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Variation of selfing rate and inbreeding depression among individuals and across generations within an admixed *Cedrus* population.

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Abstract

We investigated the variation and short-term evolution of the selfing rate and inbreeding depression (ID) at the individual level within a cedar forest that was recently established from admixture. The mean selfing rate was 9.5%, ranging from 0% to 48% among 20 seed trees. This variation was explained by individual male fecundity rather than local demography. Using paternally inherited gene markers, we investigated ID by comparing naturally produced selfed and outcrossed seeds within progenies and thus avoided maternal effects. The observed high germination and low seedling mortality rates suggest an effective purging of major recessive lethal alleles at the embryonic stage. The germination dynamics differed significantly between selfed and outcrossed seeds within progenies in the founder gene-pool. Surprisingly, selfed seeds germinated earlier than outcrossed seeds, suggesting outbreeding depression (OD) in the original admixture due to epistatic interactions. This selfing effect rapidly disappeared in the following generations, as expected under the OD hypothesis. Regarding the seedling growth traits, the ID was low but significant: 8% and 6% for height and diameter growth, respectively. These rates were stable across generations, suggesting minor gene effects. Early stage ID was lower than mother tree effects (breeding value plus maternal effects): outcrossed seedlings outcompeted their selfed relatives, but not necessarily other selfed

seedlings from other progenies. Purging these slightly deleterious genes only occurs through within-family selection. Processes that maintain a high level of genetic diversity for fitness-related traits also reduce the efficiency of purging this part of the genetic load.

Introduction

Forest trees, generally outcrossing species, are characterised by a huge genetic diversity within populations (Hamrick and Godt, 1989), including for adaptive traits (Kremer, 1994; Notivol et al, 2007). This diversity is maintained at the expense of a high mutation load (Ledig, 1986; Petit and Hampe, 2006; Scofield and Schultze, 2006), which can only be purged whenever selfing or other systems of consanguinity occur. Investigating the processes that dynamically maintain the genetic load and the adaptive genetic variation within populations cannot rely only on a theory that assumes a demographic and genetic equilibrium. This is particularly true in forestry where transient situations frequently occur: recently established forests (in terms of the number of generations), marginal areas of expansion or retraction in the distribution range, maturation phase of the silvigenesis, or simply pioneer species in disturbed areas (riparian forests, forest fires, etc.). The demo-genetic approach provides a general framework to study short-term evolution in non-equilibrium populations (Lande, 1988; Coulson et al, 2006; Benton et al, 2006; Pichot et al, 2006). In these models, the genetic variation of individual capacities such as survival, growth potential, fecundity and dispersal ability determines the demographic structure of the population. As a feedback, the demographic structure regulates competition and the mating system, thus

drinving the evolution of the genetic diversity by affecting the intensities of drift and selection.

Inbreeding results from genetic drift and particular types of mating systems, such as selfing or mating among relatives. Most studies generally consider the selfing rate through a mean value for a population but it has been shown that it can be highly variable among individuals. Under the pollen limitation hypothesis, in hermaphroditic or monoecious species with no incompatibility system, the selfing rate is determined by the ratio of autopollen vs. allopollen that is locally available for each female flower. Therefore, individual variation of the selfing rate is partly explained by the local demographic density as observed in several conifer species (Farris and Mitton, 1984; Knowles et al, 1987; Robledo-Arnuncio et al, 2004; Restoux et al, 2008). However, some species have sexual peculiarities, such as dichogamy, that also determine the individual selfing rate. One feature of the monoecious Atlas cedar, *Cedrus atlantica* Manetti, is a continuous variation of male/female allocation to reproduction among individuals, which ranges from quasi-male trees to quasi-female trees and everything in between (Krouchi et al, 2004). In such situations, both demographic and biological features may affect the mating system.

The genetic diversity responsible for inbreeding depression (ID) is generated by mutation or migration and is purged by selection or drift.

Effective purging of ID depends not only on the mating system that generates inbred individuals but also on the genetic make-up of ID (Byers and Waller, 1999). Inbreeding depression that is caused by a small number of recessive genes that have major deleterious effects on fitness is expected to vary among individuals depending on their genotype at these few major genes and, therefore, to respond rapidly to selection. Purging is less efficient when selfed offspring do not contribute to the next generation (Scofield and Schultz, 2006), or when dominance and epistatic interactions among major genes occur (Williams et al, 2003). By contrast, ID due to many genes with small individual effects that are dispersed over the genome is not expected to vary drastically among individuals and is less easily purged in the population. Furthermore, when the variation of parental breeding values overcomes ID, inbred individuals from "good" progenies still have a high fitness, which may reduce purging efficiency.

Seed abortion is often due to embryonic lethal equivalents, a number of deleterious alleles whose cumulative effects are the equivalent of one lethal allele (Lee et al, 1996; Keller and Waller, 2002). The number of embryonic lethal equivalents could be as high as 11 in conifers (Savolainen et al, 1992) and experimental results generally support the assumption of an additive effect of recessive alleles (Remington and O'Malley, 2000a). However, in Pinaceae, a stage-specific death peak occurring during early

embryogenesis could be caused by partial dominance or overdominance of deleterious genes (Williams, 2007; 2008). In *Pinus taeda* L., Remington and O'Malley (2000b) showed that embryonic lethals do not contribute to ID later in the life cycle. Beyond seed abortion, ID delays seed germination (Sorensen, 2001), increases juvenile mortality (Koelewijn et al, 1999), depresses growth (Sorensen and Miles, 1982; Bower and Aitken, 2007), causes morphological abnormalities later in the life cycle (Wilcox, 1983), and decreases seed production and fertility in young trees (Kärkkäinen and Savolainen, 1993; Durel et al, 1996). At this later stage mildly deleterious mutations or polygenic mutations, which difficult to purge, are likely the main actors (Husband and Schemske, 1996).

