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SHORT COMMUNICATION

# Clonal variation for shoot ontogenetic heteroblasty in maritime pine (*Pinus pinaster* Ait.)

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**Abstract** Pine seedling shoots undergo sharp heteroblastic changes during the early ontogenetic stages. The rate of these changes has been seen to vary between species and provenances within species, but there is a marked lack of information about its genetic control at the lower hierarchical levels. We used clonal replicates of maritime pine to determine broad-sense heritability of shoot ontogenetic heteroblasty and its correlation to rooting ability. We applied a simple ontogenetic index based on the proportion of basal nodes with secondary needles in rooted cuttings of 15 clones from 9 environmentally contrasting origins. We found a high clonal heritability for shoot ontogenetic index and a moderately high heritability for rooting ability, but both genetic and phenotypic correlations between these two traits were weak and non-significant. These results indicate that both developmental phenomena are genetically controlled, but not strictly associated in this species.

**Keywords** Development · Genetic correlation · Heritability · Rooting ability · Vegetative phase change · Vegetative propagation

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## Introduction

Pine seedlings experience an abrupt developmental transition during the first few years after germination (Cannell et al. 1976; Climent et al. 2011). This ontogenetic change starts with the formation of the first secondary needles in dwarf shoots at the axils of distal primary needles and ends with the formation of the first terminal bud containing the preformed nodes of the subsequent flush. This type of ontogenetic change is generally called vegetative phase change or heteroblastic phase change to distinguish it from the more widely known reproductive phase change (Jones 1999; Zotz et al. 2011).

The dissimilarity between ontogenetic age and chronological age in plants has been largely discussed (Poethig 2003). Since the increase of complexity linked to the unfolding of the ontogenetic programme is normally accompanied by an increase of plant size, plant height and/or biomass have been traditionally used as surrogates of the ontogenetic status (McConnaughay and Coleman 1999). Sounder proxies of ontogenetic age were attained through the plastochron index, which assumes an exponential relationship with the formation of each stem unit (Erickson and Michelini 1957) or more simply, by using node counting (Sachs 1999).

The functional and adaptive implications of the ontogenetic heteroblastic change in pines are multiple: secondary needles tend to show higher mass per unit area and lower cuticular transpiration (Pardos et al. 2009) and higher tolerance to frosts than primary needles of the same seedling (Climent et al. 2009a). Although not directly demonstrated experimentally, a greater photosynthetic efficiency of juvenile needles has been postulated based on their anatomical differences (Peters et al. 2003). Moreover, a neat reduction of seedling field mortality coinciding with

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the completion of heteroblastic change has been observed in both *Pinus canariensis* and *P. halepensis*, further supporting the adaptive (although complex) relevance of this phenomenon (Climent et al. 2006; Nieto et al. 2009). In other species and genera, biotic interactions such as herbivory have been shown to be significantly affected by plant ontogenetic heteroblasty (Boege and Marquis 2005; Karban and Thaler 1999; Donaldson et al. 2006).

The ability to produce adventitious roots in excised cuttings is another key change associated with the maturity of meristems (Greenwood 1995), but it has been scarcely compared to the morphology of shoot vegetative phase change up till know; therefore, the phenotypic and genetic correlation between shoot heteroblasty and rooting ability is poorly known.

Over recent years, significant progress has been reached in understanding the molecular control of development in model plants (Benková et al. 2009; Poethig 2003), but there is still a notable lack of information on the quantitative genetics of ontogeny in many highly relevant taxa, particularly in tree species.

Previous research has shown that heteroblastic change in maritime pine occurs at an intermediate rate between the highly delayed, thermophylic Mediterranean pines (*P. canariensis*, *P. halepensis* and *P. pinea*) and the much faster Eurasian mountain pines (*P. nigra*, *P. sylvestris* and *P. uncinata*) (Climent et al. 2011). At the intra-specific level, the vegetative phase change in *P. pinaster* was found to be highly variable both between populations and half-sib families (author's unpublished data), but there is still lack of information about the genetic control of heteroblasty at the clonal level. Moreover, fast and repeatable phenotyping methods for key developmental traits are an important bottleneck for the progress of genetic association studies of forest trees.

