredictable environments of Mediterranean ecosystems.

Key words: *Pinus pinea;* provenance trial; Nearest Neighbours Adjustment; Iterative Spatial Adjustment.

Resumen

Mayor plasticidad fenotípica que diferenciación adaptativa entre procedencias de pino piñonero

El pino piñonero, *Pinus pinea* L., está aparentemente adaptado a diferentes zonas climáticas a lo largo de los cuatro mil kilómetros de su rango de distribución en el Mediterráneo. Sin embargo, estudios moleculares recientes le han descrito como genéticamente muy empobrecido para ser un árbol de amplia distribución. En este contexto, el presente ensayo de procedencias estudió la presencia de diferenciación en características adaptativas entre 34 procedencias representativas de su rango de distribución. Debido a la presencia de autocorrelaciones espaciales en los cuatro sitios de ensayo, el análisis estadístico usó ajustes iterativos basados en los vecinos más próximos de cada árbol. No se observaron diferencias significativas entre procedencias respecto a supervivencia y desarrollo ontogénico, pero el crecimiento en altura fue ligera, aunque significativamente mayor en procedencias del interior o mayor latitud. Sin embargo, estas diferencias fueron muy inferiores al efecto del sitio o micrositio, común para todas las procedencias. Por último, se observó que su alta plasticidad ontogénica le permite al pino piñonero retrasar en condiciones desfavorables el cambio de fase vegetativa a follaje y crecimiento adulto durante varios años para sobrevivir en forma juvenil, lo que es un recurso estratégico más para ambientes limitantes e impredecibles de los ecosistemas mediterráneos.

Palabras clave: *Pinus pinea*; ensayo multilocalidad; ajuste por vecinos más próximos; ajuste especial iterativo.

Introduction

Phenotypic plasticity, defined as the ability of a genotype to express a range of different phenotypes depending on environmental conditions, is considered one of the major mechanisms allowing sessile and long-lived organisms, such as trees, to cope with environmental heterogeneity (Sultan, 2000). Though many reaction norms in plants might be passive rather than adaptive (Van Kleunen and Fischer, 2005), there are clear examples of adaptive plasticity, especially in

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invasive species (Geng *et al.,* 2006; Li *et al.,* 2006). In the context of the ongoing climate change, increased vulnerabilities of ecosystems are predicted, especially in the Mediterranean region where extreme summer droughts are expected to increase (Archaux and Wolters, 2006). Adaptability to new or stressing environments must be seen therefore not only as an important aspect for dispersion or colonisation strategies, but also in terms of its relevance for the local persistence of existing species and ecosystems (Sultan, 2000).

The complementarity of adaptation by genetic differentiation and acclimatisation by phenotypic plasticity as mechanisms for fitness in divergent or heterogeneous habitats is highlighted by the case of the Mediterranean stone pine, *Pinus pinea* L., a widespread tree species that was recently found to lack virtually any neutral genetic variation (Vendramin *et al.,* 2008). Around the Mediterranean Sea, there are about 0.7 million hectares of stone pine-dominated forests, sparsely spread over more than 4,000 km from the Atlantic coast in Portugal to the shores of the Black Sea and the Mount Lebanon. Though the stone pine is since Antiquity a cultural species in the Mediterranean landscape due to its ornamental value and its edible kernels (*cf.* Schweinfurth, 1884), still at present most of its stands are natural or naturalised forests, not cultivated plantations. However, no clear phylogenetic structure has been found within or among its widely separated populations, which are all fixed to a same haplotype for nearly all studied cpDNA and allozyme markers (Fallour *et al.,* 1997; Vendramin *et al.,* 2008). This extremely low level of diversity, truly exceptional among abundant, widespread trees, has been attributed to a severe and prolonged demographic bottleneck of stone pine long before its quaternary dispersal to its current range (Vendramin *et al.,* 2008). Even on the phylogeography of *Pinus resinosa*, considered amongst the most genetically depauperate conifers with large distribution area, clear marks were left by the ice ages (Boys *et al.,* 2005), marks that are missing in the case of the stone pine. The few studies published to date regarding quantitative genetics of *Pinus pinea* coincide in reporting only minor or non-significant differences between populations (Court-Picon *et al.,* 2004; Gordo *et al.,* 2007).

Theoretically, this putatively extremely low genetic diversity might implicate the evolutionary precariousness of the species, due to a lack not only of adaptability, but also of evolvability (*sensu* C. D. Schlichting: ability to evolve in response to natural selection). Might *P. pinea* therefore be a mere relict taxon from former Tethyan flora? But as a matter of fact, stone pine is an emblematic key species of the Mediterranean vegetation, well adapted to the severe summer drought and the poor, eroded soils that characterize its distribution area (Rodrigo *et al.,* 2007). *I.e.* whilst most stone pine populations, scattered along the Mediterranean coastline, do not normally suffer major winter frosts (annual mean temperature t 15-19°C, annual rainfall p 500-1,000 mm), its open woodlands in higher parts of inland Spanish (600-1,000 m a.s.l.) tend to be close to pseudo-steppe habitats with hot, dry summers and harsh, dry winters (t 10-14°C, p 350-450 mm, absolute minimum temperature –13 to –21ºC) and no sign for any lack of adaption is observed in stone pine in those contrasting environments.

This hardiness has made the species a widely used choice for forest ecosystem restoration and protective afforestations in semiarid zones, which have more than doubled its extension over the last two centuries. In some ecosystems, both within as well as outside its natural range, it is even considered an invasive species (Rejmánek and Richardson, 2003; Muñoz-Reinoso, 2004). Although clearly less competitive than other truly invasive plants (Grotkopp *et al.,* 2002; Peperkorn *et al.,* 2005), its lack of inbreeding depression (Ammannati, 1989) facilitates the natural colonization of new areas, on occasions by only one or just a few kin individuals, without any negative founder effects, *e.g*. after long-distance seed dispersal by birds (*cf.* Tomback and Linhart, 1990). Similarly, self-fertile pioneer or invasive herbs and forbs compensate for genetic uniformity through elevated phenotypic plasticity that enables them to occupy new, divergent habitats (Noel *et al.,* 2007).

