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Modelos de predicción genómicos para la selección de genotipos de *Eucalyptus globulus* en base a densidad de la madera y volumen

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MODELOS DE PREDICCIÓN GENÓMICOS PARA LA SELECCIÓN DE GENOTIPOS DE *EUCALYPTUS GLOBULUS* EN BASE A DENSIDAD DE LA MADERA Y VOLUMEN

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RESUMEN

La selección genómica (SG), es una metodología que ha sido bien integrada en el mejoramiento animal y también ha sido aplicada en el mejoramiento de plantas, incluyendo en especies forestales, donde diferentes estudios han sido publicados durante los últimos años. Utilizando una aceptable densidad de polimorfismos de un solo nucleótido (inglés= SNPs), distribuidos a lo largo del genoma, se estima que algunos de ellos podrían estar, ya sea, en desequilibrio de ligamiento (LD) ó podrían ser usados para estimar las relaciones genéticas entre los individuos estudiados. Por lo tanto, considerando todos estos fragmentos capturados por los marcadores en el genoma, sería posible ajustar un modelo de predicción para calcular los valores genómicos estimados de mejoramiento (inglés= GEBVs).

Hoy en día, los progresos en la secuenciación de próxima generación (inglés= NGS) y los sistemas de genotipificación, basados en la reducción de la complejidad del genoma con enzimas de restricción y SNP-Chip, permiten descubrir un gran número de SNPs con una alta eficiencia y con menores tiempos de análisis. Sin embargo, es necesario adoptar estas tecnologías para su aplicación en especies no-modelo como *Eucalyptus globulus*, del cual aún no existe gran cantidad de información genómica disponible.

En el presente trabajo, se evaluó la habilidad de genotipificación de dos tecnologías de alto rendimiento conocidas como "genotipificación por secuenciación" (inglés: GBS) y el EUChip60K-SNP chip. Después, EUChip60K fue utilizado para identificar marcadores capaces de caracterizar las relaciones genéticas entre individuos, evaluar los niveles de desequilibrio de ligamiento intra-cromosomales y ajustar un modelo de predicción de GEBVs para clones de *E. globulus*, seleccionados desde un programa de mejoramiento genético, para densidad de la madera y volumen del árbol. Los resultados mostraron que el EUChip60K, permitió estimar de una manera más realista las relaciones genéticas en comparación a la información genealógica, mostrando una distribución continua, basada en los alelos compartidos entre individuos no-relacionados, medios hermanos y hermanos completos, y que los niveles de desequilibrio de ligamiento eran bajos, como se esperaba, muy común de especies forestales. Adicionalmente, estos SNPs permitieron ajustar modelos de selección

genómica con habilidades predictivas de 0,58 y 0,75 para densidad de la madera y volumen del árbol respectivamente.

Considerando que *E. globulus* es la segunda especie forestal más relevante en Chile, especialmente para la industria de pulpa y papel, donde densidad de la madera y volumen son dos importantes características incluidas en su programa de mejoramiento, la investigación muestra el primer estudio de genotipificación mediante las tecnologías de GBS y el EUChip60K, y la primera prueba de concepto de selección genómica para una población clonal de *E. globulus* en Chile.

ABSTRACT

Genomic selection (GS) is a methodology that has been integrated for animal breeding and it is also being applied in plant breeding, including forest tree species. Several studies about this topic have been published during the past years. Using an acceptable density of single nucleotide polymorphisms (SNPs), distributed across the entire genome, some of them could be either in linkage disequilibrium (LD) with at least one gene affecting a trait of interest or be used to estimate the genetic relationships between individuals on the population studied. Therefore, considering all those fragments captured by markers across the genome, it would be possible to fit a prediction model to estimate the genomic estimated breeding values (GEBVs).

Nowadays, progresses in next generation sequencing (NGS) technologies and genotyping systems, based on reducing genome complexity with restriction enzymes (REs) and SNP-arrays to allow discover a massive number of SNPs with a high efficiency and requiring relatively little time for data analysis. However, it is necessary to adapt those methods for application in non-model species as *Eucalyptus globulus*, which has little genomic information available.

In the present work, the genotyping ability of two high throughput technologies known as "genotyping by sequencing-GBS" and "EUChip60K-SNP array" for discovering a set of polymorphic SNPs for *E. globulus* was assessed. Afterwards, EUChip60k was used to identify markers for characterizing the genetic relationship between individuals, intra-chromosomal linkage disequilibrium level and fitting a model to predict the GEBVs of *E. globulus* clones, from a genetic improvement program, according to their wood density and tree volume. Results showed that EUChip60k was better than GBS to identify polymorphic SNPs between clones with a high ability to identify mislabeled clones and family clustering. Near to 12 K polymorphic SNPs from EUChip60K allowed to estimate a more realistic genetic relationship than the pedigree information for the *E. globulus* clones, with a continuous distribution based on shared alleles between unrelated, full-sib and half-sib individuals and linkage disequilibrium levels were low as it expected, common in forest tree species. Additionally,

those SNPs allowed fitting genomic prediction models with predictive abilities of 0.58 and 0.75 for wood density and tree volume, respectively.

Considering that *E. globulus* is the second most relevant forest tree specie in Chile, specially for the pulp and paper industry, where wood density and volume are two important traits included in its breeding program, this research shows the first SNPs genotyping study by GBS and EUChip60K technologies, and the first proof-of-concept of genomic selection models using a clonal *E. globulus* population in Chile.

INTRODUCCIÓN GENERAL

Para Chile, la industria forestal es un pilar fundamental en su desarrollo económico, siendo el primer sector exportador de recursos naturales renovables, destacando productos como celulosa, tableros y madera aserrada, con destino a más de 100 países distribuidos entre los cinco continentes (Corporación Nacional Forestal 2013). Al año 2014, las plantaciones forestales en el país alcanzaban un total de 2,4 millones de hectáreas, constituidas mayoritariamente por *Pinus radiada* (1.434.085 ha - 59%) y *Eucalyptus globulus* (573.602 ha - 24%) (Instituto Forestal 2016), especies de donde derivan la mayoría de estos productos de la industria forestal.

E. globulus es originario de Australia (Eldrigde et al. 1993) y es una de las 10 especies más plantadas en el mundo, principalmente para la producción de pulpa, papel, madera y energía. Fue introducido en Chile a fines del siglo XVIII (Doughty 2000), y por lo tanto, el desarrollo genético que hoy en día tiene la especie, ha sido el resultado de años de investigación invertidos en favor de su domesticación. La variabilidad fenotípica que existe entre los individuos de *E. globulus* ha permitido implementar programas de mejoramiento genético (PMG) para la selección y evaluación del crecimiento de los genotipos en diferentes condiciones locales, incluyendo las interacciones con el medioambiente y los tratamientos silviculturales (entre otros). Tradicionalmente, estos PMG en *Eucalyptus* se han basado en características como el crecimiento volumétrico, forma del fuste (Raymond et al. 1998) y densidad de la madera; sin embargo, existen otras características de selección, basadas en propiedades químicas y físicas de la madera que afectan la productividad y calidad de la pulpa y papel (Ona et al. 2001; Wimmer et al. 2002; Ramírez et al. 2009).

Un PMG es un proceso estratégico de varios años, basado en ciclos repetidos de cruzamiento, prueba y selección (White et al. 2007). Este proceso, permite cambiar las frecuencias génicas en las poblaciones de mejoramiento, para así aumentar la proporción de individuos con genes deseables en las plantaciones comerciales. Los ciclos del PMG son repetidos en las sucesivas generaciones, lo que para especies forestales puede superar 20 años por ciclo (Instituto Forestal 2014; Ipinza 2000). Dependiendo de la calidad y rigurosidad con la que se maneja el proceso, se logrará un aumento en la productividad de las plantaciones, una mayor

adaptabilidad a diferentes sitios y la conservación de la diversidad genética, lo que finalmente se verá reflejado en un aumento de la ganancia genética (Vallejos et al. 2010).

Por años, los procesos de selección han utilizado la medición de características fenotípicas y las relaciones de parentesco entre individuos. Sin embargo, para poder realizar estas mediciones, es necesario que el árbol alcance cierta edad y/o tamaño, lo que muchas veces resulta en una lenta acumulación de ganancia genética por unidad de tiempo y costo (El-Kassaby et al. 2014). Es por ello que los mejoradores han centrado sus esfuerzos en disminuir los tiempos de ciclos de mejora, cantidad de sitios destinados a ensayos y costos asociados a la medición de rasgos expresados en edades tardías (Grattapaglia 2014).

A partir de la década de los 90's, los PMGs han impulsado implementar una estrategia basada en el uso de marcadores moleculares (MMs) conocida como "selección asistida por marcadores" (SAM) (Lande y Thompson 1990). Ésta se basa en el uso de datos de "segmentos nucleotídicos" ó MMs que explican una proporción de la variación genética de los rasgos fenotípicos (Butcher y Southerton 2007). El potencial de la SAM para mejorar la productividad de las plantaciones dependerá si se puede demostrar la relación o desequilibrio de ligamiento (DL) que existe entre estos MMs y loci de caracteres cuantitativos (QTLs), correspondientes a genes que controlan las variaciones en la característica de interés. En general, el objetivo de la SAM está principalmente enfocado en una reducción en los tiempos de selección en relación a la estrategia fenotípica, ya sea sustituyendo o asistiendo este proceso (Muranty et al. 2014), lo que implícitamente significará una reducción en los costos del PMG.

Si bien ha existido un esfuerzo enfocado en la identificación de QTLs y su uso para la selección, la SAM no ha sido exitosa para especies forestales (Strauss et al. 1992; Isik 2014). Una de las razones se debe a que los QTLs descubiertos explican una baja proporción de la varianza fenotípica (<5%) (Devey et al. 2004), ello dado principalmente a que la arquitectura genética de los rasgos cuantitativos de interés, involucra QTLs constituidos por muchos genes, cada uno con un efecto menor (Brown et al. 2003). Por otra parte, los estudios de SAM se han centrado en análisis de patrones de co-segregación para QTLs en poblaciones biparentales o

de retrocruzas, por lo que su aplicación en poblaciones forestales estaría restringido a grupos genéticos específicos, dado su limitado DL (Grattapaglia y Resende 2011). Por lo tanto, a medida que se analizan más individuos por familia y más familias, aumenta el poder de detección, se descubren más QTLs, disminuye la proporción de la variación fenotípica explicada por cada QTL y se hace más evidente la inconsistencia de estos efectos entre grupos genéticos y ambientes (Sewell y Neale 2002; Neale et al. 2002).

Dada las limitaciones del mapeo de QTLs, el enfoque llamado "genes candidatos" surgió como una alternativa capaz de identificar una variación nucleotídica específica dentro de un gen, la cual estaría fuertemente controlando el fenotipo (Neale y Savolainen 2004). La estrategia podía ser aplicada a poblaciones con estructuras familiares más complejas y ofrecer una idea atractiva de mapeo fino de QTLs en especies forestales (Neale y Kremer 2011). Sin embargo, si bien dada su mayor resolución, ésta podría extrapolarse a nuevas poblaciones con una alta diversidad nucleotídica y rápido decaimiento del DL, es difícil llegar a descubrir un gen que alcance una alta proporción de la varianza genética, por lo que hasta la fecha no ha logrado impactar el mejoramiento forestal (Plomion et al. 2016; Thuma et al. 2005; Zapata-Valenzuela y Hasbun 2011).

Durante los últimos años, una nueva estrategia llamada selección genómica (SG) (Meuwissen et al. 2001) ha cautivado el interés de mejoradores forestales, dado el éxito potencial que podría tener dentro de sus PMGs. A diferencia de la SAM, la SG no necesita conocer los genes que estén afectado una característica, ni el efecto directo de estos sobre el fenotipo. Más bien, ésta asume que, con una adecuada densidad de MMs, distribuidos a lo largo del genoma, algunos de ellos estarán en DL con algún QTL, y que por lo tanto, el efecto en conjunto de todos los MMs permitiría estimar con mayor precisión el componente genético que controla la característica, en comparación a la evaluación genética tradicional (Meuwissen et al. 2001; Calus y Veerkam 2007; Solberg et al. 2008). Y si los MMs son consistentes en la población, y explican un alto porcentaje de la varianza genética, se podría considerar que la estimación de la relación marcador-gen sería significativa (Meuwissen et al. 2001; Heffner et al. 2009).

El principio de la SG se basa en utilizar un "set de entrenamiento", el cual cuenta con individuos que han sido genotipificados por MMs y fenotipificados para alguna característica

de interés. A partir de esta información, un modelo de predicción es ajustado para estimar el fenotipo en función de los MMs (Habier et al. 2009). Posterior al ajuste del modelo, un llamado "set de validación" es utilizado para determinar el poder predictivo de la función ajustada, este grupo de muestras, corresponde a individuos que han sido genotipificados y a los cuales su fenotipo les será predicho en función de sus patrones alélico desde los MMs. Las predicciones son comparadas con el valor fenotípico real y se medirá el poder de predicción en base a sus correlaciones (Zhao et al. 2012). Es importante considerar, que el set de entrenamiento deberá ser representativo de los individuos del programa de mejoramiento donde será aplicada la selección (Heffner et al. 2009), para así lograr una mayor habilidad de predicción por parte del modelo. Una gran ventaja de utilizar la SG, se debe principalmente a que los individuos a predecir, no necesitarán esperar la evaluación fenotípica, transformándose en una reducción en los tiempo del proceso de selección y con ello también de sus costos. De acuerdo a lo publicado por Misztal (2011) respecto a la aplicación de la SG en animales, afirman que alrededor de 2.000 individuos deberían ser utilizados como set de entrenamiento para ajustar el modelo, sin embargo, cuando la progenie es más pequeña y la heredabilidad de la característica es menor, un mayor número de individuos se hace necesario; además, estiman que para un trabajo de investigación, un total de 600 individuos serían suficientes.

Hayes et al. (2009) describieron cuatro factores críticos que estarían influyendo sobre la habilidad de predicción de los modelos de SG: (1) el nivel de DL entre los MMs y los QTLs, (2) el tamaño efectivo de la población (Ne), (3) la densidad de los MMs en el genoma y (4) la heredabilidad de la característica evaluada. Sin embargo, más tarde, Grattapaglia y Resende (2011) concluyeron en sus estudios que los efectos de la heredabilidad y densidad de QTLs, serían insignificantes en la precisión de la predicción mediante SG.

Actualmente, el desarrollo de técnicas de secuenciación de segunda generación (next generation sequencing- NGS) ha impulsado la implementación de sistemas para la identificación de MMs del tipo microsatélites (single sequence repeat - SSRs) y polimorfismos de un solo nucleótido (single nucleotide polymorphisms – SNPs). Lo anterior utilizando genomas de varias especies vegetales (Wong and Bernardo 2008), principalmente no-modelo, las que carecen de genomas de referencia. Un SNP es una variación de alelos entre secuencias

en una posición específica, con una abundancia de al menos un 1% de los individuos de la población (Jeham y Lakhanpaul 2006). Los SNPs, son potencialmente el mejor tipo de MM debido a su abundancia en el genoma y su posible asociación a diferentes características (González-Martínez et al. 2006). Muchos ya han sido identificados en especies forestales (Novaes et al. 2008; Jones et al. 2009; Hamilton et al. 2011; Nelson et al. 2011; Trebbi et al. 2011) considerándose MMs valiosos entre sus análisis.

Existen diferentes rutinas de identificación de SNPs para genotipificación con potencial para aumentar la velocidad y costo-efectividad del proceso, principalmente al ser aplicados en mejoramiento genético para análisis de diversidad, estructura poblacional, identidad genética, SAM y SG (You et al. 2011; Thomson et al. 2012) entre otros enfoques. Por una parte, se encuentran las técnicas de re-secuenciación basadas en la reducción de la complejidad de los genomas mediante enzimas de restricción (ER) como la genotipificación por secuenciación (genotyping by sequencing – GBS) (Elshire et al. 2011). Po otra parte, los paneles físicos conocidos como SNP-chips, son sistemas de genotipificación basados en un set de sondas que dentro de su secuencia posee la variante alélica o SNP, y que por hibridación, permite una rápida puntación de varios miles de marcadores en paralelo entre diferentes muestras; un ejemplo es el llamado EUChip60K descrito por Silva-Junior et al. (2015a), un chip multi-especie para *Eucalyptus* con ~60 K SNPs. Sin embargo, el problema es que si bien los costos en los sistemas de secuenciación han disminuido considerablemente durante los últimos años, el genotipado por chips de SNPs aún es costoso (aproximadamente 50 US$ por muestra), considerando el alto número de muestras que se debe analizar para ser utilizados como una herramienta rutinaria de análisis en un PMG.

El presente trabajo es el primer estudio en identificación de MMs del tipo SNPs para *E. globulus* en Chile, con aplicación en un PMG de la especie. En primer lugar, 500 clones de *E. globulus* fueron genotipificados mediante GBS, información a partir de la cual, un flujo bioinformático (pipeline) de trabajo fue diseñado para la búsqueda de SNPs polimórficos. Posteriormente, basados en el EUChip60K, el perfil alélico de 140 clones de *E. globulus* fue descrito para evaluar la capacidad de genotipificación del chip mediante análisis de verificación intraclonal y familiar. Finalmente, utilizando el EUChip60K, 310 nuevos clones

de *E. globulus* fueron genotipificados y utilizados para ajustar modelos predictivos de SG y estimar los valores genómicos para densidad de la madera y volumen del fuste, permitiendo evaluar la habilidad de predicción de los SNPs.

