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Comparative mapping in *Pinus*: sugar pine (*Pinus lambertiana* Dougl.) and loblolly pine (*Pinus taeda* L.)

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Abstract The majority of genomic research in conifers has been conducted in the *Pinus* subgenus *Pinus* mostly due to the high economic importance of the species within this taxon. Genetic maps have been constructed for several of these pines and comparative mapping analyses have consistently revealed notable synteny. In contrast, little genomic research has been conducted on the *Pinus* subgenus *Strobus*, even though these pines have strong ecological relevance. We report a consensus genetic linkage map for sugar pine (*Pinus lambertiana* Dougl.) constructed with 399 single nucleotide polymorphisms markers derived from annotated genes. The map is 1,231 cM in length and organized into 19 linkage groups. Two of the mapping populations were derived from trees that were segregating for the major gene of resistance (*Cr1*) to *Cronartium ribicola*, the fungal pathogen responsible for white pine blister rust. The third mapping population was derived from a full-sib cross segregating for partial resistance to white pine blister rust. In addition, we report the first comparative mapping study between subgenera *Strobus* and *Pinus*. Sixty mapped markers were found in common between sugar pine and the loblolly pine reference map with 56 of them (93%) showing collinearity. All 19 linkage groups of the sugar pine consensus map coaligned to the 12 linkage groups of the loblolly pine reference map. The syntenic relationship observed between these two clades of pines provides a foundation for advancing genomic research and genetic resources in subgenus *Strobus*.

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Introduction

Sugar pine (*Pinus lambertiana* Dougl.) is found in mixed conifer forests of western North America, ranging from Baja Mexico to Oregon. Its abundance, large stature, and clear wood properties give it commercial importance in the lumber industry. Genetic research programs were established after World War II (Bingham 1983), primarily focused on breeding for resistance to white pine blister rust (WPBR), a disease caused by the fungal pathogen *Cronartium ribicola* A. Dietr. Worldwide, pines sub-genus *Strobus* (commonly referred to as soft pines) are susceptible to this disease (Miller et al. 1959). Fortuitously, innate immunity has been discovered in populations of many soft pines (Kinloch 1970; McDonald and Hoff 1970; Kinloch and Dupper 2002; Kinloch 2003; Sniezko et al. 2004). In

sugar pine, two inheritance patterns have been described in Kinloch et al. (2007): (1) simple resistance inherited as a major gene of resistance (MGR) in which a hyper-sensitive response is conferred by a single dominant gene, and (2) partial resistance which can be scored as a continuous trait and appears to be quantitatively inherited. To date, it is unknown how much of the observed phenotypic variation in partial resistance is controlled by genetic factors.

Genetic linkage maps provide a critical and integrative tool for investigating genomes and have been constructed for nearly all crop species and selected forest tree species, providing an important foundation for structural and functional genomics (Kole 2007). The majority of genetic maps constructed in pines have been for the *Pinus* sub-genus *Pinus* (commonly referred to as hard pines) because of their wide economic importance, with relatively little progress being made in the subgenus *Strobus*. An exception is eastern white pine (*Pinus strobus* L.) for which a full genome map was constructed using random amplified polymorphic DNA (RAPD) markers and a small number of microsatellites (Echt and Nelson 1997). Additionally, linkage analysis has been reported in western white pine (*Pinus monticola* Dougl. ex D. Don) (Liu and Ekramoddoullah 2007, 2008; Liu et al. 2006) and in sugar pine (Devey et al. 1995; Harkins et al. 1998) in which the MGR (named *Cr1* for sugar pine and *Cr2* for western white pine in Kinloch et al. 1999) was mapped to a single linkage group using RAPD markers.

The advent of high throughput sequencing has allowed for full genome sequencing in many model species in the plant and animal kingdoms (Tuskan et al. 2006; Venter et al. 2001; Lindblad-Toh et al. 2005). As pine genome sequences become available, the utility of comparative mapping among conifers will increase. Collinearity between orthologous markers will facilitate the identification of genes associated with important traits in both intra- and inter-specific comparative mapping studies (Duran et al. 2009; Devos 2005). Synteny has been reported among the hard pines such as Monterey pine (*Pinus radiata* L.; Devey et al. 1995), slash pine (*Pinus elliottii* Engel.; Brown et al. 2001), Scots pine (*Pinus sylvestris* L.; Komulainen et al. 2003) and maritime pine (*Pinus pinaster* Aiton.; Chagne et al. 2003) using loblolly pine as a reference species (Flavell 2009). Likewise, syntenic relationship was established (Neale and Krutovsky 2004) between loblolly pine and species belonging to other genera of the Pinaceae, such as Douglas-fir (*Pseudotsuga menziesii* [Mirb] Franco; Krutovsky et al. 2004) and Norway Spruce (*Picea abies* [L] Karst; unpublished), but never between the two subgenera within the genus *Pinus*.

The sugar pine mapping populations used for map construction in this study were designed for the purpose of identifying the genetic factors conferring resistance to WPBR. The individual maps for trees 5701 and 6000 reported here have been used to map *Cr1*. A third mapping population, a full-sib cross, was established for mapping quantitative trait loci (QTL) for partial resistance. A genetic map for the QTL population will provide the necessary linkage information to discover the genetic factors responsible for partial resistance. Map positions of markers from one map may be inferred or extrapolated onto another map based on the alignment of common markers or the relationship of neighboring markers (Dirlewanger et al. 2004). For example, the position of *Cr1* can be inferred in the QTL map based on the position of markers common to both species, even though *Cr1* (hypersensitive response) is not expressed or mapped in the QTL population. This ability to extrapolate information across families and species will be of great value in future studies of the soft pines.

We present here a consensus genetic map for sugar pine using mapping populations that express two inheritance patterns of resistance to WPBR. The map is composed of markers that are useful across taxonomic boundaries, have functional significance for adaptive variation, and can be used for future genomic and population genetic studies in pines of the subgenus *Strobus*. We also report the first comparative mapping analysis between the *Pinus* subgenera *Pinus* and *Strobus*. As anticipated, a syntenic relationship was observed between sugar pine and loblolly pine. It is likely that future comparative studies within *Strobus* will reveal even higher numbers of orthologous markers and will be invaluable for basic research in this subgenus.

