Theor Appl Genet (2000) 100:299–307

A. Arcade · F. Anselin · P. Faivre Rampant
M.C. Lesage · L.E. Pâques · D. Prat

Application of AFLP, RAPD and ISSR markers to genetic mapping of European and Japanese larch

Received: 15 March 1999 / Accepted: 29 March 1999

Abstract Genetic linkage maps have been increasingly developed for a wide variety of plants, using segregating populations such as F_2s or backcrosses between inbred lines. These pedigrees are rarely available in outbred species like forest trees which have long generation times. Thus genetic mapping studies have to use peculiar pedigrees and markers in appropriate configurations. We constructed single-tree genetic linkage maps of European larch (*Larix decidua* Mill.) and Japanese larch [*Larix kaempferi* (Lamb.) Carr.] using segregation data from 112 progeny individuals of an hybrid family. A total of 266 markers (114 AFLP, 149 RAPD and 3 ISSR loci) showing a testcross configuration, i.e. heterozygous in one parent and null in the other parent, were grouped at LOD 4.0, θ=0.3. The maternal parent map (*L. decidua*) consisted of 117 markers partitioned within 17 linkage groups (1152 cM) and the paternal parent map (*L. kaempferi*) had 125 markers assembled into 21 linkage groups (1206 cM). The map distance covered by markers was determined by adding a 34.7-cM independence distance at the end of each group and unlinked marker. It reached 2537 cM and 2997 cM respectively for European larch and Japanese larch, and represented respectively a 79.6% and 80.8% coverage of the overall genome. A few 3:1 segregating markers were used to identify homologous linkage groups between the European larch and the Japanese larch genetic maps. The PCR-based molecular markers allowed the construction of genetic maps, thus ensuring a good coverage of the larch genome for further QTL detection and mapping studies.

Communicated by M.A. Tigerstedt

A. Arcade · F. Anselin · P. Faivre Rampant · M.C. Lesage
L.E. Pâques · D. Prat (✉)
INRA, Unité Amélioration, Génétique et Physiologie forestières,
Avenue de la Pomme de Pin, BP 20619 Ardon,
F-45166 Olivet Cedex, France
e-mail: prat@orleans.inra.fr
Tel.: +33-2 38 41 78 16
Fax: +33-2 38 41 78 79

Keywords *Larix* · Linkage map · RAPD · AFLP · ISSR · Genetic mapping

Introduction

Genetic markers provide a valuable tool for genetic analysis and breeding research in forest trees, and they facilitate studies of genome organization, mating systems, genetic diversity and phylogeny analysis. Until recently, genetic linkage mapping suffered from a lack of genetic markers and produced only low-density genetic maps. The advent of molecular markers directly based on DNA sequences, such as RFLPs, recovered many loci and extended the linkage maps (poplar: Bradshaw et al. 1994; loblolly pine: Devey et al. 1994).

In the last decade, the development of arbitrarily primed markers based on the PCR (polymerase chain reaction) assay offered the opportunity to construct high-density genetic maps. RAPD markers (Williams et al. 1990) provided a significant advance in the construction and saturation of genetic maps. The dominant mode of inheritance of RAPD markers can be circumvented by the use of appropriate pedigrees, like conifer haploid megagametophytes (white spruce: Tulsieram et al. 1992; maritime pine: Plomion et al. 1995) or two-generation pedigrees using a two-way pseudo-testcross strategy, where each parent of the cross is simultaneously mapped (Carlson et al. 1991; *Eucalyptus urophylla*, *Eucalyptus grandis*: Grattapaglia et al. 1994). However, the RAPD technique is often criticized for its lack of reproducibility over time as well as between laboratories (Jones et al. 1997). The advent of the AFLP™ (amplified fragment length polymorphism) technique (Vos et al. 1995) provided a new class of highly polymorphic markers combining both RFLP and PCR strategies. The great number of markers generated can contribute to the saturation of existing maps. Their advantage over RAPDs is their high reproducibility (Jones et al. 1997). This technique is recent and is being increasingly developed on trees (Pinyon pine: Travis et al. 1998; *Eucalyptus globulus*, *Euca-*

lyptus tereticornis: Marques et al. 1998; Norway spruce: Paglia et al. 1998). Microsatellite (or simple sequence repeat: SSR) markers are attractive since they show co-dominant inheritance and multi-allelism. However, they still only meet a few cases (radiata pine: Smith and Devey 1994; Devey et al. 1996; *E. urophylla*, *E. grandis*: Brondani et al. 1998; Norway spruce: Paglia et al. 1998; Pedunculate oak : Barreneche et al. 1998). Inter-simple sequence repeat (ISSR) markers are generated by microsatellite-repeat-anchored primers amplifying regions between adjacent SSR loci (Zietkiewicz et al. 1994). These primers produce many polymorphic markers useful for genome mapping (Douglas-fir, sugi: Tsumura et al. 1996; Wheat: Nagaoka and Ogihara 1997; Kojima et al. 1998).

Larch belongs to the Pinaceae and is a useful pioneer species for the re-forestation of lowland areas. It is valued for fast juvenile growth, short rotation, good productivity and high quality of timber (Pâques 1989). The genetic improvement and breeding program for larch in France is especially concerned with the hybrid between European larch and Japanese larch, *Larix* x *eurolepis*, as it combines many favourable characteristics of both species (Pâques 1992).