HIPOTESIS

La identificación de SNPs entre clones de *E. globulus*, es un proceso más eficiente y reproducible si se utiliza una matriz física como el EUChip60K, en relación a la genotipificación por secuenciación (GBS). Además, los SNPs, permiten predecir valores genómicos para densidad de la madera y volumen del fuste, manteniendo una alta habilidad de predicción en ausencia del fenotipo, en clones de la especie.

OBJETIVO GENERAL

El siguiente trabajo tiene dos objetivos generales:

1. Evaluar la tecnología de genotipificación por secuenciación (GBS) y el EUChip60K para identificar SNPs entre clones de *E. globulus*.
2. Evaluar la aplicabilidad de utilizar un modelo de selección genómica para una población clonal de *E. globulus*.

OBJETIVOS ESPECÍFICOS

1. Generar un flujo de trabajo o "pipeline bioinformático" para el descubrimiento de SNPs, a partir de la GBS, entre clones de *E. globulus*.
2. Evaluar la utilidad del EUChip60K para genotipificar clones de *E. globulus.*
3. Identificar SNPs polimórficos entre individuos de una población clonal de *E. globulus* para ajustar un modelo de selección genómica.
4. Evaluar relaciones genéticas entre individuos no relacionados, medios hermanos y hermanos completos, utilizando SNPs. Y evaluar los niveles de desequilibrio de ligamiento intra-cromosomales entre SNPs.
5. Ajustar modelos de selección genómica y estimar valores genómicos para densidad de la madera y volumen del fuste, en un grupo llamado "de entrenamiento" de clones de *E. globulus.* Y validar las predicciones en un grupo llamado "de validación" de clones de *E. globulus.*
6. Correlacionar los valores genómicos y genéticos, para densidad de la madera y volumen, en los grupos de entrenamiento y validación.

CAPÍTULO I: SNP DISCOVERY IN *EUCALYPTUS GLOBULUS* BY GBS

Nicole Munnier, Ricardo Durán, Valentina Troncoso, Marta Fernández, David Neale, Sofía Valenzuela

1.1 ABSTRACT

Genotyping-by-sequencing (GBS) is a flexible and cost-effective strategy for discovery of single nucleotide polymorphisms (SNPs). However, identification of polymorphic and informative SNPs for a specific population requires a robust bioinformatics pipeline, especially for species such as *Eucalyptus globulus* that lack a reference genome sequence. In this study, 2,632 polymorphic and informative SNPs were discovered for two breeding populations of *E. globulus.* Markers were also described on the basis of their structural occurrence and their functional annotation within genes. This information provides a valuable resource for further research focused on genetic improvement of *E. globulus* using open-source bioinformatics tools.

Key Words: GBS, SNPs, reference genome, *E. globulus*,

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1.2 INTRODUCCIÓN

Single nucleotide polymorphisms (SNPs) correspond to sequence variations that involve a single nucleotide difference when two sequenced alleles from homologous chromosomes are compared, generally arising due to a copying error during cell division (Thavamanikumar et al., 2011a). In some species they represent as much as 90% of the genetic variation and they are abundant across the genome (Gupta et al., 2008). The detection of SNPs currently is a simple and cost-effective process due to the use of next generation sequencing (NGS) technologies that provides large amounts of data (Shen et al., 2005; Syvänen, 2005). The density of SNPs discovered in plant genomes varies depending on the genome region, depth of sequencing, coverage and choice of sample accessions as well as with the breeding system of the species (Nelson et al., 2011). SNP discovery in plants has been possible by the development of several genotyping platforms based on techniques that can reduce genome complexity during resequencing, one of these technologies is Genotyping by Sequencing (GBS) (Elshire et al., 2011), a simple, fast and robust method for sequencing samples. In GBS a barcoded multiplexing system and restriction enzymes (REs) are used, having several advantages including the fact that no preliminary sequence information is required and all the newly discovered markers are from the population that is being genotyped from (Deschamps et al., 2012). This technique has been described for forest species such as *Pinus contorta* (Chen et al. 2013), *Populus* (Schilling et al., 2014) and *Picea* (El-Dien et al., 2015).

During the past years, genomic studies in forest species have increased, specially focused on reference assemblies, transcriptome analysis and development of SNP databases. Therefore, considering that *E. globulus* is one of the most important forest species world-wide for the production of paper and hardwood pulp due to its excellent fiber quality (Goulao et al., 2011), it is relevant to increase the genomic resources of *E. globulus*. In this study we present a simple way to discover polymorphic and informative SNP markers using the GBS technology and a workflow for SNP discovery. Just as an exploratory analysis, markers were described by their genomic position and annotated by gene ontology (GO). Therefore, the information in this study provides a valuable resource for further research focused on genetic improvement of the *E. globulus* using open-source bioinformatic tools.

1.3 MATERIAL AND METHODS

1.3.1 Plant material, DNA extraction, library construction and sequencing

This study was carried out using two breeding populations of *E. globulus*, named "A" and "B", which contained commercial genotypes growing under field conditions, belonging to two Chilean forestry companies. Populations were from the Biobio Region, Chile (36°46′22″S, 73°3′47″O). The "A" breeding population consisted of a total of 258 genotypes belonging to 29 full-sib families, which were obtained by crossing 18 parents. The "B" breeding population, resulted from crossing 36 parents giving rise to 64 full-sib families with 248 genotypes. A total of 506 genotypes from both populations were used for the analysis. Genomic DNA was obtained starting from 100 mg of bark from each of the genotypes used in this study, by employing the commercial DNeasy Plant mini kit (Qiagen) according to the manufacturer's protocol. The DNA quantity and integrity were assayed using a 2200 Bioanalyzer TapeStation (Agilent Technology) according to the manufacturer's protocol. Samples were sent to the Institute of Genomic Diversity, Cornell University, USA, for the generation of libraries and sequencing according to the GBS protocol proposed by Elshire et al. (2011), using as restriction enzyme *Ape*KI and barcode tagging of samples followed by DNA sequencing.

1.3.2 Bioinformatics pipeline: Quality control, read alignment, variant calling and exploratory analysis for SNPs discovered

The pipeline developed for the data analysis is based on several open source programs for the bioinformatics analysis, executed in different homemade scripts. For the sequencing read quality control, FastQC (Andrews, 2010) was used. In addition, the NGS QC Toolkit package (Patel and Jain, 2012) was used for the sequence read-trimming process to eliminate barcodes in the 5´ end and bases of low quality in the 3´ end. Quality control with IlluQC_PRLL.pl of large reads with default parameters (-l 70 and –s 30) and quality values with a minimum of 25 bp and a minimum Phred score of 30 in order to improve the overall quality values for each genotype was checked. For the sequencing read data alignment, the program Bowtie2 (Langmead and Salzberg, 2012) with a minimum Phred score of 30 was used. *E. grandis* (version 1.0) data, available on Phytozome (http://www.phytozome.net/), was employed as reference genome. Alignments in SAM format were processed using SAMtools (Li et al.,

2009) available in http://samtools.sourceforge.net/index.shtml. Indexing SNP detection was performed using 'pileup' command. SNP filtering was performed in four steps: first, 'varFilter' was used to control the maximum read depth (-D 5); second, 'remove indels' to keep only SNPs; third, as part of the variant-filtering process regarding to interspecific variants (*E. grandis-E. globulus)*, a filter quality less than 30 was performed by the toolbox SnpSift, part of SnpEff v4.1 program (Cingolani et al., 2012) (http://snpeff.sourceforge.net/), and finally was used vcftools '-hardy' (Danecek et al., 2011), defining as a polymorphic variant the variant present at least 1% on the populations studied (MAF filter >0.01). SnpEff was used to identify the specific position of the SNP on the reference genome scaffolds and SNPs were described on the basis of their structural occurrence in the intronic, untranslated region (5′UTR or 3′UTR), upstream region, downstream region, splice site, or intergenic regions using results based on the annotated *E. grandis* genome**.** A functional annotation of genes was performed using *E. grandis* RefSeq database annotation info file, to obtain non-redundant annotation results. To assign a function to each putative gene identified, DAVID's Functional Annotation was used (Database for Annotation, Visualization and Integrated Discovery) (Huang et al., 2009a, 2009b), which include Gene Ontology (GO) terms and other functional themes. REVIGO (http://revigo.irb.hr/) (Supek et al., 2011) was used to summarize and visualize GO terms (Fig. S1.1). As an exploratory analysis, some SNPs discovered were selected for their role involved in cellulose, hemicellulose and lignin process.

1.4 RESULTS

1.4.1 GBS libraries treatment and mapping using E. grandis reference genome

GBS libraries generated single-end (SE) reads with an average length of 101 bp including barcodes. Total reads were similar for both populations. The “A” population had a total of 541,591,133 reads and “B” a total of 573,572,323 reads, varying from 143,157 to 15,761,239 reads per library with an average of 53% GC content. The samples were separated according to the "barcodes" that were used for sequencing. After filtering, the total reads varied in the range of 133,705 and 13,843,208 per library with an average of 53% GC content. The alignment was assessed using *E. grandis* as a reference genome considering total mapped sequences, duplication percentage, coverage and quality. A total of 224,726,205 and 292,407,331 reads for “A” and “B” population respectively were mapped (45% and 54%),

with a number of reads mapped per library between 60,590 and 7,240,450. Mapping quality average was 5.04, coverage was 2,9 and an average of 53% GC.

1.4.2 Polymorphic and informative SNPs for E. globulus

A total of 98,350 raw SNPs were identified in both populations, out of which 84,982 were classified as interspecific variants (between *E. grandis* and *E. globulus)*. A total of 13,368 SNPs corresponding to 6,986 and 6,382 SNPs for "A" and "B" were polymorphic in each population. Finally, 2,632 SNPs corresponding to 1,357 and 1,275 SNPs for "A" and "B" population" respectively, corresponded to variants that were present at least on 1% of the population studied (Table S1.1). Only 35 SNPs were common between both populations. The number of SNPs per scaffold was variable, the largest number of SNPs (173) was in scaffold 8 for the "A" population and in scaffold 2 for the "B" population (160 SNPs), while the lowest number of SNPs was present in scaffold 9 for "A" population (89 SNPs) and in scaffold 4 for "B" population (82 SNPs) (Table S1.1). After SNP calling, variant annotation was assigned for each SNP, most of which had multiple annotations; high percentages of SNPs were found in downstream regions (27.3%, 1,811 SNPs) and in upstream regions (23.5%, 1,555 SNPs). A total of 1,407 (21.2%) SNPs were found in intergenic regions, 970 (14.7%) SNPs were present in intronic regions and 557 (8.4%) in exon regions. A total of 319 SNPs corresponded to UTRs and splice site regions (Table 1.1). 1,357 SNPs located in 1,342 genes and 1,275 SNPs within 1,298 genes were annotated for the "A" and "B" population, respectively. Unique genes for "A" and "B" populations corresponded to 1,127 and 1,083 respectively. Only 215 genes were common for both populations (Table 1.1).

Table 1.1 Information of variants for "A" and "B" Population. *Fuente: Elaboración propia.*

SNP discovery	"A" Population	"B" Population
Raw SNPs	62,697	35,653
Interspecie SNPs	55,711	29,271
Polymorphic SNPs	6,986	6,382
Indels	175	156
Informative SNPs	**1,357**	**1,275**

Effects by region		
Downstream region	930	881
Upstream region	749	806
Exon region	292	265
Intergenic region	710	697
Intron region	512	458
Splice cite	28	20
3' UTR	82	90
5'UTR	71	28
Gene annotation		
Genes with SNPs	1,342	1,298
Unique genes	**1,127**	**1,083**

1.4.3 Gene ontology for SNPs discovered

The results of GO for the "A" population, showed a total of 87 terms, of which 38 were related to Biological Process (BP) with 109 genes, 17 to Molecular Function (MF) with 1,011 genes and 32 to Cellular Component (CC) with 1,286 genes. After removing redundant GO terms, this total was reduced to 63 categories, of which 29, 14 and 20 belonged to BP, MF and CC respectively. For the "B" population a total of 76 GO terms of which 38 were related to BP with 961 genes annotated, 14 to MF with 957 genes and 24 to CC with 1,240 genes. After removing redundant GO terms, this total was reduced to 54 categories, of which 28, 12 and 14 belonged to BP, MF and CC respectively. For BP, the largest number of genes was found in "protein phosphorylation" for "A" population and in "oxidation-reduction process" for "B" population. For CC, the largest number of genes was found in "integral component of plasma membrane" for both populations. For MF, the largest number of genes was found in "ATP binding" for both populations (Fig. S1.2).

1.4.4 SNPs within genes involved in biochemical pathways for E. globulus

As an exploratory analysis, SNPs discovered were annotated by gene ontology according with their BP, MF and CC, where a total of a total of 85 genes were selected for their role involved in cellulose, hemicellulose and lignin biosynthesis processes (41 described in the "A"

population and 44 in the "B" population) (Table S1.2). These genes are distributed into 25 and 22 categories for the "A" and "B" population respectively. For BP, the terms that contain the largest number of genes, in both populations, were "carbohydrate metabolic process" (13 genes), "oxidation-reduction process" (17 genes) and cell wall organization (10 genes), where important gene families such as Pectin lyase-like superfamily protein, laccase genes family (LAC), cellulose synthase family (CESA) and O-methyltransferase 1 (OMT1) were identified. For CC, in "plasma membrane" (21 genes) some *CESA* family genes were identified for both populations; for "A" population, in "extracellular region" (8 genes) and "membrane" (8 genes) terms, *LAC* family genes and expansin A13 (*EXPA13*) were respectability identified; and also to "B" population genes related with cytoplasm (15 genes) and cytosol (10 genes) were assigned such as *ELI3-1, ELI3-2* and *CAD9*. Genes assigned to MF were sorted out to "transferase activity, transferring glycosyl groups" (15 genes) for "A" and "B" population, where a large number of *CESA* genes were identified.

1.5 DISCUSSION

1.5.1 GBS library process to SNP discovery

Of the total raw reads obtained from the sequencing process only 4% of these were removed according to read quality control; being lower than similar studies as in the case of *E. camaldulensis*, where 38% of the raw sequences were removed from Illumina sequencing after filtering (Hendre et al., 2012). The high variability of reads per library could be due to different reasons: DNA sequencing quality (De Donato et al., 2013), not using the most appropriate RE (Fu et al., 2016), multiplex sequencing system (Elshire et al., 2011) and mapping error. Even though the most likely source of sample-to-sample variation in sequence coverage is the accurate quantification of high molecular weight DNA (Elshire et al., 2011), no correlations were found between the number of reads and the DNA quality for our samples (data not shown). According to Harismendy et al. (2009) an average 55% of Illumina sequences pass the filtering process, due to that some adapters or primers are not ligated in the sequencing process and those could interrupt the SNP calling (Patel and Jain, 2012). Further efforts have been made to improve the GBS efficiency in genome sampling (De Donato et al., 2013; Heffelfinger et al., 2014; Peterson et al., 2014; Schilling et al., 2014), particularly with the use of more effective RE combinations (Hamblin and Rabbi, 2014). Choosing the

appropriate RE is a critical step in developing a GBS protocol (Sonah et al., 2013), where repetitive regions of genomes can be avoided and low copy regions can be targeted with two to three fold higher efficiency (Gore et al., 2007, 2009), which tremendously simplifies computationally challenging alignment problems in species with high levels of genetic diversity (Elshire et al., 2011). Poland and Rife (2012) used a combination of REs, showing that it created shorter fragments that improved the sequencing quality and increased the coverage and depth of sequencing, therefore reducing the missing data. Sonah et al. (2013) worked with a combination of a double restriction enzyme digest (HindIII-MspI) and a selective PCR amplification to show that this combination could create shorter fragments improving the sequencing quality and increasing coverage and depth, reducing the missing data (Peterson et al., 2014).

1.5.2 Mapping reads using the Eucalyptus grandis reference genome

Almost 50% of the reads were mapped to the *E. grandis* reference genome; the remaining 50% may have been left out due to several reasons, such as the differences between species and the length of the sequences being assembled; short reads further hinder the mapping process and a low percentage of them could match sequences from cellular organelles such as mitochondria, chloroplasts with its own DNA and transposon elements or satellite DNAs (Sonah et al., 2013). Mapping quality was low (5.04), meaning that the probability to find a true alignment by using the algorithm is low (Li et al., 2008). Moreover, the coverage obtained means that the libraries represent only 2.9 times the *E. globulus* genome; being lower than the one expected for GBS, especially since the samples were digested with *Ape*KI, which is a frequent cutting RE that causes a direct effect on the number of identified variants (Lu et al., 2013). In other species such as *E. camaldulensis* a minimum coverage limit of 8x for SNPs discovery has been accepted, and coverage above 20x was required to achieve a sufficient quality of mapping to establish a 99% confidence in such mapping (Thumma et al., 2012).

