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ORIGINAL PAPER

Comparative genome mapping among *Picea glauca*, *P. mariana* × *P. rubens* and *P. abies*, and correspondence with other Pinaceae

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Abstract A composite linkage map was constructed from four individual maps for the conifer *Picea glauca* (Moench) Voss, from anonymous and gene-specific markfers (714 AFLPs, 38 SSRs, and 53 ESTPs). A total of 12 linkage groups were delineated with an average marker density of 2.7 cM. Macro-synteny and macro-colinearity comparisons with two other composite linkage maps developed for the species complex *P. mariana* (Mill.) B.S.P. × *P. rubens* Sarg., and for *P. abies* (L.) Karst. revealed an identical number of linkage groups and a remarkable conservation of the gene content and gene order of linkage groups over the million years since the split between these taxa. Identical gene order among taxa was observed for 10 of the 12 assembled composite linkage groups. The discovery of one breakdown in synteny between *P. glauca* and the other two taxa indicated the occurrence of an inter-chromosomal rearrangement involving an insertional translocation. Analysis of marker colinearity also revealed a putative segmental duplication. The combined information from these three *Picea* genomes validated and improved large-scale genome comparisons at the inter-generic level in the family Pinaceae by allowing for the identification of 11 homoeologous linkage groups between *Picea* and *Pinus*, and nine such groups between *Picea* and *Pseudotsuga menziesii*. Notably, the analysis of synteny among the three genera revealed a putative case of chromosomal fission and an inter-chromosomal rearrangement in the genome of *P. menziesii*. Both of these changes are inter-connected, indicating much instability in this part of the *P. menziesii* genome. Overall, the macro-structure of the Pinaceae genome was well conserved, which is notable given the Cretaceous origin of its main lineages.

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Introduction

Comparative mapping highlights similarities and differences in the genome organization of species and can lead to concrete applications in the domains of genome evolution and quantitative genetics (Nadeau and Sankoff 1998; Paterson et al. 2000). By the discovery of genetic processes such as duplication, deletion, insertion, inversion or translocation of genes, comparative mapping helps us to achieve a better understanding of chromosomal evolution (Ahn and Tanksley 1993; Paterson et al. 2000; Ma et al. 2005). The detection of chromosomal rearrangements may provide answers to

hypotheses concerning speciation events (Nadeau 1989; Theiβen 2005). The identification of homoeologous segments between genomes of different species can also lead to information transfer of well-studied model species to related large-genome species, thus accelerating research progress in the latter ones (Lan and Paterson 2000).

In the absence of complete genome sequences and physical maps, comparative studies of genomes can be achieved through the comparison of composite linkage maps (Foulongne et al. 2003). This is especially true for taxa harboring large genomes such as in the Poaceae (Bowers et al. 2003) and in the Pinaceae (Krutovsky et al. 2004; Pelgas et al. 2005), and where whole genome sequencing is still financially prohibitive. In this context, the development of composite linkage maps based on a large number of orthologous anchor markers becomes essential to obtain a more detailed view of whole-genome organization and thus to enable the identification and comparison of homoeologous linkage groups between more or less related taxa (Gaut 2002; Schmidt 2002). Two parameters are usually considered in comparative mapping studies among different taxa: (1) synteny, which involves the conservation of the gene content between linkage groups among taxa, leading to homoeology of linkage groups, and (2) colinearity, which corresponds to the conservation of the gene order between these homoeologous linkage groups among taxa (e.g., Gale and Devos 1998; Paterson et al. 2000; Gaut 2001).

The orthology of markers used for comparisons of homoeologous linkage groups is essential to avoid erroneous conclusions about chromosomal evolution. Such erroneous inferences could occur when marker homoplasy cannot be ruled out such as for anonymous markers (Mechanda et al. 2004), or when paralogous markers resulting from gene duplication events are mistakenly interpreted as orthologs (Gogarten and Olendzenski 1999; Remm et al. 2001; Pelgas et al. 2005). This is a particularly acute problem when considering the arbitrary criteria used to ascertain the orthology of gene markers, such as their position on linkage maps, the percent of sequence homology or the threshold of E-value for a best sequence match (Huynen and Bork 1998; Stirling et al. 2003; Delseny 2004). To facilitate the identification of orthologous markers as well as comparative mapping between different taxa, sequence-based gene markers such as ESTPs (expressed sequence tag polymorphisms) and single-locus SSRs (simple sequence repeats) are a suitable choice in the construction of linkage maps because they are usually orthologous across congeneric species and thus are more reliable than anonymous markers to anchor maps (e.g., Marques et al. 2002; Pelgas et al. 2005). As well, careful sequence homology studies should be conducted to make sure any change in synteny or colinearity is not the product of hidden paralogy (Huynen and Bork 1998; Remm et al. 2001).

In conifers, the number of available orthologous anchor markers for comparative mapping is still limited. In addition, the genome size of conifers, which is 100- to 200-fold larger than that of *Arabidopsis thaliana* (The Arabidopsis Genome Initiative 2000), and 30- to 50-fold larger than that of poplar or rice (Goff et al. 2002; Yu et al. 2002; Kirst et al. 2003), implies that the full genome sequencing of coniferous species is not feasible for the foreseeable future (Kirst et al. 2003). Despite these difficulties, a small number of comparative mapping studies have been conducted in the conifers at the interspecific level, mostly between closely related taxa in the subgenus *Pinus* of *Pinus*: *Pinus taeda* versus *Pinus radiata* (Devey et al. 1999), versus *Pinus elliottii* (Brown et al. 2001), versus *Pinus pinaster* (Chagné et al. 2003) or versus *Pinus sylvestris* (Komulainen et al. 2003). These studies have relied on maps developed mostly with AFLPs (amplified fragment length polymorphisms) or RFLPs (restriction fragment length polymorphisms), but also contained a number of SSRs and ESTPs. These studies have shown that synteny and macro-colinearity were relatively well conserved between closely related hard pine species (Brown et al. 2001). Between genera of the Pinaceae, comparisons based on orthologous markers (e.g., ESTPs) have been initiated and have permitted us to identify a few putative major chromosomal rearrangements between the genera *Pinus*, *Pseudotsuga*, and *Picea* (Krutovsky et al. 2004; Pelgas et al. 2005).

In this study, we have estimated a number of spruce genetic maps and conducted comparative mapping analyses at the intra-generic level in the genus *Picea* and at the inter-generic level in the family Pinaceae. Intra-generic comparisons in the genus *Picea* involved the eastern North American species complex *Picea mariana* (Mill.) B.S.P. × *Picea rubens* Sarg. and the widely distributed North American species *Picea glauca* (Moench) Voss. It also involved *Picea abies* (L.) Karst., a species distributed throughout Europe. Previous studies based on artificial crosses, morphology, and cpDNA restriction fragment patterns have shown that the three taxa are widely divergent in the genus (Wright 1955; Weng and Jackson 2000; Sigurgeirsson and Szmidt 1993), with a divergence time of over 10 Mya, based on a number of molecular and morphological dating approaches (Bouillé and Bousquet 2005).

In the North American boreal forests, *P. mariana* and *P. glauca* have mostly sympatric distributions extending from the Pacific to the Atlantic oceans while *P. mariana* hybridizes naturally with *P. rubens* in the northeast (e.g., Perron and Bousquet 1997), and *P. glauca* with *P. engelmannii* and *P. sitchensis* in western Canada (e.g., Sutton et al. 1991). The natural range of *P. abies* extends from the mountain ranges of central and southeastern Europe to the Ural Mountains and from Fennoscandinavia to Greece. This species can hybridize naturally with the closely related *P. obovata*, from the Ural Mountains to Finland (Krutovskii and Bergmann 1995).

The objectives of the present study were: (1) to estimate the first composite map for the North American species *P. glauca*, (2) by using a common set of reliable anchor markers, to evaluate the degree of macro-synteny and macro-colinearity with two other composite linkage maps developed for the species complex *P. mariana* × *P. rubens* (Pelgas et al. 2005) and for *P. abies* (Acheré et al. 2004), and (3) to make comparisons with maps from taxa representative of two other genera of the Pinaceae, *Pinus* spp. and *Pseudotsuga menziesii* (Brown et al. 2001; Chagné et al. 2003; Komulainen et al. 2003; Krutovsky et al. 2004). Such comparisons should allow us to detect large-scale chromosomal rearrangements in the Pinaceae and to characterize genome stability in this old family of conifer trees.

Materials and methods

Plant material

P. glauca To estimate a composite map, two outbred F1 crosses were used, each containing 118 progeny and sharing one common parent: cross #C9612856 (♀80112 × ♂80109), hereafter called F1-2856, and cross #C9612872 (♀80132 × ♂80109), hereafter called F1-2872. Both crosses were selected for their high level of heterozygosity for ESTP anchor markers and for their intermediate performance for a number of traits such as embryogenic capacity. For each *P. glauca* cross, needle tissue was collected from the two parents and their progeny, and then genomic DNA was extracted from each individual with the DNeasy Plant Mini Kit (Qiagen, Mississauga, ON, CA).

P. mariana × *P. rubens* The composite map was estimated from one backcross-like cross, hereafter called BC1 (#9920002: ♀11307-03 [♀83 × ♂425] × ♂425) including 118 individuals and one outbred F1 cross, hereafter called F1 (#S11991V: ♀422 × ♂425) including 85 individuals, as described by Pelgas et al. (2005).

P. abies One outbred F1 cross (♀TH787F × ♂Sire5) including 73 individuals was used to construct the composite map, as described by Acheré et al. (2004).

Genotyping procedure and estimation of maps

P. glauca The selection of different types of markers (AFLPs, SSRs, and ESTPs) and their specific use for mapping were as described by Pelgas et al. (2004, 2005). For AFLP markers, a total of 61 *Eco*RI/*Mse*I primer combinations with one or two selective nucleotides for the pre-amplification and three or four selective nucleotides for the selective amplification were used (Table S1**,** electronic supplementary material). For SSR markers, a total of 35 primer pairs previously developed by several authors (Pfeiffer et al. 1997; Hodgetts et al. 2001; Rajora et al. 2001; Scotti et al. 2000, 2002a, b; Besnard et al. 2003) were screened and the PCR reactions were based on the protocol of Acheré et al. (2004) with some minor modifications such as reported in Pelgas et al. (2005). According to the SSR primer pairs tested, different $MgCl_2$ concentrations and PCR programs were used in order to reduce the occurrence of multiple-banding patterns (Table S2, electronic supplementary material). Finally, a total number of 112 ESTP primer pairs previously developed from various conifer species and reported in Pelgas et al. (2004, 2005) were retained for the screening. The construction of four individual linkage maps of *P. glauca* from both crosses, the estimation of a reference map for the male parent 80109 common to the two crosses, and the assembly of a composite map for *P. glauca* were carried out according to the strategies and methods described by Pelgas et al. (2005), except that marker grouping and linked loci ordering were carried out using a LOD of 6.0 and a minimum recombination fraction (θ) of 0.35. The estimation of different genome lengths and map coverage values was conducted such as described in Pelgas et al. (2005), with a minimum LOD threshold value of 6.0.

P. abies Fifty-one additional anchor markers including 1 SSR and 50 ESTPs, which were positioned earlier on the composite linkage map of the species complex *P. mariana* × *P. rubens* (Pelgas et al. 2005) or on the composite linkage map estimated herein for *P. glauca*, were screened on both parents of the *P. abies* cross. The positioning of polymorphic anchor markers showing Mendelian segregation was conducted according to the same criteria as those used for *P. glauca*.

