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Comparing EST-based genetic maps between *Pinus sylvestris* and *Pinus taeda*

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Abstract A genetic map of *Pinus sylvestris* was constructed using ESTP (expressed sequence tag polymorphism) markers and other gene-based markers, AFLP markers and microsatellites. Part of the ESTP markers (40) were developed and mapped earlier in *Pinus taeda*, and additional markers were generated based on *P. sylvestris* sequences or sequences from other pine species. The mapping in *P. sylvestris* was based on 94 F_1 progeny from a cross between plus-tree parents E635C and E1101. AFLP framework maps for the parent trees were first constructed. The ESTP and other gene sequence-based markers were added to the framework maps, as well as five published microsatellite loci. The separate maps were then integrated with the aid of AFLPs segregating in both trees (dominant segregation ratios 3:1) as well as gene markers and microsatellites segregating in both parent trees (segregation ratios 1:1:1:1 or 1:2:1). The integrated map consisted of 12 groups corresponding to the *P. taeda* linkage groups, and additionally three and six smaller groups for E1101 and E635C, respectively. The number of framework AFLP markers in the integrated map is altogether 194 and the number of gene markers 61. The total length of the integrated map was 1,314 cM. The set of markers developed for *P. sylvestris* was also added to existing maps of two *P. taeda* pedigrees. Starting with a mapped marker from one pedigree in the source species resulted in a mapped marker in a pedigree of the other species in more than 40% of the cases, with about equal success in both directions. The maps of the two species are largely colinear, even if the species have diverged more than 70 MYA. Most cases of different locations were probably due to problems in identifying the orthologous members of gene families. These data provide a first ESTP-containing map of *P. sylvestris*, which can also be used for comparing this species to additional species mapped with the same markers.

Keywords *Pinus sylvestris* · *Pinus taeda* · ESTP · AFLP · Genetic mapping

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Introduction

Genetic maps consisting of orthologous markers are valuable tools for the study of genome evolution and for comparative quantitative trait locus (QTL) mapping. Comparative maps have shown that the rate of chromosomal evolution varies widely among different groups of organisms. For instance, gene orders of *Brassica* species and *Arabidopsis* are significantly different after divergence of 10–15 MYA (Lagercrantz 1998), whereas chromosomes of the species of the genus *Pinus* and the whole Pinaceae family seem to evolve very slowly (Prager et al. 1976; Devey et al. 1999; Brown et al. 2001). All pine species have 12 chromosomes with generally similar morphology (Sax and Sax 1933), but the genome size varies between the species (Wakamiya et al. 1993). More detailed karyotypes have only recently become available, and will allow improved comparisons between species (Doudrick et al. 1995; Hizume et al. 2002). Comparative mapping will also provide better understanding of genome evolution. The maps will also allow transfer of mapping and quantitative trait locus (QTL) mapping information across species. This can be

especially important when there are several related commercially important species, with small research communities, such as for conifers (Brown et al. 2001). One example is the comparison of wood quality QTLs between *Pinus taeda* L. and *Pinus pinaster* Ait. (Chagné et al. 2003).

Conifer genomes are large, ranging from 20 to 32 pg (Wakamiya et al. 1993), and are known to contain much repetitive DNA (Kriebel 1985) and to harbor large complex gene families (Kinlaw and Neale 1997). The earliest partial genetic maps of conifers were made using isozymes (e.g. Rudin and Ekberg 1978). The number of loci was low, but orthologous loci could usually be identified in different species without problems (e.g. Conkle 1981). This early work already suggested high genome conservation, synteny and colinearity between species (Conkle 1981). Restriction fragment length polymorphisms (RFLPs) were used to generate the first high coverage maps for *P. taeda* (Devey et al. 1994; Groover et al. 1994) and *Pinus radiata* (Devey et al. 1996). RFLPs have also allowed comparative mapping (Devey et al. 1999), even if many probes hybridize with multiple genes, causing problems in identifying orthologs. Anonymous markers such as RAPDs (Yazdani et al. 1995) and AFLPs (Remington et al. 1999; Lerceteau et al. 2001) do not reliably identify orthologous loci between species, and there may even be problems within species (Lu et al. 1995; Hurme and Savolainen 1999). Thus, they do not provide a foundation for comparative mapping. Microsatellites (SSRs) share some of the same problems of ortholog identification (Echt et al. 1999). Further, they also occur as gene families (Kostia et al. 1995; Karhu et al. 2000; Elsik and Williams 2001). Recent efforts have focused on finding markers based on sequences in coding regions, which also distinguish between members of gene families (Harry et al. 1998; Perry and Bousquet 1998; Cato et al. 2001; Temesgen et al. 2001). Such markers will be ideal for comparative mapping.

Pinus sylvestris L. (Scots pine) has the widest distribution of all pines, ranging from Spain (38°N) to northern Finland (70°N), and from Scotland (6°W) to Siberia (135°E). Because of its ecological and economical importance, its genetics has also been widely studied. It is thus important that tools are developed to transfer some of this information to other species, and from other species to *P. sylvestris*. Across the wide distribution, the species has adapted to a very broad range of environmental and especially climatic conditions, with strong genetic differentiation between populations (Mikola 1982; Hurme et al. 1997). It would clearly be interesting to compare the locations of QTLs for such adaptations across species to ascertain whether the same loci govern the adaptations in *P. sylvestris* (Hurme et al. 2000) and in other conifer species, such as in *Pseudotsuga menziesii* (Jermstad et al. 2001).

We generate here first a map of *P. sylvestris* and then compare it to the map of *P. taeda* (Brown et al. 2001), which has emerged as the reference species for pine comparative mapping. The genus *Pinus* appeared more than 100 MYA ago and diverged into hard (subgenus *Pinus*) and soft pines (subgenus *Strobus*) about 70 MYA ago. Both *P. taeda* and *P. sylvestris* belong to the subgenus *Pinus. P. taeda* is a member of subsection *Australes*, whereas *P. sylvestris* belongs to subsection *Pinus* (Price et al. 1998). The early isozyme maps of *P. taeda* (Conkle 1981) and *P. sylvestris* (Rudin and Ekberg 1978; Niebling et al. 1987; Szmidt and Muona 1989) show similar linkage for the few locus pairs which could be compared. Thus, our expectation is that we will find general conservation of gene order. The more recent RAPD (Yazdani et al. 1995; Hurme et al. 2000) and AFLP maps (Lerceteau et al. 2001) have so far not allowed even comparing maps of individual trees. Here we provide a map of *P. sylvestris* based on polymorphisms in ESTs or other well defined genetic loci, which were placed in a framework map of AFLPs. The set of markers was designed from both *P. taeda* and *P. sylvestris*. The overlapping set of 44 mapped gene markers in the two species allows identification of homologous linkage groups and will serve as a valuable tool in many comparative studies. This effort is part of a larger international project for ESTP-based comparative maps in the most important conifer species (http://dendrome.ucdavis.edu/Synteny). Such markers and maps are a shared tool for all pine geneticists, and provide a means to transfer information across different species. This shared public resource will also facilitate the access to genomes of pine species where large research efforts have so far not been initiated.