In the present study, we report the first construction of genetic maps for two species of larch, *Larix decidua* Mill. and *Larix kaempferi* (Lamb.) Carr, using a pseudo-testcross strategy. The availability of these genetic maps provides a first overview of larch genome structure and will be used for the detection of loci contributing to quantitative trait expression.

Materials and methods

Plant material and DNA extraction

The mapping population consisted of 112 F_1 individuals derived from a cross between *L. decidua* (clone 107, Czech provenance) used as the female parent and *L. kaempferi* (clone 3076 produced by a cross between full-sib trees) used as the male parent. This family was chosen among other families of a factorial mating design because of its large number of individuals (Arcade et al. 1995).

Total genomic DNA was extracted as described in Arcade et al. (1995) from needle samples from both parents and from individuals of the mapping population.

AFLP assay

AFLP analyses were carried out using the "AFLP™ Analysis System I" kit (Life Technologies, Inc.) following the manufacturer's instructions, with minor modifications: restriction digestion mixtures were incubated for 3 h at 37°C and *Eco*RI primers were labelled for 2 h at 37°C. Restriction fragments were generated by the restriction endonucleases *Eco*RI and *Mse*I. Primer labelling was performed with [γ-^{33}P]ATP (Amersham). AFLP primers contained one selective nucleotide for pre-amplification, and three selective nucleotides for amplification. PCR products were separated on 6% polyacrylamide gels (19:1 acylamide:bis; 7.5 M urea; 1 x TBE buffer). Electrophoresis was performed using a Sequi-Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad). Gels were pre-run until an adequate temperature (50°C) was reached. Five-microliter samples were loaded and electrophoresed at 90 W for 3 h. Gels were then vacuum-dried and exposed to Biomax MR films (Kodak) for 3 days.

RAPD assay

Reaction mixtures (11 µl total volume) for the RAPD reaction contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 350 µM of dNTPs, 0.3 µM of 10-base primers (obtained from Operon Technologies Inc., Alameda, California), 0.08 U of *Taq* polymerase (Appligene) and 10 ng of genomic DNA. Amplifications were performed in 96-well microplates using a MJ Research PTC-100 thermal cycler programmed for one step of 5-min denaturation at 94°C, followed by 40 cycles of 1-min denaturation at 94°C, 1-min annealing at 36°C, 2-min extension at 70°C, and a final extension step of 5 min at 70°C. Amplification products were run on 1.5% (w/v) agarose gels at 6 V/cm. After ethidium-bromide staining, gels were photographed under UV light with a MP4 Polaroid camera.

ISSR assay

Eleven ISSR primers with dinucleotide motifs were assayed. PCR amplification reactions (11 µl total volume) were performed in 10 mM TrisHCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 850 µM of dNTPs, 0.15 µg of primer, 0.13 U of *Taq* polymerase (Appligene) and 50 ng of genomic DNA. Amplification reactions were conducted for one initial step of 2-min denaturation at 94°C followed by 35 cycles of 1-min denaturation at 94°C, 1-min annealing at 50°C, 2-min extension at 72°C, and a final extension step of 7-min at 72°C. PCR products were loaded on 2% (w/v) agarose gels. Electrophoresis and detection conditions were as for RAPD assay.

Primer screening and marker scoring

In a testcross configuration, informative dominant markers should be heterozygous in one parent, absent in the other parent, and segregating 1:1 in the progeny. For a first screening, AFLP, RAPD and ISSR primers were assayed on both parents and a sample of six progeny individuals. Fragments showing polymorphism between parents and segregating in the progeny were selected for subsequent use on the whole mapping population.

Markers were scored on the 112 F_1 individuals for their presence or absence in two different data sets according to their parental origin. Only clear-cut fragments were recorded and markers with more than 25% missing data were left out of further analyses.

Linkage analysis of markers

Chi-square tests (α=0.05) of goodness-of-fit to the 1:1 expected segregation ratio were performed for all markers. Linkage analyses were done using MAPMAKER/EXP Version 3.0 (Lincoln et al. 1992) with the data type "F_2 backcross" suited for our pseudo-testcross configuration.

Markers were grouped by two-point linkage analysis using a LOD score of 4.0 and a recombination fraction θ of 0.3.

The most-likely order of markers within a linkage group was determined by multipoint analysis. The COMPARE command was used for groups with less than eight markers. For larger linkage groups, the three-point command was used to pre-compute the likelihood of all three-point crosses of each group. Then the ORDER command determined a linear order of the markers using multipoint analysis. Finally, the RIPPLE command was employed to compare the likelihoods of the original group order and those found when the order of three neighboring loci was permuted. A framework map was established when marker order was supported by a log-likelihood ratio support of 2.0.

Distances between adjacent marker loci were calculated from recombination fractions using Kosambi's mapping function.

In addition, markers present in both parents and segregating in the progeny were scored. The placement of markers showing a 3:1 segregation ratio (chi-square test, α=0.05) was achieved by two-point analysis with the LINKAGE-1 version 3.50 software (Suiter et al. 1983).

Genome length and genome map coverage were estimated using the method of moments (Hulbert et al. 1988, method No. 3 of Chakravarti et al. 1991).