Our objectives were, firstly, to check the hypothesis of a high genetic control of shoot ontogenetic heteroblasty at the clone level and, secondly, to determine the phenotypic and genetic correlation between shoot ontogeny and rooting ability.

## Materials and methods

### Plant material

For this experiment we used 15 clones of the clonal collection CLONAPIN (Majada et al. 2009) propagated by cuttings from progenies tested in field trials, belonging to nine provenances of highly contrasting conditions of origin, from Atlantic to Continental and Mediterranean (Table 1). Plants were rooted cuttings of 17 months of age, planted in 2-l pots filled with a mixture of Sphagnum peat (PINSTRUD®) and vermiculite (VERLITE®) of grade 3, with a proportion 4:1 in volume. The entire experiment was performed at SERIDA's greenhouse at Villaviciosa (Asturias, Spain). Potted plants were ferti-irrigated following the protocol by Majada et al. (2009). Prior to the assessment, all branches were pruned to trigger the formation of new axillary shoots, mostly deriving from the activation of the proventitious meristems in the extant dwarf shoots (Fig. 1). This was intended to be a "resetting" of the plants shoots, minimising the differences due to previous pruning done for cutting collection.

### Measured traits

We looked for an ontogenetic index (OS) that reflected the inherent rate of development of each genotype, rather than the ontogenetic age at a given moment. Also, this index was intended to be little affected by phenology (hence, independent of the precise moment of assessment) or by plant and shoot vigour. Former experiments in different hard pine species confirmed that the proportion of nodes that generate secondary needles in axillary dwarf shoots was a good proxy for ontogenetic age, but changed between harvests as plants grew (Climent et al. 2011). This can be of minor importance when comparisons are done between species, but can easily mask the smaller differences between genotypes within a species. Since new stem units are produced continuously in the apex during free

**Table 1** Description of sampled clone geographical origins

| Acronym | Provenance name | Province/region | Country | Latitude | Longitude |
|---|---|---|---|---|---|
| ASPE | Arenas de San Pedro | Ávila | Spain | 40°11′41″N | 5°06′58″W |
| ARMY | Armayán | Asturias | Spain | 43°18′17″N | 6°27′30″W |
| CDVO | Cadavedo | Asturias | Spain | 43°32′24″N | 6°25′04″W |
| COCA | Coca | Segovia | Spain | 41°15′17″N | 4°29′52″W |
| MIMI | Mimizan | Landes | France | 44°08′03″N | 1°18′11″W |
| ORIA | Oria | Almería | Spain | 37°31′52″N | 2°21′04″W |
| PTOV | Puerto de vega | Asturias | Spain | 43°32′53″N | 6°37′53″W |
| SCRI | San Cipriano de Ribarteme | Pontevedra | Spain | 42°07′06″N | 8°21′52″W |
| TAMR | Tamrabta | Mid Atlas | Morocco | 33°36′00″N | 5°01′00″W |

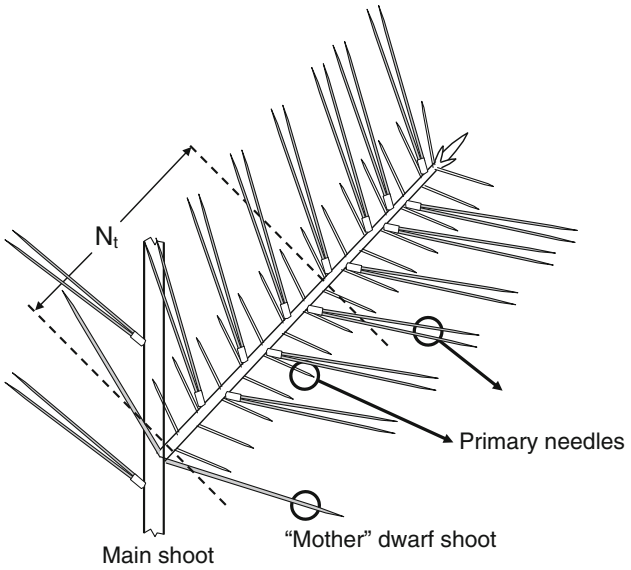


**Fig. 1** Description of node assessment for the calculation of the ontogenetic index OS, based on the proportion of basal nodes with secondary needles in axillary dwarf shoots. $N_t$ was the total number of nodes observed, 15 nodes in this case