Intra- and inter-generic map comparisons

Comparisons among the three *Picea* taxa composite maps were conducted with the help of both types of anchor markers, SSRs and ESTPs. For the inter-generic comparisons in the Pinaceae, which relied on the composite maps of each of the three *Picea* taxa and on the linkage maps of each *Pinus* spp. and *Pseudotsuga menziesii* previously reported (Brown et al. 2001; Chagné et al. 2003; Komulainen et al. 2003; Krutovsky et al. 2004), only ESTPs were retained to minimize homoplasy and because of the difficulty in transferring SSR markers between genera in the Pinaceae (Perry and Bousquet 1998a). Additional intra- and inter-generic comparisons were undertaken with individual linkage maps recently published for *P. abies* by Scotti et al. (2005). Moreover, to extend the number of inter-generic comparison points between the different maps, 27 EST sequences for markers previously developed and positioned onto seven different linkage groups (LGs) of *P. menziesii* and *Pinus taeda* (Krutovsky et al. 2004) were screened on the *P. glauca* EST database (http://www.ccgb.umn.edu/cgi-bin/spruce/blastsubform). From the consensus sequences of *P. glauca* contigs obtained, 27 new *Picea*-specific primer pairs were designed in exons or 3′ UTR regions with the program "Primer 3" (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) such that one or two introns would be included in genomic products. PCR reactions were based on the amplification protocol described by Pelgas et al. (2004), except that the primer concentration was 0.12 μM. DNA amplifications were carried out according to the PCR program #1 of Pelgas et al. (2004) with an annealing temperature of 60°C, and using a PTC-225 thermal cycler (MJ Research, Reno, NV, USA). The identification of polymorphisms was conducted on agarose gels, then by DGGE (Denaturing Gradient Gel Electrophoresis) as described elsewhere (Pelgas et al. 2004), and following different denaturing gradients.

Identification and validation of homoeologous genomic regions

The homoeology of LGs at the intra-generic level, among each of the three *Picea* consensus maps, or at the inter-generic level, among *Picea*, *Pinus*, and *P. menziesii*, was determined according to the following criteria: (1) when at least two anchor markers were positioned at the same time onto a pair of LGs being compared, these LGs were then considered as homoeologous and the common anchor markers were considered as orthologs; (2) when only one anchor marker was used to determine the homoeology between LGs or when putative chromosomal rearrangements were raised, the sequence homology of the markers considered as putative orthologs was evaluated. In this case, PCR products of the putative orthologous markers were sequenced either for the three *Picea* taxa for comparisons at the intra-generic level, or at least for two of the three genera of the Pinaceae for comparisons at the inter-generic level. In each case, amplifications were performed on DNA of a haploid megagametophyte in order to rule out paralogy cases (Pelgas et al. 2005; Lamothe et al. 2006). Because of the haploid nature of megagametophytes, any polymorphism detected in the DNA sequence of a single megagametophyte would result from distinct loci pertaining to the same gene family, thus indicating paralogy. Sequencing reactions were run on a BigDye™ Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism® 3700 Genetic Analyser (Applied Biosystems). Standard nucleotide-nucleotide searches (BLASTN; Altschul et al. 1990) between compared sequences or against the non-redundant National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) were conducted. In order to filter the BLAST alignment results and assist in the determination of orthology between two genes, the nucleotide sequence alignment had to match for at least a 150-bp stretch with a minimum identity of 95% at the intra-generic level, or at least 85% at the inter-generic level (Chagné et al. 2003; Komulainen et al. 2003; Kaló et al. 2004). These thresholds were only meant to be indicative and results were analyzed on a case-by-case basis, considering that some domains of different members of a gene family can be very conserved (e.g., Guillet-Claude et al. 2004).

Results

Development of *Picea* linkage maps

Overview for P. glauca Out of 118 genotyped individuals for the two crosses F1-2856 and F1-2872, 110 and 103 individuals with less than 10% missing data were retained for map construction, respectively. Out of a grand total of 742 and 625 markers available for crosses F1-2856 and F1-2872, respectively, 98.1% of the analyzed markers fitted the expected Mendelian ratios ($P \leq 0.01/n$; Table S3). Depending on the parent and cross analyzed, between 288 and 377 Mendelian markers were available for map construction (Table 1). LGs were generally stable for a LOD of 6.0

and a minimum recombination fraction (θ) of 0.35. A LOD value of 3.5 up to 8.5 was sometimes applied to obtain comparable groups among the different parental maps.

Individual linkage maps of cross F1-2856 of P. glauca Map construction for the female linkage map (80112) resulted in 16 major and 3 minor LGs based on a total of 295 mapped markers (243 AFLPs, 19 SSRs, and 33 ESTPs), including 8 accessory loci, and covering 2,214.6 cM (7.5 cM average marker spacing; Table 1; Fig. S1, electronic supplementary material). For the male linkage map (80109), 318 markers (264 AFLPs, 23 SSRs, and 31 ESTPs) were mapped and distributed over 15 major and 4 minor LGs, including 6 accessory loci, and covering 2,321.8 cM (7.3 cM average marker spacing; Table 1).

Individual linkage maps of cross F1-2872 of P. glauca The female linkage map (80132) resulted in 15 major and 7 minor LGs based on a total of 259 mapped markers (219 AFLPs, 22 SSRs, and 18 ESTPs), including 1 accessory locus, and covering 1,718.4 cM (6.6 cM average marker spacing; Table 1; Fig. S1, electronic supplementary material). For the male linkage map (80109), 264 markers (213 AFLPs, 22 SSRs, and 29 ESTPs) were mapped and distributed over 12 major and 8 minor LGs, including 1 accessory locus, and covering 1,867.3 cM (7.1 cM average marker spacing; Table 1).

Composite linkage map of P. glauca After merging homologous LGs of each "sub-composite" map obtained for the two crosses, 865 assigned markers were distributed among the composite LGs, and 802 were positioned (Table 1). Out of the 802 markers (714 AFLPs, 38 SSRs, and 50 ESTPs) ordered, 92 markers (35 AFLPs, 26 SSRs, and 31 ESTPs) were homologous between at least two parents. Before marker ordering on each composite LG, a test of heterogeneity of recombination frequencies was conducted between 269 homologous pairs of loci merged from both "sub-composite" maps. Out of these homologous pairs of loci, 37 showed a significant difference between their recombination frequency estimates ($P > 0.01$), so they were excluded from analyses to avoid erroneous marker positioning. Thus, the ordered markers, including a total of 88 anchor markers (38 SSRs and 50 ESTPs), were assembled in 11 LGs covering 2,168.4 cM (Haldane), for an average of 8 anchor markers per LG (Fig. 1). The average marker density was 2.7 cM, more than twofold the marker density obtained for individual linkage maps, which varied from 6.6 to 7.5 cM (Table 1). However, the LG I of the reference linkage map of the male parent 80109 could be considered as the 12th composite LG, even if no LG of individual linkage maps of the two female parents 80112 and 80132 was homologous to this reference LG I (Fig. S1, electronic supplementary material). Indeed, this LG was constructed from the two male linkage maps of each cross, over which one common anchor marker was positioned. Moreover, interspecific comparisons indicate that it may be very likely representative of the 12th chromosome of *P. glauca* (see below).

For interspecific comparisons, two additional ESTPs (*COMT1* and *Sb01*) positioned only onto the LG XI of the female parent 80112 of *P. glauca* were also taken in consideration (Fig. 2). These two ESTPs were positioned by hand onto the composite map (Alm et al. 2003) by taking into account their recombination frequencies obtained with other anchor markers positioned onto female parent 80112 and the composite map. Therefore, a total of 38 SSRs and 53 ESTPs were mapped onto the composite map of *P. glauca*. Out of these anchor markers, 26 SSRs and 44 ESTPs were useful for intra-generic comparisons (see below). At the inter-generic level, 14 and 9 ESTPs could be retained for comparisons with *Pinus* and *P. menziesii*, respectively, including four ESTPs common among the three genera (see below).

Synteny and colinearity in P. glauca Synteny was well conserved among the female individual linkage maps and the male reference linkage map (Fig. S1), since all homologous markers were placed onto the same homologous LGs. Macro-colinearity was also well conserved among homologous LGs. Indeed, marker order was the same for 33 out of 36 (91.7%) homologous markers between the reference linkage map of the male parent 80109 and the linkage map of the female parent 80112, and for 31 out of 32 (96.9%) homologous markers between the male reference linkage map and the linkage map of the female parent 80132. Between the two female linkage maps, marker order was the same for 26 out of 27 (96.3%) homologous markers (Fig. S1).

P. mariana × *P. rubens* The selection of markers as well as the genotyping procedure and the construction of the individual, male reference, and composite linkage maps for the species complex *P. mariana* × *P. rubens* were detailed elsewhere (Pelgas et al. 2004, 2005): a total of 1,124 markers, including 1,014 AFLPs, 3 RAPDs, 53 SSRs, and 54 ESTPs, were positioned onto the 12 major LGs of the composite map of this species complex.

Table 1 Parameters of individual, reference, and composite linkage maps from two crosses in *Picea glauca*

Mapping parameters	Crosses/parents				Reference map for male parent 80109[a]	Composite map
	Cross F1-2856		Cross F1-2872			
	♀ 80112	♂ 80109	♀ 80132	♂ 80109		
Total number of available markers[b]	332	383	294	294	728	1250[c]
Number of distorted markers (*Bonferroni correction;* $P \leq 0.01$/number of loci)	2	6	6	4	19	26[c]
Total number of markers without segregation distortion	330	377	288	290	709[c]	1224[d]
Number of assigned marker loci	320	368	282	283	555	865
Number of AFLP loci	268	306	238	229	492	775
Number of SSR loci	19	25	23	23	28	38
Number of ESTP loci	33	37	21	31	35	52
Number of positioned marker loci[e] (%)	295 (92.2)	318 (86.4)	259 (91.8)	264 (93.3)	512 (92.3)	802 (92.7)
Number of AFLP loci	243	264	219	213	451	714
Number of SSR loci	19	23	22	22	27	38
Number of ESTP loci	33	31	18	29	34	50
Number of positioned accessory marker loci	8	6	1	1	10	21
Number of major linkage groups ($n > 8$ markers)	16	15	15	12	12[f]	12[g]
Number of minor linkage groups ($3 \leq n \leq 8$ markers)	3	4	7	8	0	0
Number of doublets	1	0	1	0	0	0
Number of unlinked markers (%)	8 (2.4)	9 (2.4)	4 (1.4)	7 (2.4)	30 (4.1)	40 (3.2)
Map length G_F, cM (Haldane)	2214.6	2321.8	1718.4	1867.3	2297.1[h]	2168.4[i]
Map length G_F, cM (Kosambi)	1842.3	1928.2	1424.7	1533.6	1837.5[h]	1933.5[i]
Average map density, cM (Haldane)	7.5	7.3	6.6	7.1	4.1[h]	2.7[i]
Average map density, cM (Kosambi)	6.2	6.1	5.5	5.8	3.3[h]	2.4[i]
Average size for major linkage groups, cM (Haldane)	125.3	144.6	103.7	139.0	176.7[h]	197.1[i]
Average size for major linkage groups, cM (Kosambi)	115.1	128.5	95.0	127.8	141.3[h]	175.8[i]
Observed map length G_o, cM (Haldane)	2799.4	2955.6	2223.7	2394.7	-	-
Observed map length G_o, cM (Haldane) without unlinked markers	2507.0	2599.1	2089.0	2148.6	-	-
Observed map length G_o, cM (Kosambi)	2301.8	2419.7	1826.9	1950.6	-	-
Observed map length G_o, cM (Kosambi) without unlinked markers	2072.0	2143.3	1719.7	1756.0	-	-
Expected map length G_e, cM (Haldane)	3801.0	4713.9	3392.8	3523.5	-	-
Confidence interval	3578.5–4052.9	4454.7–5005.1	3172.7–3645.8	3293.2–3788.3	-	-
Expected map length G_e, cM (Kosambi)	2986.6	3655.3	2700.8	2782.8	-	-
Confidence interval	2811.8–3184.6	3454.3–3881.1	2525.6–2902.2	2601.0–2992.0	-	-
Observed map coverage C_o (G_o/G_e)[j]	73.6%	62.7%	65.5%	68.0%	-	-
Observed map coverage C_o (G_o/G_e)[j] without unlinked markers	66.0%	55.1%	61.6%	61.0%	-	-
Observed map coverage C_o (G_F/G_e)[k]	58.3%	49.3%	50.6%	53.0%	-	-
Expected map coverage C_e	91.6%	91.6%	89.4%	89.2%	-	-

