Approaches to variant discovery for conifer transcriptome sequencing

Emily Telfer1,*, Natalie Graham1, Lucy Macdonald1, Shane Sturrock1,2, Phillip Wilcox3, Lisa Stanbra1

1 New Zealand Forest Research Institute LTD. trading as Scion, Rotorua, New Zealand, 2 Real Time Genomics, Hamilton, New Zealand, 3 Department of Mathematics and Statistics, University of Otago, Dunedin, New Zealand

* emily.telfer@scionresearch.com

Abstract

There is a wide diversity of bioinformatic tools available for the assembly of next generation sequence and subsequent variant calling to identify genetic markers at scale. Integration of genomics tools such as genomic selection, association studies, pedigree analysis and analysis of genetic diversity, into operational breeding is a goal for New Zealand’s most widely planted exotic tree species, *Pinus radiata*. In the absence of full reference genomes for large megagenomes such as in conifers, RNA sequencing in a range of genotypes and tissue types, offers a rich source of genetic markers for downstream application. We compared nine different assembler and variant calling software combinations in a single transcriptomic library and found that Single Nucleotide Polymorphism (SNPs) discovery could vary by as much as an order of magnitude (8,061 SNPs up to 86,815 SNPs). The assembler with the best realignment of the packages trialled, Trinity, in combination with several variant callers was then applied to a much larger multi-genotype, multi-tissue transcriptome and identified 683,135 *in silico* SNPs across a predicted 449,951 exons when mapped to the *Pinus taeda* ver 1.01e reference.

Introduction

Radiata pine (*Pinus radiata* D.Don) is New Zealand’s most widely planted exotic forestry species [1] and breeding programmes are moving towards the implementation of genomics technologies to deliver genetic gains through selective breeding for traits of importance. Prior to the advent of Next Generation Sequencing platforms, Expressed Sequence Tag (EST) libraries, based on captured and sequenced cDNA have been a mainstay of gene discovery and functional genomics [2, 3]. Expressed Sequence Tag (EST) libraries have long been a rich resource for markers such as microsatellites or Simple Sequence Repeats (SSRs) [4, 5]. Indeed, the conserved nature of gene sequence across conifers has meant that frequently, EST based markers from one species can be studied in related species, giving insight to evolutionary processes and increasing the pool of available markers across a genus [6–8]. Fortunately, Next Generation Sequencing (NGS) is changing the face of molecular biology and marker discovery [9–11]. At its inception in 2006, the Illumina platform generated average read lengths of 35 bases and 1
Gigabase (Gb) of sequence in a single run. The latest HiSeq and Miseq instruments and associated chemistries (Illumina, San Diego, USA) are now generating read lengths up to 300 bases and up to 1500 Gb of sequence per run [12]. Long-read third-generation technologies [13, 14] are generating even more impressive sequence lengths, albeit with diminished base-calling accuracy.

Within a single NGS experiment, it is possible to 1) generate de novo sequence, and 2) perform polymorphism discovery. Ideally, genomic resources are generated via whole genome sequencing (WGS) to capture variation in both genic and non-genic space, however, due to the enormous size of conifer genomes [15–17], which can exceed 30 Gb, various methods of reduced representation sequencing [18, 19] are frequently utilised to reduce costs, while still providing a genome-wide snapshot of the variation. Focusing on transcriptomic sequence allows for the generation of contiguous consensus sequences (contigs) that can be mined for polymorphic loci and provide a catalogue of gene space, even in the absence of a species-specific reference genome [20].

The goal of genomic selection is to overcome the need for long breeding cycles, minimise dependency on expensive field trials, and increase the speed of genetic gain through selective breeding. Analysis of various deployment scenarios for genomic selection in conifers suggests potential increases in delivery of genetic gain of 80% or more, driven in particular via shortening the breeding cycle [21].

For genomic selection to be a viable strategy, sufficient numbers of well-spaced DNA markers must be identified [22, 23]. Currently, the only DNA markers present at sufficient frequency, combined with ease of identification and a variety of screening platforms [24], are single nucleotide polymorphisms (SNPs) [25]. Many markers are not suitable for genotyping, either due to technical limitations of an assay platform or due to lack of relevance to the populations of interest, therefore, an extensive resource of high quality SNPs is required for the development of high density genotyping panels, integral to genomic selection approaches [26]. In eucalypts, for example, ~47 million SNP markers were identified and screened before 60,904 were committed to a multi-species SNP array [27]. Over 500,000 SNPs were vetted to produce the OvineSNP50 bead chip [28].

Genomic selection assumes that at least some of the markers will be in linkage disequilibrium (LD) with the traits of interest [26]. Therefore, the interrogation of transcriptomes for these sequence variants assumes that much of the genetic variation of interest will be in LD with expressed genes captured in the transcriptome [29–31]. To maximise the number of SNPs detected, we investigated transcriptomes from a range of tissues and genotypes. While selection of tissue types was based on those more likely to be expressing genes regulating our key traits, growth rate, wood density and needle health, ultimately good genome coverage is more important for genomic selection than the identification of specific causative quantitative trait nucleotides (QTN) [22].

Such is the power of NGS technology that the generation of large sequencing datasets has ceased to be the research bottleneck; fast and effective bioinformatic processing of the NGS datasets is now the focus of many groups. Unlike the hardware and chemistry developed to generate these datasets, much of the analysis software being developed is freely available, including a wide variety of bioinformatics tools available for sequence assembly and in silico polymorphism discovery [32]. Therefore we sought to determine the most appropriate method for large scale analyses in multiple datasets [33]. Conifer genomes are very large and with latest estimates of gene models in excess of 50,000 in *P. taeda* [34], we compared the ease of use and performance of several publicly available global and local short read sequence alignment tools. In combination with various polymorphic prediction software, we tested a single dataset from a single genotype, prior to selecting a preferred method for application within our larger...
multi-genotype, multi-tissue transcriptome sequencing dataset. Here we report on the generation of the first large scale SNP marker resource for radiata pine, developed using this variant calling workflow.

