

# Sequence of the Sugar Pine Megagenome

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**ABSTRACT** Until very recently, complete characterization of the megagenomes of conifers has remained elusive. The diploid genome of sugar pine (*Pinus lambertiana* Dougl.) has a highly repetitive, 31 billion bp genome. It is the largest genome sequenced and assembled to date, and the first from the subgenus *Strobus*, or white pines, a group that is notable for having the largest genomes among the pines. The genome represents a unique opportunity to investigate genome “obesity” in conifers and white pines. Comparative analysis of *P. lambertiana* and *P. taeda* L. reveals new insights on the conservation, age, and diversity of the highly abundant transposable elements, the primary factor determining genome size. Like most North American white pines, the principal pathogen of *P. lambertiana* is white pine blister rust (*Cronartium ribicola* J.C. Fischer ex Raben.). Identification of candidate genes for resistance to this pathogen is of great ecological importance. The genome sequence afforded us the opportunity to make substantial progress on locating the major dominant gene for simple resistance hypersensitive response, *Cr1*. We describe new markers and gene annotation that are both tightly linked to *Cr1* in a mapping population, and associated with *Cr1* in unrelated sugar pine individuals sampled throughout the species' range, creating a solid foundation for future mapping. This genomic variation and annotated candidate genes characterized in our study of the *Cr1* region are resources for future marker-assisted breeding efforts as well as for investigations of fundamental mechanisms of invasive disease and evolutionary response.

**KEYWORDS** conifer genome; transposable elements; white pine blister rust

**T**HE gymnosperm genus *Pinus* is diverse and ubiquitous in temperate zones (Critchfield and Little 1966; Farjon and Filer 2013). Pines are often the keystone trees of terrestrial ecosystems (Richardson and Rundel 1998; Keane *et al.* 2012,

and citations therein). Typical of conifers, pines have megagenomes that vary greatly in size among species, yet their karyotype is highly conserved. *Pinus* is divided into two large, ancient monophyletic subgenera, *Strobus* and *Pinus*, “white pines” and “yellow pines,” respectively (Critchfield and Little 1966; Gernandt *et al.* 2005). The first *Pinus* genome sequence (22 Gbp) was recently reported for *Pinus taeda* L. (Zimin *et al.* 2014), a yellow pine commonly known as loblolly pine. The genomes of white pines are larger and more variable in size (Tomback 1982). Fossils allied with *Strobus* are known from the early Tertiary and late Cretaceous (Millar 1998), consistent with molecular phylogenetic dating of the crown group *Strobus* at 45–85 MYA (Willyard *et al.* 2007; DeGiorgio

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et al. 2014). Populations of a number of the majestic white pines of North America, and their associated ecosystems, have been devastated over the last century by white pine blister rust, WPBR (Kinloch 1992) caused by a highly pathogenic and invasive fungus, *Cronartium ribicola* J.C. Fischer ex Raben. While major gene resistance to this disease has been discovered in several species, and loci have been placed on the genetic maps of *Pinus lambertiana* Dougl. (Harkins et al. 1998; Jermstad et al. 2011) and *P. monticola* Dougl. ex D. Don (Liu et al. 2006), the discovery of the underlying genes, and of markers serviceable for genetic improvement in reforestation, may be greatly accelerated by the genome sequence itself.

*P. lambertiana*, commonly known as sugar pine, is a white pine native to western North America that is distributed from northern Oregon to Baja California at a wide span of altitudes. It is currently the tallest pine species, with heights reaching 76 m. The female cones of sugar pine are also gigantic, often longer than 600 mm (Kinloch and Scheuner 1990; Van Pelt 2001; American Forests 2015). *P. lambertiana* trees may live > 500 years, and the onset of the species' sexual reproduction is delayed compared to other pines, possibly due to the height and girth needed to support these massive strobili. Paralleling these oversized dimensions, the genome of *P. lambertiana* was estimated from cytometry to be 31 Gbp (see below), nearly 50% larger than that of *P. taeda* and ten times the size of the human genome. While *P. lambertiana* was historically a significant timber source, heavy harvesting, and the arrival of the devastating white pine blister rust to its range, has changed the management focus. Since this species plays important ecological roles in the maintenance of biodiversity, carbon sequestration, soil stabilization, and watershed protection (Maloney 2012), considerable effort and resources have been deployed both by the US Forest Service and the private sector to structure the genetics of reforestation to fit the ecological factors, especially WPBR (reviewed in Waring and Goodrich 2012). In particular, the screening by progeny testing of diverse seed sources for individual trees carrying the major gene for WPBR resistance, *Cr1* (Kinloch 1992), has been ongoing for more than a decade. These extra costs of collecting seeds from candidate trees throughout the species range, of progeny testing for WPBR resistance (requiring several years), and the deployment of resistant seedlings, are significant components of forest management. Genotyping by markers with strong associations to WPBR resistance has the potential to greatly reduce both the effort and time required by the ongoing approach, and could open new strategies. Here, we demonstrate that the sequencing, assembly, and annotation of the genome sequence of *P. lambertiana* greatly accelerates the discovery of such genetic tools.

### Conifer evolution and genome size

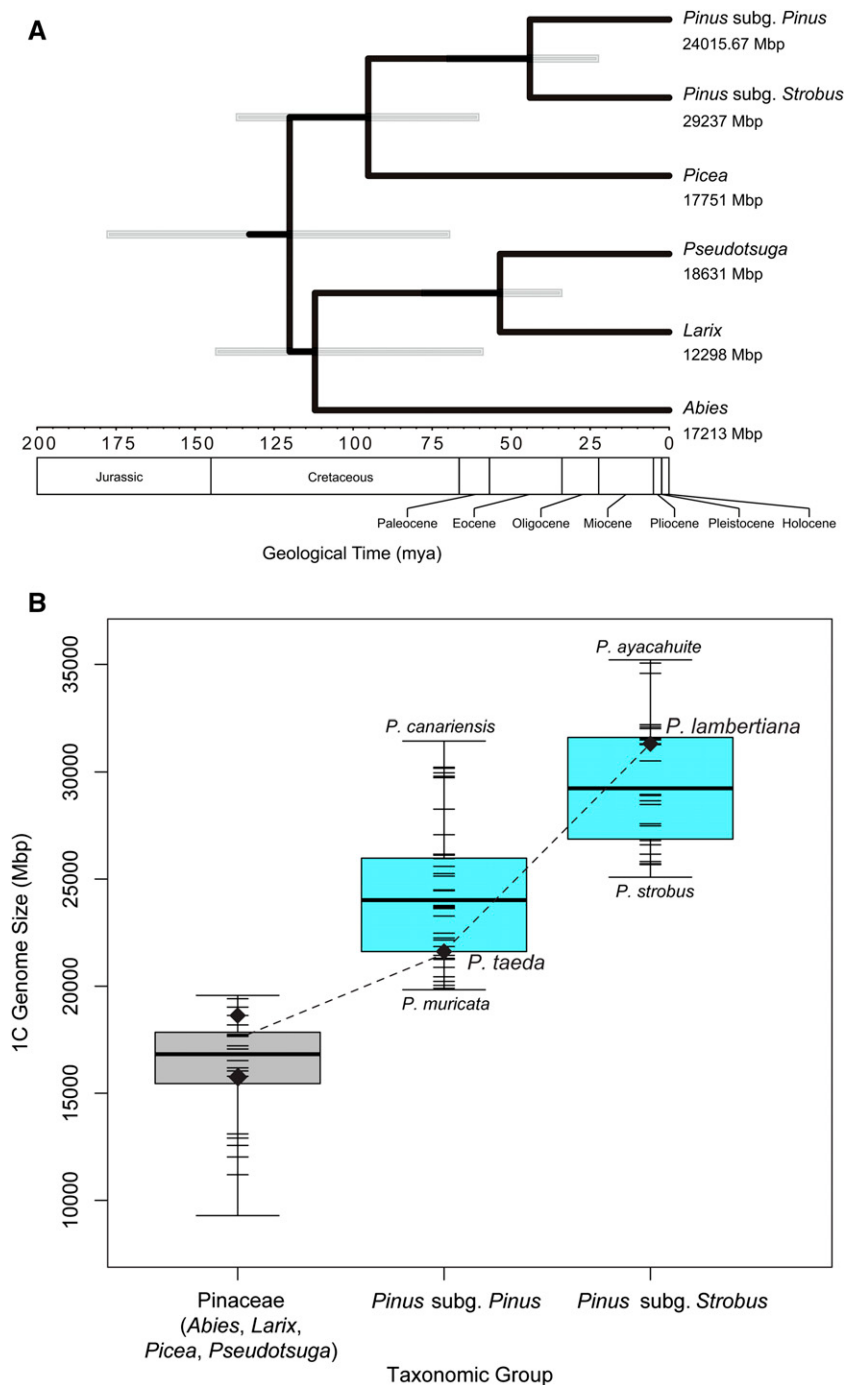
All members of the genus *Pinus* have 12 chromosomes (Saylor 1960) and are considered to be karyotypically stable throughout their evolutionary history (Sax 1960; Saylor 1964). With the exception of a potential event preceding the radiation (Li et al. 2015), whole genome polyploidy is

thought to be absent among the  $\geq 100$  species. However, the amount of nuclear DNA that comprises a single copy of a pine genome can vary widely between species. Flow cytometric estimates for the genus *Pinus* in the C-values database (Bennett and Leitch 2012) range from a low of 20 Gbp for *P. muricata* D. Don, to a high of 35 Gbp for *P. ayacahuite* Ehrenb. ex Schltdl. (Figure 1B). The correlates and causes of this variation in genome size, including in *Pinus*, are an open topic of speculation and investigation (Williams et al. 2002; Grotkopp et al. 2004; Ahuja and Neale 2005; Morse et al. 2009).

The two subgenera of *Pinus* diverged ~45–85 MYA ago (Figure 1A) (see also Willyard et al. 2007). Members of *Strobos* have an average genome size 5.2 Gbp larger than the subgenus *Pinus* (Figure 1B) (Grotkopp et al. 2004). The majority of sequenced conifer megagenomes are composed of interspersed repetitive sequences, with estimates ranging from 69% for *Picea abies* (L.) H. Karst. (Nystedt et al. 2013) to 80% for *P. taeda* (Wegrzyn et al. 2014). The evolutionary dynamics of transposable elements (TEs) have long been suspected to shape genomic change, including overall genome size, in numerous species (Orgel and Crick 1980; Hawkins 2006; Piegu et al. 2006; Tenaillon et al. 2011), including conifers (Nystedt et al. 2013). In contrast to angiosperms, where genome duplication events and LTR retrotransposon bursts are frequent, and account for most of the genome size expansions, a continual accretion of repeats may provide a better explanation of genome size variation within the genus *Pinus* (Morse et al. 2009). The genome sequence of *P. lambertiana* presents a new opportunity to address elements of the hypothesis that TE dynamics are behind these significant changes in genome size.

### White pine blister rust

WPBR, the non-native heteroecious fungus *Cronartium ribicola*, infects North American pines of the *Strobos* subgenus. An invasive species, *C. ribicola* has devastated populations of five-needle pines, including *P. strobus* L. (eastern white pine), *P. monticola* (western white pine), *P. lambertiana* (sugar pine), *P. flexilis* James (limber pine), and *P. albicaulis* Engelm. (whitebark pine), and foxtail pine, along with closely related bristlecone pines (subgenus *Pinus* subsection *Balfourianae*) since its introduction from Asia or Europe a century ago. Damage from *C. ribicola* is known to reduce reproduction and survival of the majority of white pine species (Kinloch 1970; Waring and Goodrich 2012). Exacerbated by recent outbreaks of the mountain pine beetle, decreasing pine populations have affected wildlife, biodiversity, watershed, and timber potential. Rare individuals among the white pines species exhibit innate and heritable resistance that forms the basis for various selective reforestation efforts (Kinloch 2003). A major “gene” of resistance (MGR) to WPBR was mapped in *P. lambertiana* over 40 years ago (Kinloch 1970). An apparently biallelic locus, *Cr1<sup>R</sup>/Cr1<sup>r</sup>* locus has been mapped in several *P. lambertiana* families (Devey et al. 1995; Harkins et al. 1998; Jermstad et al. 2011). In this work, we leverage these markers and the



**Figure 1** (A) The phylogeny of major genera within the Pinaceae along with genome size estimates. *P. lambertiana* falls in the *Strobus* subgenus. Inference was conducted using Bayesian analysis as implemented in BEAST ver. 2.2.0 (Bouckaert *et al.* 2014). Gray bars represent the 95% highest posterior density range for the age of the node. Data used for inference were 28 independent nuclear gene regions (see Eckert *et al.* 2013a,b), sequenced and assembled for representative taxa selected within each taxonomic group [*Pinus* subg. *Pinus*: *P. taeda*; *Pinus* subg. *Strobus*: *P. lambertiana*; *Picea*: *P. abies*; *Pseudotsuga*: *P. menziesii* (Mirb.) Franco; *Larix*: *L. decidua* Mill.; *Abies*: *A. alba* Mill.]. Details are presented in the Supplementary Methods in File S1 (B) Illustration of the genome size trends of major genera within Pinaceae. Genome sizes are from the c-values database (Bennett and Leitch 2012). Diamonds mark the estimates of genomes with a reference sequence. Point estimates in each category are shown as short horizontal lines. Species from other genera within the Pinaceae are shown in gray.

assembled *P. lambertiana* genome to identify large genomic scaffolds tightly linked to *Cr1* and SNPs in strong association with *Cr1<sup>R</sup>*. We discuss possible *Cr1* candidates among the annotated genes.

### Sequencing and assembly

The sequencing and assembly approach used here for *P. lambertiana* is an adaptation of the approach successfully used for *P. taeda* (Neale *et al.* 2014; Zimin *et al.* 2014). We have found that the haploid DNA obtainable from a single megagametophyte from the target genotype is sufficient to form

the basis of a high quality whole genome shotgun assembly. For additional contiguity, haploid megagametophyte coverage is supplemented with longer linking mate pair libraries using DNA isolated from abundantly available diploid needle tissue of the maternal parent. For additional contiguity of the gene space, we performed transcriptome-based scaffolding using deep coverage RNA-Seq data. The nearly 50% larger size of the *P. lambertiana* genome required changes to the previous software methods to make assembly tractable. The resulting draft genome sequence described here has an N50 scaffold size of 246.6 kbp and a total estimated genome size

of 31 Gbp, making it the largest genome sequenced and assembled to date.

## Materials and Methods

### Plant material

Our target tree for reference genome sequencing was *P. lambertiana* genotype 5038 in the collection of the United States Department of Agriculture (USDA) Forest Service, which is in the public domain. Haploid megagametophyte tissue was sourced from wind-pollinated seeds from grafted ramets collected in 1994 from the USDA Forest Service Badger Hill site. *P. lambertiana* needle tissue was collected in August 2011 from a ramet at the same location.

