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Original article

Towards construction of an ultra high density linkage map for *Pinus pinaster*

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Abstract – Two parental linkage maps have been constructed from the *P. pinaster* reference population (0024 × C803) based on AFLP, SSR and EST markers. Although segregating polymorphism was low due to a high degree of homozygosity in the parents, 12 linkage groups with 26 to 46 markers each were obtained for each parent. The availibility of 70 anchor points based on fragments common to both parents and based on co-dominant SSR and EST markers allowed to determine homologous chromosomes for both maps and to construct one integrated map. Total genome length of the integrated map is around 2000 cM including 1182 markers. Since some of the EST and SSR markers are also mapped in different pine species, association of linkage groups of our reference population with those of other published maps was possible.

AFLP, SSR, EST markers / genetic mapping

Résumé – Vers la construction d'une carte génétique ultra-haute densité chez *Pinus pinaster*. Deux cartes génétiques ont été construites à partir d'un croisement intra-spécifique de *P. Pinaster* (0024 Landes × C803 Corse) avec des marqueurs AFLP, SSR et EST. Malgré un faible polymorphisme dû à la forte homozygotie des parents, 12 groupes de liaison comprenant 26 à 46 marqueurs ont été obtenus pour chacune des cartes. La présence de 70 points d'ancrage déterminés à partir des fragments communs aux deux parents a permis d'identifier les chromosomes homologues des deux cartes et de construire une carte consensus d'une longueur totale d'environ 2000 cM et comprenant 1182 marqueurs. La présence de quelques marqueurs EST et microsatellites déjà cartographiés chez différentes espèces de pins a permis d'aligner un certain nombre de groupes de liaison avec cette carte de pin maritime.

marqueurs AFLP, SSR, EST / cartographie génétique

1. INTRODUCTION

Several linkage maps have been produced in a variety of forest species including *Pinus*. They are based on different marker types such as RFLPs in *Pinus taeda* [6, 19] and RAPDs in *Pinus pinaster* [4] and *Pinus radiata* [7]. Also AFLP maps are available for *Pinus pinaster* [4] , *P. radiata* [2] and *Pinus edulis* [22]. Recently a high-density map of *P. pinaster* has been constructed [5]. Other marker types such as proeins and isozymes are integrated in these maps [4, 6, 15] as well as EST [3] and SSR markers [8] which may be useful for aligning different maps.

In order to apply DNA marker technology in breeding of coniferous species a project has been initiated with the aim of constructing an ultra-high-density linkage map (UHD map) of *Pinus pinaster* based on several thousands AFLP markers and numerous published microsatellites (SSR). The reference map will be used for comparative genome and QTL analyses in different genetic backgrounds. It is the aim to align other

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published linkage maps in forest species with this reference map. Based on a reduced number of markers, comparative genome and QTL analyses will be performed in different pine species and related gymnosperms.

In this paper we present the first project results, which consist of an integrated linkage map derived from two *P. pinaster* genotypes and some associations of linkage groups of our map with those of different pine species.

2. MATERIALS AND METHODS

2.1. Plant material

The F1 reference population of *P. pinaster* descended from the cross 0024 Landes × C803 Corsica. The parental trees had been selected in cooperation by INRA-Pierroton and AFOCEL SW research stations. The cross was performed in 1987 and the 129 resulting progeny trees were established in the field by AFOCEL in 1990. A total of 80 out of the 129 progeny genotypes, established in the AFOCEL experimental stands of Troussas and Arsague (Landes; SW, France), were used for linkage mapping.

2.2. Molecular methods

Genomic DNA was extracted from needles using the DNeasy Plant Kit from Qiagen with slight modifications of the supplier's protocol.

AFLP analysis was performed according to [23] using *Eco*RI/*Mse*I adapters. Preamplification was performed with one selective nucleotide and specific amplification with 3 selective nucleotides (+1/+3 amplification). Also +2/+4 amplifications were performed. Amplification products were separated on 6 or 8% denaturing polyacrylamide gels. Different techniques were used for detecting amplification products. AFLP fragments were detected on a LI-COR 4200-S1 DNA sequencer using primers labelled with the fluorescent infrared dye IRD800 (LI-COR, Lincoln, Nebraska, USA) or on a ALFexpressII (Amersham Pharmacia Biotech, Germany) with Cy5-Amidite labelled primers (MWG-Biotech, Germany). Analysis was performed according to the manufacturer instructions in each case.

