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Use of molecular markers for estimating breeding parameters: a case study in a *Pinus pinaster* Ait. progeny trial

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Abstract The management of a genetic improvement program is based on the knowledge of the genetic parameters and their relationships to determine the genetic gains. Knowledge of the coefficient of coancestry (θ) is a requirement for efficient progeny testing scheme and for estimating additive variance components for any quantitative trait. When using open-pollinated families, most authors assume that the seedlings are related as half-sibs, but this is not always true. Our aim was to estimate a mean value of the coancestry coefficient of the families present in a maritime pine *Pinus pinaster* Ait. (maritime or cluster pine) progeny trial originating from seed collected in a clonal seed orchard and to study how deviations from the standard assumption of θ=0.125 affect heritability estimations. Five highly polymorphic microsatellite markers were scored in 125 offspring from a subsample of five families from the progeny trial. The mean value of the coancestry coefficient of the families present in this progeny trial was 0.130. Differences between the unadjusted and adjusted heritability estimates were more pronounced in wood density (0.609 and 0.586, respectively) than in diameter (0.166 and 0.154, respectively). We conclude that in the trial, the associated error in heritability estimates due to the inclusion of full-sibs, when assuming a standard coefficient of relationship among open-pollinated sibs of 0.250, was low and that this result is robust with respect to the number of families sampled, given unbiased estimates of average relationship among offspring within sib families.

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Introduction

Pinus pinaster Ait. (maritime or cluster pine) is an important commercial species in south-western Europe. In Portugal, *P. pinaster* is one of the most important native species, covering 1 Mha, it is the only source of long fiber for pulp and paper industry and the main one for solid sawn timber industries. A tree improvement program is being developed for this species since the early 1980s based on a selection of plus trees, the establishment of first-generation seed orchards and open-pollinated progeny tests (Aguiar et al. 2003), with the aim of increasing volume per hectare and quality of stem form. Progeny tests allow the estimation of genetic parameters and provide information about the ability of a species to respond to selection and thus inform the deployment strategy for that species (Zobel and Talbert 1984). To estimate genetic variance components it is necessary to establish the relationship structure among the individuals tested (Falconer and Mackay 1996; Lynch and Walsh 1997). We focus on the traditional population genetic definition of coefficient of relatedness or relationship (r) for diploid individuals, which is twice the coefficient of coancestry (θ) (Lynch and Walsh 1997; Wright 1976).

Open-pollinated families have often been used for forest tree progeny trials, due to easy operative management and simplicity of calculations (Borralho 1994), especially in first-generation breeding programs or in ecological studies where seeds are collected in natural populations. Genetic relatedness among members of a wind-pollinated family is usually assumed to be mathematically equivalent to the covariance among half-sibs, which is equal to 0.25 (Falconer and Mackay 1996). This involves assuming that families are true half-sib families, i.e., the female trees from which the progenies were collected are unrelated, crosses are based on a high effective number of unrelated males and there is no self-fertilization (Borralho 1994). However, these assumptions are not usually met due to unaccounted relatedness between parents, nonequal contributions of pollen, or the occurrence of selfed progeny (Borralho 1994; Hansen and Kjaer 2006). This failure to meet assumptions also implies that the family variance component is inflated by non-additive effects when families include some proportion of full-sibs. Random mating and panmitic equilibrium assumptions are often unrealistic for natural populations and, in many cases, for seed orchards too (El-Kassaby and Ritland 1986). For example, several studies in conifers have demonstrated that not all the clones within a seed orchard (SO) make an equal contribution to the next generation (Goto et al. 2002; Hansen and Kjaer 2006; Moriguchi et al. 2005; Plomion et al. 2001). In these conditions, the use of r=0.25 would result in a biased estimation of additive genetic variance (Squillace 1974). According to Askew and El-Kassaby (1994), any testing program that depends on wind-pollinated progenies for estimation of genetic parameters have to cautiously evaluate the factors that determine the relationships among the progeny.