Inbreeding depression has been experimentally assessed in controlled pedigrees of trees (Eriksson, 2006; White et al, 2007). Within forest tree populations, the common trend of increased heterozygosity in older age classes is considered as an indicator of previous selection against inbred individuals in younger age classes (Shaw and Allard, 1982; Farris and Mitton, 1984; Yazdani et al, 1985; Marquardt and Epperson, 2004; Jones et al, 2006). Some studies have shown increasing heterozygosity across the youngest age classes, suggesting that a rapid purging of inbred individuals occurs in these classes (Pichot et al, 2006). Here we assessed ID at the individual level within progenies, comparing the performance of

naturally produced selfed and outcrossed genotypes that we identified using chloroplast DNA markers. Molecular markers such as allozymes (Ritland and El-Kassaby, 1985) or microsatellite markers (Collevatti et al, 2001) have long been used for estimating the selfing rate in nature. In *Cedrus*, as in most Pinaceae species, the chloroplast genome is uni-parentally inherited from the male parent and does not recombine (Fady et al, 2003). Consequently, this provides an efficient tool to assess the probability of a selfing event by comparing the chloroplast genotypes of progenies and their seed trees.

We studied the *Cedrus atlantica* forest of Luberon (South Eastern France) as a model situation of a transient population of trees experiencing rapid demographic evolution. The species was introduced locally from North Africa for afforestation in the 1860s (Cointat, 1996). From the founder trees that survived (still alive), three generations have developed through natural regeneration, expanding from the initial plantation areas to a broader continuous forest, which currently shows a spatially heterogeneous pattern of density. A previous study based on isozymes revealed an initial population admixture through a transient genotypic disequilibrium in the founder generation that disappeared in the younger generations (Lefèvre et al, 2004). The three objectives of this work were (i) to assess the variation of the selfing rate at the individual level and test the predominance of

demographic or sexual parameters on this variation, (ii) to quantify early stage ID during seed germination and the first year of seedling growth by comparing selfed and outcrossed seeds within progenies, thus avoiding maternal effects, and (iii) to test the existence of purging during the first three generations after the establishment of the Luberon cedar forest.

Materials and Methods

Seed trees and seed collection

Twenty reproductive trees belonging to three different generations were sampled in different zones of the forest (Figure 1). The assignment of each tree to its corresponding generation was based on the estimate of the age of each tree from wood cores at a height of 60 cm (Lefèvre et al, 2004). The stem circumference at breast height was measured on each tree. The fecundity of *Cedrus* trees varies greatly across years and the trees show different allocations to male and female reproduction that remain relatively stable (Krouchi et al, 2004). For this reason, the male flowering intensity was scored following a 0-3 scale as an indication of the potential male fecundity of the reproductive trees, since the seeds used for the germination test resulted from pollination that occurred two years before. Spatial location of individual trees was obtained with a laser telemeter and a

compass. Local stand density around each tree was approached as the mean distance to its five nearest mature trees (Table 1).

Three cones were collected from each tree just before disarticulation, in October 2003. For each tree, 120 well-conformed seeds were sampled for the germination test, i.e., 2400 seeds for the totality of the selected trees. Non-developed seeds were assumed to correspond to non pollinated ovules (Mosseler et al, 2000) and were discarded.

Germination test

To remove dormancy, the seeds were imbibed in cryptonol (2%) for one hour and chilled. The seeds were maintained in darkness at 2-4°C for 40 days. For the germination test, a randomised block design with four blocks was used. Each 120 seed family was divided into four sub-samples of 30 seeds that were sown into each block. Each block consisted of four Petri dishes (25 cm x 25 cm). Seeds were grouped as single plot units per seed family and were randomly distributed within each Petri dish, which were also randomly distributed within each block. Petri dishes were kept in a climatic chamber at 15°C, with a cycle of 15 hours of light per day, and 40% relative humidity. During the stratification period and the germination test, seeds were laid on a thick layer of filter paper that was re-hydrated

three times a week. Petri dishes from the same block were located on the same shelf in the climatic chamber.

Three times a week, germinated seeds were identified when the radicle exceeded 2 mm in length. When this occurred, the individual germination date was recorded. The germinated seeds were then transplanted into mini-containers (40 cc). The germination of seeds lasted for 72 days after sowing. Non-germinated seeds were dissected under binoculars to check for the presence of embryos. Empty seeds or infected seeds, due to the presence of *Megastigmus sp.* larvae, were observed. Because this insect is suspected, in some cases, to induce the development of full seeds from non-pollinated ovules (Rouault et al, 2004), these full sized but infected seeds could not be assigned to either the pollinated or un-pollinated ovule groups. Therefore, we computed the germination rate as the ratio between germinated seeds and the total number of "healthy" seeds, removing all empty and infected seeds.