SSR primers developed in different species were analyzed for polymorphism and segregation. SSR developed in *Pinus pinaster* and *P. halepensis* [13] in *P. strobus* ([9, 10]; http://dendrome.ucdavis.edu/Data/echt_ssr_primers.html), in *P. radiata* and *P. sylvestris* ([11, 20, 21]; http://dendrome.ucdavis.edu/Data/hardssr.html) were used for this purpose. Furthermore, several other as yet unpublished SSR primers from *Pinus radiata* were obtained from Gavin Moran (CSIRO, Australia) and from Craig Echt (Forest Research Inc., New Zealand) and from *Picea abies* from Giovanni Vendramin (IMGPF, Italy). SSR analysis was performed as described by [13] or based on the information given in the mentioned WEB pages. EST primers were obtained from the information provided on http://www.pierroton.inra.fr/genetics/pinus/primers.htm. EST analysis was performed according to [12].

2.3. Data analysis and linkage mapping

Polymorphic DNA fragments were scored for presence or absence in parents and F1 progenies. Linkage analysis between marker fragments, estimation of recombination frequencies, and determination of linear order between linked loci including multipoint linkage analysis and the EM algorithm for handling missing data were performed as described in [16, 17]. The MAPRF program [17] was applied for the computational methods. Firstly, linkage groups were constructed based on fragments specific to either parent. Linked fragments were arranged into linkage groups using a minimum, commonly accepted LOD threshold of 3.0 between consecutive markers. Subsequently, fragments common to both parents were integrated into linkage groups as anchor points as described in [16]. Only common markers linked with recombination frequencies of zero to at least one parent (LOD > 6) and linked with a minimum LOD threshold of 3.0 to the other parent were considered for this purpose.

3. RESULTS

3.1. Generation of polymorphic DNA markers

Nearly 300 different primer combinations (PCs) were analyzed for the generation of AFLP-fragments. More than 100 fragments were often produced with specific primer combinations, with from one to 25 segregating fragments in the mapping population. However, some primer combinations produced no segregating fragments. This was generally the case for primer combinations with high AT contents. In general, better quality of gels were obtained with the +2/+4 amplification system.

A total of 239 AFLP primer combinations were used for the molecular analyses and generated 1740 segregating fragments. Thus on average, 7.3 polymorphic bands per primer combination were obtained. Approximately 39% of the segregating fragments were specific for either one parent of the cross, while 22% of the fragments were present in both parents. Around 16% of the fragments showed significant deviations ($\alpha > 5\%$) from the expected segregation ratios.

Furthermore, a total of 120 SSR and 30 EST primer pairs were used in this study. Amplification products were obtained in most cases after adapting the particular PCR conditions in each case. However, as with AFLP markers, a low degree of polymorphism between parental alleles together with a large degree of homozygosity (i.e. non segregating polymorphic fragments) was observed. Only 21 SSR and 10 EST markers showed one or more segregating bands.

3.2. Construction of linkage maps

Initially, individual linkage maps of 12 linkage groups each were obtained for the two parents of the mapping population. Their characteristics are summarised in *table I*. Details of the maps, parental AFLP profiles as well as the obtained polymorphisms are displayed on the project WEB page (http://www.neiker.net/UHDfor). Linkage groups of the P1 map (parent 0024) contained 26 to 46 individual and common markers each and were between 107.8 and 180.1 cM in length. The total P1 map length (female parent 0024) was

Table I. Characteristics of the *P. pinaster* maps from the cross 0024 × C803.