The use of molecular marker technologies for parental analysis in breeding programs can provide a solution to these uncertainties about the coancestry coefficient, because they provide a mean to infer the relationship structure among the individuals (Blouin 2003; Lynch and Ritland 1999). Some of the preferred genetic markers for obtaining precise estimates of relatedness are microsatellites markers (Gerber et al. 2000; Moriguchi et al. 2005), either from the nuclear or the chloroplast genomes, because they usually display many alleles per locus (Lynch and Ritland 1999) and are co-dominant (Hardy 2003). In pines, chloroplast microsatellites are haploid and paternally inherited. Because they do not recombine, multiple chloroplast fragments can be combined in haplotypes providing a paternal marker ideal for pollen flow studies, as they allow direct identification of paternal gametes (Plomion et al. 2001; Robledo-Arnuncio et al. 2004)

The aim of this work is to estimate a mean value of the coancestry coefficient (θ) of the families present in a progeny trial originated from seed collected in a clonal seed orchard (CSO) and to study in what way deviations from the standard assumption of θ=0.125 (i.e., r=0.25) in open-pollinated progeny tests of *P. pinaster* would affect heritability estimation. It is also an objective of this study to show how differences in coancestry coefficient across the families evaluated in a progeny test would affect quantitative genetics estimations.

Material and methods

Plant material and common garden experiment

The progeny test used in this study belongs to a series replicated at three sites and established in 1987 (Aguiar et al. 2003). The trials included 46 open-pollinated families, originated from seed collected in the Escaroupim clonal seed orchard II (Aguiar 1993). This CSO includes 49 genotypes and was established by grafting in 1975–1980, but only 46 families were considered in the progeny test due to poor seed production in the rest. The ortets were obtained from plus trees selected in Mata Nacional de Leiria by the senior forester D.H. Perry in 1963/1964. The selection criteria used was based on volume, stem form, spiral grain, and branch habits. Details about the plus phenotypes selected and the scoring system employed are described in Perry and Hopkins (1967).

The 46 families were randomly replicated in eight blocks with eight trees per plot. In 2004 (age 17), wood samples were collected from a subset of 12 trees from every family in three blocks, giving a total number of 552 trees for evaluation of wood quality traits.

A subsample of 125 offspring from five families (25 offspring per family), representative of the same seed lot used in the establishment of the progeny trial, were genotyped in our study. These five families showed extreme and contrasting values of the inter-individual variance for different quantitative traits (Gaspar et al. 2008). In this way, i.e., by selecting families from the tails of the distribution for molecular analysis, we were able to test whether quantitative genetics estimates are affected by standard assumptions on sib relationship, with a limited genotyping effort.

DNA isolation and molecular markers

Total genomic DNA was isolated from needles following the Doyle and Doyle protocol (Doyle and Doyle 1990), with some modifications. Offspring were genotyped for five microsatellite markers: two chloroplast microsatellite loci (Pt87268 and Pt1254) and three nuclear microsatellites (Itph4516, Ctg275, and Ctg4363). These markers were chosen from previous studies (see de-Lucas et al. 2008) because of their high level of polymorphism and unambiguous scoring. The amplification conditions for the different molecular markers are described in Robledo-Arnuncio et al. (2004) (cpSSRs), González-Martínez et al. (2002) (Itph4516), and Chagné et al. (2004) (Ctg275 and Ctg4363).

Microsatellite fragments were scored in an ABI-PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using GeneScan ROX-500 as internal ladder and standard running parameters.

Data analyses

Mating system parameters

Single- and multilocus estimates of outcrossing rates (t_s and t_m, respectively) per family were computed using a moment method based only on nuclear microsatellites using MLTR (see Ritland 2002, for details). Confidence intervals were obtained by bootstrapping (1,000 bootstraps). The difference between multilocus and single-locus estimates of outcrossing ($t_m - t_s$) was used to estimate biparental inbreeding (i.e., inbreeding due to mating among relatives).

The percentage of full-sibs in each progeny was estimated by computing correlated paternity within families ($r_p=2\times F_{ij}$) following Hardy et al. (2004) and using SPAGeDi 1.2 (Hardy and Vekemans 2002). This method uses molecular markers to score progeny arrays from mothers with known genotypes. In a first stage, pollen gametes are inferred by discounting the mother genotype from the offspring diploid genotypes (see Hardy et al. 2004 for details on how to deal with the classical 'double heterozygote' issue). Then, in a second stage, coancestry among inferred pollen gametes within families is computed using Nason's relative kinship estimator (see Loiselle et al. 1995). Correlated paternity (r_p) was computed separately for chloroplast and nuclear markers and averaged, as in de-Lucas et al. (2008).

