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Comparative Mapping in the Pinaceae

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ABSTRACT

A comparative genetic map was constructed between two important genera of the family Pinaceae. Ten homologous linkage groups in loblolly pine (*Pinus taeda* L.) and Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) were identified using orthologous expressed sequence tag polymorphism (ESTP) and restriction fragment length polymorphism (RFLP) markers. The comparative mapping revealed extensive synteny and colinearity between genomes of the Pinaceae, consistent with the hypothesis of conservative chromosomal evolution in this important plant family. This study reports the first comparative map in forest trees at the family taxonomic level and establishes a framework for comparative genomics in Pinaceae.

THE essence of comparative genome analysis is the extrapolation of information from one organism to another. Comparative mapping and comparative sequence analysis are the key components of comparative genomics. Comparative mapping establishes the syntenic relationships between genomes of different species, assisting in genetic map consolidation, verification of quantitative trait loci (QTL), identification of candidate genes underlying QTL, and a better understanding of genome evolution (Sankoff and Nadeau 2000; Kliebenstein *et al.* 2001; Murphy *et al.* 2001; Zhang *et al.* 2001; Schmidt 2002).

Comparative genome analysis is often performed between model and nonmodel species (for reviews, see Paterson *et al.* 2000; Hall *et al.* 2002; Schmidt 2002). For example, thale cress (*Arabidopsis thaliana*) and rice (*Oryza sativa*) are model species for dicots and monocots, respectively. Putative syntenic regions have been identified for dicots in comparisons between soybean (*Glycine max*), barrel medic (*Medicago truncatula*), cabbage (*Brassica oleracea*), potato (*Solanum tuberosum*), and *A. thaliana* (Grant *et al.* 2000; Babula *et al.* 2003; Gebhardt *et al.* 2003; Lukens *et al.* 2003; Zhu *et al.* 2003). Similar comparisons have been done for monocots between sorghum (*Sorghum bicolor*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), maize (*Zea mays*), and *O. sativa* (Klein *et al.* 2003; Ware and Stein 2003). Comparative maps have been constructed among several species within a few important families of plants, notably Brassicaceae (Paterson *et al.* 2000; Barnes 2002; Hall *et al.* 2002), Solanaceae (Doganlar *et al.* 2002), Fabaceae (Boutin *et al.* 1995; Yan *et al.* 2003), and Poaceae (Feuillet and Keller 2002; Laurie and Devos 2002; Ware *et al.* 2002; Ware and Stein 2003). A comparative map framework among these taxa facilitates the transfer of information across species and enables a taxonomic family to be viewed as a single genetic system (Freeling 2001).

Pinaceae is the most important among eight families of the order Coniferales (conifers). This family comprises 11 genera and 232 species distributed throughout the world (Frankis 1989), primarily in the temperate region of the northern hemisphere. Members of the Pinaceae have large economic importance as a source of timber, pulp, and resins. They also play a very significant ecological role by producing large biomass and creating habitat for many other organisms. Forest trees of the Pinaceae are essential for carbon sequestration that may affect global climate.

Pinaceae genomes are very large compared to nearly all other plant species and are unlikely to be completely sequenced in the near future. Pinaceae DNA contents vary from 5.8 to 32.2 pg per haploid genome (1C) with 22 pg on average for 83 species studied (Murray 1998; Leitch *et al.* 2001; Bennett and Leitch 2003). Pinaceae genomes are 6-fold larger than the human genome (3.5 pg; Morton 1991) and 100-fold larger than that of *A. thaliana* (0.18 pg; Bennett and Smith 1991). In the absence of a genome sequence for a member of the Pinaceae, comparative mapping becomes even more important as the primary tool for integrating information across species.

Loblolly pine (*Pinus taeda* L., $2n = 2x = 24$) is the

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most genetically studied conifer species and was chosen as the reference species for comparative mapping in Pinaceae. Although still far from being a true model species, loblolly pine has rich genetic resources, well-developed genetic and QTL maps (Sewell *et al.* 1999; Brown *et al.* 2001, 2003; Temesgen *et al.* 2001), and expressed sequence tag (EST) databases (Allona *et al.* 1998; Whetten *et al.* 2001; Kirst *et al.* 2003). Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco, $2n = 2x = 26$) is the most important species of the genus Pseudotsuga with well-studied genetic and QTL maps (Krutovskii *et al.* 1998; Jermstad *et al.* 1998, 2001a,b, 2003). Together, they are the most commercially important forest tree species in the United States, and a comparative map between species would have significant practical value. It might also help to establish the origin of the thirteenth chromosome pair in Douglas fir, the only species not having 12 chromosome pairs in the Pinaceae.

Orthologous markers are essential for constructing comparative maps. Fitch (1970, 2000) defined orthologous genes as homologous genes whose divergence follows a speciation event, while paralogs are defined as genes whose divergence follows a duplication event within a species. Orthologs are expected to have similar function, expression, amino acid and nucleotide sequence, and genome location in closely related species (*e.g.*, Mirny and Gelfand 2002). Two criteria, high sequence similarity and genome location, were used as evidence for orthology in this study.

Restriction fragment length polymorphism (RFLP), based on Southern hybridization with single-copy genomic clones to ensure orthology, has been used broadly for comparative mapping in plants (Ahn *et al.* 1993; Sherman *et al.* 1995; Gale and Devos 1998; Yan *et al.* 2003). Comparative mapping using single- or low-copy cDNA as hybridization probes has been also successful in the Brassicaceae (Lan *et al.* 2000; Barnes 2002; Babula *et al.* 2003), Solanaceae (Doganlar *et al.* 2002), Poaceae (Smilde *et al.* 2001; Feuillet and Keller 2002; Laurie and Devos 2002), and across different families (Davis *et al.* 1999; Gebhardt *et al.* 2003). However, RFLP methods have had limited application in conifers due to genome complexity and numerous multigene families (Kinlaw and Neale 1997). Although comparative mapping using RFLP markers has been successful in pine species (Devey *et al.* 1999), it is difficult to apply RFLPs across different genera in conifers. Many loblolly pine probes produced a complex multiband pattern in hybridization with Douglas fir genomic DNA (Jermstad *et al.* 1994, 1998). PCR-amplified EST polymorphisms (ESTPs) have emerged recently as an alternative to RFLP markers for comparative mapping (Brown *et al.* 2001). A set of orthologous ESTP markers developed in loblolly pine has been established and successfully used in comparative mapping in the genus Pinus (Brown *et al.* 2001; Chagné *et al.* 2003; Komulainen *et al.* 2003). However, only 22% of these markers were amplified in Douglas fir (Brown *et al.* 2001). Mutations in primer binding sites have made comparative mapping via a common set of PCR primers practically impossible between conifer genera. To overcome this problem a computational approach was used to identify Douglas fir ESTs with high homology, and putative orthology, to ESTPs mapped in loblolly pine. A similar approach was used in recent comparative mapping studies between tomato, potato, and Arabidopsis (Fulton *et al.* 2002; Gebhardt *et al.* 2003). The selected Douglas fir ESTs were used to design Douglas fir-specific PCR primers to amplify loblolly pine orthologs in Douglas fir for subsequent genetic mapping. This approach allowed comparative mapping to be extended to the family level and established a framework for comparative genomics in Pinaceae. This study is a part of the Conifer Comparative Genomics Project (CCGP) formed as an international collaboration at the Institute of Forest Genetics (U.S. Department of Agriculture Forest Service) to develop the orthologous genetic markers and publicly available reference mapping populations that can be shared among different laboratories to facilitate comparative mapping (http://dendrome.ucdavis.edu/ccgp).

MATERIALS AND METHODS

Mapping populations and reference maps: The loblolly pine and Douglas fir mapping populations were three-generation outbred pedigrees consisting of four grandparents, two F_1 parents, and several hundred progeny (Jermstad *et al.* 1998, 2003; Sewell *et al.* 1999). The loblolly pine reference map was based on RFLP and ESTP markers as reported in Sewell *et al.* (1999), Temesgen *et al.* (2001), and Brown *et al.* (2001). This map is a consensus map between two pedigrees. Syntenic relationships with other Pinus species were established previously for most of the 12 linkage groups (Devey *et al.* 1999; Brown *et al.* 2001; Chagné *et al.* 2003; Komulainen *et al.* 2003). These groups included 302 markers (166 RFLP, 5 isozyme, and 131 EST markers), with a total map length of 1274 cM. The Douglas fir reference map was based on 376 markers [172 RFLP, 77 randomly amplified polymorphic DNA (RAPD), and 2 isozyme markers (Jermstad *et al.* 1998) with 20 simple sequence repeat (SSR), 4 sequence-tagged site (STS), and 101 ESTP markers added in this study (see supplemental Table S1 at http://www.genetics.org/supplemental/)].