	P1 – MAP (0024)				P2 – MAP (C803)				Integrated Map				
LG	Length	IM	CM	TM	Length	IM	CM	TM	Length	IM	CM	TM	AP
1	160.4	35	9	44	173.3	27	10	37	175.6	62	12	74	7
2	153.2	28	12	40	171.1	28	10	38	168.9	56	13	69	9
3	131.8	26	9	35	174	25	6	31	176.5	51	9	60	6
4	151.2	27	10	37	190.5	23	7	30	175	50	11	61	6
5	146.3	27	8	35	187.3	30	7	37	179.3	57	11	68	4
6	107.8	20	6	26	153.7	19	4	23	151.5	39	6	45	4
7	121.5	24	7	31	116.7	23	6	29	123.2	47	8	55	5
8	135.3	22	7	29	130.5	22	5	27	142.5	44	8	52	4
9	180.1	38	8	46	181.5	27	8	35	191	65	10	75	6
10	148.7	33	7	40	115.1	24	7	31	156.5	57	9	66	5
11	149.2	27	8	35	175.1	24	12	36	182.8	51	13	64	7
12	150.3	28	7	35	172.7	32	9	41	171.4	60	10	70	6
Total:	1735.8	335	98	433	1941.5	304	91	395	1994.2	639	120	759	70
Mean:	144.7	27.9	8.2	36.1	161.8	25.3	7.6	32.9	166.2	53.3	10	63.3	5.8

+ 217 markers linked with recombination frequencies of zero to other markers
+ 206 associated fragments: Total Markers: 1182

Legend:
LG = linkage group; IM = individual markers (parent specific); CM = markers common to both parents; TM = total number of markers for linkage group; AP = number of anchor points.

1736 cM. The P2 map (male parent C803) was 1942 cM in length and made up of linkage groups with 23 to 41 markers each. The size of the linkage groups varied between 115.1 and 190.5 cM.

Further 217 fragments were linked with recombination frequencies of zero to other fragments in these linkage maps and have not been not considered in these counts.

Linkage to mapped markers on linkage groups was apparent for 206 additional markers. However, these were highly distorted or consisted of common fragments linked to other fragments with reduced LOD values and could not be placed in a single interval with high certainty. Since the standard errors of the estimates of the recombination frequencies were high, these 206 markers are included in this map as so called "associated markers", anchored to the marker with the highest probability.

Based on the integration of 70 markers common to both parents and codominant markers like SSRs and ESTs into linkage groups for both parents, it was possible to assign all 12 homologous chromosomes for P1 and P2, and to obtain in this way an integrated consensus map with a total of 759 markers (*table I, figure 1*). Linkage groups of the integrated map varied between 123.2 and 191 cM in length and contained between 45 and 74 markers each. Considering the 217 markers linked without recombination to other displayed markers and the 206 associated markers, an integrated map of 1182 markers was achieved with an average of 99 markers per linkage group.