1.5.3 SNP discovery

Of the total SNPs initially identified, only a small percentage, 2.16% and 3.58% for “A” and “B” population were considered polymorphic and informative, as result of a strict filtering process for the SNP identification. A filtering process, defined by a set of arbitrary criteria, is

generally applied to remove markers from further analysis (Easton et al., 2007; Sladek et al., 2007) and are typically based on various measures or attributes calculated to reflect the markers integrity and usefulness (Chan et al., 2009). As well, filtering strategies are applied depending on the type of the sequences and the sequencing requirements as length, quality, depth and coverage, among others. The last filter was critical to define an informative SNP, where variants present at least in 1% of the population (Brookes, 1999) were considered informative for the populations. This means that they are present in three or more individuals of the population studied, but given the low number of samples analyzed, a larger number of SNPs could be classified as polymorphic and informative if more individuals of the same population are studied. SNPs below the 1% of the population may be related to genotyping issues, such as lower genotyping rates or concerns about calling accuracy. The low number of common SNPs between both populations can be explained by the low coverage of sequencing, therefore it was unlikely that the same regions of the genome from different individuals could be compared. (Schilling et al., 2014)

Several filter settings can make a more selective SNP identification process, since some of these filters remove variants with neighboring gaps and those are not present in a minimum number of reads (depth) (Ahmad et al., 2011).

1.5.4 SNP within genes

Considering the important role of the cellulose and lignin content for *E. globulus*, a set of markers from the GO analysis showed that they are within important genes involved into the cellulose and lignin biosynthesis. Cellulose is a compound synthesized by the heteromeric cellulose synthase (cesA) complex (Somerville, 2006) where CesA genes encode the catalytic subunit of cellulose synthase. In our study, we identified SNPs within some cellulose synthase genes (*CESA4, CESA9, CES6, CSLD1 and CSLC12*) and SNPs in these genes have also been described in other species as *Pinus radiata* (*CESA3, CESA7* and *CESA1)* (Dillon et al., 2010), *Populus tomentosa (CESA4)* (Du et al., 2013) and *Pinus taeda (CESA2, CESA3, CESA4, CESA9)* (Gonzalez-Martinez et al., 2006 and Palle et al., 2013). SuSy activity is observed during wood formation (Schrader and Sauter 2002), and it is thought to be the main enzyme supplying UDP-glucose to cellulose biosynthesis. In our study *SPS3F*, *SUS3* and sucrose-6F-

phosphate phosphohydrolase family proteins were identified, as in *E. urophylla*, where a total of 46 SNPs in the sequence of sucrose synthase 1 (*SUSY1*) were found (Maleka, 2007).

Lignin is the second most abundant biopolymer after cellulose (Boudet, 2000) and is a complex of aromatic heteropolymer of monolignols (*p*-hydroxyplenyl (H), guaiacyl (G) and syringyl (S)), that are produced in the cytoplasm and moved to the cell walls (Yoon et al., 2015). Carocha et al. (2015) described eleven genes coding for enzymes involved in the monolignol biosynthesis in *E. grandis* and in this study, we identified SNPs within some of these genes, for example, SNPs within genes coding for 4-coumarate-coenzyme A ligase (*4CL*), caffeate/5-hydroxyferulate O-methyltransferase (*COMT*) and S-adenosyl-L-methionine-dependent methyltransferases superfamily protein (*CCOAOMT*) and cinnamyl alcohol dehydrogenase *(CAD)*. In *E. globulus* variation within *CAD* gene sequences were evaluated by Poke et al. (2003), where eight SNPs in *CAD2* were identified. Wegrzyn et al. (2010) described a non-coding marker from *CAD*, as well as, Dillon et al. (2010) where they described 11 SNPs that were identified within *CAD* for *P. radiata*. Southerton et al. (2010) identified a single *CAD* gene and 2 *CCoAMT* genes with SNPs, however only some SNPs showed a significant association with cellulose, microfibril angle and pulp yield in *E. nitens.*

1.6 CONCLUSIONS

A total of 2,632 new polymorphic and informative SNPs for two *E. globulus* breeding population were identified by GBS technology using different bioinformatic tools available: Considering that *E. globulus* does not have a reference genome, this strategy could be considered as an easy way for genotyping new populations. Several of these markers were involved in different biosynthesis processes which could be relevant for the genetic improvement of *E. globulus*.

1.7 REFERENCE

Ahmad R, Parfitt DE, Fass J, Ogundiwin E et al (2011) Whole genome sequencing of peach (*Prunus persica* L.) for SNP identification and selection. BMC Genomics 12:569. doi: 10.1186/1471-2164-12-569

Andrews, S (2010) FastQC: A quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Boudet AM (2000) Lignins and lignification: selected issues. Plant Physiol Bioch 38:81–96

Brookes AJ (1999) The essence of SNPs. Gene 234:177–186

Carocha V, Soler M, Hefer C, Cassan-Wang H, Fevereiro P, Myburg AA, Paiva JAP, Grima-Pettenati J (2015) Genome-wide analysis of the lignin toolbox of *Eucalyptus grandis*. New Phytol 206:1297–1313. doi: 10.1111/nph.13313

Chan EKF, Hawken R, Reverter A (2009) The combined effect of SNP-marker and phenotype attributes in genome-wide association studies. Anim Genet 40:149–156. doi: 10.1111/j.1365-2052.2008.01816.x

Chen C, Mithchel S, Elshire R et al (2013) Mining conifers' mega-genome using rapid and efficient multiplexed high-throughput genotyping-by-sequencing (GBS) SNP discovery platform. Tree genetics & genomes 9:1537-1544. doi 10.1007/s11295-013-0657-1

Cingolani P, Platts A, Wang LL et al (2012) A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. Fly 6:80–92. doi:10.4161/fly.19695

Danecek P, Auton A, Abecasis G et al (2011) The variant call format and VCFtools. Bioinformatics 27: 2156–2158. doi:10.1093/bioinformatics/btr330

De Donato M, Peters SO, Mitchell SE et al (2013) Genotyping-by-Sequencing (GBS): A novel, efficient and cost-effective genotyping method for cattle using next-generation sequencing. Plos one 8:e62137. doi: 10.1371/journal.pone.0062137

Deschamps S, Llaca V, May GD (2012) Genotyping-by-sequencing in plants. Biology 1:460–483. doi: 10.3390/biology1030460

Dillon SK, Nolan M, Li W, Bell C, Wu HX, Southerton SG (2010) Allelic variation in cell wall candidate genes affecting solid wood properties in natural populations and land races of *Pinus radiata*. Genetics 185:1477–1487. doi: 10.1534/genetics.110.116582

Du Q, Xu B, Pan W, Gong C, Wang Q, Tian J, Li B, Zhang D (2013) Allelic Variation in a Cellulose Synthase Gene (PtoCesA4) Associated with growth and wood properties in *Populus tomentosa*. G3: Genes|Genomes|Genetics 3:2069–2084. doi:10.1534/g3.113.007724

Easton DF, Pooley KA, Dunning AM et al (2007) Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 447:1087–1093. doi:10.1038/nature05887

El-Dien OG, Ratcliffe B, Klápště J et al (2015) Prediction accuracies for growth and wood attributes of interior spruce in space using genotyping-by-sequencing. BMC Genomics 16. doi:10.1186/s12864-015-1597-y

Elshire RJ, Glaubitz JC, Sun Q et al (2011) A Robust, Simple Genotyping-by-sequencing (GBS) approach for high diversity species. Plos one 6:e19379. doi:10.1371/journal.pone.0019379

Fu Y-B, Peterson GW, Dong Y (2016) Increasing genome sampling and improving SNP genotyping for genotyping-by-sequencing with new combinations of restriction enzymes. G3: Genes|Genomes|Genetics 6:845–856. doi:10.1534/g3.115.025775

Gonzalez-Martinez SC, Wheeler NC, Ersoz E, Nelson CD, Neale DB (2006) Association genetics in *Pinus taeda* L. I. Wood property traits. Genetics 175:399–409. doi: 10.1534/genetics.106.061127

Gore MA, Chia J-M, Elshire RJ et al (2009) A first-generation haplotype map of Maize. Science 326:1115–1117. doi: 10.1126/science.1177837

Gore M, Bradbury P, Hogers R et al (2007) Evaluation of target preparation methods for single-feature polymorphism detection in large complex plant genomes. Crop Sci 47:S–135. doi: 10.2135/cropsci2007.02.0085tpg

Goulao LF, Vieira-Silva S, Jackson PA (2011) Association of hemicellulose- and pectin-modifying gene expression with *Eucalyptus globulus* secondary growth. Plant Physiol Bioch 49:873–881. doi: 10.1016/j.plaphy.2011.02.020

Gupta PK, Rustgi S, Mir RR (2008) Array-based high-throughput DNA markers for crop improvement. Heredity 101:5–18. doi: 10.1038/hdy.2008.35

Hamblin MT, Rabbi IY (2014) The effects of restriction-enzyme choice on properties of genotyping-by-sequencing libraries: A study in Cassava (*Manihot esculenta*). Crop Sci 54:2603-2608. doi: 10.2135/cropsci2014.02.0160

Harismendy O, Ng PC, Strausberg RL et al (2009) Evaluation of next generation sequencing platforms for population targeted sequencing studies. Genome Biol 10:R32. doi: 10.1186/gb-2009-10-3-r32

Heffelfinger C, Fragoso CA, Moreno MA et al (2014) Flexible and scalable genotyping-by-sequencing strategies for population studies. BMC Genomics 15:979. doi: 10.1186/1471-2164-15-979

Hendre PS, Kamalakannan R, Varghese M (2012) High-throughput and parallel SNP discovery in selected candidate genes in *Eucalyptus camaldulensis* using Illumina NGS platform: High-throughput SNP discovery in *E. camaldulensis*. Plant Biotechnol J 10:646–656. doi:10.1111/j.1467-7652.2012.00699.x

Huang DW, Sherman BT, Lempicki RA (2009a) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57. doi:10.1038/nprot.2008.211

Huang DW, Sherman BT, Lempicki RA (2009b) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucl Acids Res 37:1–13. doi: 10.1093/nar/gkn923

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359. doi:10.1038/nmeth.1923

Li H, Handsaker B, Wysoker A et al (2009) The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079. doi:10.1093/bioinformatics/btp352

Li H, Ruan J, Durbin R (2008) Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res 18:1851–1858. doi:10.1101/gr.078212.108

Lu F, Lipka AE, Glaubitz J et al(2013) Switchgrass genomic diversity, ploidy, and evolution: Novel insights from a network-based SNP discovery protocol. Plos Genet 9:e1003215. doi:10.1371/journal.pgen.1003215

Maleka MF (2007) Allelic diversity in cellulose and lignin biosynthetic genes of *Eucalyptus urophylla ST BLAKE*. Doctoral Dissertation, University of Pretoria

Nelson JC, Wang S, Wu Y et al (2011) Single-nucleotide polymorphism discovery by high-throughput sequencing in sorghum. BMC Genomics 12:352. doi:10.1186/1471-2164-12-352

Palle SR, Seeve CM, Eckert AJ, Wegrzyn JL, Neale DB, Loopstra CA (2013) Association of loblolly pine xylem development gene expression with single-nucleotide polymorphisms. Tree Physiol 33:763–774. doi:10.1093/treephys/tpt054

Patel RK, Jain M (2012) NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. Plos One 7:e30619. doi: 10.1371/journal.pone.0030619

Peterson G, Dong Y, Horbach C, Fu Y-B (2014) genotyping-by-sequencing for plant genetic diversity analysis: A lab guide for SNP genotyping. Diversity 6:665–680. doi:10.3390/d6040665

Poland JA, Rife TW (2012) Genotyping-by-sequencing for plant breeding and genetics. Plant Genome. 5:92-102. doi:10.3835/plantgenome2012.05.0005

Schilling MP, Wolf PG, Duffy AM et al (2014) Genotyping-by-sequencing for *Populus* population genomics: An assessment of genome sampling patterns and filtering approaches. Plos one 9:e95292. doi:10.1371/journal.pone.0095292

Shen R, Fan J-B, Campbell D et al (2005) High-throughput SNP genotyping on universal bead arrays. Mutat Res-Fund Mol M 573:70–82. doi:10.1016/j.mrfmmm.2004.07.022

Sladek R, Rocheleau G, Rung J et al (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature 445:881–885. doi:10.1038/nature05616

Somerville C (2006) Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol 22:53–78. doi:10.1146/annurev.cellbio.22.022206.160206

Sonah H, Bastien M, Iquira E et al (2013) An improved genotyping by sequencing (GBS) approach offering increased versatility and efficiency of SNP discovery and genotyping. Plos One 8:e54603. doi:10.1371/journal.pone.0054603

Southerton SG, MacMillan CP, Bell JC, Bhuiyan N, Dowries G, Ravenwood IC, Joyce KR, Williams D, Thumma BR (2010). Association of allelic variation in xylem genes with wood properties in *Eucalyptus nitens*. Australian Forestry 73:259–264. doi:10.1080/00049158.2010.10676337

Supek F, Bošnjak M, Škunca N, Šmuc T (2011) REVIGO Summarizes and visualizes long lists of gene ontology terms. Plos one 6:e21800. doi:10.1371/journal.pone.0021800

Syvänen AC (2005) Toward genome-wide SNP genotyping. Nature Genet 37:S5–10.

Thavamanikumar S, McManus LJ, Tibbits JFG, Bossinger G (2011a). The significance of single nucleotide polymorphisms (SNPs) in *Eucalyptus globulus* breeding programs. Aus For 74:23–29. doi:10.1080/00049158.2011.10676342

Thumma BR, Sharma N, Southerton SG (2012) Transcriptome sequencing of *Eucalyptus camaldulensis* seedlings subjected to water stress reveals functional single nucleotide

polymorphisms and genes under selection. BMC Genomics 13:364. doi: 10.1186/1471-2164-13-364

Wegrzyn JL, Eckert AJ, Choi M, Lee JM, Stanton BJ, Sykes R, Davis MF, Tsai C-J, Neale DB (2010) Association genetics of traits controlling lignin and cellulose biosynthesis in black cottonwood (*Populus trichocarpa*, Salicaceae) secondary xylem. New Phytol 188:515–532. doi:10.1111/j.1469-8137.2010.03415.x

Yoon J, Choi H, An G (2015) Roles of lignin biosynthesis and regulatory genes in plant development: Roles of lignin in plant development. J Integr Plant Biol 57:902–912. doi:10.1111/jipb.12422

1.8 SUPPLEMENTARY MATERIAL

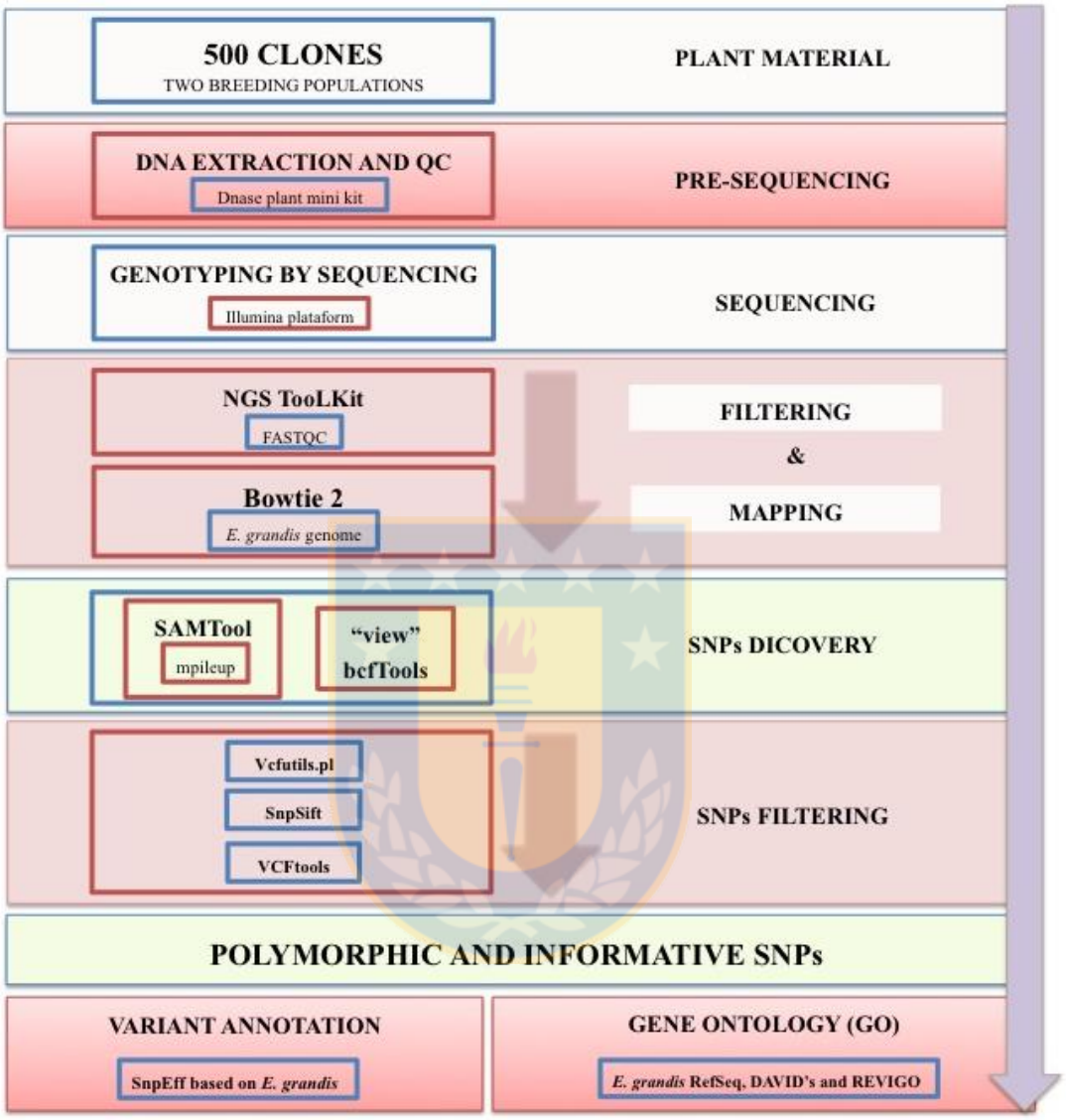

Fig. S1.1 Bioinformatic pipeline to discover SNPs for *E. globulus. Fuente: Elaboración propia*