Quantitative genetic parameters

The traits assessed were ring density determined using X-ray densitometry procedures, as described in Gaspar et al. (2008) and diameter at 1.30 m (DBH).

Traits were analyzed using the following model:

$$Y_{ijk} = \mu + B_j + F_i + B \times F_{ij} + \varepsilon_{ijk}\,, \tag{1}$$

where Y represents the phenotypic individual observation; μ is the overall mean; B_j is the effect of the jth block (fixed); F_i is the effect of the ith family (random); $B \times F_{ij}$ is the effect of the interaction between the ith family and the jth block (random); and ε is the residual error. Variance components for family (σ^2_f), family-block interaction ($\sigma^2_{f\times b}$), and residual errors (σ^2_ε), with the respective associated standard errors, were estimated by restricted maximum likelihood, using the average information REML algorithm implemented in the ASREML program (Gilmour et al. 1998).

Narrow-sense heritability (h^2) was calculated for each trait as:

$$h^2 = \frac{\sigma^2_a}{\sigma^2_P}\,, \tag{2}$$

where σ^2_a represents the additive genetic variance and σ^2_P the total phenotypic variance. Total phenotypic variance was estimated as:

$$\sigma^2_P = \sigma^2_f + \sigma^2_{f\times b} + \sigma^2_\varepsilon\,, \tag{3}$$

and estimated additive variances as:

$$\sigma^2_a = {}^1\!/_{2\theta} \times \sigma^2_f\,, \tag{4}$$

where the coancestry coefficient (θ) was obtained from estimates of outcrossing rates and correlated paternity (obtained by molecular markers) as:

$$\theta = \frac{0.2500 \times \mathrm{fs} + 0.3335 \times \mathrm{sfs} + 0.1250 \times \mathrm{hs} + 0.2040 \times \mathrm{shs}}{\mathrm{fs} + \mathrm{sfs} + \mathrm{hs} + \mathrm{shs}}\,, \tag{5}$$

where fs is the number of full-sibs, sfs the number of self full-sibs, hs the number of half-sibs, and shs the number of self half-sibs (Squillace 1974). The number of full-sibs is estimated here directly from correlated paternity ($n \times r_p$, where n is the number of offspring considered). Given that outcrossing rates are not different from 100% (see "Results"), selfing can be considered negligible. Therefore, the number of half-sibs can be estimated as $1-r_p$ while the number of self full-sibs and self half-sibs would be zero.

Coancestry coefficients estimated in this way are similar to those computed directly for each family using nuclear markers (0.0773–0.1746 using Nason's approach, depending on the family), but they are more precise as this approach also incorporates the information obtained from chloroplast haplotypes (as in de-Lucas et al. 2008). Standard errors for heritability were estimated by ASREML using a Taylor series approximation (Gilmour et al. 1998).

Simulation study

The main objective of the simulation study was to analyze the impact on heritability estimates of different coancestry coefficients (estimated with molecular markers) of the families included in a progeny trial. We simulated 46 families with different structure (measured by the coancestry coefficient). Several scenarios were then simulated for populations of 46 families presenting different degrees of correlated paternity (between 5% and 60 % of full-sibs, corresponding to coefficients of relationship of 0.26–0.40) and coefficients of variation (1%, 10%, and 25%). Although the coefficient of relationship of open-pollinated families has a lower bound in 0.25, when estimated with molecular makers it can have lower values due to statistical error. Therefore, the range of the coefficients of variation for the coefficient of relationship included in the simulations does not take into account this lower bound. The value of correlated paternity of each simulated family was drawn from a normal distribution, considering the same average (simulated) value of correlated paternity and standard deviations according to assumed among-family coefficient of variation. For each scenario, we computed narrow-sense heritability (h^2), the mean coefficient of relationship of the 46 families, and the coefficient of variation for the coefficient of relationship.

