Nouvelles observations sur quelques acacias de l'Afrique occidentale. Rev. Bot. Appl. Agric. Trop. **14**: 875–887 (1934). — CIAFFI, M., LAFIANDRA, D., PORCEDDU, E. and BENEDETTELLI, S.: Storage protein variation in wild emmer wheat *(Triticum turgidum* ssp. *dicoccides)* from Jordan and Turkey. I. Electrophoretic characterization of genotypes. Theor. Appl. Genet. **86**: 474–480 (1993). — COETZEE, J. A.: The morphology of *Acacia* pollen. S. Afr. J. Sci. **52**: 23–27 (1955). — D'OVIDIO, R., LAFIANDRA, D. and PORCEDDU, E.: Identification and molecular characterization of a large insertion within the repetitive domain of high-molecular-weight glutenin subunit gene from hexaploid wheat. Theor. Appl. Genet. **93**: 1048–1053 (1996).— EL-TINAY, A. H., KARAMALLA, K. A., EL AMIN, H. M., SHIGIDI, M. T. A. and ISHAG, K. E. A.: Serotaxonomic studies on Sudan acacias. J. Exp. Bot. **30**: 607–615 (1979).— FARRIS, J. S.: Methods for computing WAGNER trees. Syst. Zool. **19**: 83–92 (1970). — FELSENSTEIN, J.: Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**: 783–791 (1985). — GHIMPU, M. V.: Contribution à l'étude chromosomique des acacias. C. R. Acad. Sci. (Paris) **188**: 1429–1341 (1929). — GUINET, P. and VASSAL, J.: Hypotheses on the differentiation of the major groups in the genus *Acacia (Leguminosae)*. Kew Bul. **32**: 509–527 (1978). — HÄGER, K. P., BRAUN, H., CZIHAL, A., MÜLLER, B. and BÄUMLEIN, H.: Evolution of seed storage protein genes: legumin genes of *Ginkgo biloba.* J. Mol. Evol. **41**: 457–466 (1995). — HAMANT, C., LESCANNE, N. and VASSAL, J.: Sur quelques nombres chromosomiques nouveaux dans le genre *Acacia.* Taxon **24**: 667–670 (1975).— HARRIER, L. A., WHITTY, P. W., SUTHERLAND, J. M. and SPRENT, J. I.: Phenetic investigation of non-nodulating African species of *Acacia* (Leguminosae) using morphological and molecular markers. Pl. Syst. Evol. **205**: 27–51 (1997).— HUNZIKER, J. H., SAIDMAN, B. O., NARANJO, C. A., PLACIOS, R. A. and BURGHARDT, A. D.: Hybridization and genetic variation of Argentine species of *Prosopis.* For. Ecol. Manag. **16**: 301–315 (1986). — KHAN, I. R.: Study of somatic chromosomes in some *Acacia* species and hybrids. Pakis. J. For. **1**: 326–341 (1951). — LANHAM, P. G., FORESTER, B. P. and MCNICOL, P.: Seed storage protein variation in *Arachis* species. Genome **37**: 487–496 (1994). — LEONARDI, A., DAMERVAL, C., HÉBERT, Y., GALLAIS, A. and DE-VIENNE, D.: Association of protein amount polymorphism (PAP) among maize lines with performance of their hybrids. Theor. Appl. Genet. **82**: 552–560 (1991). — MANDAL, A. K. and ENNOS, R. A.: Mating system analysis in a natural population of *Acacia nilotica* subspecies *kraussiana.* For. Ecol. Manag. **79**: 235–240 (1995). — MORAN, G. F.: Patterns of genetic diversity in Australian tree species. New Forests **6**: 49–66 (1992). — MURRAY, B. E., CRAIG, I. L. and RAJHATHY, T. A.: Protein electrophoresis study of three amphidiploids and eight species of *Avena.* Can. J. Genet. Cytol. **12**: 651–665 (1970). — NAVOT, N. and ZAMIR, D.: Isozyme and seed protein phylogeny of the genus *Citrullus (Cucurbitaceae).* Pl. Syst. Evol. **156**: 61–67 (1987). — OBALLA, P. O.: Polyembryony in *Acacia karroo* HAYNE: insights from isozyme analysis. Afr. J. Ecol. **34**: 94–97 (1996). — PALMER, J. D., JANSEN, R. K., MICHAEL, H. J., CHASE, M. W. and MANHART, J. R.: Chloroplast DNA evolution and plant phylogeny. Ann. Missouri Bot. Gard. **75**: 1180–1206 (1988). — PAYNE, P. I.: Genetics of wheat storage proteins and the effect of allelic variation on bread making quality. Ann. Rev. Pl. Physiol. **38**: 141–153 (1987). — PLAYFORD, J., APPEL, R. and BAUM, B. R.: The 5S DNA units of *Acacia* species (Mimosaceae). Pl. Syst. Evol. **183**: 235–247 (1992). — PLAYFORD, J., BELL, J. C. and MORAN, G. F.: A major disjunction in genetic diversity over the geographic range of *Acacia melanoxylon* R. Br. Aust. J. Bot. **41**: 355–368 (1993). — REN, S. X., MCINTOCH, R. A., SHARP, P. J. and THE, T. T.: A storage protein marker associated with the suspensor of Pm8 for powdery mildew resistance in wheat. Theor. Appl. Genet. **93**: 1054–1060 (1996). — ROBBERTSE, P. J.: The genus *Acacia* in South Africa.I. Stipules and spines. Bothalia **11**: 473–479 (1975a). — ROBBERTSE, P. J.: The genus *Acacia* in South Africa. IV. The morphology of the mature pod. Bothalia **11**: 481–489 (1975b).— ROBBERTSE, P. J.: The genus *Acacia* MILLER in South Africa. 6. The morphology of the leaf. Boissiera **24**: 263–270 (1975c). — ROSS, J. H.: A conspectus of the African *Acacia* species. Botanical Research Institute. Memoirs of the Botanical Survey of South Africa **44**: 1–155 (1979). — ROSS, J. H.: An analysis of the African *Acacia:* their distribution, possible origins and relationships. Bothalia **13**: 389–413 (1981). — SAHNI, K. S.: Important trees of the Northern Sudan. United Nations Development Programme and FAO. Khartoum University Press, Sudan. 138 pp. (1968).— SAIDMAN, B. O., VILARDI, J. C., POCOVI, M. I. and ACRECHE, N.: Genetic divergence among species of the section *Strombocarpa,* genus *Prosopis (Leguminosae).* J. Genet. **75**: 139–149 (1996). — SHEWRY, P. R., BRADBERRY, D., FRANKLIN, J. and WHITE, R. P.: The chromosomal location and linkage relationships of the structural genes for the prolamine storage protein (secalins) of rye. Theor. Appl. Genet. **69**: 63–69 (1984). — SOLBRIG, O. T. and CANTINO, P. D.: Reproductive adaptation in *Prosopis.* J. Arnold Arbor. **56**: 185–210 (1975). — SWOFFORD, D. L.: PAUP Phylogenetic analysis using parsimony. Version 3.1.1. for Apple MacIntosh, manual and computer program. Illionois Natural History Survey Champaign, Illionois, USA (1993). — VASSAL, J.: Contribution à l'étude de la morphologie des plantules d'*Acacia*, acacias africains. Bull. Soc. Hist. Nat. Toul. **105**: 55–111 (1969). — VASSAL, J.: Apport des recherches ontogéniques et séminologiques à l'étude morphologique, taxonomique et phylogénique du genre *Acacia.* Bull. Soc. Hist. Nat. Toul. **108**: 125–247 (1972). — VASSAL, J.: Histologie comparée de téguments séminaux dans quelques espèces d'acacias africains. Boissiera **24**: 285–297 (1975). — VASSAL, J. and LESCANNE, N.: Cytologie et taxonomie dans le genre *Acacia.* Bull. Soc. Hist. Nat. Toul. **112**: 101–110 (1976).

Genetic Parameters Estimated From a Wild Cherry Diallel: Consequences for Breeding

By H. MURANTY[1]), N. SCHERMANN, F. SANTI[2]) and J. DUFOUR

Institut National de la Recherche Agronomique, Station d'Amélioration des Arbres Forestiers, F-45160 Ardon, France

(Received 11th November 1997)

Summary

Height, girth at breast height and susceptibility to cherry leaf spot were measured periodically up to seven years at three plantations of a 14-parent half diallel of wild cherry. Variance components were interpreted according to an additive-dominance genetic model and used to estimate potential gains from selection under various selection and deployment strategies. Height increment over five growing seasons, girth increment over two growing seasons and susceptibility to cherry leaf spot had moderate to high narrow and broad sense heritabilities. For these traits, the ratio of additive to total genetic variance was higher than 0.60 in the three studied tests. Genotype by site interaction was quite high for stem height increment but was low or null for girth increment and susceptibility to cherry leaf spot. Height increment and girth increment were highly genetically correlated with each other as well as with susceptibility to cherry leaf spot. Clonal means of the parents of the diallel were generally well correlated with their general combining abilities, so that gains from selection in clonal tests of parents for clonal seed orchards and for the next breeding

[1]) Present adress: Institut National de la Recherche Agronomique, Station d'Amélioration des Plantes, Domaine de la Motte, BP 29, F-35650 Le Rheu, France

[2]) Corresponding author for reprint request is F. SANTI, Institut National de la Recherche Agronomique, Station d'Amélioration des Arbres Forestiers, F-45160 Ardon, France

population should be significant : in this study, expected relative genetic gains were estimated to be between 8% and 37% of the trait mean, depending on the trait and site considered.

Key words: Prunus avium, heritability, general combining ability, specific combining ability, diallel, genetic gain, genetic correlation.

FDC: 165.3; 165.441; 181.65; 232.13; 440; 172.8 *Phloesporella padi;* 176.1 *Prunus avium*; (44).

Introduction

Wild cherry (*Prunus avium* L.) is currently more and more planted in Europe, both in view of forest enrichment and afforestation of abandoned farm land, because of its fast growth (rotation of 50 to 60 years) and its valuable wood, employed for panelling and cabinetmaking. In these plantations, intensive silviculture is generally practised, involving large planting spacing, weed and pest control, pruning, and, if necessary, protection against rodents and game.

The tree improvement program led at INRA for wild cherry began in 1978 with phenotypic selection of plus-trees in France. Altogether, around 300 of the collected clones have been tested in appropriate multisite designs. Eight clones out of 54 from the oldest plantations were selected in 1994 on vigour with a constraint of no change in branch thinness and these clones received a certification. In percentage of the plantation means, expected genetic gain from this selection are 11% for height growth, 13% for girth growth, and 31% to 33% for cherry leaf spot resistance (SANTI et al., 1998). The choice of clones to plant in a seed orchard or to combine in order to create a new breeding population for recurrent selection should be based on their combining abilities. The general combining abilities of the INRA clones have never been tested but the clonal performances, which are already known or currently tested, could be good predictors of general combining abilities. In this context, genetic parameters such as the importance of additive genetic variance in total genetic variance and the correlation between clonal values and general combining abilities should be measured.

As far as we know, no results have been published about additive genetic variance in wild cherry. Many studies on other forest trees, particularly conifers, have reported significant amounts of additive genetic variance with varying proportions of dominance genetic variance : the ratio of dominance to additive variance was not consistent throughout species (e.g. see VAN WYK, 1976; FOSTER and BRIDGWATER, 1986; COTTERILL et al., 1987; KING et al., 1988; PICHOT and TEISSIER DU CROS, 1989; SAMUEL, 1991; RAJORA et al., 1994; BOUVET and VIGNERON, 1995; YANCHUK, 1996; SCHERMANN et al., 1996).

This study reports the first results on additive and dominance genetic variances for growth, disease resistance and form traits in wild cherry; correlation between clonal value and general combining abilities for these traits are estimated in order to evaluate adequacy of clonal selection for seed orchard establishment and recurrent breeding program.

Material and Methods

Plant material and sites

Fourteen wild cherry clones, belonging to the INRA collection and originating from the northern part of France, were crossed in spring 1986 following a half-diallel mating design. The seeds were sown in March 1987 and cuttings were taken from the seedlings in June. The seedlings (ortets), their cuttings (ramets) and cuttings of the parent clones were planted in nursery and cut back during winter 1987/1988. All the plants were grown in the nursery during the 1988 growing season.

Cuttings of the parent clones and ortets or ramets of their progenies (86 out of the potential 91 families of the half-diallel) were planted in three sites in France (*Table 1*). In each site, the parents and the progenies were intermixed in a common incomplete bloc design, with three tree non-contiguous plots. In Beaumont en Beine, ortets of the seedlings were planted whereas in Val de la Nef, the vegetative copies of these ortets were planted. In Beaumont en Beine and Val de la Nef, 30 individuals per family were planted as often as possible to fit

Table 1. – Characteristics of test sites, description of study material, experimental design and ages of measurements.

Site	Beaumont en Beine	Val de la Nef	Baule
Lat	49°41'	47°38'	47°50'
Long	3°08'E	3°54'E	1°40'E
Annual rain fall (mm)	665	800	635
Potentialities	Former poplar grove; deep sandy silt, favourable	Forest site; clay silt, variable depth, sometimes only 35 cm.	Former farm land; deep silt-clay, favourable
Winter of plantation	1988-1989	1988-1989	1989-1990
Material			
number of parent clones	14	11	8
number of ramets per parent clone	15-30	12-18	9-18
number of families	85	86	33
number of individual per family	9-30	3-30	9-18
number of ortet-ramet couples	104	103	0
initial total number of trees	3600	2700	853
Design			
number of blocks	23	20	6
planting spacing	4 m x 4 m	4 m x 4 m	4,5 m x 4,5 m
Measurements at (age from plantation)			
Height	1, 2, 3, 5, 7	0, 1, 2, 5, 7	0, 1, 3, 4, 5, 6
girth at breast height	5, 7	5, 7	4, 6
leaf spot susceptibility*	5	5	4

scoring scale : 1 = almost no attack; 2 = less than 10 spots per leaf; 3 = 10 to 100 spots per leaf, few fallen leaves; 4 = more than 100 spots per leaf, many fallen leaves; 5 = almost all leaves fallen.

the recommendation of COTTERILL and JAMES (1984) and to take mortality, thinning and non measurable trees into account. Border rows and unfavourable zones were planted with plants belonging to families with large numbers of seedlings. Among these plants, about 100 ortet-ramet couples were planted contiguously. In Baule, ortets not planted in Beaumont en Beine were used in the design. These ortets belonged to progenies corresponding to a 10 parent half-diallel, with some missing families. The borders were planted mainly with individuals of families not represented in the main design. Plant material and experimental designs are described with more details in *table 1*.

Observations

From periodical measures of height and girth at breast height, we calculated all height increments between successive measurements in each site, a large height increment from the years 2 to 7 in Beaumont and Val de la Nef and from the years 1 to 6 in Baule, and the girth increment between the two measures. During summer 1993, susceptibility to cherry leaf spot, a disease caused by the fungus *Phloesporella padi*, was evaluated in the three sites, on a 1 to 5 scale (*Table 1*).

Data analysis

In a first step, data adjustment for within-site environmental effects through blocking and through an iterative moving average method (PAPADAKIS, 1937; PICHOT, 1993) were compared to each other on the basis of residual variances. The iterative moving average method, adjusting for spatial microsite effects within blocks, led to lower residual variances for most of the traits and was used for all the data.

After this adjustment, analysis of variance was performed separately for parent clones, half-diallel families and ortet-ramet couples. For each type of material, data were analysed on a site by site basis and in a multisite way. The statistical package Splus (1993, 1996) and the Modli software (KOBILINSKY, 1990) were used for these analyses.

For the half-diallel families, the model for each variable in monosite analysis was

$$Y_{ijk} = \mu + gca_i + gca_j + sca_{ij} + e_{ijk}$$

where Y_{ijk} is the adjusted phenotypic value of individual k of family ij, μ the overall mean, gca_i (gca_j) the random general combining ability (GCA) effect of parent i (j), with variance, σ^2_{gca}, sca_{ij} the random specific combining ability (SCA) effect of parents i and j, with variance σ^2_{sca} and e_{ijk} is the residual random error, with variance $\sigma^2_{w(f)}$ in this half-diallel family model. Potential reciprocal effects could not be estimated with our data (no reciprocal crosses) and were assumed negligible. The ANOVA corresponding to the model was performed according to GARRETSEN and KEULS adaptation of the HENDERSON III method (KEULS and GARRETSEN, 1977; GARRETSEN and KEULS, 1978), with a function developed under Splus.

For the multisite analysis, the model was

$$Y_{ijk} = \mu + si_i + fa_j + si.fa_{ij} + e_{ijk}$$

where Y_{ijk} is the adjusted phenotypic value of individual k of family j in site i, μ the overall mean, si_i the fixed effect of site i, fa_j the random effect of family j, with variance σ^2_{fa}, $si.fa_{ij}$ the random interaction effect between site i and family j, with variance $\sigma^2_{si.fa}$ and e_{ijk} is the residual random error, with variance $\sigma^2_{w(f)}$. Ecovalences (WRICKE, 1965) were calculated as

$$E_i = \left[\sum_j n_{ij} \left(Y_{ij\bullet} - Y_{i\bullet\bullet} - Y_{\bullet j\bullet} + Y_{\bullet\bullet\bullet} \right)^2 \right] \Big/ n_i$$

for sites and

$$E_j = \left[\sum_i n_{ij} \left(Y_{ij\bullet} - Y_{i\bullet\bullet} - Y_{\bullet j\bullet} + Y_{\bullet\bullet\bullet} \right)^2 \right] \Big/ n_j$$

for families, where n_{ij} is the number of individuals measured in site i for family j, n_i is the number of individuals measured in site i and n_j the number of individuals measured for family j.

For parent clones, the model for each variable in monosite analysis was

$$Y_{ij} = \mu + cl_i + e_{ij}$$

where Y_{ij} is the adjusted phenotypic value of ramet j of parent clone i, μ the overall mean, cl_i the random effect of clone i, with variance σ^2_{cl} and e_{ij} is the residual random error, with variance $\sigma^2_{e(cl)}$ in this clonal model.