Nursery test for selfing effects

Immediately after the germination test, a maximum of 50 seedlings were selected within each progeny to represent the whole range of germination dates. These seedlings were transplanted from mini-containers to 400cc WM containers that were filled with peat and bark substrate, and

grown in a glasshouse for one year. For an accurate comparison between selfed and outcrossed individuals within each progeny, seedlings from the same progeny were located within a single plot. As a consequence, progeny effect and micro-local environment were confounded. The whole experiment covered less than 2 x 5 m in the glasshouse. The individual height (mm from cotyledons to apical bud) and diameter (mm at the base of the stem) were recorded at the end of the first year. Any developmental abnormalities or deaths that occurred during the growing season were also recorded.

Genetic markers

To elucidate the selfed or outcrossed origin of each seedling, we used three chloroplast microsatellites: Pt71936, Pt96916 and Pt36713 (Vendramin et al, 1996). The few seeds that developed abnormally were also included in the study. DNA was extracted from 50 mg of fresh plant tissue using the Qiagen Extraction Kit. The PCRs were performed following the protocol by Fady et al (2003). Electrophoresis was conducted in a Li-Cor sequencer. Since allelic variation occurs as a single base polymorphism for these markers, we paid particular attention to avoid genotyping errors. In all gels, five repeats of the same set of three control genotypes and three ladders were regularly distributed and systematically included. Furthermore,

each progeny was distributed in the gels as groups of 10 individuals, surrounded by two samples of the corresponding mother tree, one on each side, which were also used as an extra-control among gels. Finally, 553 seedlings (18 to 39 per progeny) and all the seed trees were successfully genotyped.

Detection of selfing events

In the absence of recombination, chloroplast haplotypes were defined as the combination of the three microsatellite alleles. Outcrossed seedlings were unambiguously identified by the difference in haplotype between the seedling and mother. A seedling that shared the same haplotype with its seed tree may result either from selfing or from outcrossing, with a probability that depends on the frequency of this particular haplotype in the pollen pool. Therefore, an estimation of the haplotype frequency in the pollen pool is required to estimate the selfing rate. Thus, we developed a maximum likelihood estimate which combined both parameters (selfing rates and haplotype frequencies) as follows.

Considering $P_i^{m,j}$ the probability for mother *m* with haplotype (chlorotype) *j* to produce a seedling with haplotype *i*, we can write:

$$P_i^{m,j} = P_i^j * s_m + f_i * (1-s_m)$$

where:

- P_i^j is the probability for a mother with haplotype *j* to produce a seedling with haplotype *i* after selfing ($P_i^j = 0$ for $i \neq j$ and $P_j^j = 1$);
- s_m is the selfing rate of mother *m*;
- f_i is the frequency of haplotype *i* in the pollen pool and, assuming panmixia, $f_i * (1-s_m)$ is the probability of producing a seedling with haplotype *i* after outcrossing.

To estimate the frequency f_i of haplotype *i* in the pollen pool, we have to consider that for a mother tree of haplotype $j \neq i$, each seedling with haplotype *i* is issued from outcrossing and represents one haplotype *i* of the pollen pool. However, when the mother tree has haplotype *i*, its seedlings with haplotype *i* partly result from outcrossing and partly from selfing. In this case, it is necessary to remove the selfed seedlings from the count of *i* haplotypes in the pollen cloud. Finally, we have:

$$f_i = \frac{\sum_m [N_i^{m,j\neq i}] \square \sum_m [N_i^{m,i} - [s_m * N_\cdot^{m,i}]]}{\sum_i \sum_m [N_i^{m,j\neq i}] \square \sum_i \sum_m [N_i^{m,i} - [s_m * N_\cdot^{m,i}]]}$$

where:

- $N_i^{m,i}$ is the number of seedlings with haplotype *i* produced by mother *m* of haplotype *i*;

- $N_.^{m,i}$ is the total number of seedlings produced by mother *m* of haplotype *i* and (s_m*$N_.^{m,i}$) is the number of selfed seedlings;

- and $N_i^{m,i}$ - (s_m*$N_.^{m,i}$) is the number of purely outcrossed seedlings with haplotype *i* produced by mother *m* of haplotype *i*.

Finally, assuming that all mating events were independent, the overall probability of the observed haplotypes is:

$$P(N_i^{m,j}) = \prod_{i,m} P_i^{m,j}$$

Selfing rates (s_m) and haplotype frequencies (f_i) were jointly estimated by the maximisation of the log likelihood ($\Sigma\log(P_i^{m,j})$) using the "optim" function from the R Stats package (R version 2.6.2. in http://www.r-project.org/). Confidence intervals were estimated from 1000 bootstrap redraws of individuals within progenies from the observed data. Each seedling *k* from a mother *m* was assigned a probability $p.self_{m,k}$ of resulting from a selfing event as follows:

- $p.self_{m,k} = 0$ if the seedling and seed tree have different haplotypes;

- otherwise, the conditional probability of being selfed given the seedling and its mother-tree share the same haplotype *i*, is given by

$$p.self_{m,k} = \frac{s_m}{s_m \left[1 - s_m\right] * f_i}$$

Haplotype diversity was estimated as $H = (1- \Sigma f_i^2)$ where f_i is the frequency of haplotype *i*. We used the rarefaction method to compare the number of different haplotypes between different sub-samples (seed trees, zones, etc.).

