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A genetic map of Maritime pine based on AFLP, RAPD and protein markers

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Abstract The AFLP (amplified fragment length polymorphism) technique was adapted to carry out genetic analysis in maritime pine, a species characterized by a large genome size (24 pg/C). A genetic linkage map was constructed for one F_1 individual based on 239 AFLP and 127 RAPD (randomly amplified polymorphic DNA) markers. Markers were scored on megagametophytes (1n) from 200 germinated F_2 seedlings. Polymorphism rate, labour time and cost of both AFLP and RAPD techniques were compared. The AFLP technique was found to be twice as fast and three-times less costly per marker than the RAPD technique. Thirteen linkage groups were identified with a LOD score ≥6 covering 1873 cM, which provided 93.4% of genome coverage. Proteins were extracted from needles (2n) of the F_2 progeny and revealed by 2-DE (two-dimensional electrophoresis). Thirty one segregating proteins were mapped using a QTL detection strategy based on the quantification of protein accumulation. Two framework maps of the same F_1 individual are now available. The first map (Plomion et al. 1996) uses RAPD markers and the second map, presented in this study, uses mostly AFLP markers. Although the total genetic length of both maps was almost identical, differences among homologous groups were observed.

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Introduction

Conifers represent one of the largest group of industrial tree-plantation species worldwide. They are generally diploid species, and their haploid chromosome numbers range from n=10 to n=13. All *Pinus* species have a haploid chromosome number of n=12 (Saylor 1961; Saylor and Smith 1966) and are characterised by a large genome size, with 1C estimates ranging from 19 to 44 pg (reviewed by Newton et al. 1993). Two complementary observations explain this large DNA content: (1) a high proportion of repetitive DNA compared to single- and low-copy DNA. DNA-DNA reassociation experiments (Kriebel 1985; Dhillon 1987) have indicated a ratio of 75% (high copy) to 25% (single and low copy) in a range of gymnosperms species; and (2) the presence of multigene families (Kinlaw and Neale 1997). A high proportion of *Pinus taeda* cDNA reveals complex Southern-blot banding patterns, which suggests complex multi-locus gene families (Kinlaw and Gertula 1993; Devey et al. 1994). By comparing Southern-banding patterns, Kinlaw et al. (1996) showed that many genes are encoded by significantly wider gene families in pines than their homologues in angiosperms. Recent genetic-mapping studies demonstrated that such a large physical size did not correspond to a large genome map length. By comparing linkage data from *Pinus strobus*, *Pinus palustris* and *Pinus pinaster*, Echt and Nelson (1997) estimated genome-length at approximately 2000 cM (Kosambi) for these species.

The first saturated map of maritime pine (*P. pinaster*, n=*x*=12) was established with RAPD (random amplified polymorphic DNA) and protein markers (Plomion et al. 1995a). According to Lange and Boehnke (1982) a minimum number of 298 markers would have been expected to saturate a genome of 2000 cM with a probability $P \geq 0.95$ that a new locus might fall within d cM (d<10 cM)

of an existing marker. More markers (426), however, were necessary to reach almost complete coverage. Lange and Boehnke's calculation was based on a random distribution of markers, whereas a non-random distribution of markers has been reported in many mapping studies (reviewed by Krutovskii et al. 1998) ; hence, genome mapping remains a time-consuming process, especially with marker techniques that yield few informative markers, e.g. RFLP (restriction fragment length polymorphism) and SSR (simple sequence repeat). RAPDs, although dominant markers, have greatly facilitated linkage mapping in many forest-tree species. In maritime pine, an average of three RAPD markers were obtained per screened primer (Plomion et al. 1995a). A genetic map based on 426 markers for a family comprising 124 individuals was obtained within 6 months, including the screening step. Six months was also needed to construct RAPD-based genetic maps in eucalyptus (Verhaegen and Plomion 1996) and oak (Barreneche et al. 1998).

The more recent AFLP™ (amplified fragment length polymorphism, Vos et al. 1995) technique affords a higher effective multiplex ratio than RAPD, i.e. a higher number of polymorphic loci in a single assay/gel lane (Powell et al. 1996). Obviously, this technique should facilitate the genotyping step in mapping experiments. The AFLP technique yields mainly dominant markers and has been designed and used for species characterised by a relatively small genome size, e.g. rice (Mackill et al. 1996) and eucalyptus (Marques et al. 1998). Paglia and Morgante (1998) have described a modified AFLP protocol for species with large genomes, such as conifers. Their procedure consisted in targeting hypo-methylated regions of the genome. In this study we optimised the AFLP technology for maritime pine (24 pg/C) using an alternative strategy. AFLP data were obtained within 4 weeks for constructing an almost saturated map based on segregation in haploid megagametophytes. RAPD markers were also used as anchor points for comparison with a previous map of the same species, obtained by Plomion et al. (1995a).

As only a few percent of the pine genome consists of functional genes (Plomion et al. 1995a), we added functional information, based on protein markers revealed by two-dimensional electrophoresis (2-DE), to the DNA-based genetic map. This technique has already been used to map individual genes that control qualitative variation (presence:absence and position shift variations) of polypeptides in maritime pine (Bahrman and Damerval 1989; Gerber et al. 1993; Plomion et al. 1995a, 1997), barley (Zivy et al. 1992) or maize (De Vienne et al. 1996). Proteins were assayed on a diploid tissue (needle) and localised on the "haploid" map by QTL (quantitative trait locus) detection based on protein-spot-intensity variation.

Material and Methods

Genetic material

Two-hundred F_2 progeny of maritime pine (*P. pinaster*) were obtained by the self-pollination of a hybrid tree (accession: H12) which resulted from a cross between an individual from the Landes (accession: L146, used as female) and another from Corsica (accession: C10, used as male). The haploid megagametophyte (nutritive tissue surrounding the embryo originated from the female megaspore) of each F_2 was removed during germination and stored at –80°C before DNA extraction. Other selfed and open-pollinated progenies of this pedigree have previously been used to establish the first saturated map of this species (Plomion et al. 1995a, 1995b, 1996). This new set of seedlings is currently being used to study the physiological, genetic and molecular responses of maritime pine against drought (Costa et al., in preparation).

DNA extraction and RAPD assay

DNA was extracted from haploid megagametophytes as described by Plomion et al. (1995a). The RAPD assay followed the protocol used by Verhaegen and Plomion (1996). Twenty six oligonucleotides (10-mers) were used as single primers for the amplification of RAPD markers (Williams et al. 1990). These primers were chosen using previous information (Plomion et al. 1995a). They provided well-distributed markers in the genome of maritime pine and gave consistent scores.