Results

Average (over families) outcrossing rates were high, 1.063± 0.063, CI (95%) 0.992–1.200 and 0.911±0.039, CI (95%) 0.866–1.031 for multilocus and single-locus estimates, respectively. The high values obtained, in particular for the multilocus estimates of outcrossing (t_m=1.063±0.063), indicate absence (or at best a very low proportion) of selfed offspring. The apparent difference between single and multilocus outcrossing rates ($t_m - t_s = 0.152 \pm 0.067$) is related to biparental inbreeding (i.e., mating among relatives; Ritland 2002).

Estimates of correlated paternity within families varied between −0.0019 and 0.0738 with an average of 0.0418± 0.0273, CI (95%) 0.0174–0.0662 (Fig. 1). We did not find any trend between correlated paternity and the level of inter-individual variance shown for different quantitative traits (see Gaspar et al. 2008) by the families included in this study. Correlated paternity estimates were not significantly different, as judged by overlapping confidence intervals at 95% probability, to those obtained using other estimation methods such as the TwoGener approach [average of 0.0546±0.0297, CI (95%) 0.0290–0.0822; see description of the TwoGener method in Austerlitz and Smouse (2001) and Smouse et al. (2001)].

The effective number of males mating with a given mother tree was 24 ($N_{ep} = 1/r_p = 23.9$), which is about half the census number (49 genets). Given that similar numbers of effective males are typically found in large natural populations of the species (see de-Lucas et al. 2008), our results suggest either more even male contribution to sib families than normally expected in pine seed orchards or pollen contamination from a source outside the CSO. The mean value of the coancestry coefficient obtained considering 4% of full-sibs was 0.130, very close to the expected for half-sib progenies (0.125), with a coefficient of variation (based on estimates from molecular markers) of 2.5%.

Adjusted heritabilities and standard errors (used here as a rough approximation for inferring confidence intervals) calculated for different coancestry coefficients and associated coefficients of variation are shown for the two traits under study, diameter (DBH) and wood ring density, in Fig. 2 (it should be noted that the heritability scale is different in the two figures).

Differences between the unadjusted and adjusted heritability values were more pronounced in wood density—the

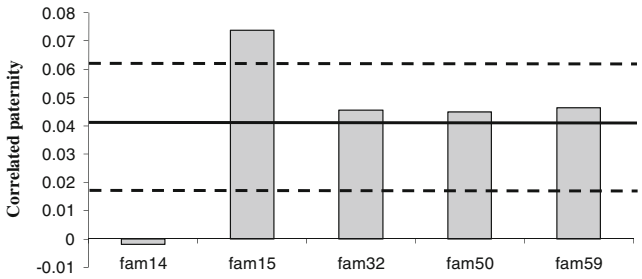

Fig. 1 Marker-based estimates of correlated paternity for each of five maritime pine families (as estimated by the approach of Hardy et al. (2004)); the average (*continuous line*) and 95% confidence intervals (*dashed lines*) are also indicated