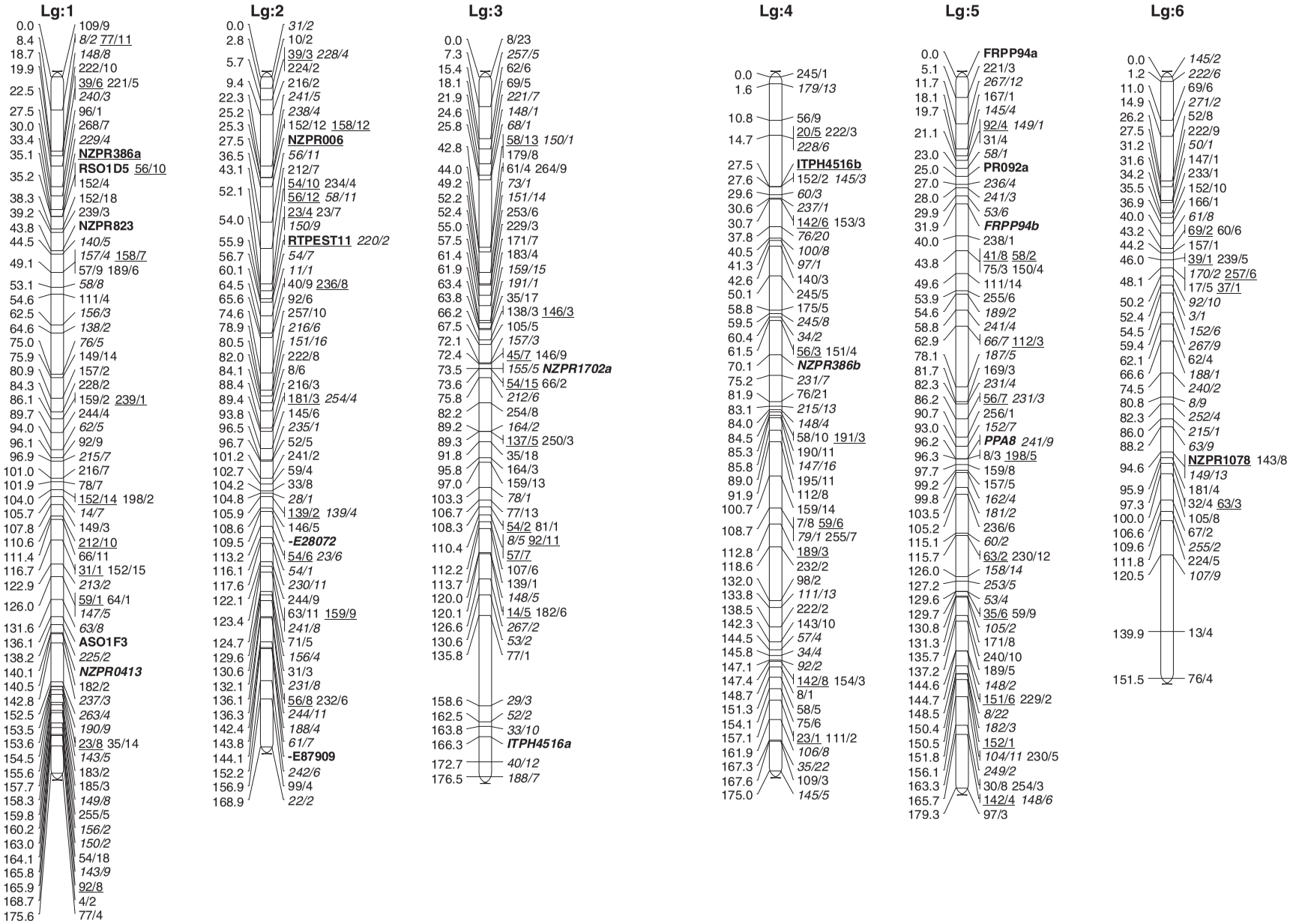
3.3. Associations of linkage groups between the reference map and other published maps

The SSR and EST markers amplified one or two loci each with variable number of alleles. A total of 14 SSR (19 loci) and 7 EST markers (7 loci) could be placed on the reference map (*figure 1*). Since some of them were also mapped in other pine species, an association of several linkage groups from our reference population with those of other published maps was possible. The summarized results are shown in *table II*.

4. DISCUSSION

4.1. The generation of segregating polymorphic DNA markers

The pine genome is known to be relatively large and contains large amounts of repetitive elements [24]. Thus a highly increased number of AFLP amplification products can be expected. It is also well known that increased AT contents in the selective nucleotides leads to a higher number of amplification products. However, the resolution of the gel is limited so that different amplification products may comigrate, hiding in this way possible segregating polymorphisms. Therefore using PCs with lower AT content and/or increasing the number of selective nucleotides in the primers to 4, potentially results in less amplification products, but in higher segregating polymorphisms of variable number of bands with good quality.