Table S1.1 Distribution SNPs by scaffold for “A” and “B” population. *Fuente: Elaboración propia.*

Scaffold	**SNPs "A" Population**	**SNPs "B" Population**
Scaffold 1	100	121
Scaffold 2	157	160
Scaffold 3	102	87
Scaffold 4	99	82
Scaffold 5	105	100
Scaffold 6	149	140
Scaffold 7	110	103
Scaffold 8	173	131
Scaffold 9	89	108
Scaffold 10	118	98
Scaffold 11	117	112
Others scaffolds	38	33
TOTAL	**1,357**	**1,275**

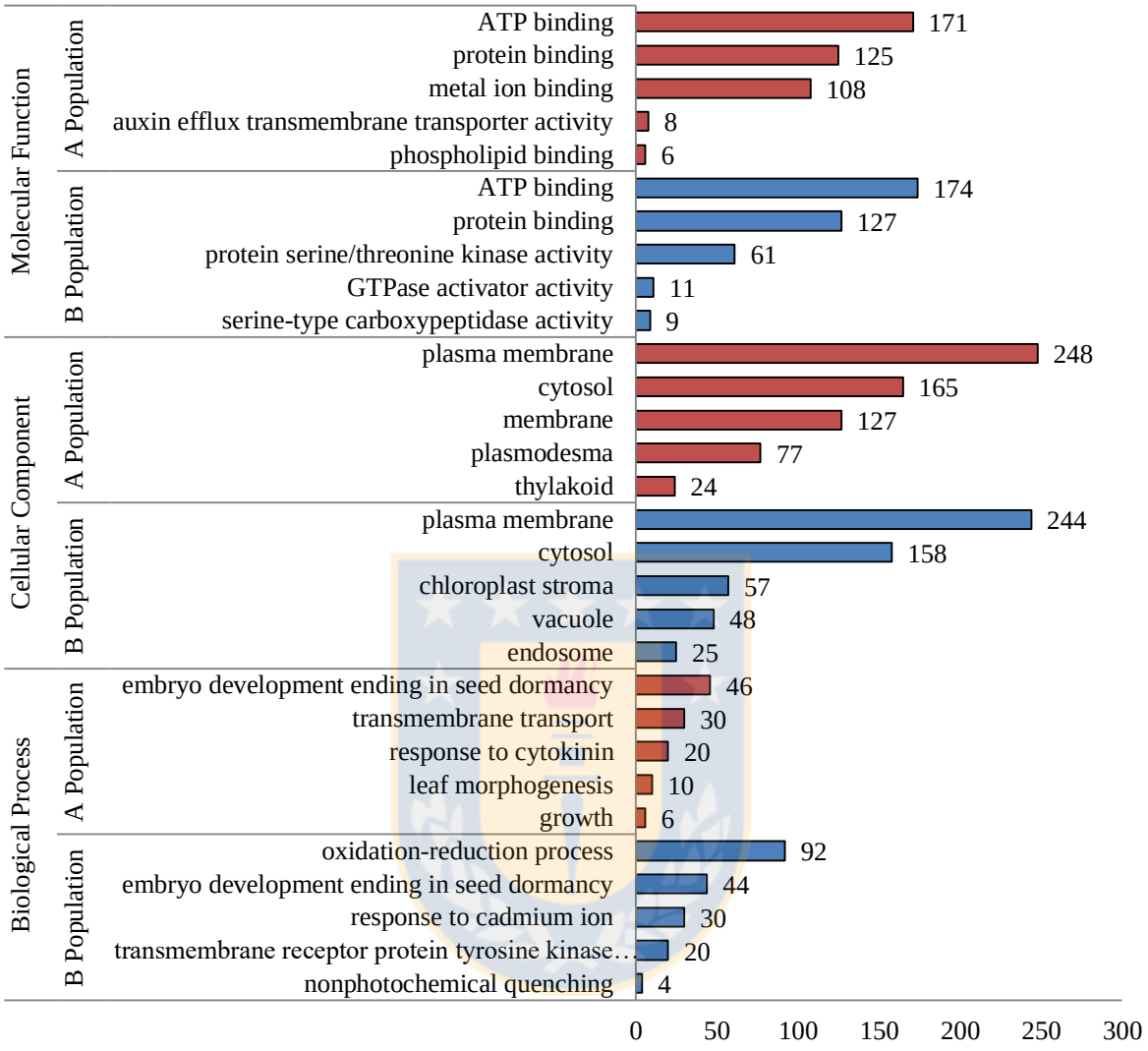

Fig. S1.2 Gene Ontology (GO) term representation for *Eucalyptus globulus*. The results are summarized in three categories: Biological process (BP), molecular function (MF) and cellular component (CC) for "A" and "B" population. Y-axis indicates the number of a specific category of genes in the main term. *Fuente: Elaboración propia.*

Table S1.2 Gene ontology annotation. *Fuente: Elaboración propia.*

	ID	Gene Name	GOTERM_BP_DIRECT	GOTERM_CC_DIRECT	GOTERM_MF_DIRECT	Eucgr Code	Scaffold number	Position	Annotation
A Population	AT1G27680	ADPGLC-PPase large subunit(APL2)	GO:0005978=glycogen biosynthetic process,GO:0019252=starch biosynthetic process,	GO:0009507=chloroplast,	GO:0005524=ATP binding,GO:0008878=glucose-1-phosphate adenylyltransferase activity,	Eucgr.F01590	scaffold_6	20216726	5_prime_UTR_variant, downstream_gene_variant
	AT4G16120	COBRA-like protein-7 precursor (COBL7)	GO:0010215=cellulose microfibril organization,GO:0016049=cell growth,	GO:0005768=endosome,GO:0005783=endoplasmic reticulum,GO:0005794=Golgi apparatus,GO:0005802=trans-Golgi network,GO:0005886=plasma membrane,GO:0009506=plasmodesma,GO:0031225=anchored component of membrane,GO:0046658=anchored component of plasma membrane,	-	Eucgr.A00190	scaffold_1	2214091	splice_region_variant&intron_variant
	AT1	Cellulase	GO:0005975=carbo	GO:0009507=chl	GO:0004553=hy	Eucgr.	scaffold_	31988910	upstream_ge

	G13130	(glycosyl hydrolase family 5) protein(AT1G13130)	hydrate metabolic process,	oroplast,	drolase activity, hydrolyzing O-glycosyl compounds,	D01800	4		ne_variant, downstream_gene_variant, intron_variant
	AT3G26130	Cellulase (glycosyl hydrolase family 5) protein(AT3G26130)	GO:0005975=carbohydrate metabolic process,	-	GO:0004553=hydrolase activity, hydrolyzing O-glycosyl compounds,	Eucgr.D01800	scaffold_4	31988911	upstream_gene_variant, downstream_gene_variant, intron_variant
	AT1G03520	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein(AT1G03520)	GO:0016051=carbohydrate biosynthetic process,	GO:0005634=nucleus,GO:0005794=Golgi apparatus,GO:0016020=membrane, GO:0016021=integral component of membrane,	GO:0008375=acetylglucosaminyltransferase activity,GO:0016757=transferase activity, transferring glycosyl groups,	Eucgr.A01787	scaffold_1	28137700	downstream_gene_variant
	AT5G61410	D-ribulose-5-phosphate-3-epimerase(RPE)	GO:0005975=carbohydrate metabolic process,GO:0009052=pentose-phosphate shunt, non-oxidative branch,GO:0009409=response to	GO:0005829=cytosol,GO:0009507=chloroplast,GO:0009570=chloroplast stroma,GO:0009579=thylakoid,GO:0009941=chloropl	GO:0004750=ribulose-phosphate 3-epimerase activity,GO:0046872=metal ion binding,	Eucgr.K02489	scaffold_11	32170818	downstream_gene_variant, intron_variant

			cold,GO:0009624=response to nematode,GO:0009793=embryo development ending in seed dormancy,GO:0019323=pentose catabolic process,GO:0044262=cellular carbohydrate metabolic process,	ast envelope,GO:0010319=stromule,GO:0048046=apoplast,					
	AT1G74670	Gibberellin-regulated family protein(GASA6)	GO:0009739=response to gibberellin,GO:0009740=gibberellic acid mediated signaling pathway,GO:0009744=response to sucrose,GO:0009749=response to glucose,GO:0009750=response to fructose,GO:0080167=response to karrikin,	GO:0005576=extracellular region,	-	Eucgr.A02289	scaffold_1	33640862	synonymous_variant, upstream_gene_variant
	AT1G54730	Major facilitator superfamily	GO:0035428=hexose transmembrane transport,GO:0046323=glucose import,	GO:0005886=plasma membrane,GO:0005887=integral	GO:0005351=sugar:proton symporter activity,GO:0005	Eucgr.B02025	scaffold_2	37934423	intron_variant

		protein(AT1G54730)		component of plasma membrane,GO:0016020=membrane,	355=glucose transmembrane transporter activity,GO:0015144=carbohydrate transmembrane transporter activity,				
	AT2G20780	Major facilitator superfamily protein(AT2G20780)	GO:0035428=hexose transmembrane transport,GO:0046323=glucose import,	GO:0005886=plasma membrane,GO:0005887=integral component of plasma membrane,GO:0016020=membrane,	GO:0005351=sugar:proton symporter activity,GO:0005355=glucose transmembrane transporter activity,GO:0015144=carbohydrate transmembrane transporter activity,	Eucgr.K00967	scaffold_11	11700638	upstream_gene_variant, downstream_gene_variant
	AT2G02400	NAD(P)-binding Rossmann-fold superfamily protein(AT2G02400)	GO:0009809=lignin biosynthetic process,	GO:0005829=cytosol,GO:0005886=plasma membrane,	GO:0003824=catalytic activity,GO:0016621=cinnamoyl-CoA reductase activity,GO:0050662=coenzyme binding,	Eucgr.G00052	scaffold_7	508528	synonymous_variant, downstream_gene_variant
	AT4G09810	Nucleotide-sugar transport	GO:0008643=carbohydrate transport,GO:00157	GO:0005794=Golgi apparatus,GO:000	GO:0005338=nucleotide-sugar transmembrane	Eucgr.I00300	scaffold_9	5838181	upstream_gene_variant, intergenic_re

		er family protein(AT4G09810)	80=nucleotide-sugar transport,	5886=plasma membrane,GO:0016020=membrane,GO:0016021=integral component of membrane,	transporter activity,GO:0022857=transmembrane transporter activity,				gion
	AT5G54160	O-methyltransferase 1(OMT1)	GO:0009809=lignin biosynthetic process,GO:0032259=methylation,GO:0051555=flavonol biosynthetic process,	GO:0005634=nucleus,GO:0005737=cytoplasm,GO:0005829=cytosol,GO:0005886=plasma membrane,GO:0009506=plasmodesma,	GO:0030744=luteolin O-methyltransferase activity,GO:0030755=quercetin 3-O-methyltransferase activity,GO:0033799=myricetin 3'-O-methyltransferase activity,GO:0046983=protein dimerization activity,GO:0047763=caffeate O-methyltransferase activity,	Eucgr.H00350	scaffold_2	7101444	upstream_gene_variant, intergenic_region
	AT1G43080	Pectin lyase-like superfamily protein(A	GO:0005975=carbohydrate metabolic process,GO:0071555=cell wall organization,	GO:0005576=extracellular region,	GO:0004650=polygalacturonase activity,GO:0016829=lyase activity,	Eucgr.F00632	scaffold_6	8330498	splice_region_variant&intron_variant, downstream

		T1G43080)							_gene_variant
	AT2G15460	Pectin lyase-like superfamily protein(AT2G15460)	GO:0005975=carbohydrate metabolic process,GO:0071555=cell wall organization,	GO:0005576=extracellular region,	GO:0004650=polygalacturonase activity,	Eucgr.F00634	scaffold_6	8342722	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT3G57790	Pectin lyase-like superfamily protein(AT3G57790)	GO:0005975=carbohydrate metabolic process,	GO:0005576=extracellular region,GO:0005774=vacuolar membrane,	GO:0004650=polygalacturonase activity,GO:0016829=lyase activity,	Eucgr.K01816	scaffold_11	22522764	synonymous_variant
	AT4G18180	Pectin lyase-like superfamily protein(AT4G18180)	GO:0005975=carbohydrate metabolic process,GO:0071555=cell wall organization,	GO:0005576=extracellular region,	GO:0004650=polygalacturonase activity,	Eucgr.C02249	scaffold_3	41618896	upstream_gene_variant, intergenic_region
	AT4G23500	Pectin lyase-like superfamily protein(AT4G23500)	GO:0005975=carbohydrate metabolic process,	GO:0005576=extracellular region,GO:0005618=cell wall,	GO:0004650=polygalacturonase activity,	Eucgr.D01821	scaffold_4	32209884	downstream_gene_variant, intron_variant
	AT1G565	Plant neutral	GO:0005987=sucrose catabolic	GO:0005739=mitochondrion,	GO:0004564=beta-	Eucgr.H0330	scaffold_8	48502830	downstream_gene_varia

	60	invertase family protein(A/N-InvA)	process,GO:0042542=response to hydrogen peroxide,GO:0048364=root development,		fructofuranosidase activity,GO:0004575=sucrose alpha-glucosidase activity,GO:0033926=glycopeptide alpha-N-acetylgalactosaminidase activity,	8			nt
	AT1G72000	Plant neutral invertase family protein(A/N-InvF)	GO:0005975=carbohydrate metabolic process,	-	GO:0004564=beta-fructofuranosidase activity,GO:0004575=sucrose alpha-glucosidase activity,GO:0033926=glycopeptide alpha-N-acetylgalactosaminidase activity,	Eucgr.D02388	scaffold_4	37749691	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT5G16490	ROP-interactive CRIB motif-containing protein 4(RIC4)	GO:0007275=multicellular organism development,GO:0009860=pollen tube growth,GO:0010215=cellulose microfibril organization,GO:00	GO:0005886=plasma membrane,GO:0009507=chloroplast,GO:0016324=apical plasma membrane,	GO:0005515=protein binding,	Eucgr.H00463	scaffold_10	12522111	downstream_gene_variant, intron_variant

			17157=regulation of exocytosis,GO:0030833=regulation of actin filament polymerization,GO:0040008=regulation of growth,GO:0051650=establishment of vesicle localization,						
	AT2G35840	Sucrose-6F-phosphate phosphohydrolase family protein(AT2G35840)	GO:0005986=sucrose biosynthetic process,GO:0046686=response to cadmium ion,	GO:0005634=nucleus,GO:0005737=cytoplasm,GO:0005829=cytosol, GO:0009506=plasmodesma,	GO:0000287=magnesium ion binding,GO:0050307=sucrose-phosphate phosphatase activity,	Eucgr.G02659	scaffold_7	44841145	missense_variant, 5_prime_UTR_variant, downstream_gene_variant
	AT1G32900	UDP-Glycosyltransferase superfamily protein(GBSS1)	GO:0019252=starch biosynthetic process,	GO:0009501=amyloplast,GO:0009507=chloroplast, GO:0009569=chloroplast starch grain,	GO:0004373=glycogen (starch) synthase activity,GO:0005515=protein binding,GO:0016757=transferase activity, transferring glycosyl groups,GO:0033	Eucgr.E01068	scaffold_5	11431833	5_prime_UTR_variant, intron_variant