Methods

Tissue collection

All trees sampled were New Zealand Forest Research Institute research trees, with the exception of tree1. Tree 1 was identified as a malformed 6 year old tree within a commercial forest of Kaingaroa Timberlands LTD, who provided permission for us to sample the tree, as malformed trees are removed as part of routine thinning operations to improve the overall quality of a forest block. In order to generate a rich transcriptomic resource, that captured a wide subset of expressed genes and genetic variation, a range of *Pinus radiata* genotypes, tissue types were collected at different developmental and temporal stages (Table 1). To prevent degradation of RNA, each sample was harvested directly into liquid nitrogen and stored at -80°C. For the pilot genotype, Tree 1, developing xylem tissue were harvested from the bent stem (Fig 1A) of a 6-yr-old tree [35] by peeling away a bark window (Fig 1B) to expose the developing cambium [36]. Xylem was similarly collected for Trees 6–8, with phloem collected for Trees 6 and 7 by removing the underside of the bark window (Fig 1B). For Trees 2, 3, and 4, bud samples were harvested from growing vegetative meristems, preferentially during the early spring flush (Fig 1C), Tree 6 buds were collected in autumn. Needles were also harvested during the spring flush for Trees 2–5. For Trees 3 and 5, needles infected with a foliar pathogen, *Phytophthora pluvialis* (Pp), were also sampled. Inoculation of this material was performed by exposing individual branches of the trees to approximately $1 \times 10^4$ zoospores in a closed bag for 24 hours [N. Table 1. Transcriptomes generated from the following tissues.

| Genotype | Tissues | Date collected | Tree Owner | Tree location |
|----------|---------|----------------|------------|--------------|
| Tree 1   | 6 year old<br>Opposite wood xylem (OW)<br>6 year old<br>Compression wood xylem (CW) | Mar 2008<br>Mar 2008 | Waimangu Forest owned by Kaingaroa Timberlands | LAT -38.258<br>LON 176.414 |
| Tree 2   | Needles (N)<br>Spring Buds (SB) | Nov 2012<br>Nov 2012 | Scion Clonal archive | LAT -38.156<br>LON 176.270 |
| Tree 3   | Needles (N)<br>Needles (infected) (NI)<br>Spring Buds (SB) | Nov 2012<br>Nov 2012<br>Nov 2012 | Scion Clonal archive | LAT -38.156<br>LON 176.270 |
| Tree 4   | Needles (N)<br>Spring Buds (SB) | Nov 2012<br>Nov 2012 | Scion Clonal archive | LAT -38.156<br>LON 176.270 |
| Tree 5   | Needles (N)<br>Needles (infected) (NI) | Nov 2012<br>Nov 2012 | Scion Clonal archive | LAT -38.156<br>LON 176.270 |
| Tree 6   | Spring xylem (SPX) 1.4 metres<br>Summer xylem (SUX) 1.4 metres<br>Autumn Buds (AB)<br>Summer phloem (Ph) 1.4 metres | Nov 2000<br>Mar 2001<br>Mar 2001<br>Mar 2001 | Scion research Trial RO 664/3<br>Forest owned by Kaingaroa Timberlands | LAT -38.622<br>LON 176.345 |
| Tree 7   | 2 year old Seedling xylem (X)<br>2 year old Seedling phloem (Ph)<br>Spring xylem (SUX) 1.4 metres | Oct 2012<br>Oct 2012<br>Mar 2001 | Scion Field Trial | LAT -38.155<br>LON 176.268 |
| Tree 8   | Summer xylem (SUX) 1.4 metres | Mar 2001 | Scion research Trial RO 664/3 Forest owned by Kaingaroa Timberlands | LAT -38.622<br>LON 176.345 |

https://doi.org/10.1371/journal.pone.0205835.t001
Fascicles with typical lesion development were collected at 7, 9 and 11 days post inoculation, and 5 cm of the proximal ends pooled into a single sample.

**RNA extraction**

Total RNA was extracted from approximately 0.5–1.5 g of tissue using a modified CTAB method [36], and stored at -80˚C in 10mM Tris-HCl buffer (pH 8.0). Approximately 25 μg of total RNA from each sample was treated with DNase I enzyme to remove contaminating genomic DNA (gDNA) prior to confirmation of RNA integrity and gDNA removal by agarose gel electrophoresis. Absence of contaminants was confirmed spectrophotometrically using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA) and sample concentrations estimated using a Qubit RNA BR kit on the Qubit v1 fluorometer (Thermo Scientific).

**RNA sequencing**

New Zealand Genomics LTD (NZGL, Dunedin, New Zealand) performed sequencing on the RNA extracted from all trees (Trees 1–8). RNA quality (18S and 26S ratios) and RNA concentration were determined using the RNA 6000 LabChip in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Thereafter, samples progressed through sequencing on the next-generation Illumina Hiseq 2000 sequencing platform (Illumina Inc. San Diego, California, USA). The total number of reads and RNA input metrics are summarised in S1 Table.