[a] The male reference map was used to obtain the best representation of the genome before assembling the final composite linkage map

[b] For individual linkage maps, only markers segregating 1:1 or 1:1:1:1 were used. Exceptionally markers in configuration 3:1 or 1:2:1 for one cross and 1:1 for the other cross were also used (see Materials and methods)

[c] Between the two crosses BC1 and F1, 104 markers were in common, from which one had distorted segregation

[d] For the composite map construction, 123 markers were in common between the two crosses

[e] Unpositioned markers correspond to markers with a recombination frequency higher than 0.35 or having a -log 10 (P) value higher than 1 that could affect marker order (see Materials and methods)

[f] This reference linkage map consisted of 12 major LGs, of which one is composed of two sub-LGs including anchor markers (total of 13 LGs)

[g] This composite linkage map consisted of 11 major composite LGs, and one reference LG (LG I) considered as the 12th composite LG, because it is likely representative of the 12th chromosome of the *P. glauca* species (see text)

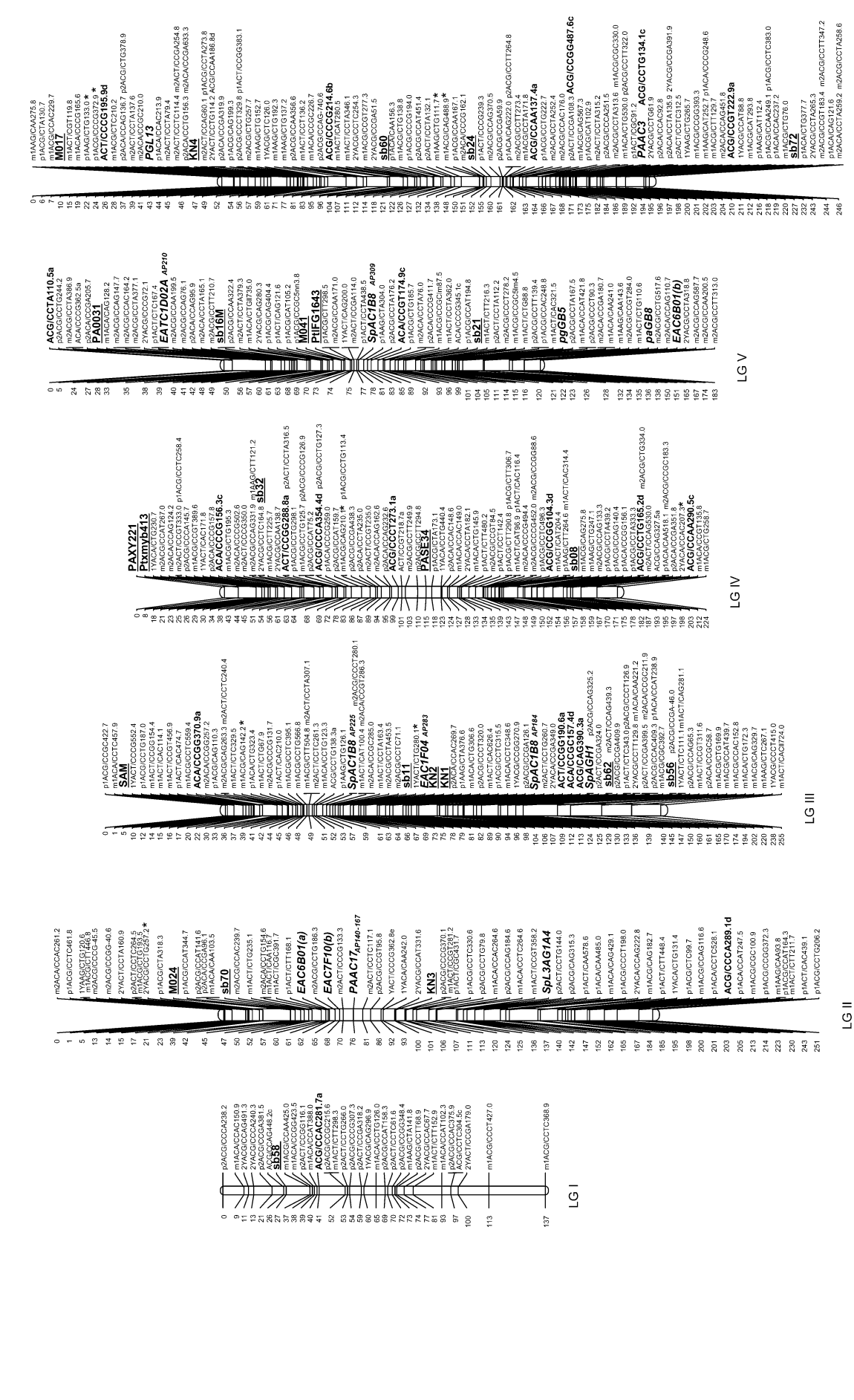
[h] Calculating from 13 major reference LGs

[i] Calculating from 11 major composite LGs, since the 12th composite LG is the reference LG I

[j] According to the method of Tani et al. (2003).

[k] According to the method of Cervera et al. (2001)

"-": Could not be calculated due to the merging of data


LG I
LG II
LG III
LG IV
LG V
LG VI

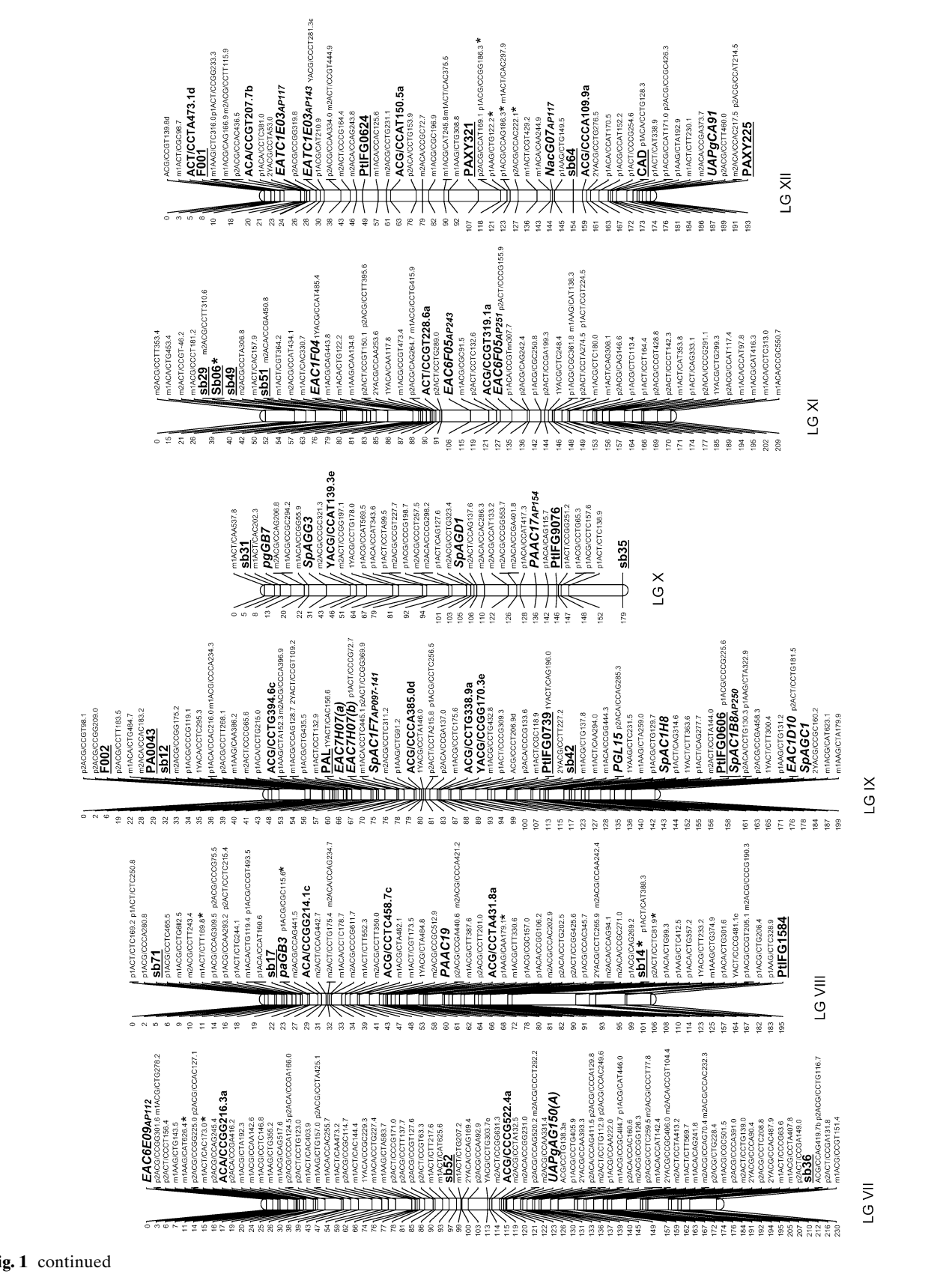


Fig. 1 continued

Fig. 1 Composite linkage map for the species *Picea glauca*. The composite map was obtained by assembly of the data sets for both crosses F1-2856 and F1-2872, and using JoinMap 3.0 (Stam 1993; Van Ooijen and Voorrips 2001). Genetic distances are on the *left* of each LG (Haldane). Markers in *bold* and *underlined* are ESTPs, markers in *bold* and *italics* are SSRs, markers in *bold* only are homologous AFLPs and all remaining markers are other AFLPs. Accessory markers (jump value > 5) are indicated with an *asterisk*. Two markers positioned side-by-side correspond to the same linkage map bar. The LG I of this composite map was not generated as the other composite linkage groups, since it corresponds to a reference linkage of the male parent 80109. However, this reference LG is considered as a composite LG and is presumably representative of the twelfth chromosome of *P. glauca* (see text)

P. abies Fourteen additional anchor markers, 1 SSR (*UAPgTG87*) and 13 ESTPs (*Sb16, Sb17, Sb18, Sb21, Sb32, Sb41, Sb50, Sb51, Sb58, Sb60, Sb67, Sb68*, and *PA0031*), were assigned and positioned onto the composite map previously developed for *P. abies* by Acheré et al. (2004). Features of the composite map were detailed elsewhere (Acheré et al. 2004; in this study Fig. 2). Thus, the total number of markers positioned onto the composite map of *P. abies* was 661 AFLPs, 75 SSRs and 32 ESTPs (Fig. 2).

