

# Ontogenetic differentiation between Mediterranean and Eurasian pines (sect. *Pinus*) at the seedling stage

José Climent · Roberto San-Martín ·  
Maria Regina Chambel · Sven Mutke

Received: 31 May 2010 / Revised: 7 September 2010 / Accepted: 10 September 2010  
© Springer-Verlag 2010

**Abstract** Heteroblastic development in pine seedlings includes extreme morphological changes with still unclear adaptive and evolutionary significance. In particular, Mediterranean and Eurasian pines (section *Pinus*) living in the Mediterranean basin seem to follow quite distinct developmental trajectories at the seedling stage. Aiming to confirm this ontogenetic differentiation we performed a nursery experiment with seedlings of five Mediterranean pines (*Pinus pinaster*, *P. brutia*, *P. halepensis*, *P. pinea* and *P. canariensis*) and three Eurasian pines (*P. sylvestris*, *P. uncinata*, and *P. nigra* subsp. *salzmannii*), also including *P. radiata* as an outgroup. After destructive analyses at two harvest times (9 and 32 weeks), we found sharp differentiation between Mediterranean and Eurasian pines in a combination of traits linked to shoot heteroblasty. In particular, Mediterranean pines showed a marked delay in the proportion of adult needles to total needles in the shoot compared to Eurasian species, especially at the second harvest. However, two Mediterranean pines, *P. pinaster* and *P. brutia* showed a slightly higher proportion of secondary needles, and a higher rate of budset at a more

advanced stage (68 weeks) compared to the other three Mediterranean species. Meaningfully, the outgroup *P. radiata* was the only species combining a high proportion of adult foliage since the first harvest with a delayed formation of the first terminal bud. We discussed the adaptive significance of these findings at the light of species' climatic niches and life histories.

**Keywords** Allometry · Hard pines · Heteroblasty · Heterophylly · Vegetative phase change

## Introduction

The term heteroblasty in plants refers to the sequence of changes in leaf characteristics, internode dimensions and fate of axillary buds along the shoot during ontogeny (Jones 1999). These changes include subtle, continuous changes of leaf morphology in most species (Winn 1999) as well as extreme or sharp changes in leaf characteristics [like in Eucalypts (Pryor 1976) or Acacia (Leroy and Heuret 2008)] or differences in the entire growth habit of the plant [such as in New Zealand's woody shrubs (Day 1998)]. This variety and complexity in related phenomena could explain the contradictory adaptive interpretation of heteroblastic ontogenetic changes among families, genera or species (Jones 1999).

Heterochrony, the heritable modification of ontogeny, is recognized as one of the major evolutionary processes in both plants and animals (McKinney and McNamara 1991) but ontogeny in the wide sense is of general concern in plant research as an important source of phenotypic variation (Wright and McConnaughay 2002). The fact that ontogenetic age can be different from chronological age in plants has long been recognized (Greenwood 1995; Poethig 2003). The Plastochron Index (PI) developed by (Erickson

---

Communicated by T. Grams.

---

J. Climent (✉) · M. R. Chambel · S. Mutke  
CIFOR, Instituto Nacional de Investigación y Tecnología  
Agraria y Alimentaria (INIA), Apto. 8111, 28080 Madrid, Spain  
e-mail: climent@inia.es

R. San-Martín  
Escuela Técnica Superior de Ingenierías Agrarias,  
Universidad de Valladolid, Avda. de Madrid, 57,  
34004 Palencia, Spain

J. Climent · R. San-Martín · M. R. Chambel · S. Mutke  
Sustainable Forest Management Research Institute (UVA-INIA)  
URL: <http://sostenible.palencia.uva.es/>

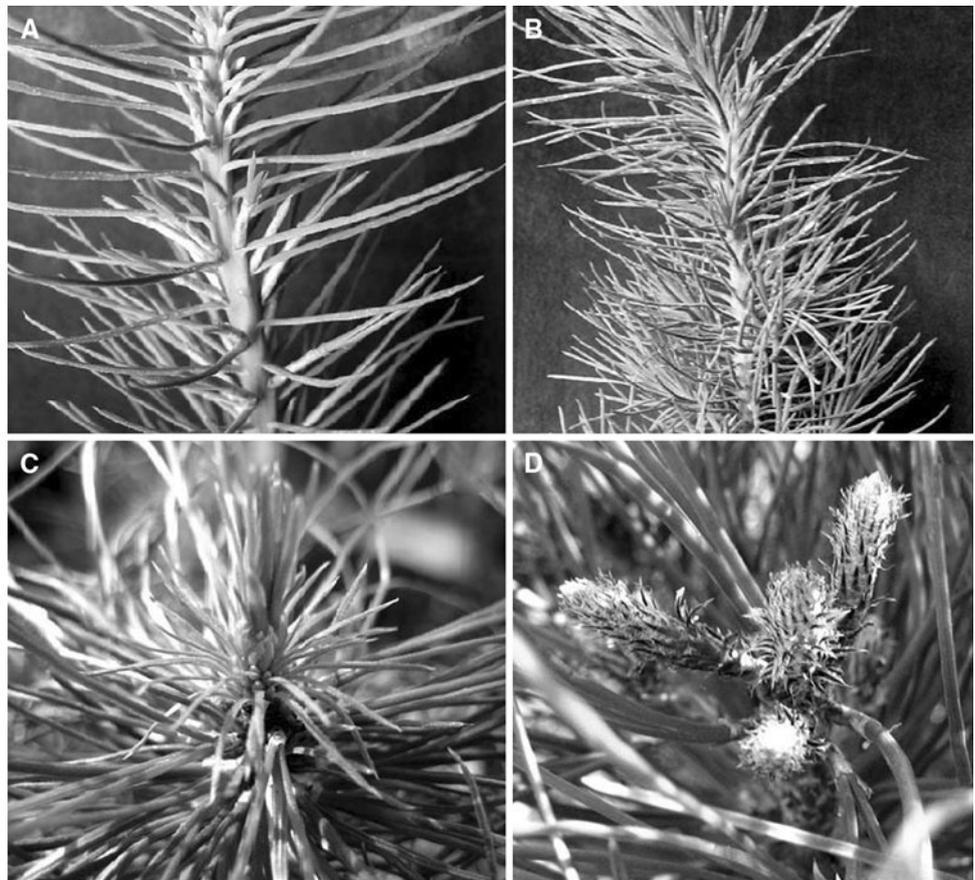
and Michelini 1957) offers a direct estimation of ontogenetic age in plants, and it has been widely used in Angiosperm studies (Ade-Ademilua and Botha 2005; Mundermann et al. 2005; Urban et al. 2008). This Index uses the number of plastochrons, i.e. the time needed to produce a node identical in size to the preceding one as a quantitative estimator of ontogenetic age. However, node counting is a simpler approach to ontogenetic age and it has proven to be closely related to ontogeny in plants (Renton et al. 2006; Sachs 1999). Destructive counting of nodes (or stem units) produced at a given age has been used in only a few studies for conifer seedlings, due to the difficulties imposed by very short internodes and numerous nodes even in young plants (Grotkopp et al. 2002; Lascoux et al. 1993). Moreover, when plant development includes distinct heteroblastic changes, a categorical or quantitative evaluation of such phenomena or the size of the plant at a given heteroblastic change can provide reasonable ontogenetic discrimination among genotypes, as demonstrated in *Eucalyptus* (Jordan et al. 2000) and in *Pinus* (Climent et al. 2006).