Materials and methods

Mapping populations

Three populations were used for genetic mapping: two open-pollinated haploid mapping populations derived from single trees (5701 and 6000) which were heterozygous for resistance to WPBR and previously used for mapping the MGR (Devey et al. 1995; Harkins et al. 1998), and a diploid mapping population derived from a controlled-cross (5038×5500) designed for the genetic mapping of QTL conferring partial resistance. Several hundred seed was collected from open-pollinated cones from each of two sugar pine trees, 5701 and 6000. Seedlings were grown in the greenhouse and megagametophytes (haploid seed tissue) were rescued upon germination, and stored at −80°C for DNA isolation (Devey et al. 1995). Eight-week-old seedlings were artificially challenged with the appropriate strain of *C. ribicola* A. Dietr. (Kinloch and Dupper 2002) using standard protocols described in Kinloch (1992). The disease phenotype (hypersensitive response) was scored present/absent for segregation analyses (Devey et al. 1995). For this study, tissue from 95 megagametophytes from each

single tree population (5701 and 6000) was excised for DNA isolation. For the QTL mapping population, needle tissue was harvested from the progeny ($n=94$) and parents (5038×5500), cut into 2 mm lengths, flash frozen in liquid nitrogen and stored at $-80°C$.

DNA isolation

Total genomic DNA was extracted from each sample using DNEasy-96 (Qiagen, Inc., Valencia, CA, USA) plant extraction kit, following the provided protocols for liquid nitrogen extraction. Prior to homogenization, megagametophyte and needle tissue were ground to a powder under liquid nitrogen in a Mixer-Mill (Qiagen Inc., Valencia, CA, USA). DNA concentrations were determined by fluorometry using Pico Green (Molecular Probes, Eugene, OR, USA) and adjusted to 50 ng/µl for single nucleotide polymorphism (SNP) genotyping.

SNP discovery and genotyping

The SNPs used in this mapping exercise were derived from the Conifer Re-Sequencing Project (CRSP; http://loblolly. ucdavis.edu/bipod/ftp/CRSP/) in which re-sequencing (Sanger and Coulson 1975) was performed with a set of primer-pairs from loblolly pine ESTs on a small panel of range-wide samples of sugar pine ($n=12$; nine from California, two from Oregon, and one from Baja, MX, USA). The set of loblolly primer-pairs were part of a larger set developed originally for the ADEPT 2 project (http://dendrome.ucdavis.edu/NealeLab/adept2/overview.php/). Primers, amplicon sequences, and annotations for the loblolly pine ESTs can be found at DiversiTree Sequence database located at TreeGenes (Wegrzyn et al. 2008; http://dendrome.ucdavis.edu/treegenes/), and contig alignments have been submitted to GenBank; PopSet Accession numbers FJ043059—FJ147084. Re-sequencing in sugar pine (CRSP, unpublished) resulted in the discovery of 4,238 SNPs which were identified using PineSap software described in Wegrzyn et al. (2009). SNPs were then rated based on Golden Gate SNP primer design scores (Illumina, San Diego, CA, USA). For design of a 1,536 SNP genotyping assay 1,508 SNPs were selected for genotyping based on (1) primer design scores (designability rank=1.0) and (2) loblolly pine EST annotations related to biotic and abiotic stress, and transcription factors. Prescreening for CRSP-derived SNP polymorphisms in our sugar pine mapping populations was not performed. An additional 28 SNPs from sequence characterized amplified regions (SCARs) linked to the MGRs *Cr1* and *Cr2* were included in the 1,536 SNP genotyping assay (RAPD-to-SCAR conversion described below). The 28 SCAR SNPs were derived from resequencing in a subsample ($n=16$) of the

three mapping populations and were included in the SNP genotyping assay if polymorphism was detected in any one population and the primer designability rank ≥ 0.5.

Genotyping was performed on all samples using the Illumina Golden Gate SNP genotyping assay, and was carried out at the DNA Technologies Core in the UC Davis Genome Center, Davis, CA, USA as described in Eckert et al. (2009a). Fluorescence intensities of assay products were quantified on the BeadArray Reader (Ilumina, Inc., San Diego, CA, USA) and analyzed using BeadStudio v. 3.1.14 (Illumina, Inc., San Diego, CA, USA). SNP assays were evaluated for quality using the Illumina software. Thresholds for data inclusion were a Gen-Call$_{50}$ score >0.35 and a call rate >0.85 (Eckert et al. 2009a). The QTL population was evaluated independent of the single tree populations in order to increase classification precision of heterozygote clusters. Manual adjustments were made to the fluorescent clusters as needed, determined by the expected allele transmission of markers in each given population. For example, a polymorphism detected at a locus in the megagamephyte haploid populations (single trees 5701 and 6000) should result in approximately 50% segregation in each of the homozygous allele classes (*aa* or *bb* cluster), but none in the heterozyogous class (*ab* cluster). The samples from the QTL population, on the other hand, were diploid and thus the heterozygous class was expected at each polymorphic locus. Markers that were monomorphic were identified and removed from further analysis.

RAPD marker to SCAR marker conversion

Two RAPD markers (BC_432_1110 and OPG_16_950) were previously positioned in close proximity to the *Cr1* in haploid populations 5701 and 6000 (Harkins et al. 1998). In order to position these dominant markers in the QTL population, they first were converted to codominant SCAR markers. RAPD bands were polymerase chain reaction (PCR) amplified in tree 5701 as described in Devey et al. (1995) and excised from 1% agarose gels. DNA was gel-purified using Qiaquick gel purification columns (Qiagen Inc., Valencia, CA, USA) and cloned using the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA, USA). DNA sequencing (Sanger and Coulson 1975) was performed with vector primers on 96 samples of insert that were the appropriate molecular weight. One unique sequence was observed for the OPG_16_950 gel band whereas, five unique insert sequences were found for the BC_432_1110 gel band. Nested primers for each candidate SCAR marker were designed for resequencing in genomic DNA from the original populations used for RAPD mapping and in the QTL population (Table 1). One of the insert sequences for BC_432_1110 and the insert sequence for OPG_16_950 co-segregated with their corresponding RAPD bands and

Table 1 Primers for resequencing SCAR amplicons

SCAR name (species)	Amplicon forward primer	Amplicon reverse primer
scarBC_432_1110 (sugar pine)	5′ ACAATTTCCTCTTGCTTTGGAG 3′	5′ GACCGTTTTATCTTCCTCAGAAAA 3′
scarOPG_16_950 (sugar pine)	5′ CATCCTATCATCTTCACCCACA 3′	5′ AGGAGACCTGATACACCGACTC 3′
Scar420[a] (western white pine)	5′ GGCCCGCTAATATGTTAT 3′	5′ GGCCGCTAATGAAGC 3′
Scar320[a] (western white pine)	5′ CCGATCTAAGCGAACTCCA 3′	5′ ACCCCACAAGCATATGTAGA 3′

[a] Liu and Ekramoddoullah 2008

also with the disease phenotype (*Cr1*), indicating success of marker conversion. BLAST (Altschul et al. 1990) queries against plant the nucleotide or protein database resulted in neither SCAR (named *scar*OPG_16_950 and *scar*BC_432_1110) having similarity with any nucleotide or translated protein sequences in GenBank (alignment score <40). SCAR SNPs were identified by resequencing in a small sample of each of the mapping populations (described above).