Materials and methods

Mapping population

P. sylvestris mapping was based on a F_1 progeny population from a cross between trees E635C and E1101 (a two-generation outbred pedigree, Finnish Forest Research Institute). Needle tissue DNA was isolated from 94 progeny of the cross with the DNeasy Plant Mini Kit (Qiagen). *P. taeda* mapping was based on two reference mapping populations, referred to as *base* and *qtl* pedigrees (http://dendrome.ucdavis.edu/Synteny/refmap.html) and the *prediction* pedigree of the Weyerhaeuser Company (Brown et al. 2001).

AFLP markers

AFLP markers for the *P. sylvestris* mapping population were generated as follows. Templates for AFLP reactions were prepared by using 500 ng of needle DNA for digestion with *Eco*RI and *Mse*I, and for ligation of adapters (Vos et al. 1995). The restriction/ligation mix was diluted 1:10. Preamplification using *Eco*RI (E) and *Mse*I (M) primers with selective nucleotides E + AC and M + CC was carried out with modifications of Remington et al. (1999). The 30 μl reaction mixture contained 3 μl of diluted restriction/ligation mix, 2 U of *Taq* polymerase (Boehringer Mannheim), 50 ng of E primer, 50 ng of M primer, 10 mM of Tris-HCl, pH 8.3, 1.5 mM of $MgCl_2$, 50 mM of KCl and 0.2 mM of each dNTP. PCR conditions were as described in Myburg et al. (2001). Preamplification products were diluted 1:40 with water.

Selective amplifications were done with combinations of E primers with three selective nucleotides and M primers with four selective nucleotides (E + 3/M + 4) from Remington et al. (1999)

Table 1 Numbers of segregating AFLP fragments by primer combination in *P. sylvestris* F_1 offspring from the cross between E635C and E1101

*Eco*RI primer	*Mse*I primer	Number of segregating fragments
ACA	CCAG	8
	CCCG	19
	CCGC	22
	CCGG	27
ACG	CCAA	42
	CCAC	32
	CCAG	19
	CCCA	28
	CCGA	23
	CCGC	16
	CCTA	25
	CCTC	20
	CCTG	27
	CCTT	34
ACT	CCCG	22
	CCGC	11
	CCGG	16
	CCTG	41
	Total	432

(Table 1). The reaction mixtures and PCR conditions were as described in Myburg et al. (2001) with single dye reactions.

AFLP fragments were resolved on polyacrylamide gels with a 4200 LI-COR automated DNA sequencer and AFLP images scored using the AFLP-Quantar software program (version 1.5, KeyGene Products B.V. Wagenigen, The Netherlands) following Myburg et al. (2001).

Microsatellites

Four *P. taeda* microsatellite markers, PtTX2123, 2146, 3013 and 3025 (Elsik et al. 2000), were analysed in the F_1 offspring. The protocols were first optimised for *P. sylvestris*. One published *P. sylvestris* microsatellite, spac7.14 (Soranzo et al. 1998), was also scored after minor modification. Microsatellite fragments labeled with fluorescent dyes were resolved on polyacrylamide gels using an ABI 377 sequencer, and the sizes of the fragments were determined with GeneScan and Genotyper software packages (Applied Biosystems) according to the manufacturer's instructions.

ESTPs and other gene sequence based markers

Genetic markers were developed from coding gene regions for comparative mapping. Sequence data from *P. taeda* EST sequencing (http://pinetree.ccgb.umn.edu) and from GenBank were used for designing primers. Polymorphism detection was done by denaturing gradient gel electrophoresis (DGGE) (Meyers et al. 1985) and single strand conformational polymorphisms (SSCP) techniques (Orita et al. 1989), and using restriction digests with endonucleases (PCR-RFLPs).

DGGE

The ESTPs developed from *P. taeda* cDNA libraries (Brown et al. 2001; Temesgen et al. 2001) were tested for amplification and segregation in the *P. sylvestris* offspring. PCR conditions were as in Harry et al. (1998), and screening for polymorphisms was with DGGE (Brown et al. 2001; Temesgen et al. 2001).

SSCPs and PCR-RFLPs

SSCP and PCR-RFLP markers were first generated for the *P. sylvestris* F_1 population. Those segregating in *P. sylvestris* offspring were also tested for amplification and segregation in *P. taeda* pedigrees (Table 2). GenBank was searched for *Pinus* cDNA, genomic and EST sequences, preferably representing single copy genes or genes belonging to low copy families. Sequences were obtained from *P. sylvestris, P. taeda* or *P. strobus*. In some cases, both primers were placed in exons, without information on intron locations. Alternatively, the forward primer was placed in an exon and the reverse primer in the 3′-untranslated region (UTR). The latter method has proved to be a useful source of well defined markers which distinguish between gene family members (Campbell 1986; Perry and Bousquet 1998; Cato et al. 2001; Temesgen et al. 2001). PCR amplifications were done in 15 or 25 µl, containing 0.2 µM of each primer, 1/10 volume of 10× reaction buffer, 200 µM of each dNTP and 1 U of *Taq* DNA polymerase. The amount of template DNA was 20 ng with the Gibco-BRL *Taq* polymerase, and 10 ng with Dynazyme EXT-enzyme (Finnzymes) and Ampli*Taq* Gold-enzyme (PE Applied Biosystems). The PCR program for the genes optimized with Gibco-BRL *Taq* DNA polymerase was: 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, 46–70 °C for 1 min and 72 °C for 1 min. Final extension was 72 °C for 10 min. For the genes optimized with Dynazyme EXT-enzyme (Finnzymes) the program was: 92 °C for 3 min, followed by 38 cycles of 92 °C for 1 min, 49–52 °C for 1 min 30 s, 72 °C for 2 min 30 s, and the final extension was 72 °C for 20 min. For the genes optimized with Ampli*Taq* Gold-enzyme (PE Applied Biosystems) the program was: 94 °C for 12 min, 38 cycles 94 °C for 1 min, 54–60 °C for 1 min 15 s, 72 °C for 1 min 45 s, and the final extension was 72 °C for 10 min.

