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Seed-protein variation in maritime pine (*Pinus pinaster* Ait.) revealed by two-dimensional electrophoresis: genetic determinism and construction of a linkage map

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Summary. Proteins from haploid megagametophytes from 18 trees were studied by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). A total of 222 seeds, an average of 12 per tree, were analysed individually. 150 protein spots appeared to segregate on the polyacrylamide gels in at least tree. Genetic interpretations were made to define the number of loci responsible for the presence versus absence, staining differences or position variation of the segregating spots. The complete covariation observed between some spots could be the result of either the separation of a single gene product into two or more constituents, very close linkage, or the action of a pleiotropic gene. Human genetics techniques were used to map the 84 putative loci detected. Sixty-five loci were organised in 17 linkage groups, whereas 19 remained unlinked.

Key words: Pine – Two-dimensional electrophoresis – Haploid megagametophyte – Linkage map

Introduction

Genetic linkage maps are important tools for studies of genome organization (Polans et al. 1985; Tanksley et al. 1988) and for marker-aided selection (Soller and Beckmann 1983). Restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) provide an unlimited number of markers, and thus, enable the construction of saturated linkage maps. Such maps are now available in an increasing number of plants (Helentjaris et al. 1986; Chang et al. 1988; McCouch et al. 1988; Gebhardt et al. 1991; Landry et al. 1991). The long generation time of tree species is an impediment to the creation of suitable pedigree for mapping. As a result, DNA markers are only beginning to be used in tree species, and especially in conifers, for mapping purposes (Neale and Williams 1991; Carlson et al. 1991).

The haploid megagametophyte of the seeds of gymnosperms provides geneticists with excellent material for studying the inheritance and linkage of genes affecting protein phenotypes. Studies on isozyme loci are numerous; for example, more than 20 loci have been identified in recent studies of *Pinus* (Niebling et al. 1987; Szmidt and Muona 1989), *Cunninghamia* (Geburek and Wang 1990) *Pseudotsuga menziesii* (Adams et al. 1990) and *Thuya* (Xie et al. 1991). The use of two-dimensional electrophoresis (2D-PAGE) of the total proteins has allowed the study of a much larger number of loci than has been possible with isozyme analysis (Bahrman and Damerval, 1989). The loci that have been detected affect several aspects of protein phenotypes, including presence/absence, modification of mobility and quantity. Quantitative polymorphism is of particular interest, as it has been found to be correlated with quantitative trait variation (Leonardi et al. 1991).

The present contribution enlarges the study of Bahrman and Damerval (1989), which was carried out on a seed lot originating from a single maritime pine (*Pinus pinaster* Ait.), and is based on 2D-PAGE of megagametophytes from 18 trees of the same species. The first aim of the study presented here was to determine the number of loci affecting spot phenotypes; the second aim was to built a genetic linkage map. In order to combine the information provided by the 18 unrelated trees, we used methods developed for mapping in human families.

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Materials and methods

Plant material

Eigtheen trees were sampled from the French breeding population of maritime pine (*Pinus pinaster* Ait., 2n=2x=24). Cones were collected on grafts of the trees in a clonal orchard planted near Bordeaux (south-west France). An average of 12 (10–14) seeds from each of 18 clones were analysed separately by two-dimensional electrophoresis (a total of 222 seeds).

Protein extraction and electrophoresis

After removal of the seed coat and the embryo from each seed, total proteins were extracted from individual megagametophytes following the protocol of Bahrman and Damerval (1989). The extraction buffer contained 3 M urea, 2% ampholytes (pH 3–10), 1% dithiothreitol and 4% FSN 100 (detergent) (Anderson et al. 1985). Aliquots of 6 µl of buffer were applied per milligram of megagametophyte, and 35 µl of the resulting homogenate was submitted to isoelectrofocusing in the first dimension followed, in the second dimension, by a sodium dodecyl sulfate electrophoresis performed in polyacrylamide gels bound to Gelbond PAG films (Granier and de Vienne 1986). Gels were then silver stained (Damerval et al. 1987a) and dried.