The effects of male flowering intensity, local density, and tree size on the individual selfing rate were tested using the following fixed linear model and type III tests:

$$f(s_m) = male_m + dens_m + \text{poly}(circ_m,2) \qquad (1)$$

where:

- $f(s_m)$ is the arc.sine(sqrt) transformation of the selfing rate of mother tree *m*;
- $male_m$ is the male flowering score (0-3) of the mother tree *m* that we treated either as a two-level factor (0 vs 1-2-3) or as a four-level factor (note that a score 0 only means no observed male strobili);
- $dens_m$ is the local density of trees around mother tree *m*;
- poly($circ_m$,2) is a quadratic function of the individual circumference of the mother tree *m*.

We included no intercept term in the model. We obtained the same results using a generalised linear model with the number of selfed and outcrossed seeds estimated from s_m as the dependant variable (matrix) and a quasibinomial distribution (data not shown).

Assessment of maternal and inbreeding depression effects

From the subset of genotyped seedlings, the effect of selfing on the seed germination time was tested using the following mixed effect ANCOVA model:

$$T_{b,m(z,g),k} = \mathcal{O} + block_b + zone_z + generation_g + a\ p.self_{m,k} + b_g\ p.self_{m,k} + A_{m(z,g)} + E_{b,m(z,g),k} \quad (2)$$

where :

- $T_{b,m(z,g),k}$ is the germination time (number of days after sowing, log transformed) of seed k from mother tree m, from zone z and generation g, grown in block b;

– $block_b$ is the fixed effect of block b in the germination experiment;

- $zone_z$ is the fixed effect of the zone z of the forest where mother tree m is located;

- $generation_g$ is the fixed effect of the generation g to which mother tree m belongs;

- $p.self_{m,k}$ is the probability of seed k from mother tree m being selfed, used as a covariate; an interaction with generation is also introduced into the model;

- $A_{m(z,g)}$ is a random term, variance var($A_{m(z,g)}$) is the variance among seed trees within zone and generation;

- var($E_{b,m(z,g),k}$) is the residual variance.

Similarly, the maternal and inbreeding effects on seedling growth beyond their effects on germination time were analysed as follows:

$$H(D)_{m(z,g),k} = \mathcal{O} + zone_z + generation_g + a\ p.self_{m,k} + b_g\ p.self_{m,k} + c\ gt_{m,k} + A_{m(z,g)} + E_{b,m(z,g),k} \quad (3)$$

where:

- $H(D)_{m(z,g),k}$ is the height (resp. basal diameter) of seedling k from seed tree m;

- $gt_{m,k}$ is the germination time of seedling k from seed tree m, used as a covariate to account for the effect of different growing periods;

- other terms are similar to that in model (2).

Considering that $p.self_{m,k}$ is also the expected proportion of true selfed individuals within the progeny, its effect represents the absolute difference between selfed and non-selfed individuals, which is a measure of ID, assumed to be constant among seed trees in this model. Alternatively, ID can also be expressed relatively to the intercept term of the model which

represents the predicted mean of outcrossed progenies (*i.e.*, for value $p.self_{m,k}$=0 of the covariate).

At the progeny level, considering that the actual progeny means are the potential progeny values without selfing (combining breeding value and maternal effects) minus the impact of selfing (selfing rate x ID), we could graphically represent ID through the relationship between the selfing rate and the difference between the actual and potential progeny values. The actual progeny values were estimated from a sub-model without the $p.self_{m,k}$ covariate, whereas the potential progeny values were estimated from the global model at the value $p.self_{m,k}$=0 of the covariate.

We used type III tests for the fixed effects and covariates. We used REML estimates of the variance components. All statistical tests were computed on R, and scripts are available upon request.

Results

Seed germination

The percentage of full seeds varied from 58% to 99% depending on the progeny. The germination rate was high and varied from 88% to 100%. Some progenies started germination immediately after the stratification period but others were delayed for up to 15 days (Figure 2), suggesting

important maternal effects on the seed families for this trait. Furthermore, the variation of germination time within progeny varied among seed trees, from just one day to 24 days before the level of germinated seeds reached 50% (Table 2). In particular, there was a significant difference between zone 1 and 2 (Wilcoxon test, P=0.034 for the date of first germination; P=0.006 for the date of 50% germination).

Polymorphism of the markers

The three microsatellites used displayed four, five and six variants for Pt36713, Pt96916, and Pt71936, respectively. Considering that the chloroplast genome does not undergo genetic recombination, a total of 35 haplotypes was identified over the whole set of genotyped individuals: seedlings and seed trees. In our sample, the highest frequencies of these haplotypes were 0.21 and 0.17, and the lowest was 0.002. The haplotype diversity was high (H = 0.90).

Selfing rate

Selfing rate estimates varied considerably among the reproductive trees, ranging from 0 to 0.479 (Table 3). Of the 20 reproductive trees, nine showed significantly positive selfing rates. These trees were dispersed in the population (Figure 1). Considering the forest as a single panmictic unit,

selfing rates and haplotype frequencies from the whole set of seed trees could be estimated regardless of their location in the forest. Then the mean selfing rate was 0.095 ± 0.140. The selfing rates were also independently estimated within zone 1 and zone 2 (the sample in zone 3 was too small for this analysis), assuming that the pollen clouds in the different zones of the forest were genetically differentiated. The within-zone estimates of selfing rates were very similar to the global estimations, suggesting a limited differentiation among zones if any (data not shown).