Results

AFLP markers

Sixty four primer combinations (8 *Eco*RI primers × 8 *Mse*I primers) were screened against both parents and six progeny individuals. Six primer pairs (9%) did not yield any amplification, six primer pairs failed to generate polymorphism between the parents and 52 primer pairs (82%) produced at least one polymorphic band. Five primer pairs were selected for subsequent use based on their ability to generate numerous polymorphic bands (Table 1). These primer combinations amplified an average of 146 bands (Fig. 1). A total of 129 polymorphic markers were recorded on the mapping population. One-hundred-and-eleven markers were selected as they fitted the 1:1 segregation ratio in the progeny. Four markers were present in both parents and showed a 3:1 segregation in the progeny. Three markers inherited from the Japanese parent departed from the 1:1 segregation ratio and were represented as accessory markers on the final map. Two distorted markers that were present in both parents could not be mapped. Lastly, three markers present in one parent fitted a 3:1 segregation ratio in the progeny. This could result either from linkage with a gene found to be lethal in a homozygous state, or from the presence in the parental pattern of two superposed AFLP bands segregating independently in the progeny. These last three markers were not used for map construction. Fifty eight fragments were inherited from the European parent and 56 from the Japanese parent.

RAPD markers

Among 600 arbitrary primers tested, 210 (35%) gave no amplification pattern and 390 (65%) primers amplified between 1 and 15 bands whose size ranged from 150 to 2500 bp. Ninety five primers (15.8%) revealed 155 polymorphic fragments. From these fragments, 147 showed a 1:1 segregation and six showed a 3:1 segregation. Only two markers departed from the 1:1 ratio and were represented as ancillary markers on the final framework maps. Sixty four polymorphic markers (44%) originated from the European parent while the 83 other markers (56%) were inherited from the Japanese parent.

Table 1 AFLP primer combinations used on the mapping population and number of bands and polymorphic fragments generated

*Eco*RI/ *Mse*I primer pair	Total number of bands	Number of polymorphic bands
E-ACC/M-CTG	154	12
E-ACG/M-CTG	101	24
E-AGC/M-CAG	180	33
E-AGC/M-CTG	124	41
E-AGG/M-CTG	172	19

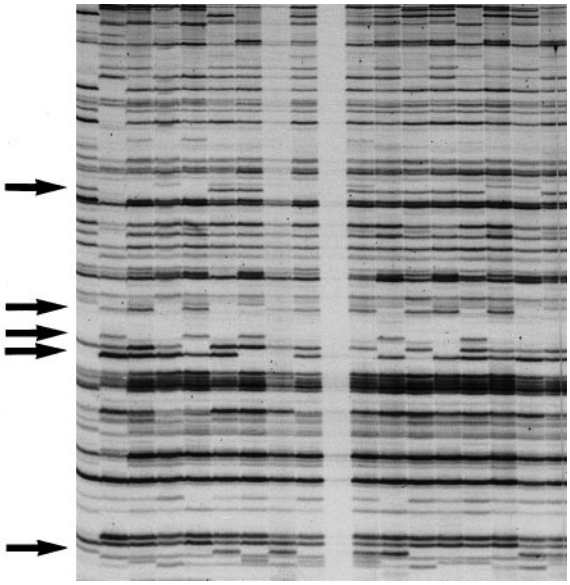


Fig. 1 Detail of an autoradiogram showing the segregation of AFLP markers in larch (E-AGC/M-CAG primer pair). The *first lane* is the female parent (*L. decidua*), the *second lane* is the male parent (*L. kaempferi*) followed by the F_1 progeny. Segregating AFLP markers are indicated by *arrows*

ISSR markers

Eleven primers were screened on both parents and six F_1 individuals (Table 2). Four primers produced clear patterns with at least one polymorphic marker, while the other primers gave a smearing pattern. We observed that clear amplification patterns with scorable bands were produced by ISSR primers with arbitrary nucleotides placed at their 3′ end. These primers are supposed to amplify sequences between adjacent SSR loci. By contrast, ISSR primers with arbitrary nucleotides at their 5′ end produced smearing patterns, presumably because they amplified both SSR and inter-SSR regions. Fragment size ranged from 200 bp to 1700 bp. ISSR primer #5 amplified five polymorphic markers and was selected for genotyping on the whole mapping population. However, only three among these five markers were clearly scorable (Fig. 2). Two of them were inherited from the European parent and the other one from the Japanese parent. They fitted the expected 1:1 segregation ratio.

Table 2 ISSR primers used for marker screening and number of bands and polymorphic markers generated

ISSR primer sequence 5′ 3′	Total number of bands[a]	Number of polymorphic bands
1 $GCGC(AC)_8$	–	–
2 $CCGG(AC)_8$	–	–
3 $GCGA(CA)_8$	–	–
4 $(CA)_8CG$	16	2
5 $(CA)_8GT$	15	5
6 $CGCG(AG)_8$	–	–
7 $CGCT(GA)_7$	–	–
8 $(GA)_8GT$	–	–
9 $(GA)_8CG$	–	–
10 $(GA)_8TC$	15	3
11 $(CA)_8TG$	9	1

[a] – indicates that no bands could be scored because the pattern showed a strong background or a smear

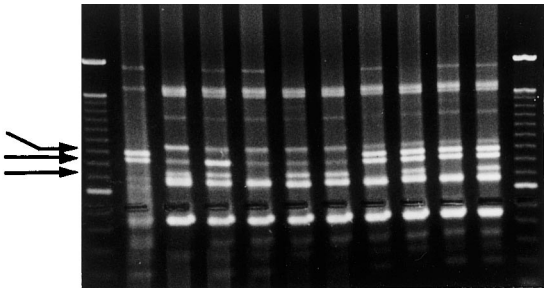


Fig. 2 Segregation of markers revealed by primer ISSR#5 in the progeny. From left to right: 100-bp size standard, female parent (*L. decidua*), male parent (*L. kaempferi*), eight F_1 progeny, 100-bp size standard. Segregating ISSR markers are indicated by *arrows*

Genetic map construction

Grouping of markers was carried out by a two-point linkage analysis with a LOD score of 4.0 and a recombination fraction θ of 0.3.