Markers analyzed: Three types of markers were used to develop the loblolly pine and Douglas fir comparative map: (1) single- or low-copy RFLP markers developed from loblolly cDNA clones, (2) ESTP markers developed in several pine and spruce (Picea) species that amplified a single locus in previous studies, and (3) ESTP and STS markers developed in Douglas fir. RFLP markers were mapped and sequenced previously in both species (Jermstad *et al.* 1998; Sewell *et al.* 1999). ESTP markers developed in pines and spruces were mapped in both species also according to methods described in Brown *et al.* (2001). ESTP and STS markers from Douglas fir are described in detail below. Briefly, EST and STS sequences were selected initially for evaluation as putative homologs to mapped loblolly pine markers on the basis of sequence similarity. One PCR primer of each pair was situated preferentially in an untranslated region to favor the selective amplification in Douglas fir of a single member of a gene family. At the intergeneric level, this strategy precluded the mapping of

Douglas fir markers in loblolly pine. However, the orthologous relationships of these loci in both species were assessed by sequence similarity of amplified fragments and their conserved mapped location.

Douglas fir EST and STS markers: A cDNA library was obtained from 1-month-old Douglas fir seedlings in collaboration with Integrated Genomics (Chicago). Total RNA was extracted from ~5 g of ground tissue following the protocol of Chang *et al.* (1993). Double-stranded cDNA was prepared using the Universal RiboClone cDNA Synthesis System (Promega, Madison, WI), filtered through a Sephacryl S-400 column, ligated into the *Eco*ICR I-cut dephosphorylated pGEM-3Z sequencing vector, and electroporated into *Escherichia coli* DH5α. A total of 5031 EST sequences were obtained and assembled into contigs. These ESTs and contigs and four Douglas fir STSs available from GenBank were used to query the December 2002 assembly of loblolly pine ESTs, which is accessible at http://pine.ccgb.umn.edu, using BLASTn and tBLASTx. The assembly contained 20,456 contigs and singletons derived from 59,430 sequences from six xylem libraries. Douglas fir sequences homologous to mapped loblolly pine loci were selected for further study, if they showed nucleotide similarity >80% and expected values of ≤*E*-15 over a minimum of 100 bp.

PCR and detection of polymorphisms: PCR primers were designed using the Douglas fir EST and STS sequences homologous to mapped loblolly pine loci. Primers were designed using the computer program GeneRunner v3.04 (Hastings Software, Hudson, NY) to yield products of 300–500 bp. A typical reaction volume was 25 μl and included 10 mm TRIS-HCl pH 8.3, 50 mm KCl, 1.5 mm $MgCl_2$, 200 μm of each dNTP, 1 μm of each primer, 12 ng of DNA template, and 0.5 units of HotStart *Taq* DNA Polymerase from QIAGEN (Valencia, CA). Following HotStart *Taq* activation (94° for 15 min), PCR amplification involved denaturation at 94° for 1 min, annealing for 0.5 min, and extension for 2 min. The annealing temperature during the initial cycles was lowered from *X* to *Y* by 1° every second cycle. Standard PCR conditions ($X = 65°$ and $Y = 60°$ or 55°) were used for most ESTP markers, although for primer pairs that failed to amplify, the stringency was reduced ($X = 60°$ and $Y = 50°$). An additional 30 cycles of amplification were performed upon reaching the final annealing temperature (*Y*) followed by a final extension at 72° for 10 min. Amplification products from the parents of the Douglas fir pedigree were screened for polymorphism in 2% agarose gels and by denaturing gradient gel electrophoresis (DGGE) using a 15–45% denaturing gradient and a DCode apparatus (Bio-Rad, Hercules, CA) according to Temesgen *et al.* (2000). Finally, ESTP segregation data were collected for the 94 progeny of the Douglas fir pedigree.

DNA sequencing: To support the possibility that PCR markers amplified in Douglas fir were orthologs to the mapped loblolly pine markers, amplifications products were directly sequenced and compared to the original Douglas fir and loblolly pine sequences. To use direct sequencing without cloning 33 markers were selected that amplified a single locus and had no amplification background, such as light additional bands that may interfere with sequencing. The selected markers represented 26 putative orthologous markers (5 loblolly pine-based ESTP, 18 Douglas fir-based ESTP, and 3 STS markers) and 7 nonorthologous Douglas fir-based ESTP markers. Four alleles at each locus were sequenced using DNA extracted from the haploid megagametophyte tissue of four Douglas fir seeds. DNA sequences were obtained from both strands with the primers used for PCR amplification and the ABI PRISM BigDye Primer Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA). Fragments were detected on an ABI 3730 DNA Analyzer at the Genomics Facility Center at the University of California, Davis. Raw sequences were base called by the PHRED program (Ewing and Green 1998; Ewing *et al.* 1998), assembled using the PHRAP program, and viewed through CONSED (Gordon *et al.* 1998, 2001).

Linkage analysis: Genotypic data were scored visually and tested for Mendelian segregation. Markers showing only slight segregation distortion ($0.01 < P < 0.05$) were not excluded from linkage analysis because recombination estimators are still valid when distortion is observed at only one locus of a linked pair of loci (Bailey 1961; Ott 1991). ESTP markers were added to the existing segregation data (Jermstad *et al.* 1998) and the linkage analysis was repeated. A sex-averaged consensus map was produced using JOINMAP versions 1.4 and 2.0 (Stam 1993; Stam and Van Ooijen 1995). Linkage groups were assigned at the LOD thresholds of 4 and 5. Grouping was almost identical at both thresholds, except a few loci unmapped at LOD = 5 and a spurious merging of two apparently independent linkage groups at LOD = 4. Therefore, we used mainly LOD = 4 for grouping, except two linkage groups that were assembled at LOD = 5. The procedure for ordering markers was the same as described in Jermstad *et al.* (1998). The Kosambi function was used to estimate map distances.

Nomenclature and informatics: Mapped loci were named according to guidelines for submitting data to the TreeGenes database (http://dendrome.ucdavis.edu/Tree_Page.htm). A mapped marker is defined by its experiment, source, accession number, and locus identifier fields. For example, an ESTP derived from the loblolly pine cDNA clone PtIFG_8732 and mapped in both loblolly pine and Douglas fir in this study is referenced as *IFGREF_estPtIFG_8732_a* and *IFGLXD_estPtIFG_8732_a* in the loblolly pine and Douglas fir maps, respectively. For brevity, however, experiment fields have been omitted (Figure 1).

RESULTS

Orthologous RFLP and ESTP markers derived from pine and spruce species: Twenty-six RFLP markers were mapped in both loblolly pine and Douglas fir (Jermstad *et al.* 1998; Sewell *et al.* 1999). Seven markers met criteria of orthologous markers. Four markers (*PtIFG_2006_a*, *PtIFG_2356_a*, *PtIFG_2988_a*, and *PtIFG_2540_a*) revealed more than a single locus, but were mapped into syntenic regions and can be also conditionally considered as orthologous markers.

The ESTP primer pairs developed from pine and spruce species amplified Douglas fir templates with variable success, ranging from 24% amplification success with primers derived from loblolly pine to 93% success with primers derived from Norway spruce, *Picea abies* (L.) Karst. (Table 1). In total, 55 ESTPs from the four species were mapped in Douglas fir, but only 11 markers met the criteria of orthologous markers.

Orthologous ESTP and STS markers derived from Douglas fir: Of 5031 ESTs analyzed, 1992 sequences were assembled into 621 contigs. There were an additional 55 singletons and 2984 singlets. All ESTs and contigs were compared to the genetically mapped loblolly pine ESTs, and Douglas fir sequences with >80% nucleotide identity were used for PCR primer design. Most primers (97%) designed in this manner amplified a single Douglas fir product of expected size, and 39 markers were mapped. Twenty-one of the 39 markers met the criteria of orthologous markers (Table 1).