In this context, we wanted to clarify in a multi-locality provenance trial whether it is possible to identify a differentiation in adaptative traits among Mediterranean stone pine provenances as a response to divergent habitats.

Materials and methods

Plant material and trial sites

Data used in this study were collected from a provenance trial started in 1994 with the exchange of Mediterranean stone pine accessions on the initiative of the French INRA within the framework of the FAO Committee *Silva Mediterranea* (Table 1; Fig. 1; Martín and Prada, 1995).

Table 1. The studied 34 Mediterranean stone pine provenances

Provenance		Country	Latitude	Longitude	Altitude (m.a.s.l.)	T (°C)	p (mm yr^{-1})
Chalkidiki Agios Nicolaos		Greece	40°14'N	23°34'E	50-400	15.0	439
Feniglia 67		Italy	42°25'N	11°17'E	25	15.8	680
Beit Mounzer (Mad. J.)	*	Lebanon	34°16'N	35°55'E	1,300-1,400	13.3	1,030
Kornaele		Lebanon	33°50'N	35°46'E	1,200-1,400	13.2	1,148
Qsaibe		Lebanon	33°51'N	35°41'E	600-700	17.1	1,098
Bkassine		Lebanon	33°34'N	35°36'E	800	14.3	977
Antalya-Kumluca-Kalmaz	*	Turkey	36°17'N	30°20'E	5	18.6	986
Yalova-Dumanlidag		Turkey	40°32'N	29°22'E	500	11.7	725
Canakkale-Eceabat		Turkey	40°11'N	26°16'E	20	15.0	626
Mugla-Yatagan-Katranci		Turkey	37°22'N	28°03'E	660	16.3	673
St. Raphaël (Var)		France	43°26'N	06°46'E	80	14.0	848
Villeneuvette (Hérault)	*	France	43°37'N	03°24'E	130-220	13.5	775
Saintes-Maries (B.du Rh.)		France	43°27'N	04°26'E	5	14.3	543
St. Aygulf (Var)		France	43°26'N	06°41'E	5	14.6	848
Brignoles-Le Val (Var)		France	43°26'N	06°05'E	310	13.1	886
Alcacer do Sal		Portugal	38°20'N	08°33'W	60	16.3	574
Serra do Minho	*	Portugal	41°00'N	08°09°W	370	14.0	1,514
Cogeces de Iscar		Spain 1	41°25'N	04°31'W	800	12.2	455
Valorio1		Spain 1	41°31'N	05°46'W	630	12.3	359
Valorio2		Spain 1	41°31'N	05°46'W	640	12.3	359
Toro		Spain 1	41°31'N	05°27'W	680	12.0	366
Vega Sicilia		Spain 1	41°38'N	04°18'W	780	11.3	461
Íscar	*	Spain 1	41°21'N	04°31'W	750	12.2	440
Tordesillas	*	Spain 1	41°30'N	04°54'W	680	12.0	432
Portillo		Spain 1	41°28'N	04°32'W	850	11.6	470
Cadalso		Spain 2	40°17'N	04°31'W	800	13.6	687
Tarazona de la Mancha		Spain 3	39°17'N	01°55'W	700	13.4	453
Doñana		Spain 4	36°55'N	06°25'W	20	17.5	575
Las Lomas		Spain 4	36°18'N	05°52'W	59	17.9	550
Cartaya	*	Spain 4	37°22'N	07°11'W	82	18.1	500
Parafrugell		Spain 6	41°57'N	03°06'E	100	15.3	665
Biar	*	Spain A	38°38'N	00°45'W	900	13.8	457
Garrovillas		Spain C	39°41'N	06°35'W	400	16.4	574
Budia		Spain R	40°38'N	02°45'W	1,000	12.9	530

Note: The provenance regions within Spain are labelled following Prada *et al.* (1997). The spring phenology was studied in the eight provenances marked by asterisk). T: mean annual temperature. p: mean annual rainfall.

Seedlings from 34 accessions spanning the range of the species were grown in Spain throughout the 1995 growing season under standard container-nursery cultural practices. Three of the analysed experimental sites (Tordesillas, Cucalón and Quintos de Mora) were planted during the winter of 1995/96 under an incomplete block design; the fourth (Trespaderne) was planted one year later in randomised complete blocks (Table 2).

Measurements

Survival, height and diameter of each tree were measured three times during the first decade. The vegetative, or heteroblastic, phase change from juvenile (short bluish-green, isolated needles and slow, free growth) to adult (paired, definitive needle type and predetermined, verticillated growth) and the reproductive phase change (appearance of first male and female strobili) were also recorded. The effect of phenotypic plasticity on these traits (survival, growth and ontogeny) reflects neatly the adaptation of plants, or the lack of it, to a given environment.