The maternal European larch map had a total of 117 markers distributed in 17 linkage groups (Fig. 3) while nine markers remained unlinked. The paternal Japanese larch map consisted of 125 markers placed in 21 linkage groups (Fig. 4) while 15 markers remained unlinked. As both larch species have n=12 chromosomes, the number of linkage groups for both maps is superior to the expected 12 groups. Lowering the LOD score threshold to 3.0 and increasing θ to 0.35 resulted in the merger of a few linkage groups and the recovery of unlinked markers. However, ordering of markers within these groups often generated conflicting linkages and suggested that mergers between linkage groups may in fact be spurious.

Ordering of markers within each linkage group was achieved by multipoint analysis. For loci ordering in the largest linkage groups, a subset of markers was chosen based on two informativeness criteria: (1) minimal determined distance between loci and (2) minimal number of missing data. The remaining markers were then added one at a time. Markers that could not be placed confidently were designed as accessory markers and were represented near the closest framework marker (Figs. 3 and 4). These ambiguous locus orderings often occurred when the distance separating markers was less than 5 cM.

For the European larch map, 100 markers were used to construct a framework map and 17 markers were placed as accessory markers. A total length of 1152 cM was recovered when adding up the distances between consecutive markers. However, a more accurate estimation of the distance covered by markers on the framework map could be determined by adding 34.7 cM (independence distance for a recombination fraction θ=0.3 using the Kosambi function) at the end of each linkage group and each isolated marker. Then, the total length of the map reached 2020 cM. Linkage group size ranged from 1.1 to 250.6 cM with an average size of 68 cM. The number of markers per linkage group varied from 2 to 20. The average distance between adjacent markers was 13.8 cM.

For the Japanese parent map, 107 markers were represented on the final framework map while 18 markers were placed as ancillary markers. The addition of all pairwise distances gave a length of 1206 cM. The addition of a 34.7-cM independence distance at the end of each group and isolated marker led to a final length of 2421 cM. The size range of linkage groups was 3.6–224.8 cM with an average size of 57.4 cM. The number of markers per linkage group varied from 2 to 21 markers. The average distance between adjacent markers was 14 cM.

Genome length estimation

Using method No.3 of Chakravarti et al. (1991), we estimated the total genome size of *L. decidua* to be 2537 cM, on the basis of the 208 pairs of markers linked at less than 34.7 cM (θ=0.3, LOD score=4.0). In *L. kaempferi*, the 213 linked pairs of markers led to an overall estimated genome size of 2997 cM. Consequently, markers covered 79.6% and 80.8% of the genome respectively for *L. decidua* and *L. kaempferi*.

Correspondence between maps

Ten markers were found to be heterozygous in both parents and segregated in a 3:1 ratio in the progeny. Two-point linkage analysis between these markers and markers already placed on the framework maps was carried out in order to locate them and to identify homologous map regions between species. Six of these markers were tightly linked ($0.00 \leq \theta \leq 0.18$) with placed markers segregating 1:1 (Figs. 3 and 4). Four other markers segregating 3:1 were linked with markers of one single map. Markers D20_420 and AE9_1800 revealed a direct homology respectively between linkage groups E1 and J1 and linkage groups E2 and J2, which, additionally, are the longest ones in both maps and show approximately the same length between species.