TABLE 1

RFLP, ESTP, and STS markers screened in Douglas fir

Marker	Species	Screened	Amplified (%)[a]	Mapped (%)[a]	Orthologous (%)[b]
RFLP	Loblolly pine (*Pinus taeda*)[c]	171	122 (71)[h]	26 (15)	11 (42)
ESTP	Loblolly pine[d]	156	37 (24)	30 (19)	6 (20)
	Maritime pine (*P. pinaster*)[e]	50	38 (76)	9 (18)	1 (11)
	Black spruce (*Picea mariana*)[f]	50	42 (84)	11 (22)	3 (27)
	Norway spruce (*P. abies*)[g]	15	14 (93)	5 (33)	1 (20)
	Total pine and spruce ESTPs	**271**	**131 (48)**	**55 (20)**	**11 (22)**
	Douglas fir (*Pseudotsuga menziesii*)	75	73 (97)	39 (52)	**21 (54)**
STS	Douglas fir	4	4 (100)	4 (100)	**3 (75)**
	Total	**521**	**330 (63)**	**124 (24)**	**46 (37)**

[a] Percentage of amplified markers out of total number of markers screened.
[b] Percentage of orthologous markers out of mapped markers.
[c] Devey *et al.* (1991, 1994); Jermstad *et al.* (1994); http://dendrome.ucdavis.edu/treegenes.html.
[d] Brown *et al.* (2001); Temesgen *et al.* (2001); http://dendrome.ucdavis.edu/Gen_res.htm.
[e] Chagné *et al.* (2003); http://www.pierroton.inra.fr/genetics/pinus/primers.html.
[f] Perry and Bousquet (1998a,b).
[g] Schubert *et al.* (2001).
[h] Number and percentage of the loblolly pine cDNA probes that cross-hybridized to the Douglas fir genomic DNAs (Jermstad *et al.* 1994).

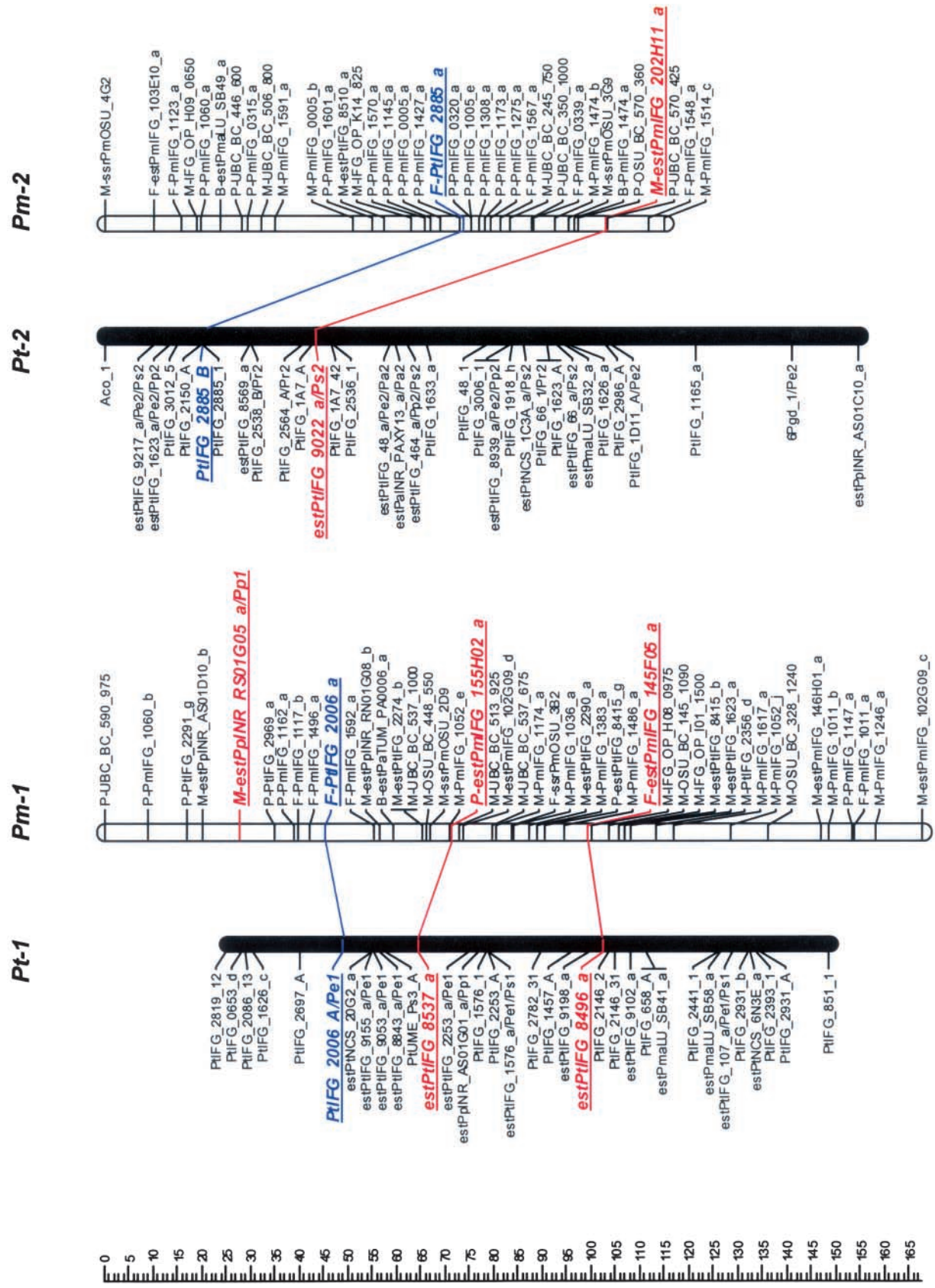
Three Douglas fir genomic sequences available from GenBank had orthologs among mapped loblolly pine ESTs. They were mapped and met the criteria of orthologous markers (Table 1).

Douglas fir linkage map: In total, 376 markers (172 RFLPs, 77 RAPDs, 20 SSRs, 2 isozymes, 4 STSs, and 101 ESTPs) were mapped to 22 linkage groups consisting of 3 or more markers (Table 1). There were 17 major linkage groups that consisted of 5 or more markers (Figure 1). The total length of the linkage map was 1664 cM for the 17 major linkage groups and 1859 cM for all 22 linkage groups.

Sequence of PCR-amplified ESTP and STS markers: PCR products for 26 putative orthologs (5 ESTPs amplified using primers based on loblolly pine ESTs, 18 ESTPs amplified using primers based on Douglas fir ESTs, and 3 STSs) and 7 nonorthologs, which were amplified and mapped in Douglas fir, were sequenced and compared to the original Douglas fir and loblolly pine EST or genomic sequences. All sequences confirmed the identity and origin of amplified markers. The orthology of all 26 putative orthologs, from which PCR amplification products were sequenced, was also confirmed (Table 2). The identity between Douglas fir and loblolly pine sequences was 89 ± 4% for 26 orthologous and 83 ± 2% for 7 nonorthologous markers on average (*t*-test $P = 0.012$).

Orthologous markers and homologous linkage groups: Comparison of Douglas fir and loblolly pine maps revealed 10 linkage groups (LG1–LG10) in loblolly pine that shared 2–10 orthologous markers with 12 apparently syntenic linkage groups in Douglas fir based on 46 orthologous markers (Table 2, Figure 1). Primer sequences and PCR conditions that were used to amplify orthologous markers are presented in the supplemental Table S2, and their homology analysis and annotation in the supplemental Table S3 (see http://www.genetics.org/supplemental/). Markers mapped in pine species other than loblolly helped to strengthen the comparative mapping. For example, the *estPpINR_RS01G05_a* marker was mapped in maritime pine (*P. pinaster* Ait; Chagné *et al.* 2003) and in Douglas fir, but not in loblolly pine. However, in both species this marker was mapped in the linkage group that was homologous to the same LG1 in loblolly pine based on other orthologous markers and therefore corroborated syntenic relationships between these groups (Table 2; Figure 1).

