African Journal of Biotechnology Vol. 9(35), pp. 5659-5664, 30 August, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.433
ISSN 1684–5315 ©2010 Academic Journals

Full Length Research Paper

Genetic linkage maps of *Pinus koraiensis* Sieb. et Zucc. based on AFLP markers

Min-Min Chen[1,3], Fujuan Feng[2], Xin Sui[1] and Shijie Han[1]*

[1]Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China.
[2]Faculty of Life Science, Northeast Forestry University, Harbin 150040, China.
[3]Academy of Sciences, Graduate University of Chinese, Beijing 100049, China.

Accepted 18 June, 2010

Genetic linkage maps provide essential information for molecular breeding. In this paper, the genetic linkage map of *Pinus koraiensis* was constructed using an F1 progeny of 88 individuals. One hundred and thirty (130) of molecular markers were mapped onto 6 linkage groups, 4 triples and 15 pairs at the linkage criteria LOD 4.0. Nine primer combinations were applied to map construction. The consensus map gained covers 620.909 cM, with an average marker spacing of 4.776 cM. The presented map provides crucial information for future genomic studies of *P. koraiensis*, in particular for QTL (quantitative trait loci) mapping of economically important breeding target traits.

Key words: Genetic mapping, Korean pine, linkage map, marker-aided selection.

INTRODUCTION

Genetic linkage maps provide essential tools for plant genomic research. The main applications of genetic maps to plants are: (1) basic knowledge of genomic structure, (2) mapping and detection of complex quantitative trait loci (QTL) (Miles et al., 2009), (3) comparative genomic study and genetic diversity study of different species. Moreover, high-density genetic maps are prerequisite for marker-aided selection (MAS) to improve the selection efficiency and accelerate breeding process. This is particularly significant for long generation species, such as forest tree genera including *Pinus*, *Eucalyptus, Populus* (Bradshaw et al., 1994; Devey et al., 1994; Grattapaglia and Sederoff, 1994). Until now, a large number of genetic maps have been reported in pine species (Costa et al., 2000; Wilcox et al., 2001; Shepherd et al., 2003; Yin et al., 2003; Zhou et al., 2003).

Pinus koraiensis (Korean pine) is a dominant tree species of the climax vegetation (broadleaved mixed forests) in North-eastern China. This tree species has important economic (excellent timber quality and delicious seeds) and ecological values (climate regulation and water conservation). But it grows more slowly and requires many years to assess most attributes. Moreover, our knowledge of Korean pine is limited in comparison with that of many other species. Thus, it is necessary to start with the molecular breeding work such as quantitative trait loci (QTL) mapping to facilitate early-stage selection.

Many marker methods have been applied in map construction of conifer species. Compared with other marker methods, AFLP (amplified fragment length polymorphism) (Vos et al., 1995) has higher polymorphic level. The method has been proven to be appropriate to saturate genetic maps in species with large genomes such as *Picea abies* (Achere et al., 2004), *Pinus tedea* (Remington et al., 1999) and *Pinus sylvestris* (Yin et al., 2003).

The present paper reports the first AFLP-based genetic map of Korean pine. The availability of the genetic map can provide more genomic information on Korean pine and will be used for the detection of loci contributing to quantitative trait expression.

MATERIALS AND METHODS

Plant materials

Higher genetic variability was found in clone 2151 (maternal, originnating from Hebei of Heilongjiang Province) and 2049 (paternal, originating from Fenglin of Heilongjiang Province) of Korean pine

*Corresponding author. E-mail: cmm0707@163.com.

Table 1. AFLP primer combinations and statistics.