In pines, the patterns of axillary shoot formation and shoot elongation, both in seedlings and adult trees have been well documented (Doak 1935; Lanner 1976; Lester 1968). The cotyledonary stage is followed by the emergence of the

epicotyl rosette, which later elongates, exhibiting exclusively primary needles. Axillary shoots consisting either of similar primary needles (juvenile long shoots) or secondary needles (dwarf shoots) appear subsequently, normally the first at basal nodes and the second at more distal nodes (Lester 1968). The proportion of both types of axillary shoots seems rather characteristic of each species (op. cit.), and its relationship to ontogeny is probably dissimilar (Bormann 1955; Lascoux et al. 1993). This heteroblastic change culminates with the formation of the first true terminal bud, which further on will generate shoots with solely secondary needles in the axils of bud scales (Fig. 1).

The “Old World” hard pines (subg. *Pinus*, section *Pinus*), comprising Mediterranean, Eurasian and two American species, form an interesting group to investigate how heteroblasty integrates with other phenotypical traits of the plant, as a first step to determine the adaptive and evolutionary significance of ontogeny in the genus. The retention of juvenile characters is typical of some Mediterranean species, as already noted by Klaus (1989), but there is no further information on the consistency of this phenomenon within this group [subsect. *Pinaster* of Germandt et al. (2005)]. Neither is it clear the degree to which Eurasian pines (subsect. *Pinus*, op. cit.) differ from Mediterranean pines in ontogenetic trajectory. Previous research

**Fig. 1** Different ontogenetic changes in hard pines: **a** first appearance of secondary needles in dwarf shoots in *Pinus pinaster* **b** profuse axillary long (juvenile) shoots in *P. halepensis* **c** juvenile apical rosette during winter in *P. radiata* **d** first true winter buds in *P. brutia*



has shown species-specific differences in cuticular transpiration and frost tolerance between primary and secondary needles of this group of species, suggesting an adaptive role of the heterophylly associated to the heteroblastic change (Climent et al. 2009; Pardos et al. 2009)

Our main objective was to test the hypothesis that Mediterranean and Eurasian hard pines form two distinct groups based on their ontogenetic heteroblasty. As a secondary objective we wanted to unveil how different developmental features linked to seedling ontogeny integrate with plant allometry. We used seedlings cultivated in a nursery experiment to capture the most relevant developmental changes from seed germination up to bud set.

## Materials and methods

### Plant material and experiment design

We chose five species as representative of Mediterranean pines: *Pinus pinaster* Ait. (Maritime pine), *P. brutia* Ten. (Turkish red pine), *P. halepensis* Mill. (Aleppo pine), *P. pinea* L. (Umbrella pine) and *P. canariensis* C. Sm. (Canary Islands pine), and three species as representative of Eurasian pines: *P. sylvestris* L. (Scots pine), *P. uncinata* Ram. ex DC. (Pyrenean black pine), and *P. nigra* subsp. *salzmannii* (Dunal) Franco (Salzmann's black pine). An extensive description of these species' ecology and distribution maps can be found in Euforgen's web site (<http://www.euforgen.org>). In addition, we included *P. radiata* D. Don (Monterey pine), now widely cultivated in mild and humid climate areas worldwide as an outgroup, representative of another Mediterranean Climate Zone (Coastal SW California), while being phylogenetically distant [classified in subsection *Australes* of section *Trifoliae* (Gernandt et al. 2005)] from genuine Mediterranean pines.

Seeds were obtained in natural stands (except for *P. radiata*, from naturalized stands in north eastern Spain) from open-pollinated trees, each separated at least 100 m in order to reduce consanguinity. All nine species were represented by a single provenance corresponding to its central ecological range (Table 1). Seed lots were an equilibrated mix from 25 to 30 mother trees of the same provenance. Previous to germination, seeds were stratified at 4°C for a month to homogenize the speed of germination. In June 2005 seeds were sown in commercial 7 l containers with a mixture of fertilized peat and perlite (3:1 v:v). Prior to sowing, growing media had been sterilized in an autoclave to avoid the presence of specific mycorrhizal fungi that could bias the response of some tested species (Grotkopp et al. 2002). Seedlings were grown in a greenhouse at North-West Spain (42°16'38"N, 7°37'32"W, 537 m a.s.l.) under natural photoperiod and carried outdoors in February before the second vegetative period. Containers were embedded in perlite to avoid excessive temperature oscillation of the rooting media. A long cultivation schedule (68 weeks) was necessary to allow all nine species to produce secondary needles and, therefore starting heteroblastic change.

Destructive sampling of 15 plants per species was done at two harvest dates, at 9 weeks (October) and 32 weeks (March) after their emergence. Roots, stems and leaves of each plant were separated; the latter sub-divided into juvenile leaves of the main shoot, juvenile leaves of axillary long shoots and secondary needles of axillary dwarfshoots (including sheath cataphylls). Each axillary shoot, both juvenile long shoots and adult dwarf shoots was counted in every plant. All plant parts were oven-dried for 48 h at 80°C before weighting. The dry weight of all components was obtained as well as their sum, i.e. the total dry weight (TDW) of each plant. With this data, the biomass fractions due to needles (LMF), stems (SMF) and roots (RMF), as well as the shoot/root ratio (S/R) were

**Table 1** Location and ecological data and of the provenances representative of each species: mean annual temperature (MAT), mean temperature of coldest month (TCM), annual rainfall (MAP)

Species	Provenance	Latitude	Longitude	Altitude	MAT (°C)	TCM (°C)	MAP (mm)	DP (month)
<i>P. brutia</i>	Taurus (Bucak)	37°29'N	30°37'E	980	13.5	3.9	600	3.0
<i>P. canariensis</i>	Tenerife	28°19'N	16°44'W	1,350	14.8	10.3	691	5.1
<i>P. halepensis</i>	Inland Eastern Spain	39°35'N	0°58'W	830	13.7	5.5	503	3.2
<i>P. pinea</i>	La Mancha	39°22'N	2°38'W	690	14.3	6.3	594	4.9
<i>P. pinaster</i>	Guadarrama Mnts.	40°34'N	4°19'W	1,230	12.7	3.2	620	2.6
<i>P. nigra</i>	Southern Iberian Range	40°43'N	2°15'W	1,280	10.3	2.2	840	1.7
<i>P. sylvestris</i>	Guadarrama Mnts.	40°51'N	4°00'W	1,520	11.0	2.0	920	0.8
<i>P. uncinata</i>	Eastern Pyrenees	42°23'N	0°32'W	1,760	5.5	0.0	1,175	0.0
<i>P. radiata</i>	Vasque Coast	43°15'N	2°05'W	220	13.0	7.5	1,390	0.0

and length of drought period (DP) corresponding to the geographical centre of the provenance

calculated (Table 2). In addition, secondary needle mass fraction (SNMF) was obtained as the dry weight ratio of dwarf shoots to total leaf biomass, and long shoot mass fraction (LSMF) as the dry weight ratio of long shoots to total aerial biomass (Table 2).

Given that at this developmental stage, every node bears at least a primary leaf, we determined the number of nodes or stem units (NSU) by counting the juvenile needles of the main stem (Lascoux et al. 1993). Then, the ratio of nodes producing axillary juvenile long shoots (LSR) or adult dwarf shoots was calculated (DSR) (Table 2).

Primary and secondary needles have significantly different specific leaf area (Climent et al. 2009; Pardos et al. 2009) thus the relative amount of each foliage type associated to the particular stage of development at the time of harvest affected leaf area traits in a very complex way. Therefore, we intentionally discarded leaf area-related traits for the purposes of this experiment.

A second set of data consisted of non-destructive measures performed 68 weeks after germination in five randomly selected plants per species. At this moment (autumn of second year, 2006), when stem elongation of the second year had already finished, height (Ht), basal diameter (Db), number of branches (Bn, at this stage mostly deriving from former juvenile long shoots), height of the most basal dwarf-shoot at the main stem (DSh), and developmental status of the terminal bud (BS) were recorded. In

accordance with earlier works by Lascoux et al. (1993), BS was assigned to the categories 0, if the bud consisted exclusively of a rosette of juvenile needles, 1 if it consisted of a bud-like structure with slightly modified juvenile needles or 2, if a true terminal bud with scales was present.