**Sequence assembly**

All programmes and software used in this analysis are listed in Table 2. All raw read outputs from the Illumina Hiseq 2000 were filtered to remove reads containing only the 3’ adaptor fragment. The remaining ‘clean’ short reads progressed to downstream sequence assembly programmes on a genotype-by-genotype basis, for alignment into contigs, scaffolds and finally unigenes. All nucleotides in raw reads from BGI were supplied with a Solexa technology-based quality score; conversions to Sanger quality scores, where required, were performed using MAQ (Mapping and Assembly with Quality) [37]. Two assembly programmes were used in this study: pilot assemblies with Tree 1 were performed using both SOAPdenovo [37] and Trinity v r2012-01-25 [38], while subsequent assemblies (Trees 2–8) were performed using only Trinity v r2013-01-25 [38]. Datasets in the Trinity assembly were treated as paired end. A final multi-genotype assembly (Trees 1–8, all tissues) was performed with Trinity v r2013-02-25 [38].
SNP discovery workflows

To evaluate a different variant calling workflows, different software for the various steps were tested in a range of combinations (Table 3). For the Tree 1 SOAPdenovo assembly, three different combinations of global alignment, local alignment and SNP discovery tools were trialled (pipelines 1, 6 and 9 in Table 3). For the pilot genotype Tree 1 Trinity-based assembly, all 9 pipelines (Table 3) were performed. All software used default parameters unless otherwise described. For the individual assemblies of Trees 2–8, three different SNP discovery pipelines were applied: rtg-GA, SAMtools [39], and Genome Analysis Toolkit (GATK) [40]. All the scripts utilised to create different combinations of software supplied as S1 File.

SAMtools (Sequence Alignment/Map) was used to reformat the outputs and identify sequence variants. All defaults were used, with the following exceptions: 1) to reduce the mapping quality for reads with a high number of mismatches, the coefficient to reduce the mapping quality of reads that had a higher number of mismatches was set to 50 as recommended for BWA alignments, 2) Bayesian inference was used to call variants using maximum-

### Table 2. Software tools used for short read sequence alignment and SNP detection.

| Software       | Function        | Version   | Reference                 |
|----------------|-----------------|-----------|---------------------------|
| SOAPdenovo     | de novo assembly|           | Li et al. (2008)          |
| SOAPdenovo-Trans| de novo assembly| 1.03      | Xie et al. (2014)         |
| Trinity        | de novo assembly| r2012-01-25 | Grabherr et al. (2011)   |
| Trinity RNASeq | de novo assembly| r2013-02-25 | Grabherr et al. (2011)   |
| Velvet         | de novo assembly| 1.2.10    | Zerbino et al. (2008)     |
| Oases          | de novo assembly| 0.2.08    | Schulz et al. (2012)      |
| BWA            | Global alignment| 0.5.9-r16 | Li and Durbin (2009)      |
| Bowtie2        | Global alignment| 2.1.0     | Langmead et al. (2012)    |
| MAQ            | Quality score conversion, global alignment, polymorphic site identification | 0.7.1 | Li, Ruan and Durbin (2008) |
| rtg-GA         | Global alignment, polymorphic site identification | 2.2.1 | www.realtimegenomics.com |
| Mosaik         | Global alignment| 1.1.0021  | Lee (2010)                |
| GATK           | Local realignment, polymorphic site identification| 1.0.5777 | McKenna et al. (2010)    |
| BLAST          | Similarity searching Basic Local Alignment Search Tool | 2.2.28+ | Altschul et al. (2012)   |
| PERL           | Scripting language for file manipulation | 5.10.1  | Christians et al. (2012) |
| SAMtools       | Polymorphic site identification | 0.1.14 | Li et al. (2009)         |
| Freebayes      | Polymorphic site identification | 0.6.5  | https://github.com/ekg/freebayes|

### Table 3. Different workflows applied for short read sequence alignment and SNP detection in Tree 1 pilot assemblies.

| Pipeline | Quality score | Global alignment software | Local realignment software | Polymorphic identification |
|----------|---------------|----------------------------|----------------------------|---------------------------|
| 1        | Solexa        | BWA                        | -                          | SAMtools                  |
| 2        | Sanger        | BWA                        | -                          | SAMtools                  |
| 3        | Sanger        | BWA                        | GATK                       | SAMtools                  |
| 4        | Sanger        | BWA                        | GATK                       | GATK                      |
| 5        | Sanger        | BWA                        | GATK                       | Freebayes                 |
| 6        | Sanger        | MAQ                        | -                          | MAQ                       |
| 7        | Solexa        | rtg-GA                     | -                          | rtg-GA                    |
| 8        | Solexa        | Mosaik                     | GATK                       | GATK                      |
| 9        | Solexa        | Mosaik                     | GATK                       | freebayes                 |

https://doi.org/10.1371/journal.pone.0205835.t002

https://doi.org/10.1371/journal.pone.0205835.t003
likelihood inference for the priors, 3) genotypes were called at the variant site, and 4) for areas of high coverage, (e.g., repeat regions), variants with a read depth greater than 100 were removed, as there is a known problem assembling abundantly expressed genes [32].

Default parameters were also used for GATK, however, some file reformatting was required. For BWA alignment files, SAMtools was first used to merge, sort and convert outputs into binary sequence alignment/map format (BAM) prior to performing local realignments in GATK. Picard (https://broadinstitute.github.io/picard/) was used to reorder files prior to running the GATK variant calling tool.

MAQ has a utility to convert Solexa quality scores to Sanger quality scores, a requirement for MAQ alignments, it also altered the BWA alignment. All defaults were used, with the exception that paired ends not mapping correctly were discarded.