AFLP assay, gel electrophoresis and scoring AFLP markers

AFLP markers were obtained from the full data set, i.e. 200 F_2s, using the following protocol: 50 ng of DNA was digested for 1.5 h at 37°C with 2 *U* of *Eco*RI (Pharmacia) and 2 *U* of *Mse*I (Biolabs) in a 1× One Phor All (OPA, Pharmacia) buffer in a total volume of 17.5 µl. Then, 2.5 µl of ligation mix [1.25 pmol of *Eco*RI adapter, 12.5 pmol of *Mse*I adapter, 0.8 mM ATP, 0.5 U *T4* DNA ligase (Pharmacia) and 1× OPA (Pharmacia)] were added to the digested DNA template and incubated for 1.5 h at 37°C and overnight at 4°C. Digested-ligated DNA fragments were diluted two-fold and used as the template for the first amplification reaction (pre-amplification). Primers were complementary to the adapters *Eco*RI and *Mse*I, with two additional selective 3' nucleotides: AC and CC, respectively. Pre-amplification was performed in 20 µl of 1× buffer (Gibco BRL), 0.2 mM of each dNTP, 0.3 mM of each primer, 1.25 mM $MgCl_2$, 0.4 U *Taq* DNA polymerase (Gibco BRL) and 3 µl of DNA diluted fragments. PCR was carried out using a Perkin Elmer 9600 thermocycler with the following procedure: preliminary denaturation (4 min, 94°C), then 28 cycles of denaturation (30 s, 94C), annealing (1 min, 60°C) and extension (1 min, 72°C). The pre-amplification products were diluted 15-fold to be used as the starting material for the second amplification reaction (selective amplification). Thirty six different primer combinations were used in the selective amplification step. Both *Eco*RI and *Mse*I primers contained the same sequences as those used in pre-amplification, with one and two additional selective nucleotides at the 3′ end respectively. The *Eco*RI primer was labelled with IRD800, a dye that is sensitive to the infrared laser of the Li-Cor automated sequencer (Li-Cor, Inc. Lincoln, Neb., USA). Selective PCR reactions were performed in 20 µl of 1× buffer (Gibco BRL), 0.1 mM of each dNTP, 0.125 mM *Eco*RI-labelled primer, 0.3 mM *Mse*I primer, 1.25 mM $MgCl_2$, 0.4 U of *Taq* DNA polymerase (Gibco BRL) and 5 µl of diluted pre-amplification fragments. Selective amplifications were carried out using a Perkin Elmer 9600 thermocycler with the following cycling parameters: preliminary denaturation (4 min, 94°C), one cycle of 30 s at 94°C, 30 s at 65°C, 1 min at 72°C, 12 cycles with the annealing temperature decreasing 0.7°C per cycle, followed by 23 cycles of 1 min at 94°C, 30 s at 56°C and 1 min at 72°C.

After selective amplification, AFLP fragments were denatured by adding an equal volume of loading-buffer (95% formamide, 10 mM EDTA pH 7.6, 0.1% bromophenol blue and 0.1% xylene cyanol) and heating for 5 min at 75°C. Finally, 1.5 µl of template was loaded on to 41 cm gels composed of 6% Long Ranger acrylamide (TEBU), 7 M urea and 1.2× TBE. The run was performed in a Li-Cor automatic DNA sequencer (model 4000 and 4000L) for 4–6 h,

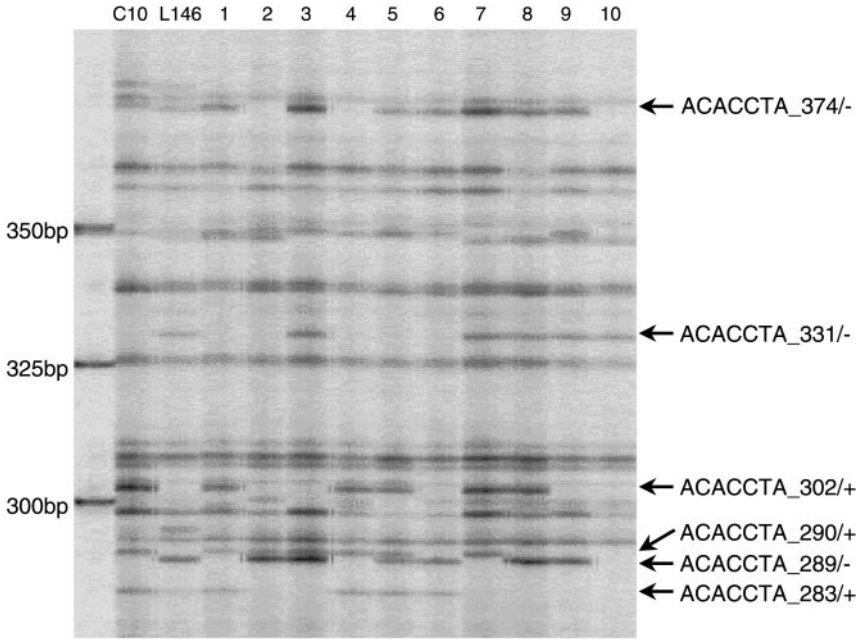

Fig. 1 Portion of an AFLP gel showing the inheritance of AFLP fragments. The PEC *Eco*RI +ACA/*Mse*I +CCTA was used in the amplification. The *first lane* is a molecular-weight standard. *Second and third lanes* correspond to the Corsican (*C10*) and the Landes (*L146*) grandparents respectively. Other lanes correspond to ten megagametophyte progeny of the hybrid parent. Arrows indicate the position of six segregating AFLP markers. The markers are named as in Materials and methods. In the marker name, a "+" denotes markers inherited from the Corsican grandparent, while a "–" denotes markers inherited from the Landes grandparent

with maximal power and a voltage of 50 W and 1500 V, respectively.

Eleven primer-enzyme combinations (PEC) were chosen among the 36 screened PEC on the basis of repeatability, pattern (i.e. ease of scoring), and level of polymorphism. Presence or absence of AFLP fragments was visually scored on the gel image (Fig. 1). Computer-assisted scoring was tested with seven PEC using the RFLP-Scan software (demonstration version 2.1, Scanalytics, CSP Inc, USA, kindly provided by Sciencetec, France). This software also allowed for estimating the molecular weight of the AFLP fragments from the PEC. Polymorphic AFLP fragments were identified: (1) by the selective nucleotides added to each primer (the first three letters corresponding to the selective nucleotides of the *Eco*RI primer and the last four letters corresponding to the selective nucleotides of the *Mse*I primer), and (2) by the molecular weight of the fragment, for the seven PEC analysed with RFLP-Scan. For example, ACACCTA_289 is a 289 bp fragment obtained with PEC *Eco*RI-ACA/*Mse*I-CCTA (Fig. 1). Polymorphic AFLP fragments that corresponded to the four remaining PEC (visually scored only) were identified as described above and numbered from bottom (low-molecular-weight fragments) to top (high-molecular-weight fragments).