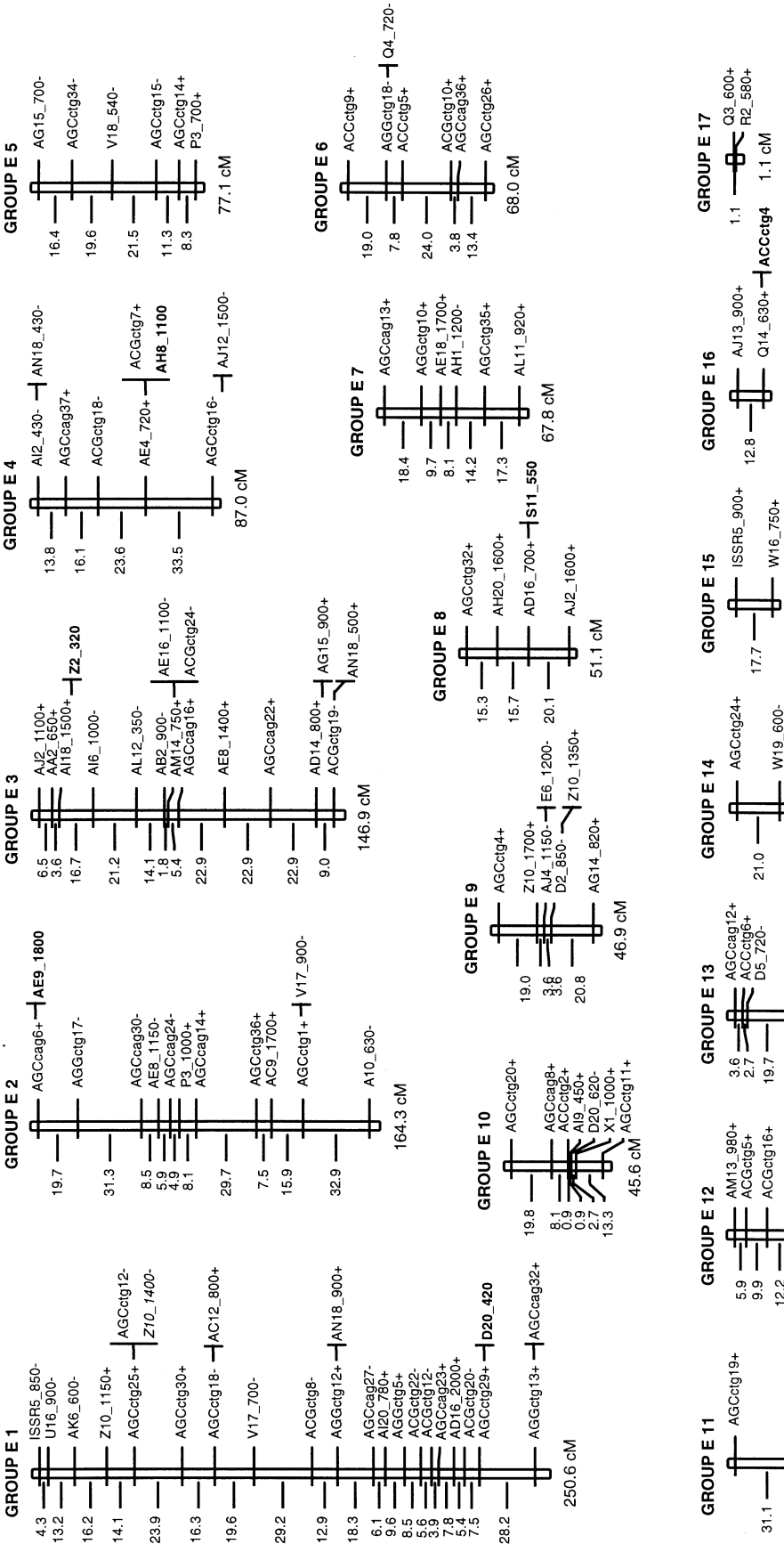
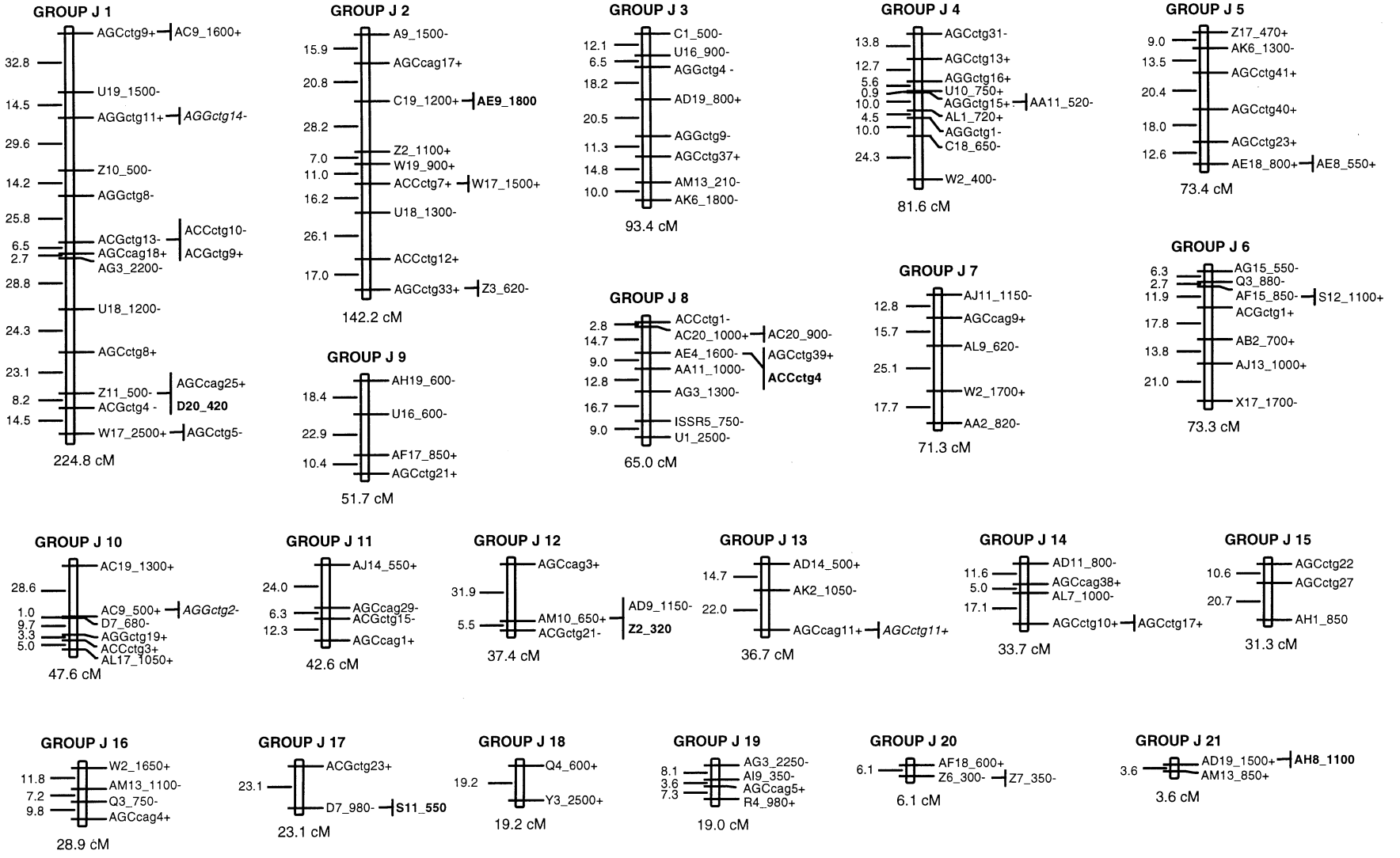