Lg:1
0.0 109/9
8.4 8/2 77/11
18.7 148/8
19.9 222/10
22.5 39/6 221/5
240/3
27.5 96/1
30.0 268/7
33.4 229/4
35.1 NZPR386a
RSO1D5 56/10
35.2 152/4
38.3 152/18
39.2 239/3
43.8 NZPR823
44.5 140/5
49.1 157/4 158/7
57/9 189/6
53.1 58/8
54.6 111/4
62.5 156/3
64.6 138/2
75.0 76/5
75.9 149/14
80.9 157/2
84.3 228/2
86.1 159/2 239/1
89.7 244/4
94.0 62/5
96.1 92/9
96.9 215/7
101.0 216/7
101.9 78/7
104.0 152/14 198/2
105.7 14/7
107.8 149/3
110.6 212/10
111.4 66/11
116.7 31/1 152/15
122.9 213/2
126.0 59/1 64/1
147/5
131.6 63/8
136.1 ASO1F3
138.2 225/2
140.1 NZPR0413
140.5 182/2
142.8 237/3
152.5 263/4
153.5 190/9
153.6 23/8 35/14
154.5 143/5
155.6 183/2
157.7 185/3
158.3 149/8
159.8 255/5
160.2 156/2
163.0 150/2
164.1 54/18
165.8 143/9
165.9 92/8
168.7 4/2
175.6 77/4
Lg:2
0.0 31/2
2.8 10/2
5.7 39/3 228/4
224/2
9.4 216/2
22.3 241/5
25.2 238/4
25.3 152/12 158/12
27.5 NZPR006
36.5 56/11
43.1 212/7
52.1 54/10 234/4
56/12 58/11
54.0 23/4 23/7
150/9
55.9 RTPEST11 220/2
56.7 54/7
60.1 11/1
64.5 40/9 236/8
65.6 92/6
74.6 257/10
78.9 216/6
80.5 151/16
82.0 222/8
84.1 8/6
88.4 216/3
89.4 181/3 254/4
93.8 145/6
96.5 235/1
96.7 52/5
101.2 241/2
102.7 59/4
104.2 33/8
104.8 28/1
105.9 139/2 139/4
108.6 146/5
109.5 -E28072
113.2 54/6 23/6
116.1 54/1
117.6 230/11
122.1 244/9
123.4 63/11 159/9
241/8
124.7 71/5
129.6 156/4
130.6 31/3
132.1 231/8
136.1 56/8 232/6
136.3 244/11
142.4 188/4
143.8 61/7
144.1 -E87909
152.2 242/6
156.9 99/4
168.9 22/2
Lg:3
0.0 8/23
7.3 257/5
15.4 62/6
18.1 69/5
21.9 221/7
24.6 148/1
25.8 68/1
42.8 58/13 150/1
179/8
44.0 61/4 264/9
49.2 73/1
52.2 151/14
52.4 253/6
55.0 229/3
57.5 171/7
61.4 183/4
61.9 159/15
63.4 191/1
63.8 35/17
66.2 138/3 146/3
67.5 105/5
72.1 157/3
72.4 45/7 146/9
73.5 155/5 NZPR1702a
73.6 54/15 66/2
75.8 212/6
82.2 254/8
89.2 164/2
89.3 137/5 250/3
91.8 35/18
95.8 164/3
97.0 159/13
103.3 78/1
106.7 77/13
108.3 54/2 81/1
8/5 92/11
110.4 57/7
112.2 107/6
113.7 139/1
120.0 148/5
120.1 14/5 182/6
126.6 267/2
130.6 53/2
135.8 77/1
158.6 29/3
162.5 52/2
163.8 33/10
166.3 ITPH4516a
172.7 40/12
176.5 188/7
Lg:4
0.0 245/1
1.6 179/13
10.8 56/9
14.7 20/5 222/3
228/6
27.5 ITPH4516b
27.6 152/2 145/3
29.6 60/3
30.6 237/1
30.7 142/6 153/3
37.8 76/20
40.5 100/8
41.3 97/1
42.6 140/3
50.1 245/5
58.8 175/5
59.5 245/8
60.4 34/2
61.5 56/3 151/4
70.1 NZPR386b
75.2 231/7
81.9 76/21
83.1 215/13
84.0 148/4
84.5 58/10 191/3
85.3 190/11
85.8 147/16
89.0 195/11
91.9 112/8
100.7 159/14
108.7 7/8 59/6
79/1 255/7
112.8 189/3
118.6 232/2
132.0 98/2
133.8 111/13
138.5 222/2
142.3 143/10
144.5 57/4
145.8 34/4
147.1 92/2
147.4 142/8 154/3
148.7 8/1
151.3 58/5
154.1 75/6
157.1 23/1 111/2
161.9 106/8
167.3 35/22
167.6 109/3
175.0 145/5
Lg:5
0.0 FRPP94a
5.1 221/3
11.7 267/12
18.1 167/1
19.7 145/4
21.1 92/4 149/1
31/4
23.0 58/1
25.0 PR092a
27.0 236/4
28.0 241/3
29.9 53/6
31.9 FRPP94b
40.0 238/1
43.8 41/8 58/2
75/3 150/4
49.6 111/14
53.9 255/6
54.6 189/2
58.8 241/4
62.9 66/7 112/3
78.1 187/5
81.7 169/3
82.3 231/4
86.2 56/7 231/3
90.7 256/1
93.0 152/7
96.2 PPA8 241/9
96.3 8/3 198/5
97.7 159/8
99.2 157/5
99.8 162/4
103.5 181/2
105.2 236/6
115.1 60/2
115.7 63/2 230/12
126.0 158/14
127.2 253/5
129.6 53/4
129.7 35/6 59/9
130.8 105/2
131.3 171/8
135.7 240/10
137.2 189/5
144.6 148/2
144.7 151/6 229/2
148.5 8/22
150.4 182/3
150.5 152/1
151.8 104/11 230/5
156.1 249/2
163.3 30/8 254/3
165.7 142/4 148/6
179.3 97/3
Lg:6
0.0 145/2
1.2 222/6
11.0 69/6
14.9 271/2
26.2 52/8
27.5 222/9
31.2 50/1
31.6 147/1
34.2 233/1
35.5 152/10
36.9 166/1
40.0 61/8
43.2 69/2 60/6
44.2 157/1
46.0 39/1 239/5
48.1 170/2 257/6
17/5 37/1
50.2 92/10
52.4 3/1
54.5 152/6
59.4 267/9
62.1 62/4
66.6 188/1
74.5 240/2
80.8 8/9
82.3 252/4
86.0 215/1
88.2 63/9
94.6 NZPR1078 143/8
149/13
95.9 181/4
97.3 32/4 63/3
100.0 105/8
106.6 67/2
109.6 255/2
111.8 224/5
120.5 107/9
139.9 13/4
151.5 76/4