In the multisite analysis, the model for each variable was

$$Y_{ijk} = \mu + si_i + cl_j + si.cl_{ij} + e_{ijk}$$

where Y_{ijk} is the adjusted phenotypic value of ramet k of parent clone j in site i, μ the overall mean, si_i the fixed effect of site i, cl_j the random effect of clone j, with variance σ^2_{cl}, $si.cl_{ij}$ the random interaction effect between site i and clone j, with variance $\sigma^2_{si.cl}$ and e_{ijk} is the residual random error, with variance $\sigma^2_{e(cl)}$. Ecovalences of sites and clones were calculated (WRICKE, 1965).

After all the analyses of variance, the verification of the postulates was examined graphically (plot of residual vs. estimated values, QQ-plots of residuals values). The plots showed that the postulates of analysis of variance were well verified for all traits in all sites.

In Beaumont en Beine and Val de la Nef, the comparison of ortets and ramets of the seedlings planted in buffer zones was based on a paired t-test.

Estimation of genetic parameters

Clonal variances and covariances were firstly considered as estimates of total genetic variances and covariances. The variances were used to estimate broad sense heritabilities in each site as

$$h^2_{bs} = \frac{\sigma^2_{cl}}{\sigma^2_{cl} + \sigma^2_{e(cl)}} \cdot$$

Other estimates of total genetic variances and covariances were then obtained from σ^2_{gca} and σ^2_{sca}. Assuming no inbreeding of the parents and no epistasis, the classical genetic model results in equations relating additive and dominance variances and covariances to between families variances and covariances as $covHS = \frac{1}{4}\sigma^2_A$ and $covFS = \frac{1}{2}\sigma^2_A + \frac{1}{4}\sigma^2_D$. The between families variances and covariances can also be expressed as $covHS = \sigma^2_{gca}$ and $covFS = 2\sigma^2_{gca} + \sigma^2_{sca}$ according to the diallel mating design. Hence, additive and dominance variances and covariances were derived from σ^2_{gca} and σ^2_{sca} as $\sigma^2_A = 4\sigma^2_{gca}$ and $\sigma^2_D = 4\sigma^2_{sca}$ and estimates of total genetic variances and covariances were calculated as $\sigma^2_A + \sigma^2_D = 4\sigma^2_{gca} + 4\sigma^2_{sca}$. These estimates were used to calculate other estimates of broad sense heritabilities as, where the denominator is the total phenotypic variance.

$$h^2_{bs} = \frac{4\sigma^2_{gca} + 4\sigma^2_{sca}}{2\sigma^2_{gca} + \sigma^2_{sca} + \sigma^2_{w(f)}}$$

Narrow sense heritabilities were calculated as

$$h^2_{ns} = \frac{4\sigma^2_{gca}}{2\sigma^2_{gca} + \sigma^2_{sca} + \sigma^2_{w(f)}} \cdot$$

Between site genotypic correlations (r_G), at the family and clonal levels, were deduced from correlations of family and clonal means between site ($r_{\bar{x}}$). Indeed,

$$r_{\bar{X}} = \frac{\mathrm{cov}(\bar{X}_1, \bar{X}_2)}{\sqrt{\sigma^2(\bar{X}_1)\sigma^2(\bar{X}_2)}} = \frac{\mathrm{cov}_G(X_1, X_2)}{\sqrt{\sigma^2_G(X_1)\sigma^2_G(X_2)}} \sqrt{\frac{\sigma^2_G(X_1)\sigma^2_G(X_2)}{\sigma^2(\bar{X}_1)\sigma^2(\bar{X}_2)}} = r_G \sqrt{h^2(\bar{X}_1)h^2(\bar{X}_2)}\,.$$

The first equality is obtained because the environmental covariance between the means is zero and the second one by replacing

$$\frac{\sigma^2_G(X_i)}{\sigma^2(\bar{X}_i)} \text{ by } h^2(\bar{X}_i)\,,$$

which is the heritability of the means (GALLAIS, 1990). These heritabilities of the means can be simply deduced from the F ratios obtained in the ANOVA. Indeed,

$$h^2(\bar{X}_i) = \frac{\sigma^2_G(X_i)}{\sigma^2_G(X_i) + \frac{1}{k}\sigma^2_E(X_i)}\,,$$

whereas the F ratio is the ratio of the genotypic mean square $MS_G(X_i)$ against the error mean square $MS_e(X_i)$ and the expectations of these mean squares are $E(MS_G(X_i)) = \sigma^2_E(X_i) + k\sigma^2_G(X_i)$ and $E(MS_e(X_i)) = \sigma^2_E(X_i)$.

Hence, an estimator of $h^2(\bar{X}_i)$ is

$$\frac{F(X_i) - 1}{F(X_i)}\,.$$

Finally, the between site genotypic correlations can be calculated as

$$r_G = \frac{r_{\bar{X}}}{\sqrt{\frac{F(X_1) - 1}{F(X_1)} \times \frac{F(X_2) - 1}{F(X_2)}}}\,.$$

Expected genetic gains

Three alternative selection and deployment schemes were compared on the basis of expected genetic gains:

(i) Selection of best parents based on their breeding values (GCA), estimated with open-pollinated half-sib families, which are much simpler to obtain than half-sib families in a diallel mating design, followed by seed production in a clonal seed orchard; for this selection method, the expected genetic gain can be calculated as

$$\Delta G_1 = 2 \times i_{GCA-SO} \frac{\sigma^2_{gca}}{\sqrt{\sigma^2_{gca} + \left(\sigma^2_{gca} + \sigma^2_{sca} + \sigma^2_{w(f)}\right)/n}}$$

where i_{GCA-SO} is the standardized selection differential corresponding to the selection rate of the best parents applied in this scheme, and n is the number of offspring per half-sib family. We considered that the results obtained from the half-diallel mating design could be used to predict genetic parameters concerning open-pollinated half-sib families.

(ii) Selection of the best tested clones, followed by seed production in a clonal seed orchard; for this selection method, the expected genetic gain can be calculated as

$$\Delta G_2 = 2 \times i_{cl-SO} \frac{\frac{1}{2}\sigma^2_A}{\sqrt{\sigma^2_{cl} + \sigma^2_{e(cl)}/r}}$$

where i_{cl-SO} is the standardized selection differential corresponding to the selection rate of the best parents applied in this scheme, and r is the number of ramets per clone.

(iii) Selection of the best tested clones followed by clonal plantations; for this selection method, the expected genetic gain can be calculated as

$$\Delta G_3 = i_{cl-cl} \frac{\sigma^2_{cl}}{\sqrt{\sigma^2_{cl} + \sigma^2_{e(cl)}/r}}$$

where i_{cl-cl} is the standardized selection differential corresponding to the selection rate applied in this scheme.

Jackknife standard errors

Standard errors of the parameter estimates (heritabilities, proportion of additive genetic variance and expected genetic gains) were obtained through a jackknife method : the data sets were divided in subsets, the parameters were estimated from the data remaining after one subset had been deleted, and this process was repeated until each subset had been deleted once; the estimates obtained from the resampled data were then used as exposed in KNAPP et al. (1989) to calculate the jackknife variances and standard errors. For the half-diallel data of each site, the subsets consisted of all replications of one full-sib family from one incomplete block as suggested by KNAPP and BRIDGES (1988); for the parent clone data, the subsets consisted of all replications of one clone from one incomplete block.

Results

Survival and grand means

The mortality rate was very low in the three sites, from 0.5% in Baule to 2.25% in Val de la Nef. The proportion of non-measurable trees was always less than 1.5% for all growth traits except the first girth measurement in Val de la Nef where it was 4% (too small trees). It was between 2.3% to 4% for cherry leaf spot susceptibility.

Growth was very variable between sites, reflecting fertility differences (*Figure 1*). Cherry leaf spot attack was more important in Val de la Nef (mean score 3.1) than in Baule (2.6) and Beaumont en Beine (2.2).

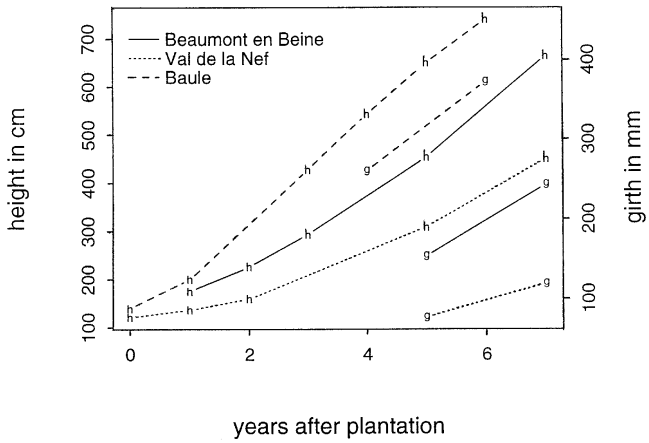


Figure 1. – Mean height (h) and girth (g) growth in the three test sites.

Analysis of variance site per site

GCA effect was significant at 5% level for almost all traits, but was not significant for initial height in Baule (*Table 2*). Significance for SCA effect was very variable. Parent clone effect was significant at 5% level, except for stem height increment

from one to two years after plantation in Beaumont en Beine and for stem height increment from plantation to one year after plantation in Val de la Nef. Coefficients of variation of residuals were generally slightly higher for families than for clones, due to the genetic variability within the families. These coefficients were particularly high for the first stem height increments obtained in the three sites (0.32 to 0.55), reflecting the plantation crisis.

Table 2. – F statistics of analysis of variance and residual coefficients of variation for each trait.

TRAITS	year	Fgca	Fsca	CVfa	Fcl	CVcl
		Beaumont en Beine				
degrees of freedom		13	71	2118	13	323
initial height	1	5.2**	1.4**	.13	4.2**	.15
stem height increment	2-7	11.4**	1.6**	.13	12.8**	.11
girth	5	6.0**	1.3ns	.20	5.9**	.19
	7	12.1**	1.9**	.18	16.7**	.16
girth increment	5-7	15.6**	3.1**	.21	32.3**	.18
leaf spot susceptibility	5	18.8**	1.9**	.37	29.1**	.31
		Val de la Nef				
degrees of freedom		13	72	1683	10	159
initial height	0	8.2**	3.8**	.24	4.1**	.21
stem height increment	2-7	7.1**	1.5*	.18	6.9**	.15
girth	5	4.6**	2.0**	.27	4.8**	.26
	7	12.9**	1.8**	.26	14.0**	.22
girth increment	5-7	27.2**	1.6**	.32	38.0**	.24
leaf spot susceptibility	5	22.0**	1.7**	.24	25.3**	.17
		Baule				
degrees of freedom		9	23	375	7	90
initial height	0	1.1ns	2.5**	.22	12.6**	.25
stem height increment	1-6	7.3**	1.9**	.10	34.5**	.07
girth	4	5.5**	1.7*	.10	19.9**	.08
	6	9.5**	1.5ns	.09	24.9**	.07
girth increment	4-6	10.6**	1.0ns	.16	5.4**	.17
leaf spot susceptibility	4	15.7**	1.3ns	.21	29.8**	.17

ns, *, ** : not significant, significant at 5%, 1% levels, resp. CVfa : within family residual coefficient of variation; CVcl : clonal residual coefficient of variation.

Heritabilities and proportions of additive genetic variance site per site (Table 3)

Narrow sense heritability (h^2_{ns}) for the large height increment was slightly higher in Baule (0.48) than in Beaumont en Beine (0.20) and Val de la Nef (0.15). h^2_{ns} were generally higher for girth and girth increment than for stem height. Moreover, h^2_{ns} for the first girth measurements were lower (0.08 to 0.34) than the h^2_{ns} for the second girth measurements (0.24 to 0.52) and for the girth increment (0.42 to 0.52). h^2_{ns} for cherry leaf spot score was always high, from 0.37 in Beaumont en Beine to 0.67 in Baule.

Broad sense heritabilities (h^2_{bs}) were first calculated with family data. For initial height h^2_{bs} was low in Beaumont en Beine (0.10) but quite high in Baule (0.46) and Val de la Nef (0.75). The girth increment showed higher h^2_{bs} in Beaumont en Beine and Val de la Nef (0.68 and 0.62, resp.) than in Baule (0.37). h^2_{bs} for cherry leaf spot was the lowest in Beaumont en Beine (0.47, vs. 0.58 and 0.74 in the other sites) where the attack was the less important.

Broad sense heritabilities were then calculated with the parent clone data. The estimates obtained were generally similar to the corresponding estimates calculated with family data. The jackknife standard errors of the heritability estimates showed that most of the observed differences were probably not significant. The only significant difference was the great difference observed for initial height in Val de la Nef: 0.75 with family data vs. 0.31 with parent clone data.

Proportion of additive genetic variance, as expressed by

$$\frac{\sigma^2_A}{\sigma^2_G},$$

ranged from 0.58 to 0.69 over the three sites for the large stem height increment. It was quite variable for girth (0.39 to 0.84) and high for the girth increment (0.65 to 1.). For cherry leaf spot susceptibility, it was high in the three sites (from 0.78 to 0.89).

Table 3. – Heritabilities, proportions of additive variance in total genetic variance and correlation coefficients between GCA and clonal effect (jackknife standard errors).

TRAITS	year	σ^2_A/σ^2_G		r(GCA -CL)	h^2_{ns}		h^2_{bs} (fa)		h^2_{bs} (cl)	
		Beaumont en Beine								
initial height	1	.63	(0.24)	.47	.08	(0.02)	.10	(0.05)	.24	(0.04)
stem height increment	2-7	.69	(0.12)	.81	.20	(0.03)	.29	(0.05)	.53	(0.06)
girth	5	.70	(0.27)	.37	.08	(0.02)	.11	(0.04)	.29	(0.05)
	7	.67	(0.11)	.89	.24	(0.03)	.35	(0.05)	.61	(0.06)
girth increment	5-7	.65	(0.07)	.94	.44	(0.04)	.68	(0.06)	.81	(0.06)
leaf spot susceptibility	5	.78	(0.07)	.93	.37	(0.04)	.47	(0.05)	.77	(0.05)
		Val de la Nef								
initial height	0	.45	(0.06)	.62	.34	(0.04)	.75	(0.07)	.31	(0.07)
stem height increment	2-7	.58	(0.16)	.76	.15	(0.03)	.24	(0.06)	.48	(0.09)
girth	5	.39	(0.12)	.48	.11	(0.03)	.27	(0.06)	.35	(0.08)
	7	.70	(0.10)	.90	.30	(0.04)	.43	(0.06)	.71	(0.08)
girth increment	5-7	.84	(0.06)	.93	.52	(0.04)	.62	(0.05)	.91	(0.05)
leaf spot susceptibility	5	.79	(0.07)	.93	.46	(0.04)	.58	(0.05)	.86	(0.06)
		Baule								
initial height	0	-		-	-		.46	(0.29)	.70	(0.13)
stem height increment	1-6	.66	(0.18)	.91	.48	(0.11)	.69	(0.16)	.94	(0.06)
girth	4	.70	(0.25)	.86	.34	(0.10)	.43	(0.20)	.86	(0.08)
	6	.84	(0.19)	.91	.52	(0.11)	.59	(0.16)	.90	(0.08)
girth increment	4-6	1	(0.32)	.95	.42	(0.10)	.37	(0.13)	.43	(0.19)
leaf spot susceptibility	4	.89	(0.14)	.93	.67	(0.10)	.74	(0.13)	.91	(0.10)

– : non significant effect

Expected genetic gains

The genetic parameter estimates were also used to calculate expected genetic gains under three alternative strategies (*Table 4*). Expected genetic gains after selection on GCA and seed production in clonal seed orchard are always lower than those expected after selection on clonal value, and the difference between these gains varies between 1% and 11% of the trait mean. Further gains, of 12% to 88% of the trait mean, are usually expected from clonal deployment after clonal selection.

Table 4. – Expected relative genetic gains, expressed in percent of each trait mean, under three selection and depolyment strategies. Estimates obtained in three sites (jackknife standard errors).

Deployment	Clonal seed orchard			Clones	
Method of test	GCA[1]		clone[2]	clone[2]	
Selection rate	30%		10%	3.3%	
Beaumont en Beine					
stem height increment 2-7	6	(1)	8	26	(2)
girth increment 5-7	17	(1)	23	69	(4)
leaf spot susceptibility 5	25	(2)	31	110	(5)
Val de la Nef					
stem height increment 2-7	6	(1)	13	31	(5)
girth increment 5-7	28	(2)	37	125	(8)
leaf spot susceptibility 5	19	(1)	30	73	(4)
Baule					
stem height increment 1-6	8	(1)	9	40	(3)
girth increment 4-6	12	(2)	21	33	(6)
leaf spot susceptibility 4	23	(3)	31	86	(9)

[1]) 30 offspring per open-pollinated half-sib family
[2]) 10 ramets per clone

Genetic correlations

As shown in *table 3*, the correlation between GCA and clonal effects was generally high for vigour related traits : from 0.76 to 0.91 for the large stem height increment in the three sites, and even higher than 0.9 for girth increment (0.93 to 0.95) and cherry leaf spot susceptibility (0.93 in the three sites). The good ellipsoid shape of point clouds corresponding to these correlations was verified graphically.

The main additive and total genetic correlations are listed in *table 5*. Initial height was rarely and then moderately correlated with the other traits. The large stem height increment and the girth increment were highly correlated in the three sites (0.56 to 0.78). Cherry leaf spot susceptibility was highly and negatively correlated with the large stem height increment and the girth increment in Beaumont en Beine and Val de la Nef (-0.53 to -0.90) but was not correlated with these traits in Baule (0.18 to -0.17).

Comparison of ortets and ramets of seedlings within Beaumont en Beine and Val de la Nef

The mean difference between ortets and ramets was significant only for the stem height increments from 3 to 5 years and 2 to 7 years in Beaumont en Beine, and from 1 to 2 years and 2 to 5 years in Val de la Nef. For these traits the difference between ortet and ramet represented 6%, 3.6%, 11.6% and 5.3% of the grand mean respectively. On the average, for these traits, the ortets were growing faster than the corresponding ramets.