The variation in selfing rate among trees was not determined by local density (Table 4). The effect of male flowering intensity was marginally significant (P=0.032), only when considered as a two-level factor (Table 4).

The number of different haplotypes in the pollen cloud, estimated after performing the rarefaction method, varied from 5.97 to 10.25 among seed trees (Table 3). There was no correlation between individual selfing rate and the diversity of haplotypes in the outcrossed progeny.

Effects of selfing on germination

As mentioned above, the final germination rate was quite stable among progenies, but germination dynamics did vary among progenies. Considering the subset of individually genotyped seedlings, which represented the whole germination period within each progeny (Figure 2),

the number of days needed for germination after stratification varied significantly among progenies (Table 5). At the individual seed level, we detected a significant effect of the probability of being selfed on the germination time and a significant interaction effect between the probability of being selfed and the generation: this selfing effect was only significant within G0 but not in the subsequent generations (Table 5). At progeny level, in generation G0, the selfing rate was not a predictor of actual progeny means but only a predictor of the difference between actual and potential progeny means (Figure 3).

Surprisingly, within progenies, the selfed seeds germinated earlier than the outcrossed seeds. Early germination seems to be positively correlated with fitness in our environmental conditions since earlier growth confers a competitive advantage by increasing the period of vegetation.

Effects of selfing on growth in nursery

Seedling survival did not appear to be affected by selfing since there was no correlation between the mortality rate (low, 0-12%) and the selfing rate at the progeny level. Morphological abnormalities and mortality appeared at a very low frequency in both self and cross-fertilised seedlings and no significant differences were observed (data not shown).

The selfing effect was significant at the individual level for both height and diameter after one year of growth, and it did not vary across generations (Table 6). The effect of the germination date on height and diameter growth was also significant but much less important than ID (Table 6). Within progenies, ID accounted for an 8% and 6% reduction in height and diameter growth, respectively. At the progeny level, the selfing rate was not a predictor of actual progeny means (Figure 3), as previously observed for the germination time.

Discussion

Variation in selfing rate among seed trees

Although the *Cedrus* population under study results from artificial introduction beyond its natural range, we found a relatively high diversity of haplotypes. Terrab et al (2006) estimated the haplotype diversity to be 0.91 in natural populations of *C. atlantica* from Morocco, which was similar to the one observed in our study. This was despite identifying twice as many different haplotypes (66) as we did with their six CpSSR markers. On the one hand, this high diversity could be due to the fact that CpSSRs show higher variability than other chloroplast regions (Provan et al, 1999). On the other hand, a large diversity was probably already present in the original

admixed gene pool (Lefèvre et al, 2004). Similar levels of diversity in these chloroplast regions were generally found in populations of different Pinaceae species (Parducci et al, 2001; Ribeiro et al, 2002; Naydenov et al, 2005). Because of the high diversity found and their paternal inheritance, CpSSRs appear as a powerful marker for monitoring gene flow and paternity analyses (Fady et al, 2003).

Traditionally, studies on ID have been carried out by performing controlled crosses. There are only a few studies that have estimated the individual selfing rates and ID under natural mating systems, by calculating the proportion of empty seeds and with biparentally inherited molecular markers such as isozymes, which need more complicated statistical estimators (Kärkkäinen and Savolainen, 1993; Ritland and Travis, 2004; Bower and Aitken, 2007). In *Cedrus*, we measured a mean selfing rate circa 0.1, which is in agreement with the absence of incompatibility mechanisms and the values reported in other conifers (Franklin, 1969; Cottrell and White, 1995; Sorensen, 1999; Restoux et al, 2008). A considerable variability among seed trees was observed, reminding us that selfing rate is not a fixed trait of the species, but that it can vary both between and within populations (Barret and Eckert, 1990).

To explain this variability, under the hypothesis of pollen limitation, a reduced number of male strobili surrounding a seed tree may restrict the

relative amount of non-self pollen and, hence, increase the proportion of self pollen proportion in the seed tree's crown. On the one hand, we found no relationship between local density of adult trees and selfing rate. Concerning the effect of local density on selfing rate, various results were obtained in similar studies on conifers (Farris and Mitton, 1984; Sorensen and Adams, 1993; Neale and Adams, 1985; Morgante et al, 1991; Parraguirre-Lezama et al, 2004; Restoux et al, 2008). Furthermore, we did not find any correlation between the selfing rate and the diversity of the allopollen cloud captured by the seed tree, as would be expected for isolated trees under the hypotheses of pollen limitation and gene mixing effects due to long distance pollen dispersal (Klein et al, 2006). On the other hand, we detected a weak but significant effect of the production of male strobili of each seed tree on its selfing rate. Therefore, individual variation of selfing rate was determined by the amount of self-pollen and not by the local amount of non-self pollen. In this case, this means that the mating system is controlled by the biology of the species and not by the demography. In *Cedrus atlantica*, sex allocation to male and female functions is highly variable and Krouchi et al (2004) have found that this variation is stable at least across several years. From an evolutionary perspective, such a tendency to dioecy in trees can be interpreted in the light of inbreeding depression avoidance (Scofield and Schultze, 2006).