With these raw data, another two derived variables were calculated, the estimated main stem volume ( $V_e$ ) assuming the stem to be conical, and the height proportion of the main shoot with dwarf-shoots, DS<sub>p</sub> (Table 2).

#### Data analysis

##### *Destructive analysis (Dataset 1)*

A principal component analysis (PCA) was performed with the data of the second harvest (32 weeks) on the five variables directly related to ontogenetic heteroblasty: SNMF, LSMF, NSU, DSR and LSR, considering the rest TDW, LMF, RMF, SMF, S/R as complementary variables. The target variables were chosen such that they represented different plant traits, although this does not imply a statistical independence, precisely due to the inherent allometric relationships between plant parts (Niklas 2004).

To check the main hypothesis, that Mediterranean and Eurasian pines formed two significantly different groups, a multivariate analysis of variance (MANOVA) was

**Table 2** Raw and derived variables, acronyms and description

Raw variables	Derived variables
Destructive analysis (Dataset 1)	
LDW, RDW, SDW, <b>TDW</b> : leaf, root, stem and total dry weight (g)	<b>LMF</b> , <b>RMF</b> , <b>SMF</b> : leaf, root, stem mass fractions LDW/TDW; RDW/TDW; SDW/TDW
DSDW: dwarf-shoot dry weight (g)	<b>RGR</b> : relative growth rate (days <sup>-1</sup> ) <b>SNMF</b> : secondary needle mass fraction DSDW/LDW
LSDW: dry weight of axillary long shoots (g)	<b>LSMF</b> : long-shoot mass fraction LSDW/(LDW + SDW) <b>S/R</b> : shoot to root mass fraction (LDW + SDW)/RDW
<b>NSU</b> : number of stem units	<b>DSR</b> : proportion of nodes with dwarf shoots DSc/NSU
DSc: dwarf-shoot count	<b>LSR</b> : proportion of nodes with long shoots LSc/NSUor
LSc: axillary long shoot count	<b>RNIR</b> : relative node increase rate (days <sup>-1</sup> )
Non-destructive measures (Dataset 2)	
<b>BS</b> : budset, developmental bud status (categorical)	<b>Bn/Ht</b> : branchiness, branches per unit height (cm <sup>-1</sup> )
Bn: branch number	<b>DS<sub>p</sub></b> : dwarf shoot height proportion (Ht-DSh)/Ht
DSh: minimum height with dwarf-shoots (mm)	
Ht (height, mm), Db (basal diameter, mm)	<b>V<sub>e</sub></b> : estimated stem volume ( $\Pi/12 \times Ht \times Db^2$ ) (mm <sup>3</sup> )

Acronyms corresponding to traits used for analyses are highlighted in bold. Units are indicated in brackets; otherwise the variable is dimensionless

performed with the coordinates of each individual on the first two principal components, with the model

$$Y_{ijk} = \mu + C_i + S_j(C_i) + \varepsilon_{ij} \quad (1)$$

where  $Y$  was the response variable,  $\mu$  the general mean,  $C$  the additive effect of the phylogenetic class (Eurasian or Mediterranean),  $S(C)$  the additive effect of the species within classes,  $\varepsilon$  was the error term. To check the hypothesis of significant differences between classes, the species within class [ $S(C)$ ] was used as the error term.

Further checking of the soundness of this classification was done through a discriminant analysis with the complementary variables TDW, LMF, RMF, SMF and S/R.

A classical plant growth analysis between the two harvests (9 and 32 weeks) was performed following the hypotheses of Causton (1991). Differences between species for relative growth rate (RGR) were checked using the mean and confidence intervals calculated with the software available from Hunt et al. (2002). Similarly, a relative node increase rate (RNIR) between the two harvest dates ( $t_2$  and  $t_1$ ) was obtained as:

$$\text{RNIR} = (\ln \text{NSU}_2 - \ln \text{NSU}_1) / (t_2 - t_1). \quad (2)$$

To check if the ontogenetic classes derived from the PCA, and species within classes underwent different ontogenetic trajectories of biomass allocation between harvests, we used a mixed model with the equation:

$$Y_{ijk} = \mu + C_i + T_j + S_k(C_i) + C_i \times T_j + T_j \times S_k(C_i) + \varepsilon_{ijk} \quad (3)$$

where  $Y$  was the response variable,  $\mu$  the general mean,  $C$  the additive effect of the phylogenetic class,  $T$  the additive effect of harvest time,  $S(C)$  the additive effect of the species within classes,  $C \times T$  the interaction between class and harvest,  $T \times S(C)$  the interaction between harvests and species within classes and  $\varepsilon$  was the error term. Errors were normally distributed and were independent. To attain model convergence, REML (restricted maximum likelihood) variances were calculated per class. A significant effect of harvest reflects a common ontogenetic trend between 9 and 32 weeks, while a significant  $C \times T$  effect reflects different ontogenetic trajectories between harvests among classes. The interaction  $T \times S(C)$  was plotted to compare the variety of morphological changes between harvests per species, grouped by their ontogenetic class.

#### Non-destructive analyses (Dataset 2)

The variables Ve, BS, Bn/Ht and DS<sub>p</sub> were analysed with the same method described for Dataset 1 (PCA and MANOVA) to check the consistency of ontogenetic groups at a more advanced developmental stage. Based on the results of the PCA, an alternative classification with three

groups was compared with the Mediterranean versus Eurasian classification. Mediterranean species were splitted into two classes, the first (MP1) comprising the more ontogenetically delayed species (*P. canariensis*, *P. pinea* and *P. halepensis*) and the second (MP2) with *P. pinaster* and *P. brutia*, with slightly faster ontogenetic development.

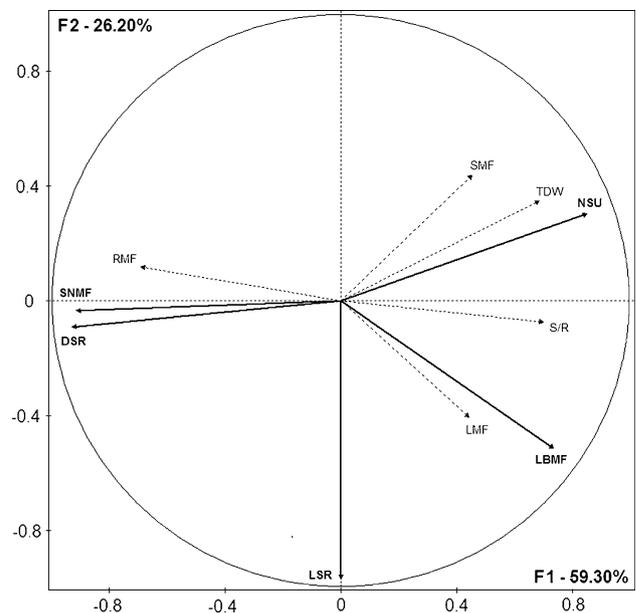
We omitted the discriminant analysis for this dataset because no other variables were available for this purpose.