The successive heights of the same plant were strongly correlated in time, as well as stem diameter and tree height at the same age, thus the analysis of plant size focuses here exclusively on the height at the last measurement. At Tordesillas, Cucalón and Trespader-

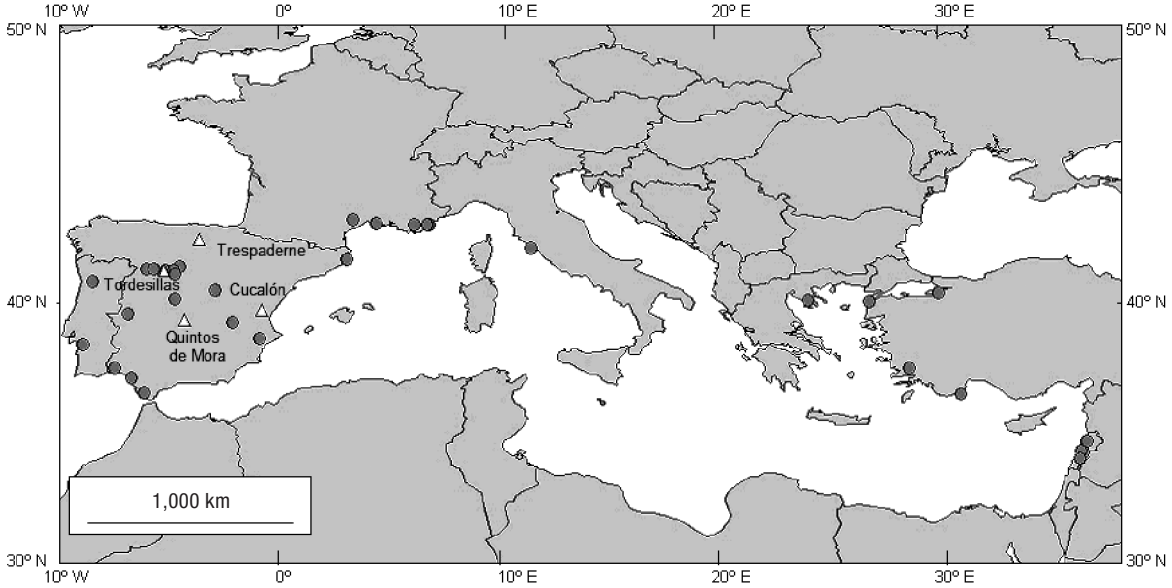


Figure 1. The 34 Mediterranean stone pine provenances (dots) and four test sites in Spain (triangles).

ne, this was at nine years, whereas due to severe deer-browsing damage, the Quintos de Mora site was excluded from the analysis until two years later, when the fencing installed to avoid further damage had allowed the saplings to recover growth.

During the spring and early summer 2001, the phenology was registered at two sites, Tordesillas on the inland plateau and Cucalón, situated in a coastal mountain range, the latter of the two having a milder and wetter winter. A sub-sample of 220 saplings from eight ecologically contrasting provenances in Lebanon, Turkey, France, Spain and Portugal were selected (Table 1); all of them presented already adult shoot growth for several years. During the spring flush (March-June) the length of the elongating leader shoot was measured twice a month, because shoot elongation is not a discrete event but a continuous process and its phenology is better assessed by growth parameters derived from repeated measurements, rather than by discrete phenostages (Chuine *et al.,* 2001).

Statistical analysis

The adjustment of a genotype-phenotype model for individual tree height [eq. 1, see below] was carried out independently for each site, because both the planting and the analysed measurement dates differed from one site to another, as did the number of accessions included (29 or 34; Table 2) and, above all, because plant

Table 2. Location and characteristics of the four Spanish experimental sites

Site	Tordesillas	Cucalón	Trespaderne	Quintos de Mora
Province	Valladolid	Castellón	Burgos	Toledo
Altitude	670 m	640 m	560 m	827 m
Latitude	41°30'N	39°47'N	42° 47'N	39°24'N
Longitude	4° 57'W	0° 38'W	3° 23'W	4° 04'W
Annual rainfall (mm)	472	592	742	400
Mean temperature (°C)	12.2	13.9	10.8	13.4
Experimental design	Incomplete blocks	Incomplete blocks	Complete blocks	Incomplete blocks
Provenances	34	34	29	29
Blocks	51	68	15	43 + 1
Plantation date	February 1996	December 1995	February 1997	April 1996
Grid	2.5 × 4 m	2.5 × 4 m	2 × 3 m	2.5 × 4 m
Surviving plants	1,759 (96%)	2,161 (88%)	1,110 (85%)	953 (61%)

growth showed clear spatial patterns at each site, that required a *post-hoc* adjustment. Although each test site occupies a flat and apparently homogeneous terrain, there was from the beginning evidence of strong positive spatial autocorrelations of plant growth in narrow neighbourhoods, a phenomenon that increased over time: *e.g.* at 9 years, tree height ranged from 0.7 to 3.5 m between patchy zones within the Tordesillas site (Fig. 2a). Standard analyses under the pre-blocking design violated hence the basic assumption of independent residual values. Loo-Dinkins (1992) recommends spatial adjustments in field trials when raw data autocorrelation among direct neighbours exceeds 0.15, a threshold well exceeded at all four sites (r 0.44-0.76).

Several approaches to *post-hoc* spatial analyses have been proposed, based on post-blocking, row-column-analysis, de-trending, response surface adjustments or nearest neighbour adjustments (NNA) such as moving averages (MA) or autoregression (AR) (Bartlett, 1978; Costa-Silva *et al.,* 2001; Gezan *et al.,* 2006). The geostatistical method of kriging, based on distance-weighted averages, gives continuous bi-dimensional interpolations that allow the mapping of the spatial patterns (Hamann *et al.,* 2002), but also simpler NNA based on MA or AR are useful for estimating a correction factor for a field trial with a regular planting grid when its experimental design has failed due to spatial autocorrelations (Mutke *et al.,* 2007).