					840=NDP-glucose-starch glucosyltransferase activity,				
	AT3G07020	UDP-Glycosyltransferase superfamily protein(SGT)	GO:0009631=cold acclimation,GO:0009813=flavonoid biosynthetic process,GO:0016125=sterol metabolic process,GO:0016126=sterol biosynthetic process,GO:0030244=cellulose biosynthetic process,GO:0030259=lipid glycosylation,GO:0048316=seed development,GO:0052696=flavonoid glucuronidation,	GO:0005886=plasma membrane,GO:0016020=membrane,GO:0043231=intracellular membrane-bounded organelle,	GO:0016757=transferase activity, transferring glycosyl groups,GO:0016758=transferase activity, transferring hexosyl groups,GO:0016906=sterol 3-beta-glucosyltransferase activity,GO:0051507=beta-sitosterol UDP-glucosyltransferase activity,	Eucgr.H01372	scaffold_8	16420801	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT3G24503	aldehyde dehydrogenase 2C4(ALDH2C4)	GO:0009699=phenylpropanoid biosynthetic process,GO:0055114=oxidation-reduction process,	GO:0005737=cytoplasm,GO:0005829=cytosol,	GO:0004028=3-chloroallyl aldehyde dehydrogenase activity,GO:0004029=aldehyde dehydrogenase (NAD) activity,GO:0050	Eucgr.C03856	scaffold_2	49434876	synonymous_variant, intron_variant

					269=coniferyl-aldehyde dehydrogenase activity,				
	AT5G22510	alkaline/ neutral invertase (INV-E)	GO:0005982=starch metabolic process,GO:0005987=sucrose catabolic process,GO:0048825=cotyledon development,	GO:0009507=chloroplast,	GO:0004564=beta-fructofuranosidase activity,GO:0004575=sucrose alpha-glucosidase activity,GO:0033926=glycopeptide alpha-N-acetylgalactosaminidase activity,	Eucgr.J00457	scaffold_10	4943071	downstream_gene_variant, intron_variant
	AT5G44030	cellulose synthase A4(CESA4)	GO:0009832=plant-type cell wall biogenesis,GO:0009834=plant-type secondary cell wall biogenesis,GO:0009863=salicylic acid mediated signaling pathway,GO:0009867=jasmonic acid mediated signaling pathway,GO:0009873=ethylene-activated signaling pathway,GO:00302	GO:0005618=cell wall,GO:0005886=plasma membrane,GO:0016021=integral component of membrane,	GO:0000977=RNA polymerase II regulatory region sequence-specific DNA binding,GO:0005515=protein binding,GO:0016757=transferase activity, transferring glycosyl groups,GO:0016759=cellulose synthase	Eucgr.A01324	scaffold_1	21280045	downstream_gene_variant, intron_variant

			44=cellulose biosynthetic process,GO:0042742=defense response to bacterium,GO:0050832=defense response to fungus,GO:0052386=cell wall thickening,GO:0071555=cell wall organization,		activity,GO:0016760=cellulose synthase (UDP-forming) activity,GO:0046872=metal ion binding,				
	AT2G21770	cellulose synthase A9(CESA9)	GO:0009832=plant-type cell wall biogenesis,GO:0009833=plant-type primary cell wall biogenesis,GO:0010214=seed coat development,GO:0030244=cellulose biosynthetic process,GO:0071555=cell wall organization,	GO:0005794=Golgi apparatus,GO:0005886=plasma membrane,GO:0016021=integral component of membrane,	GO:0000977=RNA polymerase II regulatory region sequence-specific DNA binding,GO:0016757=transferase activity, transferring glycosyl groups,GO:0016759=cellulose synthase activity,GO:0016760=cellulose synthase (UDP-forming) activity,GO:0046872=metal ion	Eucgr.I00286	scaffold_6	44742158	upstream_gene_variant, intergenic_region

					binding,				
	AT2G33100	cellulose synthase-like D1(CSLD1)	GO:0000271=polysaccharide biosynthetic process,GO:0009832=plant-type cell wall biogenesis,GO:0009846=pollen germination,GO:0030244=cellulose biosynthetic process,GO:0071555=cell wall organization,	GO:0000139=Golgi membrane,GO:0005794=Golgi apparatus,GO:0005886=plasma membrane,GO:0016021=integral component of membrane,	GO:0000977=RNA polymerase II regulatory region sequence-specific DNA binding,GO:0016757=transferase activity, transferring glycosyl groups,GO:0016759=cellulose synthase activity,GO:0016760=cellulose synthase (UDP-forming) activity,GO:0051753=mannan synthase activity,	Eucgr.H00079	scaffold_6	23439036	missense_variant, downstream_gene_variant
	AT3G03220	expansin A13(EXPA13)	GO:0009664=plant-type cell wall organization,GO:0009826=unidimensional cell growth,GO:0009828=plant-type cell wall loosening,GO:0009831=plant-type cell wall modification	GO:0005576=extracellular region,GO:0005618=cell wall,GO:0016020=membrane,	-	Eucgr.F02908	scaffold_2	44670347	upstream_gene_variant, intergenic_region

			involved in multidimensional cell growth,						
	AT4G36220	ferulic acid 5-hydroxylase 1(FAH1)	GO:0009699=phenylpropanoid biosynthetic process,GO:0009809=lignin biosynthetic process,GO:0010224=response to UV-B,GO:0055114=oxidation-reduction process,	GO:0005783=endoplasmic reticulum,GO:0016020=membrane, GO:0016021=integral component of membrane,	GO:0004497=monooxygenase activity,GO:0005506=iron ion binding,GO:0005515=protein binding,GO:0016709=oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen,GO:0020037=heme binding,GO:0046424=ferulate 5-hydroxylase activity,	Eucgr.B00712	scaffold_6	20216726	5_prime_UTR_variant, intergenic_region
	AT5G40760	glucose-6-phosphate	GO:0006006=glucose metabolic process,GO:0006098=pentose-	GO:0005737=cytoplasm,GO:0005829=cytosol,	GO:0004345=glucose-6-phosphate dehydrogenase activity,GO:0050	Eucgr.I01691	scaffold_6	12998540	intron_variant

		dehydrogenase 6(G6PD6)	phosphate shunt,GO:0009051=pentose-phosphate shunt, oxidative branch,GO:0055114=oxidation-reduction process,		661=NADP binding,				
	AT1G61800	glucose-6-phosphate/phosphate translocator 2(GPT2)	GO:0007276=gamete generation,GO:0008643=carbohydrate transport,GO:0009624=response to nematode,GO:0009643=photosynthetic acclimation,GO:0009744=response to sucrose,GO:0009749=response to glucose,GO:0010109=regulation of photosynthesis,GO:0015712=hexose phosphate transport,GO:0015713=phosphoglycerate transport,GO:0015714=phosphoenolpyruvate transport,GO:0015760=glucose-6-	GO:0005774=vacuolar membrane,GO:0009507=chloroplast,GO:0016021=integral component of membrane,GO:0031969=chloroplast membrane,	GO:0005315=inorganic phosphate transmembrane transporter activity,GO:0015120=phosphoglycerate transmembrane transporter activity,GO:0015152=glucose-6-phosphate transmembrane transporter activity,GO:0015297=antiporter activity,GO:0071917=triose-phosphate transmembrane transporter activity,	Eucgr.F00969	scaffold_6	46970158	upstream_gene_variant,intergenic_region

			phosphate transport,GO:0015979=photosynthesis, GO:0035436=triose phosphate transmembrane transport,GO:0080167=response to karrikin,						
	AT1G18140	laccase 1(LAC1)	GO:0009809=lignin biosynthetic process,GO:0046274=lignin catabolic process,GO:0055114=oxidation-reduction process,	GO:0005576=extracellular region,GO:0048046=apoplast,	GO:0005507=copper ion binding,GO:0016722=oxidoreductase activity, oxidizing metal ions,GO:0052716=hydroquinone: oxygen oxidoreductase activity,	Eucgr.B00870	scaffold_6	49797041	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT5G09360	laccase 14(LAC14)	GO:0009809=lignin biosynthetic process,GO:0046274=lignin catabolic process,GO:0055114=oxidation-reduction process,	GO:0005576=extracellular region,GO:0048046=apoplast,	GO:0005507=copper ion binding,GO:0016722=oxidoreductase activity, oxidizing metal ions,GO:0052716=hydroquinone: oxygen oxidoreductase activity,	Eucgr.F04159	scaffold_10	5905959	missense_variant, upstream_gene_variant, downstream_gene_variant
	AT5	laccase	GO:0009698=phen	GO:0005576=extr	GO:0005507=co	Eucgr.J	scaffold_	40164928	missense_va

	G60020	17(LAC17)	ylpropanoid metabolic process,GO:0009809=lignin biosynthetic process,GO:0046274=lignin catabolic process,GO:0055114=oxidation-reduction process,	acellular region,GO:0048046=apoplast,	pper ion binding,GO:0016491=oxidoreductase activity,GO:0016722=oxidoreductase activity, oxidizing metal ions,GO:0052716=hydroquinone: oxygen oxidoreductase activity,	00532	6		riant, 5_prime_UTR_variant
	AT1G43130	like COV2(LCV2)	GO:0010222=stem vascular tissue pattern formation,	GO:0005768=endosome,GO:0005794=Golgi apparatus,GO:0005802=trans-Golgi network,GO:0016021=integral component of membrane,	-	Eucgr.I00289	scaffold_9	5637172	synonymous_variant
	AT1G32100	pinoresinol reductase 1(PRR1)	GO:0009807=lignan biosynthetic process,GO:0055114=oxidation-reduction process,	GO:0005737=cytoplasm,	GO:0010283=pinoresinol reductase activity,	Eucgr.E01253	scaffold_5	13580914	upstream_gene_variant,intergenic_region
	AT3G18830	polyol/monosaccharide transporter	GO:0010311=lateral root formation,GO:0046323=glucose import,	GO:0005886=plasma membrane,GO:0005887=integral component of	GO:0005351=sugar:proton symporter activity,GO:0005354=galactose	Eucgr.G00453	scaffold_6	36375946	upstream_gene_variant, intron_variant

		5(PMT5)		plasma membrane,GO:0016020=membrane,	transmembrane transporter activity,GO:0005355=glucose transmembrane transporter activity,GO:0005365=myo-inositol transmembrane transporter activity,GO:0015144=carbohydrate transmembrane transporter activity,GO:0015145=monosaccharide transmembrane transporter activity,GO:0015148=D-xylose transmembrane transporter activity,GO:0015168=glycerol transmembrane transporter activity,GO:0015575=mannitol transmembrane transporter activity,GO:0015				

					576=sorbitol transmembrane transporter activity,GO:0015591=D-ribose transmembrane transporter activity,				
	AT1G17020	senescence-related gene 1(SRG1)	GO:0009813=flavonoid biosynthetic process,GO:0010150=leaf senescence,GO:0010260=organ senescence,GO:0055114=oxidation-reduction process,	GO:0005737=cytoplasm,	GO:0016682=oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor,GO:0016706=oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors,GO:0046872=metal ion	Eucgr.I00575	scaffold_9	11861661	upstream_gene_variant, intergenic_region

					binding,GO:0051213=dioxygenase activity,				
	AT1G04920	sucrose phosphate synthase 3F(SPS3F)	GO:0005985=sucrose metabolic process,GO:0005986=sucrose biosynthetic process,GO:0008299=isoprenoid biosynthetic process,	GO:0005634=nucleus,GO:0005794=Golgi apparatus,	GO:0016157=sucrose synthase activity,GO:0016757=transferase activity, transferring glycosyl groups,GO:0046524=sucrose-phosphate synthase activity,GO:0046872=metal ion binding,GO:0052924=all-trans-nonaprenyl-diphosphate synthase (geranylgeranyl-diphosphate specific) activity,	Eucgr.H00041	scaffold_8	406606	synonymous_variant
	AT5G06700	trichome birefringence-like protein (DUF828)(AT5G06700)	GO:0009827=plant-type cell wall modification,GO:0009834=plant-type secondary cell wall biogenesis,GO:0030244=cellulose biosynthetic	GO:0005634=nucleus,GO:0005768=endosome,GO:0005794=Golgi apparatus,GO:0005802=trans-Golgi network,GO:0016021=integral	GO:0016413=O-acetyltransferase activity,	Eucgr.B03636	scaffold_2	60745790	downstream_gene_variant, intergenic_region

			process,GO:0045489=pectin biosynthetic process,	component of membrane,					
B Population	AT4G16330	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein(AT4G16330)	GO:0009813=flavonoid biosynthetic process,GO:0055114=oxidation-reduction process,	GO:0005737=cytoplasm,GO:0005777=peroxisome,GO:0009507=chloroplast,	GO:0016491=oxidoreductase activity,GO:0016706=oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors,GO:0046872=metal ion binding,GO:0051213=dioxygenase activity,	Eucgr.B01527	scaffold_2	25322475	downstream_gene_variant, intron_variant
	AT1G65060	4-coumarate:CoA ligase 3(4CL3)	GO:0008152=metabolic process,GO:0009411=response to UV,GO:0009611=response to wounding,GO:0009	GO:0016021=integral component of membrane,	GO:0005524=ATP binding,GO:0016207=4-coumarate-CoA ligase activity,GO:0016	Eucgr.K00087	scaffold_11	1528275	downstream_gene_variant, intron_variant

			698=phenylpropanoid metabolic process,GO:0010584=pollen exine formation,GO:0050832=defense response to fungus,		874=ligase activity,				
	AT5G63380	AMP-dependent synthetase and ligase family protein(AT5G63380)	GO:0006633=fatty acid biosynthetic process,GO:0009695=jasmonic acid biosynthetic process,GO:0009850=auxin metabolic process,GO:0009851=auxin biosynthetic process,GO:0031408=oxylipin biosynthetic process,	GO:0005777=peroxisome,	GO:0004321=fatty-acyl-CoA synthase activity,GO:0005524=ATP binding,GO:0016207=4-coumarate-CoA ligase activity,GO:0016874=ligase activity,	Eucgr.B00135	scaffold_2	2223114	intron_variant
	AT4G16120	COBRA-like protein-7 precursor (COBL7)	GO:0010215=cellulose microfibril organization,GO:0016049=cell growth,	GO:0005768=endosome,GO:0005783=endoplasmic reticulum,GO:0005794=Golgi apparatus,GO:0005802=trans-Golgi network,GO:0005886=plasma membrane,GO:0009506=plasmodes	-	Eucgr.H04021	scaffold_8	58090873	synonymous_variant, upstream_gene_variant, downstream_gene_variant

				ma,GO:0031225=anchored component of membrane,GO:0046658=anchored component of plasma membrane,					
	AT4G07960	Cellulose-synthase-like C12(CSLC12)	GO:0071555=cell wall organization,	GO:0000139=Golgi membrane,GO:0005794=Golgi apparatus,GO:0009506=plasmodesma,GO:0016021=integral component of membrane,	GO:0016740=transferase activity,GO:0016757=transferase activity, transferring glycosyl groups,GO:0016759=cellulose synthase activity,	Eucgr.F00101	scaffold_6	1886751	downstream_gene_variant, intron_variant
	AT3G15350	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein(AT3G15350)	GO:0016051=carbohydrate biosynthetic process,	GO:0005794=Golgi apparatus,GO:0016020=membrane, GO:0016021=integral component of membrane,	GO:0008375=acetylglucosaminyltransferase activity,GO:0016757=transferase activity, transferring glycosyl groups,	Eucgr.F01131	scaffold_6	14484288	3_prime_UTR_variant
	AT5G60490	FASCICLIN-like arabinog	GO:0009834=plant-type secondary cell wall biogenesis,	GO:0005886=plasma membrane,GO:00	-	Eucgr.J00938	scaffold_10	10258407	missense_variant

		alactan-protein 12(FLA12)		31225=anchored component of membrane,					
	AT5G66530	Galactose mutarotase-like superfamily protein(AT5G66530)	GO:0005975=carbohydrate metabolic process,GO:0006012=galactose metabolic process,	GO:0009507=chloroplast,GO:0009570=chloroplast stroma,GO:0048046=apoplast,	GO:0004034=aldose 1-epimerase activity,GO:0016853=isomerase activity,GO:0030246=carbohydrate binding,	Eucgr.H03316	scaffold_8	48555975	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT5G41040	HXXXD-type acyl-transferase family protein(RWP1)	GO:0010345=suberin biosynthetic process,GO:0052325=cell wall pectin biosynthetic process,	GO:0005737=cytoplasm,	GO:0016740=transferase activity,GO:0016747=transferase activity, transferring acyl groups other than amino-acyl groups,GO:0050734=hydroxycinnamoyltransferase activity,	Eucgr.A01022	scaffold_1	15866153	upstream_gene_variant, intergenic_region
	AT1G75280	NmrA-like negative transcriptional regulator family	GO:0006979=response to oxidative stress,GO:0046686=response to cadmium ion,GO:0055114=oxidation-reduction	GO:0005737=cytoplasm,GO:0005886=plasma membrane,	GO:0016491=oxidoreductase activity,	Eucgr.K02656	scaffold_11	34491357	downstream_gene_variant, intergenic_region

		protein(AT1G75280)	process,						
	AT5G54160	O-methyltransferase 1(OMT1)	GO:0009809=lignin biosynthetic process,GO:0032259=methylation,GO:0051555=flavonol biosynthetic process,	GO:0005634=nucleus,GO:0005737=cytoplasm,GO:0005829=cytosol, GO:0005886=plasma membrane,GO:0009506=plasmodesma,	GO:0030744=luteolin O-methyltransferase activity,GO:0030755=quercetin 3-O-methyltransferase activity,GO:0033799=myricetin 3'-O-methyltransferase activity,GO:0046983=protein dimerization activity,GO:0047763=caffeate O-methyltransferase activity,	Eucgr.B01747	scaffold_2	31980853	downstream_gene_variant, intergenic_region
	AT1G43080	Pectin lyase-like superfamily protein(AT1G43080)	GO:0005975=carbohydrate metabolic process,GO:0071555=cell wall organization,	GO:0005576=extracellular region,	GO:0004650=polygalacturonase activity,GO:0016829=lyase activity,	Eucgr.F00632	scaffold_6	8330498	splice_region_variant&intron_variant, downstream_gene_variant
	AT4	Pectin	GO:0005975=carbo	GO:0005576=extr	GO:0004650=pol	Eucgr.	scaffold_	41618896	upstream_ge