Fig. 3 Linkage map of *L. decidua*. *Names* of framework markers are indicated on the right of the linkage groups. RAPD and ISSR loci are designated by primer identity followed by the molecular weight (in bp) of the fragment. AFLP loci are indicated by the primer combination used to detect the marker, followed by a *number* indicating the fragment position on the gel (ranking begins with high-molecular-weight fragments). The linkage phase of loci is indicated by + or –. Markers that could not be mapped with a log-likelihood support ≥2.0 are represented as accessory markers to the *right* of the nearest framework marker. Distorted markers (P<0.05) are shown in *italics*. Markers heterozygous in both parents and present in both maps are shown in *bold type*. Genetic distances in cM using the Kosambi's mapping function are indicated on the *left* of the linkage groups. The length of each linkage group (in cM) is shown below the group


GROUP J 1
AGCctg9+
AC9_1600+
32.8
U19_1500-
14.5
AGGctg11+
AGGctg14-
29.6
Z10_500-
14.2
AGGctg8-
25.8
ACGctg13-
ACCctg10-
6.5
AGCcag18+
ACGctg9+
2.7
AG3_2200-
28.8
U18_1200-
24.3
AGCctg8+
23.1
Z11_500-
AGCcag25+
8.2
ACGctg4 -
D20_420
14.5
W17_2500+
AGCctg5-
224.8 cM
GROUP J 2
A9_1500-
15.9
AGCcag17+
20.8
C19_1200+
AE9_1800
28.2
Z2_1100+
7.0
W19_900+
11.0
ACCctg7+
W17_1500+
16.2
U18_1300-
26.1
ACCctg12+
17.0
AGCctg33+
Z3_620-
142.2 cM
GROUP J 3
C1_500-
12.1
U16_900-
6.5
AGGctg4 -
18.2
AD19_800+
20.5
AGGctg9-
11.3
AGCctg37+
14.8
AM13_210-
10.0
AK6_1800-
93.4 cM
GROUP J 4
13.8
AGCctg31-
AGCctg13+
12.7
5.6
AGGctg16+
0.9
U10_750+
10.0
AGGctg15+
AA11_520-
4.5
AL1_720+
10.0
AGGctg1-
C18_650-
24.3
W2_400-
81.6 cM
GROUP J 5
9.0
Z17_470+
AK6_1300-
13.5
AGCctg41+
20.4
AGCctg40+
18.0
AGCctg23+
12.6
AE18_800+
AE8_550+
73.4 cM
GROUP J 6
6.3
AG15_550-
2.7
Q3_880-
11.9
AF15_850-
S12_1100+
ACGctg1+
17.8
AB2_700+
13.8
AJ13_1000+
21.0
X17_1700-
73.3 cM
GROUP J 7
12.8
AJ11_1150-
AGCcag9+
15.7
AL9_620-
25.1
W2_1700+
17.7
AA2_820-
71.3 cM
GROUP J 8
2.8
ACCctg1-
14.7
AC20_1000+
AC20_900-
9.0
AE4_1600-
AGCctg39+
12.8
AA11_1000-
ACCctg4
AG3_1300-
16.7
ISSR5_750-
9.0
U1_2500-
65.0 cM
GROUP J 9
18.4
AH19_600-
U16_600-
22.9
AF17_850+
10.4
AGCctg21+
51.7 cM
GROUP J 10
AC19_1300+
28.6
1.0
AC9_500+
AGGctg2-
9.7
D7_680-
3.3
AGGctg19+
5.0
ACCctg3+
AL17_1050+
47.6 cM
GROUP J 11
AJ14_550+
24.0
AGCcag29-
6.3
ACGctg15-
12.3
AGCcag1+
42.6 cM
GROUP J 12
AGCcag3+
31.9
AM10_650+
AD9_1150-
5.5
ACGctg21-
Z2_320
37.4 cM
GROUP J 13
14.7
AD14_500+
AK2_1050-
22.0
AGCcag11+
AGCctg11+
36.7 cM
GROUP J 14
11.6
AD11_800-
AGCcag38+
5.0
AL7_1000-
17.1
AGCctg10+
AGCctg17+
33.7 cM
GROUP J 15
10.6
AGCctg22
AGCctg27
20.7
AH1_850
31.3 cM
GROUP J 16
11.8
W2_1650+
AM13_1100-
7.2
Q3_750-
9.8
AGCcag4+
28.9 cM
GROUP J 17
ACGctg23+
23.1
D7_980-
S11_550
23.1 cM
GROUP J 18
19.2
Q4_600+
Y3_2500+
19.2 cM
GROUP J 19
8.1
AG3_2250-
3.6
AI9_350-
AGCcag5+
7.3
R4_980+
19.0 cM
GROUP J 20
6.1
AF18_600+
Z6_300-
Z7_350-
6.1 cM
GROUP J 21
3.6
AD19_1500+
AH8_1100
AM13_850+
3.6 cM