The spatial adjustment can be integrated into the genotype-phenotype model structure in several ways. The simplest is to rest («remove») the spatial effect estimated at each plant position from the raw values of the phenotypic response, obtaining thus a derived response variable, or even a series of new, successively derived variables if the process is iterative (Zas, 2006). However as with detrending, the biological meaning of the results, *e.g.* estimates for quantitative genetic parameters such as heritability, remains unclear when they are referred to de-trended or «de-correlated» response variables (obviously with a lessened environmental and overall variance). Conceptually, the removal of the spatial effect from the left side of the model equation is analogous to adding it to the right side as a covariate with the regression coefficient fixed to unity. The alternative approach is introducing the spatial effect as covariate in the model, like in the classic Papadakis method. Its regression coefficient is hence fitted by least square means (OLS) and the original response variable is retained, sharing this last feature with a third alternative, the variance-in-error modelling using linear mixed model regression that allows for specifying the spatial autocorrelation in the variance-covariance matrix (Costa-Silva *et al.,* 2001; Zhang and Gove, 2005; Piepho *et al.,* 2008). But the iteration of nearest-neighbour adjustments has proved to be frequently more efficient than non-iterative adjustments (Bartlett, 1978). The reason might be a lack of stationarity of the phenomenon of autocorrelation itself when patchy microsite variation occurs at smaller, heterogeneous scales. In this case, the iterative adjustment will improve the error distribution and independence mainly by gradually eliminating the hot spots or local «pockets» of non-stationarity that can be revealed using the Local Moran statistic or similar local indicators of spatial association, or LISA (*cf.* Anselin, 1995; Boots, 2002). Non-stationarity is a serious impediment to sophisticated spatial adjustment techniques that require certain assumptions to be fulfilled, as in the case of ordinary kriging based on stationary kernels. Thus Ockham's razor recommends the use of simpler, more parsimonious and robust «non-parametric» NNA techniques as long as they are efficient.

In the present case, a nearest neighbour adjustment by the iterated Papadakis method was applied in order to estimate a local mean of tree height as proxy for microsite effects (*cf.* Bartlett, 1978). An initial model for each test site included only the mean and an additive provenance effects along with an error term; the second adjustment also included a first Papadakis covariate, *i.e.* the moving average of the residuals of the former model at the eight nearest-neighbour positions in the planting grid; the third model incorporated the moving average of the obtained residuals within the same first-order neighbourhood as second covariate, etc.:

$$H_{xyp} = m + g_p + \Sigma_k (a_k M_{xyk}) + e_{xyp} \quad [1]$$

where H_{xyp} is the individual tree height from provenance p at plant position $(x;y)$, m the average height at the test site, g_p the additive effect of provenance p, a_k the linear effect of the kth spatial covariate M_{xyk}, *i.e.* the moving average of residuals from run k-1, and e_{xyp} the error term.

The iterative procedure was stopped when the F-test was non-significant for the following Papadakis covariate. In each step, the lack of multicollinearity was also assessed by the Variance Inflation Factor for each source of variation (V.I.F. values below 10), whereas the absence of significant genotype-by-environment interactions and the normality, homoscedasticity and independence of residual values were checked for the

final model. Spatial independence of residuals was assessed additionally by the standardised Localized Moran's Index *I* (Anselin, 1995; *NB* OLS-adjusted residuals average zero, thus each residual value is its own deviations from this mean):

$$I_i = n\ \Sigma_j (w_{ij} r_i r_j) * (\Sigma_i (r_i^2))^{-1} \quad [2]$$

where n is the number of observations, w_{ij} the topologic weight ($w_{ij} = 1$ if tree j is a direct neighbour of the tree i, otherwise 0), r_i the residual of tree i and r_j the residual of tree j.

Although Bartlett (1978) found that spatial adjustment does not bias the estimates of the adjusted treatment means when the number of levels of the estimated treatment is elevated (herein, 34 provenances) and there are at least three replicates (herein, 15-24 replicates at each site), it must be taken into account that in the original block design, trees were planted in groups of three trees from the same provenance in line. For this reason, in order to evaluate the possible confusion of local effects and the provenance contribution, the iterative spatial adjustment of the microsite covariate was repeated at one of the sites using local means calculated excluding any direct neighbour trees of the same provenance.

In order to compare the performance of different accessions among sites and to study common patterns of geographic variation that might be related with adaptations to ecologic conditions of each provenance, a Principal Components Analysis (PCA) was performed with the least square means estimated for each of the 29 provenances present at the four sites.

For the analysis of the phenology monitoring, time was substituted by thermal time as reference scale, given that the relative rate of shoot elongation depends in stone pine strictly on heat sums (Mutke *et al.,* 2003). The periodically measured length was hence adjusted for each shoot by non-linear regression at a degree-day (dd) scale above the species-specific threshold 1°C (*cf.* Mutke *et al.,* 2003):

$$L'(t) = 1\text{-}\exp(\text{-}\exp((D(t)\text{-}b)/c)) \quad [3]$$

where $L'(t)$ is the relative shoot length at moment t, expressing the shoot length L(t) as a proportion of total elongation from initial length (winter bud) L_0 to the final spring shoot length L_1, *i.e.* $L'(t) = (L(t)\text{-}\ L_0)/(L_1\text{-}\ L_0)$, $D(t)$ the degree day sum above 1°C accumulated at moment t since 1 March as the approximate start of the vegetative season [dd], b the heat sum corresponding to the moment of maximum growth rate (inflexion point of the cumulative function defined by eq. 3) [dd] and c a slope parameter (the smaller c, the steeper the slope) [dd^{-1}].

Analyses of variance were done for both regression parameters b and c among sites and provenances. After bud set on the preformed spring shoot in June, the growth measurements were continued until autumn in order to register possible Lammas growths/polycyclism. The differences in tendency to form Lammas shoots were assessed between sites and provenances through a logistic regression on presence/absence of a needled, completely formed second shoot.

Results

Survival and phase changes

The performance at each site was remarkably homogeneous among provenances. Survival ranged from 85 to 96% in Tordesillas, Cucalón and Trespaderne, but it was only 61% at the deer-browsed Quintos de Mora site, without any significant differences between provenances but with patchy patterns within each plot. It is especially noteworthy that no frost damage was observed in any plant or shoot apex (not even in individuals from the southern coastal provenances), although at the Tordesillas test site, for example, temperatures reached an absolute minimum of –17°C, and the last frosts occurred in most years in May, right in the middle of the spring flush.