	G18180	lyase-like superfamily protein(AT4G18180)	hydrate metabolic process,GO:0071555=cell wall organization,	acellular region,	ygalacturonase activity,	C02249	3		ne_variant, intergenic_region
	AT1G12000	Phosphofructokinase family protein(AT1G12000)	GO:0006002=fructose 6-phosphate metabolic process,GO:0006096=glycolytic process,GO:0009735=response to cytokinin,GO:0015979=photosynthesis,GO:0046686=response to cadmium ion,	GO:0005618=cell wall,GO:0005737=cytoplasm,GO:0005829=cytosol, GO:0010318=pyrophosphate-dependent phosphofructokinase complex, beta-subunit complex,GO:0016020=membrane,	GO:0003872=6-phosphofructokinase activity,GO:0005524=ATP binding,GO:0046872=metal ion binding,GO:0047334=diphosphate-fructose-6-phosphate 1-phosphotransferase activity,	Eucgr.K00768	scaffold_11	8820004	downstream_gene_variant,intergenic_region intron_variant
	AT4G26220	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein(AT4G2622	GO:0009809=lignin biosynthetic process,GO:0032259=methylation,	GO:0005829=cytosol,	GO:0042409=caffeoyl-CoA O-methyltransferase activity,GO:0046872=metal ion binding,	Eucgr.C00927	scaffold_3	14601440	upstream_gene_variant, intergenic_region

		0)							
	AT2G35840	Sucrose-6F-phosphate phosphohydrolase family protein(AT2G35840)	GO:0005986=sucrose biosynthetic process,GO:0046686=response to cadmium ion,	GO:0005634=nucleus,GO:0005737=cytoplasm,GO:0005829=cytosol, GO:0009506=plasmodesma,	GO:0000287=magnesium ion binding,GO:0050307=sucrose-phosphate phosphatase activity,	Eucgr.G02659	scaffold_7	44841145	missense_variant, 5_prime_UTR_variant, downstream_gene_variant
	AT1G12780	UDP-D-glucose/ UDP-D-galactose 4-epimerase 1(UGE1)	GO:0006012=galactose metabolic process,GO:0033358=UDP-L-arabinose biosynthetic process,GO:0045227=capsule polysaccharide biosynthetic process,GO:0046369=galactose biosynthetic process,GO:0071555=cell wall organization,	GO:0005794=Golgi apparatus,GO:0005829=cytosol,GO:0005886=plasma membrane,	GO:0003978=UDP-glucose 4-epimerase activity,GO:0046983=protein dimerization activity,GO:0050373=UDP-arabinose 4-epimerase activity,	Eucgr.D01906	scaffold_4	33014026	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT4G00110	UDP-D-glucuronate 4-epimerase 3(GAE3)	GO:0005975=carbohydrate metabolic process,GO:0009225=nucleotide-sugar metabolic process,	GO:0005794=Golgi apparatus,GO:0016020=membrane, GO:0016021=integral component of	GO:0003824=catalytic activity,GO:0050378=UDP-glucuronate 4-epimerase	Eucgr.E00471	scaffold_5	4457971	upstream_gene_variant, downstream_gene_variant, intergenic_re

				membrane,GO:0032580=Golgi cisterna membrane,	activity,				gion
	AT4G39410	WRKY DNA-binding protein 13(WRKY13)	GO:0006351=transcription, DNA-templated,GO:0006355=regulation of transcription, DNA-templated,GO:0045893=positive regulation of transcription, DNA-templated,GO:1901141=regulation of lignin biosynthetic process,GO:1904369=positive regulation of sclerenchyma cell differentiation,	GO:0005634=nucleus,	GO:0001046=core promoter sequence-specific DNA binding,GO:0003700=transcription factor activity, sequence-specific DNA binding,GO:0043565=sequence-specific DNA binding,GO:0044212=transcription regulatory region DNA binding,	Eucgr.I00305	scaffold_9	5904062	synonymous_variant
	AT2G38470	WRKY DNA-binding protein 33(WRKY33)	GO:0006351=transcription, DNA-templated,GO:0006355=regulation of transcription, DNA-templated,GO:0006970=response to osmotic stress,GO:0009408=response to heat,GO:0009409=r	GO:0005634=nucleus,	GO:0003700=transcription factor activity, sequence-specific DNA binding,GO:0005515=protein binding,GO:0043565=sequence-specific DNA binding,GO:0044	Eucgr.B01415	scaffold_11	37460733	missense_variant, downstream_gene_variant

			esponse to cold,GO:0009414=response to water deprivation,GO:0009651=response to salt stress,GO:0010120=camalexin biosynthetic process,GO:0010200=response to chitin,GO:0010508=positive regulation of autophagy,GO:0034605=cellular response to heat,GO:0042742=defense response to bacterium,GO:0050832=defense response to fungus,GO:0070370=cellular heat acclimation,		212=transcription regulatory region DNA binding,				
	AT3G48000	aldehyde dehydrogenase 2B4(ALDH2B4)	GO:0046686=response to cadmium ion,GO:0055114=oxidation-reduction process,	GO:0005739=mitochondrion,GO:0005759=mitochondrial matrix,GO:0009507=chloroplast,	GO:0004028=3-chloroallyl aldehyde dehydrogenase activity,GO:0004029=aldehyde dehydrogenase	Eucgr.B00357	scaffold_2	4871563	downstream_gene_variant, intron_variant

					(NAD) activity,GO:0005524=ATP binding,				
	AT1G61820	beta glucosidase 46(BGLU46)	GO:0005975=carbohydrate metabolic process,GO:0009809=lignin biosynthetic process,GO:1901657=glycosyl compound metabolic process,	GO:0005576=extracellular region,	GO:0004553=hydrolase activity, hydrolyzing O-glycosyl compounds,GO:0008422=beta-glucosidase activity,GO:0047782=coniferin beta-glucosidase activity,GO:0102483=scopolin beta-glucosidase activity,	Eucgr.H00071	scaffold_8	681223	upstream_gene_variant, intergenic_region
	AT5G64740	cellulose synthase 6(CESA6)	GO:0009832=plant-type cell wall biogenesis,GO:0009833=plant-type primary cell wall biogenesis,GO:0016049=cell growth,GO:0030244=cellulose biosynthetic process,GO:0043622=cortical microtubule organization,GO:00	GO:0005794=Golgi apparatus,GO:0005886=plasma membrane,GO:0010330=cellulose synthase complex,GO:0016020=membrane, GO:0016021=integral component of membrane,	GO:0000977=RNA polymerase II regulatory region sequence-specific DNA binding,GO:0005515=protein binding,GO:0008270=zinc ion binding,GO:0016757=transferase activity, transferring glycosyl	Eucgr.F04216	scaffold_6	50373294	missense_variant, upstream_gene_variant, downstream_gene_variant

			71555=cell wall organization,		groups,GO:0016759=cellulose synthase activity,GO:0016760=cellulose synthase (UDP-forming) activity,				
	AT2G21770	cellulose synthase A9(CESA9)	GO:0009832=plant-type cell wall biogenesis,GO:0009833=plant-type primary cell wall biogenesis,GO:0010214=seed coat development,GO:0030244=cellulose biosynthetic process,GO:0071555=cell wall organization,	GO:0005794=Golgi apparatus,GO:0005886=plasma membrane,GO:0016021=integral component of membrane,	GO:0000977=RNA polymerase II regulatory region sequence-specific DNA binding,GO:0016757=transferase activity, transferring glycosyl groups,GO:0016759=cellulose synthase activity,GO:0016760=cellulose synthase (UDP-forming) activity,GO:0046872=metal ion binding,	Eucgr.I00286	scaffold_5	11431833	5_prime_UTR_variant, intron_variant
	AT4G37980	cinnamyl alcohol dehydrogenase 7(ELI3-	GO:0009617=response to bacterium,GO:0009809=lignin biosynthetic	GO:0005737=cytoplasm,	GO:0008270=zinc ion binding,GO:0045551=cinnamyl-alcohol	Eucgr.I00570	scaffold_9	11733421	upstream_gene_variant, downstream_gene_variant,

		1)	process,GO:0055114=oxidation-reduction process,		dehydrogenase activity,GO:0052747=sinapyl alcohol dehydrogenase activity,				intergenic_region
	AT4G37990	cinnamyl alcohol dehydrogenase 8(ELI3-2)	GO:0009617=response to bacterium,GO:0009809=lignin biosynthetic process,GO:0055114=oxidation-reduction process,	GO:0005737=cytoplasm,	GO:0008270=zinc ion binding,GO:0045551=cinnamyl-alcohol dehydrogenase activity,GO:0046029=mannitol dehydrogenase activity,GO:0047681=aryl-alcohol dehydrogenase (NADP+) activity,GO:0052747=sinapyl alcohol dehydrogenase activity,	Eucgr.I00571	scaffold_9	11733421	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT4G39330	cinnamyl alcohol dehydrogenase 9(CAD9)	GO:0009735=response to cytokinin,GO:0009809=lignin biosynthetic process,GO:0055114=oxidation-reduction process,	GO:0005737=cytoplasm,GO:0048046=apoplast,	GO:0008270=zinc ion binding,GO:0045551=cinnamyl-alcohol dehydrogenase activity,GO:0052747=sinapyl	Eucgr.H04903	scaffold_8	69713728	downstream_gene_variant, intergenic_region

					alcohol dehydrogenase activity,				
	AT3G14570	glucan synthase-like 4(GSL04)	GO:0006075=(1->3)-beta-D-glucan biosynthetic process,GO:0008360=regulation of cell shape,GO:0071555=cell wall organization,	GO:0000148=1,3-beta-D-glucan synthase complex,GO:0005886=plasma membrane,GO:0016021=integral component of membrane,	GO:0003843=1,3-beta-D-glucan synthase activity,GO:0016757=transferase activity, transferring glycosyl groups,	Eucgr.F01403	scaffold_6	18343193	stop_retained_variant, downstream_gene_variant
	AT5G35790	glucose-6-phosphate dehydrogenase 1(G6PD1)	GO:0006006=glucose metabolic process,GO:0009051=pentose-phosphate shunt, oxidative branch,GO:0055114=oxidation-reduction process,	GO:0009507=chloroplast,GO:0009570=chloroplast stroma,	GO:0004345=glucose-6-phosphate dehydrogenase activity,GO:0005515=protein binding,GO:0050661=NADP binding,	Eucgr.K00618	scaffold_11	6878893	synonymous_variant
	AT5G13110	glucose-6-phosphate dehydrogenase 2(G6PD2)	GO:0006006=glucose metabolic process,GO:0009051=pentose-phosphate shunt, oxidative branch,GO:0055114=oxidation-reduction process,	GO:0009507=chloroplast,GO:0009570=chloroplast stroma,	GO:0004345=glucose-6-phosphate dehydrogenase activity,GO:0050661=NADP binding,	Eucgr.H04609	scaffold_8	66224126	upstream_gene_variant, intergenic_region
	AT1G096	glucuronoxylan 4-	GO:0005976=polysaccharide metabolic	GO:0000139=Golgi	GO:0030775=glucuronoxylan 4-	Eucgr.I02785	scaffold_9	38753558	downstream_gene_varia

	10	O-methyltransferase-like protein (DUF579)(GXM3)	process,GO:0009808=lignin metabolic process,GO:0032259=methylation,GO:0045491=xylan metabolic process,GO:0045492=xylan biosynthetic process,	membrane,GO:0005576=extracellular region,GO:0005794=Golgi apparatus,GO:0016021=integral component of membrane,	O-methyltransferase activity,				nt, intron_variant
	AT3G47340	glutamine-dependent asparagine synthase 1(ASN1)	GO:0006529=asparagine biosynthetic process,GO:0006541=glutamine metabolic process,GO:0009063=cellular amino acid catabolic process,GO:0009646=response to absence of light,GO:0009744=response to sucrose,GO:0009749=response to glucose,GO:0009750=response to fructose,GO:0043617=cellular response to sucrose starvation,GO:0070981=L-asparagine	GO:0005737=cytoplasm,GO:0005829=cytosol,	GO:0004066=asparagine synthase (glutamine-hydrolyzing) activity,GO:0005524=ATP binding,GO:0042803=protein homodimerization activity,	Eucgr.B02239	scaffold_2	43669556	synonymous_variant, downstream_gene_variant,

			biosynthetic process,						
	AT1G12900	glyceraldehyde 3-phosphate dehydrogenase A subunit 2(GAPA-2)	GO:0006006=glucose metabolic process,GO:0006096=glycolytic process,GO:0019253=reductive pentose-phosphate cycle,GO:0055114=oxidation-reduction process,	GO:0005737=cytoplasm,GO:0005829=cytosol,GO:0009507=chloroplast,GO:0009570=chloroplast stroma,GO:0009941=chloroplast envelope,GO:0016020=membrane, GO:0048046=apoplast,	GO:0016620=oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor,GO:0047100=glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating) activity,GO:0050661=NADP binding,GO:0051287=NAD binding,	Eucgr.I01564	scaffold_9	25592539	synonymous_variant,upstream_gene_variant,downstream_gene_variant
	AT2G13650	golgi nucleotide sugar transporter 1(GONST1)	GO:0008643=carbohydrate transport,GO:0015784=GDP-mannose transport,	GO:0000139=Golgi membrane,GO:0005794=Golgi apparatus,GO:0005886=plasma membrane,GO:0016021=integral component of membrane,	GO:0005338=nucleotide-sugar transmembrane transporter activity,GO:0005458=GDP-mannose transmembrane transporter activity,	Eucgr.H04965	scaffold_8	70546209	downstream_gene_variant, intron_variant

	AT1G35710	kinase family with leucine-rich repeat domain-containing protein(AT1G35710)	GO:0006468=protein phosphorylation,GO:0007169=transmembrane receptor protein tyrosine kinase signaling pathway,	GO:0005886=plasma membrane,GO:0016021=integral component of membrane,	GO:0004674=protein serine/threonine kinase activity,GO:0005524=ATP binding,	Eucgr.F02122	scaffold_6	28754127	missense_variant
	AT5G60020	laccase 17(LAC17)	GO:0009698=phenylpropanoid metabolic process,GO:0009809=lignin biosynthetic process,GO:0046274=lignin catabolic process,GO:0055114=oxidation-reduction process,	GO:0005576=extracellular region,GO:0048046=apoplast,	GO:0005507=copper ion binding,GO:0016491=oxidoreductase activity,GO:0016722=oxidoreductase activity, oxidizing metal ions,GO:0052716=hydroquinone: oxygen oxidoreductase activity,	Eucgr.B02438	scaffold_2	46077395	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT5G22130	mannosyltransferase family protein(PNT1)	GO:0006506=GPI anchor biosynthetic process,GO:0009793=embryo development ending in seed	GO:0005739=mitochondrion,GO:0005789=endoplasmic reticulum membrane,GO:0016021=integral	GO:0000030=mannosyltransferase activity,GO:0016757=transferase activity, transferring	Eucgr.H00040	scaffold_8	393341	synonymous_variant, downstream_gene_variant

			dormancy,GO:0009832=plant-type cell wall biogenesis,GO:0030244=cellulose biosynthetic process,GO:0051301=cell division,GO:0097502=mannosylation,	component of membrane,	glycosyl groups,GO:0016758=transferase activity, transferring hexosyl groups,GO:0051751=alpha-1,4-mannosyltransferase activity,				
	AT2G36880	methionine adenosyltransferase 3(MAT3)	GO:0006556=S-adenosylmethionine biosynthetic process,GO:0006730=one-carbon metabolic process,GO:0009809=lignin biosynthetic process,GO:0009860=pollen tube growth,	GO:0005634=nucleus,GO:0005737=cytoplasm,GO:0005829=cytosol, GO:0005886=plasma membrane,GO:0009506=plasmodesma,	GO:0004478=methionine adenosyltransferase activity,GO:0005507=copper ion binding,GO:0005515=protein binding,GO:0005524=ATP binding,	Eucgr.J00380	scaffold_2	31980853	downstream_gene_variant, intergenic_region
	AT5G51830	pfkB-like carbohydrate kinase family protein(AT5G51830)	GO:0006014=D-ribose metabolic process,GO:0019252=starch biosynthetic process,GO:0046686=response to cadmium ion,	GO:0005737=cytoplasm,GO:0005829=cytosol,	GO:0004747=ribokinase activity,GO:0005524=ATP binding,GO:0008865=fructokinase activity,GO:0016301=kinase activity,	Eucgr.K00187	scaffold_11	2277913	downstream_gene_variant, intergenic_region
	AT3	receptor-	GO:0006468=protei	GO:0005886=plas	GO:0004674=pr	Eucgr.I	scaffold_	25902876	upstream_ge