Figure 1.—Genetic maps of loblolly pine (*Pinus taeda* L.), *Pt*, and Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco), *Pm*. Orthologous markers are highlighted by larger, boldface, italicized, and underlined type and are connected by lines, except four of them that were mapped in pine species other than loblolly, but in the same syntenic linkage group (LG) [*estPpINR_RS01G05_a* was mapped in LG1 in *Pinus pinaster* (Chagné *et al.* 2003), and *estPmaLU_SB42_a*, *estPaTUM_PA0053_a*, and *estPmaLU_SB21_a* were mapped in LG6, LG8, and LG10 in *Picea abies*, respectively (our unpublished data)]. The loci were named following guidelines for the TreeGenes genome database (http://dendrome.ucdavis.edu/Tree_Page.htm; see also materials and methods). Abbreviations placed after the underscore in some loblolly pine marker names show other conifer species and syntenic linkage groups in which these markers were also mapped. For instance, Pe1 in the PtIFG_2006_A/Pe1 marker name in the linkage group *Pt-1* means that the PtIFG_2006_A marker was also mapped in the *Pinus elliottii* syntenic linkage group 1. Similarly, Pp stands for *P. pinaster*, Pr for *P. radiata*, Ps for *P. sylvestris*, and Pa for *Picea abies*.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Pm-1
Pt-2
Pm-2
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F-estPmIFG_103E10_a
F-PmIFG_1123_a
M-IFG_OP_H09_0650
P-PmIFG_1060_a
B-estPmaLU_SB49_a
P-UBC_BC_446_600
P-PmIFG_0315_a
M-UBC_BC_506_800
M-PmIFG_1591_a
M-PmIFG_0005_b
P-PmIFG_1601_a
M-estPtIFG_8510_a
M-IFG_OP_K14_825
P-PmIFG_1570_a
P-PmIFG_1145_a
P-PmIFG_0005_a
P-PmIFG_1427_a
F-PtIFG_2885_a
P-PmIFG_0320_a
P-PmIFG_1005_e
P-PmIFG_1308_a
P-PmIFG_1173_a
P-PmIFG_1275_a
F-PmIFG_1567_a
M-UBC_BC_245_750
P-UBC_BC_350_1000
F-PmIFG_0339_a
M-PmIFG_1474_b
M-ssrPmOSU_3G9
B-PmIFG_1474_a
P-OSU_BC_570_360
M-estPmIFG_202H11_a
P-UBC_BC_570_425
F-PmIFG_1548_a
M-PmIFG_1514_c
Aco_1
estPtIFG_9217_a/Pe2/Ps2
estPtIFG_1623_a/Pe2/Pp2
PtIFG_3012_5
PtIFG_2150_A
PtIFG_2885_B
PtIFG_2885_1
estPtIFG_8569_a
PtIFG_2538_B/Pr2
PtIFG_2564_A/Pr2
PtIFG_1A7_A
estPtIFG_9022_a/Ps2
PtIFG_1A7_42
PtIFG_2536_1
estPtIFG_48_a/Pe2/Pa2
estPalNR_PAXY13_a/Pa2
estPtIFG_464_a/Pp2/Ps2
PtIFG_1633_a
PtIFG_48_1
PtIFG_3006_1
estPtIFG_8939_a/Pe2/Pp2
PtIFG_1918_h
estPtNCS_1C3A_a/Ps2
PtIFG_66_1/Pr2
PtIFG_1623_A
estPtIFG_66_a/Ps2
estPmaLU_SB32_a
PtIFG_1626_a
PtIFG_2986_A
PtIFG_1D11_A/Pe2
PtIFG_1165_a
6Pgd_1/Pe2
estPpiNR_AS01C10_a
P-UBC_BC_590_975
P-PmIFG_1060_b
P-PtIFG_2291_g
M-estPpiNR_AS01D10_b
M-estPpiNR_RS01G05_a/Pp1
P-PtIFG_2969_a
P-PmIFG_1162_a
F-PmIFG_1117_b
F-PmIFG_1496_a
F-PtIFG_2006_a
F-PmIFG_1592_a
M-estPpiNR_RN01G08_b
B-estPaTUM_PA0006_a
M-estPtIFG_2274_b
M-UBC_BC_537_1000
M-OSU_BC_448_550
M-ssrPmOSU_2D9
M-PmIFG_1052_e
P-estPmIFG_155H02_a
M-UBC_BC_513_925
M-estPmIFG_102G09_d
M-UBC_BC_537_675
M-PmIFG_1174_a
F-ssrPmOSU_3B2
M-PmIFG_1036_a
M-estPtIFG_2290_a
M-PmIFG_1383_a
P-estPtIFG_8415_g
M-PmIFG_1486_a
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M-OSU_BC_145_1090
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M-PmIFG_1246_a
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PtIFG_0653_d
PtIFG_2086_13
PtIFG_1626_c
PtIFG_2697_A
PtIFG_2006_A/Pe1
estPtNCS_2G2_a
estPtIFG_9155_a/Pe1
estPtIFG_9053_a/Pe1
estPtIFG_8843_a/Pe1
PtUME_Ps3_A
estPtIFG_8537_a
estPtIFG_2253_a/Pe1
estPpiNR_AS01G01_a/Pp1
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PtIFG_2253_A
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PtIFG_2782_31
PtIFG_1457_A
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PtIFG_2146_2
PtIFG_2146_31
estPtIFG_9102_a
PtIFG_658_A
estPmaLU_SB41_a
PtIFG_2441_1
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PtIFG_2931_b
estPtNCS_6N3E_a
PtIFG_2393_1
PtIFG_2931_A
PtIFG_851_1
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165