In an attempt to infer the map position of *Cr2* in relation to *Cr1*, primers from two SCARs linked to *Cr2* in western white pine (Kinloch et al. 1999; Liu and Ekramoddoullah 2008) were used to amplify sugar pine genomic DNA in the three mapping populations (Table 1). Only *scar*420, yielded PCR product and was subsequently resequenced in sugar pine to discover SNPs for genotyping and linkage analysis.

Segregation and linkage analysis

In the two haploid mapping populations (5701 and 6000), marker segregation was expected in 1:1 (backcross configuration) ratios only, whereas in the diploid QTL population (5038×5500), both 1:1 and 1:2:1 (intercross configuration) segregation ratios were expected. Polymorphic loci were subjected to a single-locus segregation chi-square test using JoinMap v. 2.0 (Stam and van Ooijen 1995) and those showing segregation distortion at $p \leq 0.001$ were removed from the dataset. For trees 5701 and 6000, disease phenotype (*Cr1*) scores were added to the marker dataset. Linkage analyses were performed separately for each population using JoinMap v. 1.4 (Stam 1993). The Kosambi function was used to estimate map distances, and LOD thresholds of 4.0 (LinkLOD) and 0.1 (MapLOD) were used for grouping markers into linkage groups (LGs) and for ordering markers, respectively (Jermstad et al. 2001; Wheeler et al. 2005; Eckert Andrew et al. 2009b). Markers that were unlinked to any other markers or linked to only one other marker (doublet) were excluded from further analysis. Linkage groups and map order were examined with and without markers that showed distortion at $p = 0.001$ (Xian-Liang et al. 2006). For the final analysis, these distorted markers were omitted except for the *scar*420_QTLpop-124 ($p = 0.0001$) which only segregated

in population 6000. To further check for map integrity, linkage analyses were performed at higher LinkLOD thresholds (4.5 and 5.0) and the maps were re-examined. Four individual maps were constructed (LinkLOD 4; MapLOD 0.1) from the four trees of the three mapping populations: (1) 5701, (2) 6000, (3) 5038 (QTL female parent), and (4) 5500 (QTL male parent). The segregation data from the parent trees of the QTL population (5038× 5500) were then merged to construct a sex-averaged map.

For construction of the consensus map, segregation data from 5701, 6000, 5038, and 5500 were compiled into a single data segregation file. Markers that were analyzed but not positioned on the individual tree maps were included in the consensus linkage analysis, with the expectation of finding linkage with markers from alternate populations. The dataset consisted of two types of conserved orthologous sequence (COS) markers: (1) common gene amplicon but different SNP address within the amplicon (type I), and (2) common gene amplicon and common SNP address within the amplicon (type II). Prior to merging the datasets for consensus mapping, a chi-square test for heterogeneity of recombination estimates among the four parental datasets was performed using the JoinMap v. 2.0 HET module. This test was applicable to only markers with identical marker names, i.e., type II COS markers. Type II COS markers found in >1 population were merged and analyzed as a single marker by JoinMap. To detect spurious linkages, the LinkLod threshold was raised incrementally from 4.0 to 8.0. All LGs remained stable except for one group that split into two LGs at a LinkLOD 7.0 threshold. These two resultant linkage groups (6 and 10a; Suppl. Fig. 1) remained stable when analyzed with LinkLOD thresholds up to 8.0. Ultimately, the final consensus map was constructed using a LinkLOD threshold of 5.0, except for LGs 6 and 10a, which were formed with a LinkLOD threshold of 7.0 As recommended in the JoinMap manual, the MapLOD threshold was lowered to 0.001 to ease the positioning of markers by raising the goodness-of-fit measure.

Comparative mapping

All single tree maps (5701, 6000, 5038, and 5500) and the QTL sex-averaged map (5038×5500) were aligned with the

sugar pine consensus map. The consensus map LG that was split to form LG 6 and 10a at a higher LinkLOD threshold was examined and re-evaluated in the individual maps. In 5701, this group of markers was split into two LGs at LinkLOD 8.0 which aligned also with LGs 6 and 10a of the consensus map. In the sex-averaged map of the QTL population, a LG diverged into two LGs when analyzed at LinkLOD 5.0; one of which aligned with consensus map LG 10a and a second group that aligned with LG 1a. The group that aligned with consensus map LG 10a contained inter-cross markers that segregated in both parents, whereas the group that aligned with LG 1a contained markers segregating only in the male parent.

The sugar pine consensus map was subsequently aligned with and compared to the 12 linkage groups of the loblolly pine reference map described in Eckert et al. (2010) (TG091; http://dendrome.ucdavis.edu/cmap/) using type I COS markers. This was accomplished by querying the loblolly pine map with all type I COS markers (amplicon only, i.e., CL3036Contig1-01) from the sugar pine consensus map using a word processor *find function*. Once the alignments were made, the linkage groups of the sugar pine consensus map were numbered according to their collinear relationship to loblolly pine linkage groups. When more than one SP LG aligned with a loblolly pine LG, a letter suffix was appended to the LG number.

Results and discussion

SNP genotyping and single locus segregation analysis

Of the 1,536 SNP assays, 258 (17%) failed to either produce product or fluorescence in all three populations. In further evaluations, markers were omitted from each dataset because they were monomorphic or showed segregation distortion at $p \leq 0.001$ (Table 2). A surprisingly high number

Table 2 Summary of SNP genotyping in three sugar pine mapping populations

	Tree 5701 (*n*)	Tree 6000 (*n*)	*QTL* population (2*n*)
Failed reaction	258	258	258
Monomorphic	985	1011	839
Distorted $p \leq 0.001$	102	78	157
Segregating	191	189	282

The mapping populations from trees 5701 and 6000 consisted of a sample of megagametophytes (*n*=95) from open-pollinated seed. The QTL population consisted of needle tissue from a full-sib family of progeny (*n*=94) and both parents

of monomorphic loci (avg.=945; Table 2) were detected in all three populations, contributing to the low number of markers available for linkage analysis. The SNPs used in this mapping study were developed mainly for comparative genomics, genetic diversity and association studies (Eckert et al. 2010). Prescreening for polymorphisms in a small sample from our mapping populations may have resulted in an increased number of markers available for linkage analysis.