	G02130	like protein kinase 2(RPK2)	n phosphorylation,GO:0007169=transmembrane receptor protein tyrosine kinase signaling pathway,GO:0009409=response to cold,GO:0009414=response to water deprivation,GO:0009808=lignin metabolic process,GO:0009846=pollen germination,GO:0009942=longitudinal axis specification,GO:0009945=radial axis specification,GO:0010073=meristem maintenance,GO:0010152=pollen maturation,GO:0048508=embryonic meristem development,GO:0048653=anther development,GO:0051260=protein homooligomerization,	ma membrane,GO:0016021=integral component of membrane,	otein serine/threonine kinase activity,GO:0005524=ATP binding,GO:0016301=kinase activity,	01593	9		ne_variant, downstream_gene_variant, intergenic_region

	AT4G02280	sucrose synthase 3(SUS3)	GO:0005982=starch metabolic process,GO:0005985=sucrose metabolic process,GO:0005986=sucrose biosynthetic process,GO:0009414=response to water deprivation,GO:0010431=seed maturation,GO:0010555=response to mannitol,	GO:0009507=chloroplast,	GO:0008194=UDP-glycosyltransferase activity,GO:0016157=sucrose synthase activity,GO:0016757=transferase activity, transferring glycosyl groups,	Eucgr.H01094	scaffold_1	21280045	intron_variant, upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT2G18700	trehalose phosphatase/synthase 11(TPS11)	GO:0005992=trehalose biosynthetic process,	GO:0005634=nucleus,GO:0005739=mitochondrion, GO:0005829=cytosol,	GO:0003825=alpha,alpha-trehalose-phosphate synthase (UDP-forming) activity,GO:0004805=trehalose-phosphatase activity,GO:0016757=transferase activity, transferring glycosyl groups,	Eucgr.C00201	scaffold_3	4611609	upstream_gene_variant, downstream_gene_variant, intergenic_region
	AT1G78580	trehalose-6-phosphat	GO:0005991=trehalose metabolic process,GO:000599	GO:0005576=extracellular region,GO:00056	GO:0003825=alpha,alpha-trehalose-	Eucgr.F03232	scaffold_6	41535843	intron_variant

		e synthase(TPS1)	2=trehalose biosynthetic process,GO:0009793=embryo development ending in seed dormancy,GO:0009832=plant-type cell wall biogenesis,GO:0010182=sugar mediated signaling pathway,GO:0051301=cell division,	18=cell wall,GO:0005737=cytoplasm,GO:0005773=vacuole,	phosphate synthase (UDP-forming) activity,GO:0016757=transferase activity, transferring glycosyl groups,				
	AT5G57655	xylose isomerase family protein(AT5G57655)	GO:0005975=carbohydrate metabolic process,GO:0006098=pentose-phosphate shunt,GO:0042732=D-xylose metabolic process,	GO:0005737=cytoplasm,GO:0005773=vacuole,GO:0005774=vacuolar membrane,GO:0005783=endoplasmic reticulum,GO:0005794=Golgi apparatus,GO:0016020=membrane,	GO:0009045=xylose isomerase activity,GO:0046872=metal ion binding,	Eucgr.G01193	scaffold_7	19999011	downstream_gene_variant, intron_variant

CAPÍTULO II: *EUCALYPTUS GLOBULUS* CLONAL POPULATION FINGERPRINTING USING THE EUCHIP60K PIPELINE: REPRODUCIBILITY AND ABILITY

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2.1 ABSTRACT

Breeding populations normally are comprised of hundreds of parents and are the base for the development of new genetic material for propagation and to advance generations of breeding. Errors of clonal identity could have a great impact on expected gain from the breeding programs. In most breeding programs a moderate percentage of genetic material is mislabeled. Currently, this kind of error has been detected using molecular markers, such as microsatellites, and they have demonstrated being efficiently low cost for massive analysis of samples in a forest breeding program. However, they have a very low transferability across labs and the analysis can be time-consuming. In this study, we show the high reproducibility level for genotyping *Eucalyptus globulus* clones and demonstrate the high ability to identify errors between clones using the EUChip60K, a SNP panel with around 60 K SNP markers. Even though EUChip is a multi-species array for *Eucalyptus*, it could be easily applied to an *E. globulus* clonal program. This is the first case of study to evaluate the EUChip performance to evaluate identity errors in a clonal population of *E. globulus*.

Keywords: Clones, SNPs, EUChip60K, *E. globulus.*

Key Message: EUChip60K is a good option to evaluate clonal identity in a breeding population of *E. globulus.*

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2.2 INTRODUCTION

Eucalyptus globulus is one of the ten most widely planted forest species in the world. It was introduced in Chile in the last quarter of the 18th Century. However, it was not until the 1980s that its plantation area increased and genetic improvement programs were initiated in order to breed and select for important commercial traits. Nowadays, *E. globulus* is the second most important forest species in the country, with nearly 500,000 ha of plantations, distributed in the coastal and central valley of the forestland. This species has an intensive clonal breeding program, where the clones are evaluated based on phenotypic data obtained from genetic tests on the field and then, selected clones are propagated for deployment in order to establish commercial plantations. Even though this strategy has been successful until now, the efficiency of the cloning program could be increased by incorporation of molecular marker (MM) analysis in the breeding strategy, for instance by fingerprinting to reduce identity errors or the use of marker assisted selection methods (Muranty et al. 2014). A MM corresponds to a variation at the DNA level, which is detected by a chemical standard laboratory procedure, and then it can be matched to the particular phenotype. It has proven to be an efficient tool implemented to support breeding programs of different forest tree species. Among them, microsatellites (SSRs) are the most commonly used markers for genetic analysis in *Eucalyptus* species (Grattapaglia et al. 2012).

During the last years, advances in next generation sequencing (NGS) technologies have allowed to identify new SSRs and to develop new markers, like single nucleotide polymorphisms (SNPs). SNP markers are biallelic, highly conserved, and they represent the most abundant variations across the genome. Despite their limited expected heterozygosity and polymorphic information, they have an important potential due to their low error rate, high reliability and simple-fast way in which they can be analyzed with, rather than analyzing SSRs (Guichoux et al. 2011, Telfer et al. 2015). In order to apply SNPs marker data into a breeding program, the genotyping process can be performed either by whole-genome resequencing or SNP-chip panels, allowing the analysis of a large number of markers across a wide number of samples. However, SNP-Chip methods have shown a higher data reproducibility for independent experiments, laboratories and studies than reduced genomic representation

strategies as genotyping by sequencing (GBS) (Elshire et al. 2011) for heterozygous genomes where a higher sequence depth is required (Schilling et al. 2014).

SNP-chips have already been described for some forest species as *Populus trichocarpa* (Geraldes et al. 2013), *Populus nigra* (Faivre-Rampant et al. 2016), *Pinus pinaster* (Chancere et al. 2013, Plomion et al. 2016), *Picea glauca* (Pavy et al. 2013) and *Eucalyptus* (Silva-Junior et al. 2015), showing considerable advantages for genetic analysis. In particular, EUChip60K for *Eucalyptus*, is a genome-wide genotyping platform with a large set of polymorphic markers for 14 species including *E. globulus*. Therefore, given the high transferability for SNPs in *Eucalyptus*, previously evaluated by Grattapaglia et al. (2011), we tested if the EUChip60K could be a valuable tool in assisting the clonal *E. globulus* breeding program in Chile.

The objective of this study is to test a commercial SNP chip for quality of the data output in a clonal *E.globulus* population. DNA from 164 clones of *E. globulus* was genotyped by the EUChip60K. The called SNPs were evaluated according to the following pipeline: 1) Reproducibility between biological and technical duplicated samples, 2) Intraclonal and pedigree validation and 3) Comparison of two SNP generation technologies. Although SNPs are coming from a multi-species array, a simple-fast analysis can be performed by the SNP-chip, and the results showed that they were considerably robust with a high resolution and reproducibility for *E. globulus*.

2.3 MATERIAL AND METHODS

2.3.1 Plant material, DNA extraction and genotyping by EUChip60K

This study was carried out with a total of 164 samples of *E. globulus* belonging to a breeding program of the species in Chile. Either bark or leaves for each sample was collected from the field and then was used for the DNA extraction by the Qiagen kit (Hilden, Germany). Quality and quantity of DNA were assessed using the Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). A standard concentration of 15 ng of DNA from each sample was sent to genotyping with the EUChip60K system (GeneSeek, USA) reported by Silva-Junior et al. (2015) and a set

of SNPs distributed across entire genome was obtained from the genotyping process. The allelic profile by SNPs for each sample was used to make the further analyses.

2.3.2 Reproducibility analysis to genotyping by EUChip60K

A subset of 24 samples from the total sampled was used to evaluate the reproducibility of the EUChip60K between biological replicates. In this case, DNA from those samples was purified from leaf tissue in two independent work-groups (12 samples by each group) to compared reproducibility between them. Another subset of 38 samples form the total sampled was analyzed to evaluate the reproducibility between 19 pairs of technical replicates. DNA, in this case, was purified from bark of each sample. The number of missing values from the called SNPs and duplicate markers into the Euchip60K system were considered in theses analysis. Technical replicates correspond to DNA purified from the same sample and biological replicates correspond to DNA purified from different samples for the same clone. The DNA for biological and technical replicates was purified from leaves and bark tissues respectively, so DNA tissue-quality could be compared from those genotyping results.

2.3.3 Intraclonal analyisis and pedigree validation

An intraclonal evaluation was made by a clustering analysis carried out in R 3.0.2 (R Core Team 2013) environment using the Adegenet (Jombart 2008; Jombart and Ahmed 2011) package, evaluating the ability of markers to cluster samples within the clones. The total of correctly matched markers and the error rate between them were reported. The 24 samples from the biological replicates reproducibility analysis were used. Those samples were from six clones distributed between five families. All missing values between samples were removed.

Then, polymorphic markers were also determined within groups of clones and their parents. For this analysis, a total of 74 clones from the total sampled were used. Those clones and distributed between 33 families by the cross of 29 parents that were also part of the set of samples genotyped. The ability to predict the clones of each assessed family, according to their allelic profiles and the percentage of SNPs shared with their parents that were genotyped.

2.3.4 Exploratory comparison analysis between EUChip60K and GBS technology

To identify polymorphic markers, SNPs were filtered according to 1) Missing values (5%). 2) Duplicated SNPs and 3) Monomorphic SNPs. Polymorphic markers discovered for the full set of samples, were compared with a set of 12,328 SNPs previously discovered by GBS in a group of ~500 samples of *E. globulus* distributed between two subset of samples (data not shown). Markers were compared using a Venn diagram tool (Oliveros 2015).

2.4 RESULTS

2.4.1 SNP calling and reproducibility analysis

First of all, total of missing values from the EUChip genotyping was evaluated among samples. A mean of 11,790 (18%) of missing values was identified in the analysis. Afterwards, reproducibility of the called SNPs was evaluated in six clones with two biological replicates that were prepared by two different laboratories. The percentage of exact matched markers between clones was evaluated, resulting in a high percentage of SNPs called (98.8%) that were correctly assigned and there was no difference between laboratories. In fact, when SNPs from each clone-lab were compared, a high percentage of SNPs were also in common (98.3%) (Table 2.1). In addition, SNPs that were called from technical replicates were also evaluated, showing a high percentage of SNPs that matched correctly between samples, with an average of 95.8% correctly assigned (Table 2.2).

Table 2.1 Reproducibility analysis for the called SNPs between biological replicates. The codes *a-b-c-d* correspond to the biological replicates for each clone (1.-6.) from five families, evaluated in two different laboratories (Lab 1 and Lab 2). Total SNPs matched within clones and the corresponding percentages are shown. *Fuente: Elaboración propia.*

	Lab 1			**Lab 2**			**Lab 1-2**	
Family	**Clone**	**SNPs**	**%**	**Clone**	**SNPs**	**%**	**SNPs**	**%**
1	1.a / *1.b*	63,954	98.9	*1.c* / *1.d*	63,980	99	63,507	98.6
2	2.a / *2.b*	63,840	98.8	*2.c* / *2.d*	63,439	98.1	63,046	98.1

3	*3.a* / *3.b*	63,770	98.7	*3.c* / *3.d*	63,545	98.3	63,013	98
3	4.a / *4.b*	63,765	98.6	*4.c* / *4.d*	64,045	99.1	63,401	98.4
4	*5.a* / *5.b*	63,742	98.6	*5.c* / *5.d*	64,147	99.2	63,405	98.3
5	6.a / *6.b*	63,903	98.9	*6.c* / *6.d*	63,930	98.9	63,460	98.6
	Mean	63,829	98.8		63,848	98.8	63,305	98.3

Table 2.2 Reproducibility analysis for the called SNPs between technical replicates. The codes *a* and *b* correspond to the technical replicate for each sample (1-19). Total SNPs matched within clones and percentage of SNPs with match are shown. *Fuente: Elaboración propia.*

Technical replicates			**Technical replicates**		
Sample	**SNPs**	**%**	**Sample**	**SNPs**	**%**
1*a* / 1*b*	63,191	97.8	11*a* / 11*b*	62,213	96.2
2*a* / 2*b*	59,906	92,8	12*a* / 12*b*	63,767	98.7
3*a* / 3*b*	62,538	96.7	13*a* / 13*b*	63,052	97.5
4*a* / 4*b*	63,057	97.6	14*a* / 14*b*	63,189	97.8
5*a* / 5*b*	58,572	90.6	15*a* / 15*b*	63,017	97.5
6*a* / 6*b*	57,431	88.8	16*a* / 16*b*	63,046	97.5
7*a* / 7*b*	62,977	97.4	17*a* / 17*b*	62,969	97.4
8*a*	57,891	89.6	18*a*	63,336	98.0

8*b*			18*b*		
9*a* 9b	58,766	90.9	19*a* 19*b*	62,223	96.3
10*a* 10*b*	63,121	97.7	**Mean**	61,803	95.8

2.4.2 Intraclonal and pedigree validation

An intraclonal validation was made using the recorded clone information for each sample and its SNPs profile from the genotyping process. Total samples were correctly assigned with a probability equal to 1 into the clusters inferred by the analysis (Fig. 2.1). Therefore, SNP profile for each sample allowed its corresponding clonal group.

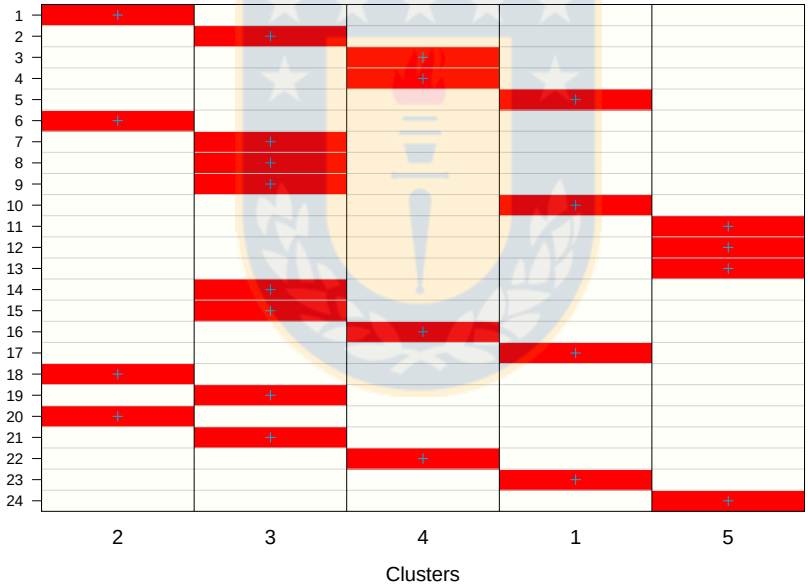

Fig. 2.1 Graphical representation for the clustering analysis where heat-colors represent membership probabilities from 0=white to 1=red for each sample (from 1 to 24) to belong to the cluster (from 1 to 5) inferred and crosses (+) represent the prior clone-cluster provided to each samples analyzed. *Fuente: Elaboración propia.*

Using the data from polymorphic markers within families, the ability of SNPs to verify the progeny of different families was evaluated. The samples with a total of 90% of SNPs correctly assigned were considered to be clones correctly generated for its family. Therefore, samples resulting with an error detected on the SNPs profile above 10% of discrepancy with

their parents were not considered to be the progeny of a particular family. A total of 21 clones distributed between thirteen families showed more than 10% of error rate when comparing their SNPs panel with those obtained for their parents. There were 53 clones distributed among 33 families correctly assigned, but only 20 of those families did not have error between their clones (Fig. 2.2).