## Results

### Destructive analysis (Dataset 1)

#### PCA

The two-first factors (F1 and F2) of the PCA with data of the second harvest explained 85.5% of the total variation (Fig. 2). F1 was negatively correlated with the SNMF (−0.92) and proportion of dwarf shoots DSR (−0.93) and positively correlated with the NSU (0.85) and juvenile LSMF (0.74, Table 3). F2 was negatively correlated with the proportion of juvenile long shoots LSR (−0.97) and juvenile LSMF (−0.51). The plot on these two axes revealed two distinct groups of cases (Fig. 3). The first factor distinguished those seedlings with a high NSU and low abundance of adult structures (DSR and SNMF), which also showed a high total biomass TDW and high stem to root ratios (Figs. 2, 3), from a group of seedlings with the

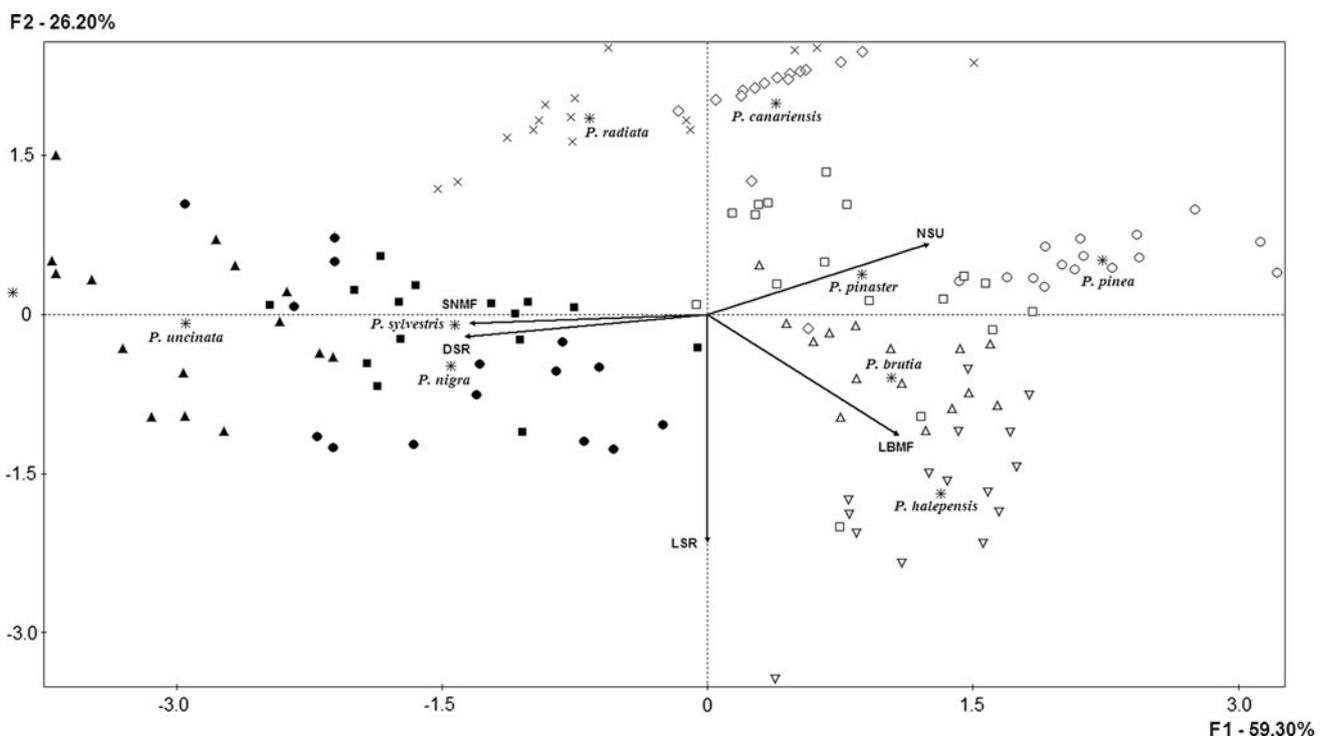


**Fig. 2** Plot of variables on the two-first PCA axes, representing 85.5% of the variation (Dataset 1, second harvest). **Bold labels** correspond to the five variables entering the PCA while the rest are complementary variables. Variable description and acronyms are explained in Table 1

**Table 3** Correlation matrix between all variables of Dataset 1 (second harvest) and the first two factors of the PCA (F1, F2)

	SNMF	DSR	LSMF	LSR	TDW	RMF	LMF	SMF	S/R	F1	F2
NSU	-0.664	-0.712	0.547	-0.294	0.911	-0.630	0.267	0.570	0.622	0.851	0.303
SNMF		0.908	-0.527	ns	-0.492	0.507	-0.286	-0.441	-0.554	-0.915	-0.036
DSR			-0.533	ns	-0.518	0.640	-0.395	-0.478	-0.676	-0.930	-0.093
LSMF				0.404	0.443	-0.623	0.616	ns	0.561	0.735	-0.513
LSR					-0.353	ns	0.354	-0.354	ns	-0.001	-0.972
TDW						-0.501	ns	0.565	0.475	0.686	0.349
RMF							-0.756	-0.457	-0.988	-0.692	0.119
LMF								-0.217	0.757	0.441	-0.406
SMF									0.474	0.452	0.437
S/R										0.700	-0.074

All coefficients are significant at  $p < 0.05$  except those indicated ns (non-significant). Variable description and acronyms explained in Table 1



**Fig. 3** Plot of cases and variables in the PCA plane with Dataset 1 (second harvest). Open symbols correspond to Mediterranean pines: open triangle *P. brutia*; open diamond *P. canariensis*; inverted open triangle *P. halepensis*; open square *P. pinaster*; open circle *P. pinea*; filled symbols correspond to Eurasian pines: filled circle *P. nigra*; filled square *P. sylvestris*; filled triangle *P. uncinata*; times *P. radiata*. Asterisks correspond to the centroids of each species

filled symbols correspond to Eurasian pines: filled circle *P. nigra*; filled square *P. sylvestris*; filled triangle *P. uncinata*; times *P. radiata*. Asterisks correspond to the centroids of each species

opposite constellation of traits, and high root mass fraction, RMF. Seedlings of Mediterranean pines clearly corresponded to the first group of cases (positive values of F1), while Eurasian pine seedlings formed the second one (negative values of F1). Moreover, F2 allowed distinguishing between those seedlings with a low proportion of juvenile long shoots, mostly corresponding to *P. canariensis* and the outgroup *P. radiata*, from seedlings with the opposite trend corresponding to *P. halepensis*, and some individuals of the Eurasian *P. nigra* and *P. sylvestris*.

The MANOVA analysis (Eq. 1) confirmed that Mediterranean and Eurasian pine seedlings were significantly different based on the two-first PCA factors F1 and F2 (Wilks'  $\lambda$ ,  $p = 0.0071$ ), even when the species within class were also highly significant (Wilks'  $\lambda$ ,  $p < 0.0001$ ).

Further discriminant analysis with the complementary variables revealed a single significant discriminant function ( $p < 0.00001$ ). Using this function, 89.2% of cases were correctly classified. Only 4 individuals out of 45 Eurasian pines were wrongly classified as Mediterranean and 9 out

of 75 Mediterranean pines seedlings were wrongly classified as Eurasian, based on their biomass ratios.