Multisite analysis

All genotypic (between families or clones) and interaction effects were significant at 1% level for large stem height increment, girth increment and cherry leaf spot susceptibility, except the family x site interaction for cherry leaf spot susceptibility (not significant). The interaction variance was higher than genotypic variance for the large stem height increment ($\sigma^2_{faxsi}/\sigma^2_{fa}$ = 1.6 and $\sigma^2_{clxsi}/\sigma^2_{cl}$ =1.7), whereas it was lower for the girth increment ($\sigma^2_{faxsi}/\sigma^2_{fa}$ = 0.4 and $\sigma^2_{clxsi}/\sigma^2_{cl}$ =0.4) and cherry leaf spot ($\sigma^2_{clxsi}/\sigma^2_{cl}$ =0.06; *Table 6*). As shown by ecovalences, Baule was the most interactive site, except for cherry leaf spot susceptibility scored on parent clones for which Baule and Val de la Nef were similarly interactive (*Table 6*). The between site genotypic correlations, at the family and clonal levels, were calculated to better understand the genotype x site interaction (*Table 6*). For the large stem height increment, the correlation between Beaumont en Beine and Baule was high (0.62 at the family level, 0.87 at the clonal level) whereas the correlation between Val de la Nef and each of the two other sites was very low or even negative (0.36 at the family level and 0.46 at the clonal level with Beaumont en Beine, -0.31 at the family level and 0.14 at the clonal level with Baule). On the other hand, for the girth increment, the correlations were high between Beaumont en Beine and Val de la Nef (0.88 at the family level and 0.82 at the clonal level), intermediate between Beaumont en Beine and Baule (0.54 at the family level and 0.72 at the clonal level) and very low between Val de la Nef and Baule (0.01 at the family level and 0.35 at the clonal level). Finally, for cherry leaf spot susceptibility, all the correlations were high (from 0.87 to 0.93 at the family level and from 0.85 to 0.98 at the clonal level).

Table 5. – Within site, additive (left column) and total genetic (right column) correlations on the basis of family data (jackknife standard errors).

TRAITS		year	initial height				stem height increment				girth increment			
			add.		gen.		add.		gen.		add.		gen.	
initial height	Beaumont en Beine	1												
	Val de la Nef	*0*												
	Baule	**0**												
stem	Beaumont en Beine	2-7	.05	(0.15)	.08	(0.17)								
height	*Val de la Nef*	*2-7*	*-.24*	*(0.13)*	*-.27*	*(0.12)*								
increment	**Baule**	**1-6**	-		**-.13**	**(0.22)**								
girth	Beaumont en Beine	5-7	-.26	(0.14)	-.07	(0.13)	.75	(0.05)	.74	(0.06)				
increment	*Val de la Nef*	*5-7*	*-.30*	*(0.09)*	*-.21*	*(0.08)*	*.78*	*(0.05)*	*.74*	*(0.06)*				
	Baule	**4-6**	-		**.44**	**(0.30)**	**.66**	**(0.13)**	**.56**	**(0.16)**				
leaf spot	Beaumont en Beine	5	.49	(0.13)	.26	(0.17)	-.53	(0.07)	-.63	(0.08)	-.86	(0.03)	-.90	(0.04)
susceptibility	*Val de la Nef*	*5*	*.34*	*(0.08)*	*.30*	*(0.07)*	*-.74*	*(0.08)*	*-.71*	*(0.10)*	*-.83*	*(0.03)*	*-.83*	*(0.04)*
	Baule	**4**	-		**.54**	**(0.23)**	**.17**	**(0.14)**	**.05**	**(0.15)**	**-.18**	**(0.17)**	**-.14**	**(0.21)**

– : non significant effect at 5% level.

Table 6. – Interaction ratio and site ecovalences in multisite analysis and between sites correlations.

		number of common genotypes		TRAITS: stem height increment (5-7 or 1-6)		girth increment (5-7 or 4-6)		leaf spot susceptibility	
		families	clones	families	clones	families	clones	families	clones
interaction ratio				1.6	1.7	0.4	0.4	0.0	0.06
site ecovalences	Beaumont en Beine			20	12	18	12	-	12
	Val de la Nef			27	42	23	30	-	44
	Baule			53	46	59	58	-	44
between sites correlations	Beaumont en Beine - Val de la Nef	85	11	.36	.46	.88	.82	.93	.95
	Beaumont en Beine - Baule	33	8	.62	.87	.54	.72	.88	.98
	Val de la Nef - Baule	33	7	-.31	.14	.01	.35	.87	.85

degrees of freedom: – for the family analysis, family effect: 85, family x site interaction: 216, error: 4189; – for the parent clone analysis, clone effect: 13, clone x site interaction: 17, error: 593.

interaction ratio: $\frac{\sigma^2_{fa\times si}}{\sigma^2_{fa}}$ for the family analysis and $\frac{\sigma^2_{cl\times si}}{\sigma^2_{cl}}$ for the parent clone analysis.

Discussion

An important feature of the results obtained is that they represent the first estimations of additive and dominance components of genetic variance in wild cherry. Nevertheless some shortcomings of these experiments can be pointed out: the parent clones were selected on flower abundance in the wild cherry collection of INRA and cannot be considered as typical clones selected for vigour, cherry leaf spot susceptibility and stem form. Apart from this restriction, the parent clones can be taken as a representative sample of the wild cherry plus-tree collection of INRA, so that the estimations of genetic parameters obtained here have a general value. The quite small number of parents (14) is also problematic vis-à-vis random model assumptions; to achieve a "complete" half-diallel mating design, 91 controlled crosses had nevertheless to be successfully realised, which is many; an incomplete half-diallel or a set of half-diallel mating designs involving more parent clones for the same number of crosses would provide more reliable estimates of genetic parameters. Indeed, NAMKOONG and ROBERDS (1974) have stated that no more than 6 full-sib families per parent would be useful to efficiently estimate genetic variance components. As a consequence, for a similar number of controlled crosses, 36 parents could have been used in 6 half-diallel designs with 6 parents without connections or 30 parents could have been used in a cyclic design with 3 circles and these designs would have given more reliable estimates of genetic parameters.

Anyway, some clear conclusions emerge from this study. Variation in vigour in wild cherry, and particularly girth growth, and also variation in cherry leaf spot susceptibility is under fairly strong genetic control (*Table 3*). Very similar results have been obtained in a previous clonal study (SANTI *et al.,* subm.), which involved 32 clones, planted in 5 sites.

Also in agreement with the clonal study (SANTI *et al.,* 1998), where weeds were well controlled, with weed-killers, in only one of the test sites (Sarrazac), we found here that genetic control of variation of stem height increments and cherry leaf spot susceptibility was stronger for traits measured in Baule than for similar traits measured in the two other sites. This can be explained by the fact that in Baule, weeds were controlled by cover-crop weeding whereas in Beaumont en Beine and in Val de la Nef weeds, shrubs and small trees (bramble, broom, poplar, willow, hornbeam, ...) could grow. On the other hand, the genetic control of variation of the girth increment was weaker in Baule, where pruning was extremely severe because of the rapid growth of the stem and branches. As a consequence, weed control is advisable in forest trials but in very fertile sites, a controlled associated vegetation should be used to avoid too big branches.

The broad sense heritabilities obtained here with the family data and with the parent clone data are generally in agreement. The assumptions used to calculate additive and dominance variances with family data, i.e. no inbreeding and no epistasis, are therefore probably not too far from reality. The only significant difference was observed for initial height in Val de la Nef. This difference can be explained by the fact that all individuals of a family were grown together in the nursery before being planted in Val de la Nef. As a consequence, the between family effect for this trait is inflated by a common-environment effect.

We tried to compare our results to results obtained with other broad-leaf species. The heritability estimates for vigour traits obtained in the present study are in the same range as those reported for *Populus trichocarpa* (PICHOT, 1993b), for hybrids between *Populus deltoïdes, P. nigra* and *P. maximowiczii* (RAJORA et al., 1994), and for hybrids between *Eucalyptus grandis*, *E. pellita* and *E. urophylla* (BOUVET and VIGNERON, 1995). However our estimates of heritabilities for girth growth are generally higher than those obtained for height growth whereas BOUVET and VIGNERON (1995) and RAJORA et al. (1994) obtained higher estimates for height than for girth. This can be explained by the very high correlation between girth growth and cherry leaf spot susceptibility and the high heritability of the latter trait. On the other hand, the high heritability obtained for cherry leaf spot susceptibility is similar to the high heritability obtained for susceptibility to a foliar disease in *Populus*, i.e. *Melampsora* leaf rust (RAJORA et al., 1994).

Additive and genetic correlations among vigour traits are generally high. Similarly, high genetic correlation between height and girth growth traits have been reported in a previous wild cherry clonal study (SANTI et al., 1998), in a nursery trial of *Populus deltoïdes* (PICHOT and TEISSIER DU CROS, 1989) and in a nursery study of hybrids between *Populus deltoïdes, Populus nigra* and *Populus maximowiczii* (RAJORA et al., 1994). As a consequence, selection for vigour can be done on an index combining height growth and girth growth in order to obtain the highest precision in the evaluation of genetic values for vigour.

Nevertheless, selection on girth growth only would be cheaper because the measure of girth is very fast and as efficient as on an index because of the high correlation and of the high heritability of girth growth. Initial height was not or negatively correlated with vigour in the forest trials, which shows that early selection on height after one year of growth in nursery would not be efficient.

The more resistant or tolerant individuals to cherry leaf spot were also the more vigorous in Beaumont en Beine and Val de la Nef. The genetic basis of this correlation is probably pleiotropy, because attacks of cherry leaf spot reduce the photosynthetic capacity of the leaves and cause their early fall so that the global photosynthetic capacity of the tree is reduced. Similar results have been reported in a previous clonal study (SANTI *et al.,* 1998). This correlation facilitates simultaneous selection for vigour and cherry leaf spot resistance or tolerance. This correlation was not observed in Baule, which can be explained by the fact that the more vigorous trees, which are the more tolerant ones, were probably also the more severely pruned, which would reduce their girth growth : the usual correlation could artificially disappear for this reason. Anyway, to evaluate the genotypic variability and the genetic parameters of vigour in absence of cherry leaf spot, trials should be planted where cherry leaf spot would be avoided with fungicide treatments or only with clones tolerant to cherry leaf spot.

The genotype x site interaction obtained for the large stem height increment is surprisingly important when compared to the results of SANTI *et al.* (1998). This important genotype x site interaction corresponds to quite weak genotypic correlations at the family or clonal level between Val de la Nef and the two other sites, which on the contrary show relatively high correlations between them. Thus the genes controlling height growth variation in Val de la Nef could be quite different from those controlling height growth variation in Beaumont en Beine or Baule. This could be explained by the great differences of fertility between the sites (*Figure 1*). The genotype x site interaction obtained for the girth increment in the present study, with a ratio of interaction variance to genetic variance of 0.4, is less important than the genotype x site interaction obtained in the clonal study (SANTI *et al.,* 1998) for girth, where the ratio was 0.8. The results obtained here show that cherry leaf spot susceptibility is very stable.

The correlation between clonal value and general combining ability is high for most selected traits, i.e. large stem height increment, girth increment and cherry leaf spot susceptibility. These traits also show intermediate or high heritabilities. Clonal seed orchards constituted by the selection of parents on clonal values should thus provide significant genetic gains. Testing on clonal value has at least two advantages as compared to testing GCA with ad hoc mating designs (i.e. open pollinated families, families obtained by polycross, diallel or factorial mating design). First, in wild cherry, vegetative propagation is much easier than controlled crossing. Secondly, less ramets per clone than individuals per family are necessary to obtain the same precision in the evaluation of average performances : as a consequence, with the same experimental means, a larger selection intensity can be applied with clonal evaluation than with family evaluation. Indeed, the estimated expected genetic gains demonstrate the advantage of clonal testing over GCA testing with open-pollinated half-sib families (*Table 4*).

The material produced in the orchards should offer large gains in comparison to material produced in selected seed stands, the current seed sources, because of the high intensity of selection of clones constituting the orchards. It should also present a larger variability because of their very various origins. On the contrary, variability inside selected seed stands, of which the surface is very limited, is not large (FERNANDEZ *et al.,* 1994), because natural suckering of wild cherry leads to clones covering varying surfaces (FRASCARIA *et al.,* 1993) : as a consequence, seeds are effectively harvested on very few genotypes in selected seed stands.

Nevertheless, the level of genetic improvement will perhaps be not sufficient for large spacing plantations currently used for wild cherry. Clonal varieties are probably the best suited to this kind of silviculture, and clonal selection is always one of the best selection strategies because it uses all genetic variability, additive and non-additive (LIBBY and RAUTER, 1984). The partitioning of genetic variance components in this experiment show that additivity is the main mode of action of the genes that control the selected traits, but the dominance part is not negligible. The ratio of additive to global genetic variance was always higher than 0.60 but often smaller than 0.8 for the large stem height increment, girth increment and cherry leaf spot susceptibility. Thus clonal varieties would offer further genetic improvement in comparison to material produced in seed orchards. For most traits, estimated expected genetic gains with clonal varieties were actually larger than those expected from seed orchard (*Table 4*). Moreover, a recurrent breeding program should be started to produce improved clones candidates to selection. The results obtained here show that the clones that will be recombined to produce the next generation population can be chosen on the basis of the current clonal test results.

References

BOUVET, J.M. and VIGNERON, P.: Age trends in variances and heritabilities in *Eucalyptus* factorial mating designs. Silvae Genetica **44**: 209-216 (1995). — COTTERILL, P.P., DEAN, C.A. and VAN WYK, G.: Additive and dominance genetic effects in *Pinus pinaster, P. radiata* and *P. elliottii* and some implications for breeding strategy. Silvae Genetica **36**: 221-232 (1987). — COTTERILL, P.P. and JAMES, J.W.: Number of offspring and plot sizes required for progeny testing. Silvae Genetica **33**: 203-209 (1984). — FERNANDEZ, R., SANTI, F. and DUFOUR, J.: Les matériels forestiers de reproduction sélectionnés de merisier (*Prunus avium* L.): classement, provenances et variabilité. Rev. For. Fr. **XLVI**-6: 629-638 (1994). — FOSTER, G.S. and BRIDGWATER, F.E.: Genetic analyses of fifth-year data from a seventeen parent partial diallel of loblolly pine. Silvae Genetica **35**: 118-122 (1986). — FRASCARIA, N., SANTI, F. and GOUYON, P.H.: Genetic differentiation within and among populations of chesnut (*Castanea sativa* MILL.) and wild cherry (*Prunus avium* L.). Heredity **70**: 634-641 (1993). — GALLAIS, A.: Théorie de la sélection en amélioration des plantes. Ed. Masson (Paris), 591 pp. (1990). — GARRETSEN, F. and KEULS, M.: A general method for the analysis of genetic variation in complete and incomplete diallels and North Carolina II (NC II) designs. II- Procedures and general formulas for the fixed model. Euphytica **27**: 49-68 (1978). — KEULS, M. and GARRETSEN, F.: A general method for the analysis of genetic variation in complete and incomplete diallels and North Carolina II designs. I- Procedures and general formulas for the random model. Euphytica **26**: 537-551 (1977). — KING, J.N., YEH, F.C. and HEAMAN, J.C.H.: Selection of growth and yield traits in controlled crosses of coastal Douglas-fir. Silvae Genetica **37**: 158-164 (1988). — KNAPP, S.J. and BRIDGES, W.C.: Parametric and jackknife confidence interval estimators for two-factor mating design genetic variance ratios. Theor. Appl. Genet. **76**: 385-392 (1988). — KNAPP, S.J., BRIDGES JR., W.C. and YANG, M.H.: Nonparametric confidence interval estimators for heritability and expected selection response. Genetics **121**: 891-898 (1989). — KOBILINSKY, A.: S Modli. BAO/ Document n 09/90, NCY/GL, Département d'informatique, Institut National de la Recherche Agronomique, 21 pp. (1990). — NAMKOONG, G. and ROBERDS, J.H.: Choosing mating designs to efficiently estimate genetic variance components for trees. Silvae Genetica **23**: 43-53 (1974). — PAPADAKIS, J.: Méthode statistique pour des expériences en champ. Thessalonique, Institut d'Amélioration des plantes, Bull. Sci. 23, 30 p. (1937). — PICHOT, C.: Analyse de dispositifs par approches itératives prenant en compte les performances des plus proches voisins. Agronomie **13**: 109-119 (1993a). — PICHOT, C.: Variabilité au stade adulte chez *Populus trichocarpa* TORR. & GRAY et prédiction juvénile-adulte chez *P. trichocarpa* et *P. deltoïdes* BARTR. Thèse Institut National d'Agronomie, Paris Grignon, 235 pp. (1993b). — PICHOT, C. and TEISSIER DU CROS, E.: Esti-

mation of genetic parameters in eastern cottonwood (*Populus deltoïdes* BARTR.). Consequence for the breeding strategy. Ann. Sci. For. **46**: 307-324 (1989). — RAJORA, O.P., ZSUFFA, L. and YEH, F.C.: Variation, inheritance and correlations of growth characters and *Melampsora* leaf rust resistance in full-sib families of *Populus*. Silvae Genetica **43**: 219-226 (1994). — SAMUEL, C.J.A.: The estimation of genetic parameters for growth and stem-form over 15 years in a diallel cross of Sitka spruce. Silvae Genetica **40**: 67-72 (1991). — SANTI, F., MURANTY, H., DUFOUR, J. and PÂQUES, L.E.: Genetic parameters and selection in a multisite wild cherry clonal test. Silvae Genetica **47**: 61-67 (1998). — SCHERMANN, N., VERGER, M. and BASTIEN, J.C.: Sélection de familles de sapin de Douglas (*Pseudotsuga menziesii*) pour leur aptitude au bouturage: conséquences pour la diffusion de variétés améliorées. Ann. Sci. For. **53**: 1113-1126 (1996). — Splus: Reference manual version 3.2. Statistical Sciences Inc., Seattle, Washington (1993). — Splus: Reference manual version 3.4. Statistical Sciences Inc., Seattle, Washington (1996). — VAN WYK, G.: Early growth results in a diallel progeny test of *Eucalyptus grandis* (HILL) MAIDEN. II. A greenhouse study. Silvae Genetica **26**: 44-50 (1977). — WRICKE, G.: Die Erfassung der Wechselwirkungen zwischen Genotyp und Umwelt bei quantitativen Eigenschaften. Zeitschrift für Pflanzenzüchtung **53**: 266-343 (1965). — YANCHUK, A.D.: General and specific combining ability from disconnected partial diallels of coastal Douglas-fir. Silvae Genetica **45**: 37-45 (1996).