Interspecific comparisons in the genus *Picea*

Between the composite map of *P. mariana* × *P. rubens* and both composite linkage maps of *P. glauca* and *P. abies*, 58 (21 SSRs and 37 ESTPs) and 40 (26 SSRs and 14 ESTPs) homologous markers were shared, respectively (Table 2). When comparing *P. glauca* and *P. abies*, 36 homologous markers (20 SSRs and 16 ESTPs) were common to both (Table 2).

Synteny among the three *Picea* composite maps was relatively well conserved for the 12 homoeologous LGs (Fig. 2; Table 2): on average, between 97.5 and 100% of all homologous markers were in synteny. However, the LG IV of *P. glauca* and the LG 7 of *P. abies* were involved in discrepancies of synteny with the LGs III and IV of *P. mariana* × *P. rubens*, respectively (Fig. 2). DNA sequence analyses conducted on each implicated taxon for the two ESTPs involved in these inconsistencies, *Ptxmyb413* and *Sb68*, revealed high levels of nucleotide identity among the taxa: 99.4% on 660 bp for *Ptxmyb413* between *P. mariana* × *P. rubens* and *P. glauca*, and 97.1% on 455 bp for *Sb68* between *P. mariana* × *P. rubens* and *P. abies*. Considering these high identity values, it might be safely assumed that each of these anchor markers targets orthologous gene loci among the three taxa. Because the anchor marker *Ptxmyb413* was monomorphic and could not be placed on the current composite map of *P. abies*, additional analyses were conducted for a second cross of *P. abies* (#C9641048) where it was found to be polymorphic. These analyses revealed that it was positioned onto the same homoeologous LG as that found for *P. mariana* × *P. rubens* (data not shown), contrary to that found for *P. glauca*. Moreover, for each of the three taxa, this anchor marker was positioned onto two individual linkage maps, confirming the positioning onto the composite maps. The sequencing on the whole length of the gene (*PgMyb4*) flagged by the anchor marker *Ptxmyb413* from the haploid DNA of a megagametophyte for each of the three spruce taxa has not revealed the presence of any paralog. Moreover, the nucleotide identity of this gene among the three taxa was relatively similar: 96.4–97.4% identity for about 1,400 bp including intronic regions, and 99.6–99.7% identity for more than 900 bp of cDNA. Consequently, these various lines of evidence suggest that *Ptxmyb413* corresponds to an orthologous gene locus among the three *Picea* taxa. Thus, its different positioning among *Picea* taxa would truly reflect an inter-chromosomal translocation. For the anchor marker *Sb68*, the chromatogram analyses of the DNA sequences obtained from a haploid megagametophyte for each *P. mariana* × *P. rubens* and *P. abies* revealed polymorphisms. Such evidence suggests that the differential positioning of this marker may in fact correspond to two paralogous gene loci.

Macro-colinearity was also well conserved among homoeologous LGs of the three *Picea* taxa because, on average, between 87.2 and 89.5% of syntenic markers were positioned in the same order (Table 2): five homoeologous LGs had the same marker order (LG I, II, IV, V, and VIII, following LG nomenclature of *P. mariana* × *P. rubens*). Minor inversions in marker order were observed, involving six SSRs and six ESTPs found in LGs III, VI, VII, IX, X, XI, and XII (Fig. 2). Of interest, a putative segmental duplication was noted on LG III of *P. glauca* and *P. mariana* × *P. rubens*, where the genes *KN1* and *KN2* were flanked by two *60S ribosomal protein L15* gene loci, *Sb11* and *Sb62* (Fig. 2).

Inter-generic comparisons within the family Pinaceae

Preliminary comparisons between the composite linkage map of *P. mariana* × *P. rubens* and maps for *Pinus* and *P. menziesii* were conducted in a recent report (Pelgas et al. 2005). In the present study, additional anchor markers were positioned onto the composite linkage map of *P. glauca* in order to increase the number of comparison points between *Picea* and the two other genera of the Pinaceae. The screening of

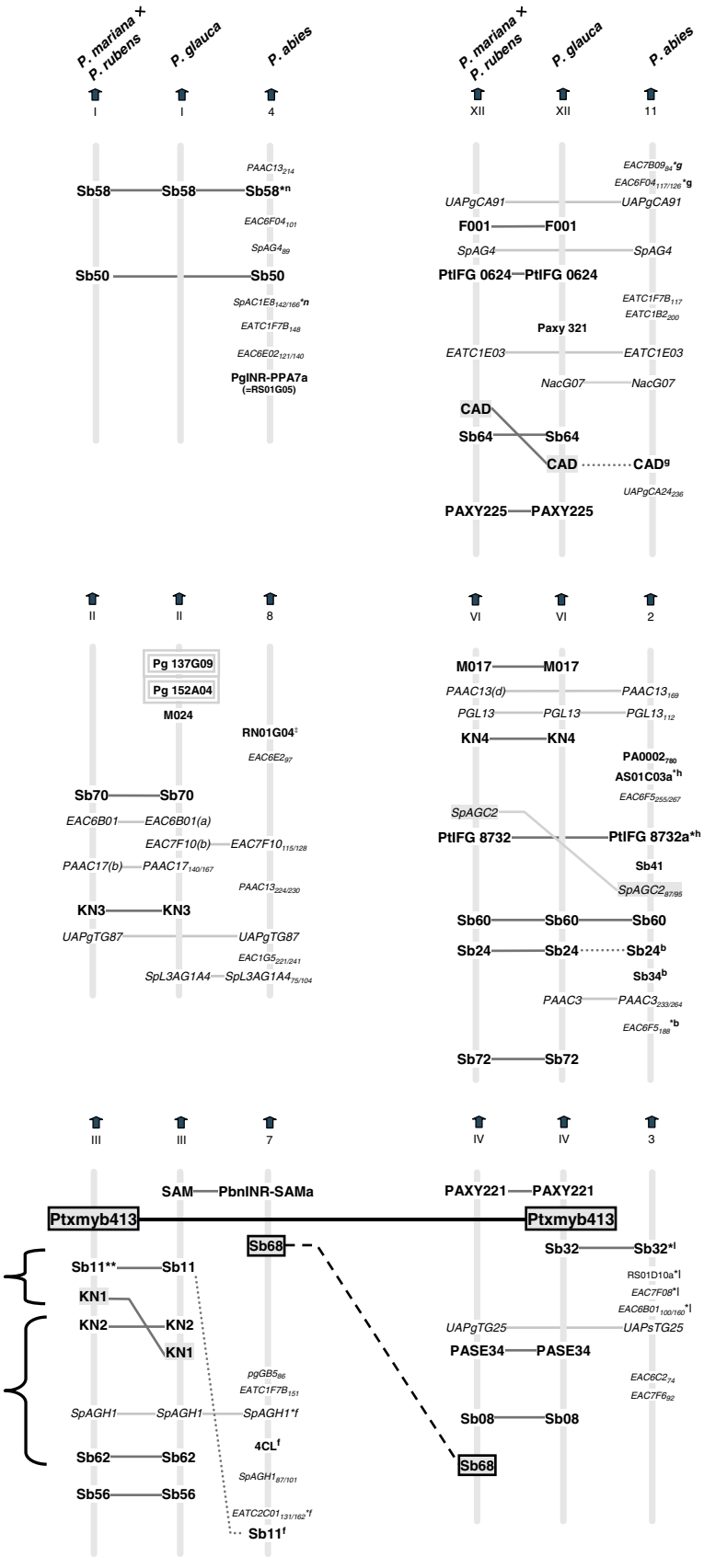

P. mariana × P. rubens
P. glauca
P. abies
I
I
4
Sb58
Sb58
Sb58*n
PAAC13214
EAC6F04101
SpAG489
Sb50
Sb50
SpAC1E8142/166*n
EATC1F7B148
EAC6E02121/140
PgINR-PPA7a (=RS01G05)
XII
XII
11
EAC7B0984*g
EAC6F04117/126*g
UAPgCA91
UAPgCA91
F001
F001
SpAG4
SpAG4
PtIFG 0624
PtIFG 0624
EATC1F7B117
EATC1B2200
Paxy 321
EATC1E03
EATC1E03
NacG07
NacG07
CAD
Sb64
Sb64
CAD
CADg
UAPgCA24236
PAXY225
PAXY225
II
II
8
Pg 137G09
Pg 152A04
M024
RN01G04‡
EAC6E297
Sb70
Sb70
EAC6B01
EAC6B01(a)
EAC7F10(b)
EAC7F10115/128
PAAC17(b)
PAAC17140/167
PAAC13224/230
KN3
KN3
UAPgTG87
UAPgTG87
EAC1G5221/241
SpL3AG1A4
SpL3AG1A475/104
VI
VI
2
M017
M017
PAAC13(d)
PAAC13169
PGL13
PGL13
PGL13112
KN4
KN4
PA0002780
AS01C03a*h
EAC6F5255/267
SpAGC2
PtIFG 8732
PtIFG 8732a*h
Sb41
SpAGC287/95
Sb60
Sb60
Sb60
Sb24
Sb24
Sb24b
Sb34b
PAAC3
PAAC3233/264
EAC6F5188*b
Sb72
Sb72
III
III
7
IV
IV
3
SAM
PbnINR-SAMa
Ptxmyb413
Sb68
PAXY221
PAXY221
Ptxmyb413
Sb32
Sb32*l
RS01D10a*l
EAC7F08*l
EAC6B01100/160*l
Sb11**
Sb11
KN1
KN2
KN2
KN1
UAPgTG25
UAPsTG25
PASE34
PASE34
pgGB586
EATC1F7B151
EAC6C274
EAC7F692
SpAGH1
SpAGH1
SpAGH1*f
Sb08
Sb08
4CLf
Sb62
Sb62
SpAGH187/101
Sb56
Sb56
EATC2C01131/162*f
Sb11f
Sb68