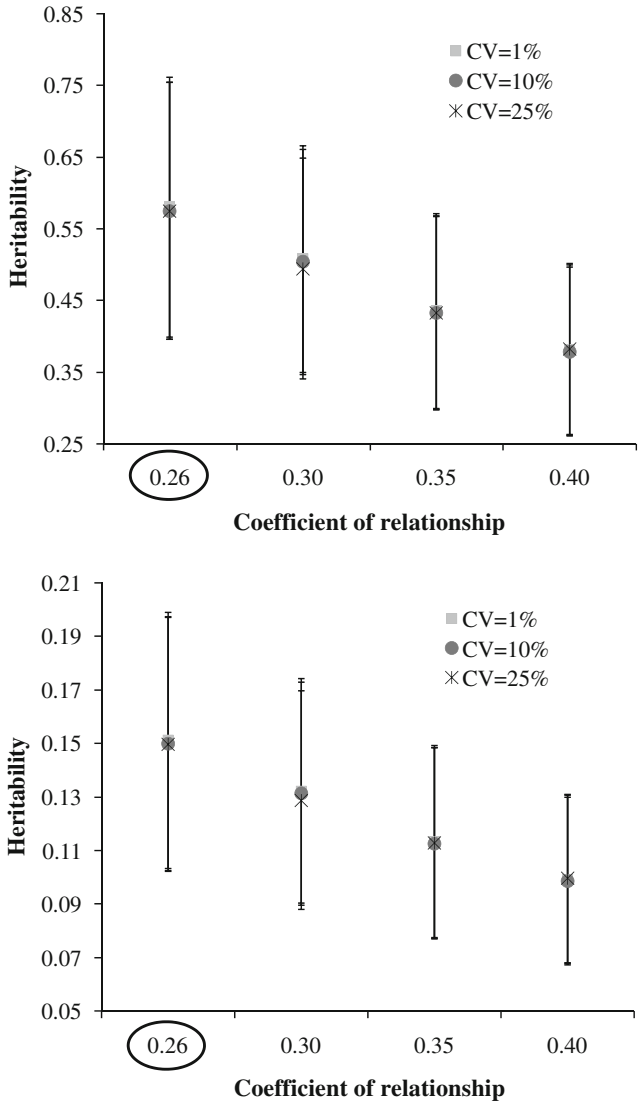

Fig. 2 Adjusted heritability and standard errors for different averages and among-family coefficients of variation (CV) of the relationship coefficient (*r*), for two quantitative traits: wood density (*top*) and diameter (*bottom*). The *circle* indicates the observed values for the coefficient of relationship in our study case

trait with higher heritability—(0.609 and 0.586, respectively) than in diameter (0.166 and 0.154, respectively), but they did not imply significant bias (bias<5%) in any of the two. The effect of the coefficient of variation was not noteworthy in any trait, indicating that it does not affect heritability estimates at all under the conditions analyzed in this experiment.

Discussion

Conifers are wind-pollinated and known to be predominantly outcrossers. Recent studies on the pine mating system indicate that, for most species, outcrossing rates are higher than 0.9 (de-Lucas et al. 2008; Fernandes et al. 2008; Robledo-Arnuncio et al. 2004; Wasieliwska et al. 2005). The multilocus outcrossing rate (t_m) of *P. pinaster* in this study, based on progenies from a SO, was 1.063±0.063 (with confidence intervals at 95% overlapping one), matching well those found in natural populations of the species. González-Martínez et al. (2003) found a value of outcrossing of approximately 0.96, based on natural regeneration (i.e., after seed germination and seedling establishment) in natural conditions. Similarly, de-Lucas et al. (2008) obtained an average (over 61 families from three populations) outcrossing rate of 0.977 based on germinated seeds (i.e., without including early natural selection). In addition, Fernandes et al. (2008), in a study performed in a *P. pinaster* CSO, concluded that the probability that a seed embryo sampled from a mother tree was derived from an outcrossing event was 90.1±2.3%. The absence of selfing found in our study was not surprising in spite of the self-fertilization rates of around 5% found in most seed orchards examined to date (Moriguchi et al. 2007). In fact, many of the seeds and seedlings produced by inbreeding have a lower viability than outcrossed individuals, so that the inbreds often remain undetected because of large-scale early mortality (Linhart 2000). In Pinus species, selection against inbreds is likely to be common at the seed stage (Ledig 1998). In *P. pinaster*, most lethal or sublethal alleles are probably eliminated during seed formation and germination as well as during the first growing season, so that the proportion of selfed offspring is expected to be very low in mature populations (González-Martínez et al. 2003). Durel et al. (1996), however, observed that *P. pinaster* survival rates after a first growing season in the nursery were the same, independently of the level of inbreeding.

Nevertheless, in cases where outcrossing is high, mating may still involve only a limited number of males or the contribution of a few males may be responsible for the majority of mating, leading to high values of correlated paternity (r_p) and, consequently, high coancestry coefficients among offspring of the same family. Several studies in different conifer species performed in seed orchards reported that contribution as pollen donor differs significantly among clones (El-Kassaby et al. 1984; Goto et al. 2002; Hansen and Kjaer 2006; Kumar et al. 2007; Moriguchi et al. 2005; Plomion et al. 2001). Possible explanations for this fact may include flowering asynchrony and differences in male flowering intensity and pollen competitive ability. In fact, Varela (1989), in a study of reproductive behavior performed in the same CSO as this study, observed flowering phenology asymmetry, which could have promoted substantial differences in the contribution of pollen donors. Fernandes et al. (2008), in a study performed in a CSO that has some families in common with our study, observed a male and female unbalanced