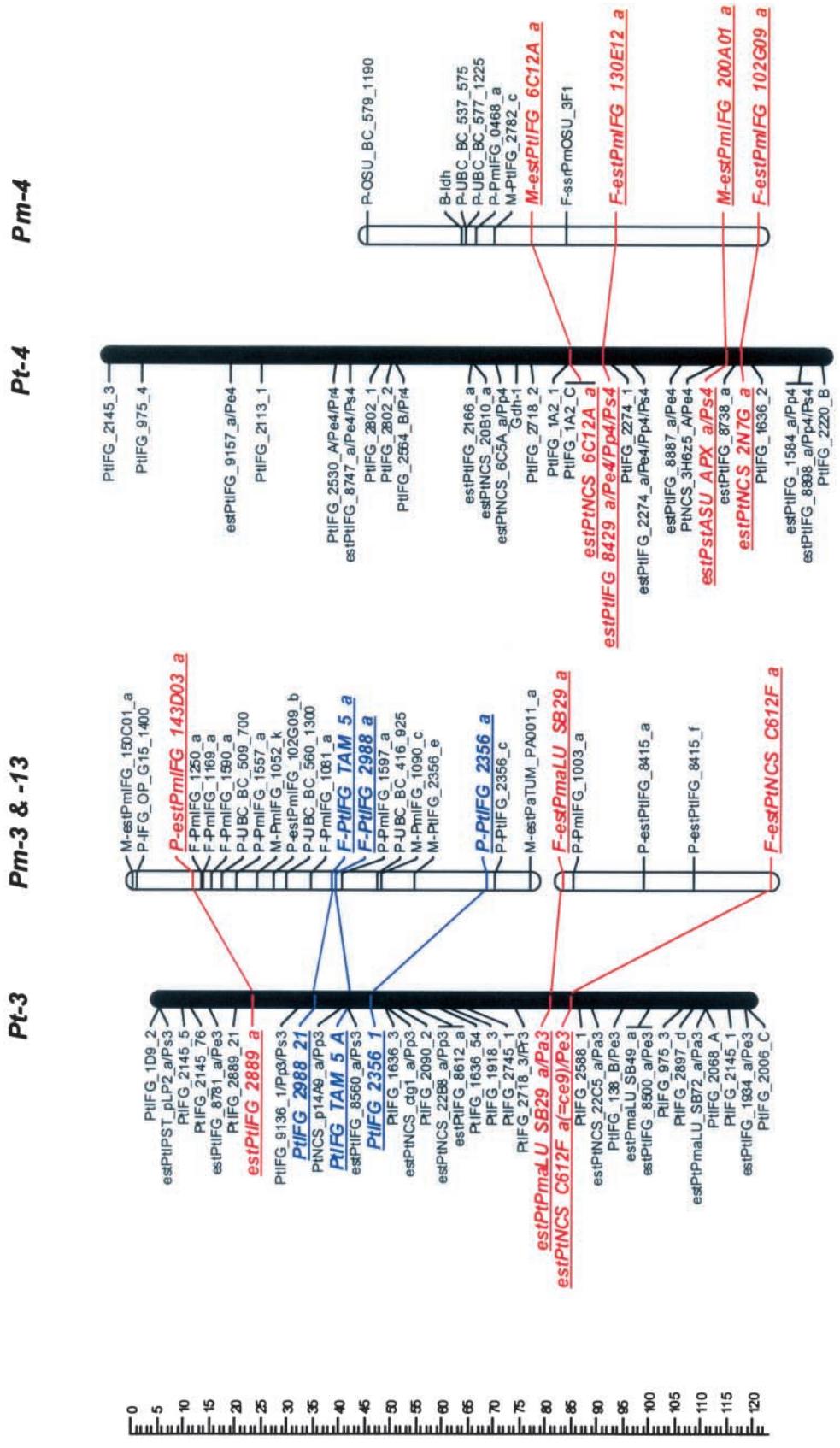


FIGURE 1.—*Continued.*

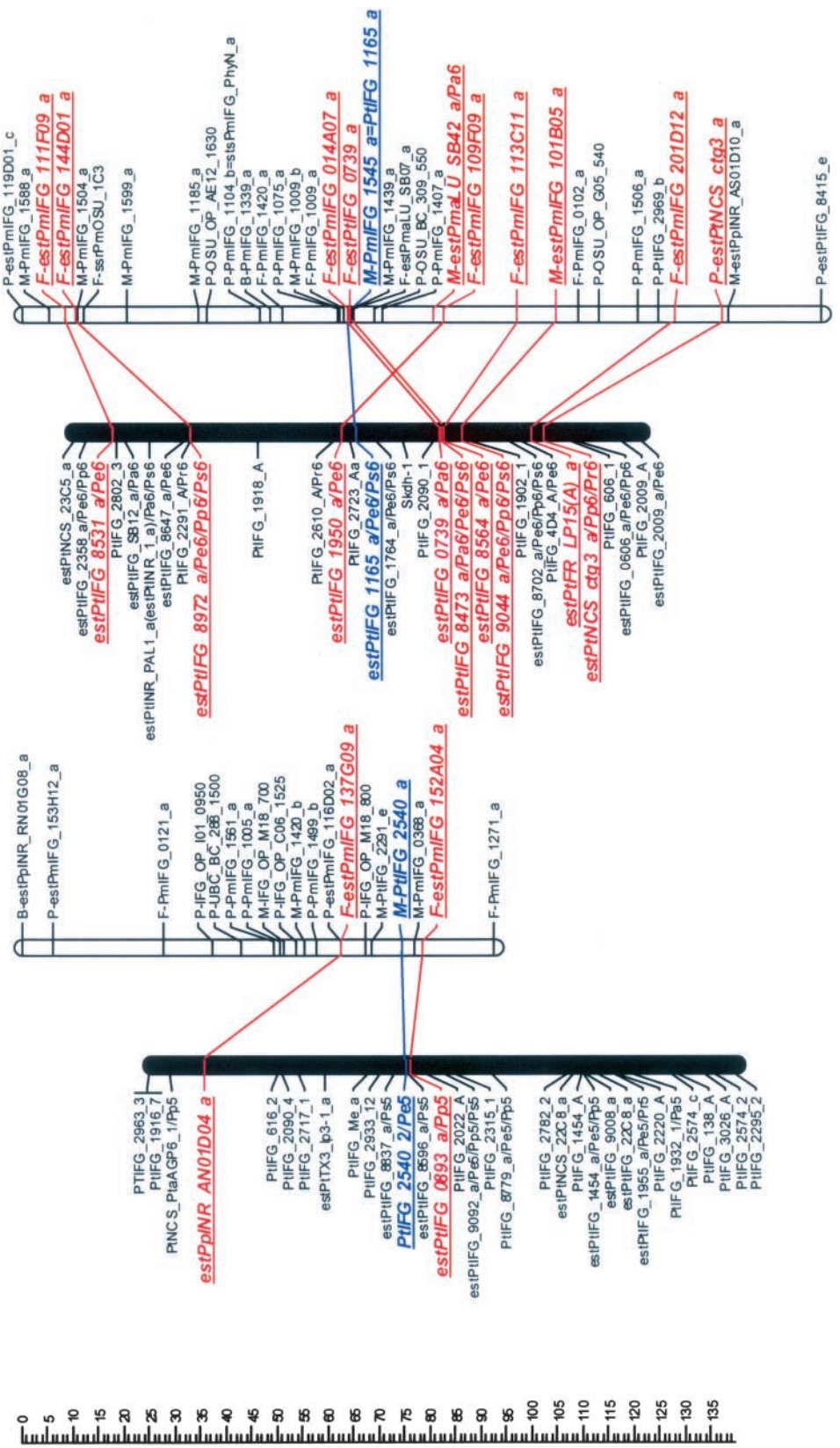


FIGURE 1.—*Continued.*

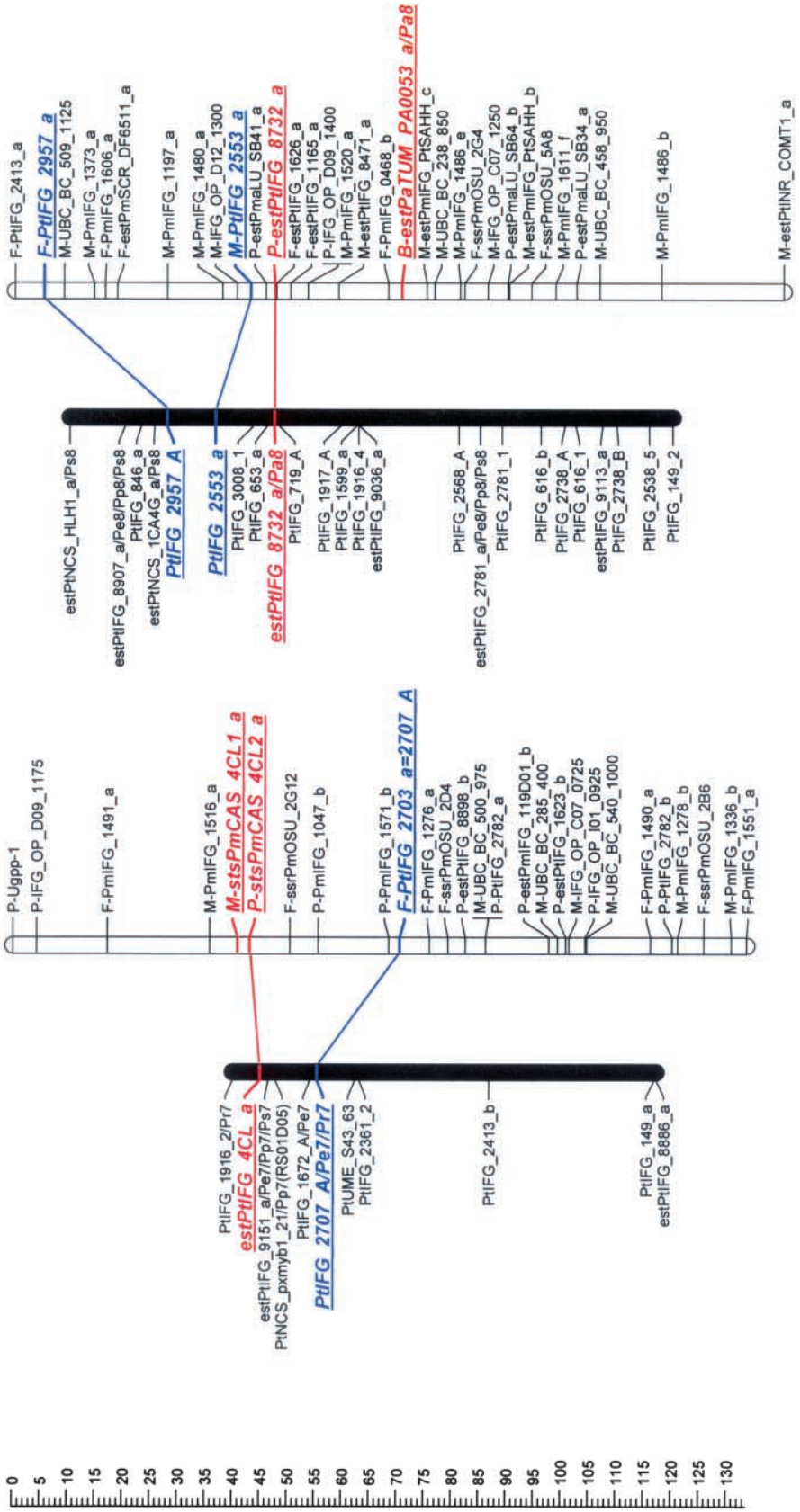
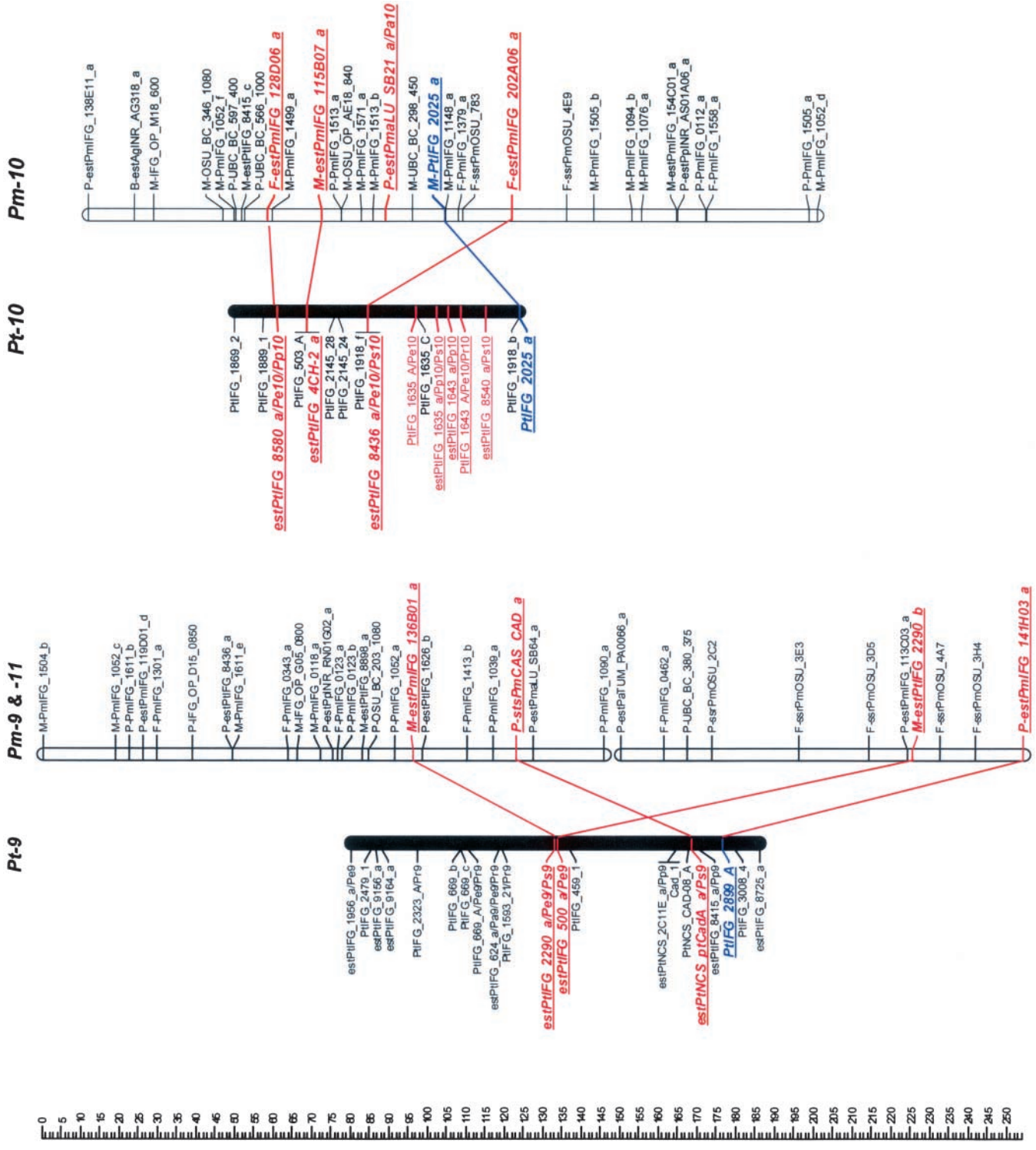