Fig. 2

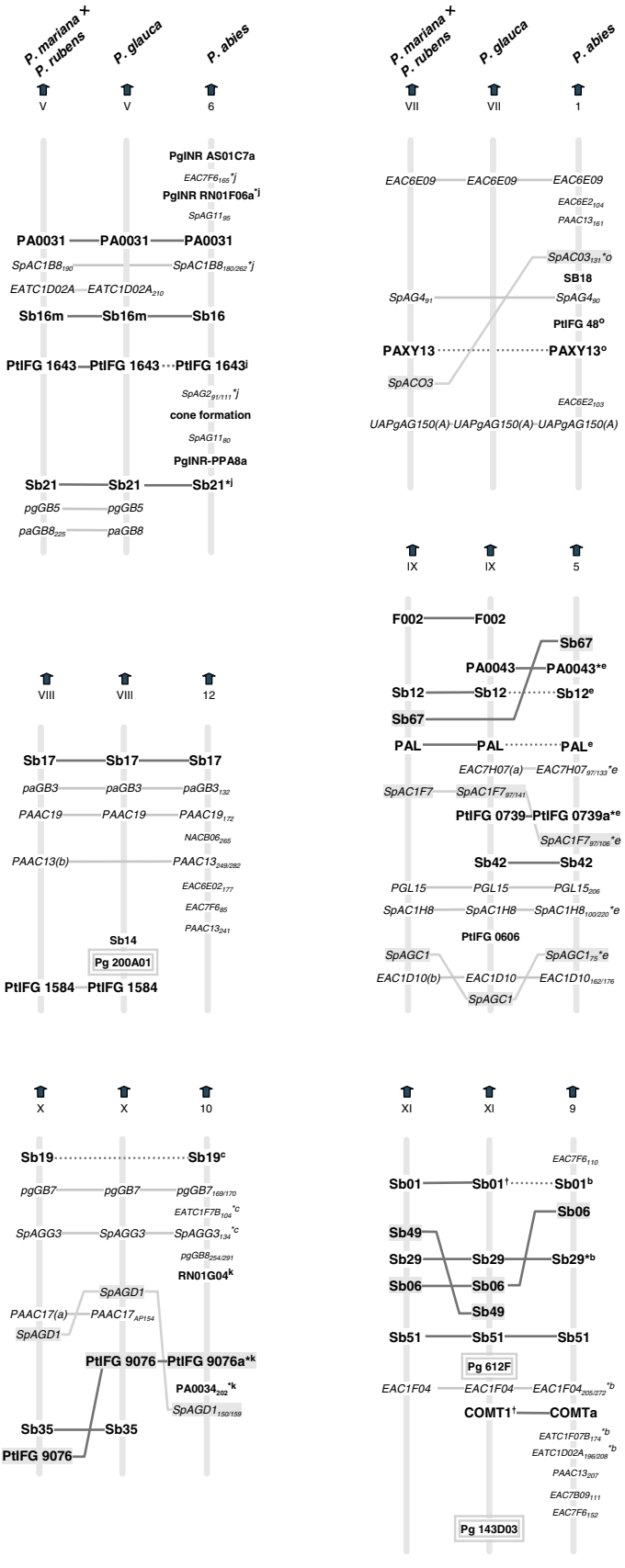


Fig. 2 continued, for legend see next page

Fig. 2 Schematic representation of homoeologous LGs of composite maps for three *Picea* taxa: *P. mariana* × *P. rubens* (on the *left*), *P. glauca* (in the *middle*), and *P. abies* (on the *right*). Only homologous anchor markers are indicated on each schematic LG of *P. mariana* × *P. rubens*. In order to present the positioning of additional markers for *P. abies*, all anchor ESTPs and SSRs of the composite map previously developed by Acheré et al. (2004) were indicated as reference points. For *P. glauca*, the positioning of five additional ESTPs useful for inter-generic comparisons was indicated by their adjacent anchor ESTPs positioned onto the individual and composite linkage maps and used as reference points. ESTPs and SSRs are indicated in *bold* and in *italics*, respectively. Orthologous markers are connected by a *solid line*, except when they are connected with homologous anchor markers positioned onto the linkage maps of *P. abies* developed by Scotti et al. (2005; *dotted line*). Orthologous markers not positioned onto homoeologous LGs are indicated with a large *solid line* and paralogous markers are connected by a *dashed line*. Anchor markers of *P. glauca* newly developed herein for inter-generic comparisons are framed by a *double line*. *Brackets* positioned on the *left* of LG III indicate a segmental duplication. For *P. abies*, anchor markers tagged by an *asterisk* followed by a letter were positioned onto the linkage maps developed by Acheré et al. (2004) and Scotti et al. (2005), whereas anchor markers tagged by letters only were reported by Scotti et al. (2005). For *P. glauca*, '†' indicates that the marker is only positioned onto LG XI of the individual linkage map of the parent 80112. Markers followed by a *double asterisk* were positioned afterwards to confirm interspecific comparisons. *Arrows* on the top indicate the LG nomenclature used for each taxon: *P. glauca* and *P. mariana* × *P. rubens* follow the nomenclature reported by Pelgas et al. (2005) and *P. abies* follows the one reported by Acheré et al. (2004)

additional markers was conducted on the cross F1-2872 of *P. glauca*. To do so, 27 new EST *Picea*-specific primer pairs were designed from the consensus sequences of *P. glauca* contigs obtained from *P. glauca* EST database (http://www.ccgb.umn.edu/cgi-bin/spruce/blastsubform). Of these, nine appeared polymorphic by DGGE, but only five were retained as anchor markers (*Pg 137G09*, *Pg 143D03*, *Pg 152A04*, *Pg 200A01*, and *Pg 612F*; Table S4), after genotyping of the progeny. These five anchor markers were positioned onto four different LGs of the composite map of *P. glauca* (Table 3; Fig. 2). To carry out inter-generic comparisons among *Picea*, *Pinus*, and *P. menziesii*, and because all anchor markers could not be mapped for every *Picea* taxon, it was necessary to produce a schematic consensus map of *Picea* at the genus level from the three composite maps evaluated at the species level (Fig. 3). The consensus map was possible because many of the loci were located on at least two of the three *Picea* composite maps, allowing us to combine the information from various species with very little ambiguity as to gene order.

Comparing Picea and Pinus Between *Picea* and *Pinus*, 29 anchor markers could be compared and 26

Table 2 Number of anchor markers in common, in synteny, and in colinearity among three *Picea* taxa[a]

Anchor marker organization	*P. mariana* × *P. rubens* vs *P. glauca*			*P. mariana* × *P. rubens* vs *P. abies*			*P. glauca* vs *P. abies*		
	SSRs	ESTPs	Total	SSRs	ESTPs	Total	SSRs	ESTPs	Total
In common/total number of anchor markers positioned in both species (%)	21/70 (30.0)	37[b]/70 (52.9)	58[b]/140 (41.4)	26/102 (25.5)	14/72 (20.8)	40/174 (23.6)	20/93 (21.5)	16[b]/68 (25.0)	36[b]/160 (23.1)
Common markers in synteny (%)	21 (100)	36[b] (97.3)	57 (98.3)	26 (100)	13 (92.9)	39 (97.5)	20 (100)	16[c] (100)	36[c] (100)
Common markers in colinearity[d] (%)	19 (90.5)	32[b] (88.9)	51 (89.5)	24 (92.3)	10 (76.9)	34 (87.2)	17 (85.0)	15[c] (93.8)	32[c] (88.9)

[a] Including all homologous markers (paralogs and orthologs)

[b] Including two anchor markers positioned only onto the reference linkage map and individual linkage map 80112 of *P. glauca*

[c] Including one anchor marker positioned only onto the individual linkage map 80112 of *P. glauca*

[d] Only syntenic markers were taken into account and when an inversion involved two markers, only one of them was retained for calculation

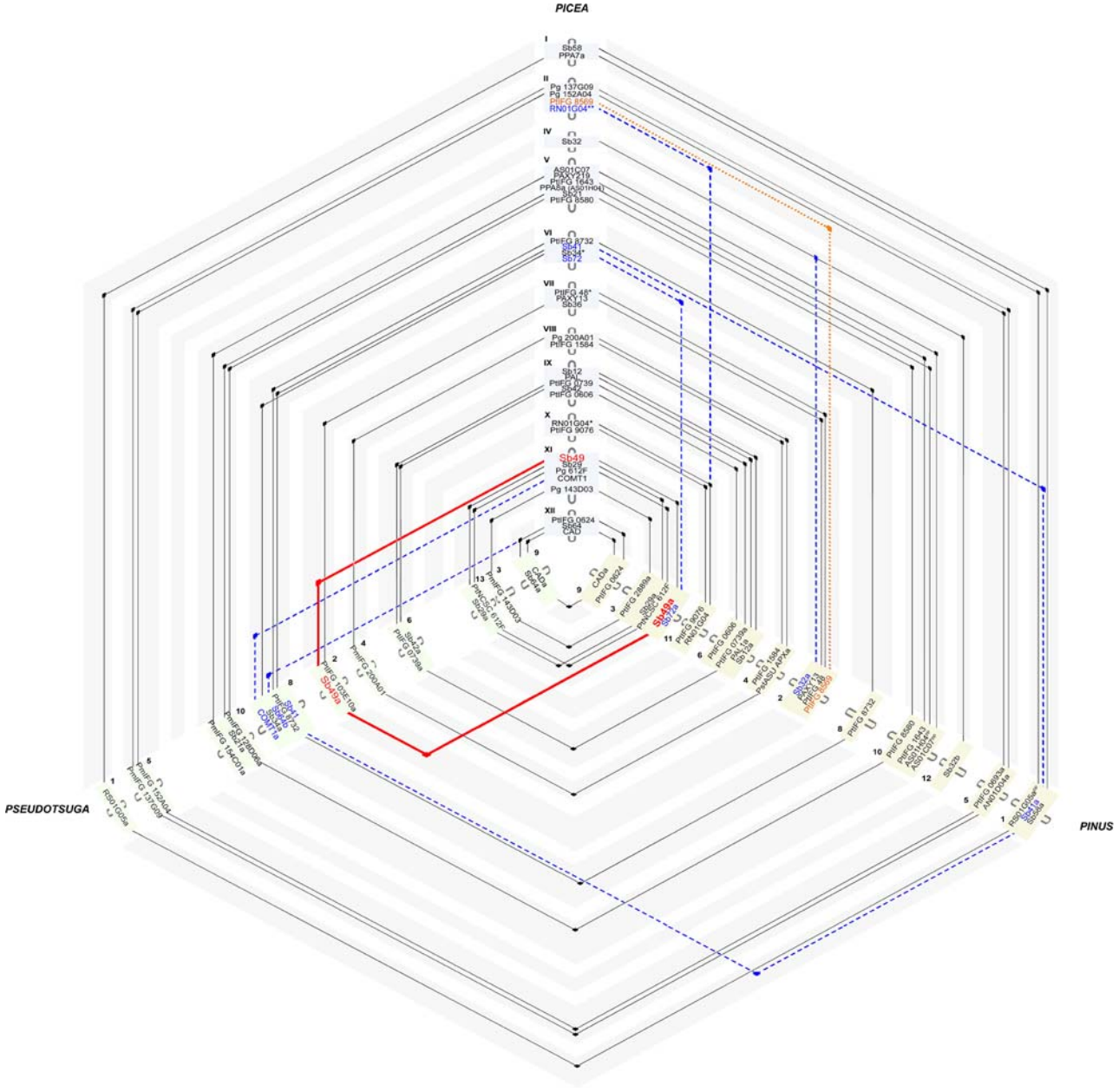


Fig. 3 Schematic representation of homoeologous linkage groups among three genera of the Pinaceae: *Pseudotsuga* (represented by *P. menziesii*; background in *green* on the *left*), *Picea* (background in *blue* in the *middle*), and *Pinus* (background in *yellow* on the *right*). Only homologous markers are indicated on each schematic LG. Orthologous markers are connected by a *solid black line*, except when they are positioned onto non-homoeologous LG (*red line*). Paralogous markers are connected by a *dashed blue line*, except for the anchor marker *PtIFG 8569* (*orange dotted line*). Anchor markers positioned only onto the linkage maps of *Picea abies* published by Scotti et al. (2005) or *Pinus pinaster* published by Chagné et al. (2003) are indicated with an *asterisk* (*) or superscript "pp" (^{pp}), respectively. The anchor marker indicated with a *double asterisk* was positioned onto the composite map of *P. abies* developed by Acheré et al. (2004). The LG nomenclature is indicated at the *top left* of each group for each genus

were found in synteny, enabling us to identify 11 homoeologous LGs (Fig. 3). In addition to previously published anchor markers (Table 4 in Pelgas et al. 2005), 15 additional anchor markers could be compared between the two genera (Table 3). Out of them, nine markers (*PPA7a*, *Pg 137G09*, *Pg 152A04*, *PtIFG 48*, *Pg 200A01*, *PtIFG 9076*, *RN01G04*, *Pg 612F*, and *Pg 143D03*) were considered as putative orthologous because they were positioned onto homoeologous LGs (Fig. 3). None of them showed any polymorphism in their haploid DNA sequences for both genera, confirming the orthology of gene loci (Table 3). For six other markers