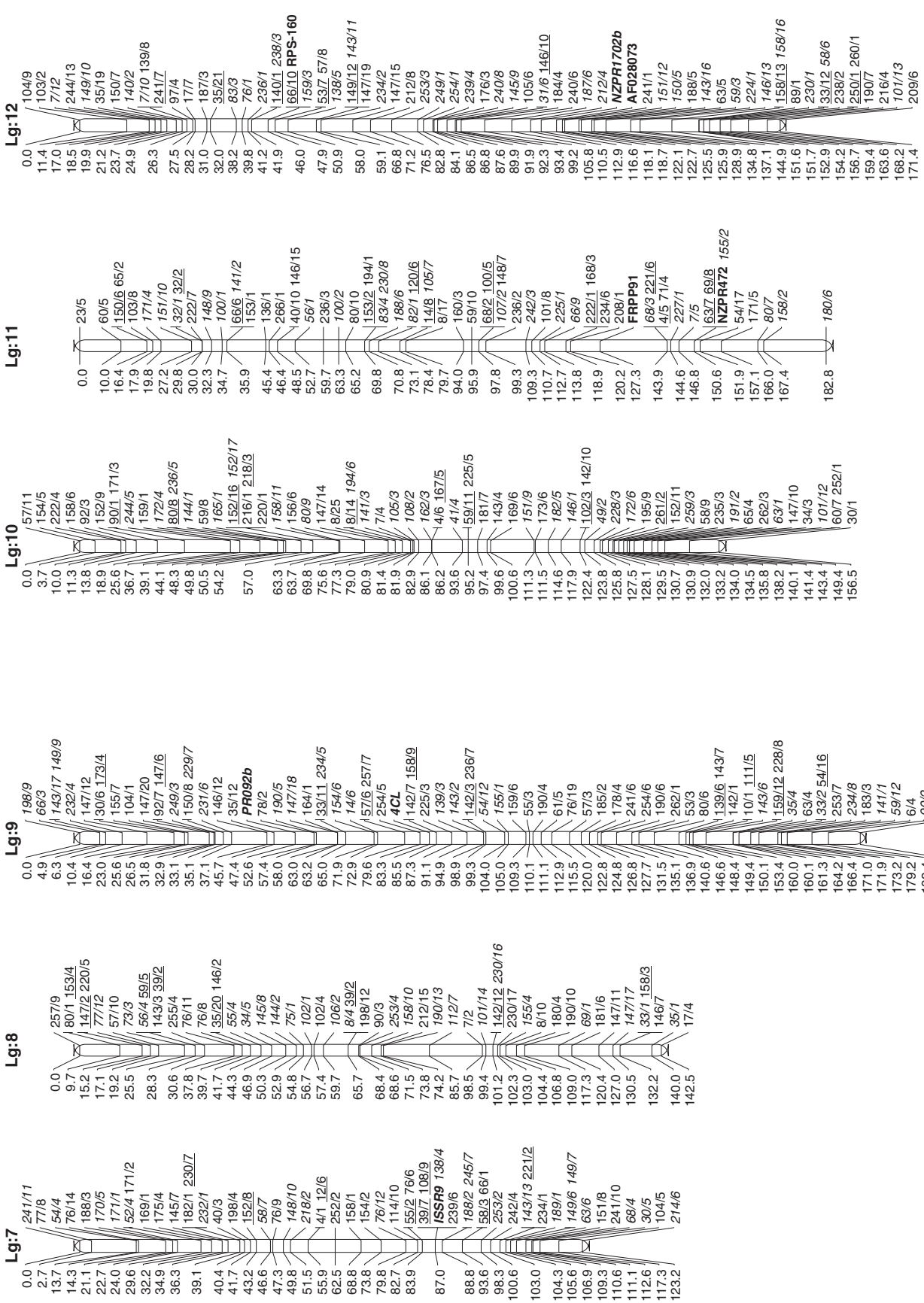


Figure 1. Integrated map of *Pinus pinaster* Cross: 0024 × C803. Positions in cM [Kosambi units] are given on the left of the linkage group bars. Marker names and fragment numbers on the right of the bar. Markers common to both parents are underlined. Most of them represent anchor points. EST and SSR markers are indicated in bold. For details on marker names and fragment numbers including parental AFLP profiles see http://www.neiker.net/UHDfor.

Table II. Locations of mapped SSR and EST markers in our *P. pinaster* reference map and in other published *Pinus* maps.

No	Name	Type	Origin	Location in reference map	Location in other published maps
1	4CL	EST	*P. taeda*	Lg 9	*P. abies* Lg6 (1)
2	AFO28073	EST	*P. taeda*	Lg 12	*P. pinaster* Lg8 (2)
3	ASO1F3	EST	*P. pinaster*	Lg 1	*P. pinaster* Lg4 (2) *P. abies* Lg4 (1)
4	E28072	EST	*P. taeda*	Lg 2	*P. pinaster* Lg5 (2)
5	E87909	EST	*P. taeda*	Lg 2	*P. pinaster* Lg8 (2)