FIGURE 1.—*Continued.*


Pm-10
Pt-10
Pm-9 & -11
Pt-9
P-estPmIFG_138E11_a
B-estAgINR_AG318_a
M-IFG_OP_M18_600
M-OSU_BC_346_1080
M-PmIFG_1052_f
P-UBC_BC_597_400
M-estPtIFG_8415_c
P-UBC_BC_566_1000
F-estPmIFG_128D06_a
M-PmIFG_1499_a
M-estPmIFG_115B07_a
P-PmIFG_1513_a
M-OSU_OP_AE18_840
M-PmIFG_1571_a
M-PmIFG_1513_b
P-estPmaLU_SB21_a/Pa10
M-UBC_BC_298_450
M-PtIFG_2025_a
M-PmIFG_1148_a
F-PmIFG_1379_a
F-ssrPmOSU_783
F-estPmIFG_202A06_a
F-ssrPmOSU_4E9
M-PmIFG_1505_b
M-PmIFG_1094_b
M-PmIFG_1076_a
M-estPmIFG_154C01_a
P-estPpiNR_AS01A06_a
P-PmIFG_0112_a
F-PmIFG_1558_a
P-PmIFG_1505_a
M-PmIFG_1052_d
PtIFG_1869_2
PtIFG_1889_1
estPtIFG_8580_a/Pe10/Pp10
PtIFG_503_A
estPtIFG_4CH-2_a
PtIFG_2145_28
PtIFG_2145_24
PtIFG_1918_f
estPtIFG_8436_a/Pe10/Ps10
PtIFG_1635_A/Pe10
PtIFG_1635_C
estPtIFG_1635_a/Pp10/Ps10
estPtIFG_1643_a/Pp10
PtIFG_1643_A/Pe10/Pr10
estPtIFG_8540_a/Ps10
PtIFG_1918_b
PtIFG_2025_a
M-PmIFG_1504_b
M-PmIFG_1052_c
P-PmIFG_1611_b
P-estPmIFG_119D01_d
F-PmIFG_1301_a
P-IFG_OP_D15_0850
P-estPtIFG_8436_a
M-PmIFG_1611_e
F-PmIFG_0343_a
M-IFG_OP_G05_0800
M-PmIFG_0118_a
P-estPdNR_RN01G02_a
F-PmIFG_0123_a
P-PmIFG_0123_b
M-estPtIFG_8898_a
P-OSU_BC_203_1080
P-PmIFG_1052_a
M-estPmIFG_136B01_a
P-estPtIFG_1626_b
F-PmIFG_1413_b
P-PmIFG_1039_a
P-stsPmCAS_CAD_a
P-estPmaLU_SB64_a
P-PmIFG_1090_a
P-estPaTUM_PA0066_a
F-PmIFG_0462_a
P-UBC_BC_380_375
P-ssrPmOSU_2C2
F-ssrPmOSU_3E3
F-ssrPmOSU_3D5
P-estPmIFG_113C03_a
M-estPtIFG_2290_b
F-ssrPmOSU_4A7
F-ssrPmOSU_3H4
P-estPmIFG_141H03_a
estPtIFG_1956_a/Pe9
PtIFG_2479_1
estPtIFG_9156_a
estPtIFG_9164_a
PtIFG_2323_A/Pr9
PtIFG_669_b
PtIFG_669_c
PtIFG_669_A/Pe9/Pr9
estPtIFG_624_a/Pe9/Pe9/Pr9
PtIFG_1593_2/Pr9
estPtIFG_2290_a/Pe9/Ps9
estPtIFG_500_a/Pe9
PtIFG_459_1
estPtNCS_2C11E_a/Pp9
Cad_1
PtNCS_CAD-08_A
estPtNCS_ptCadA_a/Ps9
estPtIFG_8415_a/Pp9
PtIFG_2899_A
PtIFG_3008_4
estPtIFG_8725_a
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250