Two-dimensional polyacrylamide-gel electrophoresis (2-DE) and gel analysis

Fully expanded needles were sampled on the first shoot cycle of 120 F_2s for protein analysis. They were flash-frozen in liquid nitrogen and stored at –80°C. Protein extraction, 2-DE, silver staining, gel scanning and protein quantification were based on the method in Bahrman et al. (1997) and Costa et al. (1998).

Framework map construction with RAPD and AFLP markers

RAPD and AFLP markers were tested for departure from the 1:1 Mendelian ratio for the presence:absence of a fragment (chi-square test with α=0.05). Linkage groups were obtained under the HAPLOID model using a minimal linkage LOD score of 6 and a maximum recombination fraction (θ) of 0.27 using the MAPMAKER v2.0 software for the Macintosh (Lander et al. 1987). A framework map was then constructed with a LOD support for marker order greater than 6, as described by Plomion et al. (1995a). Accessory markers were placed near the framework marker that had the highest LOD score value. Recombination fractions were converted to map distances using the Kosambi mapping function.

Mapping protein markers using a QTL-detection strategy

In 2-DE patterns of maritime pine, spots with qualitative variation have been revealed to be under monogenic control (Bahrman and Damerval 1989; Gerber et al. 1993, Plomion et al. 1995a, 1997). Mendelian segregation ratios, 1:2:1 and 3:1 for position shift and presence:absence variations respectively, were tested with a chi-square test (α=0.05). Significant linkage between protein markers was determined using the F_2 intercross model of MAPMAKER v2.0. Direct co-segregation between DNA and protein markers, however, was not possible, because the former were obtained from megagametophytes (1n), while the latter were obtained from needles (2n). Plomion et al. (1997) therefore genotyped both haploid and diploid tissues with the same DNA markers, enabling proteins to be localised on a "diploid" map by direct co-segregation analysis. This strategy, while successful, was costly and time consuming. In our study, proteins were assigned to the "haploid" map using an alternative strategy based on QTL detection, as described below:

(1) Polymorphic proteins were quantified using the image-analysis system Bio Image 2-D Analyzer, version 6.0.3 (BioImage Products, Millipore/Biosearch, Ann Arbor, Mich. USA). Integrated intensities were corrected for the gel-staining effect according to a linear-scaling method described by Costa (1999), to compare protein amounts among genotypes.
(2) QTL (quantitative trait locus) detection analysis was then performed for each segregating spot taking into account the whole data set, i.e. approximately 1/4 of individuals characterised by a null intensity (absence of the spot) and approximately 3/4 of individuals with non-null intensity (presence of the spot). The intensity score was used as the trait value when the spot was present. It should be pointed out that the differentiation of heterozygous from homozygous dominant genotypes is not required for the purpose of mapping the protein marker. The interval-mapping method, implemented in the QTL-cartographer software (Basten et al. 1994, 1997), was used to localise the QTL peak.
(3) When the "absent spot" allele (null intensity) was distributed mainly against one of the two alleles of a DNA marker segregat-