Morphological Traits, Microsatellite Fingerprinting and Genetic Relatedness of a Stand of Elite Oaks (*Q. robur* L.) at Tullynally, Ireland

By F. LEFORT[1)3)], M. LALLY[2)], D. THOMPSON[2)] and G. C. DOUGLAS[1)]

(Received 2nd January 1998)

Abstract

A morphological and molecular characterisation of phenotypically elite oaks (*Quercus robur* L.) which were estimated as 220 years old was undertaken to test the utility of molecular tools to examine the genetic origin of the stand. The 11 trees shared many excellent characteristics in tree form. Quantitatively, DBH ranged from 104 cm to 126.5 cm and stem height from 10 m to 25.5 m. The molecular analysis using microsatellites for nine genetic loci was on five trees. It concluded that the trees were not closely related. This small sample showed many polymorphisms and much heterozygosity. Loci AG16 and AG 9 showed 9 and 8 different alleles respectively while loci AG1/2 and Ag15 displayed 3 and 5 alleles among the five trees. At least two trees had a three-band profile for some loci indicating potential triploidy.

The historical records of the estate refers to one elite tree in 1837 and the detail of its description suggests it may correspond to one extant today. It also suggests an active silvicultural management and the practise of coppicing with standards. Such management may have resulted in this excellent stand by conversion of a natural woodland in stages starting with coppice, leading to coppice with standards, then to high forest and ultimately to a parkland stand.

Key words: microsatellite, *Quercus,* repeated DNA, SSR.

FDC: 165.3/4; 181.6; 176.1 *Quercus;* (417).

Introduction

Oak (*Quercus* spp.) is an important species for its valuable timber and its adaptability to a range of sites. It is also valued for its longevity and beauty and as a host for a wide range of organisms.

Among our oak forests are phenotypically elite specimen trees which may be characterised as having: clean stems, a round cross section with straight butts, freedom from knots or shake and with heartwood straight down the middle. They are usually found as single specimens or widely scattered in large stands. Such trees can be utilised optimally by: using them as seed sources, or propagating them vegetatively (grafting) to create clone banks or seed orchards to produce improved seeds.

At Tullynally Castle, Castlepollard, County Westmeath Ireland, there is, unusually, a group of 11 oak trees which share many elite characteristics; they are straight and of good form in a relatively small, open parkland area of approximately 3 ha. LOUDON, (1844) described one exceptionally straight and tall tree in this estate in the 1830s and which may in fact be one of the trees included in the present study. Most trees in the group are straight with strong apical dominance and two have fastigiate – type branching. Their exceptional quality might indicate some genetic relatedness or a positive combination of good genetic material together with good silvicultural practises.

When phenotypically superior material is identified in a small area, the question of its provenance or origin usually arises. We considered the possibility that the trees in this study might be closely related due to their similarity in age and their close proximity. While there are several approaches to this question, molecular techniques have been suggested as being reliable. Indeed, such techniques have been useful to determine paternity and the genetic relatedness of individuals in a stand, especially by using the analysis of microsatellite profiles (DOW and ASHLEY, 1996).

A large part of higher eukaryote genomes consists of repetitive DNA of different kinds (WEISING *et al.,* 1995). Among them repeated sequences containing iterations of short motifs are referred to as microsatellites or simple sequence repeats (TAUTZ, 1993). Each microsatellite locus consists of tandemly repeated sequences of one up to 6 nucleotides. The regions of DNA which flank each side of the repeated sequences are highly conserved and the whole represents a conserved genetic locus.

[1)] Teagasc, Agriculture and Food Development Authority, Kinsealy Research Centre, Malahide Road, Dublin 17, Ireland. GDouglas@kinsealy.teagasc.ie

[2)] Coillte Teoranta, The Irish Forestry Board, Research Laboratory, Newtownmountkennedy, Co. Wicklow, Ireland.

[3)] Laboratory of Plant Physiology and Biotechnology, Department of Biology, University of Crete, P.O. Box 2208, GR-71409 Heraklion, Crete, Greece

Although the flanking regions are conserved, there is great variability in the number of repeat units due to the mutational loss or addition of repeat units, and this accounts for a large number of distinguishable alleles and provides a basis for estimating genetic polymorphisms at the DNA level. This polymorphism can be analysed by amplification of the microsatellite loci by the polymerase chain reaction (PCR) using primers targeting the conserved flanking regions. The amplified microsatellite sequences are then separated on a sequencing gel and distinguished on the basis of their length in base pairs.

The objective of this study was to describe the important morphological traits of some elite oak trees and to demonstrate the utility of DNA analysis in determining their genetic relationships so as to allow an assessment of the contribution of genetic background and silvicultural management in producing excellent trees.

Material and Methods

Plant material

The site was visited and trees of *Quercus robur* L. numbered and measured in September 1997. Crown or epicormic shoots, where accessible, were collected previously and grafted on rootstocks and maintained in the greenhouse. Grafts were successful for five trees. Mature leaves from five grafted trees were harvested in September for DNA extraction.

Morphological scoring among the trees was rated 1 to 4 with 4 as the best condition for a number of traits (*Table 1*). The length of clean stem was measured from ground level to the first forking point of the main stem. Trees with high and wide buttresses were rated poorly as were trees with a bow or ridges in the stem, or with a large number of epicormic shoots. Trees in which the leading stem showed continuity with the main stem were rated as having high apical dominance. Tree diameter was measured using a diameter tape, and height using a clinometer.

Table 1. – Characteristics of high quality oaks at Tullynally Castle, Castlepollard, Co. Westmeath, Ireland. Rating scale 1 = poorest, 4 = best.

			Tree Form Rating 1-4				
Tree	Stem DBH	Stem Ht	Buttress Form	Stem Form	Epicormic Shoots	Apical Dominance	Total Rating
No	(cm)	(m)					
1	104	10.0	4	2	3	2	11
2	149	11.0	2	2	4	2	10
3	**108.5**	**18.5**	**3**	**4**	**2**	**4**	**13**
4	**102.9**	**16.5**	**3**	**3**	**1**	**3**	**10**
5	109.7	25.5	2	4	3	4	13
6	**103.7**	**22.0**	**4**	**4**	**1**	**3**	**12**
7	**106.4**	**20.5**	**4**	**2**	**3**	**2**	**11**
8	139.5	13.0	2	3	4	4	13
9	114.2	15.0	2	3	1	2	8
10	123.6	11.5	2	3	4	4	13
11	**126.5**	**20.5**	**2**	**2**	**1**	**4**	**9**

The trees shown in bold have been characterized by microsatellite analysis at nine loci.

DNA extraction

DNA extraction has been performed according a rapid protocol of DNA extraction developed in our laboratory for extracting DNA from mature leaves of *Acer, Fraxinus, Prunus* and *Quercus.*

Fresh plant material (100 mg of leaf) was ground in liquid nitrogen using a ceramic mortar and pestle to give a green powder. The powder was transferred to a new 1.5 ml polypropylene tube using a spatula. At this time, 1 ml of DNA extraction buffer [50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 0.7 M NaCl, 0.4 M LiCl, 1% w/v CTAB (hexadecyltrimethylammonium bromide), 1% w/v PVP 40, 2% w/v SDS] and 10 µl of β-mercaptoethanol (1% final concentration) was added. The DNA extraction buffer is a white emulsion. The mixture was mixed with the spatula, vortexed 5 seconds and then incubated 15 min. at 65 °C in a water-bath. After addition of the powdered leaf material and immersion in the 65 °C water bath, the mixture became clear in a few seconds, as soon as the different reagents interacted with proteins and phenolic compounds.

After incubation, 0.5 ml of chloroform/isoamylalcohol (24:1) was added to the tube, the mixture was agitated thoroughly until making an emulsion and centrifuged 1 min. to 5 min. in a microfuge at 17000 g (14000 rpm in an ALC microcentrifugette 4214 rotor A-12). As much as possible of the aqueous phase was transferred to a new 1.5 ml tube and centrifuged 1 min. at 17000 g; 0.8 ml of the supernatant was then transferred to a new tube and 0.8 ml of isopropanol (cold as an option) was added to the aqueous solution. The tube was swirled gently and a white DNA precipitate appeared. The tube was then centrifuged 1 min. at 17000 g and the supernatant was withdrawn. The DNA pellet was washed with 1 ml of 70% ethanol, centrifuged again 1 min. at 17000 g. The second ethanol wash is optional. Finally the supernatant was withdrawn and the pellets allowed to dry on the bench for 10 min. DNA pellets were resuspended in 50 µl to 100 µl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA. As the solution contained RNA and DNA, the protocol was followed by an RNase digestion to remove RNAs (optional). RNA digestion was performed by adding 2 µl of RNase (0.5 mg/ml) (Boehringer Mannheim, UK) and incubating for 30 min. at 37 °C. The resulting DNA mixture could be purified through a column such as the Wizard Clean-up System (Promega Biotec, Madison, Wl, USA), but could also be directly used after RNase digestion. In cases where the samples are of a brown colour, it is recommended to purify them through a column system.

Microsatellite PCR and microsatellite analysis

We used flanking primers designed for microsatellite regions described by STEINKELLNER *et al.* (1997). The loci that we analysed for this study are presented in *table 2.* PCR conditions were found different than the original conditions developed by STEINKELLNER *et al.* (1997); the main reason for this fact could be the different method of DNA extraction.

PCR conditions were the following: 50 µl reactions included 75 mM Tris-HCl pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 62.5 µM dNTPs each (Biofinex, Praroman, Switzerland), 0.2 µM to 1 µM forward primer, 0.2 µM to 1 µM reverse primer, 1.25 u to 1.5 u AmpliTaq polymerase (Perkin Elmer, Foster City, Ca, USA) and 5 ng to 50 ng DNA template. Hot-start PCR technique was performed because it was known to give better amplification of pure PCR products. Mineral oil was replaced in all PCR reactions by a home-purified pharmacy parafffin wax (LEFORT and DOUGLAS, 1997). Following an initial denaturation of 5 min. at 96 °C, 28 to 35 cycles [94 °C for 1 min., AT°C (AT = annealing temperature) for 1 min., 72 °C for 30 s] were performed, terminated by an 8 min. final extension at 72 °C. PCR products were checked on a 2% w/v agarose gel in 1xTBE and then analysed on a CastAway precast 6% polyacrylamide 7M urea sequencing gel (Stratagene Cloning Systems, La Jolla, Ca, USA). PCR samples (5 µl) were mixed (1:1) with a sequencing gel loading buffer (95% (v/v) formamide, 0.05% (v/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 10 mM NaOH). Samples were then denatured for 6 min. at 96 °C and placed on a water-ice bath prior to loading. Gels were run 1 hour to 1.5 hour at 1600 V in a CastAway Sequencing System (Stratagene

Table 2. – Microsatellite loci, primers and annealing temperatures used for microsatellite fingerprinting of elite Irish oaks.

Locus [1]	EMBL Accession Number [1]	Forward primer [1] (5'-3')	Reverse primer [1] (5'-3')	Annealing Tempearture AT
ssrQpZAG1/2	X84080	tcctccgctcactcaccatt	aaacctccaccaaaacattc	50
ssrQpZAG1/5	X84081	gcttgagagttgagatttgt	gcaacacccttaactacca	57
ssrQpZAG9	X98753	gcaattacaggcctaggctgg	gtctggacctagccctcatg	49-50
ssrQpZAG15	X98758	cgatttgataatgacactatgg	catcgactcattgttaagcac	49-50
ssrQpZAG16	X84082	cttcactggcttttcctcct	tgaagcccttgtcaacatgc	59
ssrQpZAG58	X98757	ctgcaagattcggacaagcaa	tctttttcctaatctcacctg	49-50
ssrQpZAG104	X98761	atagggagtgaggactgaatg	gatggtacagtagcaacattc	49-50
ssrQpZAG108	X98762	ctagccacaattcaggaagag	cctcttttgtgaatgaccaag	49-50
ssrQpZAG110	X98763	ggaggcttccttcaacctact	gatctcttgtgtgctgtattt	49-50

[1]) STEINKELLNER *et al.,* 1997.

Cloning Systems) and then submitted to silver staining (STREIFF and LEFORT, 1997).

After a final wash (5 min.) in ultra-pure water, they were dried in a CastAway System Gel Dryer.

Stained gels were subsequently photographed using an image analysis system (Imagestore 5000, UVP, UK).

Results

Morphological analysis

Shoot samples were obtained from nine trees and all were identified by leaf morphology as *Q. robur.* The total heights for trees 3 and 5 were estimated as 34.5 m and 26.5 m respectively. The other characteristics of each tree are given below in *table 1.*

Tree 2 had the greatest DBH and was the only tree with downward arching branch-ends with crown shoots which were accessible at ground level.

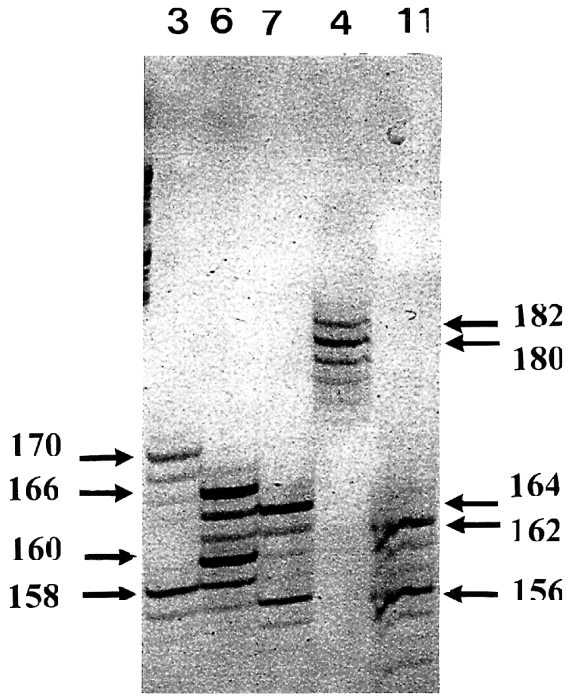


Figure 1. – Microsatellite profiles for locus AG16. The microsatellite allele is the major band. The length (bp) of some sequences are marked in the figure and all allele length are given in *table 3.*

Trees 8 and 10 were notable in having the greatest diameter combined with the best rating for tree form, while trees 5, 6 and 7 had trunk lengths over 20 m combined with high ratings for tree form. It was notable that trees 3 and 6 were almost fastigiate in nature with ascending crown branches.

The age of several trees was computed as 220 years using the method of WHITE (1994) for mature trees.

Molecular analysis

Figure 1 shows the DNA amplification pattern for trees No. 3, 4, 6, 7 and 11, obtained by using the amplifying primers for microsatellite locus AG16. Each band represents a different length in base pairs of microsatellite DNA for this locus, thus each band is an allele. The real alleles are only the darkest bands. Other bands which appear are "stutter or shadow bands" and are caused by (among others) the addition of a nucleotide to the 3' end of the amplification product by the DNA polymerase or by a slipped strand mispairing leading to deletion of repeat units during the PCR (SMITH et al., 1995; LITT et al., 1993). Stutter bands are not scored but represent a helpful ladder for allele length determination.

Two strong bands were evident for each of the trees analysed in *figure 1* and each tree was represented by alleles of different molecular weight indicating that each was heterozygous and that this locus (AG 16) was polymorphic. A total of 9 different alleles were recorded and only trees 7 and 11 had one allele (156 bp) in common. A summary of the microsatellite profiles at all loci for all trees is presented in *table 3.*

For locus AG104 in the same 5 trees, tree No. 3 and 7 shared the same alleles, each with a DNA length of 232 bp and 234 bp. Tree No. 11 had one allele in common with tree 6. Tree No. 4 has no common alleles with any of the others *(Table 3).*

An examination of the profiles at locus AG1/2, showed that trees 3, 7, 4 and 11 shared the same allele of 97 bp. There was one strong band present for trees No. 3 and No. 4 indicating that they were homozygous for this allele: however trees No. 7 and No. 11 were heterozygous while tree No. 6 was homozygous for the allele of 110 bp. The microsatellite profiles for all 9 loci are summarised in *table 3.*

An examination of locus AG9 showed the presence of 3 alleles in common for trees No. 4 and No. 11 and these are shown in *figure 2.* A three band pattern was scored when each band was clearly resolved from the others. In addition, these two trees also showed a similar 3 alleles profile for locus AG15. Furthermore tree No. 11 showed three alleles at locus AG1/5 while tree No. 4 showed two *(Table 3).*

Table 3. – Genetic polymorphisms obtained for 9 microsatellite loci with 5 elite oak trees.