Discussion

The use of AFLP, RAPD and ISSR techniques on the larch genome enabled us to generate many polymorphic markers ensuring a good coverage of the genome. However, only 15.8% of the RAPD primers tested revealed at least one polymorphic fragment, which is surprisingly low in comparison with other such mapping studies of conifer genomes, but already described for slash pine (Nelson et al. 1993). Indeed, forest trees are assumed to be highly heterozygous (Carlson et al. 1991) and many polymorphic loci are expected to segregate in the progeny. However, the AFLP technique provided a satisfactory rate of polymorphic markers per primer tested, comparable to that observed in other such studies on conifer genomes (Paglia et al. 1998; Travis et al. 1998). The lack of polymorphism in the case of RAPD markers could then be attributed to a non-optimal protocol rather than to a biological cause, such as a reduced heterozygosity of the parents or a low genetic divergence between species.

The AFLP technique was successfully applied to the genetic mapping of larch. One main advantage of this technique is the simultaneous recovery of numerous polymorphic markers (12–41 in our experiment) in the same assay, which makes it much more efficient than RAPDs. Moreover, AFLP markers are more reliable and reproducible over time as PCR reactions are carried out under stringent conditions (Vos and Kuiper 1997). However, a modification of our AFLP protocol is still required in order to decrease the number of bands and improve their readability. Vos and Kuiper (1997) have suggested to limit the number of amplified bands to 50–100. This can be achieved by developing more restrictive primers by adding one or two selective nucleotides to the ends of the primers.

ISSR primers are easy to design as they require no prior knowledge of sequence. Their likely advantage over RAPD markers is a greater repeatability due to ISSR primer length (Kojima et al. 1998) and a more stringent temperature at the annealing step. In our experiment, ISSRs produced few polymorphic markers. However, we assayed only 11 primers and many other ones are available (Tsumura et al. 1996). Moreover, the separation of ISSR fragments on non-denaturing polyacrylamide gels instead of agarose gels could enhance the resolution of ISSR bands. Another interest of this type of marker lies in its linkage to SSR loci. Indeed, microsatellites were shown to be linked to coding regions (Echt et al. 1996), so that ISSRs are susceptible to mark gene-rich regions (Kojima et al. 1998).

We observed a good homogeneity between maps of European and Japanese larch, regarding the respective number and distribution of markers, the number of linkage groups and genome length. Although the map length covered by markers reaches 80% of the estimated genome size, the number of linkage groups still exceeds the number of chromosomes (n=12 for larch), and additional markers are needed to merge them. A good distribution of markers and no evident cluster by type of marker was noticed.

The estimated length of the larch genome is in agreement with previous genetic mapping studies of conifers. Indeed, large genome sizes of 2500 cM were reported for pines (Neale and Williams 1991), which can be ascribed to a high content of repetitive DNA. Both maps of larch showed one large linkage group of respectively 251 cM and 225 cM, which was found to be homologous between species, as revealed by a RAPD marker heterozygous in both parents. Such long linkage groups are not frequently observed in other genetic maps of conifers. However, genetic mapping studies already described linkage groups reaching 200 cM for Scots pine (Yazdani et al. 1995), Pinyon pine (Travis et al. 1998) and Norway spruce (Paglia et al. 1998). Moreover, the consistency of the groups was supported by a high LOD threshold in both larch maps.

The Japanese parent results from a cross between full-sib individuals and, consequently, was expected to show less polymorphism detectable by genetic markers. This was not observed and it suggested that this individual was not affected by a significant loss of heterozygosity after inbreeding.

The application of the pseudo-testcross strategy proved to be efficient in a relatively short period of time to construct genetic maps of European larch and Japanese larch showing a good coverage of the genome. This strategy is suited to allogamous species where individuals show a high degree of heterozygosity so that many markers are expected to be in the appropriate configuration, i.e. one allele heterozygous in one parent and null in the other one, in the case of dominant markers. Another advantage of the pseudo-testcross strategy lies in the exploitation of a two-generation pedigree, which is a currently available pedigree in forest tree species (Carlson et al. 1991; Grattapaglia et al. 1994; Kubisiak et al. 1995; Verhaegen and Plomion 1996). These maps are individual-specific since most dominant markers are not transposable between maps. Nevertheless, the use of genetic maps for QTL detection first requires evenly spaced markers over the genome, and RAPD and AFLP techniques offer the opportunity to produce enough markers to reach this goal. QTL detection can be

◀ **Fig. 4** Linkage map of *L. kaempferi*. Names of framework markers are indicated on the *right* of the linkage groups. RAPD and ISSR loci are designated by primer identity followed by the molecular weight (in bp) of the fragment. AFLP loci are indicated by the primer combination used to detect the marker, followed by a *number* indicating the fragment position on the gel (ranking begins with high-molecular-weight fragments). The linkage phase of loci is indicated by + or –. Markers that could not be mapped with a log-likelihood support ≥2.0 are represented as accessory markers to the *right* of the nearest framework marker. Distorted markers ($P<0.05$) are shown in *italics*. Markers heterozygous in both parents and present in both maps are shown in *bold type*. Genetic distances in cM using the Kosambi's mapping function are indicated on the *left* of the linkage groups. The length of each linkage group is shown below the group

achieved with dominant markers, even if their informativeness is reduced.