Seven of the ten SNPs from *scar*BC_432_1110 were successfully genotyped; however, all of these were monomorphic (Table 3). Eight of the 14 SNPs derived from *scar*OPG_16_950 were successfully genoptyped in at least one population, and segregated and mapped in the population from which it was derived (Table 3). All three SNPs from *scar*420 were successfully genotyped in each population assayed, however, only *scar*420_QTLpop-124 was found to be polymorphic but only in tree 6000 (Table 3). SCAR SNPs were included in the SNP genotyping assay even if a SNP was found in only one population.

Linkage maps

The six genetic linkage maps reported here are recorded in the TreeGenes Comparative Mapping Database (Wegrzyn et al. 2008) and can be viewed at http://dendrome.ucdavis.edu/cmap/.

5701 map (TG101)

For tree 5701, 191 polymorphic SNP loci and the *Cr1* locus were used in linkage analysis (Tables 2, 4). The map for single tree 5701 (TG101) was composed of 183 markers organized into 16 linkage groups (Table 4) of ≥3 markers. All but nine markers were incorporated into the map. Map distance measured 822.1 centiMorgans (cM). The average size of linkage groups was 51.4 cM and the average distance between markers was 4.5 cM. *Cr1* and six SNP markers from *scar*OPG_16_950 mapped in close proximity to one another (Fig. 1) confirming earlier mapping studies (Harkins et al. 1998) and successful SCAR conversion.

6000 map (TG102)

For tree 6000 (#TG102), 189 polymorphic SNP loci and the *Cr1* locus were used in linkage analysis (Tables 2, 4). The map was composed of 186 markers organized into 17 linkage groups (Table 4) of ≥3 markers. All but four markers were positioned on the map. Map distance measured 849.1 cM. The average size of linkage groups was 49.9 cM and the average distance between markers was

Table 3 Results of genotyping, single locus segregation and linkage analysis of SNPs derived from SCARs associated with MGRs

SCAR marker name[a]	SNP	Genotyping success in each population		Polymorphic	Positioned on map
scarBC_432_SP_QTL-179	[A/G]	All three	No	–	–
scarBC432_SP_QTL-217	[T/C]	All three	Yes	No	–
scarBC432_SP_QTL-378	[C/G]	All three	Yes	No	–
scarBC432_SP_QTL-399	[T/G]	All three	Yes	No	–
scarBC432_SP_QTL-445	[A/C]	All three	Yes	No	–
scarBC432_SP_QTL-50	[T/C]	All three	Yes	No	–
scarBC432_SP_QTL-511	[A/C]	All three	Yes	No	–
scarBC432_SP_QTL-75	[T/C]	All three	No	–	–
scarBC432_SP5701-222	[A/G]	All three	Yes	No	–
scarBC432_SP6000-148	[T/C]	All three	No	–	–
scarOPG_16_QTLpop-242	[T/G]	QTL	Yes	Yes ♀	No
		5701	–	–	–
		6000	–	–	–
scarOPG_16_QTLpop-268	[A/G]	All three	No	–	–
scarOPG_16_QTLpop-53	[T/G]	All three	No	–	–
scarOPG_16_QTLpop-54	[A/G]	QTL	Yes	Yes ♂	Yes
		5701	Yes	No	–
		6000	No	–	–
scarOPG_16_SP5701-191	[C/G]	QTL	Yes	No	–
		5701	Yes	Yes	Yes
		6000	Yes	No	–
scarOPG_16_SP5701-212	[T/C]	QTL	Yes	No	–
		5701	Yes	Yes	Yes
		6000	No	–	–
scarOPG_16_SP5701-267	[T/C]	QTL	Yes	No	–
		5701	Yes	Yes	Yes
		6000	Yes	No	–
scarOPG_16_SP5701-348	[C/G]	QTL	Yes	No	–
		5701	Yes	Yes	Yes
		6000	Yes	No	–
scarOPG_16_SP5701-438	[T/C]	QTL	Yes	No	–
		5701	Yes	Yes	Yes
		6000	Yes	No	–
scarOPG_16_SP5701-466	[A/G]	All three	No	–	–
scarOPG_16_SP5701-469	[C/G]	All three	No	–	–
scarOPG_16_SP5701-505	[A/C]	QTL	No	–	–
		5701	Yes	Yes	Yes
		6000	No	–	–
scarOPG_16_SP5701-520	[C/G]	All three	No	–	–
scarOPG_16_SP5701-67	[T/C]	All three	No	–	–
scarOPG_16_SP6000-764	[A/G]	QTL	No	–	–
		5701	No	–	–
		6000	Yes	Yes	Yes
Scar420_QTLpop-91	[A/C]	QTL	Yes	No	–
		5701	Yes	No	–
		6000	Yes	No	–
scar420_QTLpop-124	[A/G]	QTL	Yes	No	–
		5701	Yes	No	–
		6000	Yes	Yes	No
Scar420_QTLpop-127	[T/C]	QTL	Yes	No	–
		5701	Yes	No	–
		6000	Yes	No	–

[a] The SCAR marker name includes the population from which the SNP was derived

Table 4 A summary of sugar pine linkage analyses

	Tree 5701 (TG101)	Tree 6000 (TG102)	Tree 5038 (♀) (TG103)	Tree 5500 (♂) (TG104)	QTL sex-averaged (TG105)	Consensus (TG106)
No. markers in JM input file	192	190	165	165	282[a]	457[b]
No. markers unmapped[c]	9	4	43	48	69	57
No. markers mapped	183	186	122	117	213	400
No. LGs	16	17	19	19	23	19
Map length (cM)	822.1	849.1	642.8	883.8	1,142.7	1,230.9
Cr1 mapped	√	√	–	–	–	√
*scar*OPG_16	√	√	–	√	√	√