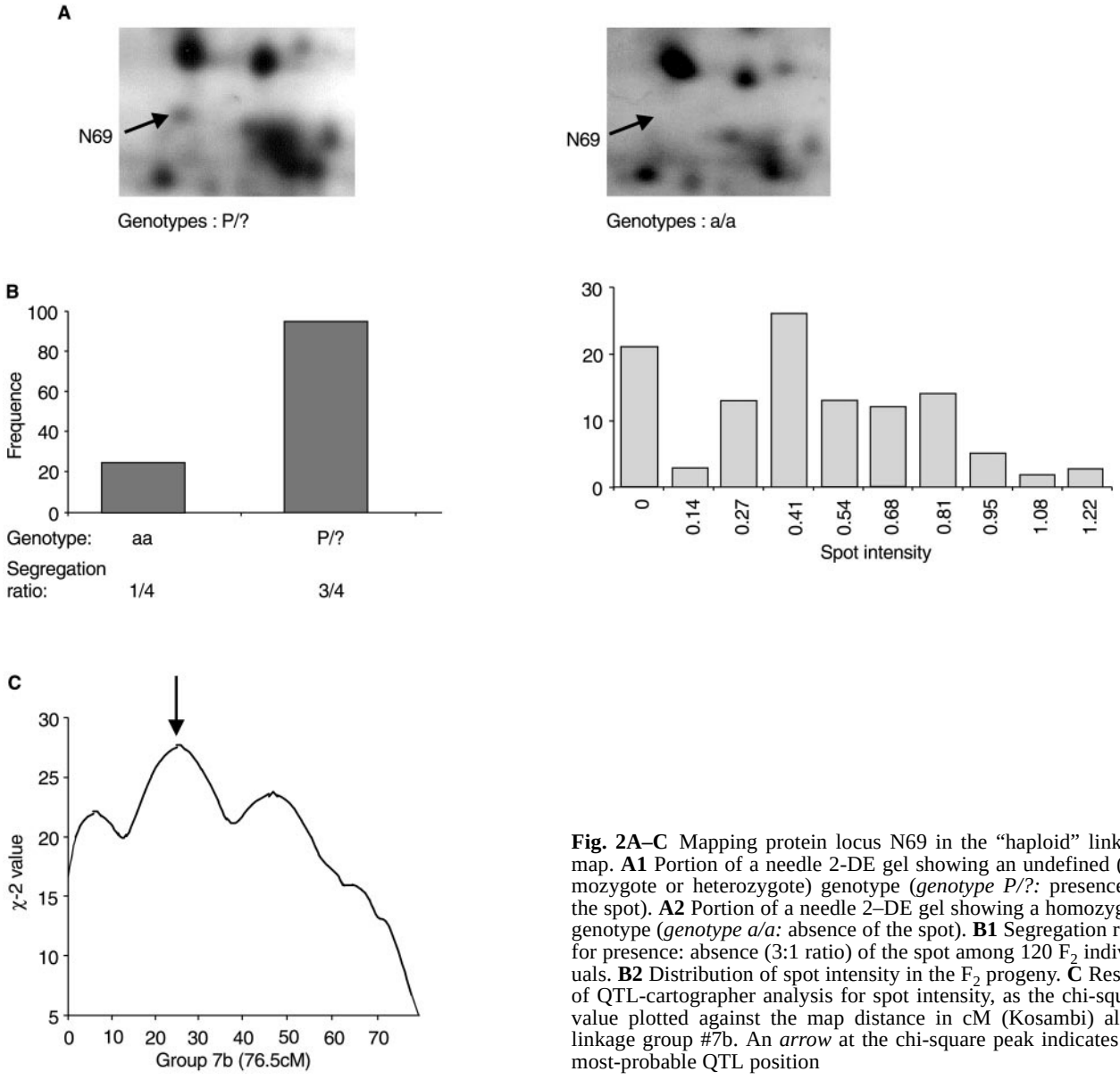

Fig. 2A–C Mapping protein locus N69 in the "haploid" linkage map. **A1** Portion of a needle 2-DE gel showing an undefined (homozygote or heterozygote) genotype (*genotype P/?:* presence of the spot). **A2** Portion of a needle 2–DE gel showing a homozygote genotype (*genotype a/a:* absence of the spot). **B1** Segregation ratio for presence: absence (3:1 ratio) of the spot among 120 F_2 individuals. **B2** Distribution of spot intensity in the F_2 progeny. **C** Results of QTL-cartographer analysis for spot intensity, as the chi-square value plotted against the map distance in cM (Kosambi) along linkage group #7b. An *arrow* at the chi-square peak indicates the most-probable QTL position

ing in the megagametophytes, a strong marker-QTL association was found. An example of such analysis is presented for spot N69 in Fig. 2.
(4) Proteins were integrated finally in the "haploid" map as accessory markers near the framework marker closest to the QTL peak.

Results and discussion

Optimisation of the AFLP assay in maritime pine

Considering its speed, sensitivity and selectivity, the AFLP technique is one of the most powerful technologies for DNA fingerprinting. It has been applied to various plant species (Åkerman et al. 1996; Mackill et al. 1996; Qi and Lindhout 1997; Cervera et al. 1998; Marques et al. 1998; Plagia et al. 1998). As conifers are characterized by large and complex genomes, the application of the original AFLP protocol requires certain modifications. Furthermore, the haploid megagametophyte, commonly used in conifer genetic studies, provides a small amount of DNA, reducing the number of possible PEC that can be used. We modified the AFLP protocol described by Vos et al. (1995) for maritime pine megagametophyte DNA as follows: (1) 50 ng of genomic DNA were used instead of 0.5 µg in the digestion-ligation step, and ligation was shortened to 1 h 30 min instead of 3 h; (2) a 1:2 dilution of the restriction-ligation product was used rather than the standard 1:10 dilution; and (3) the number of selective nucleotides used in pre-amplification and selective amplification was increased, as described recently by Travis et al. (1998) and Remington et al. (1999). Pre-amplification was carried out with *Eco*RI +2/*Mse*I +2 primers and selective amplification with *Eco*RI +3/*Mse*I +4. These modifications al-

Table 1 Polymorphism of the 11 AFLP-PEC and comparison with a RAPD assay

Technique		Number of amplified fragments	Number of polymorphic fragments
AFLP:	PEC[a]		
	ACACCAC	92	14
	ACACCTA	115	22
	ACACCGC	63	21
	ACACCGG	50	15
	ACGCCCA	81	28
	ACGCCGT	48	15
	ACGCCGC	41	11
	ACGCCAT	96	34
	ACCCCAG	101	32
	ACCCCGT	60	19
	ACCCCTG	110	29
Mean value for 11 PEC		78	22
RAPD:			
Mean value for 26 primers		11	4.8

[a] PEC: primer-enzyme combination. The first three letters correspond to the selective nucleotides of the *Eco*RI primer and the last four letters correspond to the selective nucleotides of the *Mse*I primer

lowed us to obtain repeatable AFLP-fingerprints. As reported by Remington et al. (1999) for *P. taeda*, we also observed that the number of CpG dinucleotides in the selective regions of both *Eco* and *Mse* primers had a negative significant effect on the number of AFLP fragments (Table 1).

Polymorphism rate of AFLP and RAPD markers

Using the haploid megagametophyte avoids the problem of dominance of RAPD and AFLP markers (Tulsieram et al. 1992a). The expected Mendelian segregation ratio for dominant markers in this tissue is 1:1. From a total of 366 DNA polymorphic markers (127 RAPDs and 239 AFLPs), only 24 showed segregation distortion (α<0.05), as indicated by the asterisks in Fig. 3. These markers were not used to construct the framework map but rather localized as accessory markers. As already reported by Powell et al. (1996), the AFLP technique yielded more polymorphic markers compared to the RAPD technique: i.e. 21 vs 4.8 polymorphic markers per assay respectively (Table 1). Figure 4 shows the range of molecular weight for 147 AFLP markers amplified from seven PEC. Molecular weights ranged from 50 bp to more than 1000 bp (estimated with the RFLP-Scan software). The Li-Cor sequencer made it possible to continually gather banding information over several hours (4–6 h in our study), so that low- and high-molecular-weight markers could be detected in a single run. Most markers (85%), however, were found between 51 bp and 450 bp, corresponding to about 3 h of run. The molecular-weight range of RAPD markers (200–2000 bp) in maritime pine is presented elsewhere (Plomion et al. 1995a).

Labour time and cost estimates of the RAPD and AFLP techniques

With our AFLP protocol, a single digestion-ligation assay provided enough material to run 12 pre-amplifications. Then, with one pre-amplification, 60 selective amplifications could be carried out. In our study, only one digestion-ligation and one pre-amplification were performed. After a pre-screening of 36 PEC, 11 PEC were finally used in selective amplification and the resulting PCR products run on two Li-Cor sequencers. Two weeks were needed to perform all the reactions for 200 samples. Forty eight samples were run per gel. For a single PEC, four gels were needed to genotype the whole mapping population over 2 working days. Thus, 3 weeks were needed to run all the PEC. Overall, 5 weeks were required to gather the AFLP information. The same period was also needed to run the 26 RAPD primers on the mapping population. Within the same time limit, the AFLP technique provided almost twice as much information as the RAPD technique (127 RAPD markers vs 239 AFLP markers).

Cost estimates for both techniques were based on 100% success for all of the laboratory work as well as France catalogue prices for 1998. As RAPDs and AFLPs provided information at more that one locus, costs were estimated per marker. Given that a single digestion-ligation step and pre-amplification was used in the AFLP assay, the genotyping cost for one individual with 11 AFLP-PEC was $3.30, i.e. $0.013 per marker. This can be compared to the total cost of the RAPD technique: $4.80 for 26 primers, i.e. $0.038 per marker. The AFLP was therefore three-times as economical as the RAPD technique. These calculations, however, did not include equipment prices.