From the original pilot study with the Tree 1 assemblies, rtg-GA, SAMtools [39], and Genome Analysis Toolkit (GATK) [40] variant calling algorithms were selected for both performance, and ease of use. Each algorithm was used independently to identify SNPs within each genotype. SNPs detected by multiple algorithms were only counted once when generating the total number of SNPs.

SNP markers that were homozygous within an individual were not detectable using the genotype-by-genotype approach (e.g. A/A in one genotype and T/T in another genotype). Therefore, to identify SNPs that were variable between genotypes, the raw reads from each genotype were mapped to the multi-genotype transcriptome using Bowtie2 version 2.1.0 [41], and polymorphisms identified using SAMtools [39].

**Prediction of exons**

Neves et al 2013 [42] reported that the presence of undocumented introns within target regions impacted the efficiency of sequence capture in their exome-capture genotype-by-sequencing platform. To predict intron/exon boundaries within the multi-genotype transcriptome, the assembly was aligned to the *Pinus taeda* ver 1.01e genome [43] using the Trinity assembly pipeline, as it was reported that the latest version (at that time) of Trinity outperformed SOAPdenovo with respect to % gene recovery [44].

**Filtering SNPs**

After SNP discovery via the mapping of raw reads back to the assemblies, additional filtering was performed to increase the likelihood of detecting biologically real SNPs and not sequencing or alignment errors. Firstly, SNPs had to be located more than 10 bp from the edge of contig. Secondly, read depth at SNP locations had to be greater than or equal to 10. Thirdly, as each genotype was from a diploid individual, heterozygous SNPs within an individual should display approximate allele frequencies of 0.5 across all reads; we widened our criteria to allow minor allele frequencies of no less than 0.25 within an individual.

**Results**

**Transcriptome assembly in individual genotypes**

A total of 18 transcriptome libraries were sequenced (S1 Table), and assembled (Table 4), from a total of 1.75 billion reads. The number of contigs generated per genotype ranged from 112,461 to 240,053. The N50 contig size ranged from 19,320 to 35,503 bases per genotype. The raw data files are available at NCBI (www.ncbi.nlm.nih.gov/bioproject/482145).
Assembly of multi-genotype transcriptome

The 8 individual genotypes, previously assembled independently, were combined into a large “multi-genotype radiata transcriptome” containing 194,299 contigs, ranging in size from 201 bp to 16,575 bp, with an N50 of 1,434. When mapped to the Pinus taeda ver 1.01e genome assembly, 144,007 (74%) of the radiata contigs aligned to the P. taeda genome. This enabled the prediction of 449,951 putative exons, corresponding to 46,342 P. taeda scaffolds.

Variant calling in the pilot genotype (Tree 1)

**SOAPdenovo assembly.** Three different combinations of global and/or local alignment, followed by variant calling (pipelines 1, 6 and 9 in Table 3), were tested on the Tree 1 SOAPdenovo assembly. However, many of the raw ‘clean’ reads could not be remapped onto the unigene sequence, suggesting a problem with this approach. The best result was achieved with Mosaik (combination 9), although only 36% of the raw reads remapped to the unigene sequence. This unexpectedly low rate of realignment meant that a high number of polymorphisms were likely missed. Therefore, the decision was made to perform a new assembly using Trinity, and a combined transcriptomic library contig set was created for Tree 1, comprising 137,228 different contig sequences, ranging in length from 201 to 9,175 bp.

**Trinity assembly.** Variant calling was undertaken using all nine different combinations of global alignment, and/or local alignment, and SNP identification software packages (Table 2) for the Tree 1 Trinity assembly. In all cases, the percentage of raw ‘clean’ reads that remapped was much higher than with the SOAPdenovo assembly, ranging from 82% (rtg-GA) to 93% (BWA and Mosaik). For this reason, no further analyses were performed using the SOAPdenovo assembly, and all subsequent reporting of SNPs were identified solely from the Trinity assemblies. SNPs were regarded as high confidence when all of the following criteria were
Table 5. Pair-wise analysis of SNPs predicted among pairs of pipelines. Diagonal line represents SNPs unique to that combination, with the number of total quality SNPs identified by each method shown in the final row.

| Pipeline 1 | Pipeline 2 | Pipeline 3 | Pipeline 4 | Pipeline 5 | Pipeline 6 | Pipeline 7 | Pipeline 8 | Pipeline 9 |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Pipeline 1 | 2,251     | 4,175     | 4,161     | 800       | 1,080     | 2,730     | 3,873     | 758       | 782       |
| Pipeline 2 | 46        | 32,048    | 4,670     | 7,623     | 15,470    | 21,184    | 4,117     | 4,677     |
| Pipeline 3 |           | 28        | 4,651     | 7,615     | 15,450    | 21,159    | 4,116     | 4,671     |
| Pipeline 4 |           |           | 1,663     | 1,385     | 6,106     | 6,764     | 7,808     | 863       |
| Pipeline 5 |           |           |           | 6,598     | 8,111     | 11,506    | 1,301     | 2,643     |
| Pipeline 6 |           |           |           |           | 8,684     | 41,058    | 6,325     | 7,153     |
| Pipeline 7 |           |           |           |           |           | 16,194    | 7,246     | 7,897     |
| Pipeline 8 |           |           |           |           |           |           | 21,846    | 2,060     |
| Pipeline 9 |           |           |           |           |           |           |           | 31,154    |

Total quality SNPs identified: 8,061, 32,188, 32,124, 22,892, 14,679, 63,488, 86,815, 37,575, 17,138

Quality scores: Solexa, Sanger, Sanger, Sanger, Sanger, Sanger, Sanger, Sanger, Sanger