At the two faster-growing sites Tordesillas and Cucalón, the vegetative phase change was generalised in the third or fourth year (at an average height of 25-35 cm). At Quintos and Trespaderne growth was hampered, and the vegetative phase changed successively during the following years. At the first two sites, the reproductive phase change (maturity) was achieved in 23% (female) and 27% (male) of the saplings at an age of 6-9 years. At the other two sites, none of the individuals had reached maturity at the age of 11 years. Both vegetative and sexual maturity of each plant were closely linked to its size, independently from its provenance.

Height growth

The saplings from the 34 provenances differed significantly in height growth, although the differences between provenances were masked by the strong effects of the environmental heterogeneity between sites (Ta-

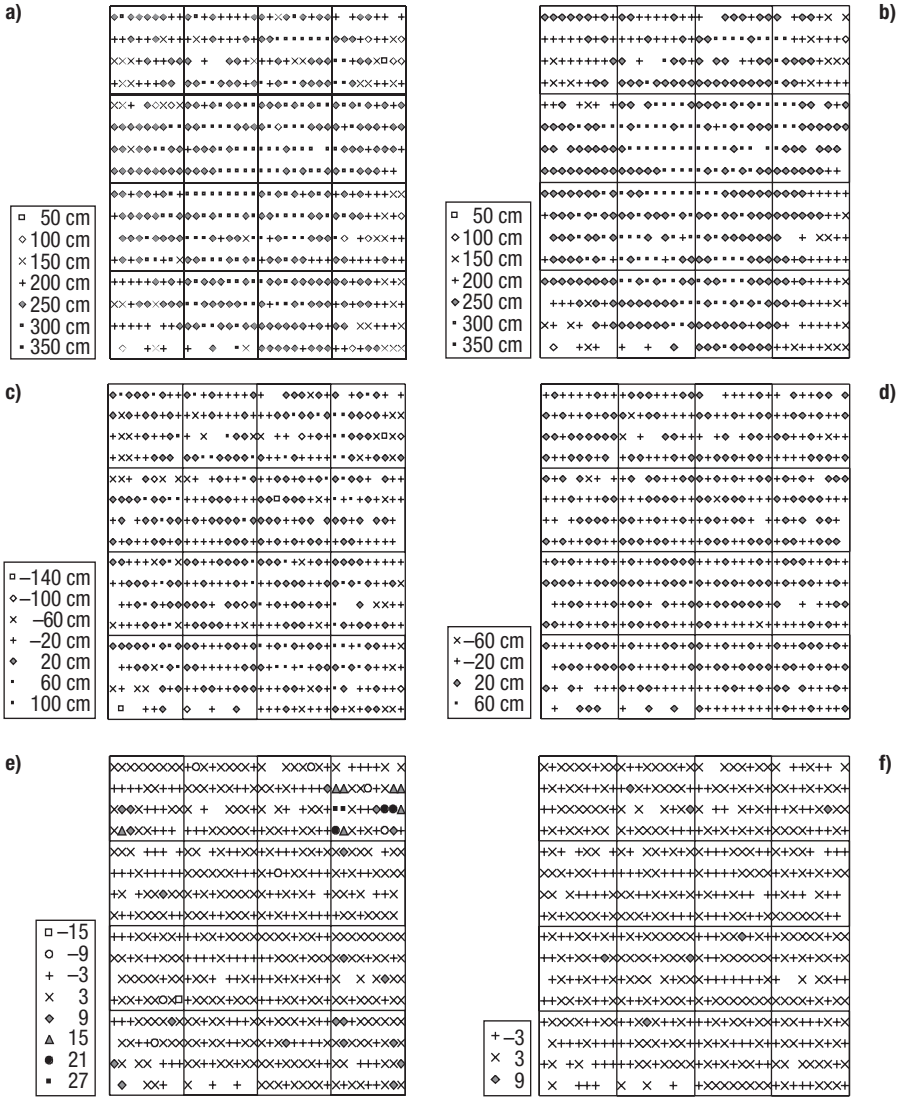


Figure 2. Spatial adjustment parameters for tree height at 9 years at the Tordesillas site, displayed in the planting grid of 16 adjoining Incomplet Blocks. *i.e.* 6 replicates. a) Observed individual tree height. b) Estimated microsite effect (nearest-neighbour-adjusted local mean height). c) Spatially auto-correlated residual values from standard analysis (Incomplete Block Design, IBD). d) Residual values from iterative spatial analysis (NNA). e) Local Moran' I for IBD's residuals. f) Local Moran' I for NNA's residuals.

ble 3) as well as within each site, the latter apparently associated with the soil texture variations in the plot (*e.g.* Fig. 2a). The coefficient of variation for the mean tree height between provenances was only 6-10% at each site, the reaction norm to site and micro-site variation being common and stable for all provenances. As said before, we desisted from adjusting a joint regression model across the four test sites, because the history of each experimental plot (planting and measurement age, deer-browsing) and, above all, their spatial patterns were estimated to be too different for considering the measured height as the expression of a same trait. For merely illustrative purpose, we might indicate that a variance component estimation among mean tree heights values for Site and Provenance (Table 3), considering both as random factors, would attribute 97.3% of the variance to differences between sites, but only 0.9% to the provenance and 1.7% to the residual variation (*i.e.* the site × provenance interaction).