Maps 5701 and 6000 were derived from megagametophytes ($n=95$) from adult open-pollinated trees that are heterozygous for *Cr1*. Maps 5038 and 5500 are derived from the parents of the QTL population, while the sex-averaged map is derived from the progeny ($n=94$) and the two parents of the QTL population. The consensus map is derived from the four adult trees

[a] 165 markers segregated in the maternal parent and 165 markers segregated in the paternal parent. Forty-eight of these markers were in the intercross configuration and should only be counted once in the sex-averaged linkage analysis because the marker data merge and map to a single position [$165+165=330-48$ (IC)$=282$]

[b] Though the sum of markers for the individual adult trees$=712$, the number of markers recognized and analyzed by JoinMap is 457. The reason for this is that type II COS marker data found in >one tree were merged and analyzed as a single locus, and thus, counted only once. Among the four trees, there were 255 type II COS markers ($712-457=255$)

[c] These are markers that linked to ≤1 marker (s)

4.6 cM. *Cr1* and one SNP from *scar*OPG_16_950 mapped in close proximity to one another confirming earlier mapping studies (Harkins et al. 1998) and successful SCAR conversion (Fig. 1). Even though *scar*420_QTL-pop-124 showed segregation distortion ($p=0.0001$), it was included in linkage analysis; however, it failed to link to any other markers and was one of the four markers not positioned on the map (Table 3).

QTL population

For the QTL mapping population, 282 polymorphic loci were entered into linkage analysis (Tables 2, 4) producing two parental maps, which in turn were merged into a sex-averaged map. There were 165 loci segregating in each of the parents, 48 of which were of the intercross (IC) configuration (1:2:1). Serendipitously, there were an equal number of loci unique to each parent (117 maternally informative and 117 paternally informative) in addition to the 48 IC loci.

Maternal map 5038 (TG103)

The maternal map (TG103) incorporated 120 markers organized into 19 linkage groups (Table 4) of ≥3 markers. Map distance measured 642.8 cM. The average size of linkage groups was 33.8 and the average distance between markers was 5.4 cM. *scar*OPG16_QTLpop-242 segregated exclusively in the female parent, but was not incorporated into the map (Table 3; Fig. 1).

Paternal map 5500 (TG104)

The paternal map (TG104) incorporated 112 markers organized into 19 linkage groups (Table 4) of ≥3 markers, which is the same number of linkage groups as in the female map. Map distance measured 883.8 cM which was 27% greater than that determined for the maternal map, even though the male map consisted of fewer markers. This difference is likely due to the higher rate of meiotic recombination typically found in the male parent (Sewell et al. 1999). The average size of linkage groups was 46.5 cM and the average distance between markers was 7.6 cM.

*scar*OPG_16_QTLpop-54 segregated exclusively in the male parent and was successfully positioned onto the paternal map (Table 3; Fig. 1).

QTL sex-averaged map (TG105)

Markers segregating in the IC configuration (1:2:1) provide the basis for merging the two parental maps into a sex-averaged map (#TG105). The map consisted of 213 markers organized into 23 linkage groups (Table 4) of ≥3 markers. Sixteen of the 22 linkage groups had markers that were segregating in both parents, whereas, seven linkage groups were uniparental. Three linkage groups consisted of markers that were segregating solely in the female parent and four linkage groups consisted of markers that were segregating solely in the male parent. Map distance measured 1,142.7 cM. The average size of linkage groups

was 49.7 and the average distance between markers was 5.4 cM. scarOPG_16_QTLpop-54 was positioned on the sex-averaged QTL map (uniparental segregation in the male) and suggests the location of the *Cr1* locus (Fig. 1) even though MGR is not expressed in this population.

Consensus map (TG106)

The consensus map (#TG106) was composed of 400 markers (399 SNPs and *Cr1*) organized into 19 linkage groups (Table 4) and map distance measured 1,230.9 cM. The loblolly pine amplicon sequences and primer pairs for the markers in the sugar pine consensus map are shown in Suppl. Table 1. The average size linkage group was 64.8 cM and the average distance between markers was 3.1 cM. Although the summation of marker data for the four individual trees (5701, 6000, 5038, and 5500) totaled 712, 255 of them were type II COS markers. JoinMap merged these orthologous markers, thereby reducing the

number of markers for analysis from 712 to 457 (Table 4). The test for heterogeneity among populations revealed 22 marker pairs with dissimilar recombination frequencies ($p \leq 0.001$). Several of the markers were represented in more than one marker pair. Methodical removal of these markers had a negligible effect on map order or map distance.