shoot growth, an ontogenetic index unaffected by plant growth must be based on the proportion of adult structures (secondary needles) within a fixed number of nodes at the basal portion of the plant (Fig. 1). Besides, it is desirable that the index is a quantitative continuous variable with the minimum experimental error, that is, unaffected by the observer.

Considering this, we used a simple ontogenetic index calculated with the formula

$$OS = (N_{ds}/N_t)^{1/2} \quad (1)$$

where $N_t$ is the total number of basal nodes observed, and $N_{ds}$ the number of nodes with the presence of axillary dwarf shoots at any developmental stage (Figs. 1, 2). In our experiment $N_t$ was fixed to 15, enough to gather the observed variation, but this figure could be changed to adjust to the type of plant material in each experiment: higher in slow-grown or ontogenetically delayed plants (like seedlings) and vice versa. The exponent was chosen to normalise the distribution; in our experiment a 0.5 exponent (square-root transformation) performed well. We also measured the basal diameter of each shoot ($D_s$) and the length of the 15 nodes ($L_{15}$) as proxies of shoot vigour and phenology. We measured three shoots of three ramets for each of the 15 clones assessed, therefore totalling 135 shoots. Though the assessment was quite straightforward and repeatable, all observations were done by the same person.

## Rooting experiment

Following previous experience, axillary shoots with an approximate length of 5 cm were collected from the plants assessed and submitted to a prophylactic process, followed by a single application of indole butyric acid at a concentration of 4 % in talcum powder, as described in Majada et al. (2011). Three mini-cuttings per ramet, hence nine cuttings per clone, were placed in 60 cc polyethylene pots


**Fig. 2** Different developmental stages of secondary needles in axillary dwarf shoots in different clones: **a** dwarf shoot buds still covered by cataphylls; **b** secondary needles starting elongation and **c** secondary needles with about a half of total elongation. Shoot **b** exhibits a low OS value (low proportion of nodes with secondary needles; Fig. 1)

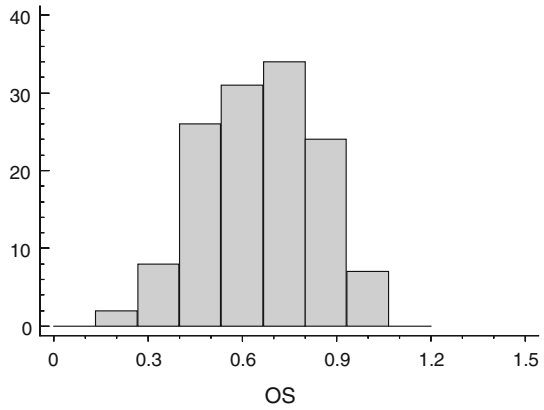


**Fig. 3** Frequency distribution for observed ontogenetic index (OS) values

(Cetap's A77) with rooting media of peat:perlite (1:1, v:v). Cuttings were arranged in randomised complete blocks and kept in the greenhouse for 4 months prior to data recording. The temperature was kept at 25 ± 2 °C with a relative humidity of 90 % and luminosity of 450 $Wm^{-2}$. These parameters were maintained by a computerised system controlled by cooling and fog systems and shade nets.