Variation in the selfing rate can also be explained by the fact that it was estimated from the the number of mature seeds and not from the number of fertilised ovules. In most conifers, during early embryogenesis, ID acts more or less severely depending on the number of embryonic lethal alleles in the parental genome (see Introduction). Kärkkäinen and Savolainen (1993) suggest that polyembryony, which occurs frequently in Pinaceae, can diminish the cost of embryonic lethals and make embryo competition possible, leading to an underestimation of the primary selfing rate. In this regard, the genus *Cedrus* displays simple polyembryony, derived from the fertilisation of about half of three to six archegonia in each female gametophyte, and cleavage polyembryony, which leads to genetically identical embryos (Wilson, 1923; Favre-Duchartre, 1970). Whereas cleavage embryos offer no selective advantage, simple polyembryony is also a weak or even nonexistent barrier against selfing because, during early embryogeny, an advantage is conferred to the embryo based on its position in the female gametophyte. The embryo closest to the corrosion cavity, which is genotype independent, is conferred the advantage (Williams, 2007).

Effects of selfing on germination and first year growth in nursery

The rate of empty seeds among well-conformed seeds, which generally indicates the presence of embryonic lethals (Savolainen et al, 1992), was low (17%) and its variation was not related to selfing rate. Furthermore, juvenile mortality was also very low in this study. These results suggest that major recessive lethal alleles with additive effects had efficiently been purged in this *Cedrus* population. This could either reflect a general feature of the species or a particular situation in this population that suffered very high mortality after their initial plantation. Similarly, juvenile mortality was not observed in studies on *Pinus pinaster* Ait. and *Pseudotsuga menziesii* (Mirb.) Franco (Durel et al, 1996; Sorensen, 1997).

Germination dynamics differed significantly among seed trees. Germination time is determined both by the genotype of the embryo, partly inherited from the seed tree, and the physiological conditions related to seed maturity when the seeds are collected. The variation in physiological conditions relates to maternal effects that include genetic and environment components as well as the interaction between them. We observed a marked difference in germination time among seed trees collected in two zones of the forest. Since we suspect that there is no genetic differentiation among these zones (Lefèvre et al, 2004; and marker data from this study), the difference in germination dynamics can be explained by the various environmental conditions that are present where the seed trees grow, which

can lead to a variation in seed maturity when the seeds were collected. Moreover, the delay between seed collection in the two zones (several days) and the variation of climatic conditions between the collecting dates can also accentuate the physiological differences observed among seed families. Furthermore, the variation in germination time also impacted first year growth of the seedlings in relation to the length of the growing season.

Within progenies (skipping maternal effects at this level), we detected a selfing effect on the germination time in the founder generation G0 but not in the following generations G1 and G2. In our controlled and artificial conditions, selfed seeds germinated earlier than outcrossed seeds, a finding that is in contradiction with results reported for other conifers, such as *Pinus contorta* var. *murrayana* (Sorensen, 2001) and *Picea abies* (L.) Karst (Skroppa, 1996). In *Pseudotsuga menziesii* and *Pinus ponderosa* Dougl. ex Laws., germination was hardly-affected by selfing (Sorensen and Miles, 1974). Therefore, we hypothesise that outbreeding depression (OD) might occur in this admixed *Cedrus* population. Seedlings from selfing are obviously a result of within-gene pool crosses. In contrast, part of the seedlings issued from outcrossing result from between-gene pool crosses and could suffer from OD. Outbreeding depression occurs in particular when hybridisation disjuncts co-adapted gene complexes (Ledig, 1986). This hypothesis is further supported by the evolution across generations. As

observed in this study, OD would not be expected to correlate to the selfed or outcrossed origin of the seeds that are produced by the next generations because of recombination among genomes.

Studies of the effects of ID on growth parameters in trees have often been performed on juvenile trees of at least four years old. This is because first-year growth can be influenced by the time of emergence, a possible effect of maternal influences, as demonstrated here. Moreover, many studies are conducted at the family level comparing selfed and outbred controlled crosses (which, by the way, also imply artificial conditions during pollination and seed development). In one-year-old *P. contorta* var. *murrayana* seedlings, Sorensen (2001) found an ID of 17.7% in height; in one-year-old *Pseudotsuga menziesii* and *Pinus ponderosa*, Sorensen and Miles (1974) observed an ID for height of 18% and 21%, respectively; in two-year-old *Pseudotsuga menziesii*, Sorensen (1997) found an ID of 30% in height and 40% in diameter. In this work, we used a different approach that consisted of a model which included maternal effects and germination time in the analysis. This approach was used to estimate ID beyond these effects at the individual seed level. In doing so, we detected a small but significant ID effect, 8% and 6% on height and diameter growth, respectively. In contrast with OD, ID did not change across the three generations, suggesting that ID mainly relates to the additive effects of

many slightly deleterious genes that are not easily purged in the population. Indeed, we detected no purge of ID at this short time scale. The effectiveness of purging is not frequently observed in plant populations, even when inbreeding occurs (Byers and Waller, 1999). Comparing selfed and crossed progenies obtained in a natural population of *Pinus sylvestris*, Koelewijn et al (1999) observed an effect of ID on survival and seed set up to an age of 23 years, but no significant differences in height and flowering, indicating that high levels of ID both for early stage (seed production) and later stages of the life cycle were maintained in the population in the long run. As explained below, our results reveal a possible mechanism that might explain low purging in trees.