6	PPA8	EST	*P. pinaster*	Lg 5	*P. pinaster* Lg10 (2)
7	RSO1D5	EST	*P. pinaster*	Lg 1	*P. pinaster* Lg7 (2)
8	FRPP91	SSR	*P.pinaster*	Lg 11	*P. pinaster* Lg9 (3)
9	FRPP94a/b	SSR	*P.pinaster*	Lg 5/Lg 5	*P. pinaster* Lg5 (3)
10	ISSR9	ISSR		Lg 7	–
11	ITPH4516a/b	SSR	*P.halepensis*	Lg 3/Lg 4	*P. pinaster* Lg3 (3)
12	NZPR0413	SSR	*P. radiata*	Lg 1	*P. pinaster* Lg4 (2) *P. radiata* Lg4 (4)
13	NZPR1702a/b	SSR	*P. radiata*	Lg 12/Lg 3	*P. pinaster* *Lg8 / Lg 11* (2) *P. radiata* Lg10 (4)
14	NZPR1078	SSR	*P. radiata*	Lg 6	*P. pinaster* Lg2 (2) *P. radiata* Lg2 (4)
15	NZPR386a/b	SSR	*P. radiata*	Lg 1/Lg 4	*P. radiata* Lg2 (4)
16	NZPR472	SSR	*P. radiata*	Lg 11	*P. pinaster* Lg1 (2) *P. radiata* Lg1 (4)
17	NZPR006	SSR	*P. radiata*	Lg 2	*P. radiata* Lg5 (4)
18	NZPR823	SSR	*P. radiata*	Lg 1	*P. pinaster* Lg5 (2) *P. radiata* Lg5 (4)
19	PR092a/b	SSR	*P. radiata*	Lg 5/ Lg 9	*P. radiata* Lg3 (5)
20	RPS-160	SSR	*P.strobus*	Lg 12	–
21	RTPEST11	SSR	*P. taeda*	Lg 2	*P. pinaster* Lg5(2)