After optimization of PCR conditions, variation between the parents was first detected by sequencing parental gene products, or with the SSCP technique as in Plomion et al. (1999), except that glycerol was added to the gels (5%) to better resolve the banding pattern.

PCR-RFLPs were generated by sequencing parental DNA for PCR products and searching for segregating restriction sites for available endonucleases. Digestion of PCR products of the parents and the progeny was done in 15 µl, containing 7 µl of PCR product, 3 U of restriction enzyme and 1× restriction buffer (Boehringer Mannheim).

Linkage analysis

First, linkage analysis for *P. sylvestris* was done separately for both trees. AFLPs segregating 1:1 were used to make framework maps with MAPMAKER version 2.0 for Macintosh (Lander et al. 1987), following the methodology of Plomion et al. (1995) and Remington et al. (1999). Grouping of AFLPs was done with LOD threshold 6.0. Framework maps were constructed for markers with a minimum log likelihood difference of 3. Linkage groups consisting of framework markers linked with LOD threshold 4 were merged. AFLPs deviating from 1:1 segregation at significance level $\alpha = 0.01$ were excluded from the analysis, whereas markers segregating at the level $\alpha = 0.05$ were included and only dropped if they mixed the orders of other markers in the group. Gene markers and microsatellites segregating 1:1 and 1:1:1:1 (coded as 1:1 for both trees separately) were located in relation to the AFLP markers.

Separate maps were integrated with the aid of 79 AFLPs segregating in both trees (dominant segregation ratios 3:1) as well as nine gene markers (six segregating 1:1:1:1 and three 1:2:1 ratios) and two microsatellites segregating 1:1:1:1 using JoinMap (Stam 1993). In the integration of the maps the orders of the 1:1 markers given by MAPMAKER were kept fixed. The Kosambi mapping function was used in all linkage analyses with MAPMAKER and JoinMap. Maps of linkage groups were drawn with MapChart (Voorrips 2001).

Table 2 Gene markers generated and mapped in *P. sylvestris* F_1 offspring from the cross between E635C and E1101, their position in linkage groups corresponding to *P. taeda*, and amplification and segregation in *P. taeda base* and *qtl* pedigrees

ESTP markers[a]	LG[b]	Function	Accession number	Species, type of sequence	PCR primers	Annealing temp. (°C), $MgCl_2$ (mM)[f]	Exp. product size/obs. size in *P. sylv.*/obs. size in *P. taeda*[i]
PsUF1_CHS	2	Chalcone synthase	X60754	*P. sylvestris* genomic	U ACTCCCCCTAATGCGGTTGA	Ps 64, 2.0	370/370/370
					L CTTGGCTGCGGCTTCTTTC	Pt 60, 1.5	
PtNCS_1C3A	2	Putative fruktokinase	AA556405	*P. taeda* EST (124/4.0E-05)	U TGCAATTACGGTTACTGAGAG	Ps 56, 2.0[g]	307/307/307[j]
					L ATTTCTTGACACCCGAATAGG	Pt 53, 2.0[g]	
PtTX3_lp3-1	Ps 2[c]	Water-stress inducible protein	U52865	*P. taeda* cDNA	U GGAGGAGAAACAGCACCAC	Ps 62, 2.0	716/716/716[j]
	Pt 5[c]				L CGGAAATCACACGAAAAGAA	Pt 57, 1.5	
PtNCS_p14A9	3	Arabinogalactan-like protein	U09556	*P. taeda* cDNA	U GAAACCCGGGGCTCTGAACT	Ps 56, 1.0	455/455/455
					L CCGCAGCGAGGAAAACAA	Pt 54, 1.5	
PtIPST_pLP2	3	Root-specific S-adenosyl methionine synthetase like protein	Ref.[e]	*P. taeda* cDNA	U GATTTGCCGTGCTGTTTGCT	Ps 58, 1.5	631/1,400/1,400[j]
					L TCGTGCTGAGTGGAGATGAGAA	Pt q 68, 2.0 Pt b 70, 2.0	
PstASU_APX	4	Ascorbate peroxidase	AF326783	*P. strobus* cDNA	U GGCTGCTGGAACCCATCA L GACGTCCATGCACCTTCAAA	Ps 63, 2.0 Pt 63 1.5	304/2,500/2,500[j]
PsTUB_GapC1	4	Glyceraldehyde-3-phosphate dehydrogenase	L07501	*P. sylvestris* cDNA	U CAGATTTCATTGGGGACAGTC	Ps 56, 2.5[g]	311/530/530[j]
					L AAGGCATGCGTTTCTTTACAT	Pt q 54, 1.0[g] Pt b 60, 1.5[g]	
PtMTU_lpPAL	6	Phenylalanine ammonialyase	U39792	*P. taeda* genomic	U TAGCCAAGAAAACCCTGAG	Ps 54, 2.0	452/452/452
					L ACTGATAGCGTCGTAAACCA	Pt 60, 1.5	
PtMTU_lp4CL-2	7	4-coumarate: CoA ligase	U39404	*P. taeda* genomic	U CCCCGTCAAATCTGGCTCCT L GGGCGCTTACTCTGCACCAC	Ps 68, 1.5 Pt 68, 2.0	1,127/1,400/1,400
PsARI_dehydrin	8	Dehydrin	AJ289610	*P. sylvestris* cDNA	U CTTCACCGCACCCACAGTT L CCGTCGGCTCACATTCATAA	Ps 57, 1.0 Pt –	685/1,020/–
PtNCS_HLH1	8	Helix-loop-helix protein 1A	AF103808	*P. taeda* genomic	U ACAGTTTTGGCACCTCTCA	Ps 58, 1.5	851/851/851[j]
					L ATTCTTTTGCACGCTTTCTT	Pt 54, 1.5	
PtWS1_PCBER-PT1	8	Phenylcoumaran benzylic ether reductase PT1	AF242490	*P. taeda* cDNA	U GAGTTCGGGAATGATGTTGA	Ps 56, 1.0	565/565/–
					L CGACGGTGGTGTATTTCACA	Pt –	
PsUPS1_PHY	8	Phytochrome P	X96738	*P. sylvestris* cDNA	U AAGCAGCACTAATACTAACAC L ATCAGTCTCCTCTAACAGTTTCCG	Ps 49, 2.0[h] Pt –	3,083/3,083/–
PtNCS_1CA4G	8	Putative thymidylate synthase	AA556223	*P. taeda* EST (177/2.0E-12)	U TAAATGAGGTGCTCTTACAA	Ps 48, 1.5	342/342/342[j]
					L GCAAACTTCTAGCCACTTA	Pt 46, 1.5	