Primer combination	*Pst*I primer extension	*Mse*I primer extension	Number of polymorphic bands scored	Number of Bands in map construction	Number of bands in LG(s)
A-1	GAA	CAA	150	15	13
A-2	GAA	CAC	127	16	11
A-6	GAA	CTC	126	27	20
A-7	GAA	CTG	113	19	13
A-3	GAA	CAG	157	29	23
C-1	GAG	CAA	134	23	12
C-5	GAG	CTA	162	25	10
D-6	GAT	CTC	124	19	14
D-8	GAT	CTT	147	14	14
Total	—	—	1240	187	130
Average	—	—	137.8	20.8	14.4

(unpublished data). Eighty-eight F1 individuals from across between clone 2049 and 2151 were used in the study. The population was generated by hand pollinating at Hongguang Forestry Farm (127°47'E, 42°31'N) in Jilin Province in 2006. Needle samples were taken from the 88 individuals, stored in ice box in the field and in -40°C in the laboratory.

DNA extraction

Total DNA was extracted from the fresh leaves using the Plant Genomic DNA Kit (DP305, TIANGEN and Beijing). The DNA preparations of each sample were quantified by 0.8% agarose gel electrophoresis. The DNA samples were stored at -20°C in the laboratory for future use.

AFLP analysis

Restriction digests and ligations were performed using the AFLP *Pst*I /*Mse*I Kit (A016, Dingguo, Beijing) with 200 ng of DNA. Pre-amplification was carried out with standard *Pst*I (P) and *Mse*I (M) adaptors with three additional nucleotides in a 25 µl reaction volume containing 100 ng of DNA, 0.2 mM of each dNTP, 0.4 mM of each primer, 2.5 µl 10 × buffers and 1 U Taq DNA polymerase. Cycling was carried out on a PE 9600 thermocycler (Perkin Elmer, USA) with a 94°C hold for 2 min followed by 30 cycles of 94°C for 30 s (denaturation), 56°C for 30 s (annealing) and 72°C for 80 s (extension) respectively, followed by a final hold at 72°C for 5 min.

Selective amplifications were carried out in a 20 µ l volume consisting of 2 µl of diluted pre-amplification template under the same reaction conditions, as pre-amplification except for 5 ng of a *Pst*I primer and 30 ng of *Mse*I primer per reaction. Selective amplification cycling was performed on a PE 9600 with the following programme: an initial cycle of 94°C for 30 s (denaturation), 65°C for 30 s (annealing) and 72°C for 80 s (extension), respectively, then 10 cycles of 94°C for 30 s (denaturation) with an annealing temperature starting at 65°C for 30 s but decreasing by 1°C each cycle, 72°C for 80 s (extension); finally, 23 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing) and 72°C for 80 s (extension) respectively, with a final hold at 72°C for 5 min.

After selective amplification, AFLP fragments were denatured by adding an equal volume of loading-buffer (98% formamide, 10 mM EDTA and 0. 25% bromophenol blue). The PCR products of selective amplification were separated on 6% denatured polyacrylamide gels (19:1 acylamide: bis; 7.5 M urea; 1 × TBE buffer). Electrophoresis was performed using an ABI PRISM 377 sequencer for 1 - 2.5 h, with a voltage of 40 - 50 W and 1200 V, respectively. Bands were analyzed and scored with Genescan 3.1 and Binthere software (developed by N.Garnhart, University of New Hampshire).

Linkage analysis

The linkage maps were constructed with JoinMap 3.0 (Voorrips and Ooijen 2001), which analyzes cross-pollinated populations derived from heterozygous parents to create a consensus linkage map. Polymorphic markers were separated into four types: (1) Those showing segregation only for the female parent (clone 2151), that is, male A1A1, female A1A2; (2) those showing segregation only for the male parent (clone 2049), that is, male A1A2, female A1A1; (3) those showing segregation for both parents (hetero-zygous in both parents), that is, male A1A2, female A1A2; and (4) non-parental bands. For each marker, a chi-quest ($p \leq 0.05$) was used to identify deviations from the expected Mendelian ratios. Markers were placed into linkage groups with the “LOD groupings” command using the Kosambi map function (Kosambi, 1944). Calculation parameters were set for a minimum LOD threshold of 4.0. Markers showing slight segregation distortion ($0.05 \leq p \leq 0.01$) were included in the final map if their presence did not alter surrounding markers’ order on the linkage group. Maps were drawn using the Mapchart version 2.0 software (Voorrips 2002).