*Specific trends between harvests*

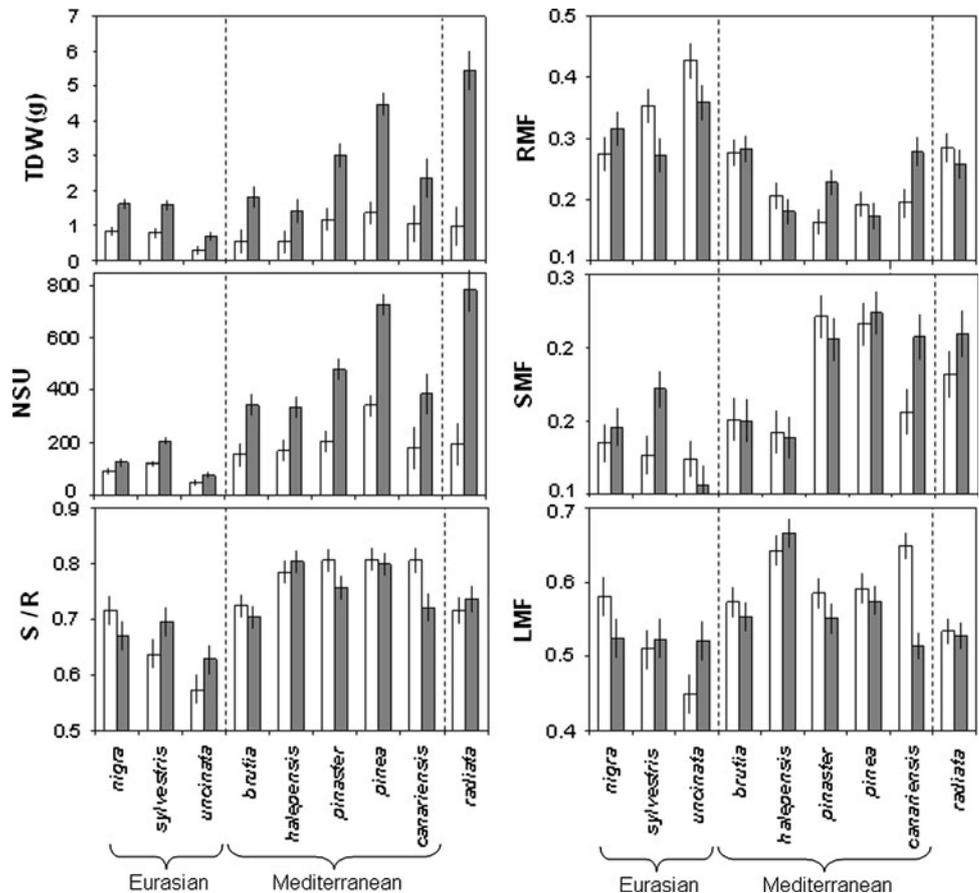
Greenhouse conditions ensured growth and development of axillary structures between harvesting dates (fall and spring) even for Eurasian species. Results of ANOVAs (mixed model of Eq. 3) showed that highly significant interaction harvest × class was for all variables except for SMF, and also a highly significant interaction harvest × species within classes, with no exceptions (Table 4). The interaction harvest × class was especially high for DSR and SNMF, the two variables related to the abundance of adult structures. Therefore, different developmental trajectories between classes and species were found (Figs. 4, 6). Eurasian pines (EP class) showed low values and small variation between harvests for TDW and NSU, and subsequently low RGRs and RNIR (Figs. 4, 5). These species allocated proportionally more biomass to roots and less to stems compared to the other ontogenetic classes. However, each of the three species showed a particular change in biomass allocation between harvests, either increasing allocation to the shoot (*P. sylvestris* and

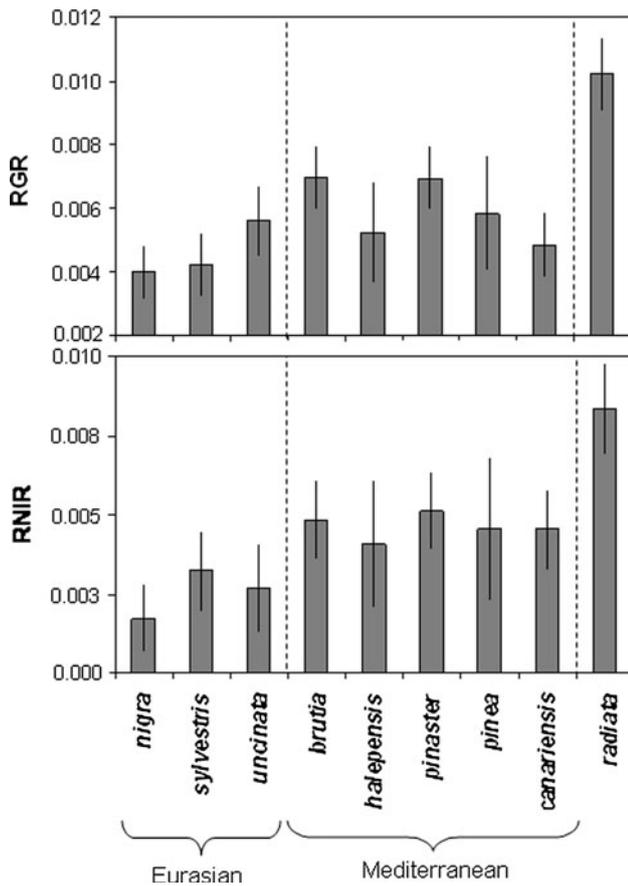
*P. uncinata*) or the opposite (*P. nigra*). However, by far the most significant morphological change between the two harvests in this class was the steep increase in secondary needles, expressed either in needle biomass or in node counts (Fig. 6). This change was accompanied in *P. nigra* and *P. sylvestris* with a significant decrease of the proportion of needle biomass in juvenile long shoots, but not in *P. uncinata* (Fig. 6).

Mediterranean pine species showed higher values for TDW and NSU, and subsequently also for RGR and RNIR (Figs. 4, 5). All five species within this class showed similarly high S/R ratio, but in three species this was due to very high biomass allocation to stems, *P. pinaster*, *P. pinea* and *P. canariensis*, while one had the highest allocation to leaves, *P. halepensis* (Fig. 4). Also, two distinct patterns of axillary shoots can be detected while all four species lacked secondary needles at the first harvest, two species, *P. brutia* and *P. pinaster* showed a significant increase towards the second harvest, while *P. halepensis*, *P. pinea* and *P. canariensis* remained with almost totally juvenile foliage (Fig. 6).

The outgroup *P. radiata* showed the highest RGRs both in biomass and in number of nodes, together with the highest biomass proportion of secondary needles of all nine species, even at the first harvest (Fig. 6).

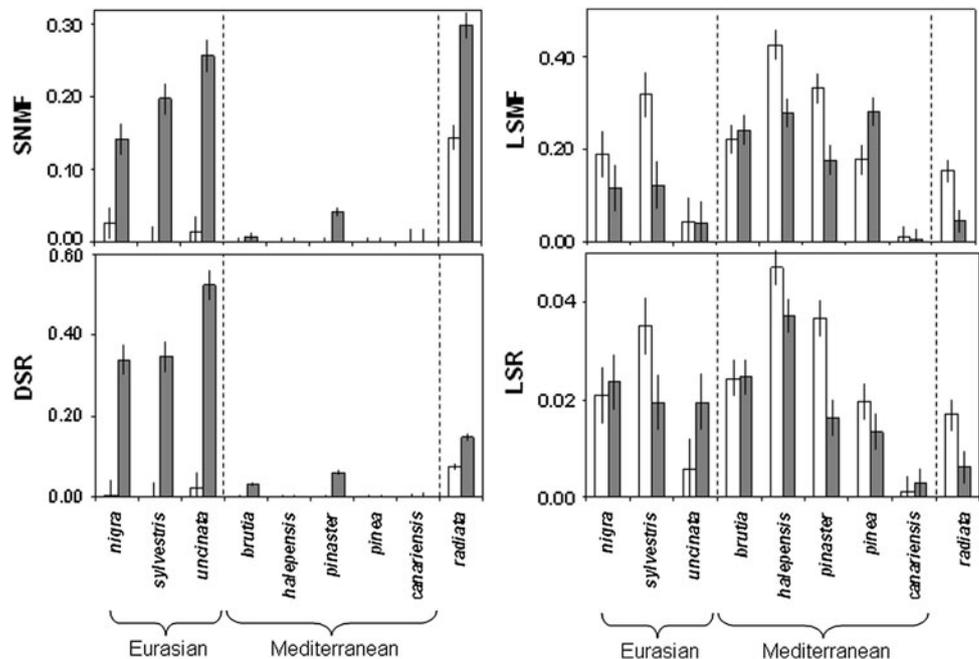
**Fig. 4** Mean plot of the interaction between species, grouped by classes, and harvests (9 weeks, white bars and 32 weeks, dark bars) for the variables TDW, NSU, S/R, RMF, SMF and LMF. Error bars represent standard errors