The precision of the information can be refined by the placement of multi-allelic co-dominant markers as microsatellites (Echt and Nelson 1997; Brondani et al. 1998; Paglia et al. 1998) or sequence-tagged-site (STS) markers (Perry and Bousquet 1998), which could lead to the construction of a unified map and allow comparisons, between experiments, of regions associated with trait expression.

The present maps of European and Japanese larch are the first reported ones in *Larix* species. They provide a basic tool for identifying and localizing QTLs. The results of further QTL studies will provide a new insight into the genetic architecture of complex traits such as growth or wood-quality parameters. These maps also provide a foundation for comparative mapping between coniferous species, provided that multi-allelic markers well-conserved among coniferous genomes are developed.

Acknowledgements We acknowledge A. Charcosset, D. Crouzillat, E. Dirlewanger, J. Gellin, H. Joly, C. Plomion, E. Teissier du Cros for helpful discussions during the Ph.D. training of A.A. We are grateful to I. Couée for his critical review of and suggestions on the manuscript. The experiments carried out in this study comply with the current laws of France.

References

Arcade A, Faivre Rampant P, Le Guerroué B, Pâques LE, Prat D (1996) Heterozygosity and hybrid performance in larch. Theor Appl Genet 93:1274–1281

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

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

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D (1998) Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *E. urophylla*. Theor Appl Genet 97:816–827

Carlson JE, Tulsieram LK, Glaubitz JC, Luk VWK, Kauffeldt C, Rutledge R (1991) Segregation of random amplified DNA markers in F_1 progeny of conifers. Theor Appl Genet 83: 194–200

Chakravati A, Lasher LA, Reefer JE (1991) A maximum-likelihood method for estimating genome length using genetic linkage data. Genetics 128:175–182

Devey ME, Fiddler TA, Liu B-H, 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

Devey ME, Bell JC, Smith DN, Neale DB, Moran GF (1996) A genetic linkage map for *Pinus radiata* based on RFLP, RAPD, and microsatellite markers. Theor Appl Genet 92:673–679

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

Echt CS, May-Marquardt P, Hseih M, Zahorchak R (1996) Characterization of microsatellite markers in eastern white pine. Genome 39:1102–1108

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

Hulbert SH, Ilott TW, Legg EJ, Lincoln SE, Lander ES, Michelmore RW (1988) Genetic analysis of the fungus, *Bremia lactucae*, using restriction fragment length polymorphisms. Genetics 120:947–958

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van de Wiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A, Karp A (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol Breed 3:381–390

Kojima T, Nagaoka T, Noda K, Ogihara Y (1998) Genetic linkage map of ISSR and RAPD markers in einkorn wheat in relation to that of RFLP markers. Theor Appl Genet 96:37–45

Kubisiak TL, Nelson CD, Nance WL, Stine M (1995) RAPD linkage mapping in a longleaf pine×slash pine F_1 family. Theor Appl Genet 90:1119–1127

Lincoln S, Daly M, Lander E (1992) Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical Report, 3rd edn

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

Nagaoka T, Ogihara Y (1997) Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor Appl Genet 94:597–602

Neale DB, Williams CG (1991) Restriction fragment length polymorphism mapping in conifers and applications to forest genetics and tree improvement. Can J For Res 21:545–554

Nelson CD, Nance WL, Doudrick RL (1993) A partial genetic linkage map of slash pine (*Pinus elliottii* Engelm. var. elliottii) based on random amplified polymorphic DNAs. Theor Appl Genet 87:145–151

Paglia GP, Olivieri 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

Pâques LE (1989) A critical review of larch hybridization and its incidence on breeding strategies. Ann Sci For 46:141–153

Pâques LE (1992) First evaluation of genetic parameters in a factorial mating design with hybrid larch (*Larix decidua*×*Larix kaempferi*). In: Weisgerber H (ed) Results and future trends in larch breeding on the basis of provenance research. IUFRO Centennial Meeting of the IUFRO Working Party. S2.02–07, pp 136–145

Perry DJ, Bousquet J (1998) Sequence-tagged-site (STS) markers of arbitrary genes: the utility of black spruce-derived STS primers in other conifers. Theor Appl Genet 97:735–743

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

Smith DN, Devey ME (1994) Occurrence and inheritance of microsatellites in *Pinus radiata*. Genome 37:977–983

Suiter KA, Wendel JF, Case JS (1983) LINKAGE-1: a PASCAL computer program for the detection and analysis of genetic linkage. J Hered 74:203–204

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

Tsumura Y, Ohba K, Strauss SH (1996) Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). Theor Appl Genet 92:40–45

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

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

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

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

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

Yazdani R, Yeh FC, Rimsha J (1995) Genomic mapping of *Pinus sylvestris* (L.) using random amplified polymorphic DNA markers. For Genet 2:109–116

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183