In this study, no effect of selfing rate on family means was observed, suggesting a previous efficient purge of the most deleterious alleles in this species. Although significant, the ID effect was weaker than that of the global seed tree effect, i.e., selfed seeds only differ from their outcrossed relatives but not from all outcrossed seeds. Because of between-family variation, selfed seedlings can still outcompete outcrossed seedlings from other progenies. The design we used in the nursery to determine growth traits was oriented towards within-family comparisons, where seed tree effects (breeding value and maternal effects) were slightly up-biased and partly confounded with micro-environmental conditions in the nursery. The

micro-environmental conditions in the nursery were still much less variable than small-scale environmental conditions in the forest. Therefore, we expect that ID is even weaker relatively to progeny effect in natural conditions, which means that purging such type of ID in nature is only possible through within-family selection. Under this scenario, seed dispersal, while affecting the spatial distribution of various progenies, would also affect purging. More generally, during the regeneration phase of the forest, when the most intense demographic reduction and selection for competing ability occur, seed dispersal is widespread enough so that competition occurs between seedlings from different progenies and, therefore, we would expect no early purging of inbred genotypes. After the regeneration phase, demography decreases more slowly and inbred trees could remain present in the forest and contribute to the next generation, even-though they have a reduced fitness. By contrast, in some particular situations such as a very low density in a colonisation front, we could expect within-family competition at the seedling stage and more efficient purging. Of course, the most deleterious mutations are more rapidly purged from the embryonic to later stages of the life cycle.

Thus, ID that has a lower effect than the variation of breeding values is maintained in the population for the long term. Part of these slightly

deleterious genes might become a reservoir of "positive diversity" when environmental conditions change.

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Titles and legends to figures

Figure 1: Location of the 20 seed trees within the Luberon forest. The nine trees with effective selfing are indicated by *.

Figure 2: Seed germination kinetics for the 20 *Cedrus atlantica* progenies. Dashed lines indicate the time to reach 50% germination; dots represent the sub-sample of genotyped seedlings. Germination rate is the ratio of seeds that germinated out of filled and non-infested seeds (ranging from 70 to 119 per progeny).

Figure 3: Top line graphs represent the relationship between individual selfing rates and corresponding progeny means for different variables (no relation). Bottom line graphs represent, for the same variables, the relationship between individual selfing rates and the differences between the actual progeny means and the potential values predicted for outcrossed seedlings only. The slopes of the lines were taken from the analysis at the individual seed level (*p.self* estimates in Table 5 and 6). They represent the selfing effect within progeny which are assumed to be homogeneous among progenies. For germination time, the selfing effect was only significant in the G0 generation which is represented here.

Table 1: Characterisation of sampled seed trees: zone of origin, generation, circumference at breast height (in cm), male flowering intensity (using a 0-3 scale) and local density. In relation to the age class, G0 included the founder trees planted in 1863, G1 was the first generation issued from G0, and G2 was the next generation that issued from G0 and G1 (Lefèvre et al, 2004). Local density was measured as the mean distance from the seed tree to the five nearest neighbour trees (in m).

Tree	Zone	Generation	Circumference	Male flowering intensity	Local density
A102	Z1	G0	291	2	5.8
A107	Z1	G1	198	3	6.6
A110	Z1	G0	285	1	7.3
B112	Z1	G1	144	1	5.6
B115	Z1	G1	134	3	3.5
B118	Z1	G2	117	0	4.0
B119	Z1	G1	144	2	5.2
C105	Z1	G2	63	0	2.5
A202	Z2	G0	203	2	4.9
A206	Z2	G0	243	2	6.4
A214	Z2	G0	263	0	7.1
B201	Z2	G1	149	0	13.5
B205	Z2	G1	159	0	6.9
B206	Z2	G1	100	0	6.1
B213	Z2	G1	96	0	5.8
B216	Z2	G1	149	3	7.0
B219	Z2	G1	92	0	6.1
C201	Z2	G2	58	0	5.5
A307	Z3	G0	219	3	12.9

A310	Z3	G0	245	1	7.1
mean(sd)			167.5 (72.0)	1.2 (1.2)	6.5 (2.6)

5

Table 2. Germination parameters for each progeny: percentage of healthy embryos (not empty, not infested), germination rate, number of days from sowing to the first germination, number of days until 50% of the seeds within the progeny germinated.

5

Tree	Zone	% healthy embryos	germination rate (%)	days to germination	days 50% germination
A102	Z1	90	99	5	24
A107	Z1	89	100	12	19
A110	Z1	96	98	1	12
B112	Z1	82	97	10	22
B115	Z1	72	94	10	24
B118	Z1	88	100	8	22
B119	Z1	80	91	1	19
C105	Z1	87	88	1	22
A202	Z2	84	100	1	15
A206	Z2	74	97	3	12
A214	Z2	63	99	1	1
B201	Z2	93	99	5	22
B205	Z2	90	99	1	8
B206	Z2	87	98	1	15
B213	Z2	58	100	1	3
B216	Z2	77	99	1	19
B219	Z2	99	100	1	10
C201	Z2	87	99	1	10
A307	Z3	90	100	1	8
A310	Z3	72	94	15	22
mean(sd)		83 (11)	98 (3)	4.0 (4.5)	15.5 (7.1)

Table 3. Selfing rate estimates (s_m) for each seed tree considering the whole forest as a panmictic unit. The number of different haplotypes in the outcrossed progeny of each seed tree was computed after rarefaction using the smallest sample size of 18, observed for B115, as a reference.