Map construction

Molecular markers obtained by both RAPD and AFLP techniques made it possible to construct a linkage map using the haploid model of MAPMAKER (Fig. 3). A total of 235 AFLP and 127 RAPD markers were mapped based on the linkage criteria defined in the Materials and methods section. Only four AFLP markers were unlinked; these consisted of highly distorted polymorphisms

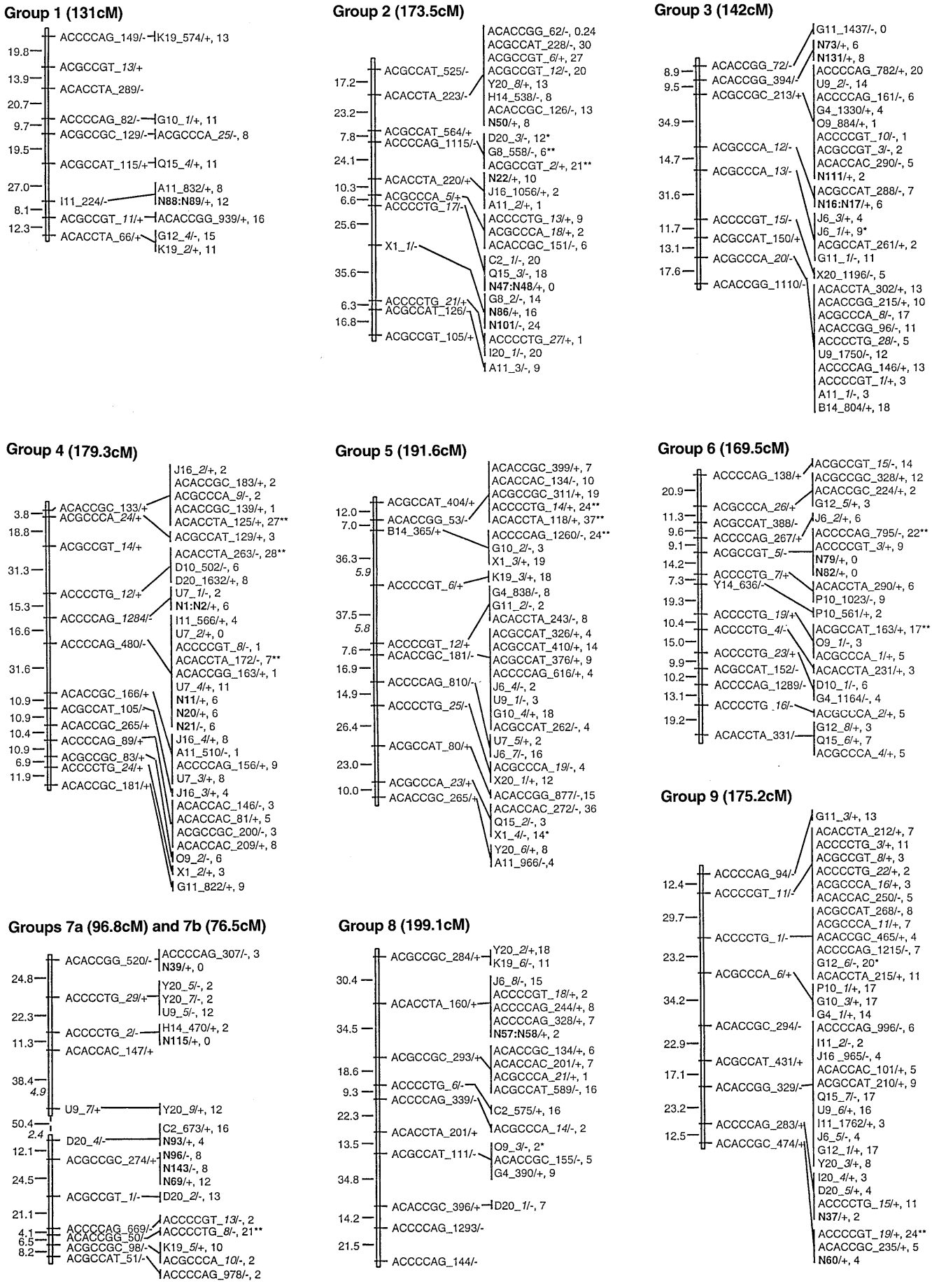

Fig. 3 Legend see page 45

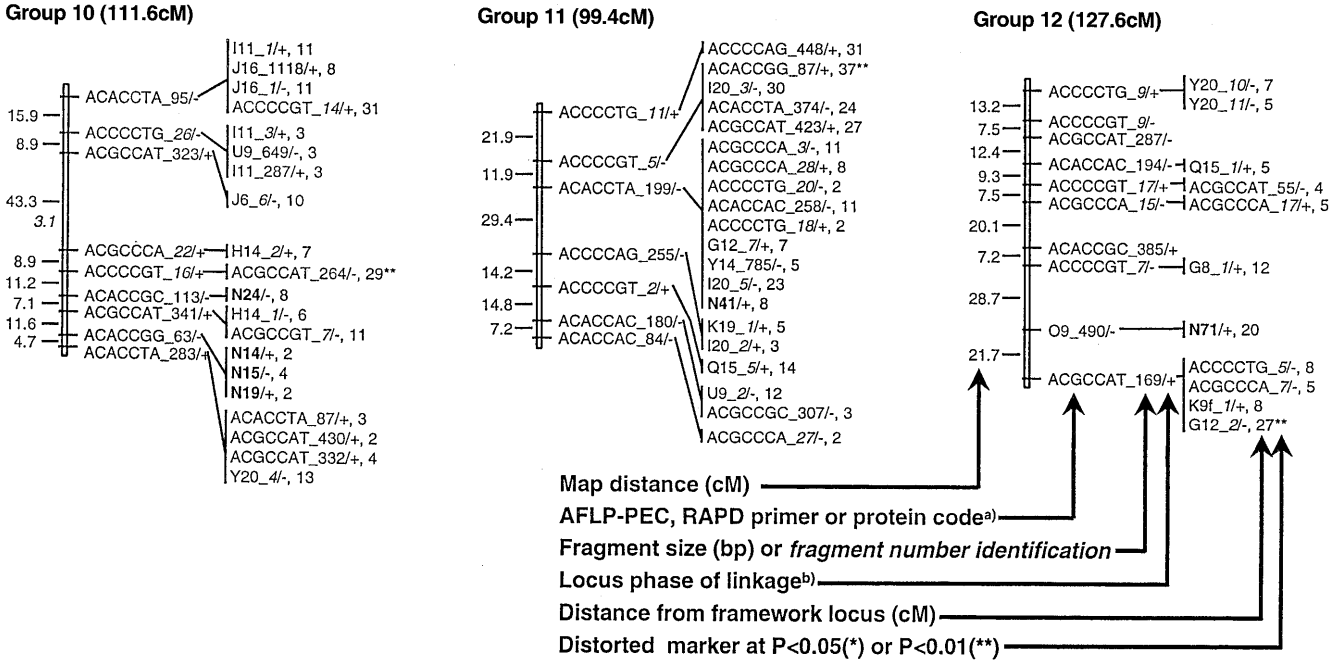

Fig. 3 Linkage map of maritime pine. Loci are listed on the right and recombination distances (cM, Kosambi) are listed on the left of each linkage group. Framework markers were grouped with a LOD≥6 or θ≤0.27. A linkage LOD for gaps that presented deviation from these values is indicated above the recombination distance of the interval (e.g. in group #5, #7 and #10). Accessory markers are listed on the right side of the framework markers. Distances from the nearest framework marker are indicated (cM). Distorted markers are indicated by *asterisks*. Proteins were mapped as described in Materials and methods. AFLP markers are identified by the PEC code (the first three letters correspond to the selective nucleotides of the *Eco*RI primer and the last four letters correspond to the selective nucleotides of the *Mse*I primer, see Table 1) and their molecular weight (base pairs) or a number. The OPERON primer code and the estimated size in base pairs or a number identify RAPD markers. Protein markers are indicated in bold and named as in Table 2. "+" denotes markers inherited from the Corsican grandparent, "–" denotes markers inherited from the Landes grandparent

(*P*<0.001). Out of these 362 polymorphisms, 124 framework markers (mostly AFLP markers) were assigned to 13 linkage groups resulting in an 1873 cM framework map. Linkage groups #7a and #7b could be considered as two pieces of a single linkage group (#7). Although a genetic distance of 50.4 cM (i.e. independence) was found between markers U9_7/+ (#7a) and D20_4/– (#7b), the associated linkage LOD was 2.4, which made this linkage probable. Furthermore, a RAPD marker (H14_470/+) in group #7a, a RAPD marker (C2_673/+) and a protein (N69), both belonging to group #7b, were previously located in linkage group #7 (Fig. 1 in Plomion et al. 1996). These considerations resulted in 12 linkage groups that corresponded to the haploid chromosome number of the maritime-pine genome. The average distance between two framework loci was 15 cM. The average size of linkage groups was 156±38.5 cM. The map contained four gaps ≥36 cM (in group #5, #7a and #10) but their associated linkage LODs were greater than 3.

Estimating the total map length of several pine species, including maritime pine, gave a result of nearly 2000 cM (Echt and Nelson 1997). The 1873-cM map would cover 93.4% of the genome. According to Lange and Boehnke (1982) if markers are randomly distributed on a 15 cM mean distance, 398 markers are expected to cover 95% of the linkage map. The expected number was close to the number in our experiment (362 markers).

Localisation of protein markers in the "haploid" map