Table 2 (continued)

ESTP markers[a]	LG[b]	Function	Accession number	Species, type of sequence	PCR primers	Annealing temp. (°C), $MgCl_2$ (mM)[f]	Exp. product size/obs. size in *P. sylv.*/obs. size in *P. taeda*[i]
PsUOU_adhB	9	Alcohol dehydrogenase	–	*P. sylvestris* genomic	U CAGTGTATAGTGTTTTATCCC L GAAAGGAAATTCTTCAGTATC	Ps 52, 2.5[h] Pt 50, 2.5[h]	466/466/460[j]
PsUOU_adhC	9	Alcohol dehydrogenase	–	*P. sylvestris* genomic	U GTGGGGGAGCAGTATTCTC L GTACTTCAGCTCAAGGAAAA	Ps 54, 2.5 Pt 59, 2.0	701/701/701
PsUOU_adhF	9	Alcohol dehydrogenase	–	*P. sylvestris* genomic	U TGTTAGACATCTGACCTGGTG L GTTGCTAAAACTGAGTGTGGT	Ps 58, 2.0 Pt 56 2.5	888/888/700
PsUOU_adhH	9	Alcohol dehydrogenase	–	*P. sylvestris* genomic	U TGAAATGGCTGATACTGCTTT L GTTGCTAAAACTGAGTGTGGT	Ps 50, 3.0[h] Pt 50, 2.0[h]	1,965/1,965/2,200
PtNCS_ptCadA	9	Cinnamyl alcohol dehydrogenase	Z37991	*P. taeda* cDNA	U ACGTGACGGTTATCAGTTC L AAGACTTGCCATTGGATTA	Ps 59, 2.0 Pt 58, 2.0	686/2,040/2,400[j]
PsUMU_plNEab21	9	LHC1 type II chlorophyll a/b binding protein	X58516	*P. sylvestris* cDNA	U GTAGGCAGGGCTGTGAGT L TACCCAACATCAGTTCCAGTC	Ps 56, 2.5[g] Pt 53, 2.0[g]	482/780/780[j]
PsUPS2_PST13	10	Superoxide dismutase (chloroplastic form)	X58579	*P. sylvestris* cDNA	U TCCGTTTGACAGGATTGACT L CCCCAGGTCATCCTCTAACT	Ps 60, 2.0 Pt 62, 2.5	284/950/950[j]
PtNCS_4C1C	12	Putative phosphofructo-kinase	AA556465	*P. taeda* EST (255/8.0E-22)	U GCAAATGCAAAAACCATAAC L AAAATAAGCAGGCAAAATGAC	Ps 56, 2.5[g] Pt 53, 2.0[g]	431/500/500[j]
PsUF2_NIR	E1101a[d]	Nitrite reductase	X74949	*P. sylvestris* cDNA	U AAAGCAAGAGCCCTGAAAAT L CCTGATGGCTCCAAAGTG	Ps 57, 2.0 Pt 58, 1.5	303/303/303

[a] ESTP markers are given with their source fields and clone names
[b] Location corresponding to *P. taeda* linkage groups. Denoting with [c]refers to location in different linkage groups of lp3-1 in *P. taeda* (Pt 5 = linkage group 5) and *P. sylvestris* (Ps 2 = linkage group 2). The *P. sylvestris* group denoted with [d]had no correspondence to *P. taeda* linkage groups
[e] Reference: Chang et al. 1996
[f] Annealing temperatures and $MgCl_2$ concentrations of PCR amplifications for *P. sylvestris* (Ps) and *P. taeda* (Pt). Conditions for the *P. taeda* qtl (q) and base (b) pedigrees are given separately in the cases of different optimums. Amplifications with another enzyme than Gibco-BRL *Taq* DNA polymerase are denoted with [h] for Ampli*Taq* Gold (PE Applied Biosystems) and [g] for Dynazyme EXT (Finnzymes)
[i] Product lengths (bp) in PCR amplification: expected lengths on the basis of GenBank sequences/observed PCR product lengths in *P. sylvestris*/observed PCR product lengths in *P. taeda*. Markers denoted with [j]segregated in *P. taeda*. In *P. taeda* all markers were SSCP markers, in *P. sylvestris* three markers (clones 1C3A, 4C1C and plNEab21) were PCR-RFLPs, all other markers were SSCPs

Nomenclature and informatics

Locus nomenclature is according to the guidelines of the TreeGenes database (http://dendrome.ucdavis.edu/TreeGenes). Markers are defined by the experiment, source field, accession number field and the locus identifier. The ESTP markers developed and mapped in *P. taeda* (Brown et al. 2001) have full names such as IFGTXS_estPtIFG_107_a for ESTP marker 107, and those also mapped in *P. sylvestris* as UOUPSY_estPtIFG_107_a. For brevity, in the map the markers are denoted with the accession numbers and the locus identifiers only (e.g. 107_a). ESTP markers developed in our laboratory or in INRA, France, are given with their source fields, clone names (that is the accession numbers) and the locus identifiers in Table 2. Other ESTPs developed in other laboratories have experiment fields and source fields denoted in the map. RFLP markers in the *P. taeda* map are denoted with accession number fields and locus identifiers (e.g. 2819_12 is shortened from the full name IFGTXS_PtIFG_2819_12), and enzyme loci with their abbreviations (Brown et al. 2001).

For AFLP markers the nomenclature omits the experiment and source fields (e.g. acg/ccag_225 is an abbreviation of UOUPSY_aflp_acg/ccag_225). For microsatellites we use the original nomenclature (e.g. PtTX3025 and spac 7.14).

Homology assessment by sequencing

For loci that mapped to different locations or amplified different numbers of fragments between the two *Pinus* species, homology was assessed by sequencing. The PCR fragments were sequenced directly from purified PCR products when possible. If several fragments were observed either on agarose gels or by direct sequencing, the PCR products were cloned with the TOPO TA cloning Kit (Invitrogen) and then sequenced (BigDye, PE Applied Biosystems). All sequencing reactions (both PCR fragments and clones) were sequenced from both strands and three clones were sequenced to eliminate PCR errors. Sequences were run with PE Applied Biosystems ABI PRISM 377 (×l96), and edited and aligned with Sequencher-software. The nucleotide differences were calculated with the DnaSP program (ver. 3.53) (Rozas and Rozas 1999).