### DNA isolation

As described by Zimin *et al.* (2014), our sole source of haploid DNA was a single megagametophyte. Prior to DNA extraction, seeds were immersed in water for 4 days, after which individual haploid megagametophytes were dissected from each seed. DNA was subsequently extracted from dissected megagametophytes as described in Zimin *et al.* (2014). For diploid DNA, large-scale extractions were prepared from *P. lambertiana* needles. For long insert mate pair libraries, nuclei were isolated and DNA was extracted and quantified at University of California (UC) Davis using the methods previously reported (Zimin *et al.* 2014). The resulting DNA was treated with 0.33  $\mu$ l PreCR Repair Mix (New England Biolabs) per microgram DNA prior to use in library construction. DNA for the *P. lambertiana* fosmid pools was isolated and quantified at CHORI using the slightly modified method previously reported (Zimin *et al.* 2014). Further details can be found in the Supplemental Material, File S1 (Supplementary Methods).

### Error correction

Paired end reads were error corrected using QuorUM (Marçais *et al.* 2013), as packaged in the MaSuRCA 2.3.0 assembly pipeline (Zimin *et al.* 2013). Only *k*-mers from the haploid sequences were used in constructing the error correction database. Detailed error correction results are given in Tables S1–S3 in File S1.

### Super-read construction

Error-corrected data were used to construct super-reads (Zimin *et al.* 2013), which are longer, nonredundant, and overall much more compact than the original read data. For the *P. lambertiana* paired end sequence data, the super-reads procedure reduced the 6.36 billion error-corrected read pairs to 148 million super reads (Figure 2). The average length of the super-reads was 502 bp with a total length of 75 Gbp. By comparison, the average super-read length for *P. taeda* was 362 bp (Zimin *et al.* 2014).

### Mate pair cleaning and filtering

Mate pairs from diploid libraries were cleaned and filtered as follows. (1) Mate pair sequence were error corrected by

QuorUM, using a *k*-mer database from the haploid data. This step had the secondary effect of enriching for our target haplotype. (2) Nonjunction fragments, “short innies,” were detected and removed using a procedure that attempted to connect pairs by *k*-mer extensions (again using *k*-mers from the haploid data) off the “wrong” ends. (3) Reads <100 bp were extended via unique *k*-mers to a length of 64–100 bp. If both reads in a pair could not be extended to at least 64 bp, the pair was discarded.

### Initial assembly

The preprocessed reads from both the haploid and diploid libraries were then assembled with SOAPdenovo2 (Luo *et al.* 2012) using a *k*-mer size of 99. Paired end libraries (Table S2 in File S1) were divided into three progressively less reliable fragment sizes: <200, 200–400, and >400 bp. Mate-pair libraries (Table S4 in File S1) were divided into two groups: <10 and >10 kbp.

### Gap closing

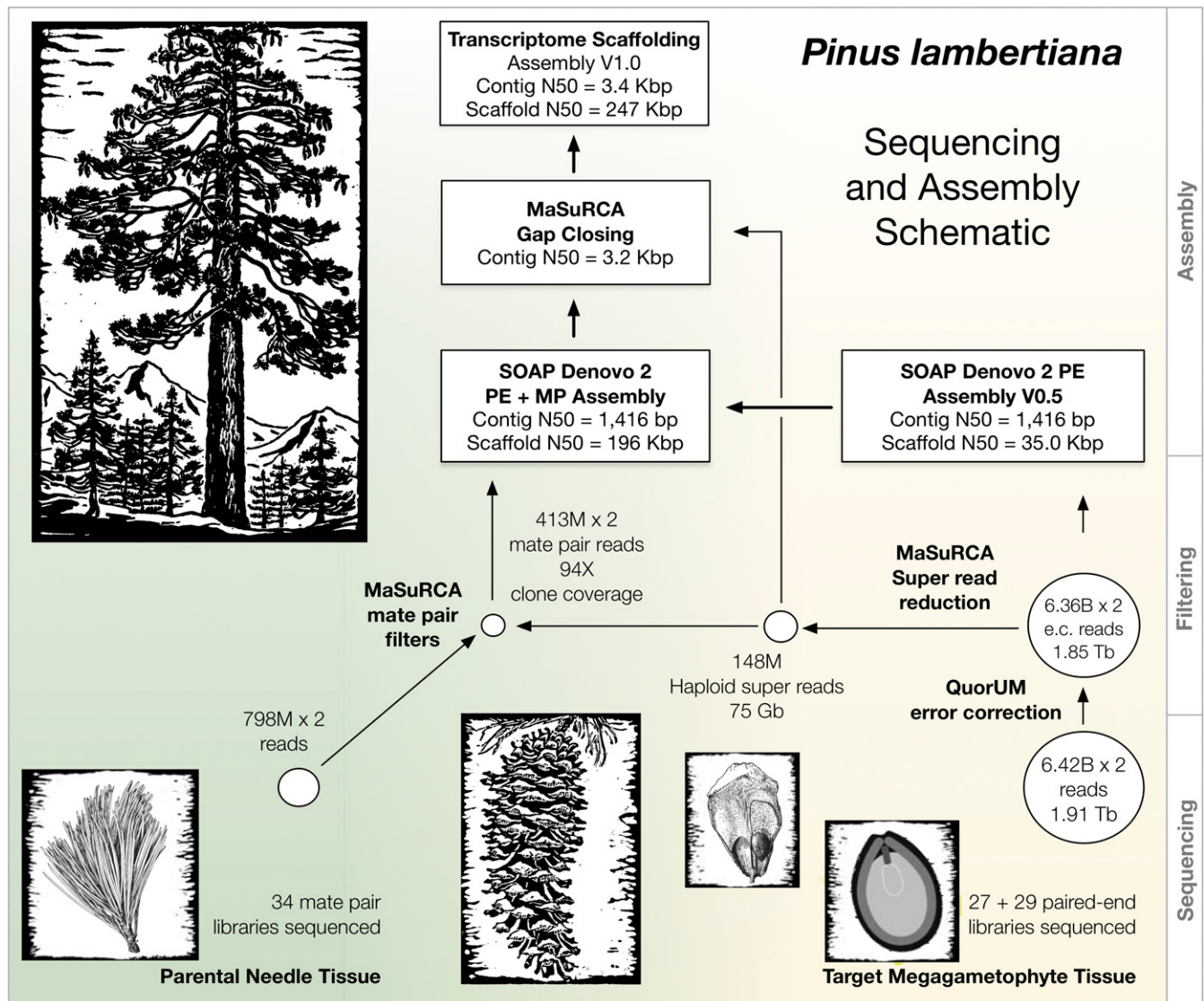
To increase contiguity, gap closing was performed on the output of the SOAPdenovo assembler using the MaSuRCA gap closer, plus the super-read sequences to “patch” gaps in the SOAP assembly.

### Transcriptome scaffolding

Additional scaffolding steps used a set of transcript sequences assembled from Pacific Biosciences (PacBio) and Illumina RNA-seq data (Table S7) from Gonzalez-Ibeas *et al.* (2016). We aligned transcript sequences to the whole genome shotgun (WGS) scaffolds using both *nucmer* (-maxmatch -nosimplify -l 45 -c 4) (Kurtz *et al.* 2004) and *bwa-mem* (-k 45 -O 60 -E 10) (Li 2013). We then merged alignments that were adjacent on both the transcript and the corresponding scaffold. For pairs of scaffolds that were aligned adjacently to the same transcript, we subsequently created a link. We sorted the links in descending order according to intron size. Next, we built a graph by visiting links in order. Each link corresponds to a potential edge in the graph between vertices corresponding to scaffolds. We added a link/edge to the graph if it did not create a cycle or a vertex degree >2. Upon completion, the graph consisted only of paths, which we converted to superscaffolds that contained one or more of the original assembly scaffolds.

### Transcriptome assembly

Thirty-one tissue-specific samples, including needle, root, stem, pollen, cone, strobili, and embryonic tissues, were used for the construction of cDNA libraries. A variety of treatments were applied to seedlings before RNA extraction, including: cold/heat shock, flood/drought stress, wounding, and salicylic or methyl jasmonate exposure. RNA sequencing was done using Illumina MiSeq and HiSeq to generate short (100–300 bp) reads, and PacBio Iso-Seq reads, which range from 1000 to over 6000 bp. Seven MiSeq libraries, nine HiSeq, and 18 PacBio libraries were created, and a total of 40 SMRT cells (1–4 SMRT cells per library) was sequenced.



**Figure 2** Overview of sequencing and assembly strategy for *P. lambertiana*. Woodcut images used with permission from “The trees of Yosemite; a popular account.” Library of Congress call number QK484.C2 T7 1932.

Quality trimmed reads were used for assembly with Trinity (Haas *et al.* 2013), and protein coding sequences (CDS) were identified with Transdecoder (Haas *et al.* 2013). All CDSs were clustered at 95% sequence identity with Uclust (Edgar 2010) (usearch v8.1.1861) to generate a nonredundant set of transcripts.

#### Identification of genomic scaffolds and mapping in the *Cr1* region

Jermstad *et al.* (2011) reported the sequences from cloned RAPD bands OP\_G16 and BC\_432 that were linked to *Cr1*. To identify these genomic loci, the representative consensus sequence for each RAPD band was aligned to the *P. lambertiana* genome assembly using *gmap* (Wu and Watanabe 2005). In both cases, a unique top hit (path1) was observed identifying target scaffolds, which we used to develop new markers.

Target scaffolds were masked for annotated simple and interspersed repeats (see Supplementary Methods in File S1). We designed pairs of nested PCR primers using PRIMER3 (Rosen and Skaletsky 1999) for unique regions in these two target scaffolds. All of the PCR assays used standard PCR reaction conditions: 2.0 mM MgCl<sub>2</sub>, 0.2 mM each of dNTPs, 0.5 mM each of forward and reverse primers, 1 U of *Taq* polymerase, and 50 ng of DNA. For validation purposes, we used the available primer sequences of PCR amplicon, UMN\_3258\_01 (<http://treegenesdb.org/ftp/CRSP/>) to develop a new marker, *cr11C*.

#### Gene annotation

Annotations were generated using the automated genome annotation pipeline MAKER-P (Campbell *et al.* 2014). Inputs and training sets for MAKER-P included the *P. lambertiana* genome assembly, a *P. lambertiana* transcriptome assembly

(see Supplementary Methods in File S1), ESTs from spruce and pine (1,027,297 downloaded from GenBank), protein sequence data from *Vitis vinifera* L. (25,665), *Amborella trichopoda* Baill. (25,354), *Populus trichocarpa* Torr. and A.Gray ex Hook (38,655), *Picea abies* (22,721), *Picea sitchensis* (Bong.) Carrière (17,841), *Pinus taeda* (34,059), and RNA-seq data from *P. lambertiana*. Default MAKER-P mapping parameters were used (80% coverage and 85% identity threshold for EST-genome alignments, and 50% coverage and 40% identity for protein-genome alignments). More details can be found in the Supplementary Methods in File S1.

### Interspersed repeat annotation

To find interspersed repeat elements, we used both similarity and *de novo* based approaches (Figure S3 in File S1). RepeatModeler combines two complementary *de novo* repeat element prediction algorithms: RECON (Bao *et al.* 2002) and RepeatScout (Price *et al.* 2005). To make the RepeatModeler computation tractable, we used only the Illumina sequenced fosmid pools (above), along with the longest 2.5% of genomic scaffolds. We also used a combination of TEclass (Abrusán *et al.* 2009), CENSOR (Kohany *et al.* 2006), and manual characterization to identify the uncharacterized elements from the repeat library produced by RepeatModeler. We used this library, along with the plant Repbase library (Jurka *et al.* 2005) (plant component only, v19.01) as the reference database for RepeatMasker (Tarailo-Graovac *et al.* 2009). Full-length elements were determined by applying a cut-off of 80-80-80 (80% sequence similarity, and 80 bp minimum length) (Wicker *et al.* 2007).

### Data availability

The *P. lambertiana* assembly and annotation are available from GenBank as accession GCA\_001447015.2 and BioProject 174450, and also from <http://www.pinegenome.org/pinerefseq>. Genomic DNA and RNA reads are also available under BioProject 174450.

## Results

### Sequencing

Our sequencing strategy for conifer genomes has taken advantage of the haploid tissue of the conifer megagametophyte (Neale *et al.* 2014; Zimin *et al.* 2014). Fortunately the observed correlation over the evolutionary diversity of gross seed weight with genome size (Wakamiya *et al.* 1993) (Grotkopp *et al.* 2004) in the genus *Pinus* worked to our advantage. Our collection of *P. lambertiana* megagametophytes had an average weight of 225 mg compared to only 23.5 mg for *P. taeda*. This translated into substantially larger yields of haploid genomic DNA from single seeds. From our target *P. lambertiana* megagametophyte, we were able to obtain 36.2 mg of DNA, from which we generated 1.91 trillion base pairs of sequence (Figure 2 and Table 1), representing ~62× coverage of the 31 Gbp haploid genome.

**Table 1 Characteristics of the *P. lambertiana* sequence data and 1.0 assembly, compared to known cytometric and cytological properties**

| Cytometric Genome Size               | 31 Gbp                                                                  |
|--------------------------------------|-------------------------------------------------------------------------|
| Chromosome number                    | 12                                                                      |
| Assembly V1.0                        |                                                                         |
| Total size                           |                                                                         |
| Scaffolds ≥ 200 bp                   | 4,259,911 scaffolds<br>27.6 Gbp including gaps<br>25.5 Gbp without gaps |
| Scaffolds ≥ 500 bp                   | 1,089,992 scaffolds<br>26.9 Gbp including gaps<br>24.7 Gbp without gaps |
| Contigs < 200 bp ("chaff")           | 54,147,744 contigs<br>6.5 Gbp                                           |
| N50 scaffold size (31 Gb)            | 246.6 kbp                                                               |
| N50 contig size (31 Gb)              | 4.25 kbp                                                                |
| Sequence data                        |                                                                         |
| Number of paired-end libraries       | 56                                                                      |
| Paired end sequencing depth          | 1,910 Gbp (61.5×)                                                       |
| By platform                          |                                                                         |
| HiSeq 2000 (125 bp + 125 bp)         | 2.8 × 10 <sup>11</sup> bp (9.0×)                                        |
| HiSeq 2500 (150 bp + 150 bp)         | 1.4 × 10 <sup>12</sup> bp (45.1×)                                       |
| GAllx (160 bp + 156 bp)              | 1.8 × 10 <sup>11</sup> bp (5.8×)                                        |
| MiSeq (255 bp + 255 bp)              | 4.7 × 10 <sup>10</sup> bp (1.5×)                                        |
| By fragment size                     |                                                                         |
| [200 bp, 400 bp]                     | 9.6 × 10 <sup>11</sup> bp (31.0×)                                       |
| [400 bp, 600 bp]                     | 4.6 × 10 <sup>11</sup> bp (15.0×)                                       |
| [600 bp, 900 bp]                     | 4.8 × 10 <sup>11</sup> bp (15.6×)                                       |
| Long fragment libraries (1.5–25 kbp) | 34                                                                      |
| Long fragment coverage               |                                                                         |
| Illumina TruSeq                      | 22.5× physical coverage                                                 |
| Nextera mate pair                    | 71.2× physical coverage                                                 |

N50 statistics were calculated using an estimated genome size of 31 Gbp. Paired end sequencing depth represents the raw output prior to error correction. Physical coverage estimated by MaSuRCA (including the inferred DNA fragmentation) is reported here for all libraries by chemistry (see Supplementary Methods in File S1).