**Fig. 5** Mean plot of relative growth rates (RGR) and relative rate of node formation (RNIR) between harvests (9 and 32 weeks); species grouped by classes. Error bars represent 95% confidence intervals

**Fig. 6** Mean plot of the interaction between species, grouped by classes, and harvests (9 weeks, white bars and 32 weeks, dark bars) for the variables related to axillary shoots SNMF, DSR, LSMF and LSR



Non-destructive analysis (Dataset 2)

The PCA analysis for non-destructive measures at 68 weeks showed two significant factors, accumulating 81.1% of the total variation. The first factor (F1<sub>2</sub>) was positively correlated with bud set (BS) (0.92, Table 5) and dwarf shoot height proportion DS<sub>p</sub> (0.88) and negatively correlated to branchiness Bn/Ht (-0.57) and stem volume Ve (-0.46). Most individuals of Eurasian pines showed high positive values of F1<sub>2</sub> while individuals of the Mediterranean *P. halepensis*, *P. pinea* and *P. canariensis* (and also the outgroup *P. radiata*) showed negative values. By contrast, individuals of *P. pinaster* and *P. brutia* were mostly intermediate between these two groups. The second factor (F2<sub>2</sub>) was positively correlated to branchiness (0.66) and negatively correlated to stem volume (-0.81). This axis mostly separated some especially branchy individuals of *P. halepensis* from scarcely branched plants of *P. canariensis*, *P. pinaster* and the outgroup *P. radiata*.

Attending to the grouping of cases in this biplot and the results of the previous analysis between harvests, we compared the MANOVAs with the former classification (Eurasian vs. Mediterranean) with an alternative classification with three categories, splitting the five Mediterranean species into MP1 (ontogenetically delayed, *P. canariensis*, *P. halepensis* and *P. pinea*), and MP2 (the more accelerated *P. pinaster* and *P. brutia*). Meaningfully, while the initial classification performed well for the MANOVA of F1<sub>2</sub> and F2<sub>2</sub> (Wilks'  $\lambda$ ,  $p = 0.0045$ ), the alternative classification showed a more significant

**Table 4** Summary of the results of mixed-model analyses for the main ontogenetic and biomass allocation traits (Dataset 1): degrees of freedom (*df*), *F* values and significance level

Trait	Harvest	Class	Species (class)	Harvest × class	Harvest × species (class)
<i>df</i>	1	2	6	2	6
NSU	373.79***	854.39***	73.79***	169.39***	9.17***
SNMF	439.41***	468.26***	17.02***	354.8***	18.68***
DSR	686.70***	622.94***	28.74***	565.90***	26.64***
LSMF	31.66***	44.31***	100.66***	5.39***	20.36***
LSR	7.07***	1.46 ns	88.12***	7.27***	11.86***
TDW	377.39***	179.18***	50.46***	70.23***	10.74***
RMF	0.67 ns	291.39***	26.58***	18.01***	10.44***
LMF	5.44***	124.94***	22.00***	12.99***	15.27***
SMF	8.00***	163.40***	33.95***	0.49 ns	7.47***
S/R	0.47 ns	330.60***	22.59***	15.61***	7.59***

Variable description and acronyms explained in Table 1

**Table 5** Correlation matrix between all variables of Dataset 2 (non-destructive measures taken 68 weeks after germination) and the first two factors of the PCA ( $F_{12}$ ,  $F_{22}$ )

	Bn/Ht	DSP	BS	$F_{12}$	$F_{22}$
Ve	ns	ns	−0.517	−0.458	−0.809
Bn/Ht		−0.487	−0.309	−0.572	0.656
DSP			0.755	0.884	−0.229
BS				0.923	0.225

All coefficients are significant at  $p < 0.05$  except those indicated ns (non-significant). Variable description and acronyms explained in Table 1

between-class differentiation compared to species within-class variation (Wilks'  $\lambda$ ,  $p = 0.0007$ ).

## Discussion

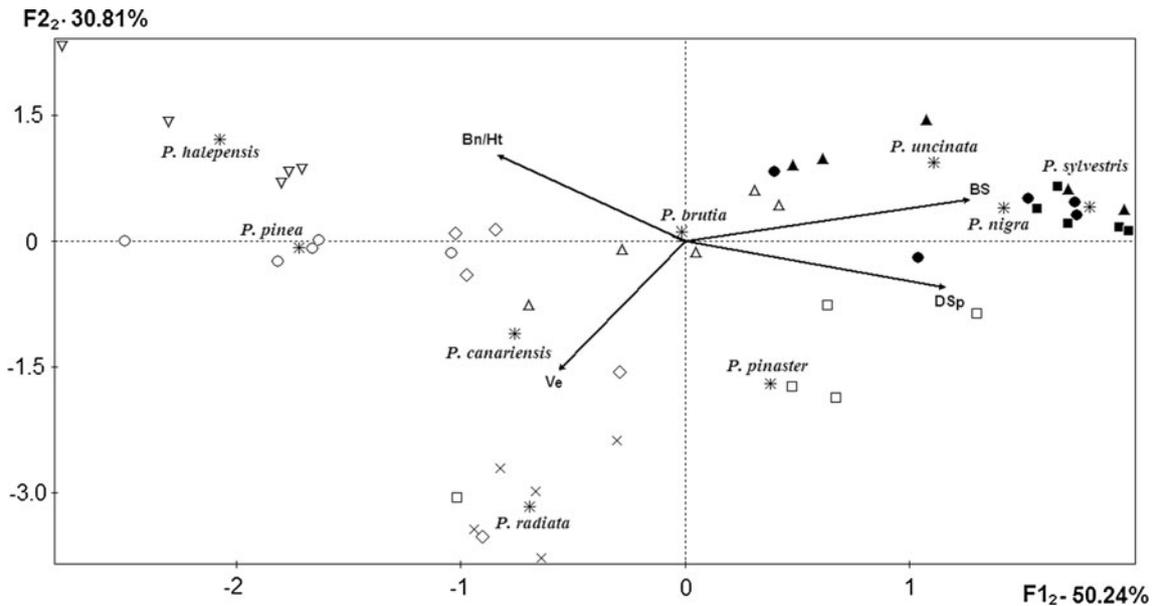
Our study deals with the ontogenetic differentiation between and within Eurasian and Mediterranean pines and how heteroblasty relates to seedling allometry in these two species' groups. The results confirmed that Mediterranean pines as a whole show a marked ontogenetic delay compared to Eurasian pines at the seedling stage, but some differentiation within the Mediterranean group was also evident.

The multivariate nature of ontogeny in pines

The nine studied species showed extremely different developmental characteristics within the first 12 months after germination, under favourable greenhouse conditions. Although this is an expectable result considering previous information (Chambel et al. 2007; Klaus 1989; Lester

1968), the correlations among traits suggest rather different developmental pathways whose adaptive and evolutionary meaning deserves some further close attention. The results of multivariate analyses confirmed that the (NSU, i.e. the total number of nodes in the main shoot), was closely linked to the increase in total plant size, but inversely related to proportion of secondary needles indicating the amount of heteroblastic change. On the other hand, the formation of long shoots ("juvenile" branching) was rather independent from both node number and secondary needle proportion amongst the studied pine species. Hence, the nine species studied showed different combinations of these two basic traits: high or low number of nodes (associated to high or low growth rates and size), and high or low proportion of nodes producing adult axillary structures (dwarf-shoots) or, alternatively, juvenile long shoots. This highly different combination of axillary structures between species had already been postulated by (Lester 1968), but it has been mostly neglected in other studies, probably due to the use of very young seedlings (Grotkopp et al. 2002; Strauss and Ledig 1985).