Microsatellite locus	Microsatellite length in base pairs	Estimated number of alleles per microsatellite				
		Tree Number				
		3	6	7	4	11
Locus AG 1/2	97	2	0	1	2	1
	99	0	0	1	0	0
	101	0	2	0	0	1
Locus AG 1/5	169	0	0	1	0	1
	170	1	1	0	1	0
	171	0	1	0	0	0
	172	0	0	0	1	1
	174	0	0	1	0	0
	175	1	0	0	0	0
	176	0	0	0	0	1
Locus AG 9	186	0	0	1	1	1
	188	1	0	0	0	0
	192	0	1	0	1	1
	196	0	1	1	0	0
	198	1	0	0	0	0
	200	0	0	0	1	1
Locus AG 15	110	0	1	0	1	1
	112	1	0	1	1	1
	114	0	0	1	1	1
	118	1	0	0	0	0
	144	0	1	0	0	0
Locus AG 16	156	0	0	1	0	1
	158	1	0	0	0	0
	160	0	1	0	0	0
	162	0	0	0	0	1
	164	0	0	1	0	0
	166	0	1	0	0	0
	170	1	0	0	0	0
	180	0	0	0	1	0
	182	0	0	0	1	0
Locus AG 58	154	1	0	0	0	0
	156	0	0	0	2	0
	160	0	0	1	0	0
	163	0	1	0	0	0
	166	0	0	1	0	0
	171	0	0	0	0	2
	192	1	0	0	0	0
	196	0	1	0	0	0
Locus AG 104	186	0	0	0	1	0
	200	0	0	0	1	0
	206	0	1	0	0	1
	220	0	1	0	0	0
	232	1	0	1	0	0
	234	1	0	1	0	0
	240	0	0	0	0	1
Locus AG 108	211	1	1	0	0	1
	217	0	0	1	0	0
	223	0	1	0	0	1
	225	0	0	0	1	0
	233	1	0	1	0	0
	237	0	0	0	1	0
Locus AG 110	194	0	0	0	0	1
	206	1	0	0	0	0
	208	0	0	2	2	1
	210	0	1	0	0	0
	220	1	0	0	0	0
	234	0	1	0	0	0

1 = 1 allele at the microsatellite indicated.
2 = 2 alleles at the microsatellite indicated (homozygous).

It can be deduced from the microsatellite profiles that the five trees analysed do not share a common mother or father. For example, analysis of AG1/5 suggest that trees No. 3, No. 6 and No. 4 may be related since they have one allele of 170 bp in common. However, analysis of locus AG16 shows that each is polymorphic *(Table 3).* Similarly, for trees 7 and 11, that share a common allele of 156 bp at locus AG 16 but for locus AG104 no alleles are in common.

As shown in *table 3*, the number of alleles differed greatly from one locus to another, ranging from 3 alleles for AG1/2 up to 9 alleles for AG16 and this, with a small sample of just five trees. Locus AG15 displayed only 5 different alleles whereas locus AG58 displayed 8. AG16 has been shown to be a very polymorphic locus displaying 20 alleles within a sample of 17 elite Irish oaks from 10 different provenances (unpublished).

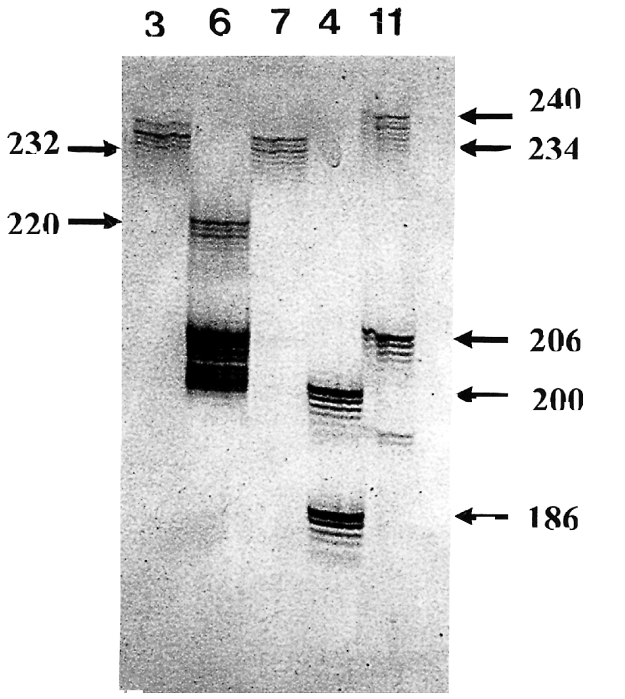


Figure 2. – Microsatellite profile for locus AG104. The microsatellite allele is the major band. The length (bp) of some sequences are marked in the figure and all allele length are given in *table 3.*

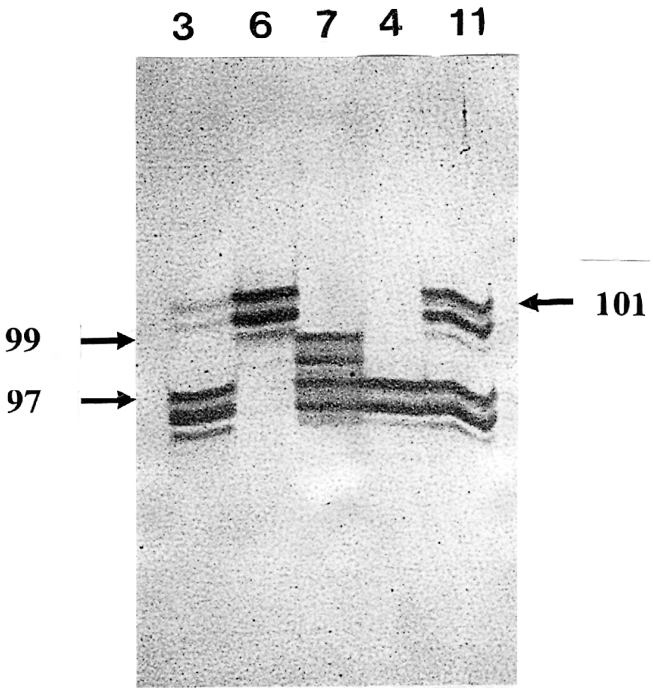


Figure 3. – Microsatellite profile for locus AG1/2. The microsatellite allele is the major band. The length (bp) of some sequences are marked in the figure and all allele length are given in *table 3.*

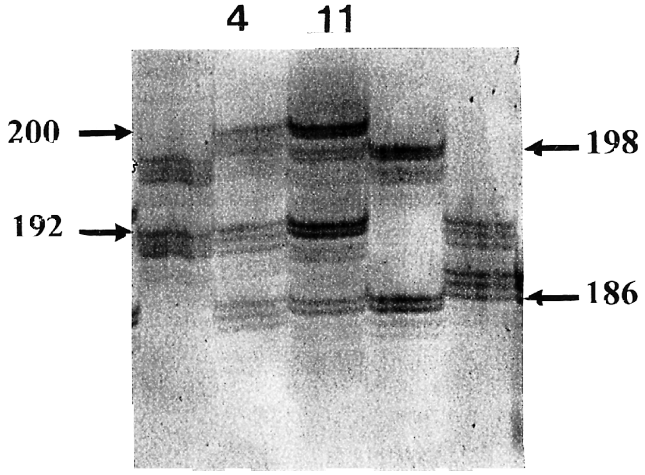


Figure 4. – Microsatellite profile of trees No. 4 and No. 11 for locus AG9 showed a 3-band pattern. The microsatellite allele is the major band. The length (bp) of some sequences are marked in the figure and all allele length are given in *table 3*.

Discussion

Analysis of microsatellite profiles is a useful and accurate tool to confirm genetic relatedness to the level of siblings in tree populations (DOW and ASHLEY, 1996). However, molecular markers such as isozymes or RAPDs were not very useful for discriminating between provenances or even species since this genus seems to be highly hetreozygous and has not yet undergone intense specialisation (KLEINSCHMIDT, 1993; KREMER and PETIT, 1993).

We have shown that none of the five trees analysed could be genetically related as siblings or half siblings, using two very polymorphic loci such as AG16 (*Fig. 1*) and AG104 (*Table 3*). A similar conclusion could be deduced by examining other loci which displayed a low or moderate polymorphism. The trees displayed a high degree of heterozygosity and this is typical of oaks which have been shown by recent analyses of molecular markers to disperse pollen over great distances (DOW *et al.,*1995; DOW and ASHLEY, 1996).

Some trees displayed a 3-band pattern for some loci. Tree No. 4 showed a 3-band pattern at loci AG9 and AG15 and tree No. 11 at loci AG1/5, AG9 and AG15. A three-band pattern could result from the presence of three copies of the haploid genome provided the tree was heterozygous at every locus i.e. triploidy. Triploids probably arise from unreduced female or male gametes. If the 2 n female gamete was heterozygous at any given locus and fertilised by pollen carrying a third allele, a three-band pattern will appear as the microsatellite profile. Triploids would give a two-band pattern if, for example, the 2 n female gamete was homozygous and the pollen carried a different allele. Indeed, triploids could give a one-band pattern if each gamete carried the same allele. It is not possible to assay the dosage effect of two microsatellite alleles which co-migrate to the same point on the gel since PCR is not quite quantitative. The three-band pattern for some loci shown with trees No. 4 and No. 11 may indicate that these trees are triploids. It could also be due to the presence of one (or more) extra-chromosomes (aneuploidy) or a duplication of one region of the genome. Previous studies provide evidence for triploidy and aneuploidy in oaks. JOHNSSON (1946) found three triploids among 726 oaks (0.0041%) in Sweden. In another study, isozyme analysis and size distribution of stomata indicated triploidy in one tree among 400 oaks (0.0025%) in Germany and cytological analysis showed the most frequently counted number of chromosomes as 33 to 35 (NAUJOKS *et al.,* 1995). BUTORINA (1993) presented a cytogenetic study of two *Q. robur* trees showing that 3 n cells were predominant with occasional diploid, hypoaneuploid and hyperaneuploid cells. Although such trees were considered triploids, they were mixoploids *stricto sensu.*

In addition, OHRI and AHUJA (1990) reported a few cases of aneuploidy in karyotyping *Q. petraea, Q. robur* and *Q. rubra* by Giemsa C-banding and the presence of B-chromosomes (JONES, 1981). The hypothesis of triploidy in two trees which gave a three-band pattern for some loci (trees No. 4 and No. 11) will be further tested by measurement of stomatal size and DNA quantification by flow cytometry (FAVRE and BROWN, 1996).

If these analysis confirm triploidy among our sample of five elite trees, it may point to a higher frequency of tripioids among elite trees than previously believed. Indeed, the two triploids studied by BUTORINA (1993) were noticed because they were unusually large. The 90 years old triploid among 400 trees analysed by NAUJOKS *et al.* (1995) was fertile and had all the characteristics of an elite tree, having been described as "27 m high with an excellent straight stem form and a diameter of 42 cm at 1.30 m. Its crown started at 18.5 m".

The development of this high quality stand at Tullynally was probably assisted by silvicultural practises and the historical record of the estate suggests that the owners showed much interest in their woodlands.

One outstanding specimen was measured in 1836 or 1837 and a description of it is given in a publication in 1844, however the first edition of this book is 1838 (LOUDON, 1844). This describes one tree in the estate as "A *Q. pedunculata* is 80 ft. high, with a trunk perfectly clear from knots or branches for 31 ft.; girthing 12 ft. at 1 ft. from the ground and 6 ft. at 31 ft., just below the swelling of the branches. The trunk is perfectly straight, and the tree which is in a healthy and growing state is about 96 years old." (LOUDON, 1844). This tree may well be tree No. 1 of the present study since it is the only tree with first branches at 10.0 m (33 feet) and is situated closest to the driveway which followed the same route as today (PAKENHAM, pers. commun.). Subsequently, in 1861 a report by the forester FRASER was commissioned and it gives the recommendation for oaks that "the best trees should be selected as standards, pruned and treated accordingly" (PAKENHAM, 1998). This suggests that the estate woodlands may have been managed as a coppice with standards i.e. a two storey forest with a coppice underwood comprising a scattering of trees (standards) to be grown for timber size. Such a management strategy was commonplace and was the legally required way of management in the time of HENRY VIII (EVANS, 1984). Oak was and still is the most common species grown in this way and planted stock is rare in such situations. Standards exhibit large open crowns with a rapid growth in stem diameter similar to that under free growth conditions, but they often develop vigorous epicormic branches (EVANS, 1984). The straightness of the trees at Tullynally and the absence of epicormic branches on the main trunk even in trees which have an inherent high capacity for epicormic shoot production *(Table 2)*, strongly suggest that the stand may have been converted from coppice and were pruned regularly.

Our study shows that there is no close relationship between the trees at the genetic level and that their good form was the result of the capacity of good genotypes to respond to good silvicultural practises which seem to have been applied throughout the life of the stand in relation to thinning and pruning.

It also suggests that triploidy and mixploidy should be investigated by cytogenetic studies in elite phenotypes, especially when microsatellite profiling raises such a possibility. Microsatellite fingerprinting is also shown to be a potential tool for cytogenetics.

Acknowledgements

Dr. FRANCOIS LEFORT was supported by European Union FAIR contract N°CT 965004, we thank COFORD for additional support. We are grateful to Mr. THOMAS PAKENHAM, Tullynally Castle Castlepollard Co. Westmeath for providing access to the elite trees and for researching the historical records and Dr. REJANE STREIFF, INRA, Cestas, France, for the helpful discussion on microsatellite analysis.

References

BUTORINA, A. K.: Cytogenetic study of diploid and spontaneous triploid oaks, *Quercus robur* L. Ann. Sci. For. **50**(s1): 144s–150s (1993). — DOW, D. D. and ASHLEY, M. V.: Microsatellite analysis of seed dispersal and parentage of saplings in bur oak, *Quercus macrocarpa.* Mol. Ecol. **5**: 615–627 (1996). — DOW, D. D., ASHLEY, M. V. and HOWE, H. F.: Characterization of highly variable (GA/CT)n microsatellites in the bur oak *Quercus macrocarpa.* Theor. Appl. Genet. **91**: 137–141 (1995). — EVANS, J.: Coppice silviculture. Chapter 7. In: The silviculture of broadleaved woodland. Forestry Commission Bulletin **62**, pp. 73–81 (1984). — FAVRE, J. M. and BROWN, S.: A flow cytometric evaluation of the nuclear DNA content and GC percent in genomes of European oak species. Ann. Sci. For. **53**: 915–917 (1996). — JOHNSSON, H.: Chromosome numbers of twin plants of *Quercus robur* and *Fagus silvatica.* Hereditas **32**: 469–472 (1946). — JONES, R. N.: Wide spread occurence of B-chromosomes. Bionature **1**: 63–73 (1981). — KLEINSCHMIDT, J.: Intraspecific variation of growth and adaptive traits in European oak species. *In:* Genetics of oaks. KREMER, A., SAVILL, P. S. and STEINER, K. C., editors. Ann. Sci. For. **50**, Supp. 1, 166s–185s (1993). — KREMER, A. and PETIT, R.: Gene diversity in natural populations of oak species. *In*: Genetics of oaks. KREMER, A., SAVILL, P. S. and STEINER, K. C., editors. Ann. Sci. For. **50**, Supp. 1, 186s–202s (1993). — LEFORT, F. and DOUGLAS, G. C.: A simplified method to purify a pharmaceutical grade wax for use in the polymerase chain reaction (PCR). Biologia **52**(6): 803–806 (1997). — LITT, M., HAUGE, X. and SHARMA, V.: Shadow bands seen when typing polymorphic dinucleotide repeats: some causes and cures. BioTechniques **15**: 280–284 (1993). — LOUDON, J. C.: In: "Arboretum et Fruticum Britannicum". 2nd Ed., Vol. 111, page 1842 (1844). — NAUJOKS, G., HERTEL, H. and EWALD, D.: Characterisation and propagation of an adult triploid pedunculate oak *(Quercus robur* L.). Silvae Genetica **44**: 282–286 (1995). — OHRI, D. and AHUJA, M. R.: Giemsa C-banded karyotype in *Quercus* L. (oak). Silvae Genetica **39**: 216–219 (1990). — PAKENHAM, T.: Personal communication. Report in the archive of the estate, Tullynally Castle, Castlepollard, Co Westmeath, Ireland (1998). — SMITH, J. R., CARPTEN, J. D., BROWNSTEIN, M. J., GHOSH, S., MAGNUSON, V. L., GILBERT, D. A., TRENT, J. M. and COLLINS, F. S.: Approach to genotyping errors caused by nontemplated nucleotide addition by *Taq* DNA polymerase. Genome Research **5**: 317–321 (1995). — SREIFF, R. and LEFORT, F.: A protocol for higher contrasted DNA silver staining. CastAway Times **6**: 2 (1997). — STEINKELLNER, H., FLUCH, S., TURETSCHEK, E., LEXER, C., STREIFF, R., KREMER, A., BURG, K. and GLÖSSL, J.: Identification and characterization of $(GA/CT)_n$-microsatellite loci from *Quercus Petraea.* Plant Mol. Biol. **33**: 1093–1096 (1997). — TAUTZ, D.: Notes on the definition and nomenclature of tandemly repetitive DNA sequences. *In*: DNA fingerprinting: state of the science. PENA, S.D.J., CHAKRABORTY, R., EPPLEN, J. T. and JEFFREYS, A. J. eds.: Birkhäuser Verlag, Basel, Switzerland. Pp. 21–28. ISBN 3-7643-2781-2 (1993). — WEISING, K., NYBOM, H., WOLFF, K. and MEYER, W.: DNA fingerprinting in plants and fungi. CRC Press, Boca Raton, Florida, USA. ISBN 0-8493-8920-8 (1995). — WHITE, J.: Estimating the age of large trees in Britain. Research Information Note 250, Forestry Commission, 231 Corstorphine Road, Edinburgh EH 12 (1994).

MENDELian Inheritance and Tissue Expression of RAPD-markers in *Picea abies* (L.) KARST.

By E. SKOV

Royal Veterinary and Agricultural University, Department of Economics and Natural Resources, Arboretum, Kirkegårdsvej 3 A, DK-2970 Hørsholm, Denmark

(Received 10th February 1998)

Abstract

Due to a need to identify DNA-markers well suited for genome mapping in Norway spruce, an investigation was carried out with RAPD markers. The plant material – controlled crosses – made it possible to study MENDELian inheritance of the applied RAPD markers in the following tissues: Needles and buds (2n) as well as megagametophytes (n) of parent individuals, germinating embryos, young seedlings as well as 15 year old progenies of the progeny generation (2n). Prior to the MENDELian study, the RAPD assay from DNA extraction through PCR to agarose gel electrophoresis was optimized and an extensive primer screening revealed the available level of polymorphism. The RAPD markers behaved as a rule as reproducible dominant markers and was expressed in all tested tissues, haploid as well as diploid. Occasional occuring RAPD fragments in the haploid megagametophytes, not present in either of the parents, can in mapping projects where megagametophytes are the mapping population be discarded by including a reference sample of the diploid female parent.