### Estimating genome size

We analyzed the *k*-mer distribution of the paired reads to derive an independent estimate of the haploid size of the genome for coverage estimates. Using the jellyfish program (Marçais and Kingsford 2011), we computed distributions of *k*-mer depth for *k* = 24 and *k* = 36 for all the paired sequences derived from our megagametophyte. We estimated genome size from the *k*-mer distribution as described previously (Zimin *et al.* 2014), using both the mean and the mode of the distributions for *k* = 24 and *k* = 31. As shown in Table 2, all four estimates of the genome size are in close agreement, ranging from 30.9 to 31.9 Gbp.

Our haploid library based estimates were in the range of previous experimental estimates in the literature. The Gymnosperm DNA C-values Database release 6.0 (Bennett and Leitch 2001) contains three flow cytometry-based estimates for the genome size of *P. lambertiana*: 33.4 Gbp (Grotkopp *et al.* 2004); 31.1 Gbp (Williams *et al.* 2002); and 29.4 Gbp (Wakamiya *et al.* 1993). The authors of the 33.4 Gbp estimate noted that their genome size estimates of various species were consistently higher than values already in the literature. The mean of these experimental estimates, 31 Gbp, is in close

**Table 2 Estimates of the genome size of *P. lambertiana* based on the distribution of *k*-mers in the paired read data**

|                                    | <i>k</i> = 24         | <i>k</i> = 31         |
|------------------------------------|-----------------------|-----------------------|
| Total <i>k</i> -mers               | $1.56 \times 10^{12}$ | $1.47 \times 10^{12}$ |
| Erroneous <i>k</i> -mers           | $1.20 \times 10^{10}$ | $2.20 \times 10^{10}$ |
| Total correct <i>k</i> -mers       | $1.55 \times 10^{12}$ | $1.45 \times 10^{12}$ |
| E(unique <i>k</i> -mer depth) mode | 49.72                 | 46.77                 |
| Estimated genome size              | 31.1 Gbp              | 30.9 Gbp              |
| E(unique <i>k</i> -mer depth) mean | 48.53                 | 46.02                 |
| Estimated genome size              | 31.9 Gbp              | 31.4 Gbp              |

Erroneous *k*-mers refer to *k*-mers that were identified as likely to contain errors, and these were removed from the calculation.

agreement with our sequenced-based estimates, and therefore we chose this value as the estimated total size of the genome.

### Assembly

Super-reads (Zimin *et al.* 2013) played a fundamental role in the assembly of *P. lambertiana*, where they allowed us to dramatically reduce the size of the input to subsequent assembly steps (Figure 2). Nevertheless the CABOG assembler (Miller *et al.* 2008) used for the 22 Gbp genome of *P. taeda* could not process the larger *P. lambertiana* genome, so we instead used the *de Bruijn* graph-based SOAPdenovo 2 assembler (Luo *et al.* 2012) for initial contig and scaffold construction. Following this step, we reassembled the contigs, with SOAPdenovo 2, adding the 93× coverage from long-fragment libraries, yielding scaffolds with an N50 size of 196 kbp. We then ran a separate gap-closing procedure to reduce the number of intrascaffold gaps, which closed 12.6 million out of 26.2 million gaps in the assembly. This reduced the total gap length by ~780 Mbp, and increased the N50 contig size to 3.4 kbp.

Finally, we used transcript sequences to improve contiguity in the vicinity of genes. We aligned a set of 17,167 assembled transcripts (see *Materials and Methods*) to the scaffolds. We joined scaffolds together if the links created were consistent with a colinear transcript alignment. In total, 32,619 scaffolds were merged during this step. The resulting assembly (version 1.0) has an N50 scaffold length of 246.6 kbp. The combined length of the assembly, including all scaffolds and contigs >200 bp, is 27.6 Gbp (Table 1). The assembly contains another 6.48 Gbp in contigs, and scaffolds ≤200 bp that were not considered for most analyses.

### Validation

As an independent assessment of assembly quality, we sequenced four pools of 48 fosmid each using the PacBio RS II platform (see Supplementary Methods in File S1). We collected deep coverage (>250×) of each pool. The vector-trimmed HGAP3-assembled pools are reported in Table 3. Most of the assembled contigs appeared to span the full length of a fosmid, ~40 kbp (Table 3, and Table S6 in File S1). Overall, the PacBio fosmid assemblies were 98.8% identical to the WGS assembly, which covered >95% of their total length. Because the haploid fosmids were constructed from

**Table 3 Assemblies of the four fosmid pools sequenced with PacBio technology**

| Pool  | Contigs | Minimum | Mean  | Maximum | Length  | N50   |
|-------|---------|---------|-------|---------|---------|-------|
| SPPB1 | 61      | 979     | 31983 | 45177   | 1950994 | 34685 |
| SPPB2 | 54      | 586     | 33949 | 44946   | 1833274 | 35595 |
| SPPB3 | 58      | 586     | 29525 | 43039   | 1712462 | 35375 |
| SPPB4 | 73      | 551     | 27960 | 43934   | 2041131 | 35324 |

Each pool contained 48 fosmids.

diploid needle tissue, at most half were expected to match exactly. Thus, the 1.2% divergence represents an upper bound on alignment and assembly errors, or, alternatively, half the heterozygosity rate.

As a measure of the correctness of the WGS assembly, we looked for large insertions, deletions, or rearrangements between the PacBio and WGS assemblies. The comparison yielded only one noncolinear alignment, and one WGS scaffold with a large 7.6 kbp deletion, for which we could not rule out haplotype differences. A second scaffold with a 5.3 kbp deletion was clearly a heterozygous insertion of an LTR element in the assembled fosmid.

For further validation, we examined the alignment of the WGS scaffolds just prior to transcriptome scaffolding to our collection of 12,533 PacBio and 4634 Illumina assembled transcripts; >99% of these alignments were consistent. When examining the 1% that were not colinear, we found that these were dominated by Illumina-based transcripts, leading to the conclusion the most of these represented errors in the transcript assembly rather than the WGS assembly.

### Gene content

Annotation yielded 13,936 high-quality gene models and 71,117 low-quality models, the presence of direct RNA evidence being the primary distinction between the two classes (Supplementary Methods in File S1). A total of 11,769 scaffolds were annotated with at least one high-quality gene model, ranging from one to eight models per scaffold (1.2 models/scaffold on average). Only 33 scaffolds were annotated with five or more models. Completeness of the gene space evaluated with BUSCO (Simão *et al.* 2015) was 53% when using the high-quality models, and 58% when the low-quality models were included. Alternatively, DOGMA (Dohmen *et al.* 2016) estimated a coverage of 94% for their Conserved Domain Arrangements, For comparison, when run on the complete set of *P. taeda* gene models, BUSCO estimated 50% completeness and DOGMA estimated 61% (Table S8 in File S1).

In total, 11,595 of the 13,936 gene models were functionally annotated with a characterized plant protein sequence. A total of 2041 were classified as uninformative (protein alignment with no functional assignment), and 300 showed no homology to characterized proteins. As expected, *Vitis vinifera*, *Arabidopsis thaliana* (L.) Heynh., and *Ricinus communis* L. were the species that contributed the most to the functional annotations. The largest *P. lambertiana* intron, at 578 kbp, is the second largest (after one in *P. taeda*) found in

**Table 4** Comparison of gene metrics among sequenced conifer genomes and select angiosperms

|                             | <i>Pinus taeda</i> | <i>Picea abies</i>  | <i>Pinus lambertiana</i> | <i>Picea glauca</i> | <i>Arabidopsis thaliana</i> | <i>Populus trichocarpa</i> | <i>Vitis vinifera</i> | <i>Amborella trichopoda</i> |
|-----------------------------|--------------------|---------------------|--------------------------|---------------------|-----------------------------|----------------------------|-----------------------|-----------------------------|
| Genome size (Mbp)           | 20,148             | 19,600              | 31,000                   | 20,000              | 135                         | 423                        | 487                   | 706                         |
| Chromosomes                 | 12                 | 12                  | 12                       | 12                  | 5                           | 19                         | 19                    | 13                          |
| Gc+cC content (%)           | 38.2               | 37.9                | 35.1                     | 31.1                | 35                          | 33.3                       | 36.2                  | 35.5                        |
| TE content (%)              | 74                 | 70                  | 79                       | N/A                 | 15.3                        | 42                         | 41.4                  | N/A                         |
| Number of genes             | 9,024              | 26,359 <sup>a</sup> | 13,936                   | 14,462              | 27,160                      | 36,393                     | 25,663                | 25,347                      |
| Average CDS length (bp)     | 1,562              | 931                 | 1,330                    | 1,421               | 1,102                       | 1,143                      | 1,095                 | 969                         |
| Average intron length (bp)  | 12,875             | 1,020               | 8,039                    | 603                 | 182                         | 366                        | 933                   | 1,538                       |
| Maximum intron length (bps) | 8,91,919           | 68,269              | 5,78,081                 | 1,19,319            | 10,234                      | 4,698                      | 38,166                | 1,75,748                    |

<sup>a</sup> High confidence genes from the Congenie project.

a plant genome to date (Table 4), although the draft state of the genome means that larger introns are highly likely to be scattered among multiple scaffolds.

### Transposable elements

TE sequences constitute 79% of the *P. lambertiana* genome, higher than the 74% found in *P. taeda* (see Supplementary Methods in File S1). Of these, 67% of the transposable sequences in *P. lambertiana* are LTR retrotransposons. The distribution of transposable element families is very similar in the two species (see Figure 3). The most substantial difference in repeat content observed between the genomes is a 35% greater proportion of Gypsy elements in *P. lambertiana*. The distributions of estimated insertion times among LTR retrotransposons are congruent with those reported for spruce in Nystedt *et al.* (2013) (Figure S5). The median LTR insertion time for *P. lambertiana* (16.0 MYA) is younger than that of *P. taeda* (17.4 MYA). As a class, *P. lambertiana* Gypsy elements are significantly younger (14.5 MYA;  $P < 1.5 \times 10^{-12}$ ), consistent with their increased numbers and a lineage-specific expansion. These observations are consistent with the hypothesis that TEs make up the bulk of the enlarged genomes of subgenus *Strobus*, with much of the expansion in *P. lambertiana* attributable to Gypsy.

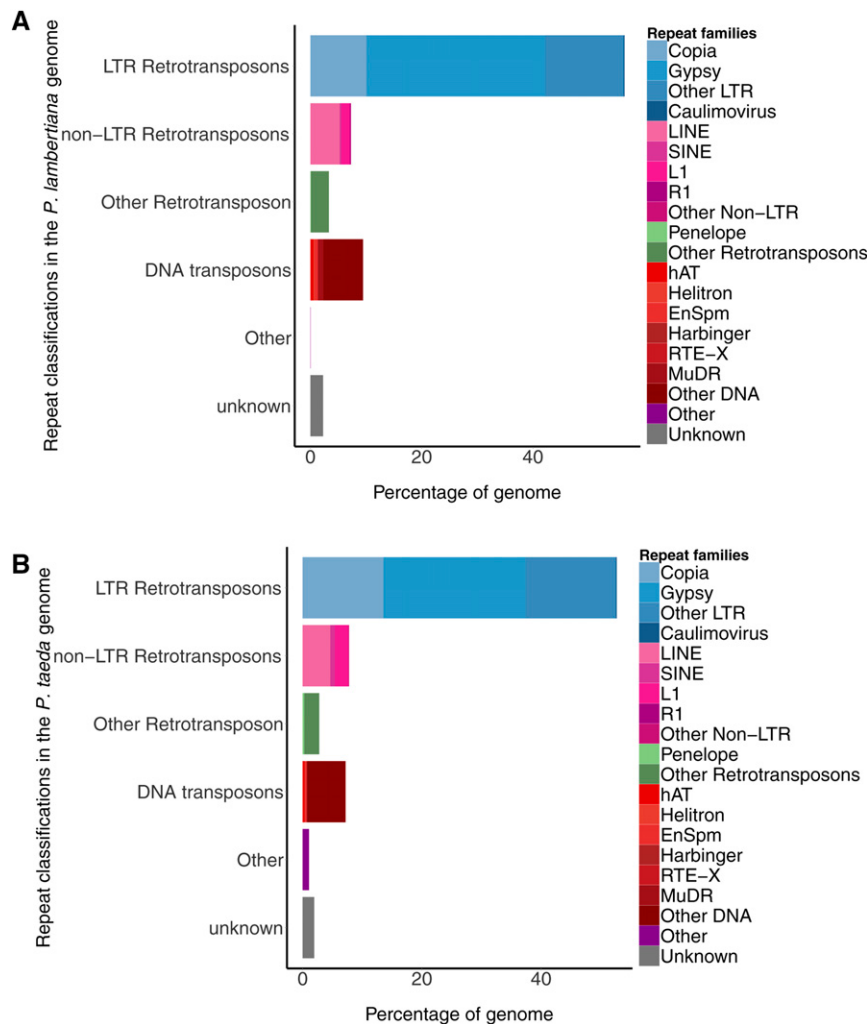
The similarity in TEs among the sequenced conifer genomes supports the hypothesis that conifers have experienced massive expansion of TEs throughout their history (Neale *et al.* 2014), likely including the period prior to the radiation of *Pinus*, yielding their large and varied sizes. The bulk of TE sequences are ancient and diverged. Consistent with this, we observed that partial elements are far more abundant than full-length sequences in *P. lambertiana*, representing 67.3% of the genome, and 87% of the total repetitive content. And while the vast majority of LTRs were ancient and inactive, we did find evidence of recent transposition in the form of a recently inserted heterozygous TE. We observed a complete heterozygous insertion of a PARTC element in a genomic segment captured in an assembled fosmid clone. Heterozygosity is inferred from the insertion of the element, and the presence of a target site duplication in the alignment to the alternate haplotype (Figure S6). Previous analysis of the many copies of the PARTC subfamily suggested that it was dead (Zuccolo *et al.* 2015). However, this copy has identical LTR sequences, and apparently functional proteins.