RESULTS

AFLP markers

Sixty four primer combinations (8 *Pst*I primers × 8 *Mse*I primers) were screened against both parents and 10 progeny individuals. Among these primer combinations, 9 were selected for subsequent use based on the ability to generate numerous polymorphic bands and to show better repeatability (Table 1). These primer combinations amplified a total of 1240 polymorphic bands (138 on average). Around 659 markers (53.1%) which were the non-parental polymorphic bands were excluded from further linkage analysis. Of the 225 testcross markers (18.1%), 138 were heterozygous in “2049” and the remaining 87 in

Table 2. Main characteristics of markers and genetic distances (GD) per linkage group in *Pinus koraiensis*.

Linkage group markers	Total markers	Genetic distance (cM)	Average distance (cM)	Distorted (0.05≤P ≤0.01)
LG1	55	43.120	0.784	0
LG2	6	43.067	7.178	0
LG3	5	10.200	2.040	5
LG4	5	50.302	10.060	1
LG5	8	69.958	8.745	0
LG6	9	58.102	6.456	1
LG7	3	34.263	11.421	0
LG8	3	19.232	6.411	1
LG9	3	22.776	7.592	1
LG10	3	3.224	1.075	3
LG11	2	6.365	3.183	2
LG12	2	13.268	6.634	0
LG13	2	28.369	14.185	0
LG14	2	18.990	9.495	0
LG15	2	22.293	11.147	1
LG16	2	14.304	7.152	1
LG17	2	6.411	3.206	1
LG18	2	25.972	12.986	1
LG19	2	8.167	4.084	0
LG20	2	29.004	14.502	1
LG21	2	35.657	17.829	0
LG22	2	24.757	12.379	1
LG23	2	21.091	10.546	0
LG24	2	1.606	0.803	2
LG25	2	10.411	5.206	0
Total	130	620.909	4.776	22

“2151”. Three-hundred and fifty-six markers (28.7%) were heterozygous in both parents.

One-hundred and eighty-seven markers (20.8 on average) (Table 1) were selected for the map construction. Eighty four (44.9%) and 103 (55.1%) ($p \leq 0.01$) out of the 187 detected AFLP markers segregated in the 1:1 and 3:1 ratios, respectively. The chi-square test revealed biased segregation, 65 out of 187 markers (34.8%) showed slight distortion ($0.05 \leq p \leq 0.01$) from the expected Mendelian segregation ratios. The number of paternal markers (51) was higher than that of the maternal markers (33). Among the 187 polymorphic markers, 130 were mapped onto the genetic map (Table 1).

Parental maps

For the integrated map, 187 markers were used to construct a framework map. One hundred and thirty molecular markers were assembled into 6 groups, 4 triples and 15 pairs. Twenty five linkage groups (pairs) were established covering a total map distance of 620.909 cM, with an average space of 4.77 cM between any two adjacent markers (Table 2 and Figure 1). The size of the linkage groups (pairs) ranged from 1.606 to 69.958 cM (4.776 cM on average). The number of markers per linkage group varied between 2 and 55. Twenty two of the mapped markers (16.9%) showed delete distorted segregation. Fifty seven markers could not be linked to any other linkage groups at a LOD threshold of 4.0.

DISCUSSION

Mapping population

Segregation populations obtained from crosses between inbred lines are usually utilized to construct genetic linkage maps. However, such populations are generally not known for trees because of their long growth generation (Grattapaglia and Sederoff, 1994). In this study, the F1-mapping population which had been widely adopted in trees for their higher heterozygosity (Wu et al., 2000; Achere et al., 2004) was utilized. The marker data were analyzed according to the double pseudo-test cross strategy. The separated parental maps (data not shown)