Results

Single locus polymorphism and analysis of Mendelian segregation

AFLPs

The total number of segregating AFLPs was 432 (see Table 1). Of these, 353 were segregating 1:1 (172 segregating in tree E1101 and 181 in E635C) and 79 segregating 3:1 (heterozygous in both trees). In E1101, 16 (9%) of the 1:1 markers deviated from the 1:1 ratio at the level $\alpha = 0.01$ and were discarded from the linkage analysis. In E635C the number of discarded markers was 6 (3.3%). Altogether 331 testcross markers (1:1), 156 in E1101 and 175 in E635C, were used for linkage analysis (Table 3).

Table 3 Numbers of all marker types in *P. sylvestris* F_1 offspring from the cross between E635C and E1101

Item	Total	E1101	E635C	Both parents
AFLPs	**410**	**156**	**175**	**79** (3:1 segregation)
Coding areas	**63**	**38**	**34**	**9**
SSCPs	20	10	11	1
PCR-RFLPs	3	1	2	–
ESTPs	40	27	21	8
Microsatellites	**5**	**3**	**4**	**2**

Microsatellites

All five microsatellite loci segregated in a regular Mendelian fashion, two of them in both parents (Table 3). The number of alleles at these two loci was four at spac7.14, three at PtTX2146 and two alleles in a testcross configuration at the remaining loci.

Gene markers from the P. taeda map: ESTPs

Altogether 90 ESTs developed for *P. taeda* were previously checked for amplification and 45 were determined to segregate in the *P. sylvestris* pedigree (Brown et al. 2001). Only 38 ESTPs were used in this study, as the remaining seven ESTPs did not yield high enough quality banding patterns. These 38 resulted in 40 markers that were analyzed in the F_1 population, as one primer pair amplified three segregating fragments in *P. sylvestris*. Of these, 27 were segregating in E1101 and 21 in E635C. Eight markers were heterozygous in both trees, three of which were segregating 1:2:1.

Gene markers developed for P. sylvestris: SSCPs, PCR-RFLPs

Altogether 90 primer pairs were designed, of which 60 resulted in PCR success and 24 (27%) segregated in the progeny (Table 4). This includes some genes for which

Table 4 Success of marker development in *P. sylvestris*, and marker transfer between *P. sylvestris* and *P. taeda*

Marker origin – species tested	Number of loci studied	Number (percentage) of loci amplifying	Number (percentage) of loci segregating
P. sylvestris-P. sylvestris[a]	90	60 (67%)	24 (27%)
P. taeda-P. sylvestris[b]	90	77 (86%)	40 (44%)
P. sylvestris-P. taeda (qtl)[c]	23	19 (83%)	10 (43%)
P. sylvestris-P. taeda (base)[c]	23	19 (83%)	7 (30%)

[a] SSCP and PCR-RFLP ESTP markers
[b] DGGE ESTP markers
[c] SSCP and PCR-RFLP ESTP markers in *P. sylvestris*, all applied with the SSCP technique in *P. taeda*

primers were designed earlier (Plomion et al. 1999). In the end, 23 genes were scored in the F_1 progeny (20 with the SSCP technique, three with PCR-RFLP). Eleven of these were segregating in E1101 and 13 in E635C (Table 3). One of the genes (APX) was segregating in both trees with four alleles in total. We attempted to transfer these 23 segregating markers to *P. taeda*. Of the 23 segregating SSCP and PCR-RFLP markers, seven and ten were segregating in the two *P. taeda* pedigrees, using the SSCP technique (Tables 2 and 4).

The nature of the marker variation in *P. sylvestris* was studied by sequencing parts of 12 genes. In eight of these, only very small sequence differences were found. These did not cause any changes in restriction enzyme cut sites, and could not be detected in the SSCP either. Thus, only four of these loci were developed into markers. The sequence differences included base substitutions, and in two cases there were also insertions and deletions in the noncoding region. The success of mapped marker transfer from *P. sylvestris* to individual pedigrees of *P. taeda* was thus 30% and 43%, similar to transfer success from *P. taeda* to *P. sylvestris* (44%).

Linkage analysis of *P. sylvestris*

Separate maps of the parent trees were constructed from the 1:1 AFLPs, gene markers and microsatellites. The E1101 map consisted of 112 AFLP markers in 16 linkage groups in E1101 and the E635C map of 120 AFLP markers in 21 linkage groups (data not shown). In addition, of the 40 markers from *P. taeda*, 38 were linked in the *P. sylvestris* map and two remained unlinked (one of which was segregating 1:2:1). The total map lengths were 1,452 cM for E1101 and 929 cM for E635C.

The integrated map consisted of 12 groups corresponding to the *P. taeda* linkage groups, and additionally three and six smaller groups for E1101 and E635C, respectively (Fig. 1). The number of gene markers on the map is 61, six of which segregated in both trees with more than two alleles and two in a 1:2:1 ratio. In addition, the number of microsatellites is four, of which two segregated in both trees in a 1:1:1:1 ratio. The total length of the integrated map was 1,314 cM.