Comparison of the gels

The dried gels were compared in a pairwise manner while superimposed on a light box. Due to the small size of the seeds, a single gel was generally run per megagametophyte. In a first step, the gels were compared within each genotype to detect the spots exhibiting apparent segregation; in a second step, the gels from the 18 genotypes, a total of 222 gels, were compared.

To assess the relative positions of spots in different gels of the same genotype, or in different genotypes, pairs of samples were mixed in equal quantity for co-electrophoresis. The resulting gel was then compared with the individual gels from each sample. Each spot was identified by four numbers, the two first digits indicating its approximate position in a given region of the gel, and the second two numbers its number in the region.

Spot variations and genetic hypotheses

The diploid genotype of a tree could be inferred from the segregation observed among its gametes. With only ten gametes per tree, however, segregation distortion could not be tested. When a spot was found with two forms among the electrophoresed total proteins of a single tree, the tree was assumed to be heterozygote at a locus with two alleles determining the two forms of the spot. Two different types of phenotypes were detected:

1) Some spots had two classes of staining intensity. Additional classes may have been present in some cases, but these were difficult to assess by eye and were thus discarded.
2) Some spots were alternatively present or absent.

Among the present/absent spots, series of spots could be seen that were position variants. These spots were generally close to each other on the gel and differed by their isoelectric point and sometimes by their apparent molecular mass. A genotype in which two spots of the series were found to be alternatively present but never detected simultaneously on a gel was interpreted to be heterozygous at a locus with two alleles determining the two position variants (Table 1 part A). Additional alleles corresponding to new position variants could be found through genotypes, as well as silent alleles (Table 1 part B).

To avoid redundancy, we compared the patterns of all pairwise combinations of spots on the 222 gels using FORTRAN programs. Some pairs of spots co-varied precisely in their phenotypes. We assumed that a single locus was responsible for the co-variation of such pairs, though two tightly linked loci may also have been the cause. When the covariation in several spots could be explained by the action of a single locus, the spot with the lowest number was usually chosen to give its name to the locus, and subsequently, written in italics.

Table 1. Examples of mobility variation and its genetic interpretation: part A, biallelic locus; part B, triallelic locus and null allele

A	Different types of gels observed[a]						
	Spot x		Spot y				
Tree x1	•		∅	or	∅	•	
Tree x2	•		∅		(All gametes)		

Interpretation:
A biallelic locus determines two position variants, spot x [left allele (1)], spot y [right allele (r)]

Tree x1 Heterozygous genotype l/r
Tree x2 Homozygous genotype l/l

B	Spot x	Spot y	Spot z				
Tree x1	•	∅	∅	or	∅	•	∅
Tree x2	∅	•	∅	or	∅	∅	•
Tree x3	∅	∅	•	or	•	∅	∅

Interpretation:
A triallelic locus determines three position variants, spot x [left (1)], spot y [middle (m)], spot z [right (r)]

Tree x1 Heterozygous genotype l/m
Tree x2 Heterozygous genotype m/r
Tree x3 Heterozygous genotype l/r

If an additional kind of variation is observed:

Tree x4	∅	∅	∅	or	∅	•	∅

We assume the occurrence of a null allele (0) in this genotype:

Tree x4 Heterozygous genotype m/0

[a] •, Presence of the spot; ∅, absence of spot

Linkage analysis

The linkage map of the loci was calculated with a multipoint linkage analysis technique based on maximum likelihood provided by the MAPMAKER computer package (Lander et al. 1987). The 18 trees of our sample are unrelated and thus not heterozygous at the same loci. The linkage between 2 loci can be studied only in "informative" genotypes, which are heterozygous at these 2 loci. The number of informative genotypes will vary according to the pair of loci considered. Moreover, the linkage phases of the heterozygous genotypes are unknown, thus a gamete cannot be classified as being in a parental or recombinant phase. Similar situations are found in human genetics, where maps are constructed with several unrelated families with a limited number of children per family (Lander and Green 1987).