The 2-DE gels obtained from the 120 F_2s revealed a dominant mode of inheritance (3:1 segregation ratio) for 26 spots and a co-dominant mode of inheritance (1:2:1 segregation ratio) for six pairs of protein spots. A "diploid" map, based solely on proteins, was constructed under the F_2 intercross model of MAPMAKER. This map covered 436 cM and comprised 11 linkage groups (data not shown) and six unlinked protein. The location of the protein markers in the "haploid" map was obtained using the QTL strategy described in Materials and methods and presented in Fig. 2. Members of allelic pairs (co-dominant markers) were considered independently, which gave a total of 38 segregating spots.

Based on spot intensity (null intensity when the spot was absent and actual intensity when the spot was present), a QTL with a very high chi-square value was identified, except in two cases where no QTL was detected (Table 2). The map location of the protein marker was reinforced by the following observations: (1) proteins that were linked in the "diploid" map presented QTL peaks close to each other in the "haploid" map; (2) QTLs detected for both alleles of a co-dominant marker were also co-located in the "haploid" map, (3) QTLs for proteins previously localized by direct co-segregation with

Table 2 Location of the protein markers in the maritime-pine genetic map. The position of the QTL peak and its corresponding chi-square value in the "haploid" map is given for each protein. Linkage information in the "diploid" map as determined by MAPMAKER under the F_2 intercross model is also provided in the last column

Protein marker[a]	Molecular weight (kDa)	Type of variation[b]	Map distance (cM) from the nearest framework marker	Chi-square[c]	Linkage group	Linkage in the "diploid" map[d]
N88*	23	S	0 (I11_224/+)	11.51	1	Unlinked
N89*	23	S	12 (I11_224/+)	13.28	1	Unlinked
N50	50	P	8 (ACACCTA_223/+)	35.63	2	Unlinked
N22	71	P	10 (ACACCTA_220/+)	30.83	2	A
N47+	37	S	0 (ACCCCTG_17/+)	51.27	2	A
N48+	37	S	0 (ACCCCTG_17/+)	68.51	2	A
N86	29	P	16 (X1_1/–)	16.41	2	A
N101	21	P	24 (X1_1/–)	28.54	2	A
N131	33	P	8 (ACACCGG_394/+)	25.30	3	B
N73	34	P	6 (ACACCGG_394/+)	34.50	3	B
N111	24	P	2 (ACGCCGC_213/+)	36.86	3	B
N16†	70	S	10 (ACGCCCA_13/-)	31.67	3	B
N17†	70	S	10 (ACGCCCA_13/–)	21.17	3	B
N1¥	61	S	6 (ACCCCAG_1284/–)	58.44	4	C
N2¥	61	S	6 (ACCCCAG_1284/–)	31.27	4	C
N11	66	P	6 (ACCCCAG_480/–)	58.51	4	C
N20	68	P	6 (ACCCCAG_480/–)	42.31	4	C
N21	68	P	6 (ACCCCAG_480/–)	42.36	4	C
N79	28	P	0 (ACGCCGT_5/–)	49.89	6	D
N82	27	P	0 (ACGCCGT_5/–)	33.12	6	D
N39	34	P	0 (ACACCGG_520/–)	34.94	7a	E
N115	24	P	0 (ACCCCTG_2/–)	36.39	7a	E
N93	20	P	4 (D20_4/–)	28.07	7b	F
N96	16	P	8 (ACGCCGC_274/+)	14.46	7b	F
N143	19	P	8 (ACGCCGC_274/+)	16.99	7b	F
N69	31	P	12 (ACGCCGC_274/+)	27.45	7b	F
N57‡	37	S	18 (ACACCTA_160/+)	24.44	8	Unlinked
N58‡	36	S	2 (ACACCTA_160/+)	39.71	8	Unlinked
N37	52	P	2 (ACCCCAG_283/+)	51.32	9	G
N60	32	P	4 (ACACCGC_474/+)	39.49	9	G
N24	52	P	8 (ACACCGC_113/–)	45.73	10	H
N14	72	P	2 (ACACCTA_283/+)	20.96	10	H
N15	71	P	4 (ACACCGG_63/–)	88.94	10	H
N19	74	P	4 (ACACCTA_283/+)	35.97	10	H
N41	34	P	8 (ACACCTA_199/–)	32.22	11	Unlinked
N71	31	P	20 (O9_490/–)	40.42	12	Unlinked