The iterative spatial adjustment (ISA) placed the sum of squares attributed to differences between provenances at 3-12% of the overall variation at each site,

Table 3. Adjusted mean tree height (cm) of the provenances at 11 years at Quintos de Mora, at 9 years at the other test sites

	Provenance	Tordesillas	Cucalón	Trespaderne	Quintos de M.
GR	Chalkidiki Agios Nicolaos	251^{ab}	173^{klmn}	67^{fghijk}	126^{a}
I	Feniglia 67	238^{cdefgh}	228^{a}	—	—
RL	Beit Mounzer (Mad. J.)	241^{bcdef}	198^{cdefg}	—	—
RL	Kornaele	223^{ijklm}	190^{efghijk}	55^{lm}	117^{ab}
RL	Qsaibe	227^{ghijkl}	192^{efghijk}	—	—
RL	Bkassine	212^{mno}	182^{hijklm}	—	—
TR	Antalya-Kumluca-Kalmaz	199^{op}	174^{lmn}	61^{jkl}	120^{ab}
TR	Yalova-Dumanlidag	249^{abc}	185^{ghijklm}	52^{m}	120^{ab}
TR	Canakkale-Eceabat	235^{cdefghi}	190^{efghijk}	60^{klm}	124^{a}
TR	Mugla-Yatagan-Katranci	219^{lmn}	159^{n}	64^{hijk}	91^{c}
F	St. Raphaël (Var)	198^{p}	175^{jklmn}	66^{ghijk}	129^{a}
F	Villeneuvette (Hérault)	221^{klm}	174^{lmn}	68^{efghij}	114^{abc}
F	Saintes-Maries (B.du Rh.)	230^{fghijkl}	192^{efghij}	72^{bcdefg}	111^{abc}
F	St. Aygulf (Var)	220^{lmn}	186^{ghijkl}	72^{bcdefgh}	121^{ab}
F	Brignoles- Le Val (Var)	242^{bcdef}	204^{bcde}	70^{cdefgh}	118^{ab}
P	Alcacer do Sal	226^{ghijkl}	183^{ghijklm}	66^{ghijk}	103^{bc}
P	Serra do Minho	246^{abcd}	186^{fghijklm}	69^{defghi}	114^{abc}
E-1	Cogeces de Iscar	248^{abcd}	197^{cdefgh}	74^{bcde}	124^{a}
E-1	Valorio1	245^{abcde}	202^{bcdef}	78^{ab}	115^{ab}
E-1	Valorio2	258^{a}	216^{ab}	75^{bcd}	123^{ab}
E-1	Toro	235^{defghij}	211^{abcd}	85^{a}	124^{a}
E-1	Vega Sicilia	238^{bcdefgh}	211^{bd}	71^{bcdefgh}	118^{ab}
E-1	Íscar	237^{cdefgh}	200^{bcdefg}	70^{cdefgh}	126^{a}
E-1	Tordesillas	235^{defghijk}	192^{efghij}	72^{bcdefg}	114^{abc}
E-1	Portillo	219^{lmn}	179^{ijklm}	67^{efghijk}	120^{ab}
E-2	Cadalso	239^{bcdefg}	205^{bcde}	76^{bc}	111^{abc}
E-3	Tarazona de la Mancha	245^{abcde}	193^{efghi}	71^{bcdefgh}	115^{ab}
E-4	Doñana	207^{nop}	173^{lmn}	65^{ghijk}	116^{ab}
E-4	Las Lomas	223^{ijklm}	190^{efghijk}	61^{ijkl}	122^{ab}
E-4	Cartaya	207^{nop}	192^{efghijk}	62^{ijkl}	118^{ab}
E-6	Parafrugell	232^{efghijkl}	195^{cefgh}	74^{bcdef}	119^{ab}
E-A	Biar	226^{hijklm}	193^{efghi}	66^{ghijk}	129^{a}
E-C	Garrovillas	221^{jklm}	170^{mn}	66^{ghijk}	116^{ab}
E-R	Budia	231^{efghijkl}	172^{lmn}	—^{fghijk}	—
	Site mean	230	190	68	118

Note: Values with the same letter at each site are not significantly different at p = 0.05 level (Bonferroni's post-hoc comparison).

compared to 67-80% attributed to the spatial covariable (Fig. 2b). The coefficients of determination (R^2) of the fitted models ranged from 0.81 to 0.89 for the three sites measured at nine years, somewhat lower (0.75) at the Quintos de Mora site due to the additional variance from deer-browsing damages (not included explicitly in the model). Contrarily, the standard analysis following the pre-blocking experimental design would have suggested a minor or even non-significant provenance effect due to the large residuals (R^2 about only 0.5 for Incomplete, 0.3 for Complete Block Designs) - in any case, those analyses are invalidated by the presence of spatial correlations (Fig. 2c-e).

When repeating the ISA for the Tordesillas data excluding the 1-2 neighbours from the same provenance as the target tree, in order to assess the likelihood of confusion between genetic and spatial effects, the LSM estimates for genetic effects were consistent with those previously found (r 0.99), the maximal deviation between both being ± 5 cm, thus a possible confusion between effects can be ruled out.

In Cucalón, where initial seedling height had been measured also just after planting in 1995, the means of the different provenances correlated significantly (r 0.67) with their percentage of nursery-germinated seeds, a proxy for seed quality (Martín and Prada, 1995). However, during the following years, this correlation

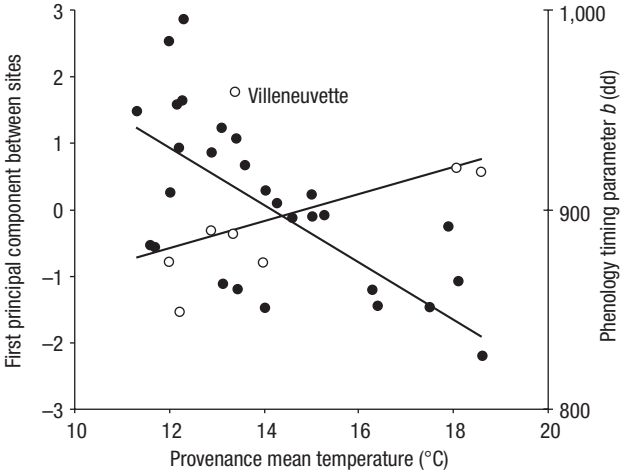


Figure 3. The first Principal Component of 29 provenances (adjusted mean heights at four experimental sites; filled diamonds; left scale) and the phenology timing parameter *b* of 8 studied provenances (open squares; right scale) against their annual mean temperatures.

decreased and became non-significant: at nine years it was less than 0.1 for all sites.