Global alignment software: BWA, BWA, BWA, BWA, BWA, MAQ, rtg-GA, Mosaik, Mosaik

Local alignment software: - , - , GATK, GATK, GATK, rtg-GA, GATK, GATK

Polymorphism identification: SAMtools, SAMtools, SAMtools, GATK, Freebayes, MAQ, rtg-GA, GATK, Freebayes

Table 6. Frequency of SNP detection across all 9 discovery pipelines.

| Number of pipelines detecting the same SNP | Number of SNPs |
|------------------------------------------|----------------|
| 9                                        | 6              |
| 8                                        | 74             |
| 7                                        | 626            |
| 6                                        | 2,991          |
| 5                                        | 7,208          |
| 4                                        | 11,021         |
| 3                                        | 15,888         |
| 2                                        | 37,867         |
| 1                                        | 88,464         |
| Total SNPs                                | 164,145        |

https://doi.org/10.1371/journal.pone.0205835.t005

https://doi.org/10.1371/journal.pone.0205835.t006

Approaches to variant discovery for conifer transcriptome sequencing

satisfied: 1) more than 10 bases from the edge of the unigene, 2) allele frequency between 0.25 and 0.75, 3) a read depth of 10 or more sequences at that SNP position, and 4) at least 60 bases clear of other polymorphic features on at least one side of the SNP.

The number of high confidence SNPs (Table 5) ranged from 8,061 (pipeline 1) to 86,815 (pipeline 7), with 34,996 being the average number of SNPs detected across all combinations. Merely the conversion of sequence quality scores from Solexa to Sanger caused an additional 24,127 SNPs to be detected (pipeline 1 vs 2), while the addition of a subsequent local realignment step did not markedly change the SNPs detected (pipeline 2 vs 3). Changing the final polymorphic identification software from SAMtools to either GATK or Freebayes reduced SNP numbers by 9,232 or 19,445, respectively (pipeline 3 vs 4 or 5). Use of two other independent packages, MAQ (pipeline 6) and rtg-GA (pipeline 7), both without a local realignment step, gave the highest SNP predictions at 63,488 and 86,815, respectively. Pipelines 8 and 9 both used the original Solexa sequence quality scores and a Mosaik global alignment followed by a GATK local realignment, but differed in the SNP prediction software used. Approximately half as many SNPs were predicted using Freebayes in pipeline 9 (17,138) than for GATK in pipeline 8 (37,575).

SNPs predicted multiple times using different software package combinations were expected have a higher likelihood of being a true polymorphic event, therefore, we aimed to
distinguish SNPs common to multiple pipelines from those predicted in only one pipeline. Using a pair-wise combination approach, each pipeline was compared to the others (Table 5). Pipelines 6, 7, 8 and 9 each predicted greater than 7,000 unique SNPs, i.e. SNPs not shared with any other combination.

A total of 164,145 different SNPs were predicted across all pipelines, with only six SNPs predicted in all 9 software combinations (Table 6). There were 10,905 SNPs detected in 5 or more combinations, only 6.6% of the total SNPs predicted. A total of 37,814 (23%) SNPs are predicted by at least 3 or more pipelines. A total of 88,464 SNPs (53.9%) were identified by only a single software pipeline. Such SNPs should be considered with less confidence for downstream applications, however, without further testing, we cannot rule out the fact that these predicted SNPs may actually be true polymorphisms.

SNP discovery in individual genotypes

For SNP discovery within each of the genotypes, we selected the three best SNP prediction tools identified in the pilot study: rtg-GA, GATK and SAMtools [39]. Using individual Trinity v 2.0 [38] assemblies for each genotype, with Bowtie2-mapped raw reads [41], three pools of SNPs were generated per genotype using each of the three SNP prediction tools. These pools were screened using the same quality criteria as described for Tree 1 to identify high confidence SNPs. A cumulative total of 683,135 unique SNPs were identified across all pipelines in the 8 genotypes analysed (Table 7). The rtg-GA software predicted the greatest number of SNPs, followed by SAMTools then GATK. As SNP calling was performed within individuals, some redundancies are to be expected within this cumulative total; SNP discovery across genotypes will alter this total as SNPs are identified in multiple genotypes (decreasing the total number) and new SNPs are detected that were homozygous SNPs within genotypes (increasing the total number).

SNP discovery in multi-genotype transcriptome

For the final round of SNP discovery within the multi-genotype Trinity v 2.0 [38] assembly, raw reads were mapped back to the contigs using Bowtie2 [41] and SAMtools [39] used to
predict polymorphisms, and use of rtg-GA discontinued. Filtering criteria were applied to remove SNPs that were identified in sequences with a read depth of less than 10 and/or or less than 10bp from the edge of a contig. A total of 328,981 unique SNP markers were identified within the multi-genotype assembly, including 59,424 between-genotype SNPs which were only identified when multiple genotypes were compared.

**Discussion**

SNPs identified from tissue-specific transcriptomes can be an ideal resource [45] for candidate gene SNP discovery or genome-wide SNP identification using platforms such as Illumina’s Infinium [46] or Affymetrix’s Axiom (www.affymetrix.com) [20, 33]. During the development and evaluation of the various SNP discovery pipelines described in this work, genotyping platforms that utilise genotype-by-sequencing (GBS) of reduced representation genomic DNA, either through restriction enzyme digestion [47], or targeted exome capture [42] became more widely available. These methods capture and sequence all SNP markers within target region, rather than focussing on specific SNPs as with fixed array SNP chips. Therefore, the accuracy of in silico SNP predictions became less critical with these alternative genotyping platforms as preselected SNP markers were not required. However, the prediction of high confidence SNPs, described here, did influence the selection of genomic sites to target for an exome capture GBS assay.