(1) http://www.pierroton.inra.fr/genetics/Picea/
(2) http://www.pierroton.inra.fr/genetics/pinus/primers.html and Chaumeil P., Développement de marqueurs hypervariables (microsatellites) chez le pin maritime (*Pinus pinaster* Ait.) et applications en génétique, 2001, DEA Biologie Forestière, Université de Nancy (several markers are only cited in the DEA but will be published on this web site).
(3) Mariette et al., 2001.
(4) *P. radiata* map aligned with *P. taeda* reference population [1]; Phil Wilcox and Craig Echt, personal communication.
(5) Devey et al., 1999.

Independently of these findings, the unexpected low degree of polymorphism of AFLP, SSR and EST markers observed in our progeny is surprising considering the well marked differentiation between the original provenances of the parents [14] and the similar level of genetic diversity encountered in *P. pinaster* and other *Pinus* species [9, 20]. Many polymorphic fragments exist between the parents of our mapping population, which represent different ecotypes from Landes and Corsica, respectively. However, a large degree of homozygosity exists, since parent specific fragments do not segregate. This increased homozygosity is probably due to a low degree of biodiversity, which exist at the specific sites (i.e., trees are quite different between sites but very similar within a site).

4.2. Arrangement of DNA markers into linkage maps

The analysis of segregating DNA markers established twelve independent linkage groups for the *P. pinaster* genotypes 0024 and C803, respectively (i.e., lateral markers were not statistically linked to any other lateral marker of any other linkage group). These 12 linkage groups may correspond to the 12 chromosomes of the haploid pine genome (2n = 2x = 24). Moreover, the presence of common markers made it possible to identify all homologous chromosomes in each parent. With several common markers present in the same order on chromosomes of both parents, it is possible to combine the information of markers from different individuals as described in [17]. In this way the number of markers available per chromosome can be increased.

The total length of linkage maps did not differ between the parents of the mapping population and is in agreement with other linkage maps obtained in this species. Our *P. pinaster* reference map represents one of the maps with the highest number of markers in forest species.

4.3. Alignment with other *Pinus* maps

Alignment between different linkage maps can be achieved, if identical markers have been used in these maps

and if comigrating bands map to identical positions. SSR and EST markers are mainly codominant, highly polymorphic and represent powerful tools for different genetic analyses. Since they seem to be conserved among species and to a certain degree also within families, they have been used for mapping and alignment of linkage maps in several forest species [1, 3, 8, 13]. We have evaluated numerous SSR and EST markers in our study and several could be used to associate linkage groups in different parents (*table II*). However, the low level of polymorphism of EST and SSR markers observed in our reference population has led to association of linkage groups between maps. Since this goal is crucial for the usefulness of our map, additional SSR/EST primers will be evaluated in order to achieve a complete alignment.

Alignments between maps were achieved also with comigrating AFLP markers in potato, involving different *Solanum* species [18]. However, it will be necessary to prove if this is also possible for pine species by comparing parental profiles and map locations of comigrating fragments from AFLP primer combinations which have been used in different mapping populations.

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