### Identification of genomic scaffolds and mapping in the *Cr1* region

Using the draft WGS assembly, we succeeded in anchoring the cloned RAPD sequences, *scar*OPG16\_950 and *scar*BC432\_1110, which had previously been mapped near the *Cr1* locus (Jermstad *et al.* 2011), to two distinct scaffolds (Table S13). No longer limited to designing PCR primers within those cloned sequences, we utilized the entire repeat masked scaffolds as a resource, and were able to identify many clear single nucleotide polymorphisms (SNPs) in each flanking amplicon, including that adjacent to *scar*BC432\_1110, which had previously yielded no scorable SNPs (Jermstad *et al.* 2011).

PCR primers were designed to amplify two small genomic loci, one in scaffold 223,058 and the other in 370,413 (Table S10). The amplicons of successful primer pairs were sequenced and tested for segregation in a small sample of both *Cr1<sup>R</sup>* and *Cr1<sup>r</sup>* segregant megagametophytes from maternal tree 5701 (*Cr1<sup>R</sup>/Cr1<sup>r</sup>*), for which the rescued embryos were genotyped for *Cr1*. Note, the pollen parent of the rescued embryos was assumed to be *Cr1<sup>r</sup>/Cr1<sup>r</sup>* because the frequency of *Cr1<sup>R</sup>* is assumed to be rare. Alternative haplotype sequences were found for both amplicons that segregated (see Supplementary Methods in File S1 for the Fasta sequence), and appeared to be linked to one another and to the *Cr1* locus.

A large sample of megagametophytes was efficiently genotyped using Cleaved Amplified Polymorphic Sequence (CAPS) assays (Konieczny and Ausubel 1993). We developed two new CAPS markers, *cr1A* and *cr1B*, based on the sequence variation in these two amplicons physically linked via the assembly to the previously reported RAPD markers (see Table S13 in File S1). Genotyping of *cr1A* and *cr1B* on a sample of 245 megagametophytes from maternal tree 5701 yielded two apparent single crossovers between markers *cr1A* and *cr1B* (both *cr1A<sup>R</sup> - cr1B<sup>r</sup>*), and 225 non-recombinants (Table S12 in File S1). We were not able to confirm the Harkins *et al.* (1998) gene order BC\_432\_1110 – *Cr1* – OPG\_16\_950. For the RAPD markers BC\_432\_1110 and OPG\_16\_950, Harkins *et al.* (1998) reported recombination fractions of 3%, and genetic map distances of 1.2 cM between both markers and *Cr1* for maternal tree 5701. For our data, Harkins *et al.* (1998) gene order results in



**Figure 3** Comparison of repetitive content between transposable element repeat families in *P. lambertiana* (top) and *P. taeda* (bottom).

12 putative double recombinants, which can alternatively be interpreted as *Cr1* genotyping error.

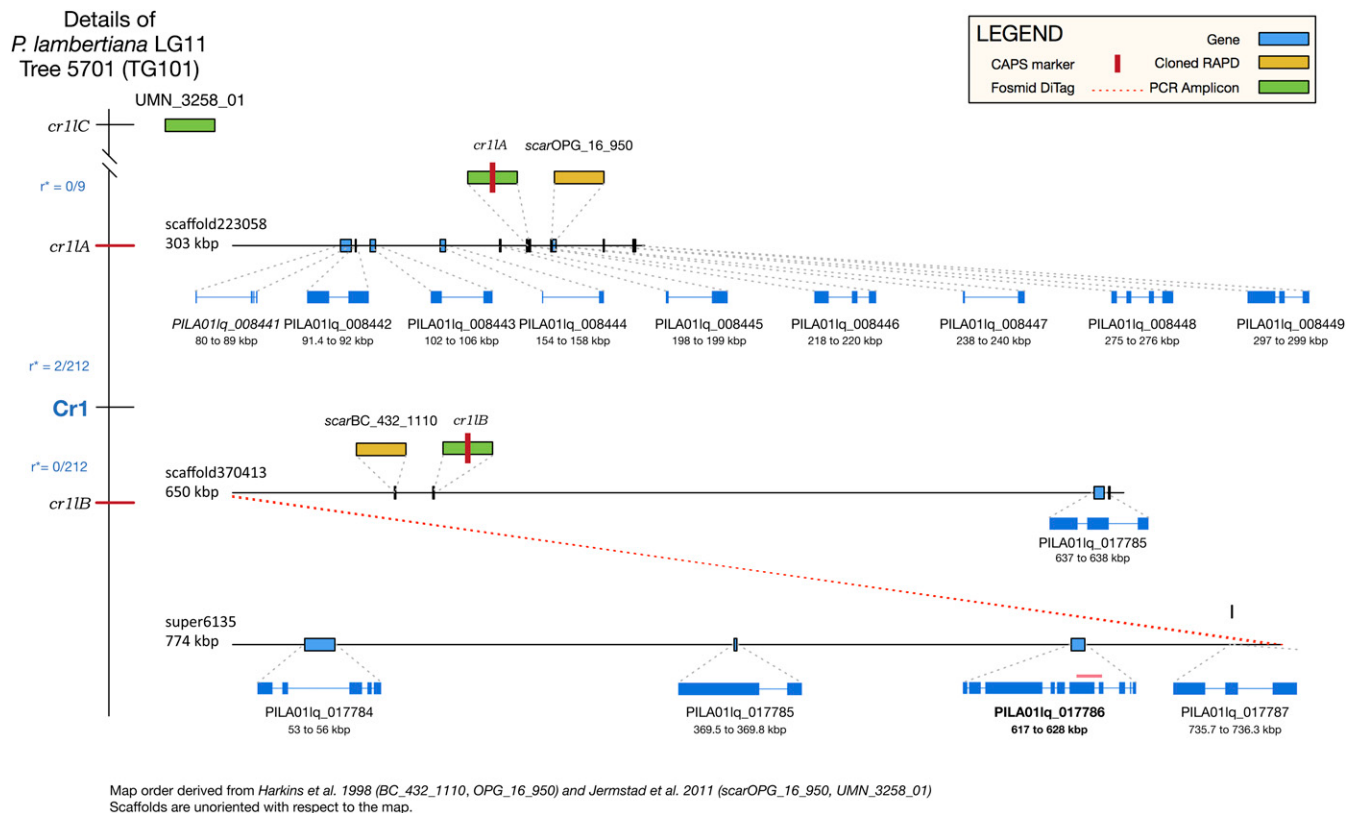
Our two crossovers between *cr1A* and *cr1B* indicate that *Cr1* is closer to *cr1B*. To validate this result, and place it in a slightly broader mapping context, we added a new marker *cr1C* to the genetic map, derived from a previously characterized PCR amplicon (Jermstad *et al.* 2011; UMN\_3258\_01; <http://treegenesdb.org/ftp/CRSP/>) Genotyping with SNPs derived from this amplicon placed it closest to scar-OPG\_16\_959 on the side away from *Cr1* in the Jermstad *et al.* (2011) map for maternal tree 5701 at a distance of ~12 cM. We genotyped *cr1C* in the two *cr1A* - *cr1B* recombinant megagametophytes from 5701, in three randomly selected *Cr1<sup>r</sup>* nonrecombinants, and four randomly selected *Cr1<sup>R</sup>* nonrecombinants to further refine marker order. Two distinct *cr1C* haplotypes were determined among these progeny. None were recombinant between *cr1A* and *cr1C*, thus placing *Cr1* outside of these loci (Figure 4, left), consistent with the gene order (*cr1C* - *cr1A*) - (*Cr1* - *cr1B*).

### Increasing the *Cr1* genomic region

To expand our annotated intervals linked to *Cr1*, we walked outward from the two marker-anchored scaffolds using

physical linkage inferred from one or more aligned fosmid DiTag reads not included in the assembly. Using this approach, an additional gene-containing scaffold was physically linked to one of our anchored scaffolds by two fosmid DiTags (Figure 4).

The genome assembly allowed a more targeted identification of potential gene candidates for *Cr1*. Figure 4 shows a total of 14 gene annotations on the two scaffolds genetically linked to *Cr1*, and a third scaffold that was physically linked by fosmid DiTags. Of the 14 linked genes, PILA\_lg017786 stands out as a candidate because it contains both the NB-ARC and LRR domains that are common elements of disease-resistance genes. We looked for direct evidence of expression in transcriptome assemblies and found only one transcript (TR43508|c1\_g1\_i2|m.82078; see Supplementary Methods in File S1) assembled from a library constructed from a WPBR resistant tree. The transcript overlaps two exons of the candidate gene (red bar above the gene in Figure 4). The most similar known gene is in *P. monticola* (Western white pine), a TIR-NBS-LRR protein (GI:321530320). The closest well-annotated gene appears to be the disease resistance protein RGA2 in the grass *Aegilops tauschii* Coss. (GI:475615320).



**Figure 4** Annotated scaffolds and elements linked to *Cr1*. On the left is a tentative map of the *Cr1* region of chromosome 11 showing the positions of identified markers. The gene order shown was derived from Harkins *et al.* (1998) (BC\_432\_1110 labeled *cr1B*, *Cr1*, OPG\_16\_950 labeled *cr1A*) and Jermstad *et al.* (2011) (*Cr1*, scarOPG\_16\_950, UMN\_3258 labeled *cr1C*). To the right are five scaffolds and 14 gene annotations that are linked to the *Cr1* gene. The evidence of expression of PILA\_lg017786 was a single transcript (red bar) assembled from a library constructed from a resistant tree (Supplementary Methods in File S1). Scaffold super6135 is physically linked to scaffold 370413 that harbors *cr1B* by two fosmid DiTags.

### *Cr1* association

To both confirm the tight linkage of *cr1B* to *Cr1* and to provide a potential resource for marker-assisted selection, small, representative samples of *Cr1*-genotyped trees were genotyped by sequencing the amplicon from a single megametophyte. A total of six *Cr1<sup>R</sup>/Cr1<sup>R</sup>*, 12 *Cr1<sup>R</sup>/Cr1<sup>r</sup>*, and 22 *Cr1<sup>r</sup>/Cr1<sup>r</sup>* genotyped sugar pine seed trees from the center of the species' range were assayed (Table S15 in File S1). Genotyping of the diploid parent for *Cr1* was done by the Forest Service at the El Dorado National Forest, Placerville Nursery, using their standard protocol of germinating, and scoring at least 56 exposed seed trees for WPBR resistance. These *Cr1<sup>R</sup>/Cr1<sup>R</sup>* and *Cr1<sup>R</sup>/Cr1<sup>r</sup>* trees were previously reported in Vangestel *et al.* (2016).

We selected one megagametophyte each from a maternal parent that had been genotyped for resistance. The *cr1B* primers appeared to work well outside of their original context (maternal genotype 5701) and the haploid nature of the DNA afforded additional confirmation of the sequencing results. Evaluating only sequences associated with known *Cr1* alleles (*i.e.*, transmitted from *Cr1<sup>r</sup>/Cr1<sup>r</sup>*, *Cr1<sup>R</sup>/Cr1<sup>R</sup>*, or phenotyped progeny of 5701) we identified a five-site motif that predicted the *Cr1* allele nearly completely (see Figure 5). All

seven of our *Cr1<sup>R</sup>* associated haplotypes (six transmitted from *Cr1<sup>R</sup>/Cr1<sup>R</sup>* and the *Cr1<sup>R</sup>* linked haplotype of 5701) had the motif "TTACT." Furthermore, 23 out of 24 of our *Cr1<sup>r</sup>/Cr1<sup>r</sup>* transmitted haplotypes had the alternate *Cr1<sup>r</sup>* linked motif "GCGGC." The association is almost complete; the differences in the frequencies of the two haplotypes transmitted with known *Cr1* genotypes is statistically significant,  $P < 10^{-5}$  by  $\chi^2$  with 2 d.f. Both motifs segregated in the progeny of one *Cr1<sup>r</sup>/Cr1<sup>r</sup>* parent. The observation of this single heterozygous tree is consistent with a low frequency of "recombinant" haplotypes. Still the association of *Cr1* with SNPs in the *cr1B* amplicon on scaffold 370,314 is strong.

### Discussion

A key step in the sequencing strategy for *P. lambertiana* was the generation of deep sequencing coverage of the haploid genome. Even so, the unprecedented amount of data, two trillion bases, required an alternative strategy in order to assemble the genome in a reasonable time frame. The contiguity of the *P. lambertiana* assembly, as measured by the N50 scaffold size, is higher than previous conifer genome assemblies (Birol *et al.* 2013; Nystedt *et al.* 2013; Neale *et al.* 2014;

|                             |                                                         | 1 | 2 | 3 | 4 | 5 |           |
|-----------------------------|---------------------------------------------------------|---|---|---|---|---|-----------|
| Reference                   |                                                         | A | A | G | T | C | G G G C G |
| 5701 <i>Cr1<sup>r</sup></i> |                                                         | A | A | G | G | C | G G G C G |
| SP-1156-00091.1             |                                                         | A | A | G | A | C | A G G C G |
| SP-K-0139-U.1               |                                                         | A | C | G | G | C | G G G C G |
| SP-0356-00043.1             |                                                         | A | A | G | A | C | A G G C G |
| SP-1553-00115.1             |                                                         | G | C | G | G | C | G G G C A |
| SP-K-0121-U.1               |                                                         | G | C | G | G | C | G G G C A |
| SP-1151-AD-00015.1          |                                                         | G | C | G | G | C | A G G C G |
| SP-1154-00087.1             |                                                         | G | C | G | G | C | A G G C G |
| SP-0353-00060.1             |                                                         | A | A | G | T | C | G G G C G |
| SP-K-0132-U.1               |                                                         | A | A | G | T | C | G G G C G |
| SP-1156-00068.1             |                                                         | A | A | G | T | C | G G G C G |
| SP-1154-00216.1             |                                                         | A | A | G | A | C | A G G C G |
| SP-1153-00226.1             |                                                         | A | A | G | A | C | A G G C G |
| SP-0355-00159.1             |                                                         | G | C | G | G | C | A G G C G |
| SP-0351-00303.1             |                                                         | G | C | G | G | C | A G G C G |
| SP-K-0145-U.1               |                                                         | A | A | G | G | C | G G G C G |
| SP-K-0144-U.1               |                                                         | A | A | G | T | C | G G G C G |
| SP-K-0149-U.1               |                                                         | A | A | G | T | C | G G G C G |
| SP-0355-00162.1             |                                                         | G | C | G | G | C | G G G C A |
| SP-K-0155-U.1               |                                                         | G | C | G | G | C | G G G C A |
| SP-0351-00218.1             |                                                         | G | C | G | G | C | G G G C A |
| SP-1154-DFC-00272.1         |                                                         | G | C | G | G | C | G G G C A |
| SP-K-0142-U.1               |                                                         | A | A | G | T | C | G G G C G |
| SP-K-0142-U.2               |                                                         | G | C | T | T | T | A A C T A |
| 7646.1                      | transmitted from <i>Cr1<sup>r</sup>/Cr1<sup>r</sup></i> | A | A | G | T | C | G G G C G |
| 6202.1                      |                                                         | A | A | G | G | C | G G G C G |
| 6554.1                      |                                                         | A | A | G | G | C | G G G C G |
| 5062.1                      |                                                         | G | C | G | G | C | G G G C A |
| 18852.1                     |                                                         | G | C | T | T | T | A A C T A |
| 18875.1                     |                                                         | G | C | T | T | T | A A C T A |
| 19600.1                     |                                                         | G | C | T | T | T | A A C T A |
| 19188.1                     |                                                         | G | C | T | T | T | A A C T A |
| 19601.1                     |                                                         | G | C | T | T | T | A A C T A |
| 19409.1                     |                                                         | G | C | T | T | T | A A C T A |
| 5701 <i>Cr1<sup>R</sup></i> |                                                         | G | C | T | T | T | A A C T A |
| 6351.1                      |                                                         | G | C | T | T | T | A A C T A |
| 6200.1                      |                                                         | G | C | T | T | T | A A C T A |
| 5892.1                      |                                                         | G | C | T | T | T | A A C T A |
| 6902.1                      |                                                         | G | C | T | T | T | A A C T A |
| 6352.1                      |                                                         | G | C | T | T | T | A A C T A |
| 6353.1                      |                                                         | G | C | T | T | T | A A C T A |
| 7519.1                      |                                                         | G | C | T | T | T | A A C T A |
| 7453.1                      |                                                         | G | C | T | T | T | A A C T A |

**Figure 5** Multiple alignment of association samples showing the most variable sites, 40% or more consensus differences. The numbered five site *Cr1* linked motif is seen as two haplotypes, the *Cr1<sup>r</sup>* linked GCGGC and the *Cr1<sup>R</sup>* linked TTACT. One haplotype (SP-K-0142-U.2) transmitted from a *Cr1<sup>r</sup>/Cr1<sup>r</sup>* parent genotyped as a putative *Cr1<sup>R</sup>* linked "TTACT" recombinant.