The principal component analysis of provenances' mean tree heights at the four test sites retained two components with an eigenvalue above unity, the first accounted for 52% of the observed variance, particularly at the three sites measured at nine years (loadings between 0.74 and 0.90), somewhat less for the Quintos de Mora site measured two years later (loading 0.35). The second principal component (25% explained variance) separated this last site (loading –0.92) from the others three (loadings from –0.09 to 0.31). Most provenances from the inland Spain and one from France achieved the highest growths across sites, whereas especially coastal provenances from southern Turkey, southern Spain and the French Côte d'Azur grew less. The ranking of other provenances was not consistent among sites (Table 3), but the first principal component correlated significantly with the mean annual temperature as climate proxy for each provenance (Fig. 3: r –0.66, *p* 0.0001).

Phenology

The shoot elongation, studied in 2001 in a sample of 220 trees at two sites, reached 50% of the final length on the 1st May at Cucalón, 18 days later at Tordesillas. However, this apparently plastic phenology between sites proved to be completely canalised on a thermal time scale. The half-elongation of the shoot corresponded to nearly identical heat sums, 796 and 812 degree day (dd), whilst the adjusted mean effect of each site was 897 ± 20 and 908 ± 17, respectively (non-significant in Table 4). Grouping together the eight studied provenances into five Northern or inland/upland ones (from altitudes above 900 m a.s.l. at latitudes below 40°N, above 300 m if northerner) and three Southern or coastal ones, the latter group needed a slightly higher degree-day sum for shoot development: the parameter *b*, corresponding to the heat sum for reaching the moment of maximum spring growth rate, averaged (+SE) in the Southern group 931 ± 11 dd above 1°C after 1 March versus 874 ± 7 dd in the Northern group. This delay of 57 degree days corresponds to about 5 days of the time scale. There were no significant differences between provenances within each group or between sites. The correlation between parameter *b* and the mean annual temperature in origin of each accession was non-significant (r 0.50) due to the aberrant value of accession 12 (Fig. 3; Villeneuvette). Excluding this French provenance, r rises to 0.88 (p 0.008). Nevertheless, there was a high residual variability among trees of each provenance (R^2 0.10; Table 4). The speed of the spring flush expressed in thermal time did not differ significantly between groups or provenances, the common slope parameter *c* being 235 ± 2 dd^{-1}.

The mean spring shoot length for the sampled trees was 32.5 cm in Tordesillas and 24.6 cm in Cucalón. At Tordesillas, in 2001, most trees of each provenance presented a complete needled summer shoot with a second whorl and terminal bud; the mean distance between the spring shoot whorl and the final shoot tip

Table 4. Analysis of variance for the shoot-growth phenological parameter *b* of [eq. 3]

Source of variance	Sum of squares	d.f.	Mean square	p-value
Site	5,946	1	5,946	0.3928
Group	140,002	1	140,002	0.0000
Provenance(Group)	45,423	6	7,571	0.4717
Residuals	1,710,754	211	8,108	
Total	1,903,241	219		R^2 0.10

(its own bud or that of the Lammas shoot) was 14.1 cm when the annual growth had stopped in September. In Cucalón, due to the milder autumn at this site, the terminal buds continued elongating during the autumn, reaching a final mean length of 12.4 cm in December. The presence of fully developed, needled and whorled Lammas shoots was less common in Cucalón. The logistic regression for the presence of Lammas growth indicated that both the effect of Site and of N/S Group were significant (p-value < 0.0001 and 0.03, respectively), attributing 24% of the deviance to the difference between Sites and 2% between Groups. Provenances within each Group did not differ significantly. The likelihood of Lammas growth was 82% for northern and 59% for southern provenances in Tordesillas, 7% and 2% respectively in Cucalón.

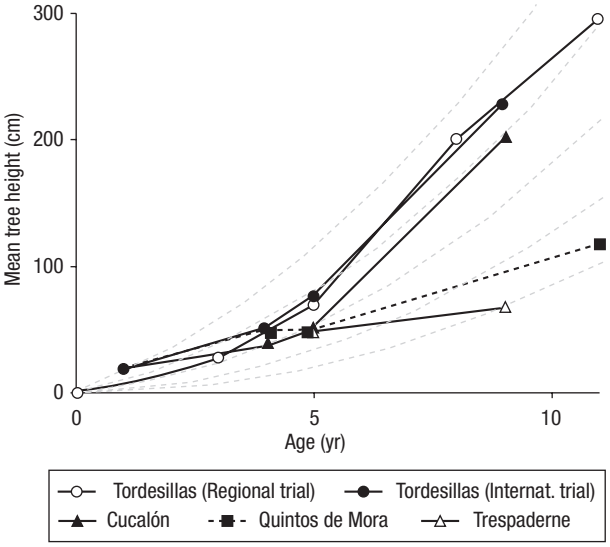


Figure 4. Mean tree height at the four test sites, including also the Tordesillas site of a regional stone pine trial (Gordo *et al.,* 2007). Fine dotted lines correspond to the site-index model curves for stone pine in Spain for dominant heights of 19, 17, 15, 13 and 11 m at 100 years, respectively (Calama *et al.,* 2003).