**Sequence assembly**

We tested a number of sequence assembly software packages, including Trinity, SOAPdenovo, and Velvet/Oases [48, 49] (data not shown), although the latter required more RAM than was available to us at the time. The unexpectedly low rate of realignment in the SOAPdenovo assembly (only 36% of the raw reads remapped to the unigene sequence) meant that a high number of polymorphisms were likely missed. This has been reported for other assemblers as well as SOAPdenovo when mapping back on assembled contigs [32]. The Trinity package therefore outperformed during the remapping of the raw reads back against the assembled contigs, despite generating 43% fewer contigs than the SOAPdenovo assembly with a smaller maximum contig length (9,175 vs 16,502). Remapping was an essential component of SNP discovery, therefore the best performing assembly package for this step was selected ahead of the usual quality metrics of contig number, length or N50.

**Variant calling**

The variation in SNP discovery observed in a single dataset, channelled through a range of bioinformatic pipelines, varied by over an order of magnitude, (8,061 SNPs with pipeline 1, up to 86,815 SNPs for pipeline 7), and highlights the extent of variation among SNP calling pipelines, with 54% of the 164,145 SNPs discovered in the Tree 1 pilot study being unique to a single pipeline. A similar study comparing SNP discovery pipelines in Antarctic fur seals [33] showed that, between the 4 different methods compared, only 51% of SNP markers were detected in more than one pipeline. The filtering criteria applied post variant calling is also an important consideration, as low representation of an allele within a total read depth could be a dubious variant or sequencing error [19]. However, using these tools in combination can provide a more robust pipeline for SNP discovery, and we are starting to see this approach of applying multiple variant calling tools to a sequencing dataset being adopted [20, 50]. A single variant tool can still be suitable depending on the downstream application, or where added confidence can be gained through other approaches, such as stringent mapping of segregating markers in full-sibling populations [51–53].
There are two processes that current NGS algorithms can employ in the discovery of polymorphic loci: (a) global and local sequence alignment, and (b) polymorphism detection. The Burrows-Wheeler alignment (BWA) can be used to assemble short sequence reads [54] then SAMTools can be applied for polymorphism discovery [39]. The Genome Analysis Toolkit (GATk) can be used for quality control, local and global sequence assembly [40], as well as for polymorphism discovery [55]. A third program, MOSAIK, is another open-source global assembly tool [56] which can be used in conjunction with FreeBayes to use Bayesian methods for detection of polymorphisms within short read alignments [57].

**Conclusion**

The purpose of this work was to evaluate bioinformatic workflows and combinations of software for identification of polymorphic loci and the development of a resource for a number of genomic tools for the radiata pine industry, with a requirement for varying SNP densities. A large, complex genome and incomplete reference resources precluded whole genome resequencing for SNP marker discovery in radiata pine. Nine different pipelines applied to a single pilot transcriptome identified SNPs at a rate the ranged over an order of magnitude. However, the utilisation of transcriptomic RNA sequencing in combination with several variant calling pipelines and quality filtering, has identified 683,135 in silico SNP markers and 449,951 exome templates, the first large-scale SNP resource reported for this species. In addition, the *P. radiata* multi-genotype transcriptome assembly is proving to be a valuable resource and being utilised in multiple downstream projects, including facilitating the assembly of a *P. radiata* reference genome, various gene discovery programmes, pedigree reconstruction and genomic selection.

**Supporting information**

S1 Table. RNA sequencing summary.
(DOCX)

S1 File. Individual scripts used to perform analysis in this study.
(TGZ)

**Acknowledgments**

The authors would like to thank Nicole Silvester, for upload of data to NCBI SRA. We thank Nari Williams, Sheere Cato, Lorelle Phillips, Armin Wagner, Christina Weinberg, Heather Flint, Toby Stovold, Kane Fleet and Mark Miller for collection of tissue samples for RNA extraction in the field. We thank Tancred Frickey and Steffi Fritsche as well as anonymous reviewers, for their thoughtful critique and feedback.

**Author Contributions**

**Conceptualization:** Emily Telfer, Natalie Graham, Lucy Macdonald, Phillip Wilcox, Lisa Stanbra.

**Data curation:** Lucy Macdonald.

**Formal analysis:** Emily Telfer, Lucy Macdonald.

**Investigation:** Lisa Stanbra.

**Methodology:** Natalie Graham.
Project administration: Emily Telfer.

Software: Lucy Macdonald, Shane Sturrock.

Supervision: Emily Telfer.

Writing – original draft: Emily Telfer, Lisa Stanbra.

Writing – review & editing: Emily Telfer, Natalie Graham, Phillip Wilcox, Lisa Stanbra.