Warren *et al.* 2015). A combination of factors, including deeper sequence coverage, more physical coverage from new linking mate pair library chemistries, and better computational methods, all likely contributed to the advance. Like other con-

ifers, a critical biological aspect of the *P. lambertiana* genome that allows it to be assembled, is the accumulated divergence among the ancient repeats comprising the majority of the genome. This increased contiguity of the *P. lambertiana* assembly

clearly suggests that the contiguity of conifer genome assemblies will continue to increase as scalable, long-range linking methods become available.

The characterization of *P. lambertiana* transposable element sequences supports the hypothesis advanced by Nystedt *et al.* (2013) that an ancient accretion of mostly inactive TEs at a rate faster than they are removed, explains the majority of the increased genome size observed in the *Pinus* subgenus *Strobus*. Given the huge genome sizes, the time scale involved, and the still sparse sampling of genome sequences of conifer species, recent TE dynamics (if such exist) are difficult to detect. Nevertheless, we made two observations relevant to the hypothesis. First, sequences of gypsy families are more abundant in the *P. lambertiana* genome lineage, and this likely contributed to the increase in genome size. This hypothesis is supported by Gypsy families having increased fractions of repeat sequences with younger age. Second, we detected what appears to be an actively transposing *Part-C* element, based on its fully intact coding genome, and its heterozygous insertion state. These observations are consistent with the simplest hypothesis that the many transposon families remain an active but small cohort, and that their sequences accumulate over millions of years because their replicative transposition rate exceeds their removal rate. So far, there is no evidence for any very recent huge expansion of specific families. We did detect the signature of recent duplication in the *P. lambertiana* genome in the *k*-mer distribution, perhaps evidence of nonhomologous crossover. However such duplications were not abundant enough to explain the difference in genome sizes. While ancient genome duplication (Li *et al.* 2015) may also have played a role, the hypothesized event predates the radiation of *Pinus*.

The immense size and repetitive nature of the conifer genome, especially that of *P. lambertiana*, has been, and remains, a daunting barrier to genetic analyses, especially the investigation of pathogen resistance. And this challenge, compounded with those inherent to the long generation time, as well as resource requirements, have translated into strenuous efforts to achieve modest advances in understanding and impacts on the genetics of reforestation. This reference genome brings new powerful tools to genetics/genomic research in *P. lambertiana*. We sought to apply the new reference genome sequence to the characterization of the genetics of resistance to WPBR, building on the rich previous research, and indeed the availability of genomic samples from now classic efforts to genetically map a major disease resistance gene. Also (as discussed above) strong ecological and economic considerations motivate the pursuit of both new knowledge, and effective practical tools that can be applied to forest management (Waring and Goodrich 2012). Large scaffolds in the assembly of *P. lambertiana* bearing short sequences previously linked to *Cr1* (Harkins *et al.* 1998; Jermstad *et al.* 2011) were identified, validated as linked to *Cr1*, and annotated as containing a promising candidate gene. Of substantial immediate practical relevance is the strong association between SNPs anchored in one of these

scaffolds and *Cr1* in natural populations. Genotyping with such SNPs is a long-sought-after tool that will increase the efficiency of ongoing and future WPBR-resistant reforestation. The present expensive and time consuming process of identifying candidate trees, collecting seed (during a narrow period), and waiting 2 years for infection bioassay results, does ultimately identify trees heterozygous (or rarely homozygous) for *Cr1<sup>R</sup>* that can then be harvested for seeds to go into reforestation. But the efficiency is low, and the cost to identify a single such tree is thousands of dollars [see the estimated replacement costs in a 2013 supplement to a US Forest Service Handbook (page 5), available at <http://www.fs.fed.us/im/directives/field/r5/fsh/2409.18/r5U2409U18U50U2013U1.doc>]; furthermore, the supply is not always adequate or ecologically optimal. Ongoing efforts to develop these and other SNPs as practical tools for sugar pine forest management have great promise, and may lead the way to similar tools for other white pines.

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*Note added in proof:* See Gonzalez-Ibeas *et al.* 2016 (pp. 3787–3802) in *G3: Genes, Genomes, Genetics* for a related work.

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## Supplementary Methods, Tables, and Figures

### Sequencing and Assembly

#### *Paired-end libraries from megagametophytes*

Paired-end libraries were constructed as described in Zimin *et al.* (2014). Briefly: approximately 5 µg of DNA from our target megagametophyte was fragmented by sonication, end-repaired, and A-tailed. Universal Illumina paired-end adapters were ligated to the fragments and agarose gel size selection was used to collect a series of ligation-product fractions with mean insert sizes ranging from 180 to 880 bp. Ten ng of each fraction was used as template for a 10-cycle enrichment PCR with barcoded primers. Libraries were quantified on an Agilent Bioanalyzer 2100 and sequenced on the GAIIX and HiSeq 2500 platforms.

Two enrichment PCR chemistries were used: the Illumina-recommended Phusion HF master mix (New England Biolabs) and KAPA HiFi HotStart master mix (Kapa Biosystems). In a side-by-side comparison of k-mer depth distributions the Kapa Biosystems chemistry demonstrated a lower variance in coverage and it was therefore used for all remaining library construction.

#### *Paired-end sequencing*

**Table S1** Paired end sequencing results by platform. The majority of paired end sequence data came from the HiSeq 2500 platform which replaced the GAIIX as a high throughput longer-read solution achieving an average error-corrected read length just 3 bp shorter than the GAIIX. ('C. len' is corrected length in bp).

| Platform   | Read length | Reads sequenced | Reads after E.C. | %    | Bases sequenced | Bases after E.C. $\geq 31$ bp | %    | C. len | %    |
|------------|-------------|-----------------|------------------|------|-----------------|-------------------------------|------|--------|------|
| MiSeq      | 255+255     | 191329972       | 190012005        | 99.3 | 47165405920     | 44250142585                   | 93.8 | 234    | 91.9 |
| HiSeq 2500 | 150+150     | 3704633253      | 3670172611       | 99.1 | 5.55695E+11     | 5.4229E+11                    | 97.6 | 148    | 98.5 |
| HiSeq 2500 | 151+151     | 5577432158      | 5518035319       | 98.9 | 8.42192E+11     | 8.20401E+11                   | 97.4 | 149    | 98.5 |
| HiSeq 2000 | 125+125     | 2250040534      | 2220695615       | 98.7 | 2.81255E+11     | 2.71663E+11                   | 96.6 | 122    | 97.9 |
| GAIIX      | 160+156     | 1134732636      | 1127425204       | 99.4 | 1.81557E+11     | 1.71896E+11                   | 94.7 | 152    | 96.5 |

**Table S2** Paired end sequencing results by insert size. We observed a slight reduction in the efficiency of error correction for the longer insert libraries.

| Insert size    | Libraries | Reads      | Reads after E.C. | %    | Bases sequenced | Bases after EC $\geq 31$ bp | %    |
|----------------|-----------|------------|------------------|------|-----------------|-----------------------------|------|
| [200bp, 400bp) | 32        | 6686446005 | 6634318205       | 99.2 | 9.59584E+11     | 9.32758E+11                 | 97.2 |
| [400bp, 600bp) | 12        | 2961998624 | 2936847083       | 99.2 | 4.64692E+11     | 4.52177E+11                 | 97.3 |
| [600bp, 900bp) | 12        | 3209723924 | 3155175466       | 98.3 | 4.83589E+11     | 4.65565E+11                 | 96.3 |

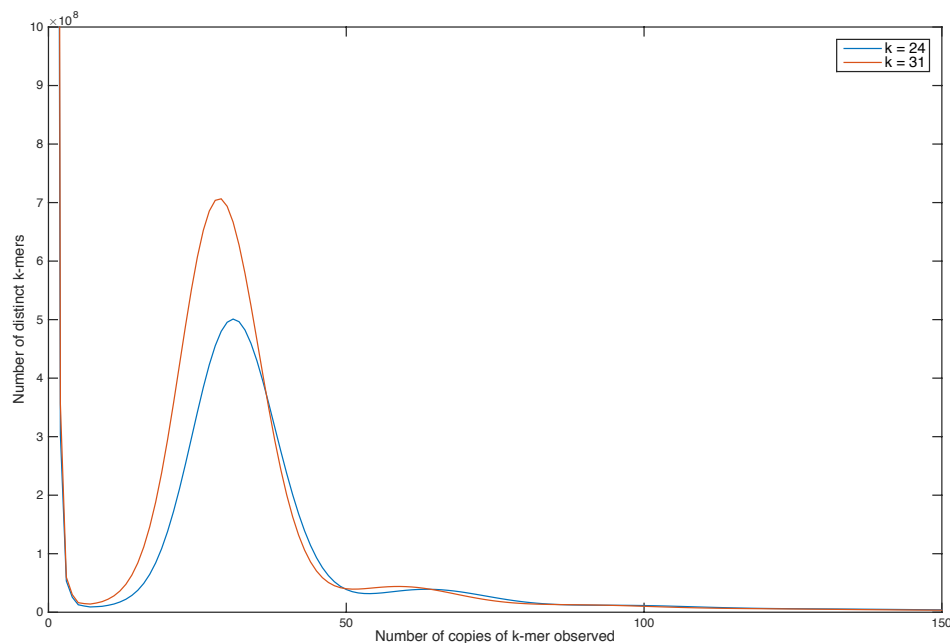
### Paired-end super-reads

The  $k$ -mer size for the construction of paired-end super reads was optimized to maximize the number of distinct  $k$ -mers in the error-corrected paired-end data. The value of 89 was chosen using a grid search, implemented by repeatedly running super-read construction, and identifying a local maximum (Table S3).

**Table S3** Selecting a value of  $k$  for the MaSuRCA assembler.

| $k$ | Distinct $k$ -mer count | $k$ -unitig count | Average $k$ -unitig length |
|-----|-------------------------|-------------------|----------------------------|
| 79  | 26,999,996,380          | 585,474,925       | 125.12                     |
| 89  | 27,134,295,936          | 458,204,603       | 148.22                     |
| 99  | 27,105,725,056          | 350,678,093       | 176.30                     |

### $K$ -mer histogram for error-corrected paired-end reads



**Figure S1** The  $k$ -mer histogram of the error-corrected *P. lambertiana* paired-end reads shows a strong distinct peak at 1C depth consistent with haploid DNA. The peak at roughly twice the expected coverage (putative recent duplications) represents approximately 7% of the genome and appears more pronounced than in *P. taeda* (Zimin *et al.* 2014). We observed that 39.4% of the 31-mers were in the more highly repeated tail, to the far right. In *P. taeda* a slightly smaller fraction (34.1%) of 31-mers were in this tail.

### Mate pair libraries

For all mate pair libraries input DNA was first treated with 0.33  $\mu$ l PreCR Repair Mix (New England Biolabs) per microgram of DNA following the manufacturer's guidelines. Jumping libraries were constructed using two methods. Initially libraries were constructed as in (Zimin *et al.* 2014) using the Illumina Mate Pair Library v2 protocol. Later we switched to Illumina's Nextera Mate Pair kit because it gave superior results, particularly for longer-range linkage. Nextera Mate Pair libraries were constructed following the "gel-plus" method in the kit instructions but with the following modifications: input DNA amounts and reagent/reaction volumes for steps up to agarose gel size-selection were tripled in order to achieve increased yields. For longer-range libraries (i.e. > 10 Kbp) the amount of tagmentation enzyme was reduced to 1  $\mu$ l per microgram input DNA, which shifted the fragment-length distribution to higher molecular weights. Bst polymerase (8 U/ $\mu$ l; New England Biolabs) was sometimes substituted for Strand Displacement Polymerase when kit volumes ran short. PCRClean DX beads (Aline Biosciences) were substituted for Ampure XP beads throughout. 0.6% MegaBase agarose gels were run overnight using a Bio-Rad FIGE Mapper. Shearing of circularized molecules was performed using a Diagenode Bioruptor NGS at high power for 8 cycles of 15 seconds on/90 seconds off. Fifteen cycles of enrichment PCR were performed.

#### *Diploid mate pair sequencing and pre-processing*

Deep fragment coverage from long-range paired reads is essential for constructing large scaffolds (Gnerre *et al.* 2011; Ross *et al.* 2013; Zimin *et al.* 2014). Fragment or "clone" coverage refers to the coverage of the genome represented by the entire DNA fragment. Thus if a pair of 100-bp reads is sequenced from both ends of a 5000-bp fragment, the fragment coverage will be 25 times deeper than the actual read coverage. In total, 20 modified Illumina TruSeq and 14 Illumina Nextera mate pair libraries were constructed from diploid maternal genomic DNA. We monitored library complexity during the sequencing process as described in Zimin *et al.* (2014). An initial investigation determined that our modified Illumina TruSeq libraries would be impractical for obtaining deep coverage on the larger genome, particularly for longer fragment sizes. After an evaluation of Illumina's Nextera mate pair libraries, in which we observed deeper per-library coverage, we chose these libraries for the bulk (76%) of our long-fragment sequencing.