Moreover, another heteroblastic phenomenon, bud set, seems to add independent information to the studied variation trends at higher developmental stages as seen in the non-destructive assessment, 68 weeks from germination. The ontogenetic nature of the formation of the first true terminal bud is undoubted, since it implies new foliar structures (the bud scales) and the onset of a new growth habit: the preformed shoot growth (Lanner 1976; Lascoux et al. 1993). Preformation has been postulated to have deep adaptive and evolutionary meaning and it must have been subjected to strong selection pressures (Diggle 1997; Jones and Watson 2001). In our experiment, bud set was generally correlated to the abundance of dwarf shoots (or secondary needles), with the meaningful exception of *P.*



**Fig. 7** Plot of cases and variables on the two-first PCA axes, gathering 81.1% of the variation, for Dataset 2. Open symbols correspond to Mediterranean pines: open triangle *P. brutia*; open diamond *P. canariensis*; inverted open triangle *P. halepensis*; open

square *P. pinaster*; open circle *P. pinea*; filled symbols correspond to Eurasian pines: filled circle *P. nigra*; filled square *P. sylvestris*; filled triangle *P. uncinata*; times *P. radiata*. Asterisks correspond to the centroids of each species

*radiata*. This species was the only one combining a significant presence of secondary needles early in seedling development with a delayed bud set (Fig. 8). This feature is related to the tendency of this species to form continuous shoot growth cycles, extending shoot elongation along the year, a trait typical of species from frost-free subtropical habitats (Cannell et al. 1976; Dougherty et al. 1994).

#### Relationship between ontogenetic heteroblasty and biomass allocation

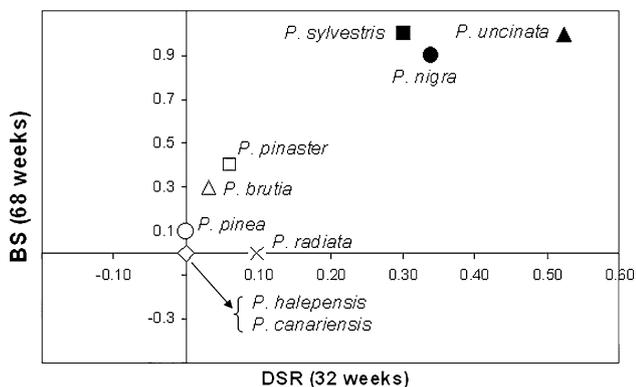
Among species, the multivariate analysis revealed that a high proportion of juvenile long shoots was associated to high biomass allocation to leaves, while a high proportion of adult, dwarf shoots was related to high allocation to roots. However, the comparison between harvests revealed that two Eurasian pines (*P. sylvestris* and *P. uncinata*) combined a sharp increase of adult foliage with a significant decrease in the biomass allocated to roots between the two harvests. By contrast, Mediterranean pines tended to increase (in *P. canariensis* and *P. pinaster*) or maintain (rest of species) allocation to roots between harvests. This suggests that the formation of adult foliage in species showing accelerated ontogenetic change, and the subsequent formation of the terminal bud both imply a shift of biomass allocation towards the shoot at the expense of a lower investment in roots. This was postulated to explain the intra-specific variation in the *P. canariensis*, whose dry provenances showed a marked ontogenetic delay in

common garden tests (Climent et al. 2006). However, *P. halepensis* showed a similarly delayed formation of secondary needles (Fig. 6) and delayed BS (Fig. 7) than *P. canariensis*, but unlike this species, *P. halepensis* maintained a very high allocation to leaves across harvests. This contrasting allometry is highly consistent with the differences in life-history between these two species, following the postulations by Strauss and Ledig (1985) for the genus *Pinus*: a negative association between mature tree height and investment in foliage and a positive association between root biomass, age at reproduction and tree lifespan. *P. canariensis* can grow as tall as 65 m and live more than 500 years, and is a late reproducer (Climent et al. 2004). On the contrary, *P. halepensis* embraces a short-living strategy, based in a fast accumulation of aerial biomass and a rapid onset of the reproductive stage, at the expenses of a reduced size (Tapias et al. 2001). This simple pair-wise comparison supports that the relationship between biomass allocation patterns and ontogenetic heteroblasty between species is not straightforward, and the first seems more linked to life history strategies along its entire lifespan (Strauss and Ledig 1985).

#### An adaptive interpretation of heteroblasty in pines

Significant functional differences between juvenile and mature leaves have been reported for pines and other conifers (Bormann 1955; Greenwood 1995; Miller et al. 1995). A prolonged juvenile phase has been postulated to

increase fitness in xeric environments, through the production of low-cost needles with high photosynthetic efficiency, allowing higher carbon allocation to roots in *Juniperus occidentalis* (Miller et al. 1995). On the other hand, differences in cuticular transpiration between juvenile and adult leaves, associated to the different rates of development have been found in the same species studied in this paper, such that the more delayed heteroblastic change is associated to higher resistance of secondary needles to water loss compared to primary needles (Pardos et al. 2009). In addition, primary needles are generally more sensitive to frost damage (Climent et al. 2009), but probably have a higher photosynthetic efficiency due to their higher proportion of photosynthetic tissues (Bormann 1955; Peters et al. 2003). Therefore, it seems plausible that delayed heteroblastic change might have selected in drought-tolerant pines to construct drought-resistant foliage and, in some cases, a massive stem and a strong root system (like in *P. canariensis*). On the other hand, a fast ontogeny combining high proportion of secondary needles and early bud-set is probably advantageous in cold-tolerant pines like the three Eurasian species analysed. It is noteworthy that the two Mediterranean pines living in colder habitats, *P. pinaster* and *P. brutia* (Table 1) appeared as intermediate between Eurasian and the more thermophilous Mediterranean pines in the two main traits related to ontogenetic heteroblasty: the proportion of nodes with secondary needles and BS (Fig. 8). Also interesting is the fact that this inter-specific trend mimics the intra-specific trend found among populations of *P. canariensis* (Climent et al. 2006) and *P. pinaster* (Alía et al., in prep.). This consistency suggests that heterochrony, as the heritable alteration of the rate of development (McKinney and McNamara 1991; Smith 2003), might be a source of adaptation in hard pines. To check this hypothesis it will be necessary to carry out a similar experiment with a much



**Fig. 8** Biplot of mean values per species for the proportion of nodes with secondary needles (DSR) at 32 weeks and budset (BS) at 68 weeks. *Open symbols* correspond to Mediterranean pines; *closed symbols* correspond to Eurasian pines

wider sampling of the genus, which would enable separating phylogenetic constraints from adaptive evolutionary trajectories (Grotkopp et al. 2002).

Of course, besides the possible adaptation to the abiotic environment, the inclusion of biotic interactions in a broader phylogenetic study would enlarge our interpretation of the evolution of ontogeny in pines. Particularly, herbivory has been postulated to be strongly influenced by plant phase change in different genera (Karban and Thaler 1999; Lawrence et al. 2003; Loney et al. 2006). The inclusion of abiotic and biotic factors would probably help us understanding why almost every pine species considered represents such an original combination of developmental features at a particular timing along the developmental programme.

**Acknowledgments** Thanks to S. Iglesias, J. Peñuelas and J.L. Nicolas (DGMNPF, Spain) for providing the seeds, to N. Godoy, F. del Caño, S. Herrera (INIA, Madrid) for laboratory work, to F. Salgado, I. Santos and G. Ruiz (TRAGSA, Spain) for nursery work and to M. Pardos, R. Alía and M. Verdú for criticism and advising. Research funded by projects AT-05 002 (INIA) and AGL2005-07440 REPROFOR (CYCIT). The English was kindly revised by Marcas Ó Murchú, Derry, Ireland.