We converted the 18 trees and each of their megagametophyte progeny into 18 fictitious human families (Table 2). The genotype of the mother of the family was inferred from the segregation observed among megagametophytes. A fictious father that was recessive homozygous at all loci was also created.

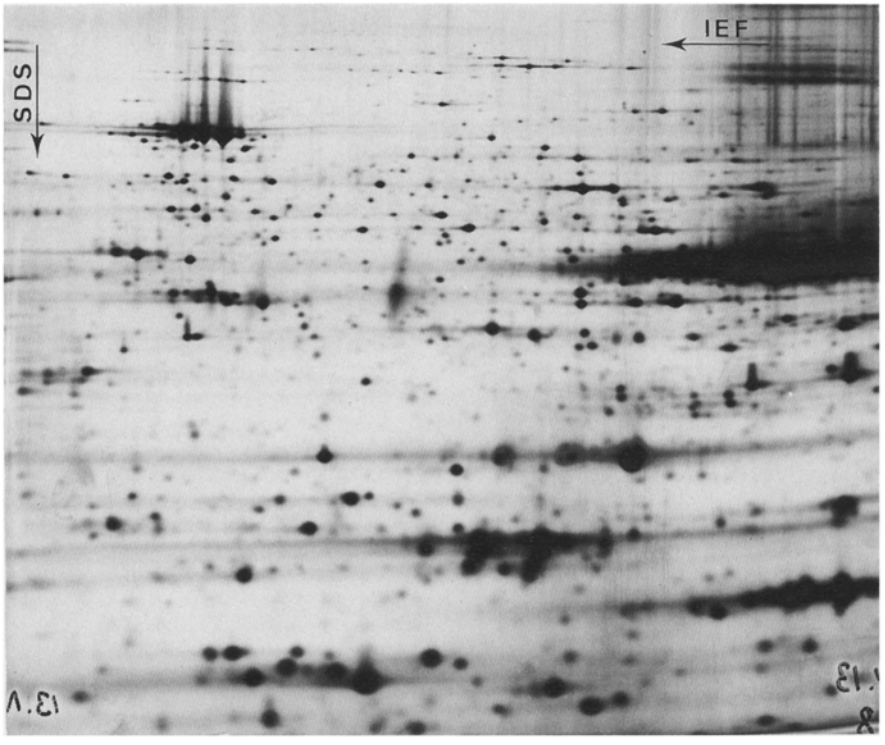

Fig. 1. Two-dimensional electrophoresis gel of proteins extracted from the haploid megagametophyte of maritime pine

Table 2. Creation of a fictitious human family

Tree x:

Alleles expressed in the gels for one locus. Spot present (p allele) or absent (a allele)

Gel no. 1	Gel no. 2	Gel no. 3	Gel no. 4	Gel no. 5	Gel no. 6
p	p	a	p	a	a

Equivalent "human family": mother, genotype p/a; father, genotype Y/Y

Genotype of the children

Child no. 1	Child no. 2	Child no. 3	Child no. 4	Child no. 5	Child no. 6
p/Y	p/Y	a/Y	p/Y	a/Y	a/Y

Thus, each gel could be interpreted as providing the maternal genotype of each "child". The human data type option of MAPMAKER was then used to compute our map, using the Haldane function to calculate map distances.

On the basis of two-point analysis, apparent linkage groups were first constructed authorizing a minimum lod-score of 4 and a maximum recombination rate of 0.3. The "best" order of the markers was then determined. The positions of the remaining loci were tested relative to these groups. Loci being at least a 100 times more likely to be linked than unlinked were added to the group (Donis-Keller et al. 1987).

Results

Amount of polymorphism detected

More than 600 spots could be seen on a gel when the total protein of an haploid megagametophyte was electrophoresed; the isoelectric point ranged from 4.5 to 7 and the molecular mass from 18–94 kDa (Fig. 1). We found 150 spots that exhibited variation in at least 1 of the 18 trees studied (Fig. 2): (1) 125 spots were alternatively present or absent; (2) 15 spots exhibited staining intensity variation, and (3) 10 spots exhibited both kinds of variation.