Table 3 Homologous anchor markers positioned onto the linkage groups for the genera *Picea*, *Pinus*, and for *Pseudotsuga menziesii*

Anchor markers	Linkage group number			Sequence comparisons			
				Picea vs *Pinus*		*Picea* vs *P. menziesii*	
	Picea[a]	Homoeologous in *Pinus*	Homoeologous in *P. menziesii*	% identity (BLASTN)	Aligned length (bp)	% identity (BLASTN)	Aligned length (bp)
Sb58	I	1	–	Pelgas et al. (2005)		Pelgas et al. (2005)	
PPA7a (RS01G05)	I	1[b]	1	94.2[b]	359[b]	93.3	387
Pg 137G09	II	5 *(AN01D04)*	5 *(PmIFG 137G09)*	94.0[c]	433[c]	93.3[d, e]	520[d, e]
Pg 152A04	II	5 *(PtIFG 0893)*	5 *(PmIFG 152A04)*	87.8[c]	262[c]	94.6[c, f]	37[c, f]
PtIFG 8569	II	2	–	96.2	211	–	–
Sb32	IV	2 or 12	–	86.3–95.2	96–147	–	–
AS01C07a[g]	V	10	–	90.3	186	–	–
PAXY 219	V	–	10 *(PmIFG 154C01a)*	–	–	81.4	161
PtIFG1643	V	10	–	Pelgas et al. (2005)		Pelgas et al. (2005)	
PPA8 (AS01H04a)[g]	V	10	–	87.2	290	–	–
Sb21	V	–	10	Pelgas et al. (2005)		Pelgas et al. (2005)	
PtIFG 8580	V	10	10 *(PmIFG 128D06a)*	Pelgas et al. (2005)		Pelgas et al. (2005)	
PtIFG 8732	VI	5 or 6 or 8	8	Pelgas et al. (2005)		Pelgas et al. (2005)	
Sb41	VI	1	8	91.6	405	91.4	454
Sb34[h]	VI	–	8	–	–	85.6	981
Sb72	VI	3	–	Pelgas et al. (2005)		Pelgas et al. (2005)	
PtIFG 48[h]	VII	2	–	95.0	221	–	–
PAXY13	VII	2	–	Pelgas et al. (2005)		Pelgas et al. (2005)	
Sb36	VII	–	2 *(PmIFG103E10a)*	–	–	84.9	278
Pg 200A01	VIII	4 *(PstASU APXa)*	4 *(PmIFG 200A01)*	93.4[i]	439[i]	85.5[d]	235[d]
PtIFG 1584	VIII	4	–	Pelgas et al. (2005)		Pelgas et al. (2005)	
Sb12	IX	6	–	Pelgas et al. (2005)		Pelgas et al. (2005)	
PAL	IX	6	–	Pelgas et al. (2005)		Pelgas et al. (2005)	
PtIFG 0739	IX	6	6	88.7	231	90.8	238
Sb42	IX	–	6	–	–	86.5–87.8	340–378
PtIFG 0606	IX	6	–	89.5	544	–	–
RN01G04	X[h] or II[j]	11	–	95.6	180	–	–
PtIFG 9076	X	11	–	88.0	192	–	–
Sb49	XI	3	2	Pelgas et al. (2005)		Pelgas et al. (2005)	
Sb29	XI	3	13	Pelgas et al. (2005)		Pelgas et al. (2005)	
Pg 612F	XI	3 *(PtNCSC 612F)*	13 *(PtNCSC 612F)*	85.1[k]	338[k]	85.3[d]	928[d]
COMT1	XI	–	8	-	-	89.6–91.4	413–416
Pg 143D03	XI	3 *(PtIFG 2889)*	3 *(PmIFG 143D03)*	87.5[c]	272[c]	83.8[c]	309[c]
PtIFG 0624	XII	9	–	Pelgas et al. (2005)		Pelgas et al. (2005)	
Sb64	XII	–	9 or 8	Pelgas et al. (2005)		Pelgas et al. (2005)	
CAD	XII	9	9	Pelgas et al. (2005)		Pelgas et al. (2005)	

[a] In reference to nomenclature of *P. mariana* × *P. rubens* and *P. glauca* LGs

[b] Positioned onto the LG 1 of *P. pinaster* and compared with DNA sequence of *P. pinaster* (GenBank accession number: AL750905)

[c] Comparisons completed with the DNA sequences of *P. taeda* or *P. menziesii* published in GenBank

[d] Amplified for both compared genera with new primer pairs developed for the genus *Picea*

[e] The new *Picea* primer pair (*Pg 137G09*) has been designed in another portion of the gene *Pm-AT1_412m2 alpha tubulin 1 (AT1)* of *P. menziesii* (GenBank accession number: AY832610)

[f] For the rest of the sequence, nucleotide identity decreased to 74.8% (222 bp)

[g] Comparison was done between *P. pinaster* (Chagné et al. 2003) and *P. abies*

[h] Positioned only onto the linkage map of *P. abies* developed by Scotti et al. (2005)

[i] *PstASU APXa* versus *Pg 200A01* primer pairs did not amplify the same gene portion. A new primer pair (*Pg 200A01*) was designed from the DNA sequence of contig 6182 (*Picea*-CCGB EST library), which showed 93.4% (439 bp) nucleotide identity with the sequence *PtIFG APX* of *P. taeda*

[j] Positioned only onto the linkage map of *P. abies* developed by Acheré et al. (2004)

[k] *PtNCSC 612F* versus *Pg 612F* primer pairs did not amplify the same gene portion. A new primer pair (*Pg 612F*) was designed from the DNA sequence of contig 4057 (*Picea*-CCGB EST library), which showed 85.1% (388 bp) nucleotide identity with the sequence *PtNCSC612F* of *P. taeda*

(*Sb32*, *AS01C07a*, *AS01H04a*, *Sb41*, *PtIFG 0739*, and *PtIFG 0606*), polymorphisms were observed in the chromatogram analyses of haploid DNA sequences of megagametophytes from *Picea* or *Pinus*, suggesting that paralogous sequences had been amplified from a same gene family. However, five of them (*Sb32, AS01C07a*, *AS01H04a*, *PtIFG 0739*, and *PtIFG 0606*) were positioned onto homoeologous LGs in both genera and for four of them, their respective neighboring anchor markers have already been characterized as orthologous gene loci. Thus, such evidence suggests that these five anchor markers correspond to orthologous gene loci rather than to paralogs (Table 3; Fig. 3). For the remaining locus *Sb41* (LG VI), it was not positioned onto homoeologous LGs between *Picea* and *Pinus*, strengthening the case for paralogy (Fig. 3). Out of the 26 anchor markers found in synteny between *Picea* and *Pinus*, two (*Sb32* and *RN01G04*) enabled us to identify putative paralogous gene loci in one of the two genera. For the anchor marker *Sb32*, one locus was found for *Picea* taxa, whereas two paralogous gene loci were previously reported in *Pinus* onto LGs 2 and 12 (Krutovsky et al. 2004). On the contrary, for the anchor marker *RN01G04*, two putative paralogous anchor markers were found for *Picea* taxa, whereas only one locus was reported in *Pinus* (Fig. 3). Thus, for these two markers in particular, homoeology of LGs could contribute to the identification of orthologous gene loci.

Besides the anchor marker *Sb41* for which paralogous evidence between *Picea* and *Pinus* did not support the hypothesis of an inter-chromosomal translocation (above), there were two other anchor markers not in synteny between *Picea* and *Pinus*, *Sb72* and *PtIFG 8569*. Previous evidence from sequencing haploid DNA from a megagametophyte indicated that *Sb72* is presumably paralogous between *Picea* and *Pinus* (Pelgas et al. 2005). As for *PtIFG 8569*, further DNA sequencing from a haploid megagametophyte in *P. glauca*, in *P. mariana* × *P. rubens*, and in *P. taeda* did not reveal any evidence for paralogy, and nucleotide identity was high between *Picea* and *Pinus*, with 96.2% over a 211-bp stretch, suggesting orthology.

For homoeologous LGs with more than two orthologous anchor markers in common between *Picea* and *Pinus* (LGs V, IX, and XI), colinearity could be evaluated and appeared relatively well conserved except for two small inversions, the first one involving *PtIFG 1643* and *AS01H04a* (LG V) and the second one involving *Pg 612F* and *Sb29* (LG XI; Fig. 3). It is possible that these inversions represent artifacts of consensus map construction unless they are confirmed on at least one individual linkage map for each genus. For the first putative case of inversion, the relative position of both anchor markers on the consensus map of *Picea* (genus-level) could not be ascertained at the species level because *AS01H04a* and *PtIFG 1643* could not be mapped together on the same individual linkage map in any of the three *Picea* taxa. For the second putative inversion between *Picea* and *Pinus*, which involved *Pg 612F* and *Sb29*, both anchor markers were positioned in the same order onto one individual linkage map of *P. glauca*. The positioning of both markers on the same individual linkage map in *P. taeda* could confirm that this inversion is not an analytical artifact.

Comparing Picea and P. menziesii Between *Picea* and *P. menziesii*, 20 anchor markers could be compared and 15 were found in synteny, enabling us to identify nine homoeologous LGs (Fig. 3). In addition to previously published anchor markers (Table 4 in Pelgas et al. 2005), 13 new anchor markers could be compared between both genera (Table 3). Out of these, nine markers (*PPA7a*, *Pg 137G09*, *Pg 152A04*, *Sb41*, *Sb34*, *Sb36*, *Pg 200A01*, *Pg 612F*, and *Pg 143D03*) were considered as putative orthologs because of their positioning onto homoeologous LGs (Fig. 3) and because no polymorphisms were observed in their haploid DNA sequences for both genera (Table 3). Analyses of haploid DNA sequences for the remaining four anchor markers (*PAXY219*, *PtIFG 0739*, *Sb42*, and *COMT1*) revealed polymorphisms in either *Picea* or *P. menziesii*, indicating putative paralogy. However, *PAXY219*, *PtIFG 0739*, and *Sb42* were each positioned onto homoeologous LGs between both genera, suggesting that they should correspond to true orthologs (Table 3; Fig. 3). The anchor marker *PAXY219* would be orthologous to the anchor marker *PmIFG 154C01a* of *P. menziesii*. This putatively orthologous anchor marker was discovered by comparison of the haploid DNA sequence of *PAXY219* with sequences from the *P. menziesii* EST database (http://www.almaren.vbi.vt.edu:8080/estap/servlet/ProjectList). Sequence comparisons revealed an identity of 81.4% over a 161 bp stretch with the anchor marker *PmIFG 154C01a*, which was positioned onto the LG 10 of *P. menziesii*, a group found to be homoeologous to *Picea* LG containing *PAXY219* (Table 3; Fig. 3). By contrast, for *COMT1*, the differential positioning onto non-homoeologous LGs between *Picea* (LG XI) and *P. menziesii* (LG 8) is likely the consequence of the positioning of different members of the *COMT* gene family. Consequently, *COMT1* should be considered as paralogous between the two genera. Thus, out of 20 anchor markers available between *Picea* and *P. menziesii*, 19 could be considered as orthologous. One of these, *Sb64*, was previously shown to locate on two LGs in *P. menziesii*

(Krutovsky et al. 2004) while on one LG in *Picea* (LG XII, Fig. 3), it helped us to identify one homoeologous group between the two genera (Fig. 3).