Key words: Picea abies, RAPD, tissue expression, MENDELian segregation, repeatability, primer-screening.

FDC: 165.3; 165.41; 174.7 *Picea abies.*

Introduction

Dealing with identification of DNA-markers suitable for genome mapping in *Picea abies* (L.) KARST., a research programme has been initiated in Denmark. This programme serves as a part of a larger project concerning localizing of quantitative trait loci (QTL) expressed later in the development of coniferous trees.

Initial point to clarify is the type of methodology to select, which again causes a wide range of questions to be answered before deciding which markers to apply.

A retrospective view reveals that a lot of attempts have been made to examine the large sized and relative anonymous nuclear genomes of conifers.

Several fragments of genome maps have been constructed in an early period based on isozyme markers (GURIES et al., 1978; CONKLE, 1981; KING and DANCIK, 1983; POULSEN et al., 1985; O'MALLEY et al., 1986; MUONA et al., 1987). Although isozymes are a useful tool for forest genetics and tree improvement research, the small number of mapped loci provides only a limited view of the organization of the conifer genome (NEALE and WILLIAMS, 1991).

A major event in the history of marker methodology is the introduction of the DNA marker, restriction fragment length polymorphism – RFLP – (BOTSTEIN et al., 1980) which has been used to construct linkage maps for identifying quantitative trait loci – QTL – in a number of crop species (PATERSON et al., 1988; LANDER and BOTSTEIN, 1989; KJÆR, 1994) and a few tree species (DEVEY et al., 1991; NEALE and WILLIAMS, 1991; BRADSHAW and FOSTER, 1992; GROOVER et al., 1994). RFLP is a powerful technique considering detection of polymorphism but seems not quite suitable for our purpose due to several facts: i) The large size of the conifer genomes and a great deal of repetitive DNA sequences make standard RFLP analysis difficult (NEALE and WILLIAMS, 1991; WEINING and LANGRIDGE, 1991; WILKIE et al., 1993; NEALE et al., 1994). ii) The method is time consuming and involves technically complex laboratory procedures. iii) The DNA quantity for RFLP exceeds the DNA amount available in the megagametophytes and early – stage seedlings of *Picea abies.*

Recent advances of DNA technology resulted in the development of the polymerase chain reaction – PCR (SAIKI et al., 1985) and later, based on this the random amplified polymorphic DNA (RAPD) methodology entered the research field of genome mapping in 1990 (WELSH and MCCLELLAND, 1990; WILLIAMS et al., 1990). The RAPD technique provides a new way of detecting polymorphism at the DNA level. The assay is based on DNA amplification using only single primers of arbitrary nucleotide sequence. There is no prior requirement for template DNA sequence information and only nanogramme quantities of template DNA is necessary.

Despite a lot of criticism concerning repeatability and dominance (RIEDY et al., 1992; ELLSWORTH et al., 1993) and MENDELian inheritance (HEUN and HELENTJARIS, 1993; LU et al., 1997), the RAPD assay has developed into a widespread and generally accepted powerful tool for genome mapping in the plant kingdom (GRATTAPAGLIA et al., 1992; TINGEY and TUFO, 1993; DAVIS et al., 1995).

Pinaceae constitute an evident opportunity to test the inheritance of the dominant RAPD markers. The megagametophytes are haploid and are derived from the same single mothercell after meiosis which also produces the corresponding egg cell. In this way it is possible to analyse a DNA fragment expressed in diploid tissue for homo- or heterozygosity and to use the segregating loci in megagametophytes as a mapping population. Recently three publications on tissue expression and MENDELian segregation have been published, namely CARLSON et al. (1991) in *Picea glauca* and *Pseudotsuga menziesii,* BUCCI and MENOZZI (1993) in *Picea abies* and LU et al. (1995) in *Pinus sylvestris.*

A number of partial genome mapping attempts has been published in *Pinaceae* based on the segregation of RAPD-markers in the haploid megagametophytes of individual mother trees: *Pinus taeda* (GRATTAPAGLIA et al., 1991), *Picea glauca* (TULSIERAM et al., 1992), *Pinus elliottii* (NELSON et al., 1993), *Pinus palustris* (NELSON et al., 1994), *Picea abies* (BINELLI and BUCCI, 1994), *Pinus brutea* (KAYA and NEALE, 1995), *Pinus sylvestris* (YAZDANI et al., 1995), *Pinus pinaster* (PLOMION et al., 1995a and b).

This report describes the Danish version of optimization of the RAPD assay on four different tissue types (needles, leaf primordia, seedlings and megagametophytes) in *Picea abies.* Based on the obtained confidence in the development of the technique, successive investigations of level of polymorphism, repeatability of the method and MENDELian inheritance are reported.

Materials and Methods

Plant material

In this preliminary investigation, 4 available tissue types (needles, leaf primordia dissected from buds, seedlings and the corresponding megagametophytes) have been tested from two *Picea abies* L. KARST. pedigrees based on controlled crosses *(Fig. 1).*

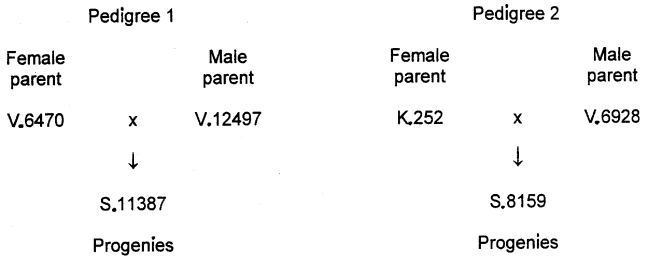


Figure 1. – The two applied pedigrees initiated by controlled crosses.

The female clones used, K 252 and V 6470, are non-related members of different sub populations in the Danish Norway spruce breeding programme (WELLENDORF et al., 1994). Male clones are unrelated with their partners and mutually unrelated as well.

Experiments performed

(1) Optimization procedures involved all 4 tissue types from both applied female clones. These were tested according to a number of variables concerning DNA isolation and the PCR-RAPD procedure *(Table 1).*

(2) Primer screening was carried out through a subset of 5 megagametophytes (n) from clone K 252 using 118 different 10-base primers to demonstrate the level of polymorphism obtained by the optimized procedure.

(3) Repeatability. To test the reliability of the whole process from DNA isolation through DNA-amplification, subsequent gel electrophoresis and observations on specified RAPD fragments, the following experiment was set up. DNA samples from one leafprimordia (2n) and from 5 haploid megagametophytes (no: 58,59,60,61,62) originating from clone V 6470, were analysed against 28 different 10-base primers. The repeatability was investigated by replication of the basic procedures 4 months later. χ^2 analyses of frequencies of identical/non-identical observations as well as frequencies of missing PCR at the first or second observation were carried out.

(4) MENDELian inheritance and tissue expression. Two controlled crossings, pedigree 1 and 2 shown in *figure 1* were utilized to determine the segregation pattern of the dominant RAPD markers in the parents, their megagametophytes as well as in the offspring and simultaneously demonstrate if agreement existed between the different tissue types.

Table 1. – Summary of experience with optimization of 10 components of the RAPD assay in the 4 investigated tissues in *Picea abies*. The underscored alternative was the best suited.

COMPONENTS	TESTED ALTERNATIVES	REMARKS
1) DNA isolation procedures	Sharp et al. 1988 (S-buffer method)	No DNA precipitation
	Qiagen method	No DNA precipitation
	Doyle and Doyle 1990	DNA isolated, but insufficient amount in megagametophytes
	Neale and Williams 1991	Excellent on needle tissue
	Carlson et al. 1991	Works well in all tested tissue types
	Tulsieram et al. 1992	DNA isolated, but PCR not reproducible using megagametophytes
2) Template DNA concentration	0.5 ng, 1.0 ng, 2.0ng, 10ng and 20ng	Below 2 ng PCR not reproducible, higher than 10 ng no reaction.
3) $MgCl_2$ concentration	1.5 mM, 2.0 mM, 2.5mM and 3.0 mM	Lesser background and brighter bands with 2.0 mM
4) Decamer primer concentration	8 ng , 16 ng and 32 ng / reaction	8 ng → ampl. products larger, 32 ng → ampl. products shorter
5) Amplitaq polymerase conc.	0.3 units and 0.5 units / reaction	0.5 units recommended by purchaser, and gives the best results
6) Brand of DNA polymerase	Supertaq versus Amplitaq	Supertaq resulted in too many amplification products to secure correct scoring
7) Individual thermocyclers	Two Perkin Elmer 480s tested	No differences observed
8) Wells in the heating block	Different positions in the heating block	No differences observed
9) Mineral oil in the heating block	+ /–	Only marginal differences observed
10) Type of reaction tubes	Thin walled tubes versus ordinary tubes	Thin walled tubes require another optimized cycler program

DNA isolation

Among several DNA isolation procedures tested (see *Table 1*), the CTAB procedure recommended by BOUSQUET et al. (1990) and modified by CARLSON et al. (1991) with some minor adjustments, proved to be the most efficient to obtain genomic DNA from needles, leaf primordia, seedlings and megagametophytes. The criterion for judging the applied DNA extraction was consistent amplification by PCR.

3 to 5 needles per vial, 2 to 7 leaf primordia (depending on developmental stage) per vial, a single megagametophyte per vial or a whorl of primary leaves from the seedlings per vial are sufficient quantity of material to isolate DNA for a large number of RAPD reactions.

The tissues were transferred aseptically into 1.5 ml sterile microcentrifuge tubes, with 150 µl CTAB isolation buffer (2% (w/v) CTAB (cetyltrimethylammonium bromide, Sigma), 1.4 M NaCl, 20 mM EDTA, 1% (w/v) PEG 8000 (polyethylene glycol, Sigma) and 100 mM TRIS-HCl (pH 9.5)). The tubes were frozen in liquid nitrogen for 30 sec., followed by grinding of the tissue with a fitted glass rod. The glass rod was rinsed with 700 µl CTAB buffer, in the microcentrifuge tube to be mixed with the homogenate and then incubated at 65 °C for 30 min. Afterwards the homogenate was extracted with 500 µl chloroform: isoamyl alcohol (24:1) and then placed at a shaking table (slow vibrations) for 30 min followed by a centrifugation in a microcentrifuge at 13,000 rpm for 5 min at 4 °C. DNA was precipitated from the aqueous phase by mixing with 500 µl isopropanol and pelleting by centrifugation at 4 °C for 5 min. The DNA pellet was rinsed in 70% ethanol, vacuum dried, and resuspended in 100 µl 1 x TE buffer (10 mM Tris, 0.25 mM EDTA, pH 8.0).

Concerning DNA isolation from needle tissue: The needles were washed in alcohol and grounded to powder in liquid nitrogen using sterile mortar and pestle. The powder was then transferred to separate microcentrifuge tubes and the above description was followed until the point of extraction, which was done twice or more on needle tissue.

Finally the DNA concentrations were measured using a fluorometric assay (CESARONE et al., 1979), and the samples were diluted to equal concentrations.

RAPD analysis

The optimal RAPD reaction conditions were established after testing a range of different factors (see *Table 1*) resulting in the following procedure.

Amplification reactions were performed according to WILLIAMS et al. (1990) with minor modifications. The reaction volumes of 20 µl contained 2 ng to 5 ng template DNA, 0.5 unit Amplitaq DNA polymerase (Perkin Elmer, Cetus), 2 µl 10 x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl), 200 µM each dATP, dCTP, dGTP and dTTP (Pharmacia), 2.0 mM MgCl2 and 16 ng of a 10-base random primer (Operon technologies, Alameda CA). The reactions were prepared on ice and transferred to a 93 °C thermocycler (Perkin Elmer, Cetus, 480) programmed for an initial denaturation stage at 93 °C in 5 min and subsequent 45 cycles consisting of 1 min at 93 °C (denaturation), 1 min at 36 °C (primer annealing) and 2 min at 72 °C + extension time 1 sec/cycle from the 6. cycle (primer extension). The fastest attainable transitions between each temperature were employed. After cycling the reactions were soaked at 4 °C until recovery.

A volume of 16 µl of amplification product was added to 4 µl loading buffer ll and analyzed by electrophoresis in 1.4% agarose TBE gels with ethidium bromide (SAMBROOK et al., 1989). Bands were visualized by UV light and photographed with Polaroid film for instant interpretation.

Results

Optimization of the RAPD assay for Picea abies

According to *table 1* the procedures of CARLSON et al. (1991) were conclusive, at least in our laboratory, for DNA isolation because of its effectiveness in all tissue types tested. A striking demand to the method to be suitable for genome mapping in conifers based on haploid megagametophyte populations, is a sufficient amount of isolated DNA from individual megagametophytes to run a large number of RAPD reactions. This point was fulfilled with yields averaging 4 µg/megagametophyte. The method proposed by NEALE and WILLIAMS (1991) was excellent when used on needle tissue. It could be recommended if the demand is large quantities of DNA and the marker type co-dominant, for instance if the aim is RFLP linkage maps based on known pedigrees.

Within the frame given by WILLIAMS et al. (1990), different components in the RAPD assay were adjusted one by one, keeping the remaining components constant (see *Table 1*).

Template DNA concentrations were varied between 0.5 ng and 20 ng per reaction. The most critical tissue type – the megagametophytes – reacted with unreproducible amplification products in concentrations below 2 ng. Utilizing DNA concentrations higher than 10 ng, no amplification was observed. DNA from needles, leafprimordia and seedlings showed the same tendency although less pronounced. The optimal concentrations of template DNA were 2 ng to 5 ng/reaction.

In tests of $MgCl_2$ concentrations in the range of 1.5 mM to 3.0 mM, no crucial dissimilarities were found. All amplification products were reproduced in each concentration used, and no new products appeared. $MgCl_2$ concentration at 2.0 mM was chosen according to the brightness of the fragments.

Three different concentrations of decamer primer, 8.0 ng, 16 ng and 32 ng per reaction were tested. A general tendency appeared. Using high decamer concentrations the amplified fragment lengths were displaced in a direction of shorter products and low decamer concentrations amplified larger fragments. This is a key point in narrow defined jobs where specified fragments are sought. It might be useful in joint mapping projects where one may be forced to reproduce specific RAPD fragments run at other laboratories. To a certain degree variable success was obtained in the assay depending on the primer used, indicating that a few primers needed separate optimization.

Concerning Amplitaq DNA polymerase, the most expensive ingredient in the RAPD-assay, we unsuccessfully tried a reduction of the recommended concentration given by the purchaser. When moving from 0.5 to 0.3 units per reaction, several amplification products disappeared and by using that option we needed to run far more primers to reach the same level of polymorphism.

Attempts were made to change Amplitaq DNA polymerase with SuperTaq (HT BioTechnology). Amplification succeeded to a degree where interpretation was complicated by too many bands. Careful examination made it possible to re-create the fragments observed in Amplitaq DNA polymerase runs.

Two Perkin Elmer Thermal 480 Cyclers were utilized interchangeable and no differences were observed. Wells in the heating block of the thermocyclers were tested for uniformity regularly. No dissimilarities were found.

Primer screening

To identify the level of polymorphism achieved by the above mentioned optimization, a subset of DNA from 5 megagametophytes (n) belonging to clone K 252 was screened against 118 different decamer primers. Segregation of markers in haploid tissue is expected to follow a 1 : 1 MENDELian ratio of presence/absence of marker band and therefore the use of 5 megagametophytes will give reasonable confidence (94% of the time) in detecting heterozygotic loci in the maternal parent tree.

Each primer produced a unique banding pattern. Of 118 primers tested, 88 were found to be usable in the prospect of genome mapping. The 88 segregating primers gave rise to 223 potential polymorphic loci, which is an average of 2.5 polymorphism/primer. 8 primers were monomorphic and in 9 primers no reactions were found. The remaining group of 13 primers expressed a weak reaction which indicated that a further optimization of these primers might be necessary. Typically a suitable primer gave rise to 5 to 14 bands ranging 300 to 2600 base pairs.

Repeatability

We needed to demonstrate that under the same reaction conditions we could reproduce identical amplification products.

In order to separate tests of repeatability of the double-observations and the total response pattern, two sets of contingency tables were lined up: a) Test of repeatability, + : identical observations, – : non-identical observations, see *table 2* and *3*, and b) Test of total response pattern including missing observations ~ 0 : *table 4* and *5*. In each of the contingency tables, the standard G^2-test (SOKAL and ROHLF, 1981) was applied to test independence of factors (tissue and RAPD bands respectively) and the response counts. The χ^2 for each table has d.f. = (r-1)(c-1), where r is the number of rows and c is the number of columns in the contingency tables.

Table 2. – Observations on repeatability on tissue.

Tissue	Numbers of		
	+	–	total
Buds (2N)	80	2	82
MG58 (N)	88	4	92
MG59 (N)	74	3	77
MG60 (N)	92	2	94
MG61 (N)	71	5	76
MG62 (N)	86	4	91
Total	491	20	509

+ : identical observations in two successive analyses;
– : different observations in two successive analyses;
0 : missing observations due to fall-out of PCR.

Table 3. – Observations on repeatability on RAPD bands.

RAPD bands	Numbers of		
	+	–	total
1	5	0	5
2	5	0	5
3	4	1	5
–	–	–	–
–	–	–	–
95	5	0	5
Total	491	20	509

+ : identical observations in two successive analyses;
– : different observations in two successive analyses;
0 : missing observations due to fall-out of PCR.

Table 4. – Observations on total response pattern on tissue.

Numbers of			
+	–	0	total
80	2	13	95
88	4	3	95
74	3	18	95
92	2	1	95
71	5	19	95
86	4	5	95
491	20	59	570

+ : identical observations in two successive analyses;
– : different observations in two successive analyses;
0 : missing observations due to fall-out of PCR.