## References

- Ade-Ademilua OE, Botha CEJ (2005) A re-evaluation of Plastochron Index determination in peas—a case for using leaflet length. *S Afr J Bot* 71:76–80
- Bormann FH (1955) Primary leaf as an indicator of physiological condition in short-leaf pine. *For Sci* 1:189–192
- Cannell MGR, Thompson S, Lines R (1976) An analysis of inherent differences in shoot growth within some north temperate conifers. In: Cannell MGR, Last FT (eds) *Tree physiology and yield improvement*. Academic Press, London, pp 173–205
- Causton DR (1991) Plant growth analysis: the variability of relative growth rate within a sample. *Ann Bot* 67:137–144
- Chambel MR, Climent J, Alía R (2007) Divergence among species and populations of Mediterranean pines in biomass allocation of seedlings grown under two watering regimes. *Ann For Sci* 64:87–97
- Climent J, Tapias R, Pardos JA, Gil L (2004) Fire adaptations in the Canary Islands pine (*Pinus canariensis*). *Plant Ecol* 171:185–196
- Climent J, Chambel MR, López R, Mutke S, Alía R, Gil L (2006) Population divergence for heteroblasty in the Canary Islands pine (*Pinus canariensis*, Pinaceae). *Am J Bot* 93:840–848
- Climent J, Costa e Silva F, Chambel MR, Pardos M, Almeida H (2009) Freezing injury in primary and secondary needles of Mediterranean pine species of contrasting ecological niches. *Ann For Sci* 66:407–415
- Day JS (1998) Light conditions and the evolution of heteroblasty (and the divaricate form) in New Zealand. *NZ J Ecol* 22:43–45
- Diggle PK (1997) Extreme preformation in alpine *Polygonum viviparum*: an architectural and developmental analysis. *Am J Bot* 84:154–169
- Doak CC (1935) Evolution of foliar types, dwarf shoots, and cone scales of *Pinus*. *Ill Biol Monogr* 13:1–106
- Dougherty PM, Whitehead D, Vose JM (1994) Environmental influences on the phenology of pine. *Ecol Bull* 43:64–75

- Erickson RO, Michelini FJ (1957) The plastochron index. *Am J Bot* 44:297–305
- Germandt DS, Lopez GG, Garcia SO, Liston A (2005) Phylogeny and classification of *Pinus*. *Taxon* 54:29–42
- Greenwood MS (1995) Juvenility and maturation in conifers: current concepts. *Tree Physiol* 15:433–438
- Grotkopp E, Rejmanek M, Rost TL (2002) Toward a causal explanation of plant invasiveness: seedling growth and life-history strategies of 29 pine (*Pinus*) species. *Am Nat* 159:396–419
- Hunt R, Causton DR, Shipley B, Askew AP (2002) A modern tool for classical plant growth analysis. *Ann Bot* 90:485–488
- Jones CS (1999) An essay on juvenility, phase change, and heteroblasty in seed plants. *Int J Plant Sci* 160:S105–S111
- Jones CS, Watson MA (2001) Heteroblasty and preformation in mayapple, *Podophyllum peltatum* (Berberidaceae): developmental flexibility and morphological constraint. *Am J Bot* 88:1340–1358
- Jordan GJ, Potts BM, Chalmers P, Wiltshire RJE (2000) Quantitative genetic evidence that the timing of vegetative phase change in *Eucalyptus globulus* ssp *globulus* is an adaptive trait. *Aust J Bot* 48:561–567
- Karban R, Thaler JS (1999) Plant phase change and resistance to herbivory. *Ecology* 80:510–517
- Klaus W (1989) Mediterranean pines and their history. *Plant Syst Evol* 162:133–163
- Lanner RM (1976) Patterns of shoot development in *Pinus* and their relationship to growth potential. In: Cannell MGR, Last FT (eds) *Tree physiology and yield improvement*. Academic Press, London, pp 223–243
- Lascoux D, Paino E, Sierra de Grado R, Kremer A, Dormling I (1993) Maturation of maritime pine (*Pinus pinaster* Ait.) seedlings after exposure to a period of continuous light. *Tree Physiol* 12:363–378
- Lawrence R, Potts BM, Whitham TG (2003) Relative importance of plant ontogeny, host genetic variation, and leaf age for a common herbivore. *Ecology* 84:1171–1178
- Leroy C, Heuret P (2008) Modelling changes in leaf shape prior to phyllode acquisition in *Acacia mangium* Willd. seedlings. *Comptes Rendus Biologies* 331:127–136
- Lester D (1968) Developmental patterns of axillary meristematic activity in seedlings of *Pinus*. *Bot Gaz* 129:206–210
- Loney PE, McArthur C, Potts BM, Jordan GJ (2006) How does ontogeny in a *Eucalyptus* species affect patterns of herbivory by Brushtail Possums? *Funct Ecol* 20:982–988
- McKinney M, McNamara K (1991) *Heterochrony: the evolution of ontogeny*. Plenum Press, New York
- Miller PM, Eddleman LE, Miller JM (1995) *Juniperus occidentalis* juvenile foliage: advantages and disadvantages for a stress-tolerant, invasive conifer. *Can J For Res* 25:470–479
- Mundermann L, Erasmus Y, Lane B, Coen E, Prusinkiewicz P (2005) Quantitative modeling of *Arabidopsis* development. *Plant Physiol* 139:960–968
- Niklas KJ (2004) Plant allometry: is there a grand unifying theory? *Biol Rev Camb Philos Soc* 79:871–889
- Pardos M, Calama R, Climent J (2009) Difference in cuticular transpiration and sclerophylly in juvenile and adult pine needles relates to the species-specific rates of development. *Trees Struct Funct* 23:501–508
- Peters J, Morales D, Jimenez MS (2003) Gas exchange characteristics of *Pinus canariensis* needles in a forest stand on Tenerife, Canary Islands. *Trees Struct Funct* 17:492–500
- Poethig RS (2003) Phase change and the regulation of developmental timing in plants. *Science* 301:334–336
- Pryor LD (1976) *Biology of Eucalyptus*. Edward Arnold, London
- Renton M, Guedon Y, Godin C, Costes E (2006) Similarities and gradients in growth unit branching patterns during ontogeny in ‘Fuji’ apple trees: a stochastic approach. *J Exp Bot* 57:3131
- Sachs T (1999) ‘Node counting’: an internal control of balanced vegetative and reproductive development. *Plant Cell Environ* 22:757–766
- Smith KK (2003) Time’s arrow: heterochrony and the evolution of development. *Int J Dev Biol* 47:613–621
- Strauss SH, Ledig FT (1985) Seedling architecture and life-history evolution in pines. *Am Nat* 125:702–715
- Tapias R, Gil L, Fuentes-Utrilla P, Pardos JA (2001) Canopy seed banks in Mediterranean pines of southeastern Spain: a comparison between *Pinus halepensis* Mill., *P. pinaster* Ait., *P. nigra* Arn. and *P. pinea* L. *J Ecol* 89:629–638
- Urban O, Sprtova M, Kosvancova M, Tomaskova I, Lichtenthaler HK, Marek MV (2008) Comparison of photosynthetic induction and transient limitations during the induction phase in young and mature leaves from three poplar clones. *Tree Physiol* 28:1189–1197
- Winn AA (1999) The functional significance and fitness consequences of heterophylly. *Int J Plant Sci* 160:S113–S121
- Wright SD, McCaughay KDM (2002) Interpreting phenotypic plasticity: the importance of ontogeny. *Plant Species Biol* 17:119–131