Raw sequence from mate pair libraries was processed through a special module of MaSuRCA (Zimin *et al.* 2013) to make the reads match the target haplotype. We used a database of haploid 24-mers to correct errors and single-nucleotide polymorphisms in the diploid read pairs. This correction procedure yielded over 93X fragment coverage in paired reads where both reads had been corrected to match the haploid data (Table S4). This represents more than twice the fragment coverage obtained for *P. taeda* (Zimin *et al.* 2014).

**Table S4** Mate pair libraries, MaSuRCA-processed reads, and estimated physical coverage by insert size.

| Insert Size<br>Range | Count | Processed<br>reads | Physical<br>coverage |
|----------------------|-------|--------------------|----------------------|
| [1Kbp, 5Kbp)         | 14    | 358,618,948        | 18.8X                |

|                |    |             |       |
|----------------|----|-------------|-------|
| [5Kbp, 10Kbp)  | 10 | 268,825,892 | 30.3X |
| [10Kbp, 15Kbp) | 7  | 157,651,636 | 32.1X |
| [15Kbp, 25Kbp) | 3  | 41,269,998  | 12.6X |

### *Illumina sequenced fosmid pool*

For use in repeat-library construction, a pool of approximately 5000 *P. lambertiana* fosmid clones (0.5% of the genome) was prepared and sequenced following our previous method (Wegrzyn *et al.* 2013; Zimin *et al.* 2014). Paired-end and Illumina mate pair libraries were prepared as described above. Both libraries were sequenced in a single HiSeq 2500 lane in high-throughput mode (Table S5). Data were processed with RTA 1.17.21.3 and CASAVA 1.8.2. Sequence was subsequently filtered and assembled with SOAPdenovo2 using the method reported in Wegrzyn *et al.* (2013) yielding a 159 Mbp assembly containing 4963 scaffolds greater than 20 Kbp (a fosmid may generate only one of these).

**Table S5** Illumina sequencing of fosmid pools.

| Library type | Insert size | Number of paired 150 bp reads (Millions) | Number of bases (Mbp) | Estimated coverage    |
|--------------|-------------|------------------------------------------|-----------------------|-----------------------|
| paired-end   | 400 bp      | 46.4                                     | 13,928                | 67X                   |
| mate pair    | 3 Kbp       | 22.5                                     | 6,750                 | 32X physical coverage |

### *PacBio sequenced fosmid pools*

Four identical fosmid pools of 48 fosmids each were constructed from the larger pool above. These were prepared and sequenced using PacBio RS II for validation purposes. Additional details on the sequencing depth and alignment assembled pools to the WGS assembly are given here.

**Table S6** PacBio sequencing of fosmid pools.

| Fosmid Pool | Number of reads | Mean read length | N50 read length | Number of bases | Estimated coverage |
|-------------|-----------------|------------------|-----------------|-----------------|--------------------|
| SPPB1       | 82,563          | 7,421            | 10,863          | 612,739,994     | 255X               |
| SPPB2       | 91,904          | 6,974            | 9,815           | 640,943,357     | 266X               |
| SPPB3       | 106,393         | 6,333            | 8,969           | 673,810,507     | 280X               |
| SPPB4       | 92,381          | 6,312            | 9,023           | 583,153,465     | 242X               |

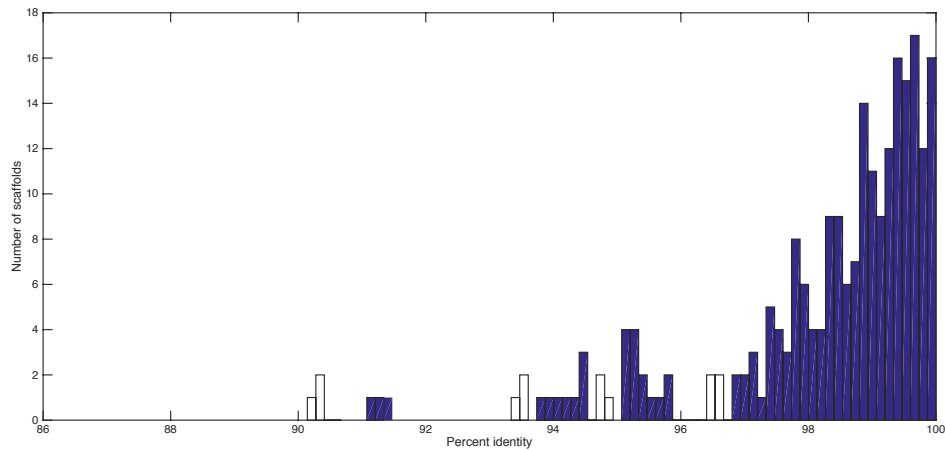


Figure S2 Histogram of %identity weighted across the nucmer alignment for each scaffold when comparing fosmid assemblies to the WGS assembly. The median %identity for an aligned scaffold was 98.82%.**Annotation (genes and transposable elements)**

*Libraries used for gene annotation and transcriptome scaffolding*

A subset of the libraries and sequence described in Gonzalez-Ibeas *et al.* (2016) were used to construct the transcripts used for scaffolding and annotating the genome. Additional information about those libraries is available here.

**Table S7.** *P. lambertiana* RNA libraries used in this paper. More details are available in Gonzalez-Ibeas *et al.* (2016). Sequence data is available at GenBank under the NCBI Bioproject 174450.

| Library ID and Description                                     | Library type | Sequencing           | Transcriptome Scaffolding | Gene Annotation |
|----------------------------------------------------------------|--------------|----------------------|---------------------------|-----------------|
| K, from pollen                                                 | RNA-seq      | MiSeq                | X                         | X               |
| M, from early female cones (2 weeks before pollination)        | RNA-seq      | MiSeq                | X                         | X               |
| Embryo, from germinating sugar pine seed                       | RNA-seq      | HiSeq, MiSeq, PacBio | X                         | X               |
| Basket, from "basket stage" seedling (root, stem, and needles) | RNA-seq      | MiSeq                | X                         | X               |
| S, from 2-cm female cones                                      | RNA-seq      | HiSeq, PacBio        | X                         | X               |
| V, from female cones at the time of pollination                | RNA-seq      | HiSeq, PacBio        | X                         | X               |

|                                                       |         |               |   |   |
|-------------------------------------------------------|---------|---------------|---|---|
| DCS, from stem of control plants (no treatment)       | RNA-seq | HiSeq, PacBio | X | X |
| BRN, from Blister Resistant needles (LCO2-03)         | RNA-seq | HiSeq         | O | X |
| DCR, from root of control plants (no treatment)       | RNA-seq | HiSeq         | O | X |
| JASS, from stem after Methyl jasmonate treatment      | RNA-seq | HiSeq         | O | X |
| NACLR, from root after NaCl treatment                 | RNA-seq | HiSeq         | O | X |
| WS, from stem after wounding                          | RNA-seq | HiSeq         | O | X |
| BRS, Blister Resistant stem (LCO2-03)                 | RNA-seq | MiSeq         | O | X |
| SDN, from needles of seedling slowly drought-stressed | RNA-seq | MiSeq         | O | X |
| P, from pollen cones                                  | RNA-seq | MiSeq         | O | X |

### *Gene model identification and annotation*

Annotation of the *P. lambertiana* genome was performed with MAKER-P. Models that did not contain at least one protein domain as defined in Pfam/Panther via InterProscan were removed. For the high quality set, due to the potential high content of pseudogenes, only multi-exonic models supported by RNAseq data were considered, and remaining models were moved to the low quality set. Manual inspection of gene coordinates of the high quality set and comparison with transcriptome data revealed that the genes could have been split during the identification process (that is, the gene is fragmented in several parts which are counted as independent consecutive gene models sorted on the same genomic area). The problem of genes fragmented into >1 *loci* within the same scaffold during gene prediction has been also reported for other conifers . We followed a merging strategy by combining MAKER gene predictions that were mapped under the same transcript source (that is, after mapping the transcript on the genome, it overlapped with split consecutive models). This way, 5,133 original MAKER models were collapsed, resulting in 1,454 merged models. Additionally, we rescued 807 mono-exonic MAKER models by using more stringent criteria (they were full-length, with a recognizable protein domain, supported by RNA-seq data and protein evidence from species relatives and whose *Arabidopsis* counterpart is also mono-exonic (TAIR10 database, e-value cut-off 1e-09)) to be added to the high quality set. Transcripts that were not used by MAKER were aligned to the genome using GMAP and included (1,745 models). In total, 13,936 gene models were

considered the final high-quality set (combined categories) for downstream analysis, and 71,117 were flagged as low quality (Table S8). Categories of the high-quality set included 1) original MAKER predictions (being 9,930 non-merged multi-exonic and 807 mono-exonic, both with RNA-seq support but different selection criteria), 2) 1,454 merged MAKER models, and 3) 1,745 models built from RNA-seq data.

Gene models were subsequently functionally annotated with a characterized plant protein sequence via our in-house annotation pipeline, enTAP (<https://github.com/SamGinzburg/WegrzynLab>)

**Table S8.** *P. lambertiana* gene models

| Category                                 | Gene models              |                    |
|------------------------------------------|--------------------------|--------------------|
|                                          | <i>Pinus lambertiana</i> | <i>Pinus taeda</i> |
| 1) MAKER models with RNA support         | <b>10737</b>             | <b>5877</b>        |
| 2) Models added from RNAseq data         | <b>1745</b>              | <b>1466</b>        |
| 3) Total merged models                   | <b>1454</b>              | <b>1681</b>        |
| <b>Total high quality gene models</b>    | <b>13936</b>             | <b>9024</b>        |
| BUSCO gene space completeness (%)        | 53                       | 30                 |
| Models without RNA support (low quality) | 71117                    | 75528              |
| Total gene models                        | 85053                    | 84552              |
| BUSCO gene space completeness (%)        | 58                       | 50                 |
| DOGMA gene space completeness (%)        | 94                       | 61                 |

### *Tandem repeat identification*

*P. lambertiana* genome v1.0 scaffolds greater than 400 bp were used for tandem repeat analysis. A total of 1,184,160 scaffolds were present in the resulting dataset. Tandem repeat finder (Benson 1999) was used to detect simple repeats across the full genome. Tandem repeats that overlapped interspersed repeats were removed. Tandem repeats

were categorized as microsatellites (2-8bp), minisatellites (9-100bp), or satellites (>100bp). Mononucleotide repeats were excluded as less reliable.

### *Interspersed repeat identification*

To find interspersed repeat elements, we used both similarity and *de novo* based approaches (Supplementary Figure S3). RepeatModeler combines two complementary *de novo* repeat element prediction algorithms: RECON (Bao *et al.* 2002) and RepeatScout (Price *et al.* 2005). To make the RepeatModeler computation tractable, we used only the Illumina sequenced fosmid pools (above) along with the longest 2.5% of genomic scaffolds. We also used a combination of TEclass (Abrusán *et al.* 2009), CENSOR (Kohany *et al.* 2006), and manual characterization to identify the uncharacterized elements from the repeat library produced by RepeatModeler. We used this library along with the plant Repbase library (Jurka *et al.* 2005) (plant component only, v19.01) as the reference database for RepeatMasker (Tarailo-Graovac *et al.* 2009). Full-length elements were determined by applying a cut-off of 80-80-80 (80% sequence similarity and 80 bp minimum length) (Wicker *et al.* 2007).



**Figure S3.** Methodology for identification of repeat elements in the *Pinus lambertiana* and *P. taeda* genomes. Both *de novo* repeat methodology algorithms such as RECON and RepeatScout as well as similarity search using RepeatMasker were used. Full-length repeat

datasets were obtained by using a cut-off of 80% sequence similarity and a minimum of 80bp alignment length (Wicker *et al.* 2007).

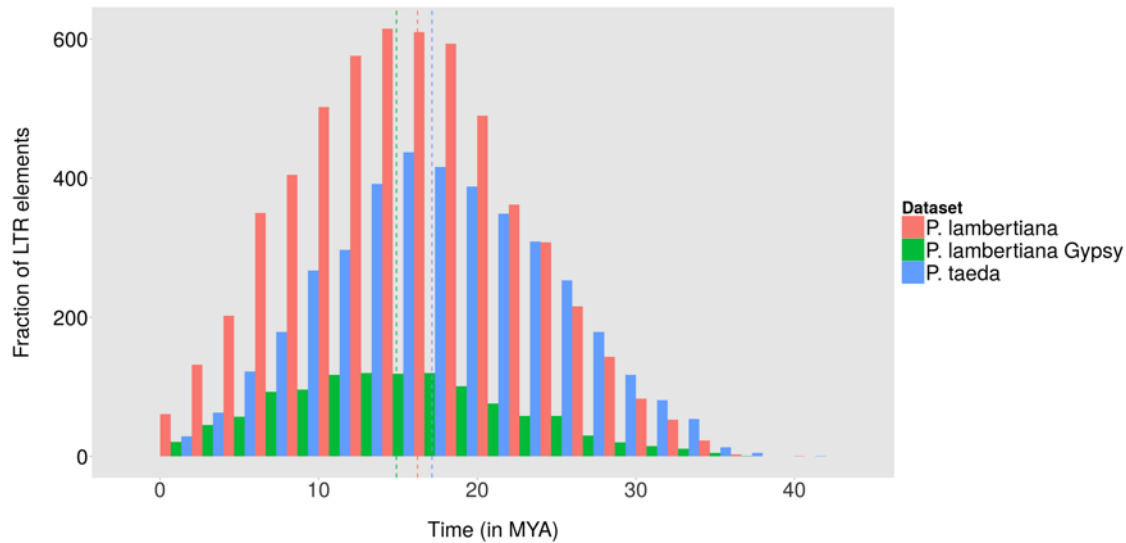
**Table S9.** Full-length and partial repeat elements in *P. lambertiana*

| Repeat classification | Percentage of full-length repeat elements | Percentage of partial-length repeat elements |
|-----------------------|-------------------------------------------|----------------------------------------------|
| LTR/Gypsy             | 4.740                                     | 27.390                                       |
| LTR/Copia             | 1.480                                     | 8.570                                        |
| other LTR             | 2.070                                     | 12.010                                       |
| Caulimovirus          | 0.025                                     | 0.150                                        |
| LINE/L1               | 0.220                                     | 1.290                                        |
| LINE/R1               | 0.020                                     | 0.118                                        |
| other LINE            | 0.770                                     | 4.490                                        |
| other SINE            | 0.045                                     | 0.260                                        |
| other Non-LTR         | 0.009                                     | 0.049                                        |
| Penelope              | 0.013                                     | 0.081                                        |
| other Retrotransposon | 0.480                                     | 2.734                                        |
| hAT                   | 0.079                                     | 0.462                                        |
| EnSpm                 | 0.084                                     | 0.489                                        |
| Helitron              | 0.036                                     | 0.206                                        |
| MuDR                  | 0.147                                     | 0.852                                        |
| other DNA             | 1.054                                     | 6.041                                        |
| other repeat elements | 0.006                                     | 0.035                                        |