Table 5. – Observations on total response pattern on RAPD bands.

Numbers of			
+	–	0	total
5	0	1	6
5	0	1	6
4	1	1	6
–	–	–	–
–	–	–	–
5	0	1	6
491	20	59	570

+ : identical observations in two successive analyses;
– : different observations in two successive analyses;
0 : missing observations due to fall-out of PCR.

The results of the 4 G^2-tests are shown in *table 6*. The mosaic plot in *figure 2* shows the proportion of + , – , and 0 observations corresponding to the total response pattern for tissues. Concerning conclusions on repeatability, i.e. the frequency of identical observations of defined RAPD bands, the overall frequency is 491 / 509 = 96.5%. The rare non-identical observations seem to be randomly distributed to tissues and primers. Concerning conclusions of the total response pattern, i.e. including the distribution of missing reactions during the first or second run, highly significant effects of the tested tissues appeared. However, according to *figure 2* the dependence seems not associated with the distinction between diploid or haploid tissue.

MENDELian inheritance and tissue expression

The genetic and experimental design used made it possible in one investigation to expose if discrepancies from MENDELian segregation and deviations between tissue types occurred.

An example of the original observations on two RAPD-fragment in pedigree 1 is presented in *figure 3* and the corresponding interpretation is presented in *table 7*.

Each RAPD fragment analysis included tissue from the female parent represented by replicated runs of DNA from needles (2N) and leafprimordia from dormant buds (2N) besides DNA from 7 megagametophytes (N) from her seeds. The male parent was subject to a similar sampling. Seedlings (2N) descending from a controlled cross between the involved parent trees were tested in order to follow the inheritance of specific RAPD fragments.

Although the sample size is too small for critical tests of distortions, the observed segregations are in accordance with expectations of dominant markers.

As is demonstrated here, crossings between the heterogeneous members of the wild, outcrossing base-populations results in all type of situations, two of which are realized here, i.e. a heterozygote x homozygote recessive (a test-cross situation for locus A) and a heterozygote x heterozygote (a F_2 situation for locus B).

Concerning tissue expression, agreement seems to occur:

V 6470 has the + phenotype in the two loci in both diploid tissues, needles and leafprimordia. The segregation in the female haploid megagametophytes indicates, that the female is heterozygotic in both loci.

V 12497 shows the phenotype of a 'silent' allele for locus A in both diploid tissues indicating that the male is homozygotic recessive. For locus B, the male shows the + phenotype in both diploid tissues. Looking at the male megagametophyte segregation the homozygotic recessive status of locus A is confirmed by lack of + phenotypes. For the other locus B, the male shows up to be heterozygotic as + and – phenotypes segregate.

In progeny S 11387 (V 6470 x V 12497) sampling in the diploid tissue of early-stage seedlings shows the expected segregations mentioned above and shown in *figure 3* and *table 7*.

A summary of 25 such tests of MENDELian inheritance is presented in *table 8*. For situations where segregation is expected in the progeny, G^2-tests are performed to check if the proportions are in accordance with the expected, i.e. 1:1 for the backcross to the homozygote recessive (test-cross situations) and 3:1 for the F_2-situations.

Backcross to the homozygotic dominant parent and two dominant homozygotes crossed with each other ("hom dom selfed") are not expected to generate segregation in the progeny if complete dominance is in operation.

A closer interpretation of the material in *table 7* and *8* confirms the general experience of RAPD fragments as fully dominant markers closely following MENDELian inheritance.

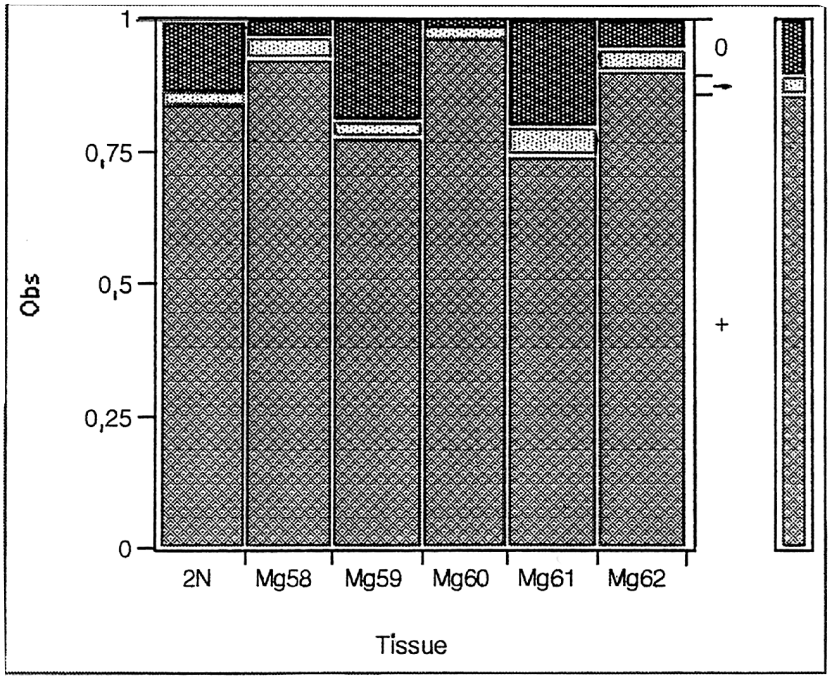
These markers are expressed in diploid tissues: needles, leafprimordia from dormant buds and early stage seedlings as well as in haploid tissue, the megagametophytes.

Conclusions and Discussion

Concerning the RAPD assay, establishment of standard conditions in *Picea abies* proved to be critical due to the sensi-

Table 6. – 4 tests of independence between i) tissue and response counts and ii) RAPD bands and response counts.

Respons Factors	Test of repeatability 2 response classes: + / –			Test of total response pattern 3 response classes: + / – / 0		
	X^2	d.f.	p	X^2	d.f.	p
i) Tissue	2.80 NS	5	.730	42.04***	10	.000
ii) RAPD bands	78.08 NS	94	.882	170.95NS	188	.809



0: Missing PCR at first or second run; –: non-identical observations; +: identical observations at first and second run.

Figure 2. – Mosaic plot of repeatability investigation.

tivity of the system to experimental variables. Consequently, amplification of random genomic sequences in a reproducible way is only possible with rigorously optimized reaction conditions as demonstrated in the present investigation. The importance of this general experience is shared by numerous authors.

With the developed RAPD assay a reproducibility of 96.5% in the present investigation matches remarkable well with the relative few other reported reproducibility figures (WEEDEN et al., 1992; HEMMAT et al., 1994; and SKROCH and NIENHUIS, 1995).

Concerning identification of components of the RAPD assay which are the most critical, a certain discrepancy appears between reports.

In the present investigation in *Picea abies* on different tissues, DNA extraction – influencing DNA purity and amount – as well as DNA concentration showed up to be key points. Other investigators agree on the importance of DNA purity (WEEDEN et al., 1992), whereas the same authors for template DNA find that the concentration could be varied 10-fold without seriously affecting the RAPD-pattern.

In *Picea abies* the importance of fluorometric or spectrophotometric analyses to standardize the DNA concentrations across all individuals is stressed.

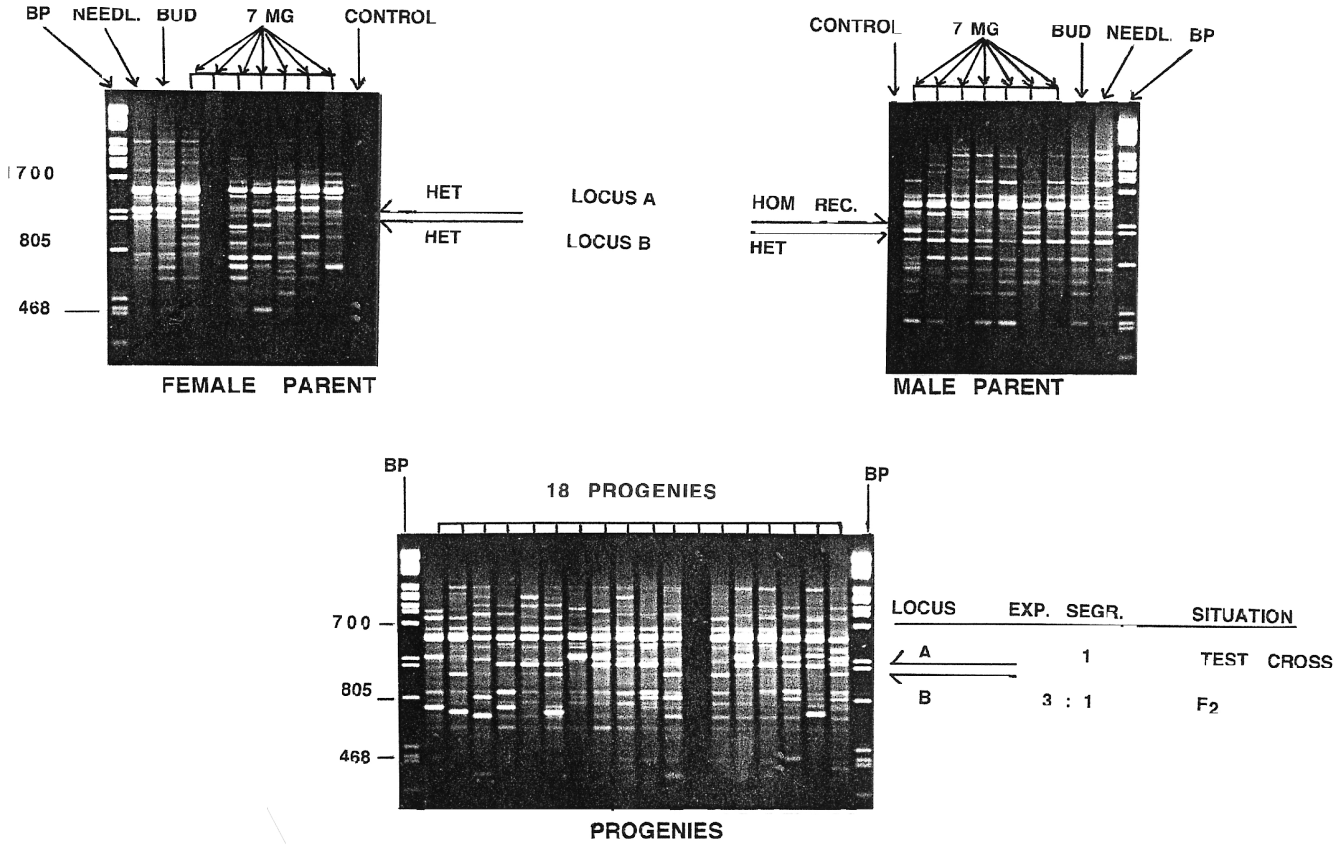
In principle each oligonucleotide primer requires optimization with respect to the amplification conditions, concentration of primer relative to the template, and magnesium concentra-

Table 7. – MENDELian inheritance and tissue expression. Example of two RAPD loci.

		Female parent - V 6470														Male parent – V 12497									
Loc	Zyg	2n tissue		n tissue (MG no)							n tissue (MG no)							2n tissue		Zyg
		needl	bud	1	2	3	4	5	6	7	1	2	3	4	5	6	7	needl	bud	
A	ht	+	+	+	0	–	+	–	+	+	–	–	–	–	–	–	–	–	–	hm r
B	ht	+	+	+	0	+	+	–	+	+	+	+	+	+	–	+	–	+	+	ht

18 progenies - S 11387 - seedlings																				
	2n tissue - (individual no)																		Segregation +/–	
Loc	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Situation	Obs	Exp
A	+	–	+	–	+	+	–	–	+	+	+	0	+	–	–	+	–	+	test cross	10 : 7	1 : 1
B	–	–	–	+	+	+	–	+	+	+	+	0	–	+	+	+	+	–	F_2	11 : 6	3 : 1

MG: Megagametophyte;
Loc: locus; Zyg: Zygosity; ht: heterozygote; hm r: homozygote recessive (–); +: band present; –: band absent; 0: missing observation (PCR-reaction drop-out).



BP: Base pair ladder, λ digested with Pst 1; MG: megagametophytes – haploid (n); HOM-REC: Homozygotic recessive; HET: Heterozygotic; TEST-CROSS: Back-cross to a homozygotic recessive.

Figure 3. – MENDELian inheritance and tissue expression. Example of two RAPD loci.

tion. However, in practice the great majority of primers requires nearly identical experimental conditions and these should therefore be followed strictly by the default.

With the RAPD method of fragment amplification an often occurring artefact on agarose gels is the resolution of some minor bands that are not repeatable. These unstable bands have been suggested to result from the formation of artificial heteroduplexes between multiple amplified fragments (AYLIFFE et al., 1994) or from non specific amplification, that is, amplification when primer/template homology is not perfect. These artefacts were minimized by optimizing PCR components.

Occasional, more bands were observed in megagametophyte DNA than expected from the parents DNA phenotype observed in their diploid tissue. Further investigations are needed to determine the cause of these aberrant patterns.

In the stage of demonstrating the MENDELian inheritance and tissue expression through a generation turn-over, the developed RADP assay seems to be reliable for subsequent genetic analyses. For a specified sample of RAPD fragments the inheritance and tissue expression have been checked and were found to be in accordance with expectations for a dominant marker. This infers monitoring the markers through the generation turn-over from the parents diplophase over the female parents haplophase to the diplophase of the progenies.

On this background, it is recommended to apply a diploid parent reference sample when analyzing segregation and recombinations in megagametophyte populations. In this way one avoid scoring loci that do not occur in diploid tissue.

The cited conclusions are in general agreement with other investigations of the formal genetics of RAPD fragments in *Picea glauca* and *Pseudotsuga menziesii* (CARLSON et al., 1991), in *Picea abies* (BUCCI and MENOZZI, 1993) and in *Pinus sylvestris* (LU et al., 1995).

A number of investigators have used RAPDs in megagametophytes from *Pinaceae* individuals as mapping populations (GRATTAPAGLIA et al., 1991; TULSIERAM et al., 1992; NELSON et al., 1993; BINELLI and BUCCI, 1994) and others. Implicit in these investigations are 1 : 1 tests of segregation of presence or absence of RAPD fragments in the mapping population. These observations are therefore supplying us with further information of the formal MENDELian genetics of specified RAPD fragments in *Pinaceae.*

The general conclusion then is, that amplification products in the form of RAPD fragments defining polymorphisms exhibit MENDELian inheritance and is expressed in diverse tissues.

The developed assay for RAPD markers thus fulfils the required demand for a type of DNA marker well suited for dense genome mapping in *Picea abies* and other *Pinaceae.* The expression in diverse tissues is important for use in mapping of quantitative trait loci (QTL), as we in such projects need to determine the genotype in successive developmental stages. For application in subsequent marker aided selection (MAS) it is furthermore convincing that RAPD markers are expressed in available tissues associated with somatic embryogenesis (ISABEL et al., 1993).

Table 8. – Summary table of performed tests of MENDELian inheritance of 22 RAPD fragments in two pedigrees.

RAPD fragment	Female parent			Male parent			Progeny population				
							Situation	Segregation		G^2 test	
Primer - locus	hom dom	het	hom rec	hom dom	het	hom rec		Observed	Expected	χ^2	d.f.
	K 252			V 6928			S 8159				
C 07 -a		x			x		F_2	12:5	3:1	0.1702	1
C 07 -b		x				x	BC to hom rec	8:9	1:1	0.0294	1
C 07 -c		x			x		F_2	12:5	1:1	0.1702	1
C 07 -d	x			x			self hom dom	17:0	1:0		
C 09 -a		x			x		F_2	15:3	3:1	0.7280	1
C 09 -b		x			x		F_2	13:5	3:1	0.0724	1
C 09 -c		x			x		F_2	13:5	3:1	0.0724	1
C 15 -a		x				x	BC to hom rec	9:9	1:1	0.0000	1
C 15 -b		x			x		F_2	13:5	3:1	0.0724	1
C 15- c		x			x		F_2	11:7	3:1	1.6802	1
D 20 -a	x					x	F_1	18:0	1:0		
D 20- b		x		x			BC to hom dom	18:0	1:0		
D 20- c		x				x	BC to hom rec	10:8	1:1	0.1113	1
D 20-d		x			x		F_2	12:6	3:1	0.6254	1
	V 6470			V 12497			S 11387				
A 02 -a	x			x			self hom dom	18:0	1:0		
A 02 -b		x				x	BC to hom rec	10:8	1:1	0.1113	1
A 02 -c		x			x		F_2	14:4	3:1	0.0760	1
A 02 -d			x		x		BC to hom rec	9:9	1:1	0.0000	1
B 12 -a		x		x			BC to hom dom	17:0	1:0		
B 12 -b		x				x	BC to hom rec	10:7	1:1	0.2661	1
B 12 -c			x		x		BC to hom rec	9:8	1:1	0.0294	1
B 12 -d	x			x			self hom dom	17:0	1:0		
C 15 -a		x			x		F_2	13:4	3:1	0.0199	1
C 15 -b		x				x	BC to hom rec	8:9	1:1	0.0294	1
C 15 -c		x			x		F_2	12:5	3:1	0.1702	1
Grand total	4	19	2	5	12	7				4.4342 NS	19

hom dom: homozygote dominant; het: heterozygote; hom rec: homozygote recessive; BC: backcross; 5% fractile for χ^2-tests with 1 d.f. is 3.84, i.e. none of the individual χ^2-tests contradicts MENDELean inheritance. The total χ^2 for all the independent tests is far from any upper limit fractile, i.e. the overall pattern is in accordance with MENDELean expectations.

Acknowledgements

This study has been carried out at the Risø National Laboratory, Department of Plant Genetics and Resistance Biology in the period 1991 to 1994. Financing has been provided by the Danish Research Council to the project 'Identification of DNA markers in Norway spruce well suited for genome mapping' initiated by the Hørsholm Arboretum, Royal Veterinary and Agricultural University, which also has supplied part of the applied laboratory equipment. Consultancy on statistics has been provided by H. WELLENDORF. Finally, VIBEKE SIMONSEN has kindly reviewed the manuscript.