[a] Protein codes followed by the same upper character indicate allelic products of the same protein (e.g. N88 and N89 correspond to two allelic products of the same protein)

[b] S: position shift variation (1:2:1 segregation ratio); P: presence/absence variation (3:1 segregation ratio)

[c] a chi-2=10.827 corresponds to a *P*-value of 0.001 (1ddl)

[d] Each letter represents a linkage group of the "diploid" map

DNA markers (see Plomion et al. 1997) were again detected within the same linkage groups. Although protein intensity did not follow a normal distribution, the uniformity of these results validated our approach for mapping protein markers in the "haploid" map. An analysis of variance also confirmed these results (data not shown).

Comparison of maritime-pine linkage maps

Two framework maps of maritime pine are now available for the same pedigree. The first map (Map 1) is based on RAPD and protein markers (Plomion et al. 1995a, 1996, 1997), while the map presented in this paper (Map 2) is based on RAPD, AFLP and protein markers. A total of 33 RAPD markers and seven proteins contained in both data sets were mapped and enabled identical linkage groups to be identified. When more than two anchor markers were identified in a group, their order was identical. The total length of both maps was also similar (1860 cM for Map 1 and 1873 cM for Map 2). The mean length difference between identical groups in both maps was 6±32.7 cM. This high standard deviation (±32.7 cM) could be a result of statistical variations caused by the different sample sizes used for constructing the maps (124 gametes in Map 1 vs 200 gametes in Map 2), or different numbers of framework markers (224 markers in Map 1 vs 124 markers in Map 2). Biological effects could also create differences between identical linkage groups. Even if both framework maps covered about 95% of genome, the covered chromosomal regions could be different, because we did not used the same markers (mainly RAPDs for Map 1 and AFLPs for Map 2). In addition, both mapping samples were obtained in a different pollination year (1980 and 1993 for mapping populations 1 and 2 respectively). Environmental factors

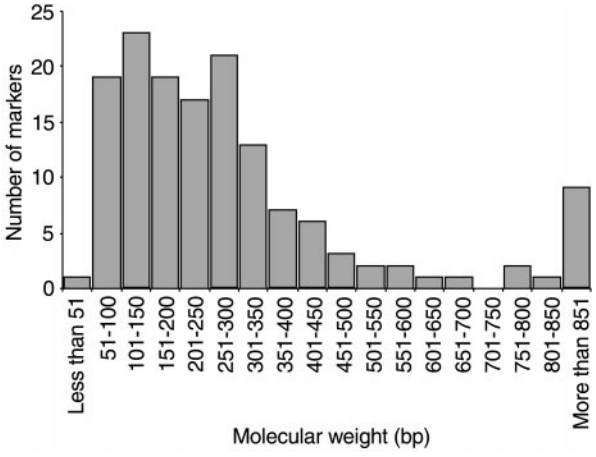

Fig. 4 Distribution of 147 AFLP markers belonging to seven primer-enzyme combinations according to their molecular weight (bp), estimated with the RFLP-Scan software

have already been shown to affect recombination rate (Zhuchenko and Korol 1983; Tulsieram et al. 1992b).

Both maps were finally aligned, and the result can be viewed at URL http://www.pierroton.inra.fr/genetics/pinus/. A total of 66 protein markers (42 in Map 1 and 31 in Map 2) are now placed on the maritime-pine genome. Hyperlinks can be used to retrieve the position of protein markers in needle 2D gels from the linkage map and vice versa. This first step toward the mapping of the expressed genome in maritime pine will be achieved further by the characterizing protein function. Routine identification of proteins excised from 2-DE gels enabled the identification of 63 needle and xylem proteins (Costa et al. 1999). Bahrman and Petit (1995) have already reported 218 allelic variations in three different tissues of maritime pine. This information furthers greatly the perspective of 2-DE towards a comprehensive analysis of the expressed genome in this species.

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References

Åkerman S, Tammisola J, Regina M, Kauppinen V, Lapinjoki S (1996) Segregation of AFLP markers in *Betula pendula* (Roth). For Genet 3:117–123

Bahrman N, Damerval C (1989) Linkage relationships of loci controlling protein amounts in maritime pine (*Pinus pinaster* Ait) . Heredity 63:267–274

Bahrman N, Petit R (1995) Genetic polymorphism in Maritme pine (*Pinus pinaster* Ait.) assessed by two-dimensional gel electrophoresis of needle, bud and pollen proteins. J Mol Evol 41:231–237

Bahrman N, Plomion C, Petit RJ, Kremer A (1997) Contribution to maritime pine genetics using proteins revealed by two-dimensional electrophoresis. Ann Sci For 54:225–236

Barreneche T, Bodenes C, Lexer C, Trotin JF, Fluch S, Streiff R, Plomion C, Roussel G, Steinkellner H, Burg K, Favre JM, Glössl J, Kremer A (1998) A genetic linkage map of *Quercus robur* L. (pedunculate oak) based on RAPD, SCAR, microsatellite, minisatellite, isozymes and 5S rDNA markers. Theor Appl Genet 97:1090–1103

Basten CJ, Weir BS, Zeng ZB (1994) Zmap-a QTL cartographer. In: Smith C, Gavora JS, Benkel B, Chesnais J, Fairfull W, Gibson JP, Kenedy BW, Burnside EB (eds) Proc of the 5th World Congr on Genetics Applied to Livestock Production: Computing Strategies and software. Guelph, Ontario, Canada, vol 22, pp 65–66

Basten CJ, Weir BS, Zeng ZB (1997) QTL cartographer: a reference manual and tutorial for QTL mapping. Department of Statistics, North Caroline State University, Raleigh, N.C., USA

Cervera MT, Cabezas JA, Sancha JC. Martínez de Toda F, Martínez-Zapater JM (1998) Application of AFLPs to the characterization of grapevine *Vitis vinifera* L. genetic resources. A case study with accessions from Rioja (Spain). Theor Appl Genet 97:51–59