### Data analysis

A general linear model was obtained with the formula

$$Y_{ijk} = \mu + C_i + R_j(C_i) + Z_k + \varepsilon_{ijk} \quad (2)$$

where $\mu$ is the general mean, $C_i$ the effect due to clone, and $R_j(C_i)$ the effect of ramet nested within each clone. $Z_k$ is a covariate to correct for differences in shoot vigour and/or phenology ($D_s$ or $L_{15}$) and $\varepsilon$ is the error term. Clone and ramet were considered as random factors. Rooting ability RR was evaluated through the function arcsine of square root of the ratio between living and planted cuttings.

With the variance components obtained for random factors, we calculated the broad-sense heritabilities for ramets $\left(H_i^2\right)$ and clone means $\left(H_C^2\right)$ with the formulas (Mullin et al. 1992):

$$H_i^2 = \sigma_C^2/\left(\sigma_C^2 + \sigma_r^2 + \sigma_e^2\right) \quad (3)$$

$$H_C^2 = \sigma_C^2/\left[\sigma_C^2 + \sigma_r^2/r + \left(\sigma_e^2/rb\right)\right]. \quad (4)$$

Genetic (clonal) correlations between OS (mean values per ramet) and rooting ability were assessed with the following formulas (Falconer and Mackay 1996):

$$r_{g(XY)} = \mathrm{Cov}_{C(XY)}/\left(\sigma_{C(X)}^2\sigma_{C(Y)}^2\right)^{0.5} \quad (5)$$

with $\mathrm{Cov}_{C(XY)}$ the covariance between traits $X$ and $Y$, and $\sigma_{C(X)}^2$ and $\sigma_{C(Y)}^2$ the clonal variances of these same traits. $\mathrm{Cov}_{C(XY)}$ was calculated from the variance components obtained for the sum of variables $X$ and $Y$ using the equation (Rice 1988):

$$\mathrm{Cov}_{C(XY)} = \left[\sigma_{C(X+Y)}^2 - \left(\sigma_{C(X)}^2 + \sigma_{C(Y)}^2\right)\right]/2 \quad (6)$$

The standard error of the heritability was calculated following the classical approach from Osborne and Paterson and standard errors of genetic and phenotypic correlations were calculated following the Tallis general approach (Visscher 1998).

We further calculated the best linear unbiased predictors (BLUPs) for OS per clone.

## Results

The ontogenetic index averaged 0.65, with a minimum of 0.25 and maximum of 1.0. The distribution was close to normal, with a variance of 0.028 and a coefficient of variation of 25.7 % (Fig. 3). Rooting rate ranged from 0 to 1, with an average of 0.30, variance 0.092 and coefficient of variation as wide as 98 %.

### Genetic variation and heritability

The clonal effect on OS was highly significant ($P < 0.000001$), while ramet within clone was not significant ($P > 0.1$) when the length of 15 nodes ($L_{15}$) was included as a covariate with highly significant ($P < 0.0005$) effect (Table 2). By contrast, shoot basal diameter was not significant ($P > 0.1$) as a covariate for OS and thus it was excluded from further analyses. The complementary ANOVAs for $L_{15}$ and shoot basal diameter showed highly significant effects of both clone and ramet within clone (Table 2).

Consistently with the former results, broad-sense heritability for OS increased when $L_{15}$ was included as a covariate, since this variable absorbed a great part of error variance compared to clone variance component (Table 3). Among the variables analysed, OS showed the highest heritabilities both at the individual and clonal level. Clone estimates (BLUPs) for OS corrected by $L_{15}$ ranged from 0.25 up to 0.50 (Fig. 4). Plant basal diameter ($D_p$) and rooting rate (RR) also displayed moderate or high heritability at both levels (Table 3).

### Phenotypic and genetic correlations between traits

Both phenotypic and genetic correlations of OS with the other traits considered were not significantly different from zero ($P > 0.1$, Table 4). Nevertheless, the sign of the correlations between OS and RR (rooting) was negative, while the correlation between OS and $L_{15}$ was positive.