Genotyping in multiple populations and genetic backgrounds increases the probability of mapping a larger collection of polymorphic markers. Thus, we were hopeful that the number of linkage groups in the consensus map would resemble the true number of chromosomes for pines ($n=12$). However, it is not uncommon for first generation maps to have a higher number of LGs than expected. In development of the first consensus loblolly pine map, linkage analysis using 581 genetic markers from four adult trees resulted in 20 LGs (Sewell et al. 1999) with 14 of the LGs containing orthologous markers. In order to reduce the number of LGs in the sugar pine consensus map, additional type II COS markers (SNPs) that are evenly distributed
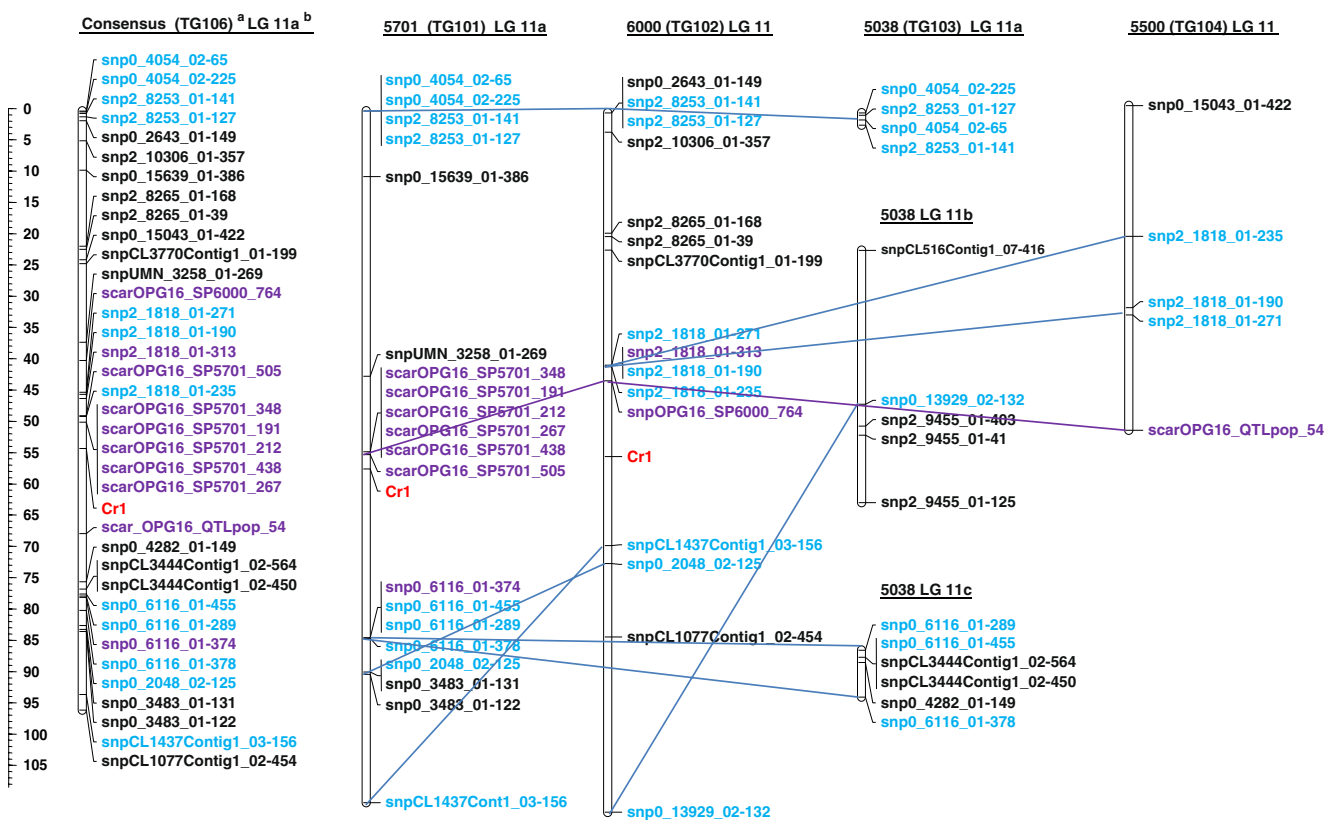
[a] Accession number assigned to maps in the TreeGenes Comparative Mapping Database (http://dendrome.ucdavis.edu/cmap/)

[b] Maps with > 1 linkage group aligning with the *P. taeda* map are given letter suffixes, such as "a", "b", etc.

Fig. 1 Linkage group 11a of the sugar pine consensus map (TG106) is shown aligned with the same linkage group from the four corresponding individual linkage maps. Fourteen of the 50 SNP markers in LG 11a of the consensus map were unique to an individual map (non-orthologous) and are shown in *black font*. Type I COS markers (amplicons) that coaligned among two or three trees are

shown in *blue font*. Type II COS markers (SNP) that coaligned among two or three trees are shown in *purple font*. All of the SCAR markers were type I COS markers with the unique SNPs mapping specifically to the populations from which they were derived. The major gene for resistance to white pine blister rust (*Cr1*) is shown in *red font*

throughout the genome are needed. Segregation data from this type of orthologous marker are analyzed by JoinMap as a single locus, and allows for the merging of LGs from multiple populations.

Comparative mapping in sugar pine

All of the individual maps were of similar length with more marker incorporation in the two single tree maps 5701 and 6000 (Table 4). The parental maps of the QTL population (5038 and 5500) resulted in an equal number of LGs with the paternal map having the greater map length (Table 4). It is not uncommon for parental maps to differ in the number of linkage groups and map length (Groover et al. 1994; Jermstad et al. 1998) due to marker configurations and abundance. Differences in meiotic recombination frequencies in the male and female parents can also influence map length, with higher recombination estimates being reported for the male parent (Sewell et al. 1999). There were no LGs in any of the four individual maps that did not find alignment with the consensus map.

Intra-specific comparative mapping is useful in map construction by identifying spurious linkages and gaps where marker information may be non-existent or weak. We were able to align both type I and type II COS markers among the sugar pine maps. It was not known a priori which SNPs would segregate in multiple mapping populations, therefore, genotyping of several SNPs from the same amplicon was necessary for successful consensus and comparative mapping. In general, the number of type II COS markers in common between trees was higher than the number of type I COS markers between trees (Table 5). Type I COS markers (amplicons) were counted only once in pair-wise calculations, regardless of how many type II COS markers (SNPs) were derived from it. By aligning COS markers, we were able to validate map organization among the various individual maps (Xian-Liang et al.

Table 5 Pair-wise calculations of orthologous markers found in common among the four individual sugar pine maps

Tree	5701 (TG101)	6000 (TG102)	5038 ♀ (TG103)	5500 ♂ (TG104)
5701	–	59	39	35
6000	64	–	49	47
5038 ♀	41	40	–	35
5500 ♂	38	47	32	–

Type I COS markers (amplicons) found in common among the four individual sugar pine maps are reported in the top half of the matrix. Type II COS markers (SNPs) found in common among the four individual sugar pine maps are reported in the bottom half of the matrix

2006). We were also able to infer the position of *Cr1* in the sex-averaged map and the final consensus map (Fig. 1) because of collinear positioning of the *scar*OPG_16_950 markers and several other orthologous markers. For example, markers snp0_6116_01, snp2_8253_01 and snp2_1818_01 showed syntenic relationship to each other in multiple trees, and aligned to the same linkage group on which *Cr1* was positioned in the 5701 and 6000 maps (Fig. 1).

Comparative mapping between sugar pine and loblolly pine

Of the 399 SNP markers positioned on the sugar pine consensus map, a total of 60 type I COS markers (amplicons) were in common with the loblolly pine map, with 56 (93%) showing collinearity in map position (Suppl. Fig. 1). A larger proportion of type I COS markers might have been discovered had our markers been selected primarily for comparative mapping. However, the priority for marker selection was set on genes with function related to stress resistance and future population and comparative genomic studies in sugar pine. A proportion of the SNP primers failed in the genotyping assay (258/1536; 17%), despite selection of SNP primers with high designability scores. However, the highest cause of data attrition in this study was due to the unexpected high proportion (945avg/1536; 62%) of monomorphic loci (Table 2).