contribution to the progeny. In our study, a relatively low level of correlated mating (4–5%) was obtained, which is similar to the values obtained in natural populations of *P. pinaster* (de-Lucas et al. 2008). This result was not expected, given the low number of males available for mating in the CSO studied (only 49 genotypes) in comparison to the large natural populations studied by de-Lucas et al. (2008) and the typically uneven male contributions in pine seed orchards. However, according to Askew and El-Kassaby (1994), the intrusion of foreign pollen usually increases the effective size of the paternal population. The same authors note that wind-pollinated seed orchards dominated by effectively large foreign pollen pools would produce the 'idealized' seed crops of virtually all half-sib relationships. Numerous studies have reported the occurrence of high pollen contamination in conifer seed orchards (Adams et al. 1997; Kaya et al. 2006; Moriguchi et al. 2007; Plomion et al. 2001; Slavov et al. 2005). Fernandes et al. (2008), in a *P. pinaster* CSO installed side by side with the CSO from which the trees of the present trial originated, found gene immigration rates from outside the CSO of 52.4%. The very possible existence of pollen contamination may explain why the r_p value is so low in our case study and so very close to the natural-population values.

Our results are similar to those expected from half-sib families. This situation is not always the case when the seeds are collected in natural populations, as mating can differ among the different populations. In populations that are a source of plus trees, pedigrees are usually unknown, and it is assumed that all plus trees are genetically unrelated (Kumar and Richardson 2005). This lack of relatedness may not always be true, causing the occurrence of biparental inbreeding within the families in the following stages of the breeding program, as it may be the case in the present progeny trial. Comparison of multi-locus and single-locus outcrossing rates in our families revealed some amount of biparental inbreeding, although it probably does not contribute much of the inbreeding coefficient. In contrast, Fernandes et al. (2008) obtained a minimum estimate of biparental inbreeding of 21.7%. These authors note that it is a very high value considering the care taken in the selection of plus trees; however, as they were collected from the same provenance, the existence of some family relationship among different first selections is still a reasonable hypothesis.

The coancestry coefficient was calculated based on outcrossing rates (t_m) and correlated paternity (r_p) estimates from molecular markers. We did not find any trend in r_p values when comparing families showing extreme and contrasting values of the inter-individual variance for different quantitative traits. The average value obtained (θ=0.130) was not very different from that expected for half-sib progenies (0.125). The mean value of the genetic covariance coefficient of the families present in this progeny trial was then 0.260. Differences between the unadjusted and adjusted heritability values were more pronounced in wood density (0.609 and 0.586, respectively) than in diameter (0.166 and 0.154, respectively), but were not significantly biased (<5%). Nevertheless, a relatively low number of full-sibs (~10%; i.e., a covariance coefficient of 0.28) would be enough to produce heritability overestimations of about a 10%.

Attention should be given to the fact that, as the number of full-sibs increase, the value of heritability does not depend only on the additive variance but also on the dominance variance, so that the value of heritability would be further inflated. According to Borralho (1994), when dominance effects are large and selfing rates vary significantly among families, heritabilities can be substantially overestimated, especially for low heritability traits. On the other hand, bias does not appear to be important when dominance effects are small and heritabilities are moderate to high. This author also refers that the magnitude of the bias due to overdominance seems small compared with the potential bias from assigning a wrong genetic correlation among open-pollinated sibs.

The effect of the coefficient of variation (CV) of coancestry coefficients among families was not notable for either of the traits studied, indicating that even if larger variation among families was present, as far as the mean correlated paternity is correctly estimated (unbiased), increasing the number of families studied (for instance, from five to ten or more) would not, in our case, affect heritability estimates. The low importance of the CV in our results suggests that only under extreme conditions (high incidence of full-sib relationships) or extremely diverse values among families, the heritability estimates can be affected. Therefore, molecular markers, even scored only in a subsample of the families included in the test, can be a valuable tool in assessing mean coancestry coefficient for the estimate of quantitative genetic parameters in common garden experiments. We can conclude that in *P. pinaster* open-pollinated progeny tests (and probably in other species with a similar mating system), the associated error in heritability estimates due to the inclusion of full-sibs, when assuming a standard coefficient of relation among open-pollinated sibs of 1/4, is low and that this result is robust with respect to the number of families sampled with genetic markers, given unbiased estimates of average relationship among offspring within sib families.