**Table 2** Resume of general linear models for analysis of variance applied for ontogenetic index (OS), length of basal 15 nodes ($L_{15}$) and shoot basal diameter ($D_s$)

| Variable | Source | SS | df | MS | $F$ | $P$ |
|---|---|---|---|---|---|---|
| OS | Clone | 1.99252 | 14 | 0.14232 | 5.6913 | 0.00000 |
| | Ramet (clone) | 0.87287 | 30 | 0.02910 | 1.1635 | 0.28880 |
| | Covariate $L_{15}$ | 0.38447 | 1 | 0.38447 | 15.374 | 0.00018 |
| | Error | 2.15061 | 86 | 0.02501 | | |
| | Total | 5.40046 | 131 | | | |
| $L_{15}$ | Clone | 8,055.9 | 14 | 575.4 | 5.522 | 0.00000 |
| | Ramet (clone) | 7,110.3 | 30 | 237 | 2.275 | 0.00165 |
| | Error | 9,065 | 87 | 104.2 | | |
| | Total | 24,200.6 | 131 | | | |
| $D_s$ | Clone | 9.062 | 14 | 0.647 | 2.225 | 0.01262 |
| | Ramet (clone) | 17.132 | 30 | 0.571 | 1.963 | 0.00817 |
| | Error | 25.306 | 87 | 0.291 | | |
| | Total | 51.575 | 131 | | | |

**Table 3** Variance components for clone ($\sigma^2_C$), ramet ($\sigma^2_r$) within clone and error ($\sigma^2_e$) for the ontogenetic index (OS), length of basal 15 nodes ($L_{15}$), shoot basal diameter ($D_s$), plant basal diameter ($D_p$) and rooting ability of cuttings (RR)

| Variables | $\sigma^2_C$ | $\sigma^2_r$ | $\sigma^2_e$ | $H^2_i$ | $H^2_C$ |
|---|---|---|---|---|---|
| OS | 0.01029 | 0.00301 | 0.01629 | 0.35 (0.12) | 0.79 (0.10) |
| OS* | 0.00827 | 0.00080 | 0.01357 | 0.37 (0.12) | 0.82 (0.08) |
| $L_{15}$ | 38.28 | 45.37 | 103.86 | 0.20 (0.12) | 0.59 (0.19) |
| $D_s$ | 0.00880 | 0.09734 | 0.28892 | 0.02 (0.08) | 0.12 (0.41) |
| $D_p$ | 0.39691 | – | 0.51214 | 0.44 (0.16) | 0.70 (0.14) |
| RR | 0.92778 | – | 1.36099 | 0.37 (0.17) | 0.63 (0.17) |

Heritabilities at the individual ($H^2_i$) and clonal level ($H^2_C$) and their standard errors in brackets. OS* corresponds to the model with $L_{15}$ as a covariate

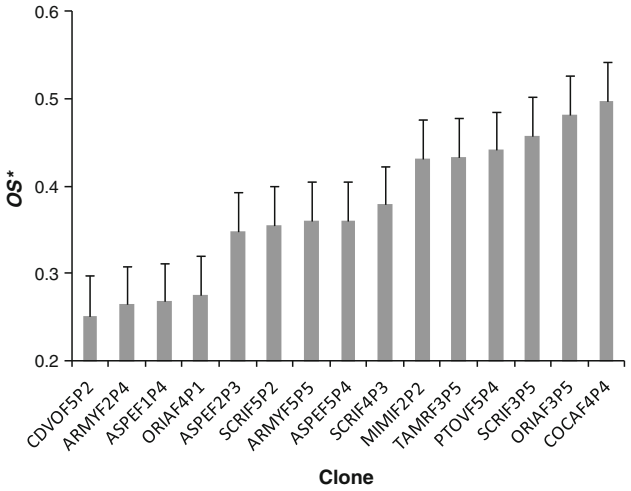


**Fig. 4** BLUP values obtained from mixed models and standard errors for ontogenetic index OS* (adjusted for covariate $L_{15}$) for each of the 15 clones evaluated

## Discussion

This experiment is one of the very few providing experimental evidence of clonal variation of shoot ontogenetic heteroblasty in a tree species. This evidence of total genetic variation sums up to a high additive variation found between half-sib progenies in field trials of maritime pine (author's unpublished data). High narrow-sense heritability was also found in field trials of *Eucalyptus globulus* (Jordan et al. 1999).