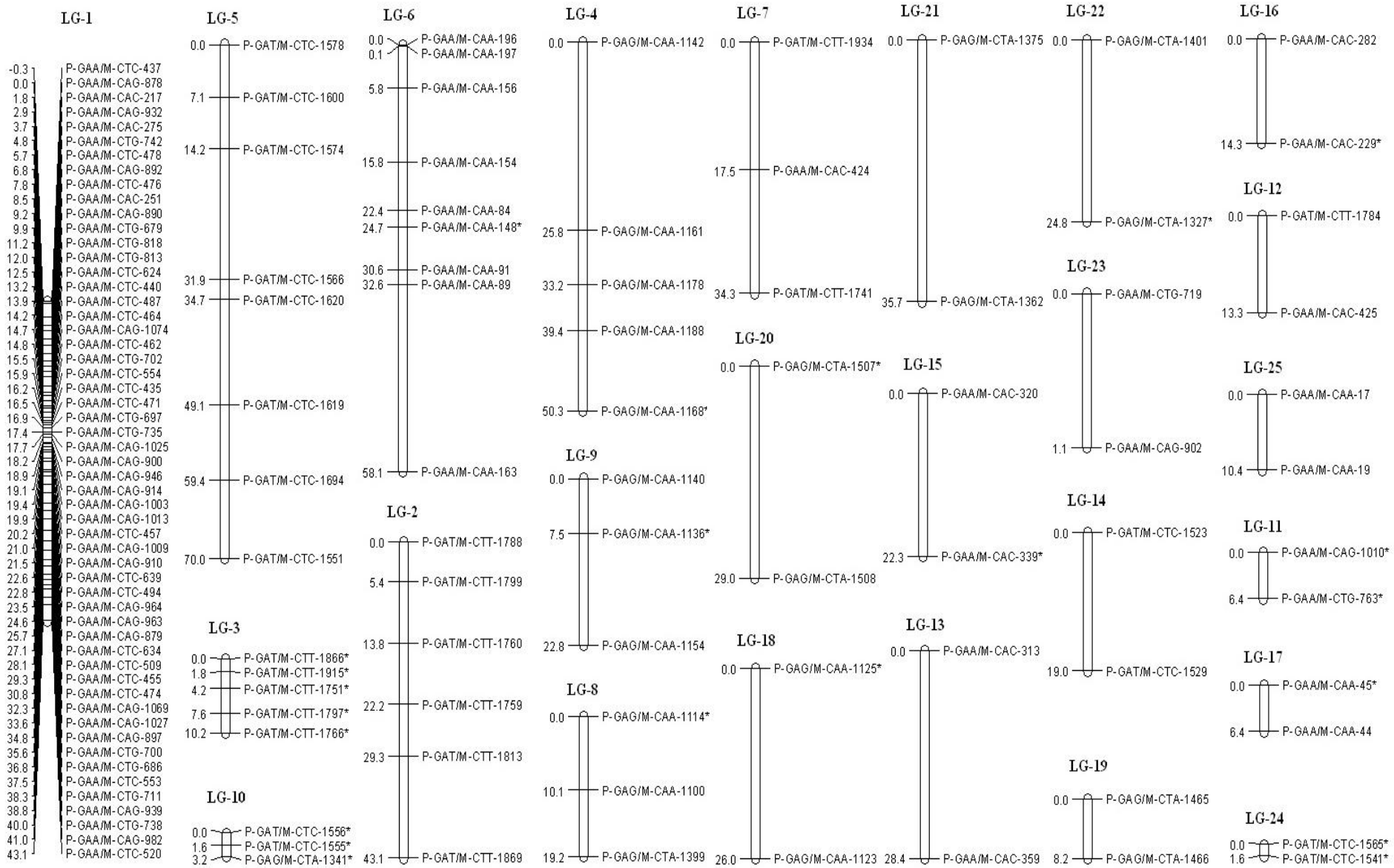

Figure 1. Linkage map of *Pinus koraiensis*. Loci are listed on the right and recombination distances (cM, Kosambi) are listed on the right of each linkage group. Markers showing slight distorted segregation are labeled with one asterisk depending on the significance level ($0.05 \leq p \leq 0.01$) of the χ^2 test. AFLP markers are identified by the PEC (primer-enzyme combinations) code (the first three letters correspond to the selective nucleotides of the *Pst*I primer and the last three letters correspond to the selective nucleotides of *Mse*I primer).

were integrated using the 3:1 dominant markers (Figure 1).