Regardless of the relatively small proportion of orthologous markers detected between loblolly pine and sugar pine, each of the 12 loblolly pine linkage groups coaligned with one or more sugar pine linkage groups and all 19 sugar pine linkage groups found placement within the loblolly pine map. All sugar pine LGs had ≥2 markers that aligned with markers in the loblolly pine reference map, except for LG 6, which had only one marker in common with the loblolly pine map. In four cases, there were inconsistencies in linkage group assignment of type I COS markers (snp0_12190_02 and snpCL3862Contig1_06 markers on *Pl* LG 3a; snp0_15361_1 marker on *Pl* LG 10b; and snp2_6130_01 markers on *Pl* LG 1a; Suppl. Fig. 1). These markers showed no segregation distortion. In trees 5701 and 5500, the marker pair snp0_15361_1-64: snp0_9284_02-822 showed significant deviance from the chi-square heterogeneity test ($p \leq 0.001$). However, the most common observation for these markers was that they were mapped in only one of the four trees. Removing each of these four markers heuristically from linkage analysis had little or no effect in map order, so they were not omitted from the final consensus map. It remains unknown why these markers did not coalign, but a plausible cause is that an alternate member of a dispersed gene family (paralog) was

amplified instead of a sequence orthologous to the loblolly pine amplicon. The amplicon sequences used for developing SNP markers in this study were derived from the 3′ untranslated region of functional genes with the assumption that this region is unique to individual members of gene families, thereby reducing the occurrence of amplifying parologous sequences. The difficulty of resequencing and mapping paralogs versus orthologs will remain a challenge until a reference genome sequence for pines is available. A second explanation, chromosomal rearrangement within the genome, is possible but presents a difficult conjecture to support.

We were successful in inferring the relative position of *Cr1* in loblolly pine. The sugar pine LG containing scar*OPG_16_950* markers and *Cr1* aligned with loblolly pine LG 11 with two collinear markers showing good relative positioning (Fig. 1). This alignment implies that a potential *Cr1* ortholog is centrally located on LG 11. Loblolly pine is currently scheduled for full genome sequencing, which may enable identification of the *Cr1* ortholog and neighboring genes.

Genomics and simple resistance to white pine blister rust

Several SNPs from scar*OPG_16* were successfully mapped in sugar pine and were linked to *Cr1*, (Table 3) corroborating earlier mapping studies (Harkins et al. 1998). We were unsuccessful in our attempt to map SCARs that were shown to be linked to *Cr2* in western white pine. The genotyping assay either (1) failed, (2) resulted in monomorphism, or (3) resulted in segregation distortion. It remains unknown whether *Cr2* is allelic to *Cr1* or is another locus, linked or unlinked. In many plant species, it is common for resistance gene analogs (RGAs) to be duplicated and organized in localized clusters (McHale et al. 2008). RGAs have been reported in several pines (Meyers et al. 1999; Liu and Ekramoddoullah 2007; Jermstad et al. 2006), however, the sugar pine nucleotide sequence of *Cr1* remains unknown. Possible strategies to obtain the sequence for *Cr1* include: (1) whole genome sequencing, (2) electronic northern analyses on transcripts derived from differentially treated tissues, (3) microarray analysis. The use of *Cr1* as a molecular diagnostic marker would enable forest land managers to monitor the frequency of *Cr1* in wild populations and expedite the identification of resistant trees for use in breeding programs. Currently, candidate *Cr1* seed trees are identified through cultivation and artificial-inoculation screening of seedlings under nursery conditions, a process requiring more than a year to accomplish. A genetic marker for *Cr1* would require needle sampling for determining the zygosity at the *Cr1* locus. The reduction in cost and labor would allow for larger samples of trees to be screened (Dirlewanger et al. 2004).

Conclusion

We have developed a consensus linkage map for sugar pine using populations that are segregating for resistance to WPBR. We have positioned a major gene of resistance (*Cr1*) onto the map as well as 399 SNP markers representing annotated genes. In addition, we have shown a syntenic relationship between subgenus *Strobus* and a reference species from subgenus *Pinus*. The comparative map presented here is the first of several pending comparative mapping studies using SNP high-throughput markers from candidate gene amplicons mapped in loblolly pine (CRSP and WhiSP; http://dendrome. ucdavis.edu/NealeLab). The research presented here will quickly advance the development of genomic resources for sugar pine and other soft pines facing environmental stressors, and will enhance the ability to effectively define and manage forest genetic resources.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bingham RT (1983) Blister rust resistant western white pine for the Inland Empire: the story of the first 25 years of the research and development program. USDA Forest Service General Technical Report INT-146. Intermountain Forest and Range Experiment Station, Ogden, Utah

Brown GR, Kadel EE III, Bassoni DL, Kiehne KL, Temesgen B, van Buijtenen JP, Sewell MM, Marshall KA, Neale DB (2001) Anchored reference loci in loblolly pine (*Pinus taeda* L.) for integrating pine genomics. Genetics 159:799–809

Chagne D, Brown GR, Lalanne C, Madur D, Pot D, Neale DB, Plomion C (2003) Comparative genome and QTL mapping between maritime and loblolly pines. Mol Breed 12:185–195

Devey ME, Delfino-Mix A, Kinloch BB Jr, Neale DB (1995) Random amplified polymorphic DNA markers tightly linked to a gene for

resistance to white pine blister rust in sugar pine. Proc Natl Acad Sci 92:2066–2070

Devos K (2005) Updating the 'Crop circle'. Curr Opin Plant Biol 8:155–162

Dirlewanger E, Graziano E, Joobeur T, Garriga-Calderé F, Cosson P, Howad W, Arús P (2004) Comparative mapping and marker-assisted selection in Rosaceae fruit crops. Proc Natl Acad Sci 101:9891–9896

Duran C, Edwards D, Batley J (2009) Genetc maps and the use of synteny. In: Methods of molecular biology, Plant Genomics. New York: Springer

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

Eckert AJ, Bower AD, Wegrzyn JL, Pande B, Jermstad KD, Krutovsky KV, St Clair JB, Neale DB (2009a) Association genetics of coastal Douglas fir (Pseudotsuga menziesii var. menziesii, Pinaceae). I. Cold-hardiness related traits. Genetics 182:1289–1302

Eckert Andrew J, Pande B, Ersoz ES, Wright MH, Rashbrook VK, Nicolet CM, Neale DB (2009b) High-throughput genotyping and mapping of single nucleotide polymorphisms in loblolly pine (Pinus taeda L.). Tree Genet Genomes 5:225–234