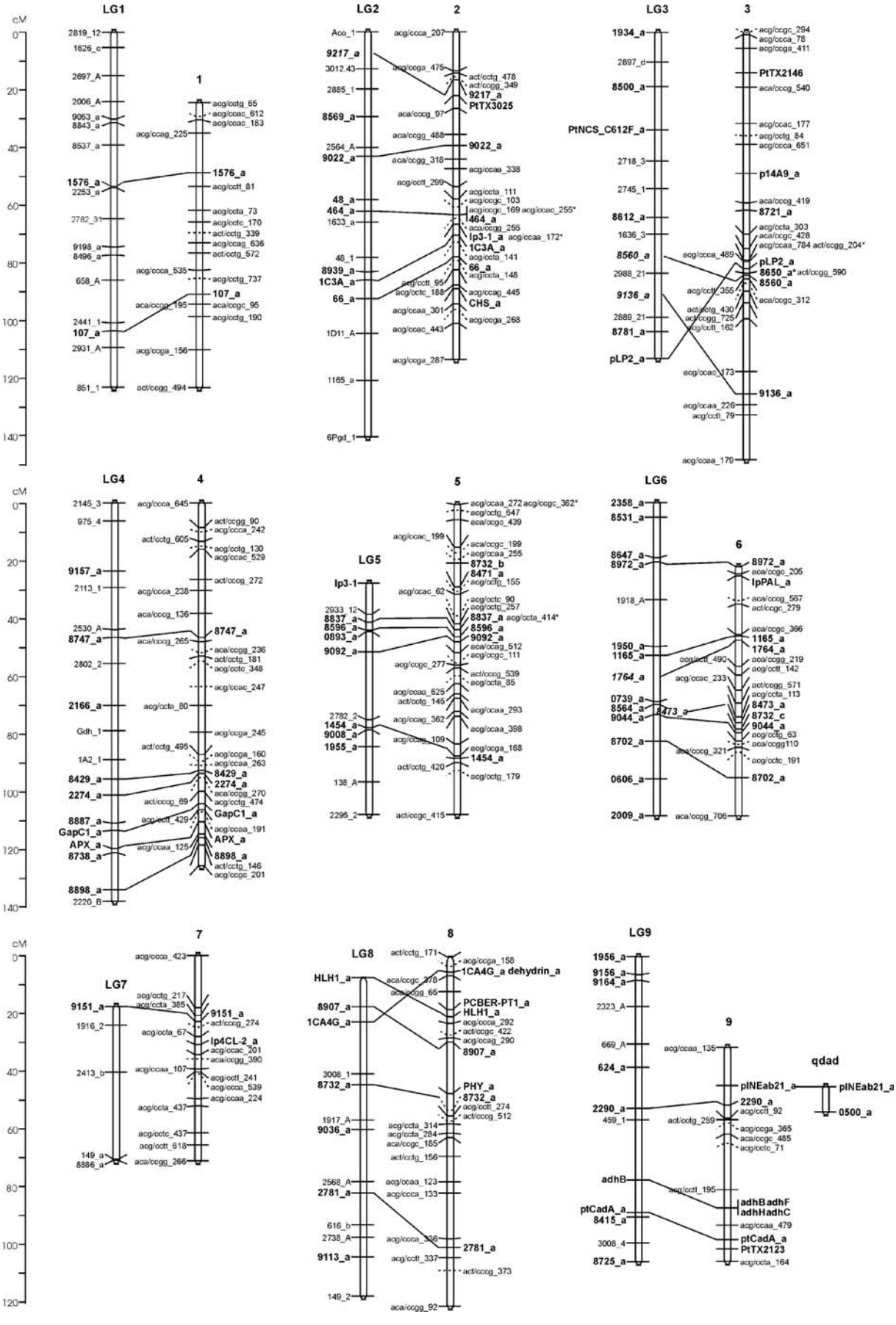
The estimates of total genome size, using the method of Chakravarti et al. (1991), are 2,147 cM and 1,974 cM for E1101 and E635C, respectively, estimated on the basis of the separate maps with AFLPs and the gene-based markers. The average spacing of the gene-based and AFLP framework markers in E1101 was 11.5 cM and 9.1 cM in E635C. In estimating genome coverage, we added these numbers to the distal ends of each linkage group, resulting in estimates of genome coverage of 85% and 66% in E1101 and E635C, respectively. An approximately similar number of markers gave rise to a longer genetic map in the male tree E1101 than in the female tree E635C.

Comparing the maps of *P. sylvestris* and *P. taeda* and homology assessments

We found considerable similarity between the maps. Twelve homologous linkage groups could be identified based on one to six orthologous markers per linkage group (Fig. 1). Marker order was the same between species except for three cases. For two of these, in LG3 and LG8, the genes in the region were segregating in different parental trees of *P. sylvestris*. Thus, the gene order is not based on the analysis of the pair of loci in meioses of a single tree. On the other hand, in LG10 ESTP markers 1635_a and 8436_a, both originate from the map of E1101, but they are in reverse orders in *P. sylvestris* and *P. taeda*. However, the position of 8436_a in *P. taeda* is only an approximation, based on the homologous flanking markers among the three *P. taeda* pedigrees (Brown et al. 2001).

There were also some cases of potential gene family members. Gene families are common in pines (Perry and Furnier 1996; Kinlaw and Neale 1997). The orthologous members must be identified in the different species. We know from earlier studies that the random accumulation of mutations in the two orthologs separated by speciation has resulted in a low level of divergence (approximately 5%) (Dvornyk et al. 2002), i.e. 95% of similarity between *P. sylvestris* and *P. taeda* in silent sites. If two loci are more diverged then it is likely that they are paralogous and have arisen already before the speciation. The larger difference is because of longer separation time between the loci. Hence, when the same primer set produced amplification products that were approximately 95% similar at synonymous sites in the two species, we considered that they could be orthologs. We mapped four *adh* loci in *P. sylvestris*, which has at least 11 copies of these loci (Mikkonen et al., unpublished). Different loci were detected with locus specific primers, and they all mapped into an identical position. For the genes that mapped to different locations, loci that were clearly less than 95% similar were regarded as paralogous, i.e. different members of the family, based on the homology assessments by sequencing (Table 5). *P. taeda* has 8732_a on LG8, whereas the same primers revealed three loci in *P. sylvestris*. 8732_a (1:2:1 marker), with a similar electrophoretic mobility to the *P. taeda* gene was mapped in the region corresponding to the *P. taeda* LG8. The genes are probably orthologous loci with reasonably low sequence differentiation. The two other loci, 8732_b located in LG5 and 8732_c in LG6, probably represent different members of the same gene family.

There are also cases when individual loci behave like different members of the gene family in the two species. The locus lp3-1_a is on LG 2 in the parent E635C, but on LG5 in *P. taeda*. Based on the differences between the two sequences (5.2%), the representatives of lp3-1 may be different members of the same gene family. ESTP marker 624_a from LG 9 of *P. taeda* map remained unlinked in the *P. sylvestris* map, even though the map coverage in the area was reasonably good. Sequencing revealed two