References

AYLIFFE, M. A., LAWRENCE, G. J., ELLIS, J. G. and PRYOR, A. J.: Heteroduplex molecules formed between allelic sequences cause nonparental RAPD bands. Nucleic Acids Research **22**(9): 1632–1636 (1994). — BINELLI, G. and BUCCI, G.: A genetic linkage map of *Picea abies* KARST., based on RAPD markers, as a tool in population genetics. Theoretical and Applied Genetics **88**: 283-288 (1994). — BOTSTEIN, D., WHITE, R. L., SKOLNICK, M. H. and DAVIS, R. W.: Construction of a genetic map in man using restriction fragment length polymorphisms. American Journal of Human Genetics **32**: 314–331 (1980). — BOUSQUET, J., SIMON, L. and LALONDE, M.: DNA amplification from vegetative and sexual tissues of trees using polymerase chain reaction. Canadian Journal of Forest Research **20**: 254–257 (1990). — BRADSHAW, H. D. and FOSTER, G. S.: Marker aided selection and propagation systems in trees: advantages of cloning for studying quantitative inheritance. Canadian Journal of Forest Research **22**: 1044–1049 (1992). — BUCCI, G. and MENOZZI, P.: Segregation analysis of random amplified polymorphic DNA (RAPD) markers in *Picea abies* KARST.. Molecular Ecology (2): 227–232 (1993). — CARLSON, J. E., TULSIERAM, L. K., GLAUBITZ, J. C., LUK, V. W. K., KAUFFELDT, C. and RUTLEDGE, R.: Segregation of random amplified DNA markers in F1 progeny of conifers. Theoretical and Applied Genetics **83**: 194–200 (1991). — CESARONE, C. F., BOLOGNESI, C. and SANTI, L.: Improved Microfluorometric DNA Determination in Biological Material Using 33258 Hoechst. Analytical Biochemistry **100**: 188–197 (1979). — CONKLE, M. T.: Isozyme Variation and Linkage in Six Conifer Species. Symposium on Isozymes of North American Forest Trees and Forest Insects, Berkeley, California (1981). — DAVIS, T. M., YU, H., HAIGIS, K. M. and MCGOWAN, P. J.: Template mixing: a method of enhanching detection and interpretation of codominant RAPD markers. Theoretical and Applied Genetics **91**: 582–588 (1995). — DEVEY, M. E., JERMSTAD, K. D., TAUER, C. G. and NEALE, D. B.: Inheritance of RFLP loci in loblolly pine three-generation pedigree. Theoretical and Applied Genetics **83**: 238–242 (1991). — DOYLE, J. J. and DOYLE, J. L.: Isolation of DNA from small amounts of plant tissue. Focus **12**: 13–15 (1990). — ELLSWORTH, D. L., RITTENHOUSE, K. D. and HONEYCUTT, R. L.: Artifactual Variation in Randomly Amplified Polymorphic DNA Banding Patterns. BioTechniques **14**(2) (1993). — GRATTAPAGLIA, D., O'MALLEY, D. and SEDEROFF, R.: Multiple applications of RAPD markers to genetic analysis in *Eucalyptus sp.* IUFRO Conference, S2.02–08, Breeding Tropical Trees, Cartagena and Cali, Columbia, CAMCORE (1992). — GRATTAPAGLIA, D., WILCOX, P., CHAPARRO, J. X., O'MALLEY, D. M., MCCORD, S., WHETTERN, R., MCINTYRE, L. and SEDEROFF, R.: A RAPD map of loblolly pine in 60 days. International Society for Plant Molecular Biology, International Congress, Tucson, Arizona (1991). — GROOVER, A., DEVEY, M., FIDDLER, T., LEE, J., MEGRAW, R., MITCHEL-OLDS, T., SHERMAN, B., VUJCIC, S., WILLIAMS, C. and NEALE, D.: Identification of Quantitative Trait Loci Influencing Wood Specific Gravity in an Outbred Pedigree of Loblolly Pine. Genetics **138**: 1293–1300 (1994). — GURIES, R. P., FRIEDMAN, S. T.

and LEDIG, F. T.: A megagametophyte analysis of genetic linkage in pitch pine *(Pinus rigida)*. Heredity **40**: 309–314 (1978). — HEMMAT, M., WEEDEN, N. F., MANGANARIS, A. G. and LAWSON, D. M.: Molecular Marker Linkage Map for Apple. Journal of Heredity (January/February): 4–11 (1994). — HEUN, M. and HELENTJARIS, T.: Inheritance of RAPDs in F1 hybrids of corn. Theoretical and Applied Genetics **85**: 961–968 (1993). — ISABEL, N., TREMBLAY, L., MICHAUD, M., TREMBLAY, F. M. and BOUSQUET, J.: RAPDs as an aid to evaluate the genetic integrety of somatic embryogenesis-derived populations of *Picea mariana* (MILL.) B.S.P. Theoretical and Applied Genetics **86**: 81–87 (1993). — KAYA, Z. and NEALE, D. B.: Utility of Random Amplified Polymorphic DNA (RAPD) Markers for Linkage Mapping in Turkish Red Pine (*Pinus brutia* TEN.). Silvae Genetica **44**(2–3): 110–116 (1995). — KING, N. J. and DANCIK, B. P.: Inheritance and linkage of isozymes in white spruce *(Picea glauca)*. Canadian Journal of Genetics and Cytology **25**: 430–436 (1983). — KJÆR, B. J.: Use of molecular markers to identify quantitative trait loci in barley. The Royal Veterinary and Agricultural University. PhD thesis (1994). — LANDER, E. S. and BOTSTEIN, D.: Mapping MENDELian factors underlying quantitative traits using RFLP linkage maps. Genetics **121**: 185–199 (1989). — LU, M.-Z., SZMIDT, A. E. and WANG, X.-R.: Inheritance of RAPD fragments in haploid and diploid tissues of *Pinus sylvestris* (L.). Heredity **74**(6): 582–589 (1995). — LU, M.-Z., WANG, X.-R. and SZMIDT, A.E.: Molecular properties in RAPDs in *Pinus sylvestris* (L.) and their implications for genetic analysis. Forest Genetics **4**(4): 227–234 (1997). — MUONA, O., YAZDANI, R. and LINDQUIST, G.: Analysis of linkage in *Picea abies*. Hereditas **106**: 31–36 (1987). — NEALE, D. B., KINLAW, C. S. and SEWELL, M. M.: Genetic Mapping and DNA sequencing of the Loblolly Pine Genome. Forest Genetics **1**(4): 197–206 (1994). — NEALE, D. B. and WILLIAMS, C. G.: Restriction fragment length polymorphism mapping in conifers and applications to forest genetics and tree improvement. Canadian Journal of Forest Research **21**: 545–554 (1991). — NELSON, C. D., KUBISIAK, T. L., STINE, M. and NANCE, W. L.: A Genetic Linkage Map of Longleaf Pine (*Pinus palustris* MILL.) Based on Random Amplified Polymorphic DNAs. Journal of Heredity **85**(6): 433–439 (1994). — NELSON, C. D., NANCE, W. L. and DOUDRICH, R. L.: A partial genetic linkage map of slash pine *(Pinus elliottii* ENGELM. var. *elliottii)* based on random amplified polymorphic DNAs. Theoretical and Applied Genetics **87**: 145–151 (1993). — O'MALLEY, D., GURIES, R. P. and NORDHEIM, E. V.: Linkage analysis for 18 enzyme loci in *Pinus rigida* MILL. Theoretical and Applied Genetics **72**: 530–535 (1986). — PATERSON, A. H., LANDER, E. S., HEWITT, D. J., PETERSON, S., LINCOLN, S. E. and TANKSLEY, S.D.: Resolution of quantitative traits into MENDELian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature **335**: 721–726 (1988). — PLOMION, C., BAHRMAN, N., DUREL, C.-E. and O'MALLEY, D. M.: Genomic mapping in *Pinus pinaster* (maritime pine) using RAPD and protein markers. Heredity **74**: 661–668 (1995a). — PLOMION, C., O'MALLEY, D. M. and DUREL, C. E.: Genomic analysis in maritime pine *(Pinus pinaster)*. Comparison of two RAPD maps using selfed and open-pollinated seeds of the same individual. Theoretical and Applied Genetics **90**: 1028–1034 (1995b). — POULSEN, H. D., SIMONSEN, V. and WELLENDORF, H. : The Inheritance of Six Isozymes in Norway Spruce (*Picea abies* (L.)KARST.). Forest Tree Improvement **16**: 12–33 (1985).— RIEDY, M. F., ILL, W. J. H. and AQUADRO, C. F.: Excess of non-parental bands in offspring from known primate pedigrees assayed using RAPD PCR. Nucleic Acids Research **20**(4): 918 (1992). — SAIKI, R. K., SCHARF, S., FALLONA, F., MULLIS, K., HORN, G. T., ERLICH, H. A. and ARNHEIM, N.: Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**: 1350–1354 (1985). — SAMBROOK, J., FRITSCH, E. F. and MANIATIS, T.: Molecular cloning. A laboratory manual. Cold Spring Habor, New York, Cold Spring Habor Laboratory Press (1989). — SHARP, P. J., KREIS, M., SHEWRY, P. R. and GALE, M. D.: Location of α- amylase in wheat and its relatives. Theoretical and Applied Genetics **75**: 286–290 (1988). — SKROCH, P. and NIENHUIS, J.: Impact of scoring error and reproducibility of RAPD data on RAPD based estimates of genetic distance. Theoretical and Applied Genetics **91**(6–7): 1086–1091 (1995). — SOKAL, R. R. and ROHLF, F. J.: Biometry. The Principles and Practice of Statistics in Biological Research. San Francisco, W.H. Freeman and Company (1981). — TINGEY, S. V. and TUFO, J. P.: Genetic Analysis with Random Amplified Polymorphic DNA markers. Plant Physiology **101**: 349–352 (1993). — TULSIERAM, L. K., GLAUBITZ, J. C., KISS, G. and CARLSON, J. E.: Single tree genetic linkage mapping in conifers using haploid DNA from megagametophytes. Bio/Technology **10**(June): 686–690 (1992). – WEEDEN, N. F., TIMMERMAN, G. M., HEMMAT, M., KNEEN, B. E. and LODHI, M. A.: Inheritance and Reliablility of RAPD markers. Applications of RAPD Technology to Plant Breeding, Minneapolis, Minnesota. USA. Crop Science Society of America, American Society for Horticultural Science, American Genetic Association (1992). — WEINING, S. and LANGRIDGE, P.: Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction. Theoretical and Applied Genetics **82**: 209–216 (1991). — WELLENDORF, H., SKOV, E. and KJÆR, E.: Suggested updating of improvement strategy for Danish grown Norway spruce. Forest Tree Improvement **25**: 1–12 (1994). — WELSH, J. and MCCLELLAND, M.: Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research **18**: 7213–7218 (1990). — WILKIE, S. E., ISAAC, P. E. and SLATER, R. J.: Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theoretical and Applied Genetics **86**: 497–504 (1993). — WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. and TINGEY, S. V.: DNA polymorphisms Amplified by Arbitrary Primers Are Useful as Genetic Markers. Nucleic Acids Research **18**: 6531–6535 (1990). — YAZDANI, R., YEH, F. C. and RIMSHA, J.: Genomic Mapping of *Pinus sylvestris* (L.) Using Random Amplified Polymorphic DNA Markers. Forest Genetics **2**(2): 109–116 (1995).

Timing of DNA Extraction from Megagametophytes for PCR during Initial Steps of Seedling Development in *Picea abies* (L.) KARST.

By E. SKOV

Royal Veterinary and Agricultural University, Department of Economics and Natural Resources, Arboretum, Kirkegårdsvej 3 A, DK-2970 Hørsholm, Denmark

(Received 10th February 1998)

Abstract

Within the framework of QTL investigations and subsequent marker aided selection in *Pinaceae,* a need to grow seedlings with saved megagametophytes for DNA extraction has evolved. The present investigation reports a timing experiment in which the optimum stage of seedling development is identified. DNA amount proved to be relative independent of seedling development, but successful PCR was, probable due to the occurrence of fat and carbohydrates, only possible at relative late stages of seedling development when the role of the megagametophyte as a nutrient source has decreased. Removing the megagametophyte at this stage is not a problem for the germinating seedlings, which then are relying on their own root uptake and photosynthesis.

Key words: DNA extraction, megagametophytes, RAPD, *Picea abies,* seedling development.

FDC: 165.3/4; 181.525; 174.7 *Picea abies.*

Introduction

An important field of supportive research for conifer breeding is to map major genes behind breeding objectives. By using DNA-markers for genome mapping it is possible to study linkage between these markers and quantitative trait loci – QTL – in for instance *Picea abies* (L.) KARST..

The PCR based random amplified polymorphic DNA markers – RAPD – appear to be well suited for genetic mapping in *Pinaceae* species, because it is possible to utilize the tiny megagametophytes of individual seeds of a single tree as a mapping population.

Pinaceae seeds, when fertilized, consist of a haploid nutritive tissue, the megagametophyte, and the diploid developing embryo. The megagametophytic tissue and the corresponding egg cell – female founder of the embryo – originate from a single mother cell as a product of a meiotic division, and therefore posses identical genotypes (CHAMBERLAIN, 1935).

A main drawback of the RAPD system is the dominant expression (absent/present) of this marker type. Usually there is only two alleles in a certain locus and heterozygotes cannot be distinguished from homozygotes in diploid tissue. This can be circumvented by using haploid tissue. By recording markers in the haploid megagametophytes, we can identify the mother contribution to the genotype of the progeny and we can map the markers of individual mother trees by performing classical linkage studies on recombinations of markers directly expressed in the haploid megagametophytes.

QTL-mapping can then be performed by studying co-segregation of markers in the megagametophytes - the mother contribution – and quantitative traits in the corresponding germinating half-sib or full-sib offspring population, which later develops into juvenile and mature trees (WILCOX et al., 1996).

An important component in such QTL experiments is therefore the survival rate of the seedlings with rescued megagametophytes as well as purity of the extracted DNA from the corresponding megagametophytes.

Although RAPDs are simple to perform, the early experience with amplification of RAPD fragments from megagametophytic DNA did not turn out convincing at least in our laboratory. Often the resultant amplified product was missing or not complete and not reproducible at a sufficient high rate. In order to refine the combined process of RAPD-analyses on saved megagametophytic tissue and secure undisturbed further growth of the corresponding seedling, a developmental stage/PCR experiment was initiated.

The reported study is aiming to uncover the two-sided problem encountered:

- Development of a DNA extraction method tailored to megagametophytes of germinating seedlings.
- Development of a safe method for growing seedlings when megagametophytes are rescued for DNA extraction.

Materials and Methods

Handling of plant material

Seeds from clone number V6470 were germinated on sterile filter paper according to the method of KNUDSEN (personal communication), see *figure 1.*

During the process of early germination megagametophytes were rescued from a sample of at least 5 germinating seedlings

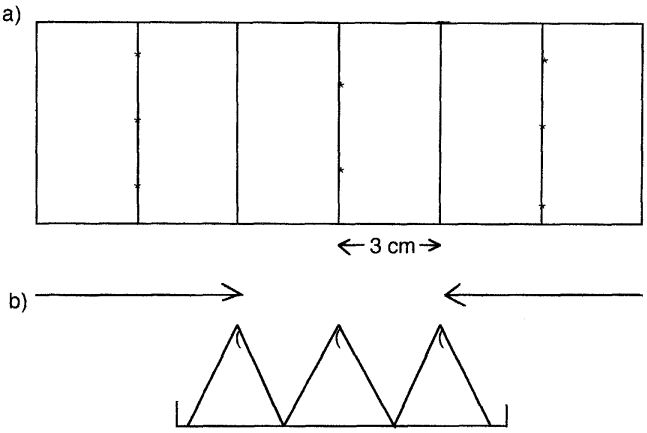


Figure 1. – Germination in Petri dishes in folded filterpaper according to the "KNUDSEN method". Surfaced-treated seeds are placed at regular intervals on sterile filterpaper. a) Seen from above – b) side view. After folding the filterpaper and moistening with water, the germination will be initiated and seedlings develop an erect growth pattern suitable for later transplanting after megagametophyte rescuing.

when they had reached 5 successive stages of development, see *figure 2.* Stage 1: radicle not seen, stage 2: radicle 1 cm, stage 3: radicle 2 cm, stage 4: radicle 4 cm, and stage 5: full-grown seedling, just before spontaneous release of the megagametophyte. In the different stages mentioned, the seed coat was removed and the megagametophytes were separated from the embryos / seedlings and stored at -80 °C in distinct sterile microcentrifuge tubes until later DNA extraction. The seedlings from stage 3 to 5 were transplanted to containers in mini-greenhouses to maintain undisturbed growth and survival.

DNA extraction, - amplification, and gel electrophoresis

The applied materials and methods are descibed by SKOV (1998).

Results

Results of the timing experiment are presented in *figure 2.*

DNA-extraction and subsequent PCR of megagametophytic DNA

The following main interpretations of the observations are drawn:

- Total DNA amount in the megagametophytes shown in the second column – "DNA-extraction" – seems to be stable through all investigated stages of seedling development.
- At the early germination stages 1 to 3 detectable amounts of DNA are left in the gel-wells when total DNA is applicated on agarose gels for initial electrophoresis. This coincides with poor Polymerase Chain Reactions shown in the third column – "PCR".

Determination of the DNA concentration using an fluorometric assay gives an estimate of the amount of DNA extracted, but actually tells nothing about the purity of the DNA (HENGEN, 1994).

It is to a certain degree possible to dilute the DNA samples and thereby the contaminants with distilled water and in this way be able to amplify the DNA (data not shown). However results were not reproducible probably due to the poorer genome representation in the reaction mixes prepared for PCR.

Inclusion of a proteinase-K step in the extraction procedure helps overcoming part of the problem with contaminants in the