Seed-tree	Selfing rate Forest	Number of haplotypes
A102	0.092	8.01 ± 0.79
A107	0	8.62 ± 1.06
A110	0	8.77 ± 1.09
B112	0.273	6.69 ± 0.88
B115	0.479	10
B118	0	6.88 ± 0.89
B119	0	8.63 ± 0.83
C105	0.331	5.97 ± 0.8
A202	0.158	7.27 ± 0.7
A206	0.109	7.07 ± 0.77
A214	0	9.48 ± 0.6
B201	0.107	10.18 ± 0.95
B205	0	7.95 ± 0.22
B206	0	8.68 ± 0.46
B213	0	6.19 ± 0.73
B216	0	10.25 ± 1.11
B219	0	7.41 ± 0.93
C201	0.054	9.62 ± 1.06
A307	0.288	-
A310	0	-
mean(sd)	0.095 ± 0.141	

Table 4. Effects of male flowering intensity (treated as a two-level factor in (a), or as a four-level factor in (b)), local density, and tree circumference (quadratic effect) on the individual selfing rate (arc.sine(sqrt) transformed). The model included no intercept and type III tests were performed.

(a)	Num d.f.	Den d.f.	*F*	*P-value*	
male flowering [0/+]	1	16	*5.53*	*0.0318*	*
local density	1	16	*0.61*	*0.4480*	*ns*
poly(circumference,2)	2	16	*1.68*	*0.2175*	*ns*

(b)	Num d.f.	Den d.f.	*F*	*P-value*	
male flowering [0-3]	4	13	*1.26*	*0.3326*	*ns*
local density	1	13	*0.29*	*0.5995*	*ns*
poly(circumference,2)	2	13	*0.91*	*0.4263*	*ns*

Table 5. Mixed effect ANCOVA analysis of germination time (in days, log transformed) following model (1) and type III tests (see Materials and Methods). The covariate *p.self* is the probability of each seedling being selfed, which provides an estimate of inbreeding depression. The random variation among progenies includes variation of genetic values of the mother trees and maternal effects.

Fixed effects and covariates	Num d.f.	Den d.f.	*F*	*P-value*	
block	3	527	*0.89*	*0.4462*	*ns*
zone	2	15	*4.15*	*0.0368*	*
generation	2	15	*0.18*	*0.8407*	*ns*
p.self	1	527	*7.28*	*0.0072*	**
*generation * p.self*	2	527	*6.31*	*0.0020*	**
Selfing effect within each generation	Estimate		95% Conf. Int.		
p.self within G0	-1.010		[-1.440 ; -0.581]		
p.self within G1	0.053		[-0.392 ; +0.497]		*ns*
p.self within G2	-0.151		[-0.672 ; +0.370]		*ns*
Variance components	s.d. estimate		95% Conf. Int.		
among progenies (at *p.self* = 0)	0.583		[0.397 ; 0.856]		
residual	0.769		[0.724 ; 0.817]		

Table 6. Mixed effect ANCOVA analysis of seedling growth in height and diameter following model (2) and type III tests (see Materials and Methods). The covariate *p.self* is the probability of each seedling being selfed, which provides an estimate of inbreeding depression. The covariate *gt* is the time before germination of the original seed, accounting (negatively) for the length of the growing period. The random variation among progenies includes variation of genetic values of the mother trees and maternal effects.

a) Height growth

Fixed effects and covariates	Num d.f.	Den d.f.	*F*	*P-value*	
zone	2	15	*0.30*	*0.7500*	*ns*
generation	2	15	*1.30*	*0.3005*	*ns*
p.self	1	500	*6.41*	*0.0116*	*
gt	1	500	*78.6*	*<.0001*	***
*generation * p.self*	2	500	*2.28*	*0.1032*	*ns*
Selfing and germination time effects	Estimate		95% Conf. Int.		
p.self	-20.675		[-37.364 ; -3.986]		
gt	-1.687		[-2.067 ; -1.307]		
Variance components	s.d. estimate		95% Conf. Int.		
among progenies (at *p.self* = 0)	24.588		[16.444 ; 36.767]		
residual	45.646		[42.910 ; 48.557]		

(continued next page)

Table 6. (continued)

b) Diameter growth

Fixed effects and covariates	Num d.f.	Den d.f.	*F*	*P-value*	
zone	2	15	*1.98*	*0.1725*	*ns*
generation	2	15	*1.33*	*0.2953*	*ns*
p.self	1	500	*7.22*	*0.0075*	**
gt	1	500	*52.24*	*<.0001*	***
*generation * p.self*	2	500	*0.48*	*0.6206*	
Selfing and germination time effects	Estimate		95% Conf. Int.		
p.self	-0.288		[-0.493 ; -0.082]		
gt	-0.017		[-0.022 ; -0.013]		
Variance components	s.d. estimate		95% Conf. Int.		
among progenies (at *p.self* = 0)	0.162		[0.095 ; 0.276]		
residual	0.579		[0.544 ; 0.616]		