LG1
LG2
2
LG3
3
LG4
4
5
LG5
LG6
6
7
LG7
8
LG8
LG9
9
cM
1576_a
107_a
9217_a
PtTX3025
9022_a
464_a
1C3A_a
66_a
CHS_a
PtTX2146
p14A9_a
8721_a
pLP2_a
8650_a
8560_a
9136_a
8747_a
2166_a
8429_a
2274_a
GapC1_a
APX_a
8898_a
8732_b
8471_a
Ip3-1_a
8837_a
8596_a
9092_a
1454_a
1955_a
8732_c
8473_a
9044_a
IpPAL_a
1165_a
1764_a
8702_a
0606_a
2009_a
9151_a
Ip4CL-2_a
HLH1_a
1CA4G_a dehydrin_a
PCBER-PT1_a
8907_a
PHY_a
8732_a
2781_a
9113_a
adhB
adhBadhF
adhHadhC
ptCadA_a
PtTX2123
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pINEab21_a
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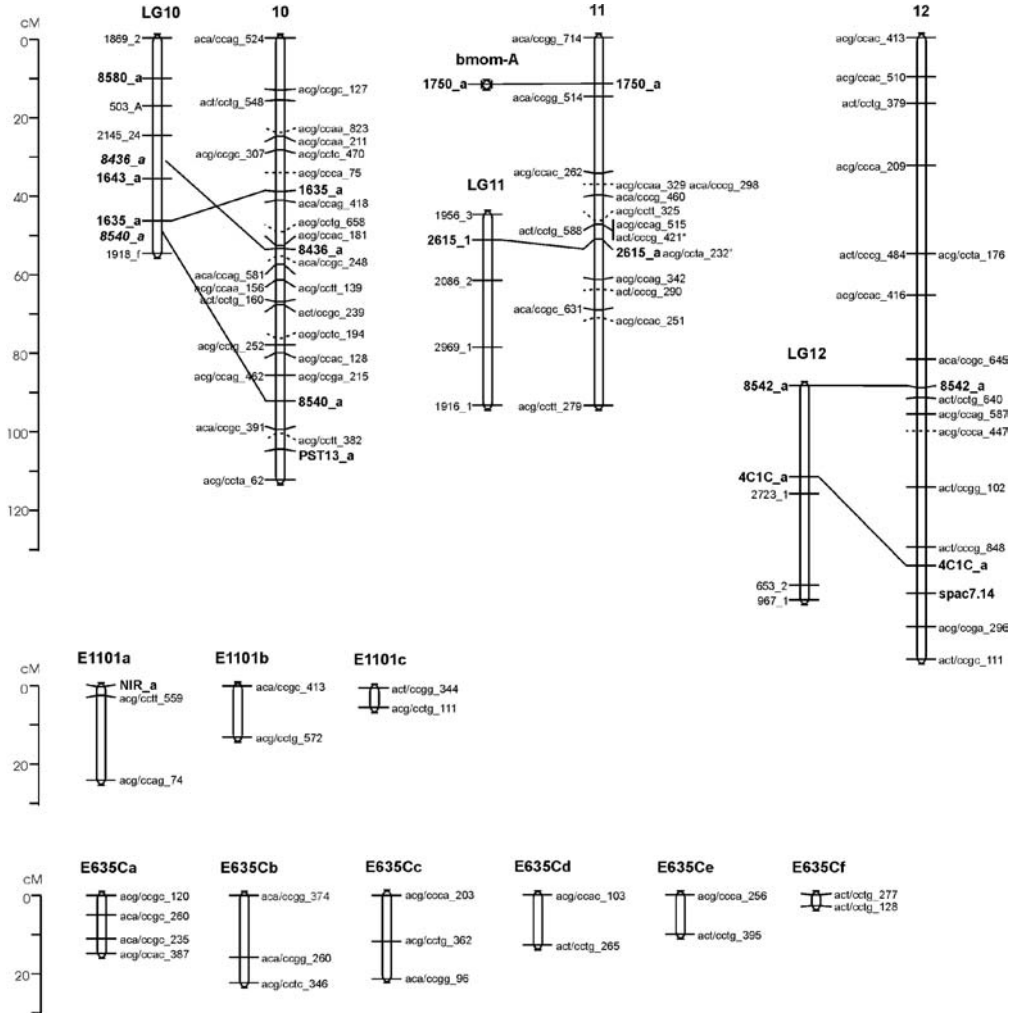

Fig. 1 Genetic maps of *P. taeda* and *P. sylvestris*. *P. taeda* linkage groups are on the left and *P. sylvestris* linkage groups of the integrated map on the right. The AFLP frameworks for separate parent trees are on the different sides of the groups; E1101 AFLPs on the left and E635C AFLPs on the right. Gene-based markers are denoted in *bold*. ESTP markers developed in *P. taeda* in the Institute of Forest Genetics laboratory and tested for amplification and segregation in *P. sylvestris* are denoted with the *clone number and the locus identifier*, like 1576_a (abbreviation of the full name IFGTXS_est PtIFG_1576_a). ESTPs developed for *P. sylvestris* in the University of Oulu or in INRA, France, are denoted with the clone name and the locus identifier (clone names and the source fields are shown in Table 2.). If the ESTP marker is from another source than the Institute of Forest Genetics laboratory or the University of Oulu or INRA, the *name of the laboratory* is in the marker name (PtNCS_C612F_a). ESTPs with different locus identifiers in the *P. sylvestris* map (8732_a, 8732_b) are markers that amplified more than one locus in *P. sylvestris*. The positions of ESTP markers in *italics* are only estimated. AFLP and ESTP markers segregating in 3:1 are marked with *dashed lines* on the *P. sylvestris* linkage groups, or alternatively if the 3:1 marker is located at the same position with some other marker it is denoted with *. RFLP markers from *P. taeda* are denoted with *number codes*, e.g. 2819_12 is IFGTXS_PtIFG_2819_12, and enzyme loci with their abbreviations. Spac 7.14 and markers beginning with letters PtTX in the *P. sylvestris* map are *P. sylvestris* (Soranzo et al. 1998) and *P. taeda* (Elsik et al. 2000) microsatellites, respectively. *Connecting lines* represent potentially orthologous markers between maps

fragments of nearly identical sizes in *P. sylvestris*, one of which may be orthologous to 624_a of *P. taeda*. In DGGE, the overlapping sizes of the fragments may have interfered with the mapping of the gene in *P. sylvestris*. Another case was found with 8471_a, which was mapped in LG5 in *P. sylvestris*, but remained unlinked in *P. taeda*. However, the gene was segregating only in the *base* pedigree, which did not have any linkage information in the area. Sequencing revealed two fragments of nearly identical sizes in *P. taeda* one of which is probably orthologous to the gene mapped in *P. sylvestris*. SOD-chl_a in LG10 of *P. sylvestris* could not be mapped in *P. taeda*. However, the genes are probably orthologous, and the gene could not be located in *P. taeda* most likely due to the low coverage of the map in the corresponding area. Finally, 8721_a that was located in LG3 of E635C did not segregate in the *P. taeda* pedigrees available. However, in *Pinus elliottii*, this locus is found in an area corresponding to LG3 (Fig. 1, Brown et al. 2001).