Some spots were organised in "charge trains" (about 2–15 spots) with the same molecular mass and different but close isoelectric points. Since these "charge trains" always varied as a unit they were considered to be a single spot (see, for example, spot no. 1205, 2430, 2402 or 3315 on Fig. 2).

Genetic interpretations

Among the 125 spots alternatively present or absent, 93 could be interpreted as position variants of the same proteins. Variation in these spots was attributed to 35 polymorphic loci with as many alleles as the number of different positions: (1) 19 biallelic loci, (2) 12 triallelic loci; (3) 2 four-allelic loci including a "silent" allele; (4) 1 five-allelic locus including a "silent" allele; (5) 1 six-allelic locus.

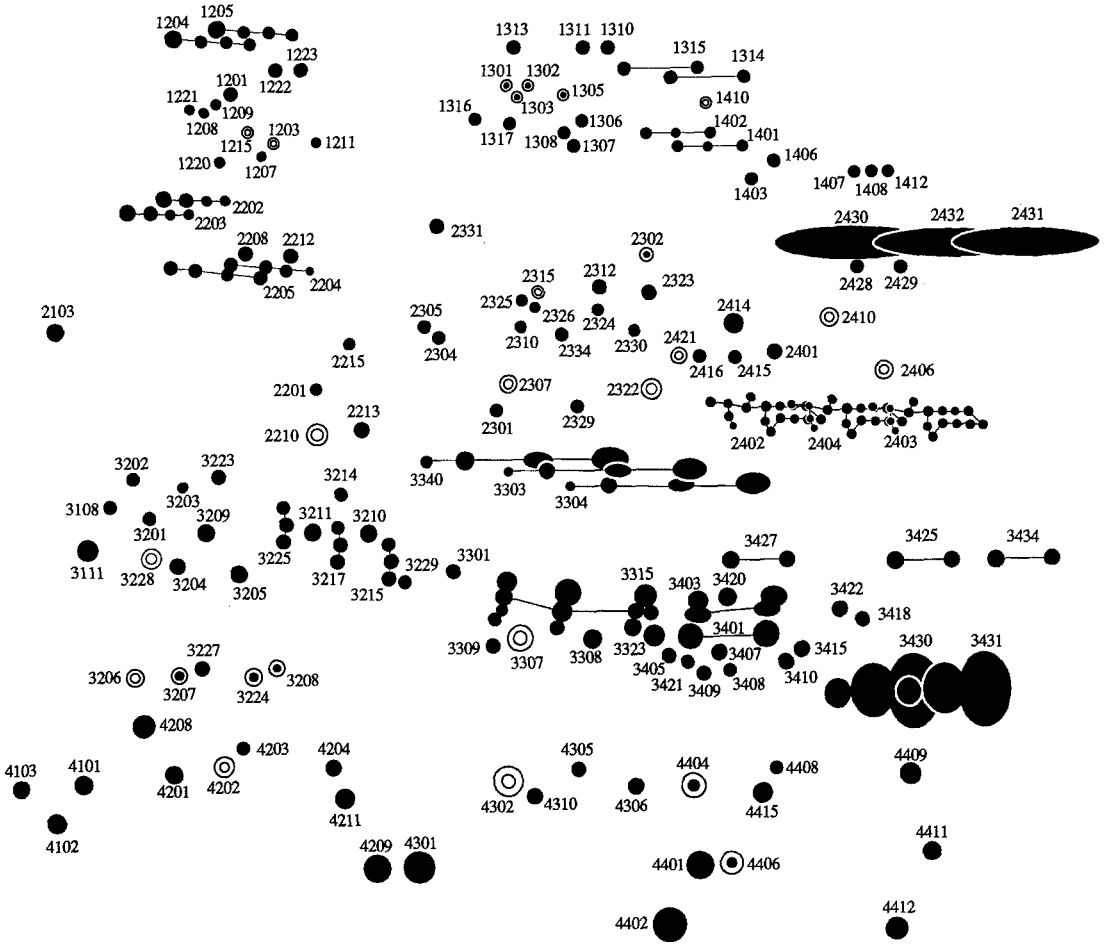

Fig. 2. A schematic diagram of a gel identifying spots that segregated with two forms in at least one tree. ●, Spot alternatively present or absent; ◎, spot with two staining intensities; ⊙, spot exhibiting a variation in both presence/absence and staining intensity

The allelic products of these loci are presented on Fig. 3 tied together by a continuous line and by a small-dotted line when an additional silent allele was detected in the corresponding locus. The variation observed for the remaining 32 present/absent spots and for the 15 spots with two staining intensities were considered to be under the control of 47 biallelic loci.