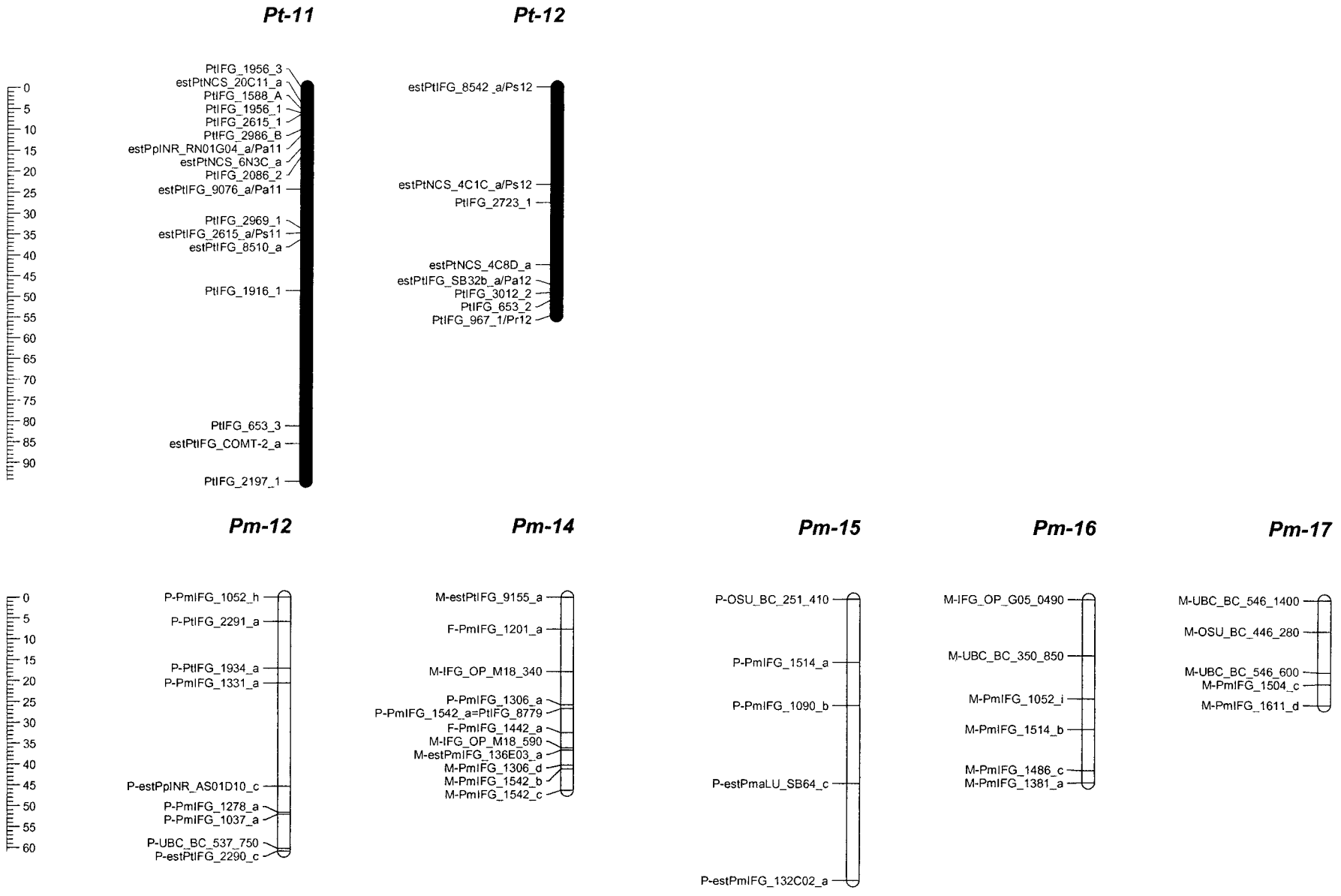


FIGURE 1.—*Continued.*

Similarly, the *estPmaLU_SB42_a* marker was mapped in Norway spruce (our unpublished data) and Douglas fir, but not in loblolly pine. The linkage group containing this marker in both Norway spruce and Douglas fir was homologous to LG6 in loblolly pine based on other markers (Table 2; Figure 1). The total lengths of the syntenic linkage groups that shared orthologous markers were 1125 cM for 10 groups in loblolly pine and 1421 cM for 12 groups in Douglas fir.

Seven of 12 linkage groups in Douglas fir had three or more orthologous markers that allow inspection of colinearity. Gene order was completely colinear in the syntenic regions of 5 of these 7 groups (LG1, LG3, LG4, LG5, and LG8) and partly colinear in LG6 (Figure 1). Local noncolinearity was observed only in two groups, LG6 and LG10 (Figure 1). There were two cases when two linkage groups in Douglas fir showed synteny with a single linkage group in loblolly pine: (1) *Pm-3* and *Pm-13 vs. Pt-3* (LG3) and (2) *Pm-9* and *Pm-11 vs. Pt-9* (LG9).

DISCUSSION

Comparative mapping in Pinaceae: The macrosyntenic relationships between species of two genera of the family Pinaceae were established for nearly all major linkage groups. Ten homologous linkage groups were identified in loblolly pine that shared two or more orthologous markers with Douglas fir linkage groups (Figure 1). The same 10 homologous linkage groups were identified between loblolly pine and maritime pine (Chagné *et al.* 2003). All 12 homologous linkage groups were identified between loblolly pine and Scots pine, but only 10 linkage groups shared two and more orthologous markers (Komulainen *et al.* 2003). Therefore, the intergeneric maps in this study have identified practically all the same homologous linkage groups as were found in the intrageneric pine maps.

Chromosomal evolution in Pinaceae: The high level of synteny and colinearity among species within the Pinaceae supports the general hypothesis based on cytogenetic data that major chromosomal rearrangements have not been frequent in the evolution of the Pinaceae (Prager *et al.* 1976). Except in a few species, there is no evidence for major chromosomal rearrangement or polyploidy in Pinaceae (Prager *et al.* 1976). We also observed substantial colinearity between genera at the macrosyntenic level. Local noncolinearity was observed only in LG6 and LG10 and could be easily explained by a single inversion (Figure 1). However, it would be preliminary to speculate about local rearrangements and microcolinearity from these data. Local noncolinearity might also result from mapping paralogs and/or other members of multigene families in the same syntenic regions. Approximately 40% of all ESTP, STS, and RFLP markers tested could serve as orthologous loci. Some of the remaining nonorthologous markers were assayed under less stringent conditions and may therefore have revealed paralogs or different members of multigene families. This can be easily expected due to the complexity of conifer genomes (Kinlaw and Neale 1997). This is also supported by lower levels of identity observed between Douglas fir and loblolly pine sequences for nonorthologous (83%) *vs.* orthologous (89%) markers. There were a few ESTP primers that amplified two products that were mapped in the same region, showing that tandem duplication might be common in Pinaceae genome evolution. Paralogs complicate construction of comparative maps in Pinaceae, but are also of great interest for studying evolution of multigene families.

Pseudotsuga and Larix are the next closest genera to Pinus after Picea on the basis of phylogenetic studies (Wang *et al.* 2000; Rydin *et al.* 2002). However, the karyotype of Douglas fir is unique in Pinaceae. It has 13 chromosome pairs ($2n = 26$), while all other species, including closely related Pseudotsuga and Larix species, have only 12 pairs ($2n = 24$). The karyotype of Douglas fir includes 2 telocentric chromosomes that are strikingly dissimilar to the other 11 chromosomes (5 metacentric and 6 submetacentric chromosomes). Their length is also less than one-half that of the metacentric chromosomes (Doerksen and Ching 1972), suggesting that these two chromosomes originated by centromeric fission of one of the metacentric chromosomes. We could not resolve unambiguously the question of the origin of the thirteenth chromosome pair in Douglas fir, but our data allow some speculation. Douglas fir linkage groups *Pm-3* and *Pm-13* were syntenic with loblolly pine LG3, as were *Pm-9* and *Pm-11* with LG9 (Figure 1). One of these syntenic linkage pairs could represent two different chromosomes in Douglas fir. Orthologous markers mapped in these groups would be good candidates for FISH to resolve this question.

Potential applications of comparative-mapping results: Comparative mapping is an important tool for integrating genetic data among related taxa. It helps to consolidate genetic maps and bridge linkage gaps. For instance, comparative mapping has helped to assign several small unlinked groups to the larger homologous linkage groups in pine species (Brown *et al.* 2001; Komulainen *et al.* 2003). The loblolly pine × Douglas fir comparative map now integrates the mapping data between different genera of the family Pinaceae and also between the two most important tree species in North America. For example, the QTL mapped in loblolly pine (Sewell *et al.* 2000, 2002; Brown *et al.* 2003) and in Douglas fir (Jermstad *et al.* 2001a,b, 2003) can now be compared across different genera the same way as it was done across different species within genus Eucalyptus (Marques *et al.* 2002) and Pinus (Chagné *et al.* 2003). Mapped orthologous markers that consistently associated with the same QTL across different species can be used to confirm and verify QTL and to identify candidate genes for quantitative traits. The orthologous markers that have been developed and mapped in this study could also