#### *LTR insertion time estimation*

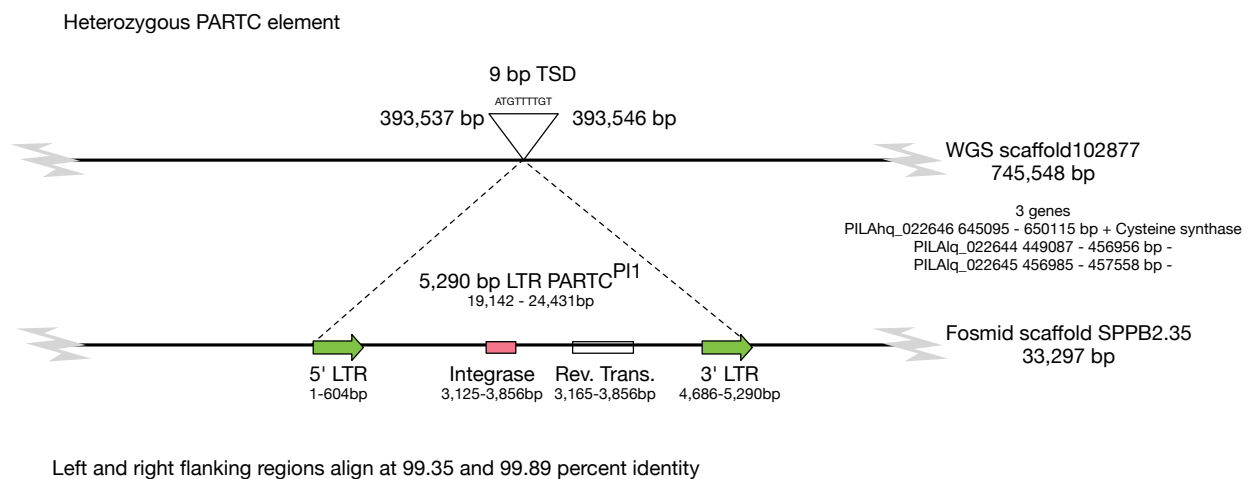
We used LTR Harvest (Ellinghaus *et al.* 2008) to identify long terminal repeats (LTRs) in

probed for their respective LTR regions by searching for LTR harvest hits that were subsets of the full-length hits from RepeatModeler (or vice-versa). LTR Harvest alignments that fulfilled this criteria were aligned with MUSCLE (Edgar 2004) and percent identity between the LTR regions at two ends of the retro-transposon was computed. Divergence was calculated from the percent identity using the Jukes-Cantor formula (Chor *et al.* 2006). The insertion time was calculated from the divergence values as described by SanMiguel *et al.* (1998). The nucleotide substitution rates were used as described in the case of *Picea abies* (Nystedt *et al.* 2013).



**Figure S4.** Histogram depicting insertion times of various retrotransposons in the combined fosmid dataset of *P. lambertiana* and *P. taeda*. The dotted lines represent the average insertion time of the respective datasets. Histograms have been created using substitution rates of  $2.2 \times 10^{-9}$  mutations per year from Nystedt *et al.* (2013). Dotted lines represent the average insertion time of their respective datasets in the histogram

#### *Evidence of a recent LTR insertion*



**Figure S5.** Evidence of a heterozygous and active PARTC element: PARTC<sup>P11</sup> The alignment of genomic scaffold102877 to fosmid scaffold SPPB2.35 reveals a single large structural difference, the insertion of a PARTC into the fosmid scaffold. The 5' and 3' LTR sequences are identical. The coding regions of integrase and reverse transcriptase appear to be functionally conserved (no frameshift or stop codon mutations). At the site of insertion, there are 6-bp duplications at each end of the PARTC<sup>P11</sup> element.

## Genomics of the *C. ribicola* Resistance Gene *Cr1*

### *Megagametophyte DNA Prep*

Prior to DNA extraction, megagametophytes were stored at -80°C. Approximately 1/6 of each megagametophyte was ground with two glass beads on a Mini BeadBeater 8 (BioSpec) at maximum speed for 2 minutes. DNA was extracted following a Qiagen DNeasy mini prep kit (Qiagen) with the addition of proteinase K. Quality and quantity were measured using Picogreen dye on a Qubit fluorometer (Invitrogen).

### *Identification of genomic loci of interest*

Jermstad *et al.* (2011) reported the sequences from cloned RAPD bands OP\_G16 and BC\_432 that were linked to *Cr1*. To identify these genomic loci, the representative consensus sequences for each RAPD band were aligned to the V1.0 draft *P. lambertiana* genome using *gmap* (Wu and Watanabe 2005). In both cases, a unique top hit (path1) was observed and reported.

### *Primer design and sequencing*

We designed nested PCR primers using PRIMER3 (Rosen and Skaletsky 1999) on the reference genome repeat masked changing RepeatMasker annotated repeats to N (Figure S3). Table S9 gives a list of primers and conditions. All of the PCR assays used standard PCR reaction conditions: 2.0 mM MgCl<sub>2</sub>, 0.2 mM each of dNTPs, 0.5 mM each of forward and reverse primers, 1U of Taq and 50 ng of DNA.

For validation purposes, we used the available primer sequences of PCR amplicon, UMN\_3258\_01 (<ftp://dendrome.ucdavis.edu/ftp/CRSP/>) to develop a new marker *cr1C*. In our case, genotype was determined by sequencing UMN\_3258\_01 and subsequent *phred* and *phrap* analysis as described below.

### *SNP discovery*

The DNA sequences for each PCR amplicon were processed and assembled with *phred* and *phrap* (Greene *et al.* 1992) with default parameters. The resulting contigs were subsequently inspected with *consed*. If a single contig was produced, SNPs and short indels were determined by inspection for high-quality discrepancies with the consensus sequence. Most segregating loci produced two scaffolds. SNPs and short indels were identified by alignment of the sequences with *muscle* (Edgar 2004).

**Table S10.** PCR primer details

| Scaffold | Primer name | Primer sequence      | [Mg <sup>2+</sup> ] | Annealing |            | Size (bp) | Comment          |
|----------|-------------|----------------------|---------------------|-----------|------------|-----------|------------------|
|          |             |                      |                     | Temp (C)  | Time (sec) |           |                  |
| 370413   | cr1B_F1     | GATAGGGAGGTTACAGGCC  | 2.00                | 57        | 30         | 1083      | External primers |
| 370413   | cr1B_R1     | TAGTGGATAGGAACCGTGCC |                     |           |            |           |                  |
| 370413   | cr1B_nF1b   | ACAAGAATCTTACCTGGGCC | 1.50                | 56        | 30         | 482       | Nested primers   |
| 370413   | cr1B_nR1b   | GTCTATTTAAGCCACGCCCC |                     |           |            |           |                  |
| 223058   | cr1A_F2     | ATTTTCACGCCTTCTACGCC | 2.00                | 57        | 30         | 1064      | External primers |
| 223058   | cr1A_R2     | TTGCTAAGGACCCAGATCCC |                     |           |            |           |                  |
| 223058   | cr1A_nF2a   | AGCTTTGAATTGCGCTAGGG | 1.50                | 58        | 30         | 577       | Nested primers   |
| 223058   | cr1A_nR2a   | CGCTGAGTACCCATATCCCC |                     |           |            |           |                  |
| 277631   | 277631_F1   | GGGGAGGGGTGTCATTGTTA | 2.00                | 57        | 30         | 932       | External primers |
| 277631   | 277631_R1   | CCCAATGTTTGTGACCCAG  |                     |           |            |           |                  |
| 277631   | 277631_nF1a | CCACCCTAGCTCCAAAGTGA | 1.00                | 57        | 30         | 420       | Nested primers   |
| 277631   | 277631_nR1a | GCATCTCCATTTGTTGCGGA |                     |           |            |           |                  |

*Cleaved amplified polymorphic sequence (CAPS) assays*

The two distinct haplotypes per loci that were identified with consed were mapped for restriction sites using RestrictionMapper (<http://www.restrictionmapper.org/>). We identified specific restriction enzymes that detect polymorphic cutting sites producing readily discernable banding patterns. Conditions and size distributions are described in Table S10. A set of 99 megagametophytes from randomly selected open-pollinated seeds of parent 5701 (*Cr1<sup>R</sup>/Cr1<sup>r</sup>*) were initially genotyped for the CAPS markers. A second expanded search for recombinants was made by pre-screening a larger set of 1054 megagametophytes for RAPD markers BC\_432\_1110 and OPG\_16\_950 used in Harkins *et al.* (1998). This screen resulted in an enriched subset of 146 proposed recombinants.

We expect the assignment of *Cr1* genotypes to be susceptible to error (Harkins *et al.* 1998) and we did observe a small number of ‘double crossovers’ based on their proposed gene order, OP\_G16 – *Cr1* - BC\_432. (Table S12). These were removed from downstream analysis.

**Table S11.** Restriction digest markers

| Marker      | Restriction enzyme | Sequence | Reaction conditions | Inactivation conditions | Haplotype 1 | Haplotype 2    |
|-------------|--------------------|----------|---------------------|-------------------------|-------------|----------------|
| <i>cr1B</i> | MseI (10 µl)       | TTAA     | 37°C for 15 min     | 65C for 20 min          | 322 bp      | 116 and 164 bp |
| <i>cr1A</i> | RsaI (10 µl)       | GTAC     | 37C for 15 min      | 65C for 20 min          | ~290 bp     | 204 and ~290bp |

**Table S12** Restriction digest genotyping results

| <i>cr1B</i> | <i>cr1A</i> |
|-------------|-------------|
|-------------|-------------|

| <i>MseI</i> (TTAA) | <i>Cr1</i> | <i>RsaI</i> (GTAC) | Count |
|--------------------|------------|--------------------|-------|
| 116,164            | R          | 204,292            | 74    |
| 322                | r          | 284,292            | 138   |
| 322                | r          | 204,292            | 2     |
| 116,164            | r          | 204,292            | 5     |
| 322                | R          | 284,292            | 7     |

**Table S13.** Sequenced cloned RAPD markers anchored to the assembly (top), and the corresponding cloned amplified polymorphic sequence (CAPS) assays (bottom).

| RAPD/SCAR        | Scaffold ID | Scaffold length (bp) | Position begin (bp) | Position end (bp) | Coverage    | Identity      |
|------------------|-------------|----------------------|---------------------|-------------------|-------------|---------------|
| (scar)OPG16_950  | 223058      | 303,049              | 221,124             | 221,124           | 98.1%       | 97.2%         |
| (scar)BC432_1110 | 370413      | 655,271              | 119,205             | 119,205           | 99.3%       | 95.7%         |
| CAPS marker      | Linked RAPD | Restriction enzyme   | Cut site            | Amplicon size     | Haplotype 1 | Haplotype 2   |
| <i>cr1A</i>      | OPG16_950   | <i>MseI</i>          | TTAA                | 577               | 322 bp      | 116bp, 164bp  |
| <i>cr1B</i>      | BC432_1110  | <i>RsaI</i>          | GTAC                | 482               | ~290 bp     | 204bp, ~290bp |

### Linking in additional scaffolds

We used Fosmid DiTag linking libraries not included in the assembly to link in additional scaffolds. The libraries were constructed using a refinement of the approach used in *Zimin et al.* 2014, modified so that library inserts containing a junction motif could be enriched by hybridization<sup>1</sup>. The Fosmid DiTag libraries were aligned to the genome using *bwa mem* (Li and Durbin 2010). Alignments were kept if their mapping quality exceeded a minimum threshold of 40 and both sequences aligned within 40 kbp to the end of a scaffold with an implied distance of less than 55 Kbp. We had the highest confidence in the link between scaffold370413 and super6135 which was witnessed by two DiTag pairs (Table S14).

**Table S14.** Linking fosmid DiTags in the *Cr1* region.

| DiTag pair | Target scaffold ID | Alignment start (bp) | Mapping quality | Scaffold length (bp) | Offset from beginning (end) (bp) |
|------------|--------------------|----------------------|-----------------|----------------------|----------------------------------|
| 2          | scaffold370413     | 15586                | 60              | 655271               | 15586                            |
| 2          | super6135          | 770368               | 43              | 772474               | (2106)                           |
| 3          | scaffold370413     | 6392                 | 60              | 655271               | 6392                             |
| 3          | super6135          | 760342               | 60              | 772474               | (12132)                          |

<sup>1</sup> <http://www.idtdna.com/pages/docs/default-source/xgen-libraries/xgen-lockdown-protocols/hybridization-capture-protocol-xgen-lockdown-probes-and-reagents.pdf>

**Table S15.** Megagametophytes sequenced for the population sample. With one exception one megagametophyte from each phenotyped seed tree was sequenced. All 8 available megagametophytes were sequenced from SP-K-0142-U.

| Seed Tree ID      | Resistance Phenotype | National forest | Ranger district | Elevation |
|-------------------|----------------------|-----------------|-----------------|-----------|
| 19600             | RR                   | Tahoe           | Downieville     | 5500      |
| 19188             | RR                   | Sierra          | Minarets        | 5981      |
| 19409             | RR                   | Stanislaus      | Groveland       | 4500      |
| 19601             | RR                   | Tahoe           | Downieville     | 5500      |
| 18875             | RR                   | n/a             | n/a             | n/a       |
| 18852             | RR                   | n/a             | n/a             | n/a       |
| 6351              | Rr                   | Shasta-Trinity  | Mt. Shasta      | 5600      |
| 6200              | Rr                   | Six Rivers      | Lower Trinity   | 4900      |
| 5892              | Rr                   | Klamath         | Goosenest       | 6100      |
| 6902              | Rr                   | Lassen          | Hat Creek       | 5600      |
| 6352              | Rr                   | Shasta-Trinity  | Mt. Shasta      | 5800      |
| 5062              | Rr                   | Klamath         | Happy Camp      | 3700      |
| 7646              | Rr                   | Sierra          | Pine Ridge      | 5600      |
| 6353              | Rr                   | Shasta-Trinity  | Mt. Shasta      | 5900      |
| 7519              | Rr                   | Eldorado        | Georgetown      | 3000      |
| 6202              | Rr                   | Six Rivers      | Lower Trinity   | 4800      |
| 6554              | Rr                   | Shasta-Trinity  | Weaverville     | 5100      |
| 7453              | Rr                   | Tahoe           | Foresthill      | 4600      |
| SP-1151-AD-00015  | rr                   | Plumas          | Beckwourth      | 7000      |
| SP-0356-00043     | rr                   | Eldorado        | Placerville     | 7000      |
| SP-0353-00060     | rr                   | Eldorado        | Georgetown      | 3500      |
| SP-1156-00068     | rr                   | Plumas          | Quincy          | 6200      |
| SP-1154-00087     | rr                   | Plumas          | Feather River   | 3000      |
| SP-1156-00091     | rr                   | Plumas          | Quincy          | 7000      |
| SP-1553-00115     | rr                   | Sierra          | Pine Ridge      | 6500      |
| SP-K-0121-U       | rr                   | Klamath         | Ukonom          | 5500      |
| SP-K-0132-U       | rr                   | Klamath         | Ukonom          | 1500      |
| SP-K-0139-U       | rr                   | Klamath         | Ukonom          | 1020      |
| SP-K-0142-U       | rr                   | Klamath         | Ukonom          | 2030      |
| SP-K-0144-U       | rr                   | Klamath         | Ukonom          | 3070      |
| SP-K-0145-U       | rr                   | Klamath         | Ukonom          | 1250      |
| SP-K-0149-U       | rr                   | Klamath         | Ukonom          | 3601      |
| SP-K-0155-U       | rr                   | Klamath         | Ukonom          | 4507      |
| SP-0355-00159     | rr                   | Eldorado        | Pacific         | 6000      |
| SP-0355-00162     | rr                   | Eldorado        | Pacific         | 5500      |
| SP-1154-00216     | rr                   | Plumas          | Feather River   | 3500      |
| SP-0351-00218     | rr                   | Eldorado        | Amador          | 4500      |
| SP-1153-00226     | rr                   | Plumas          | Feather River   | 2400      |
| SP-1154-DFC-00272 | rr                   | Plumas          | Feather River   | 4000      |
| SP-0351-00303     | rr                   | Eldorado        | Amador          | 6500      |