The pattern of variation of the 10 spots expressing both quantitative variation and presence/absence was genetically interpreted according to the segregation observed within each genotype. If a given protein is detected with two different forms on gels of one genotype, the action of 1 biallelic locus can be suggested since the parental genotypes studied here are diploid. Among the 10 spots, some had three forms among the gels of single genotypes. In 2 cases, we had thus to assume the action of 2 different loci. In contrast, 6 cases were interpreted as being a result of the action of triallelic loci. These loci were associated with alleles responsible for quantitative differences and with alleles determining absence or position variants.

A total of 92 loci were identified that accounted of the pattern of variation of the 150 spots.

Covariable spots

Some spots were found to vary in a precisely identical manner regardless of tree: 22 spots showed this behaviour (see large-dotted lines in Fig. 3). Groups of covariable spots were explained by the action of a single locus.

A total of 84 loci were retained for the linkage analysis.

Genetic mapping

MAPMAKER estimated the presence of 17 linkage groups that consisted of 2–8 loci, whereas 19 loci remained unlinked (Fig. 4). The 65 mapped loci covered 530 cM.

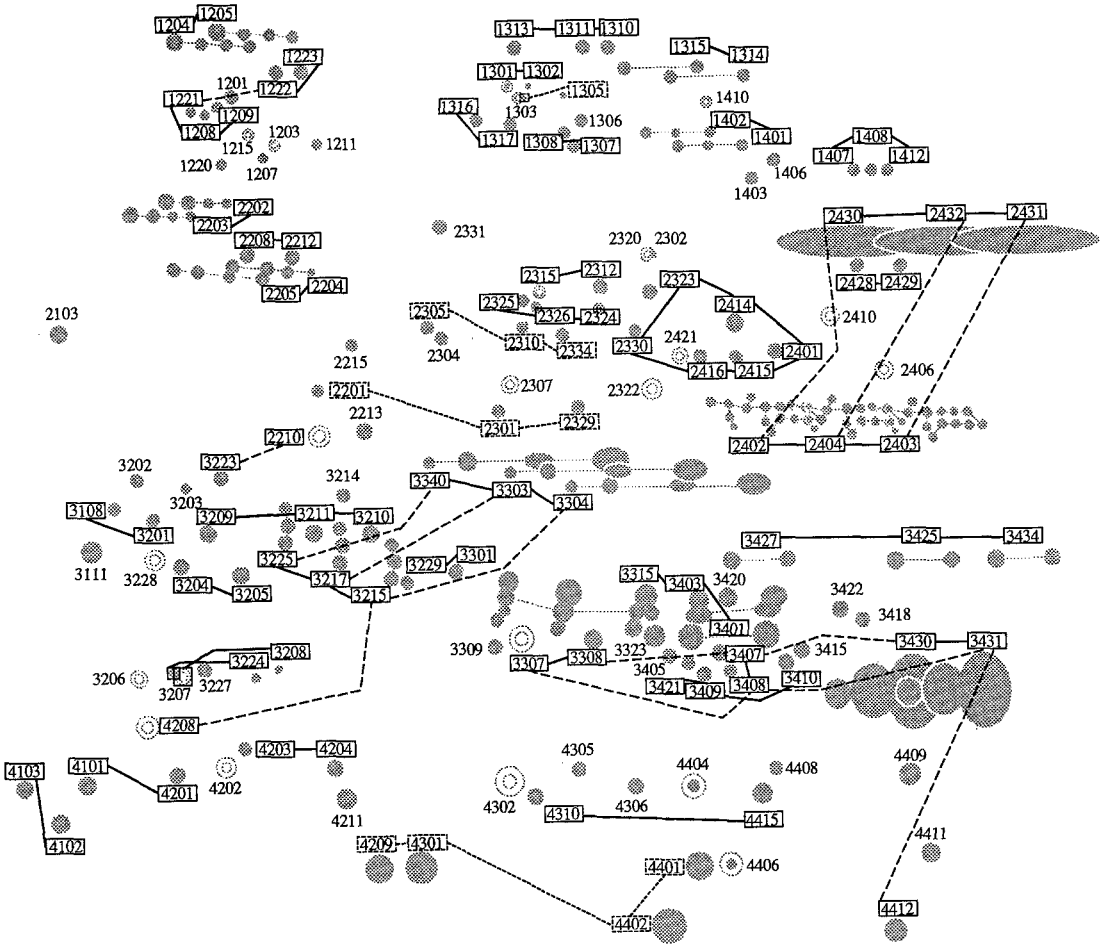

Fig. 3. Relationships between spots. —□—□—, series of position variants; ···□···□··· , series with a null allele; ···□···□··· , covariable spots

Discussion

Charge trains