References

1. Ministry for Primary Industries & Forest Owners Association. Facts & Figures. 2016.
2. Mann IK, Wegryn JL, Rajora OP. Generation, functional annotation and comparative analysis of black spruce (Picea mariana) ESTs: An important conifer genomic resource. BMC Genomics. 2013; 14(1).
3. Liu L, Zhang S, Lian C. De Novo transcriptome sequencing analysis of cDNA library and large-scale unigene assembly in Japanese red pine (Pinus densiflora). International Journal of Molecular Sciences. 2015; 16(12):29047–59. https://doi.org/10.3390/ijms16126139 PMID: 26690126
4. Du J, Zhang Z, Zhang H, Junhong T. EST–SSR marker development and transcriptome sequencing analysis of different tissues of Korean pine (Pinus koraiensis Sieb. et Zucc.). Biotechnology and Biotechnological Equipment. 2017;1–11.
5. Liu JJ, Hamnett C. Development of novel polymorphic microsatellite markers by technology of next generation sequencing in western white pine. Conservation Genetics Resources. 2014; 6(3):647–8.
6. Lesser MR, Parchman TL, Buerkle CA. Cross-species transferability of SSR loci developed from transcriptome sequencing in lodgepole pine. Molecular Ecology Resources. 2012; 12(3):448–55. https://doi.org/10.1111/j.1755-099x.2011.03102.x PMID: 22171820
7. Leonarduzzi C, Spanu I, Labriola M, González-Martínez SC, Piotto A, Vendramin GG. Development and Characterization of Three Highly Informative EST-SSR Multiplexes for Pinus halepensis mill. and their Transferability to Other Mediterranean Pines. Plant Molecular Biology Reporter. 2016; 34(5):993–1002.
8. Chagné D, Chaumeil P, Ramboer A, Collada C, Guevara A, Cervera MT, et al. Cross-species transferability and mapping of genomic and cDNA SSRs in pines. Theoretical and Applied Genetics. 2004; 109(6):1204–14. https://doi.org/10.1007/s00122-004-1683-z PMID: 15448894
9. Ronaghi M, Uhlen M, Nyren P. A Sequencing Method Based on Real-Time Pyrophosphate. Science. 1998; 281(5375):363. PMID: 9705713
10. Applied Biosystems. The SOLiD System: Next-Generation Sequencing 2011 [Available from: http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/solid-next-generation-sequencing.html.
11. Illumina. Technology / Solexa Technology 2011 [Available from: http://www.illumina.com/technology/solexa_technology illmn.
12. Illumina. HiSeq 2500 performance specifications 2016 [Available from: http://www.illumina.com/systems/hsieq_2500_1500/performance_specifications.html.
13. Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, et al. Real-time DNA sequencing from single polymerase molecules. Science. 2009; 323(5910):133–8. https://doi.org/10.1126/science.1162986 PMID: 19023044
14. Branton D, Deamer DW, Marziali A, Bayley H, Benner SA, Butler T, et al. The potential and challenges of nanopore sequencing. Nature Biotechnology. 2008; 26(10):1146–53. https://doi.org/10.1038/nbt.1495 PMID: 18846088
15. De La Torre AR, Birol I, Bousquet J, Ingvason PK, Jansson S, Jones SJM, et al. Insights into Conifer Giga-Genomes. Plant Physiology. 2014.
16. Ahuja MR, Neale DB. Evolution of genome size in conifers. Silvae Genetica. 2005; 54(3):126–37.
17. Wakamiya I, Newton RJ, Johnston JS, Price HJ. Genome size and environmental factors in the Genus Pinus American Journal of Botany. 1993; 80(11):1235–41.
18. Davey JW, Hohenlohe PA, Etter PD, Boone QJ, Catchen JM, Blaxter ML. Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nat Rev Genet. 2011; 12(7):499–510. https://doi.org/10.1038/nrg3012 PMID: 21681211
19. De Wit P, Pespeni MH, Palumbi SR. SNP genotyping and population genomics from expressed sequences—Current advances and future possibilities. Molecular Ecology. 2015; 24(10):2310–23. https://doi.org/10.1111/mec.13165 PMID: 25809893
20. Canales J, Bautista R, Label P, Gómez-Maldonado J, Lesur I, Fernández-Pozo N, et al. De novo assembly of maritime pine transcriptome: Implications for forest breeding and biotechnology. Plant Biotechnology Journal. 2013.

21. Li Y, Dungey HS. Simulation of the impact of genomic selection on the breeding cycle of Pinus radiata D. Don. IN prep. 2017.

22. Grattapaglia D, Resende MDV. Genomic selection in forest tree breeding. Tree Genetics and Genomes. 2011; 7(2):241–55.

23. Isik F. Genomic selection in forest tree breeding: The concept and an outlook to the future. New Forests. 2014; 45(3):379–401.

24. Mammadov J, Aggarwal R, Buyarapu R, Kumpatla S. SNP markers and their impact on plant breeding. International Journal of Plant Genomics. 2012:2012.

25. Wang DG, Fan JB, Siao CJ, Berno A, Young P, Sapolsky R, et al. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science. 1998; 280(5366):1077–82. PMID: 9582121

26. Meuwissen THE, Hayes BJ, Goddard ME. Prediction of Total Genetic Value Using Genome-Wide Dense Marker Maps. Genetics. 2001; 157(4):1819–29. PMID: 11290733

27. Silva-Junior OB, Faria DA, Grattapaglia D. A flexible multi-species genome-wide 60K SNP chip developed from pooled resequencing of 240 Eucalyptus tree genomes across 12 species. New Phytologist. 2015; 206:1527–40. https://doi.org/10.1111/nph.13322 PMID: 25684350

28. Consortium ISg. Sheep HapMap 2009 [Available from: http://www.sheepmap.org/genseq.php.

29. Lamara M, Raherison E, Lenz P, Beaulieu J, Bousquet J, Mackay J. Genetic architecture of wood properties based on association analysis and co-expression networks in white spruce. New Phytologist. 2016; 210(1):240–55. https://doi.org/10.1111/nph.13762 PMID: 26619072

30. Li Y, Wilcox P, Telfer E, Graham N, Stanbra L. Association of single nucleotide polymorphisms with form traits in three New Zealand populations of radiata pine in the presence of genotype by environmental interactions. Tree Genetics and Genomes. 2016; 12(4).

31. Dillon SK, Brawner JT, Meder R, Lee DJ, Southerton SG. Association genetics in Corymbia citriodora subsp. variegata identifies single nucleotide polymorphisms affecting wood growth and cellulosic pulp yield. New Phytologist. 2012.