Eckert AJ, van Heerwaarden J, Wegrzyn JL, Nelson CD, Ross-Ibarra J, González-Martínez SC, Neale DB (2010) Patterns of population structure and environmental association of aridity across the range of loblolly pine (Pinus taeda L., Pinaceae)

Flavell R (2009) Role of model plant species. In: Methods of molecular biology, plant genomics. New York: Springer

Groover A, Devey M, Fiddler T, Lee J, Megraw R, Mitchel-Olds T, Sherman B, Vujcic S, Williams C, Neale D (1994) Identification of quantitative trait loci influencing wood specific gravity in an outbred pedigree of loblolly pine. Genetics 138:1293–1300

Harkins DM, Johnson G, Skaggs AP, Mix A, Dupper G, Devey M, Kinloch B Jr, Neale D (1998) Saturation mapping of a major gene for resistance to white pine blister rust in sugar pine. Theor Appl Genet 97:1355–1360

Jermstad KD, Bassoni DL, Wheeler NC, Neale B (1998) A sex-averaged genetic linkage map in coastal Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco var 'menziesii') based on RFLP and RAPD markers. Theor Appl Genet 97:762–770

Jermstad KD, Bassoni DL, Jech KS, Wheeler NC, Neale DB (2001) Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. I. Timing of vegetative bud flush. Theor Appl Genet 102:1142–1151

Jermstad KD, Sheppard LA, Kinloch BB, Delfino-Mix A, Ersoz ES, Krutovsky KV, Neale DB (2006) Isolation of a full-length CC-NBS-LRR resistance gene analog candidate from sugar pine showing low nucleotide diversity. Tree Genet Genomes 2:76–85

Kinloch BB Jr (1970) White pine blister rust: simply inherited resistance in sugar pine. Science 167:193–195

Kinloch BB Jr (1992) Distribution of a gene for resistance to white pine blister rust in natural populations of sugar pine. Can J Bot 70:1319–1323

Kinloch BB (2003) White pine blister rust in North America: past and prognosis. Phytopathology 93:1044–1047

Kinloch BB, Dupper GE (2002) Genetic specificity in the white pine-blister rust pathosystem. Phytopathology 92:278–280

Kinloch BB Jr, Sniezko RA, Barnes GD, Greathouse TE (1999) A major gene for resistance to white pine blister rust in western white pine from the western cascade range. Phytopathology 889:861–867

Kinloch BB, Davis DA, Burton D (2007) Resistance and virulence interactions between two white pine species and blister rust in a 30-year field trial. Tree Genet Genomes 4:65–74

Kole C (ed) (2007) Genome mapping and molecular breeding in plants, volume 7, forest trees. Springer, Berlin

Komulainen P, Brown GR, Mikkonen M, Karhu A, Garcia-Gil MR, O'Malley D, Lee B, Neale DB, Savolainen O (2003) Comparing EST-based genetic maps between Pinus sylvestris and P. taeda. Theor Appl Genet 107:667–678

Krutovsky KV, Troggio M, Brown GR, Jermstad KD, Neale DB (2004) Comparative mapping in the Pinaceae. Genetics 168:447–461

Lindblad-toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL et al (2005) Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 438:803–819

Liu JJ, Ekramoddoullah AKM (2007) The CC-NBS-LRR subfamily in Pinus monticola: targeted identification, gene expression, and genetic linkage with resistance to Cronartium ribicola

Liu JJ, Ekramoddoullah AKM (2008) Development of leucine-rich repeat polymorphism, amplified fragment length polymorphism, and sequence characterized amplified region markers to the Cronartium ribicola resistance gene Cr2 in western white pine (Pinus monticola). Tree Genet Genomes 4:601–610

Liu JJ, Ekramoddoullah AKM, Hunt RS, Zamani A (2006) Identification and characterization of random amplified polymorphic DNA markers linked to a major gene (Cr2) for resistance to Cronartium ribicola in Pinus monticola. Phytopathology 96:395–399

McDonald GI, Hoff RJ (1970) Resistance to Cronartium ribicola in Pinus monticola: early shedding of infected needles. USDA Forest Service Research Note INT-124. Intermountain Forest and Range Experiment Station, Ogden, p 8

McHale L, Tan X, Koehl P, Michelmore RW (2008) Plant NBS-LRR proteins: adaptable guards. Genome Biol 7:212

Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J 20:317–332

Miller DR, Kimmey JW, Fowler MR (1959) White pine blister rust. USDA Forest Service, Forest Pest Leaflet, p 36

Neale D, Krutovsky K (2004) Comparative genetic mapping in trees: the group of conifers. In: Lörz H, Wenzel G (eds) Biotechnology in agriculture and forestry, vol. 55, Molecular marker systems in plant breeding and crop improvement. Springer, Berlin, pp 267–277

Sanger F, Coulson AR (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol 94(3):441–448

Sewell MM, Sherman BK, Neale DB (1999) A consensus map for loblolly pine (Pinus taeda L.). I. Construction and integration of individual linkage maps from two outbred three-generation pedigrees. Genetics 151:321–330

Sniezko RA, Bower A, Kegley A (2004) Variation in Cronartium ribicola field resistance among Pinus monticola and 12 P. lambertiana families: early results from Happy Camp. In: Breeding and genetic resources of five-needle pines; growth, adaptability, and pest resistance; 2001 July 23–27; Medford, OR, USA. US Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO, pp 203–208

Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package. Plant J 6:739–744

Stam P, van Ooijen JW (1995) JoinMap™ version 2.0: software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A et al (2006) The genome of black cottonwood, Populus trichocarpa (Torr. And Gray). Science 313:1596–1604

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q,

Kodira CD et al (2001) The sequence of the human genome. Science 291:1304–1351

Wegrzyn JL, Lee JM, Tearse BR, Neale DB (2008) TreeGenes: a forest tree genome database, Int J Plant Genom, vol. 2008, Article ID 412875, 7 pages, 2008. doi:10.1155/2008/412875

Wegrzyn JL, Lee JM, Liechty J, Neale DB (2009) PineSAP—pine alignment and SNP identification pipeline. Bioinformatics 25:2609–2610

Wheeler NC, Jermstad KD, Krutovsky K, Aitken SN, Howe GT, Krakowski J, Neale DB (2005) Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. IV. Cold-hardiness QTL verification and candidate gene mapping. Mol Breed 15:145–156

Xian-Liang S, Xue-Zhen S, Tian-Zhen Z (2006) Segregation distortion and its effect on genetic mapping in plants. J Agric Biotech Environ 14:286–292