Among these 19 orthologous anchor markers, three were not in synteny between *Picea* and *P. menziesii* (*Pg 612F*, *Sb29*, and *Sb49*). The first two markers were grouped together on a small LG in *P. menziesii* (LG 13), not shared by *Picea* nor *Pinus*, suggesting that LGs 3 and 13 from *P. menziesii* are the result of a chromosomal fission (Fig. 4). The third marker, *Sb49*, which was located on LG XI of *Picea*, was not found on the homoeologous LG 3 of *P. menziesii*. Rather, it was translocated to LG 2 of *P. menziesii*, suggesting a case of inter-chromosomal rearrangement (Fig. 4). For homoeologous LGs with more than two orthologous anchor markers in common between *Picea* and *P. menziesii*, such as LGs V and VI, colinearity was conserved for LG V and one inversion was observed between *Sb41* and *Sb34* on LG VI (Fig. 3). However, to confirm this inversion, additional evidence of the relative positioning of these two markers should be sought at the level of individual linkage maps for each genus. Indeed, *Sb41* and *Sb34* could not be mapped together in the same individual linkage map in the same *Picea* species (*Sb41* was positioned only onto the composite map developed by Acheré et al. (2004) and the information for the marker *Sb34* was only provided by the linkage maps developed by Scotti et al. (2005)) and a similar verification would need to be conducted for *P. menziesii*.

Discussion

P. glauca genetic linkage maps

For *P. glauca*, the construction of individual parental and male reference linkage maps from both crosses with one common parent was based on an approach described by Pelgas et al. (2005) for *P. mariana* × *P. rubens*. The evaluation of synteny and macro-colinearity among all individual and reference linkage maps enabled us to confirm the ordering of anchor markers for all LGs before assembling the composite map representative of each taxon. Another benefit of using two crosses for the assemblage of a composite map is the substantial increase in the number of anchor markers positioned (Sewell et al. 1999; Pelgas et al. 2005). Indeed, for *P. glauca*, the use of a second cross resulted in an average increase of 23% of anchor markers positioned. Out of them, about 65% (SSRs and ESTPs) and 58% (ESTPs only) were useful for interspecific and inter-generic comparisons, respectively (see below). These results are quite similar to those

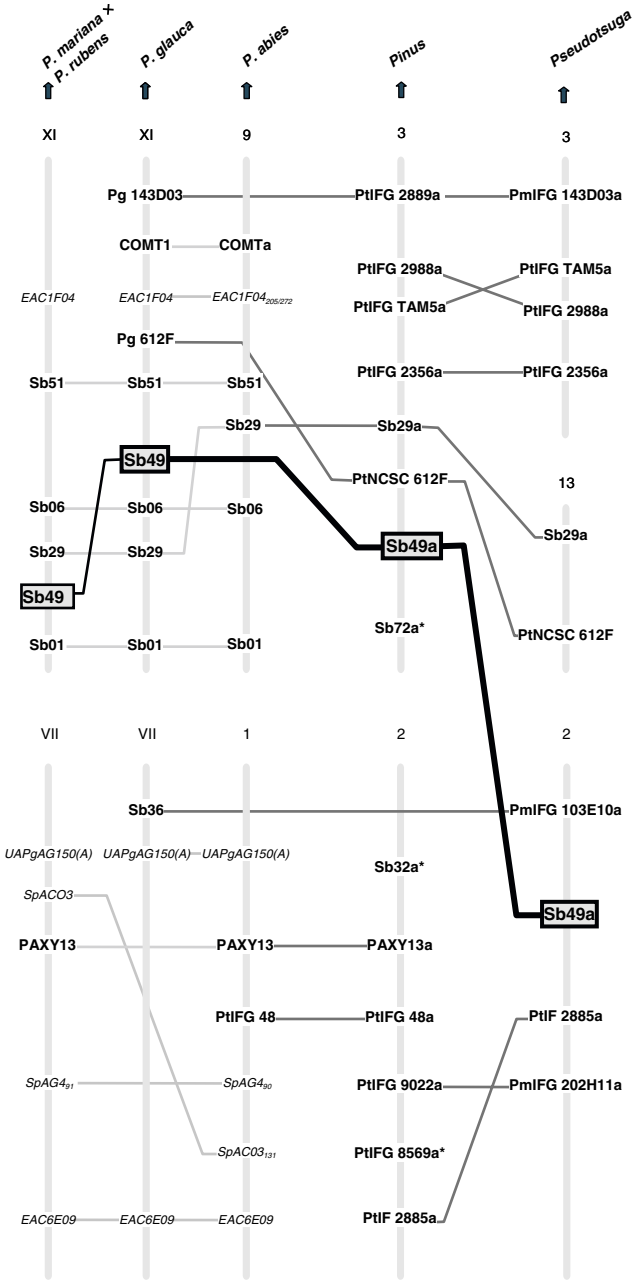


Fig. 4 Schematic representation of homoeologous LGs for three *Picea* taxa (*P. mariana* × *P. rubens*, *P. glauca*, and *P. abies*), for *Pinus* spp. and for *Pseudotsuga menziesii* (adapted from Krutovsky et al. 2005) were involved in putative inter-chromosomal rearrangements in the genome of *P. menziesii*. Only homologous intra- and inter-generic anchor markers are indicated on each schematic LG: in *italics*, microsatellites, and in *bold*, ESTP markers. Markers connected with large *lines* are not syntenic between *Picea/Pinus* and *P. menziesii*. Markers tagged by an *asterisk* are involved in inter-generic comparisons with other LGs than those shown here. Orthologous markers are connected by a *solid line*: *light gray lines* correspond to intra-generic comparisons and *dark gray lines* correspond to inter-generic comparisons. *Arrows* on the *top* indicate the LG nomenclature used for each taxon or genus

obtained for *P. mariana* × *P. rubens* (Pelgas et al. 2005). Moreover, out of 19 new *P. glauca* anchor markers that were useful for inter-generic comparisons, 42% and 26% were only obtained in one or the other of both crosses of *P. glauca*. These results compare well with those obtained from both crosses in *P. mariana* × *P. rubens* (Pelgas et al. 2005), where 18 anchor markers were useful for inter-generic comparisons with *Pinus* or *P. menziesii* with two (11%) and six (33%) obtained from only one or the other of both crosses of *P. mariana* × *P. rubens*. Because these taxa are highly heterozygous for ESTP markers (Perry and Bousquet 1998a, 1998b), the increase in the recovery of useful anchor markers for inter-generic comparisons from the use of a second cross would be even more significant for taxa with reduced levels of heterozygosity.

The average map length (G_F) of individual linkage maps of *P. glauca* was in the same range as that observed for *P. mariana* × *P. rubens* (Pelgas et al. 2005) and slightly smaller than those estimated in previous studies for *P. glauca* and *P. abies* (Gosselin et al. 2002; Acheré et al. 2004). For the composite map of *P. glauca*, G_F was similar to estimated values obtained for *P. mariana* × *P. rubens* and for *P. abies* (Acheré et al. 2004; Pelgas et al. 2005). Except for the male 80109 (F1-2856), the expected map lengths (G_e) of individual linkage maps were in agreement with those of previous studies conducted for *P. glauca* (Gosselin et al. 2002), *P. mariana* × *P. rubens* (Pelgas et al. 2005), and *P. abies* (Paglia et al. 1998). The different expected map length (G_e) obtained for one of the individual linkage maps of male parent 80109 could be attributed to the higher number of LGs obtained for this individual from the cross F1-2856, compared with that obtained from the cross F1-2872. Indeed, the number of LGs directly influences the estimation of G_e. Observed as well as expected map lengths of individual linkage maps of *P. glauca* were smaller for the parents of the cross F1-2872 (Table 1) than for the other cross (F1-2856). In fact, for overall map length values G_F, G_o and G_e, an average reduction of 23% was observed from cross F1-2856 to cross F1-2872. Such variation is comparable to that obtained by analyses of different populations in a same species (e.g., Beavis and Grant 1991; Yogeeswaran et al. 2005). Numerous causes have been proposed to explain such discrepancies, including variation in some genetic or environmental factors affecting recombination frequencies (Liu 1998; de Vienne 2003).

Choice of markers for comparative mapping

For each spruce taxon, composite linkage maps of high density have been obtained essentially with anonymous DNA markers such as AFLPs (this study; Acheré et al. 2004; Pelgas et al. 2005), which are typically difficult to compare across species (Pelgas et al. 2005). Consequently, we sought to position codominant PCR markers such as ESTPs (expressed gene-specific markers) and SSRs to allow for intra-generic or inter-generic map comparisons. However, our own experience from this study and in previous ones (Pelgas et al. 2005) indicates that the transferability of SSR markers across genera is much lower than that of ESTPs. This can be partly explained by the presence of null alleles resulting from the modifications in the repeat or flanking regions of some SSR loci (Peakall et al. 1998; Karhu et al. 2000). These disadvantages should be overcome with the advent of new SSR markers developed from EST libraries (Jany et al. 2003; Chagné et al. 2004; Yu et al. 2004), although the frequency of these markers across expressed regions remains to be determined. Accordingly, only ESTP markers have been used herein for inter-generic comparisons. Therefore, in order to increase the number of inter-generic anchor markers among *Picea, Pinus*, and *P. menziesii*, a number of ESTP markers common to *Pinus* and *P. menziesii* were additionally developed and positioned onto the composite linkage map of *P. glauca*.

Marker orthology in the Pinaceae

The distinction between orthologs and paralogs is fundamental for the success of genome comparative studies (e.g., Gogarten and Olendzenski 1999; Pelgas et al. 2005), which rely greatly on the comparisons of orthologous gene content (synteny) and orthologous gene order (colinearity) between different taxa. However, as indicated previously by Huynen and Bork (1998): "There is not a single, simple, and perfect solution to the question of orthology". To increase primer specificity and reduce the risk of paralogous amplification, most of our primer pairs were designed with a primer matching in the 3′ UTR gene region (e.g., Perry and Bousquet 1998b; Brown et al. 2001). We also relied extensively on sequence comparisons among taxa and on resequencing from haploid megagametophyte DNA, where double peaks on sequence chromatograms should indicate paralogous polymorphisms (e.g., Pelgas et al. 2005).

Interspecific comparisons in the genus *Picea*

The composite maps developed herein for *P. glauca* and *P. abies* allowed us to confirm the positioning of about half of the anchor markers previously positioned

onto the composite map of *P. mariana* × *P. rubens* (Pelgas et al. 2005). The alignment of homoeologous LGs among widely divergent *Picea* taxa revealed many inversions in marker order. However, these inversions, involving tightly linked orthologous markers, were most likely the result of analytical artifacts generated by the integration of different parental maps rather than the consequence of true chromosomal rearrangements. Indeed, among individual linkage maps of each *Picea* taxon, the same discrepancies could be observed. Such a pattern has also been observed in other conifers (e.g., Sewell et al. 1999). Only a larger array of progeny (Liu 1998) as well as a higher number of markers surrounding these markers might help to establish their positioning onto linkage maps.

The location of some of the anchor markers already positioned herein onto the composite map of *P. abies* has been confirmed in a recent study of the genome of *P. abies* (Scotti et al. 2005). This study has resulted in additional comparison points to validate the homoeology of LGs among *Picea* taxa, and with the other Pinaceae genera. Indeed, seven and eight anchor markers positioned onto individual linkage maps of *P. abies* (Scotti et al. 2005) were also positioned onto homoeologous LGs in *P. glauca* and *P. mariana* × *P. rubens*, respectively, including six markers common among the three taxa (Fig. 2). Three of them (*Sb34*, *PtIFG 48*, and *RN01G04*) appeared useful for inter-generic comparisons.