Table 5 Confirmation of locus orthology by nucleotide sequencing

Locus	Linkage group[a]	*P. sylvestris*/*P. taeda* differences		
		Sequenced fragment length (bp)[b]	Percentage of similarity (differences/sites)[c]	Indel lengths (bp)[d]
SODchl	LG10	881 Ps 872 Pt	97.3 (19/712)	7, 2, 1, 1, 1
lp3-1	LG2 Ps LG5 Pt	630 Ps 627 Pt	94.9 (18/355)	9, 14, 8
624	LG9 Pt	820 Ps 792 Ps 819 Pt	95.7 (27/626) 94.3 (34/597)	1, 1, 3, 2, 2 4, 2, 32
8471	LG5 Ps	156 Ps 156 Pt 153 Pt	 93.6 (10/156) 97.4 (4/153)	 – 3
8732	a LG8 Ps b LG5 Ps c LG6 Ps a LG8 Pt	224 Ps 225 Ps 83 Ps 224 Pt	95.1 (10/203) 91.7 (17/204) 55.4 (33/74)	– 1 14, 58, 1, 5, 61, 1, 1

[a] Ps refers to the locus mapped in *P. sylvestris* and Pt to *P. taeda*
[b] Fragment lengths in bp in *P. sylvestris* (Ps) and in *P. taeda* (Pt)
[c] Silent differences (both synonymous and non-coding regions) between the *P. sylvestris* and *P. taeda* sequences
[d] The numbers refer to the lengths of individual indels between the *P. sylvestris* and *P. taeda* sequences

Discussion

The *P. sylvestris* genetic map

We developed a map containing AFLPs, microsatellites and ESTPs for *P. sylvestris*. The AFLP markers were first used to develop a framework map for both parental trees. Separate maps were then integrated with the aid of AFLPs and other markers segregating in both trees.

The length of the male map was longer than the female map, 1,452 cM and 929 cM, respectively. Part of this difference may be due to higher recombination in males than in females, a result found on many occasions (e.g. Sewell et al. 1999). Estimates using RAPDs or AFLPs have often resulted in much larger maps, for example 2,638 cM for *P. sylvestris* (Yazdani et al. 1985). Genotyping errors result in upward bias of map size. The careful choice of framework markers may have lessened this bias. The length of the male map (1,452 cM) is similar to what has been obtained for several other pine maps using RFLPs or ESTPs. Additional ESTP markers may give improved estimates of genome length.

Comparison of *P. taeda* and *P. sylvestris* and other pine maps

As conifer karyotypes are known to evolve slowly (Prager et al. 1976), comparative mapping starts with the hypothesis of high synteny and colinearity. We have examined our data with the view of finding evidence against this genome conservation. Where comparisons were possible, most linkage groups had a similar content. This similarity allows identification of homologous linkage groups between the species. Further, the gene order within the linkage groups was also maintained, with three exceptions.

This level of conservation of linkage groups and gene order can be compared to comparative mapping between *P. taeda* and *P. radiata* based on 60 RFLPs and nine microsatellite loci (Devey et al. 1999). Only small deviations from colinearity were found, even if the species are in different subsections of the subgenus *Pinus Australes* and *Attenuatae*). Within the subsection *Australes*, between *P. elliottii* and *P. taeda*, linkage group content and colinearity were also maintained apart from small deviations, based on a comparison of 60 markers (Brown et al. 2001). The present comparison was also between species of two different subsections, which gives further support for the wide conservation of gene order.

Gene families are thought to be abundant in conifers (Perry and Furnier 1996; Kinlaw and Neale 1997). EST projects suggest that the number of expressed gene family members may not be very high, but the number of related nonexpressed pseudogenes is higher than in many other plant groups. However, until now only few tissue types of conifers have been used to generate ESTs. When discrepancies occur in gene order, the parsimonious explanation may be that paralogous copies of gene families in different locations in the genome have been detected. In one case a primer pair that amplified a single locus in *P. taeda* appears to have amplified three loci (8732_a, 8732_b and 8732_c) in *P. sylvestris*. While the primer pair may be gene family member-specific in one species, it may amplify several members, or a single paralog in a different location in another species. If the paralogous loci are in tandem, this might not be detected. For instance, the *adh* loci of *Pinus banksiana* are found on the same chromosome in two closely linked locations (Perry and Furnier 1996). In *P. sylvestris*, two isozyme

Adh loci have been mapped earlier within 1 cM of each other (Szmidt and Muona 1989). In our study, the *adh* gene family members were all located together, meaning they were tandemly duplicated in the scale detectable in this mapping pedigree. In some gene families, the members seem to be located in tandem (e.g. *adh*) while there is evidence for others that mapped into different linkage groups (e.g. lp3-1,). Further detailed studies of gene families are needed to assess which of these two options is more common.

The issue of gene families is important also with RFLPs, because probes may detect several loci. Furthermore, the loci detected by these "multilocus" probes have proved to be the ones with highest heterozygosity (Devey et al. 1999).

Success of ESTP-based comparative mapping

The *P. sylvestris* map contains 61 ESTPs or other gene-based markers. Relative to anonymous markers such as AFLP or microsatellites, these have a much wider area of use. The markers in such maps can serve as candidate genes for explaining phenotypic differences in interesting traits. Forest trees are predominantly outcrossing and many populations are expected to have a low level of linkage disequilibrium. This predicts a low level of disequilibrium even at short physical distances, as has been found in a study of *P. sylvestris* (Dvornyk et al. 2002). Thus, associations between a candidate gene and a phenotypic trait are best searched for with variation right at the gene, and not linked anonymous markers.

The most common form of comparative mapping in general is to use the markers from a model species in other species. *P. taeda* has emerged as a reference species, as there has been an intensive mapping effort (Sewell et al. 1999; Brown et al. 2001). The set of *P. taeda* markers has proved very useful with RFLPs in *P. radiata* and ESTPs in *P. elliottii*. Some of the *P. radiata* derived markers have been mapped also in *P. taeda*. This similar approach, of using markers that are developed for one species in other species, has been also used in developing markers for *P. sylvestris* and *P. pinaster* (Plomion et al. 1999). The rate of success in transferring the markers has been high enough that it is more efficient to start with markers mapped in other species rather than by developing a whole new set of markers for each species. The colinear maps of pines facilitate the construction of a genus wide pine map, which will be available in the near future.

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