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References

Adams WT, Hipkins VD, Burczyk J, Randall WK (1997) Pollen contamination trends in a maturing Douglas-fir seed orchard. Can J For Res 27:131–134

Aguiar A (1993) Análise da variação do crescimento em altura de descendências maternais de pinheiro bravo. Silva Lusitana 1:267–274

Aguiar A, Alemeida MH, Borralho N (2003) Genetic control of growth, wood density and stem characteristics of *Pinus pinaster* in Portugal. Silva Lusitana 11:131–139

Askew GR, El-Kassaby YA (1994) Estimation of relationship coefficients among progeny derived from wind pollinated orchard seeds. Theor Appl Genet 88:267–272

Austerlitz F, Smouse PE (2001) Two-generation analysis of pollen flow across a landscape. II. Relation between Phi(ft), pollen dispersal and interfemale distance. Genetics 157:851–857

Blouin M (2003) DNA-based methods for pedigree reconstruction and kinship analysis in natural populations. Trends in Ecology and Evolution 18:503–511

Borralho NMG (1994) Heterogeneous selfing rates and dominance effects in estimating heritabilities from open-pollinated progeny. Can J For Res 24:1079–1082

Chagné D, Chaumeil P, Ramboer A, Collada C, Guevara A, Cervera MT, Vendramin GG, Garcia V, Frigerio JMM, Echt C, Richardson T, Plomion C (2004) Cross-species transferability and mapping of genomic and cDNA SSRs in pines. Theor Appl Genet 109:1204–1214

de-Lucas AI, Robledo-Amuncio JJ, Hidalgo E, González-Martínez SC (2008) Matting system and pollen gene flow in mediterraneam maritime pine. Heredity 100:390–399

Doyle J, Doyle J (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Durel CE, Bertin P, Kremer A (1996) Relationship between inbreeding depression and inbreeding coefficient in maritime pine (*Pinus pinaster*). Theor Appl Genet 92:347–356

El-Kassaby YA, Ritland K (1986) The relation of outcrossing and contamination to reproductive phenology and supplemental mass pollination in a Douglas-fir seed orchard. Silvae Genet 35:240–244

El-Kassaby YA, Fashler AMK, Sziklai O (1984) Reproductive phenology and its impact on genetically improved seed production in a Douglas-fir seed orchard. Silvae Genet 33:120–125

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics. Longman Scientific & Technical edn, New York

Fernandes L, Rocheta M, Cordeiro J, Pereira S, Gerber S, Oliveira M, Ribeiro MM (2008) Genetic variation, mating patterns and gene flow in a *Pinus pinaster* Aiton clonal seed orchard. Ann For Sci 65:706

Gaspar MJ, Louzada JLPC, Silva ME, Aguiar A, Almeida MH (2008) Age trends in genetic parameters of wood density components in 46 half-sibling families of *Pinus pinaster* Ait. Can J For Res 38:1470–1477

Gerber S, Mariette S, Streiff R, Bodenes C, Kremer A (2000) Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. Mol Ecol 9:1037–1048

Gilmour A, Gogel B, Cullis B, Welham S, Thompson R (1998) ASREML users manual. VSN International, New south Wales Agriculture, Orange

González-Martínez SC, Gerber S, Cervera MT, Martínez-Zapater JM, Gil L, Alía R (2002) Seed gene flow and fine-scale structure in a Mediterranean pine (*Pinus pinaster* Ait.) using nuclear microsatellite markers. Theor Appl Genet 104:1290–1297

González-Martínez SC, Gerber S, Cervera MT, Martínez-Zapater JM, Alía R, Gil L (2003) Selfing and sibship structure in a two-cohort stand of maritime pine (*Pinus pinaster* Ait.) using nuclear SSR markers. Ann For Sci 60:115–121

Goto S, Miyahara F, Ide Y (2002) Identification of the male parents of half-sib progeny from Japanese black pine (Pinus thunbergii Parl.) clonal seed orchard using RAPD markers. Breeding Science 52:71–77

Hansen OK, Kjaer ED (2006) Paternity analysis with microsatellites in a Danish Abies nordmanniana clonal seed orchard reveals dysfunctions. Can J For Res 36:1054–1058