Two-dimensional electrophoresis of megagametophytes revealed several spots associated in charge trains that covaried as single spots. These charge trains could be due either to post-translational modifications of a protein or to differences existing in the structural genes. On 2-D electrophoresis gels, storage proteins show patterns similar to those of the charge trains described here. They are usually constituted by the assembly of subunits, themselves formed by the association of several heterogeneous polypeptides differing by isoeletric points and/or molecular mass (Higgins 1984; Vedel and Delseny 1987). The explanation of this heterogeneity was found at the DNA level, and no case of post-translational modifications was observed. In maize, the heterogeneity of one zein fraction was due to a sequence variation in a tandem repetition (Heidecker and Messing 1986). In rye, among 42 repeats for prolamin, only 5 sequences were found to be identical to the consensus; the others differed as a result of amino-acid substitution and/or deletions (Hull et al. 1991). The spots organised in charge trains on our gels were almost all found with two or three position variants through the gels of the different genotypes (Fig. 3).

Presence/absence and mobility variation

The presence/absence variation observed for some spots on our gels can be interpreted in two ways. The disappearance of a spot can be due to the presence of a "silent" allele preventing the expression of the protein. However, the absence of a spot may result when the quantity of the protein drops below the detection level. A quantitative variation would then be interpreted as a presence/absence variation.

The interpretation of mobility variation of a spot is less ambiguous. This phenomenon is commonly observed in plants as well as in animals; for example, wheat