### Transcript evidence for linked and associated genes

Candidate transcripts were found by BLASTX search using the candidate genes. Transcripts were kept if the reciprocal best gmap alignment of the candidate transcript to the genome overlapped the candidate gene. The candidate transcript TR43508|c1\_g1\_i2|m.82078 was identified in a library constructed from needles of a resistant genotype inoculated with the fungus . The library was prepared, sequenced with the HiSeq platform, and analyzed by the same method described in Gonzalez-Ibeas *et al.* (2016). This library was not included neither in the scaffolding nor the annotation transcriptome sets.

**Figure S6.** Candidate transcript from a resistant library overlapping gene candidate PILA\_017786.

```
>TR43508|c1_g1_i2|m.82078
AAACTCAGAAACCTTCAATACATCGATTTGGAAGGTGCTTCTAATTTGCAGATGCTTCCA
AATTCATTTGGGGATTAACTCAACTCAAACATCTAATTTTGGAAAGGTGCTCTAATTTG
ACCATCTCCAGCGAAGCACTTGGAATATTACCACGCTTAAAAGCTTAGATCTTTCATAT
TGTAACCAGGTGAAAGACGTGCCCTCCCAAGTCACACGTCAACTGTCCTTGCAAACTTA
TATTGAATGGATCAAAGTTAAAGAATTGCCGAGCAATATTGGAGTCCCTCTGCAATTTG
GAAGTTCTGCATTTAGGTAGCGATTGTTGGAAGCGCTGCCAGATGGTCTTGGTGCCTG
AATAGTTTGAAGAGATTATCACTCTCTTCTCGCCGCGAGTTGAAATCCTTGCCGGATTCC
ATTGGACTATTGACTCAGTTGAGAGTACTGGTCATAGAATCTTGCGGACTAGAACCTTA
CCAAAAGAAATTTTCAAGATGAGTAATCTGAGAAGTTTAATGATACGGAATTGTCCGTTG
CGGGAACACCCATTTAGAAAGGAGTTTGAAGGAGTAAGAGAAACGCACCTTATTATTGGAA
GGGAAAGTGCCTTGAATAATTTGAACTCCTCCAATCACAGACGCATGTTTGGGCTCAAG
TGGTTAACCTGTGTCAGGCACAGAAATAAGGGAGGTATTTTTTGTGAGGGCGTTTTCCCC
TGCGTTCAACAATAATGTTCTAGACTGCCCTGAGATACGTAAGTTGTCAGTGGAACAT
TTAATTCTTTGGAGAATTTGGTTGTTGCGCAATGCAAGAATCTCCAGAGCATACTAGGG
TTGAGGCAGCTCACACAGCTTACAGAACTACATGTTTATGGATGCCCTGAGATACGAGAG
CTGCCAGGTGTGGAACAATTGTTTCTTTGGAGATGTTGAAAATTGGGGAATGC
```

**Table S16** Gene annotation for scaffolds linked to the *Cr1* locus.

| Scaffold       | Gene ID/Name | Annotation                                                                                                                                                                |
|----------------|--------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| scaffold370413 | PILA_071809  | Alias=uninformative,<br>Interpro:IPR000757,PANTHER:PTHR31062,PANTHER:PTHR31062:S<br>F18,Pfam:PF00722, note:partial                                                        |
| scaffold223058 | PILA_008442  | Alias=putative MYB DNA-binding domain superfamily<br>protein,Interpro:IPR001005,PANTHER:PTHR10641,PANTHER:PTHR<br>10641:SF460,Pfam:PF00249,note:partial                   |
| scaffold223058 | PILA_008443  | Alias=ATPUP11, putative,Interpro:IPR004853,Interpro:IPR012946,<br>Interpro:IPR030182,PANTHER:PTHR31376,PANTHER:PTHR31376:S<br>F2,Pfam:PF03151,Pfam:PF07983, note:complete |
| Scaffold223058 | PILA_008444  | Alias=adenosylhomocysteinase/s-adenosyl-l-homocysteine<br>hydrolase,Interpro:IPR000043,<br>Interpro:IPR015878,PANTHER:PTHR23420,Pfam:PF00670,<br>note:complete            |
| scaffold223058 | PILA_008445  | Alias=non-annotated model,<br>Interpro:IPR000043,Interpro:IPR015878,PANTHER:PTHR23420,Pfa<br>m:PF00670, note:complete                                                     |
| scaffold223058 | PILA_008446  | Alias=PREDICTED: transcription factor MYB108-like,<br>Interpro:IPR001005,PANTHER:PTHR10641,PANTHER:PTHR10641:S                                                            |

|                |             |                                                                                                                                                                                        |
|----------------|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                |             | F484,Pfam:PF00249, note:complete                                                                                                                                                       |
| scaffold223058 | PILA_008447 | Alias=RAB GTPase homolog A4C,<br>Interpro:IPR001806,PANTHER:PTHR24073,PANTHER:PTHR24073:S<br>F437,Pfam:PF00071, note:complete                                                          |
| scaffold223058 | PILA_008448 | Alias=PREDICTED: alpha-galactosidase-like isoform X1,<br>Interpro:IPR000111,PANTHER:PTHR11452,PANTHER:PTHR11452:S<br>F18,Pfam:PF02065, note:partial                                    |
| scaffold223058 | PILA_008449 | Alias=R2R3-MYB transcription factor,<br>Interpro:IPR001005,PANTHER:PTHR10641,PANTHER:PTHR10641:S<br>F494,Pfam:PF00249, note:complete                                                   |
| super6135      | PILA_017784 | Alias=PREDICTED: probable xyloglucan<br>endotransglucosylase/hydrolase protein 32-like,<br>Interpro:IPR000757,PANTHER:PTHR31062,PANTHER:PTHR31062:S<br>F18,Pfam:PF00722, note:complete |
| super6135      | PILA_017785 | Alias=putative DNAJ heat shock protein,<br>Interpro:IPR002939,PANTHER:PTHR24077,Pfam:PF01556,<br>note:complete                                                                         |
| super6135      | PILA_017786 | Alias=uninformative,Interpro:IPR001611,Interpro:IPR002182,<br>Interpro:IPR026906,PANTHER:PTHR23155,Pfam:PF00560,Pfam:PF<br>00931,Pfam:PF13306,Pfam:PF13504, note:complete              |
| super6135      | PILA_017787 | Alias=uninformative,Interpro:IPR001452,Pfam:PF00018,<br>note:complete                                                                                                                  |
| super6135      | PILA_017787 | Alias=uninformative,<br>Interpro:IPR001452,Pfam:PF00018,note:complete                                                                                                                  |

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## Pinaceae phylogenetic tree estimation

A multitude of studies has examined phylogenetic patterns within genera, as well as among genera. The vast majority of these studies, however, are based on chloroplast DNA (cpDNA;

2013) or handfuls of nuclear loci with or without inclusion of cpDNA (e.g., Wang et al. 2000; Syring et al. 2005; Willyard et al. 2007). Most studies have identified a broadly supported backbone for branching patterns for the phylogeny of the Pinaceae (Fig. 1). More contentious, however, is the estimation of divergence times, due not only to use of fossils in questionable placements in the phylogeny (Eckert and Hall, 2006; Willyard et al., 2007; Gernandt et al., 2008), but also to limited information about branch lengths across multiple, independent loci. Here, we utilize the resource provided in this paper to estimate a multilocus phylogeny for the Pinaceae based on 28 nuclear genes using the BEAST ver. 2.20 software (Bouckaert et al. 2014). Specifically, we explored estimates of divergence times in a six-taxon tree (*Pinus* subg. *Pinus*, *Pinus* subg. *Strobus*, *Picea*, *Larix*, *Pseudotsuga*, and *Abies*) representing approximately 55% of the genus-level diversity within the Pinaceae. Divergence times were estimated under two models of molecular evolution, each assuming an HKY+G substitution model - (1) a global, strict molecular clock and (2) a global, relaxed molecular clock parameterized with a lognormal distribution.

Parameters for both models were estimated using MCMC with  $1.1 \times 10^8$  steps, a burn-in of  $1.0 \times 10^7$ , and a thinning interval of  $1.0 \times 10^4$ . Convergence was assessed for each model through comparisons of three independent runs of the MCMC routine, while mixing for each run was assessed using effective sample size (ESS) calculations based on the autocorrelation of parameter estimates along the Markov chains. Models were compared using Bayes factors (BFs) based on the marginal likelihoods for each model (Suchard et al. 2001). For comparison, we also report modified AIC values for each model (Baele et al.

**Table S17.** Summary of the 28 loci used for phylogenetic inference of divergence times within the Pinaceae. Putative homologs were identified via blastx analysis of the expressed sequence tag (EST) contig against the Reference Protein database housed at NCBI. More information about these loci is available in the DiversiTree database housed at the Dendrome website (<https://dendrome.ucdavis.edu/DiversiTree/>). Information about the assembly and sequencing of loci across the Pinaceae can be found in Eckert *et al.* (2013a, 2013b). Loci with NA in the E-value column did not have a putative homolog found in the Reference Protein database via blastx analysis of the EST contig listed in the second column of the table.

| Locus id   | EST contig id | Homolog      | Gene Product                                            | E-value   |
|------------|---------------|--------------|---------------------------------------------------------|-----------|
| 0_846_01   | 0_846         | NM_129800    | bZIP transcription factor                               | 6.00E-14  |
| 0_5038_01  | 0_5038        | XP_010248353 | Phloem protein 2-Like A10-like protein                  | 5.00E-21  |
| 0_6448_02  | 0_6448        | NM_099986    | ATP-dependent helicase (DCL1)                           | 4.00E-130 |
| 0_8642_01  | 0_8642        | XP_003635538 | Elongation factor G-2, chloroplastic-like               | 6.00E-125 |
| 0_9383_01  | 0_9383        | NM_106563    | Ubiquitin thiolesterase                                 | 7.00E-53  |
| 0_10706_01 | 0_10706       | NM_179945    | Uncharacterized protein                                 | 3.00E-08  |
| 0_11772_01 | 0_11772       | XP_003554743 | Probable tRNA N6-adenosine threonylcarbamoyltransferase | 5.00E-139 |
| 0_12745_01 | 0_12745       | NM_122578    | Kelch repeat-containing F-box family protein            | 5.00E-59  |
| 0_13240_01 | 0_13240       | NM_121480    | L-aspartate oxidase                                     | 6.00E-68  |
| 0_14122_02 | 0_14122       | NM_113125    | Uncharacterized protein                                 | 4.00E-75  |
| 0_15075_01 | 0_15075       | NM_129383    | CAX-interacting protein                                 | 6.00E-51  |

|                  |               |              |                                                                      |           |
|------------------|---------------|--------------|----------------------------------------------------------------------|-----------|
| 0_15762_01       | 0_15762       | NA           | NA                                                                   | NA        |
| 2_1501_01        | 2_1501        | XP_016463965 | Uncharacterized protein                                              | 2.00E-42  |
| 2_1528_01        | 2_1528        | XP_010497358 | Mediator of RNA polymerase II transcription subunit 33B-like protein | 4.00E-55  |
| 2_3742_03        | 2_3742        | XP_010269982 | LAG1 longevity assurance homolog 2-like protein                      | 2.00E-35  |
| 2_8011_02        | 2_8011        | NM_116232    | Scarecrow-like transcription factor                                  | 2.00E-27  |
| 2_8443_01        | 2_8443        | XP_016647698 | Glycosyltransferase family protein 64 C5 isoform                     | 7.00E-126 |
| 2_9456_01        | 2_9456        | XP_006844460 | E3 ubiquitin-protein ligase ORTHUS 2 isoform X1                      | 5.00E-12  |
| CL149Contig3_04  | CL149Contig3  | NM_112485    | L-asparaginase                                                       | 2.00E-70  |
| CL516Contig1_07  | CL516Contig1  | XP_009410369 | Pyrophosphate-energized vacuolar membrane proton pump-like protein   | 0.0       |
| CL1064Contig1_02 | CL1064Contig1 | XP_006844510 | Protein bicaudal C homolog 1                                         | 3.00E-25  |
| CL2472Contig1_01 | CL2472Contig1 | XP_010919153 | Lysine-specific histone demethylase 1 homolog 3                      | 1.00E-32  |
| CL3148Contig1_04 | CL3148Contig1 | XP_002318094 | Leucine-rich repeat transmembrane protein kinase                     | 3.00E-115 |
| CL3770Contig1_01 | CL3770Contig1 | XP_008219652 | Uncharacterized protein                                              | 3.00E-16  |
| CL4354Contig1_01 | CL4354Contig1 | XP_002270378 | Serine/threonine-protein phosphatase PP2A-2                          | 3.00E-114 |
| CL4481Contig1_04 | CL4481Contig1 | NP_564202    | OB-fold nucleic acid binding domain-containing protein               | 3.00E-57  |
| CL4511Contig1_02 | CL4511Contig1 | XP_013592268 | Protein HHL1, chloroplastic-like isoform X2                          | 2.00E-67  |
| UMN_1023_01      | UMN_1023      | XP_00685278  | F-box/LRR-repeat protein 14                                          | 3.00E-103 |

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