TABLE 2

Orthologous markers mapped in both Douglas fir and loblolly pine

Type	Marker mapped in Douglas fir	Douglas fir linkage group	Position (cM)	GenBank accession no.	Orthologous marker mapped in loblolly pine	Syntenic linkage group in loblolly pine	GenBank accession no.	Comparison between Douglas fir and loblolly pine sequences	
								Nucleotide identity (%)	BLASTN *e*-value
ESTP	*estPpINR_RS01G05_a*[a]	1	27.5	AL750905	*estPpINR_RS01G05_a* in *Pinus pinaster*	1	AL750905	NA[a]	NA[a]
RFLP	*PtIFG_2006_a*[b]	1	45.1	H75041 H75042	*PtIFG_2006_A*	1	H75041 H75042	NA[b]	NA[b]
ESTP	*estPmIFG_155H02_a*[c]	1	71.4	CN637754	*estPtIFG_8537_a*	1	AA739563	89	1.E-107
ESTP	*estPmIFG_145F05_a*[c]	1	99.2	CN637175	*estPtIFG_8496_a*	1	AA739536	82	9.E-17
ESTP	*estPmIFG_202H11_a*[c]	2	11.8	CN638460	*estPtIFG_9022_a*	2	AI725138	93	1.E-160
RFLP	*PtIFG_2885_a*[b]	2	41.3	NA	*PtIFG_2885_B*	2	NA	NA[b]	NA[b]
RFLP	*PtIFG_2356_a*[b]	3	8.3	H75085	*PtIFG_2356_a*	3	H75085	NA[b]	NA[b]
RFLP	*PtIFG_2988_a*[b]	3	37.6	NA	*PtIFG_2988_a*	3	NA	NA[b]	NA[b]
RFLP	*PtIFG_TAM_5_a*[b]	3	38.2	NA	*PtIFG_TAM_5_a*	3	NA	NA[b]	N/A[b]
ESTP	*estPmIFG_143D03_a*[c]	3	65.1	CN637040	*estPtIFG_2889_a*	3	H75234	85	7.E-19
ESTP	*estPmaLU_SB29_a*[d]	13	0.0	AF051222	*estPmaLU_SB29_a*	3	AF051222	NS[d]	NS[d]
ESTP	*estPtNCS_6C12F_a*[c]	13	39.9	AA556811	*estPtNCS_6C12F_a*	3	AA556811	85[e]	3.E-30[e]
ESTP	*estPmIFG_102G09_a*[a]	4	0.0	CN634675	*estPtNCS_2N7G_a*	4	AA556198	84	3.E-50
ESTP	*estPmIFG_200A01_a*[c]	4	6.8	CN638230	*estPtIFG_APX*	4	AF326783	93	2.E-86
ESTP	*estPmIFG_130E12_a*[c]	4	27.9	CN636293	*estPtIFG_8429_a*	4	AA739505	91	1.E-57
ESTP	*estPtIFG_6C12A_a*[c]	4	44.2	AA556806	*estPtIFG_6C12A_a*	4	AA556806	93[e]	1.E-103[e]
ESTP	*estPmIFG_137G09_a*[d]	5	62.6	CN636731	*estPpINR_AN01D04_a*	5	AL749558	92[f]	0[f]
RFLP	*PtIFG_2540_a*[b]	5	74.4	H75131	*PtIFG_2540_a*	5	H75131	NA[b]	NA[b]
ESTP	*estPmIFG_152A04_a*[c]	5	78.6	CN637533	*estPtIFG_0893_a*	5	H75118	87	4.E-08[g]
ESTP	*estPmIFG_111F09_a*[c]	6	8.4	CN635180	*estPtIFG_8531_a*	6	AA739558	82	3.E-57
ESTP	*estPmIFG_144D01_a*[c]	6	10.5	CN637093	*estPt_8647_a*	6	AA739625	95	1.E-22
ESTP	*estPmIFG_014A07_a*[a]	6	63.8	CN634509	*estPtIFG_8473_a*	6	AA739526	84	7.E-16
ESTP	*estPtIFG_0739_a*[c]	6	64.2	H75167 H75168	*estPtIFG_0739_a*	6	H75167 H75168	92[e]	1.E-22[e]
RFLP	*PmIFG_1545_a*	6	64.4	AA701802 H75180	*PtIFG_1165_a*	6	AA701802 H75180	86[h]	1.E-10[h]
ESTP	*estPmaLU_SB42_a*[a]	6	80.3	AF051232	*estPmaLU_SB42_a* in *Picea abies*	6	AF051232	NA[a]	NA[a]
ESTP	*estPmIFG_109F09_a*[c]	6	82.5	CN635076	*estPtIFG_1950_a*	6	H75126	84	3.E-11
ESTP	*estPmIFG_113C11_a*[c]	6	96.8	CN635266	*estPtIFG_8564_a*	6	AA739580	95	5.E-55
ESTP	*estPmIFG_101B05_a*[c]	6	104.3	CN634590	*estPtIFG_9044_a*	6	AA739876	94	1.E-115
ESTP	*estPmIFG_201D12_a*[c]	6	127.8	CN638348	*estPtFR_LP15_a*	6	AF013803	82	9.E-25
ESTP	*estPtNCS_ctg3_a*[c]	6	137.0	AF036095	*estPtNCS_CCoAOMT_a*= *estPtNCS_ctg3_a*	6	AF036095	83[e]	2.E-26[e]
RFLP	*PtIFG_2703_a* (=*PtIFG_2707_A*)[b]	7	63.1	H75113 H75227 H75228	*PtIFG_2707_A* (=*PtIFG_2703_a*)[b]	7	H75113 H75227 H75228	NA[b]	NA[b]

(*continued*)

TABLE 2

(Continued)

Type	Marker mapped in Douglas fir	Douglas fir linkage group	Position (cM)	GenBank accession no.	Orthologous marker mapped in loblolly pine	Syntenic linkage group in loblolly pine	GenBank accession no.	Comparison between Douglas fir and loblolly pine sequences	
								Nucleotide identity (%)	BLASTN *e*-value
STS	*stsPmCAS_4CL2_a*[c]	7	90.5	AF144507	*estPtIFG_4CL_a*	7	U12012 U39405	88	1.E-173
STS	*stsPmCAS_4CL1_a*[c]	7	92.7	AF144506 AF144508	*estPtIFG_4CL_a*	7	U12012 U39405	87	1.E-166
RFLP	*PtIFG_2957_a*[b]	8	5.3	NA	*PtIFG_2957_a*	8	NA	NA[b]	NA[b]
RFLP	*PtIFG_2553_a*[b]	8	42.9	H75210	*PtIFG_2553_a*	8	H75210	NA[b]	NA[b]
ESTP	*estPtIFG_8732_a*[c]	8	47.3	AA739680	*estPtIFG_8732_a*	8	AA739680	90[e]	7.E-19[e]
ESTP	*estPaTUM_PA0053_a*[a]	8	70.5	AJ132535	*estPaTUM_PA0053_a* in *Picea abies*	8	AJ132535	NA[a]	NA[a]
STS	*stsPmCAS_CAD_a*[c]	9	22.7	AF145985 AF145986	*estPtNCS_ptCadA_a*	9	Z37991	90	2.E-92
ESTP	*estPmIFG_136B01_a*[c]	9	49.4	CN636620	*estPtIFG_500_a*	9	H75151	86	2.E-51
ESTP	*estPmIFG_141H03_a*[c]	11	0.0	CN636962	*PtIFG_2899_a*	9	H75245	95	2.E-71
ESTP	*estPtIFG_2290_b*[a]	11	28.8	H75067	*estPtIFG_2290_a*	9	H75067	NA[a]	NA[a]
ESTP	*estPmIFG_202A06_a*[c]	10	72.8	CN638390	*estPtIFG_8436_a*	10	AA739508	93	2.E-87
RFLP	*PtIFG_2025_a*[b]	10	88.8	H75045 H75046	*PtIFG_2025_a*	10	H75045 H75046	NA[b]	NA[b]
ESTP	*estPmaLU_SB21_a*[d]	10	103.0	AF051216	*estPmaLU_SB21_a* in *Picea abies*	10	AF051216	NS[d]	NS[d]
ESTP	*estPmIFG_115B07_a*[c]	10	118.2	CN635370	*estPtIFG_4CH-2_a*	10	AF096998	89	3.E-52
ESTP	*estPmIFG_128D06_a*[c]	10	131.1	CN636156	*estPtIFG_8580_a*	10	AA739590	87	1.E-22

[a] Direct sequencing of the PCR product was not applicable (NA) to these markers because their PCR pattern included additional background bands amplified. Those additional bands were mostly light, but they usually interfere with sequencing and produce unreadable sequence runs. However, all of these markers were mapped also as orthologs in several other species, supporting their orthology in Douglas fir.

[b] Nucleotide identity and *e*-values were not available (NA) for the RFLP markers because Douglas fir genomic DNA, which hybridized with the loblolly pine cDNA probes, was not sequenced.

[c] The identity of the ESTP marker to the corresponding EST sequence, which was used to design primers, was confirmed by sequencing the PCR product amplified in Douglas fir.

[d] The PCR product, which was amplified in Douglas fir for these markers, was not sequenced (NS), but all of these markers were mapped also as orthologs in several other species, supporting their orthology in Douglas fir.

[e] The values are based on comparison between the sequence of the PCR product amplified in Douglas fir and the EST sequence of the ESTP marker mapped in loblolly pine using the same PCR primers.

[f] Comparison was done between the maritime pine (*Pinus pinaster*) cDNA clone AN01D04 sequence and the Douglas fir contig sequence that included the EST sequence of the cDNA clone 137G09.

[g] The *e*-value was unsatisfactory because the Douglas fir EST sequence was very short and contained only partial coding sequence. However, the similarity between this partial coding region and loblolly pine counterpart was relatively high at the amino acid level (93%) and qualified this ESTP marker as a potential ortholog.

[h] Based on comparison between *PmIFG_1545* (AA701802) and *PtIFG_1165* (H75180) cDNA orthologous sequences.

be used with species of the remaining genera of Pinaceae that also have worldwide importance, such as Picea, Larix, Abies, and Tsuga. For example, these markers have recently been used to construct a comparative map between loblolly pine and Norway spruce (our unpublished data) that will further contribute toward a deeper understanding of the evolution of conifer genomes.

Establishing Pinaceae as a genetic system: We were able to establish basic syntenic relationships in Pinaceae. High synteny and conserved gene order found in this study for such distantly related species as Douglas fir and loblolly pine open the possibility of comparative mapping at the family level and establish a comparative genomics framework in Pinaceae that can now be viewed as a genetic system. However, more studies are needed to establish a comparative genomics system that would rival the existing model systems. ESTs represent a good alternative to complete genome sequencing for nonmodel organisms or organisms with large genomes, such as conifers. As more conifer EST and genomic sequences become available in public databases their homology can be tested and more orthologs can be established.

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