Hardy OJ (2003) Estimation of pairwise relatedness between individuals and characterization of isolation-by-distance processes using dominant genetic markers. Mol Ecol 12:1577–1588

Hardy OJ, Vekemans X (2002) SPAGEDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. Mol Ecol Notes 2:618–620

Hardy OJ, González-Martínez SC, Colas B, Fréville H, Mignot A, Olivieri I (2004) Fine-scale genetic structure and gene dispersal in *Centaurea corymbosa* (Asteraceae) II. Correlated paternity within and among sibships. Genetics 168:1601–1614

Kaya N, Isik K, Adams WT (2006) Mating system and pollen contamination in a *Pinus brutia* seed orchard. New Forests 31:409–416

Kumar S, Richardson TE (2005) Inferring relatedness and heritability using molecular markers in radiata pine. Molecular Breeding 15:55–64

Kumar S, Gerber S, Richardson TE, Gea L (2007) Testing for unequal paternal contributions using nuclear and chloroplast SSR markers in polycross families of radiata pine. Tree Genetics & Genomes 3:207–214

Ledig FT (1998) Genetic variation in Pinus. In: Richardson DM (ed) Ecology and Biogeography of Pinus. Cambridge University Press, Cambridge, pp 251–279

Linhart YB (2000) Variation in woody plants; molecular markers, evolutionary processes and conservation biology. In: Jain SM, Minocha SC (eds) Molecular biology of woody plants. Kluwer academic, Dordrecht, pp 341–373

Loiselle BA, Sork VL, Nason J, Graham C (1995) Spatial genetic-structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). Am J Bot 82:1420–1425

Lynch M, Walsh B (1997) Genetics and analysis of quantitative traits. Sinauer Associates, Massachusetts

Lynch M, Ritland K (1999) Estimation of pairwise relatedness with molecular markers. Genetics 152:1753–1766

Moriguchi Y, Tani N, Itoo S, Kanehira F, Tanaka K, Yomogida H, Taira H, Tsumura Y (2005) Gene flow and mating system in five *Cryptomeria japonica* D. Don seed orchards as revealed by analysis of microsatellite markers. Tree Genetics & Genomes 1:174–183

Moriguchi Y, Tsuchiya S, Iwata H, Itoo S, Tani N, Taira H, Tsumura Y (2007) Factors influencing male reproductive success in a Cryptomeria japonica seed orchard revealed by microsatellite marker analysis. Silvae Genet 56:207–214

Perry D, Hopkins E (1967) Importation of breeding material of *Pinus pinaster* Ait. from Portugal. For Dep W A Bulletin 75

Plomion C, LeProvost G, Pot D, Vendramin G, Gerber S, Decroocq S, Brach J, Raffin A, Pastuszka P (2001) Pollen contamination in a maritime pine polycross seed orchard and certification of improved seeds using chloroplast microsatellites. Can J For Res 31:1816–1825

Ritland K (2002) Extensions of models for the estimation of mating systems using n independent loci. Heredity 88:221–228

Robledo-Arnuncio JJ, Smouse PE, Gil L, Alia R (2004) Pollen movement under alternative silvicultural practices in native populations of Scots pine (Pinus sylvestris L.) in central Spain. Forest Ecol Manag 197:245–255

Slavov GT, Howe GT, Adams WT (2005) Pollen contamination and mating patterns in a Douglas-fir seed orchard as measured by simple sequence repeat markers. Can J For Res 35:1592–1603

Smouse PE, Dyer RJ, Westfall RD, Sork VL (2001) Two-generation analysis of pollen flow across a landscape. I. Male gamete heterogeneity among females. Evolution 55:260–271

Squillace AE (1974) Average genetic correlations among offspring from open-pollinated forest trees. Silvae Genet 23:149–156

Varela C (1989) Comportamento reprodutivo de *Pinus pinaster* Ait. num pomar clonal de sementes. Universidade Técnica de Lisboa, Lisbon, p 107

Wasieliwska M, LKlemm M, Burczyk J (2005) Genetic diversity and mating system of Scots pine plus trees. Dendrobiology 53:57–62

Wright JW (1976) Introduction to forest genetics. Academic, New York

Zobel BJ, Talbert J (1984) Applied forest tree Improvement. Wiley, New York