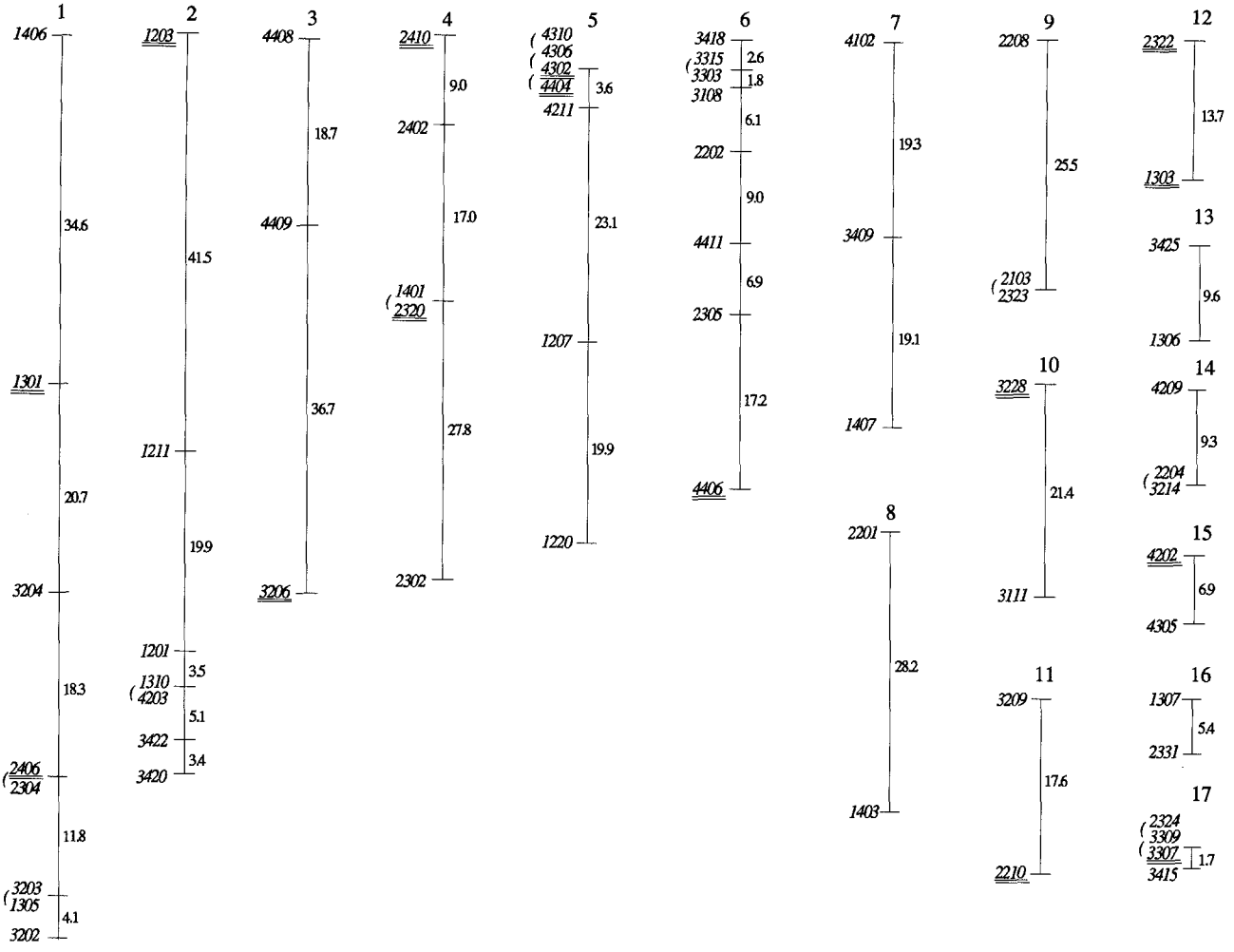

Fig. 4. Linkage map generated by MAPMAKER. Distances are indicated in centimorgans on the *right side of the lines*; loci are shown in *italics at left of lines*; loci responsible for quantitative variations are *underlined*

(Zivy et al. 1983), maize (Leonardi et al. 1987, 1988) and primates (Goldman et al. 1987). Most of the position variants observed for the proteins present in the megagametophyte of maritime pine corresponded to horizontal shifts, and thus to an isoelectric point modification of the protein; apparent molecular mass modifications were less frequent (Fig. 3). The presence, at the same locus, of alleles responsible for position variants and alleles causing the absence of the protein was observed in 4 cases (loci no. *1305, 2305, 2201* and *4209* on Fig. 3). A combination of 2 alleles corresponding to different sizes of the protein and a null allele at the globulin-1 gene in maize has been described at the molecular level (Belanger and Kriz 1991). The production of a larger protein was due to an insertion in an exon, whereas another insertion caused a translational frameshift and resulted in a null allele. Similar mechanisms may cause the polymorphism of pine seed-proteins discussed here.

Co-variations of protein phenotypes

Several spots had perfectly correlated patterns of variation over all of the 222 gels compared (Fig. 3). Three hypotheses can be proposed to explain this.