Marker identification

A genetic linkage map, especially a high-density genetic linkage map, requires not only an appropriate mapping population but also a large number of molecular markers. The present paper is the first attempt to use AFLP as a basis molecular marker for Korean pine genetic maps' construction. Although more parameters are required for AFLP (Wang et al., 2004), this technique can recover numerous polymorphic markers (14-29 in our experiment) (Table 1) in the same assay. This merit makes it much more efficient than RAPD. Moreover, PCR reactions of AFLP technique are more reliable and reproducible as they are carried out under stringent conditions (Vos and Kuiper, 1997). As a result, we can generate a large amount of good-quality polymorphic markers using this method.

In this study, 659 non-parental polymorphic bands were found, accounting for a large number of the polymorphic bands (53.1%). Such kind of non-parental bands were firstly reported in RAPD molecular markers in offspring from both baboon and human CEPH pedigrees (Riedy et al., 1992). According to Ayliffe et al. (1994), the non-parental products were heteroduplex molecules formed by the annealing of two amplified allelic sequences, which differs in length. This kind of molecules represent PCR artifact potentially capable of confusing RAPD-based analysis (Ayliffe et al., 1994). No other rational explanations were reported in molecular genetics for the reason of the appearance of non-parental bands in AFLP, so this kind of bands were discarded from further analysis.

Map construction

Based on karyotype analysis, pine species are known to contain 12 (similar-sized) pairs of homologous chromosomes (Saylor, 1972; Kormutak, 1975). Inconsistent with these previous reports, our map contains 6 groups, 4 triples and 15 pairs (Figure 1 and Table 2). But the number of linkage groups identified in our maps (25) (Table 2 and Figure 1) is comparable with previously published findings for other Pinus species (Plomion et al., 1995; Echt and Nelson, 1997; Hayashi et al., 2001; Shepherd et al., 2003;Yin et al., 2003; Kim et al., 2005).

In our study, some clustering of the AFLP markers appeared in some portions of the linkage groups as previously reported (Wu et al., 2000; Cavalcanti and Wilkinson, 2007). This can be explained by the uneven distribution of restriction sites of *Psti*-based markers throughout the plant genome (regions around centromeres and telomeres) (Boivin et al., 1999). The uneven distribution may reduce the frequency of crossover during meiosis and cause marker clustering (Luckaszewski and Curtis, 1993).

High-density genetic map should be a valuable reference for detecting important QTL on the genetic maps that will facilitate marker-assisted selection (MAS) of important traits. MAS are helpful for improving the selection efficiency of breeding program and allowing inferior seedlings to be removed early in growth. In the long term, the present study is helpful in positioning cloning when linkages between molecular markers and important genes are established.

REFERENCES

Achere V, Faivre-Rampant P, Jeandroz S, Besnard G, Markussen T, Aragones A, Fladung M, Ritter E, Favre JM (2004). A full saturated linkage map of Picea abies including AFLP, SSR, ESTP, 5S rDNA and morphological markers. Theor. Appl. Genet. 108: 1602-1613.

Ayliffe MA, Lawrence GJ, Ellis JG, Pryor AJ (1994). Heteroduplex molecules formed between allelic sequences cause nonparental RAPD bands. Nucleic Acids Res. 22: 1632-1636.

Boivin K, Deu M, Rami JF (1999). Towards a saturated sorghum map using RFLP and AFLP markers. Theor. Appl. Genet. 98: 320-328.

Bradshaw HDJ, Villar M, Watson BD, Otto KG , Stewart S, Stettler RF (1994). Molecular genetics of growth and development in *Populus.* A genetic linkage map of a hybrid poplar composed of RFLP, STS, and RAPD markers. Theor. Appl. Genet. 89: 167-178.

Cavalcanti JJV, Wilkinson MJ (2007). The first genetic maps of cashew (*Anacardium occidentale* L.). Euphytica, 157: 131-143.