The ontogenetic index used in our analyses was based on adult node counting within a fixed basal node number. Node counting has been recognised as a simple and accurate measure of developmental status, closely related to the real ontogenetic age of the plant (Sachs 1999) and highly coherent with the modular nature of plant development (de Kroon et al. 2005). Previously published ontogenetic indexes were based on a destructive observation of the ratios of secondary needles to total needles, either in leaf number or biomass terms (Climent et al. 2009b; Lascoux et al. 1993). Since those measures were not based on a fixed node number, the ratios changed during seedling development, which means a lower discriminating power between genotypes.

Our experiment has also shown moderately high broad-sense heritability for rooting ability in maritime pine, in parallel with results in other species. For example, broad-sense

**Table 4** Genetic and phenotypic correlations between ontogenetic index (OS) and basal 15 nodes ($L_{15}$), shoot basal diameter ($D_s$), plant basal diameter ($D_p$) and rooting ability of cuttings (RR)

| | $L_{15}$ | $D_s$ | $D_p$ | RR |
|---|---|---|---|---|
| OS-gen. | 0.414 (0.235) | −0.612 (0.204) | 0.714 (0.182) | −0.341 (0.242) |
| OS-phen. | 0.55 (0.124) | 0.00 | 0.33 (0.142) | −0.155 (0.147) |

clonal heritability was 0.55 in lodgepole pine (Fries and Kaya 1997). In *Eucalyptus*, despite a relatively large within-family variation, the best rooters belonged predominantly to a single family (Ruaud et al. 1999). By contrast, the lack of significant correlation between ontogenetic heteroblasty and rooting ability did not confirm a close link between these two traits as it could be assumed by the literature as part of the broad concept of plant maturation (Greenwood 1995; Zhang et al. 2003).

Shoots developed from proliferated dwarf shoots, like those used in our study, have been successfully used as cuttings for vegetative propagation (Browne et al. 1997), but we have found no reference comparing cutting rooting ability with a morphological index of shoot ontogeny, but rather statements of a general decrease of rooting ability with the age of the donor plant (for example, Hamann 1998). Our results suggest that both maturation processes have considerable genetic variation and a moderately high genetic control, but both are genetically decoupled at the individual and clonal levels, as some authors had already suggested (Jones 1999). Other key maturation process such as reproductive ability seems to be largely decoupled from vegetative phase change in woody plants when it has been properly assessed (Jones 1999; Jordan et al. 1999). However, in Eucalyptus some interaction between genetic and morphological indexes of maturation was observed (Ruaud et al. 1999), and the small size of our experiment obliges us to take the lack of correlation with caution.

The functional and practical implications of selecting clones with fast or slow shoot ontogeny clearly needs further research, but an accurate index is a good start. Interspecific comparisons of the behaviour of primary and secondary needles in hard pines suggest that a fast formation of secondary needles can be linked to higher frost tolerance (Climent et al. 2009a). By contrast, the relationship with drought tolerance is not straightforward, since juvenile needles are more sensitive to water loss through cuticular transpiration (Pardos et al. 2009), but the production of resistant secondary needles means less resources allocated to roots or stems that are also critical for drought avoidance (Aranda et al. 2010; Poorter and Nagel 2000). Moreover, the ontogenetic index used in this experiment reflects one of the important changes in pine seedling ontogeny—the formation of secondary needles in the shoot—but there is another crucial change: the first bud set and the subsequent onset of preformed growth. Both phenomena are only partially correlated between pine species (Climent et al. 2011). The genetic and phenotypic correlations between different ontogenetic changes occurring during the early stages in pines should be investigated to progress in our understanding of their life histories, with deep implications for the sustainable management of their genetic resources. Given the small size of our experiment, new lager clonal experiments will be assessed in the future to confirm the heritability values we provided.

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