Costa P (1999) Etude de la réponse moléculaire, physiologique et génétique chez le pin maritime soumis à un stress hydrique. PhD thesis, Université de Nancy I, France

Costa P, Bahrman N, Frigerio JM, Kremer A, Plomion C (1998) Water-deficit-responsive proteins in maritime pine. Plant Mol Biol 38:587–596

Costa P, Pionneau C, Bauw G, Dubos C, Bahrman N, Kremer A, Frigerio J-M, Plomion C (1999) Separation and characterization of needle and xylem maritime pine proteins. Electrophoresis 20:1098–1108

De Vienne D, Burstin J, Gerber S, Leonardi A, Le Guilloux M, Murigneux A, Beckert M, Bahrman N, Damerval C, Zivy M (1996) Two-dimensional electrophoresis of proteins as a source of monogenic and codominant markers for population genetics and mapping the expressed genome. Heredity 76:166–177

Devey ME, Fiddler TA, Liu B-H, Knapp SJ, Neale BD (1994) An RFLP linkage map for loblolly pine based on a three-generation outbred pedigree. Theor Appl Genet 88:273–278

Dhillon SS (1987) DNA in tree species. In: Bonga JM, D Durzan J (eds) Cell and tissue culture in forestry. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, vol 1, pp 298–313

Echt CS, Nelson CD (1997) Linkage mapping and genome length in eastern white pine (*Pinus strobus* L). Theor Appl Genet 94:1031–1037

Gerber S, Rodolphe F, Bahrman N, Baradat P (1993) Seed-protein variation in maritime pine (*Pinus pinaster* Ait) revealed by two-dimensional electrophoresis: genetic determinism and construction of a linkage map. Theor Appl Genet 85:521–528

Kinlaw CS, Gerttula SM (1993) Complex pine gene families. In: Proc 22nd Southern For Tree Improvement Conf, Atlanta, Georgia, USA, pp 275–283

Kinlaw CS, Neale DB (1997) Complex gene families in pine genomes. Trends Plant Sci 2:356–359

Kinlaw CS, Ho T, Gerttula SM, Gladstone E, Harry DE, Quintana L, Baysdofer C (1996) Gene discovery in loblolly pine through cDNA sequencing. In: Ahuja MR, Boerjan MR, Neale DB (eds) Somatic cell genetics and molecular genetics of trees. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 175–182

Kriebel HB (1985) DNA sequence components of the *Pinus strobus* nuclear genome. Can J For Res 15:1–5

Krutovskii KV, Vollmer SS, Sorensen FC, Adams WT, Knapp SJ, Strauss SH (1998) RAPD genome maps of douglas-fir. J Hered 89:197–205

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lange K, Boehnke M (1982) How many polymorphic genes will it take to span the human genome?. Am J Hum Genet 34:842–845

Mackill DJ, Zhang Z, Redona ED, Colowit PM (1996) Level of polymorphism and genetic mapping of AFLP markers in rice. Genome 39:969–977

Marques CM, Araújo JA, Ferreira JG, Whetten R, O'Malley DM, Liu BH, Sederoff R (1998) AFLP genetic maps of *Eucalyptus globulus* and *E. tereticornis*. Theor Appl Genet 96:727–737

Newton RJ, Wakamiya I, Price HJ (1993) Genome size and environmental stress in gymnosperm crops. In: Dekker M (ed) Handbook of plant and crop stress. Inc NY, USA, pp 321–343

Paglia GP, Morgante M (1998) PCR-based multiplex DNA fingerpriting techniques for the analysis of conifer genomes. Mol Breed 4:173–177

Paglia GP, Oliveri AM, Morgante M (1998) Towards second-generation STS (sequence-tagged sites) linkage maps in conifers: a genetic map of Norway spruce (*Picea abies* K.). Mol Gen Genet 258:466–478

Plomion C, Bahrman N, Durel C-E, O'Malley DM (1995a) Genomic mapping in *Pinus pinaster* (maritime pine) using RAPD and protein markers. Heredity 74:661–668

Plomion C, O'Malley DM, Durel CE (1995b) Genomic analysis in maritime pine (*Pinus pinaster*). Comparison of two RAPD maps using selfed and open-pollinated seeds of the same individual. Theor Appl Genet 90:1028–1034

Plomion C, Bahrman N, Durel CE, O'Malley DM, Kremer A (1996) Genetic dissection of phenotypic traits in maritime pine using RAPD and protein markers. In: Ahuja MR, Boerjan W, Neale DB (eds) Somatic cell genetics and molecular genetics of trees. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 223–231

Plomion C, Costa P, Bahrman N, Frigerio JM (1997) Genetic analysis of needle proteins in maritime pine. 1. Mapping dominant and co-dominant protein markers assayed on diploid tissue, in a haploid-based genetic map. Silvae Genet 46:161–165

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalsky A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellites) markers for germplasm analysis. Mol Breed 2:225–238

Qi X, Lindhout P (1997) Development of AFLP markers in barley. Mol Gen Genet 254:330–336

Remington DL, Whetten RW, B-H Liu, DM O'Malley (1999) Construction of an AFLP genetic map with nearly complete genome coverage in *Pinus taeda*. Theor Appl Genet 98:1279–1292

Saylor LC (1961) A karyotypic analysis of selected species of *Pinus*. Silvae Genet 10:77–84

Saylor LC, Smith BW (1966) Meiotic irregularity in species and intraspecific hybrids of *Pinus*. Am J Bot 53:453–468

Travis SE, Ritland K, Whitham TG, Keim P (1998) A genetic map of Pinyon pine (Pinus edulis) based on amplified fragment length polymorphisms. Theor Appl Genet 97:871–880

Tulsieram LK, Glaubitz JC, Kiss G, Carlson J (1992a) Single-tree genetic linkage mapping using haploid DNA from megagametophytes. Bio/Technology 10:686–690

Tulsieram L, Compton WA, Morris R, Thomas-Compton M, Eskridge K (1992b) Analysis of genetic recombination in a maize population using molecular markers. Theor Appl Genet 84: 65–72

Verhaegen D, Plomion C (1996) Genetic mapping in *Eucalyptus urophylla* and *Eucalyptus grandis* using RAPD markers. Genome 39:1051–1061

Vos P, Hogers R, Bleeker M, Reijans M, van der Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531–6535

Zhuchenko AA, Korol AB (1983) Ecological aspects of the recombination problem. Theor Appl Genet 64:117–185

Zivy M, Devaux P, Blaisonneau J, Jean R, Thiellement H (1992) Segregation distorsion and linkage studies in a microspore-derived double haploid of *Hordeum vulgare* L. Theor Appl Genet 83:663–672