Costa P, Pot D, Dubos C, Frigerio JM, Pionneau C, Bodenes C, Bertocchi E, Cervera M-T, Remington DL, Plomion C (2000). A genetic map of Maritime pine based on AFLP, RAPD and protein markers. Theor. Appl. Genet. 100: 39-48.

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

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

Grattapaglia D, Sederoff R (1994). Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics, 137: 1121-1137.

Hayashi E, Kondo T, Terada K, Kuramoto N, Goto Y, Okamura M, Kawasaki H (2001). Linkage map of Japanese black pine based on AFLP and RAPD markers including markers linked to resistance against the pine needle gall midge. Theor. Appl. Genet. 102: 871-875.

Kim YY, Choi HS, Kang BY (2005). An AFLP-based linkage map of Japanese red pine (*Pinus densiflora*) using haploid DNA samples of megagametophytes from a single maternal tree. Mol. Cells, 20: 201-209.

Kormutak A (1975). Karyological structure of some *Pinus* species. Biologia, 30: 545-550.

Kosambi DD (1944). The estimation of map distances from recombination values. Ann. Eugen. 12: 172-175.

Luckaszewski AJ, Curtis CA (1993). Physical distribution of recombination in B-genome chromosomes of tetraploid wheat. Theor. Appl. Genet. 86: 121-127.

Miles LG, Isberg SR, Glenn TC, Lance SL, Dalzell P, Thomson PC, Moran C (2009). A genetic linkage map for the saltwater crocodile (*Crocodylus porosus*). BMC Genomics, 10: 339.

Plomion C, O'Malley DM, Durel CE (1995). 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.

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

Riedy MF, Hamilton WJ, Aquadro CF (1992). Excess of non-parental

bands in offspring from known primate pedigrees assayed using RAPD PCR. Nucleic Acids Res. 20: p. 918.
Saylor LC (1972). Karyotype analysis of the genus *Pinus*-subgenus *Pinus*. Silvae Genet. 21: 155-163.
Shepherd M, Cross M, Dieters MJ, Henry R (2003). Genetic maps for *Pinus elliottii* var. *elliottii* and *P. caribaea* var. *hondurensis* using AFLP and microsatellite markers. Theor. Appl. Genet. 106: 1409-1419.
Voorrips RE, Van Ooijen JW, (2001). JoinMap version 3.0: software for the calculation of genetic linkage maps. Plant Research International, Wageningen, The Netherlands. http://www.kyazma.nl/index.php/mc.JoinMap.
Voorrips RE (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. J. Hered. 93: 77-78.
Vos P, Hogers R, Bleeker M, Reijans M, Van De 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.
Vos P, Kuiper M (1997). AFLP analysis. In: Caetano-Anolles G., Gresshoff PM, eds. DNA markers: protocols, applications and overviews. Wiley-Liss, Inc., New York, pp. 115-131.
Wang S, Bao Z, Pan J, Zhang L, Yao B, Zhan A, Bi K, Zhang Q (2004). AFLP linkage map of an intraspecific cross in *Chlamys farreri*. J. Shellfish Res. 23: 491-499.
Wilcox PL, Richardson TE, Corbet GE, Ball RD, Lee JR, Djorovic A, Carson SD (2001). Framework Linkage Maps of *Pinus radiata* D. Don based on pseudotestcross markers. For. Genet. 8: 109-117.
Wu RL, Han YF, Hu JJ, Fang JJ, Li L, Li ML, Zeng ZB (2000). An integrated genetic map of *Populus deltoides* based on amplified fragment length polymorphisms. Theor. Appl. Genet. 100: 1249-1256.
Yin TM, Wang XR, Andersson B, Lerceteau-Köhler E (2003). Nearly complete genetic maps of *Pinus sylvestris* L. (Scots pine) constructed by AFLP marker analysis in a full-sib family. Theor. Appl. Genet. 106: 1075-1083.
Zhou Y, Gwaze DP, Reyes-Valdés MH, Bui T, Williams CG (2003). No clustering for linkage map based on low-copy and undermethylated microsatellites. Genome, 46: 809-816.