32. Honaas LA, Wafula EK, Wickett NJ, Der JP, Zhang Y, Edger PP, et al. Selecting Superior De Novo Transcriptome Assemblies: Lessons Learned by Leveraging the Best Plant Genome. PLOS ONE. 2016; 11(1):e0146062. https://doi.org/10.1371/journal.pone.0146062 PMID: 26731739

33. Humble E, Thorne MAS, Forcada J, Hoffman JL. Transcriptomic SNP discovery for custom genotyping arrays: Impacts of sequence data, SNP calling method and genotyping technology on the probability of validation success. BMC Research Notes. 2016; 9(1).

34. Wegrzyn JL, Liechty JD, Stevens KA, Wu LS, Loopstra CA, Vasquez-Gross HA, et al. Unique features of the loblolly pine (Pinus taeda L.) megagenome revealed through sequence annotation. Genetics. 2014; 196(3):891–909. https://doi.org/10.1534/genetics.113.199996 PMID: 24653211

35. Mast S, Peng L, Jordan TW, Flint H, Phillips L, Donaldson L, et al. Proteomic analysis of membrane preparations from developing Pinus radiata compression wood. Tree Physiology. 2010; 30(11):1456–68. https://doi.org/10.1093/treephys/tpq084 PMID: 21030408

36. Cato S, McMillan L, Donaldson L, Richardson T, Echt C, Gardner R. Wood formation from the base to the crown in Pinus radiata: Gradients of tracheid wall thickness, wood density, radial growth rate and gene expression. Plant Molecular Biology. 2006; 60(4):565–81. https://doi.org/10.1007/s11103-005-5022-9 PMID: 16525892

37. Li H, Ruan J, Durbin R. Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Research. 2008; 18(11):1851–1871. https://doi.org/10.1101/gr.078212.108 PMID: 18714091

38. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011; 29(7):644–52.

39. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009; 25(16):2078–9. https://doi.org/10.1093/bioinformatics/btp352 PMID: 19950943

40. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The genome analysis toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. Genome Research. 2010; 20(9):1297–303. https://doi.org/10.1101/gr.107324.110 PMID: 20644199

41. Langmead B S S. Fast gapped-read alignment with Bowtie 2. Nature Methods. 2012; 9:357–9. https://doi.org/10.1038/nmeth.1923 PMID: 22388286
42. Neves LG, Davis JM, Barbazuk WB, Kirst M. Whole-exome targeted sequencing of the uncharacterized pine genome. Plant Journal. 2013.

43. Zimin A, Stevens KA, Crepeau MW, Holtz-Morris A, Koriabine M, Marçais G, et al. Sequencing and Assembly of the 22-Gb Loblolly Pine Genome. Genetics. 2014; 196(3):875–90. https://doi.org/10.1534/genetics.113.159715 PMID: 24653210

44. Yang Y, Smith SA. Optimizing de novo assembly of short-read RNA-seq data for phylogenomics. BMC Genomics. 2013;328. https://doi.org/10.1186/1471-2164-14-328 PMID: 23672450

45. Fernandez-Pozo N, Canales J, Guerrero-Fernandez D, Villalobos D, Diaz-Moreno S, Bautista R, et al. EuroPineDB: a high-coverage web database for maritime pine transcriptome. BMC Genomics. 2011; 12:366. https://doi.org/10.1186/1471-2164-12-366 PMID: 21762488

46. Illumina. Illumina custom genotyping options. Illumina; 2013.

47. Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, et al. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS ONE. 2011; 6(5).

48. Zerbino DR, Birney E. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Research. 2008; 18(5):821–9. https://doi.org/10.1101/gr.074492.107 PMID: 18349386

49. Schulz MH, Zerbino DR, Vingron M, Birney E. Oases: Robust de novo RNA-seq assembly across the dynamic range of expression levels. Bioinformatics. 2012; 28(8):1086–92. https://doi.org/10.1093/bioinformatics/bts094 PMID: 22968243

50. Geraldies A, Pang J, Thiessen N, Cezard T, Moore R, Zhao Y, et al. SNP discovery in black cottonwood (Populus trichocarpa) by population transcriptome resequencing. Molecular Ecology Resources. 2011; 11(SUPPL. 1):81–92.

51. Cokus SJ, Gugger PF, Sork VL. Evolutionary insights from de novo transcriptome assembly and SNP discovery in California white oaks. BMC Genomics. 2015; 16(1).

52. Chopra R, Burow G, Farmer A, Mudge J, Simpson CE, Wilkins TA, et al. Next-generation transcriptome sequencing, SNP discovery and validation in four market classes of peanut, Arachis hypogaea L. Molecular Genetics and Genomics. 2015; 290(3):1169–80. https://doi.org/10.1007/s00438-014-0976-4 PMID: 25663138

53. Liu JJ, Schoettle AW, Sniezko RA, Sturrock RN, Zamanya A, Williams H, et al. Genetic mapping of Pinus flexilis major gene (Cr4) for resistance to white pine blister rust using transcriptome-based SNP genotyping. BMC Genomics. 2016; 17(1).

54. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009; 25(14):1754–60. https://doi.org/10.1093/bioinformatics/btp294 PMID: 19451168

55. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nature Genetics. 2011; 43:491–8. https://doi.org/10.1038/ng.806 PMID: 21478669

56. Lee W. Mosaik Aligner 2010 [Available from: http://code.google.com/p/mosaik-aligner/.

57. Garrison E. FreeBayes 2010 [Available from: https://github.com/ekg/freebayes.