1) The two proteins come from a single polypeptide, post-translationally modified. For instance, the left, the middle or the right position variant of 1 protein (spot no. 2430, 2432, 2431; Fig. 3) was present on the gels simultaneously with the left, middle or the right position variant of a different protein (spot. no. 2404, 2404, 2403). The same situation was found with spot no. 3340, 3303, 3304 and no. 3225, 3217, 3215. Both pairs of triplets correspond to the most abundant proteins of the gels and probably have a storage function. Each pair may represent large and small subunits of storage proteins, which often result from a post-translational cleavage of a unique polypeptide, that may be assembled later by a disulphide

bridge (Higgins 1984; Collada et al. 1991). According to Allona et al. (1992) glutelins of maritime pine have a dimeric structure. The two subunits, joined by a disulphide bridge, are heterogeneous and are both basic. Spots nos. 2430 (or 2432, 2431) and 2402 (or 2404, 2403) appear to fit this model.

2) A single pleiotropic gene controls the expression of several different proteins. An example of this was illustrated (with 2D-PAGE) by Gottlieb and de Vienne (1988) for pea. Yeast cells transformed by a single multicopy plasmid revealed unexpected amplication of several proteins on 2D-PAGE, also suggesting the pleiotropic action of a single gene (Thoraval et al. 1990).

3) The modifications of the two co-variable spots are due to polymorphism at 2 thightly linked loci. Consequently, for a heterozygous tree, only parental gametes are observed since the size of our sample was too small to detect rare recombinants. However, the co-variations we observed are independent of the tree considered: a given form of a first spot was strictly associated with that of a second spot in all 222 gametes. If the hypothesis of the presence of 2 linked loci is correct, the 18 trees would be in the same phase (complete linkage disequilibrium) for all the pairs of loci studied.

Tight linkages

A set of loci affecting proteins of about the same molecular mass but different isoelectric point were found to be tightly linked (nos. *4302, 4310, 4306* and *4404* on linkage group 5). In the present case, the phases of the 18 genotypes are different for these loci. An additional locus (no. *4211*), separated by 3.6 cM from this group, was responsible for the modification of a protein located on the same horizontal line further away on the gel. Several pairs of loci responsible for the variations of proteins with same apparent molecular mass and close isoelectric points were also found to be linked (nos. *4406–4411, 4408–4409, 3418–3315, 3422–3420, 3307–3309, or 3111–3228*).

We also noticed strong linkage between loci affecting spots that had no obvious resemblance on the gel and which exhibited a different type of variation (nos. *3203* and *1305* on linkage group 1, no. *1401* and *2320* on group 4, nos. *2103* and *2323* on group 9, nos. *2204* and *3214* on group 14). This corroborates observations made by Bahrman and Damerval (1989), though with loci affecting different spots than the ones we observed.

Loci affecting protein amount

The loci responsible for modifications of the intensity of the spots appeared to be dispersed throughout all of the linkage groups (Fig. 4). Leonardi et al. (1987) observed that the loci affecting protein amount could be either closely linked or at a position distant from the structural genes of the affected protein. Bahrman and Damerval (1990) found 2 loci, the first responsible for the mobility variation and the second for the quantitative variation of the same spot, on 2 different linkage groups. In our data, the same situation was found in 1 case, with loci nos. *1303* and *1305*. In contrast, loci nos. *2320*, affecting the amount, and *2302*, determining the presence or absence of the same spot, were located in the same linkage group but separated by 27.8 cM.

Conclusion

2-D electrophoresis of haploid megagametophytes of a pine allowed a large number of protein polymorphisms to be detected. The number of loci causing this variation was estimated. The present study confirmed and enlarged an earlier investigation of Bahrman and Damerval (1989) made with seeds of a single tree. We also demonstrated that mapping techniques developed for human genetics could be easily applied in a study dealing with several unrelated progenies of gymnosperm trees.

The relationship between allozyme genotype and quantitative traits in conifers has been investigated by several researchers (El-Kassaby 1982, Ledig et al. 1983, Strauss 1986). However, no general conclusion can be derived from the different studies, which all included a limited number of loci. Recent data derived from 2-D electrophoresis in maize (Damerval et al. 1987 b, Leonardi et al. 1991) indicated that polymorphism in protein amount could explain part of the phenotypic variation of quantitative traits. The relationship between the growth potential of the 18 trees, evaluated on progeny tests, and their genotype for the genetic markers described is currently under investigation.

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