Discussion

The fact that the vegetative phase change from juvenile to adult growth and foliage depends in stone pine on a critical tree size rather than age is similar to findings in Canaries Pine (Climent *et al.,* 2006), where it was interpreted as capability to survive under unfavourable conditions as low-cost juvenile phenotype for years, in stone pine even for several decades. On the contrary, other species of the subgenus *Pinus* have the vegetative phase change fixed at the end of the first or second growth season and would perish if the adult plant's resources demand exceeds environmental limitations (Klaus, 1989). The capacity of the stone pine to postpone the phase change in order to survive in limiting environments can thus be seen as an adaptative trait within a conservative resource use strategy, similar to delayed stem-growth patterns in the Canaries pine, pine species with grass stage or other lignotuberous plants, shaped by the environmental unpredictability of Mediterranean ecosystems (Valladares *et al.,* 2002). Furthermore, after the phase change, the stone pine gradually loses also other juvenile traits, like the capacity to sprout adventitious juvenile shoots from needle axils, *e.g.* in response to foliage loss due to drought or herbivory, traits that are relevant and adaptative in the critical first establishment stage.

Previous stone pine field trials have found a similar lack of genetic differentiation in quantitative traits as here, as well as a strong dependence on soil conditions (Court-Picon *et al.,* 2004; Mutke *et al.,* 2007). A local-scaled trial was planted at seven sites through a gradient of different soil types in order to compare nine regional seed stands within the inner Spanish provenance region E-1, six of which coincided with accessions of the here analysed international provenance trial (Gordo *et al.,* 2007). The results coincide substantially: in the regional experiment, differences between sites and microsites led to 84% of the observed variation in tree height, whilst the differences between seed lots were absolutely non-significant (0.02%); particularly, compact clay or silt soils were found to hamper root development (Gordo *et al.,* 2007). One replicate of the regional trial, sited at Tordesillas less than 200 m from the international trial plot, displayed a nearly identical average age-height curve (Fig. 4). In another stone pine provenance trial, established in 1994 by the INRA in southern France (Court-Picon *et al.,* 2004), sharing 15 accessions with the Spanish trial analysed here, the tree development was also strongly influenced by soil heterogeneity. In this case, no significant differences were found in tree height at the age of 6 years between provenances, appreciating only a certain gradient between inland/upland and coastal provenances, the former growing somewhat more vigorously, with earlier budburst and longer terminal shoots (Court-Picon *et al.,* 2004). Also in several grafted stone pine orchards, the effect of microsite variation was consistently (2 to 5-fold) higher than the differences between clones, both

on tree growth and on reproductive effort, the reproductive allometry being stable among genotypes and the influence of other factors like the rootstock only minor (Mutke *et al.,* 2007).

Spatial autocorrelations have been observed in ecological and forest trials of many species and have been the object of growing attention in their statistical analyses (Costa-Silva *et al.,* 2001; Dutkowski *et al.,* 2002; Hamann *et al.,* 2002; Zhang and Gove, 2005; Dutkowski *et al.,* 2006; Gezan *et al.,* 2006; Zas, 2006, 2008). But the exceptionally high degree of microsite influence on Mediterranean stone pine growth must be stressed. In the case of tree height, microsite accounted for as much as 67-80% of the total variation at all four test sites, compared to a residual variation of only 11-25% between trees, comprising any genetic differences within provenances as well as random effects or measurement errors. In other, genetically less homogeneous species, the differentiated response of each genotype (among and within accessions) to microenvironment would probably not allow such a smooth estimation of a common local mean. Even in later stages of the same trial, negative autocorrelations due to competition probably will counteract and mask the common microsite effect (Fox *et al.,* 2008).

All the evidence appears to suggest that this observed common reaction norm on (micro-)site conditions can be put down to a mere strong sensibility of the stone pine to soil properties, rather than an adaptative capability (with positive ecological-evolutionary connotations). Apparently, this species suffers from root constraints in compact soils, though ongoing soil analyses will also unveil possible correlations with soil chemistry or mycorrhizal flora. To our knowledge, similar micro-spatial effects on tree growth are only well known in clonal plantation forestry, *e.g.* reduced height growth or the risk of chlorosis in hybrid poplars on alkaline sites (St. John, 2001). In view of the postulated severe and prolonged Quaternary bottleneck that might account for the lack of genetic variation in Mediterranean stone pine (Vendramin *et al.,* 2008), this sensitivity might be a collateral effect of the hypothesised genetic depletion.

The observed differences between distant, isolated provenances around the Mediterranean were, in any case, scarce compared to this strong, common reaction norm. Provenances did not group phylogeographically along the east-western major axis of the species range, but ecologically along a latitudinal/altitudinal gradient, similar to findings in *P. canariensis* (López *et al.,* 2007) or *P. contorta* (Chuine *et al.,* 2006). There is also a certain parallelism to the effects of epigenetic memory of cold and warm embryo formation described in other conifers, which are interpreted as a mechanism of adaptative phenotypic plasticity that improves the fitness of the same genotype in different environments (Besnard *et al.,* 2008; Kvaalen and Johnsen, 2008; Søgaard *et al.,* 2008). Against this background one might wonder whether, and to what degree, those moderate differences found between the studied stone pine provenances, distinguishable only after major spatial adjustments (that reflect a common, very stable microsite response of each and every genotype), might also be due to an epigenetic acclimatisation (Vendramin *et al.,* 2008).

Concluding, differentiations in adaptative traits regarding survival, phenology, growth and ontogeny were found to be minor or even absent between the studied provenances that span the entire range of the species, findings concording with the results in molecular genetics. The most relevant aspects of adaptative phenotypic plasticity during the establishment stage of the Mediterranean stone pine can be resumed in: (a) the vegetative phase change, canalised in most Eurasian pine species in the first or second year after germination, can be delayed in this species for several or many years in order to survive in limiting environments, until a sufficient size is achieved, (b) a pronounced sensitivity to soil properties that hampers its growth and development. Whereas the latter appears to be a merely passive response, related putatively to the genetic depletion during the Quaternary bottleneck, the flexibility of life cycle and structural acclimatisation offered by the former mechanism gives an adaptative advantage in the environmental unpredictability of Mediterranean ecosystems.

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