Overall, macro-synteny and macro-colinearity of anchor markers among the three *Picea* taxa appeared relatively well conserved, despite two major differences in synteny. Because lack of synteny or lack of colinearity does not necessarily indicate lack of orthology, further sequence analyses were conducted to confirm the discrepancies observed among the three *Picea* genomes. Sequences from haploid megagametophyte DNA were obtained for the anchor markers *Sb68* and *Ptxmyb413* positioned onto non-homoeologous LGs among taxa. Sequence comparisons revealed that *Sb68* likely represents paralogous gene loci between *P. mariana* × *P. rubens* and *P. abies*, whereas *Ptxmyb413* appears to represent an orthologous gene locus among the three *Picea* taxa. In the latter case, orthology was not in any doubt, even if the targeted gene, *PgMyb4*, belongs to the MYB, a large gene family in the conifers (e.g., Xue et al. 2003) and in *Arabidopsis* (Romero et al. 1998). For the change of synteny involving *Ptxmyb413*, a plausible explanation would imply an inter-chromosomal insertional translocation between LG III and LG IV. It might have occurred in the lineage leading to *P. glauca* or in a common ancestor to the two other taxa because *Ptxmyb413* was positioned on the same homoeologous LG in *P. mariana* × *P. rubens* and *P. abies*. While *Ptxmyb413* could not be placed on the main *P. abies* composite map, such a placement was confirmed in *P. abies* from the analysis of a subset of markers on a second cross (data not shown). Given the large phylogenetic divergence between *P. mariana* × *P. rubens* and *P. abies* (Sigurgeirsson and Szmidt 1993; M. Bouillé and J. Bousquet, unpublished data), it is likely that such a translocation occurred in the lineage leading to *P. glauca*, and not in a common ancestor to *P. mariana* × *P. rubens* and *P. abies*. Such a chromosomal rearrangement could result from various causes, including the presence of transposable elements, which have been shown to decrease chromosome stability (e.g., Zhang and Peterson 1999, 2004; Wicker et al. 2003; Lai et al. 2004). Further positioning of this anchor marker in *Pinus* or in *P. menziesii* would indicate which chromosomal structure is ancestral, that in *P. glauca* or that in *P. mariana* × *P. rubens* and *P. abies*. Results obtained regarding the anchor markers *Sb68* and *Ptxmyb413* well illustrate the utility of analyzing haploid DNA sequences to confirm orthology when a lack of synteny is observed (Pelgas et al. 2005).

A second feature related to the LG III of *P. glauca* and *P. mariana* × *P. rubens* is worth mentioning. The anchor markers positioned onto this LG suggested a segmental duplication, most likely predating the split between the two *Picea* taxa. The two *knox-I* genes *KN1* and *KN2* have been shown to result from a duplication event predating the split between *Picea* and *Pinus* (Guillet-Claude et al. 2004). In the present study, these genes were found to be flanked by two *60S ribosomal protein L15* gene loci (*Sb11* and *Sb62*), suggesting a segmental duplication rather than just a gene duplication. Thus, the duplication event involving *KN1* and *KN2*, which was dated by Guillet-Claude et al. (2004) at 160–171 Mya using rates of nonsynonymous and synonymous substitutions, respectively, would have involved a large chromosome segment also carrying the ribosomal protein locus. It is likely that this segmental duplication is also shared by *Pinus*, as *Picea* and *Pinus* diverged later (Guillet-Claude et al. 2004). Such segmental duplications have been reported in the genomes of Angiosperms (Salse et al. 2004; Mayerhofer et al. 2005).

Inter-generic comparisons in the family Pinaceae

Previous comparisons between the genome of *P. mariana* × *P. rubens* and those of *Pinus* spp. and *P. menziesii* (Pelgas et al. 2005) were considered as a useful starting point for this study. With the help of the new composite maps for *P. glauca* and *P. abies* developed herein, additional comparisons could be made among the genomes

of the three conifer genera, resulting in a doubling of the number of comparison points. Chromosome homoeology between *Picea* and *Pinus* previously reported by Pelgas et al. (2005) was confirmed for nine LGs (I, V, VI, VII, VIII, IX, X, XI, and XII) using the new composite maps of *P. glauca* and *P. abies*. Moreover, the anchor markers positioned onto the LG II of *P. glauca* and the LG IV of both *P. glauca* and *P. abies* allowed us to detect homoeology with LG 5 and LG 12 of *Pinus*, respectively. Thus, homoeology could be established for 11 of the 12 chromosomes between *Picea* and *Pinus*. As for *P. menziesii*, homoeology with *P. mariana* × *P. rubens* was previously reported by Pelgas et al. (2005) only for four chromosomes, involving respectively LGs V, VI, XI, and XII of *P. mariana* × *P. rubens* and LGs 10, 8, 13-2, and 9 of *P. menziesii*. These homoeologies were confirmed by the new composite maps of *P. glauca* and *P. abies*. These maps further allowed us to establish five new or additional homoeologies for LGs II, VII, VIII, IX, and XI of *Picea* with LGs 5, 2, 4, 6, and 3 of *P. menziesii*, respectively.

Out of the 29 anchor markers that could be compared between *Picea* and *Pinus*, three were not found in synteny (*Sb41, Sb72*, and *PtIFG 8569*). For the two first markers, DNA sequencing from a haploid megagametophyte revealed polymorphisms, suggesting potent cases of paralogy. For the third marker, no polymorphism could be detected from such sequencing and nucleotide identity was high between *Picea* and *Pinus*, suggesting orthology and a potential case of inter-chromosomal translocation. However, the marker *PtIFG 8569* might also be targeting paralogous gene loci between *Picea* and *Pinus*, because it corresponds to a highly conserved area of the *alpha tubulin* gene family. Indeed, nucleotide identity varies between 77.5 and 98.6% among six different *alpha tubulin* genes of *Arabidopsis* (*TUA1, TUA2, TUA3, TUA4, TUA5* and *TUA6*), suggesting that the identity value of 96.2% obtained between the *Picea* and *Pinus* gene sequences might indicate paralogy. Supplementary analyses of DNA sequence identity over the entire length of the genes or at least over more variable parts should be undertaken in conjunction with phylogenetic analyses to confirm marker orthology and the potential inter-chromosomal translocation depicted by this marker. Such analyses are particularly recommended when gene markers target conserved domains of different members of a gene family (Huynen and Bork 1998; Salse et al. 2002; Delseny 2004). As for colinearity, it was relatively well conserved between *Picea* and *Pinus*. At this time, we cannot ascertain that the two proximal inversions observed between *Picea* and *Pinus*, one between LGs V and 10, and another one between LGs XI and 3, reflect true chromosomal rearrangements rather than analytical artifacts. Similarly, for the proximal inversion in the marker order observed between *Picea* and *P. menziesii* on LG VI, it is likely to result from the merging of information collected from the diverse maps developed in the genus *Picea*. To confirm these various inversions, densifying genetic maps or the positioning of these pairs of anchor markers on the same individual linkage map would need to be pursued, at least in *Picea*.

Out of the 20 anchor markers that could be compared between *Picea* and *P. menziesii*, sequence analyses revealed only one case of potent paralogy for *COMT1*. Among the remaining orthologous markers, three were not in synteny (*Pg 612F*, *Sb29*, and *Sb49*). The positioning of *Pg 612F* and *Sb29* on a separate linkage group (LG 13) in *P. menziesii* confirms that a chromosomal fission has presumably occurred in this species (Krutovsky et al. 2004; Pelgas et al. 2005). Indeed, LGs 3 and 13 of *P. menziesii* were found to correspond to LG XI of *Picea*, for which several orthologous anchor markers were available. This chromosomal fission may have played a central role in generating the difference in basic chromosome number between *P. menziesii* ($n = 13$) and the other Pinaceae ($n = 12$). Another difference was detected between *Picea* and *P. menziesii* and also involved LG XI of *Picea*. The marker *Sb49* was found to be translocated to LG 2 of *P. menziesii*, which is homoeologous to LG VII of *Picea* (Fig. 4). This inter-chromosomal rearrangement suggests an insertional translocation from LG 13 to LG 2 of *P. menziesii*. Thus, this change of position of *Sb49* would not result directly from the chromosomal fission discussed above, as suggested earlier based on a more limited number of anchor markers (Pelgas et al. 2005). This translocation suggests that LG 13 of *P. menziesii* might be unstable. Altogether, these various changes indicate that much instability has affected the ancestral Pinaceae chromosome corresponding to LG XI of *Picea* and LG 3 of *Pinus* and *P. menziesii*, particularly in the lineage leading to *P. menziesii*.

No other inter-chromosomal rearrangements could be safely inferred among the three Pinaceae genera because most cases of break in synteny could be explained by paralogy of the anchor markers involved. Consequently, when considering the actual data, the macro-structure of the three Pinaceae genomes appears relatively similar. Such a trend is surprising for the Pinaceae family where the split among the major lineages, including the genera *Pinus* and *Picea*, is thought to have happened as early as the beginning of the Cretaceous (Florin 1963; Miller 1976, 1977). Similar stability in genome macro-structure appears to be rare in the

Angiosperms, even among plant genera with reportedly much more recent divergence. Although no major rearrangements were reported between the oak and the chestnut genomes, which may have diverged about 60 Myr ago (Casasoli et al. 2006), about 90 chromosomal rearrangements were inferred between *Brassica nigra* and *A. thaliana*, during a period of about 10 to 35 Myr since divergence (Lagercrantz 1998). Between maize and sorghum, which diverged between 12 and 20 Myr ago, nine chromosomal rearrangements were inferred (Whitkus et al. 1992; Gaut and Doebley 1997; Swigoňová et al. 2004). Our observations suggest that the genome macro-structure of the Pinaceae looks much the way it used to more than 100 Myr ago, at the time of the split between *Pinus* and the other major lineages of the Pinaceae.

Prospects

Inferences about the apparent macro-colinearity observed among the various Pinaceae genomes compared herein should not be extended to the level of intervening genes. As suggested from genome comparisons between some monocots and dicots, apparent regions of colinearity may reflect the presence of several smaller gene regions that have been reshuffled by intra-chromosomal inversions (Paterson et al. 1996). Recent studies with a high number of gene-specific markers reported that micro-rearrangements have occurred between regions that appeared essentially colinear at the macro-level (e.g., Kurata et al. 1994; Choi et al. 2004; Lai et al. 2004; Peng et al. 2004). A major difficulty for such a more in-depth evaluation of synteny and colinearity among conifer genomes resides in the limited number of gene-specific markers and the difficulty to define orthologous gene loci in the presence of gene families. To further investigate genome structure at a finer scale, hundreds of additional conserved orthologous markers will be necessary (e.g., Salse et al. 2004). The use of single nucleotide polymorphisms (SNP) of orthologous genes appears promising, with the advent of high-throughput SNP genotyping technologies (Bell et al. 2002; Shen et al. 2005) and with the recent availability of extensive collections of gene sequences in the genera *Picea* (Pavy et al. 2005) and *Pinus* (Egertsdotter et al. 2004). However, in spite of such progress, the identification of orthologous gene sets will remain a main challenge. Indeed, plant gene families are large and sequence homology varies extensively between members of a same gene family (e.g., Guillet-Claude et al. 2004), a problem encountered often in setting the criteria for the definition of gene contigs from large-scale EST sequencing projects (Pavy et al. 2005). Complete sequencing of small regions of the genome might help us get around this problem and contribute to a better evaluation of the stability of the genome micro-structure in the Pinaceae.

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