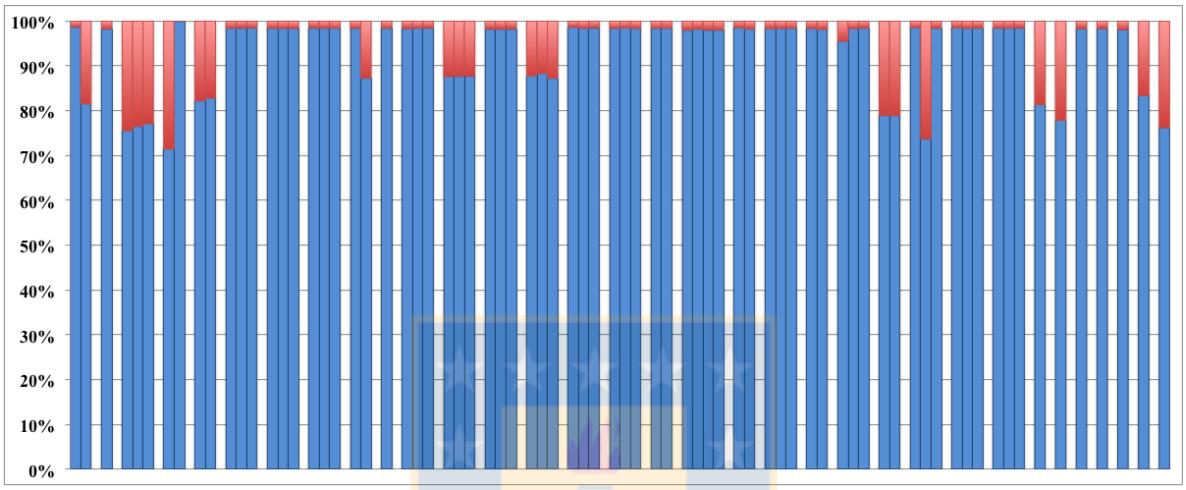

Fig. 2.2 Pedigree validation of 74 clones distributed between 33 families. Each family ranged from one to four clones. Percentage of correctly assigned SNPs is showed in blue bars. Red bars show discrepancy between clones and their parents. *Fuente: Elaboración propia.*

2.4.3 Comparison between EUChip60k and GBS technologies

Polymorphic SNPs were identified and compared. In this case, all the 164 clone-samples were considered for the analysis whatever the pedigree information of each sample was previously documented. Duplicated SNPs from EUChip60K were removed and only SNPs up to 5% of missing values were considered for the analysis, where a missing value corresponded to all those markers without their genotype information from the SNP called. A total of 13,669 SNPs were considered polymorphic with a minimum allele count of five between all samples.

A total of 13,669 polymorphic SNPs was compared with a set of SNPs discovered using GBS technology from two groups (A and B) of clones of *E. globulus*. In the case of GBS system, DNA libraries were sequenced and analyzed by a homemade bioinformatics workflow for marker discovery using *E. grandis* as a reference genome for mapping and SNP calling

process (data not shown). A total of 2,597 polymorphic SNPs were identified by GBS, and only 35 were common between both groups. When GBS-SNPs technologies were compared with the set of polymorphic markers from EUChip60K, 11 and 12 SNPs were common with the A-GBS and B-GBS group respectively, and one SNP was common between under the 3 conditions. Unique SNPs were also found, where a total of 13,645 SNPs were exclusive for EUChip, 1,229 and 1,310 SNPs were found for A and B groups respectively. A graphical representation of this analysis is showed in the Fig. 2.3.

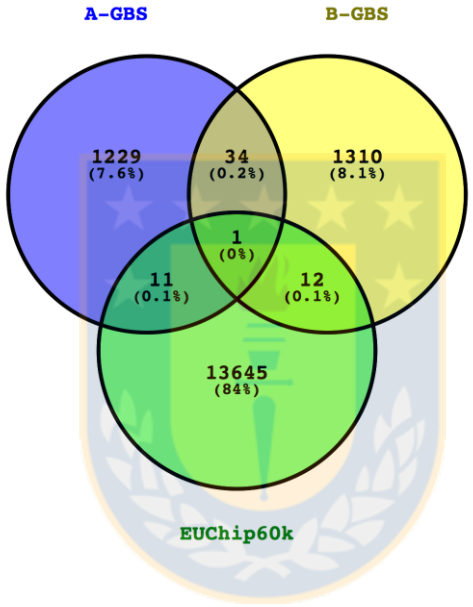

Fig. 2.3 A Venn diagram representing a comparison analysis between polymorphic SNP markers discovered by the EUChip (EUChip60k) and GBS (A-GBS and B-GBS groups) technological approaches. *Fuente: Elaboración propia.*

2.5 DISCUSSION

Fingerprinting is a critical process in plant propagation that has been increasingly assisted by molecular markers (Nybom et al. 2014). During last years the continuous advances in reduced DNA re-sequencing and SNP-chips, have resulted in a massive amount of SNPs that have been fixed in arrays for different crops and non-model species (Yan et al. 2009, Hyten et al. 2010, McCouch et al. 2010, Li et al. 2014) including trees (Livingstone et al. 2015, Bianco et al.2016).

In this study, a set of clones of *E. globulus* was genotyped with 60K SNPs by the EUChip to evaluate the opportunity to use this panel of markers in improvement of the information quality used in the breeding program of the species. The multi-species array displayed had a high performance to identify polymorphic and informative SNPs for *E. globulus*, since it was one of the main species used to create the array. The results obtained are consistent with those previously reported by Silva-Junior et al. (2015) who reported around 19K polymorphic and transferable SNPs across Eucalyptus Symphymyrtus subgenus and later reported by Telfer et al. (2015) the fact that a set of markers selected from EUChip for *E. nitens* were robust and informative. When the chip-reproducibility was evaluated comparing biological and technical replicates, a high percentage of SNPs were correctly matched between samples in both types of replicates. Also, DNA samples extracted from different laboratories did not show any difference in the SNP genotyping results. The DNA was extracted using the same protocol but each protocol had slight modifications according with the laboratory requirements and it did not have an impact in the analysis. When biological and technical replicates were compared, there was a slight difference in the percentage of SNPs matched correctly, considering that DNA was extracted from leaf and bark. Previous studies in our laboratory have shown that bark-DNA has a considerably lower quality than leaf-DNA (data not shown), using DNA quality control by fluorometric quantification and SSR-genotyping, but apparently in this analysis, it would not be affecting the genotyping process. It would not be necessary to have high quality DNA as previously published by others (Bayés and Gut 2011). The number of missing values was very consistent across all called samples, differently to those reported in pine studies where protocols and the quality of DNA had an impact in genotyping (Telfer et al. 2013).

Assignment test performed by SNP marker data was efficient to confirm each group of clones. There were no intraclonal mislabeling according to this analysis. In agreement with this finding, reported studies in cacao for a set of 53 SNPs was evaluated and showed a robust and accurate genotyping for identification of errors between 160 tree clones (Takrama et al 2014). Commercial clonal plantations are more frequently planted, so if mislabeled or incorrectly identified clones are carried to the field, the economical losses could be damaging. An error in

the genotyping process occurs if an observed genotype for an individual does not correspond to the true genotype (Bonin et al. 2004) and can be detected by comparing replicate genotyping (Taberlet et al. 1999). The main causes of genotyping error results either from variation in DNA sequences, low quantity and quality of DNA, biochemical artefacts or human errors (Pompanon et al. 2005). Therefore it is a really good alternative to consider technical replicate samples when a genotyping process is being used, it would reduce probabilities of discarding samples that they were not really off-types and demonstrate that the results obtained are reliable. Parentage analysis can also support the assignment test, is our case, we did not have information about the SNP profile for parents of clones previously evaluated, however, a different set of samples was used, where parents and their progeny were genotyped to evaluated the ability of SNP to identify off-type intraclones, verifying genetic identity and pedigree information. Parent profiles are used as a kind of reference to know if progeny alleles are or are not from parents. Around 28% of clones were mislabeled, having more than 10% of SNPs unmatched between parents and progeny. Sometimes, these levels of mislabeling can arise from contamination of the crosses (Takrama et al. 2012) or missing data. In this study, we considered more than 10% of unmatched SNPs as clonal misidentification, however, it is going to depend to the on the number of markers used for genotyping (Wang et al. 2015). Greenhill et al. (2017) screened 113 clones, represented by 2 or more samples, with 31 SNPs and 24 clones exhibited some kind of mislabeling error.

When polymorphic markers of the EUChip were compared with the set of SNPs obtained from GBS, a low number of them were common between them. The idea was to identify the exact same group of SNPs across independent technologies and consider these markers as a more specific group to *E. globulus*. However, it is difficult and probably impossible considering the sequencing techniques as GBS; not having reference haplotypes considering the high level of heterozygosity and that rare identical by descent (IBD) segments are not available yet (Silva-Junior et al. 2015).

2.6 CONCLUSION

According with the current study, the use of the EUChip as a fingerprint tool in *E. globulus* constitutes a simple, fast and robust technology for clonal identity analysis. The pipeline that

has been presented can be applied to any other tree plant scenario. The EUChip method showed not only a high reproducibility between biological and technical replicates, but it had a very high efficiency to identify off-type samples within clones using two simple profile SNPs analysis that could be applied routinely for checking. Those analyses would be more difficult if a sequencing technique such as GBS was used, considering its lower reproducibility between samples in *Eucalyptus* species (De Faria 2012). Moreover, we have to consider that SNPs can not only be used to fingerprint, since EUChip tool may be applied in marker assisted breeding strategies as genomic selection, where hundreds of thousands of markers are used to fixing a prediction model that allows the early genomic estimation of breeding values of clones. A recent study where EUChip was used for genotyping a set of *E. globulus* clones, showed that 12 K polymorphic SNPs were identified with a good predictive ability results between its models (Duran et al. 2017).

The results have shown a moderate cost-effective way using the EUChip60K, as a new opportunity in a breeding program of *E. globulus.* However, it should be stated that further work needs to be carried out with the EUChip markers panel in a larger set of samples from the breeding program to validate these results.

2.7 REFERENCES

Bayés M, Gut IG (2011) Overview of genotyping. Molecular Analysis and Genome Discovery Second Edition 1:23

Bianco L, Cestaro A, Linsmith G, Muranty H, Denancé C et al (2016) Development and validation of the Axiom® Apple480K SNP genotyping array. The Plant Journal 86:62-74

Bonin A, Bellemain E, Bronken P, Pompanon F, Brochmann C, Taberlet P (2004) How to track and assess genotyping errors in population genetics studies. Molecular ecology, 13:3261-3273

Chancerel E, Lamy JB, Lesur I, Noirot C, Klopp C et al (2013) High-density linkage mapping in a pine tree reveals a genomic region associated with inbreeding depression and provides clues to the extent and distribution of meiotic recombination. BMC Biology 11:50

De Faria, DA, Grattapaglia, D (2014) Milhares de SNPS genotipados por sequenciamento de alto desempenho (GBS-"Genotyping By Sequencing") em espécies de *Eucalyptus*. Heringeriana 6:23-25

Duran R, Isik F, Zapata-Valenzuela J, Balocchi C, Valenzuela S (2017) Genomic predictions of breeding values in a cloned *Eucalyptus globulus* population in Chile. Tree Genet Genomes 13:74. doi:10.1007/s11295-017-1158-4

Elshire RJ, Glaubitz JC, Sun Q et al (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. Plos one 6:e19379. doi:10.1371/journal.pone.0019379

Faivre-Rampant P, Zaina G, Jorge V, Giacomello S, Segura V et al (2016) New resources for genetic studies in *Populus nigra*: genome-wide SNP discovery and development of a 12k Infinium array. Molecular ecology resources 16:1023-1036

Geraldes A, Difazio SP, Slavov GT, Ranjan P, Muchero W et al (2013) A 34k SNP genotyping array for *Populus trichocarpa*: design, application to the study of natural populations and transferability to other populus species. Molecular Ecology Resources 13: 306–323

Grattapaglia D, Vaillancourt RE, Shepherd M, Thumma BR, Foley W et al (2012) Progress in Myrtaceae genetics and genomics: *Eucalyptus* as the pivotal genus. Tree Genet Genomes 8: 463–508

Grattapaglia D, Silva OB, Kirst M, de Lima BM, Faria DA, Pappas GJ (2011) High-throughput SNP genotyping in the highly heterozygous genome of eucalyptus: assay success, polymorphism and transferability across species. BMC Plant Biology 11: 65

Greenhill R, Mollison E, Dowman C, Johnson L et al (2017) Utility of single nucleotide polymorphism markers in clonal profiling of *Hevea brasiliensis*. Acta Horticulturae 429:436

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O et al (2011) Current trends in microsatellite genotyping. Molecular Ecology Resources 11: 591–611. doi: 10.1111/j.1755-0998.2011. 03014.x PMID: 21565126

Hyten DL, Choi IY, Song Q, Specht JE, Carter TE et al (2010) A high density integrated genetic linkage map of soybean and the development of a 1536 universal soy linkage panel for quantitative trait locus mapping. Crop Sci 50:960–968.

Jombart, T (2008) Adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403-1405. doi:10.1093/bioinformatics/btn129

Jombart T, Ahmed I (2011) Adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. Bioinformatics doi: 10.1093/bioinformatics/btr521

Li X, Han Y, Wei Y, Acharya A, Farmer AD et al (2014) Development of an Alfalfa SNP Array and Its Use to Evaluate Patterns of Population Structure and Linkage Disequilibrium. Plos one 9: e84329. doi:10.1371/journal.pone.0084329

Livingstone D, Royaert S, Stack C, Mockaitis K, May G (2015) Making a chocolate chip: development and evaluation of a 6K SNP array for *Theobroma cacao*. DNA research, 22:279-291

McCouch S, Tung CW, Zhao K, Wright M, Kimball J et al (2010) Development of genome-wide SNP assays for rice. Breeding Sci 60:524–535

Muranty H, Jorge V, Bastien C, Lepoittevin C, Bouffier L, Sanchez L (2014) Potential for marker-assisted selection for forest tree breeding: lessons from 20 years of MAS in crops. Tree Genet Genomes 10:1491–1510. doi: 10.1007/s11295-014-0790-5

Nybom, H, Weising K, Rotter B (2014). DNA fingerprinting in botany: past, present, future Investigative genetics 5:1. https://doi.org/10.1186/2041-2223-5-1

Oliveros JC (2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html

Pavy N, Gagnon F, Rigault P, Blais S, Deschenes A et al (2013) Development of high- density SNP genotyping arrays for white spruce (*Picea glauca*) and transferability to subtropical and nordic congeners. Molecular Ecology Resources 13: 324–336

Pompanon F, Bonin A, Bellemain E, Taberlet P (2005) Genotyping errors: causes, consequences and solutions. Nature reviews Genetics 6:847

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Viena, Austria

Schilling MP, Wolf PG, Duffy AM, Rai HS, Rowe CA et al (2014) Genotyping-by-sequencing for *Populus* population genomics: An assessment of genome sampling patterns and filtering approaches. Plos one 9:e95292. doi:10.1371/journal.pone.0095292

Silva-Junior OB, Faria DA, Grattapaglia D (2015) A flexible multi-species genome-wide 60K SNP chip developed from pooled resequencing of 240 *Eucalyptus* tree genomes across 12 species. New Phytol 206:1527–1540. doi: 10.1111/nph.13322

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap Trends Ecol Evol 14:323–327

Takrama J, Dadzie AM, Opoku SY, Padi FK, Adomako B et al (2012) Applying SNP marker technology in the Cacao breeding programme in Ghana. African Crop Science Journal 20:67–75

Takrama J, Kun J, Meinhardt L, Mischke S, Opuku SY (2014) Verification of genetic identity of introduced cacao germplasm in Ghana using single nucleotide polymorphism (SNP) markers. African Journal of Biotechnology 13:2127-2136

Telfer E, Graham N, Stanbra L, Manley T, Wilcox P (2013) Extraction of high purity genomic DNA from pine for use in a high-throughput Genotyping Platform. New Zealand Journal of Forestry Science 43:3

Telfer EJ, Stovold GT, Li Y, Silva-Junior OB, Grattapaglia DG, Dungey HS (2015) Parentage Reconstruction in *Eucalyptus nitens* Using SNPs and Microsatellite Markers: A Comparative Analysis of Marker Data Power and Robustness. Plos one 10:e0130601. doi:10.1371/journal.pone.0130601

Wang B, Tan HW, Fang W, Meinhardt LW, Mischke S, Matsumoto T, Zhang D (2015) Developing single nucleotide polymorphism (SNP) markers from transcriptome sequences for identification of longan (*Dimocarpus longan*) germplasm. Hortic Res 2:14065.

Yan JB, Yang XH, Shah T, Sanchez-Villeda H, Li JS et al (2009) High-throughput SNP genotyping with the GoldenGate assay in maize. Mol Breed 25:441–451

CAPÍTULO III: GENOMIC PREDICTIONS OF BREEDING VALUES IN A CLONED *EUCALYPTUS GLOBULUS* POPULATION IN CHILE

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3.1 ABSTRACT

In Chile, an intensive *Eucalyptus globulus* clonal selection program is being carried out to increase forest productivity for pulp production. A breeding population was used to investigate the predicted ability of single nucleotide polymorphism (SNP) markers for genomic selection (GS). A total of 310 clones from 53 families were used. Stem volume and wood density were measured on all clones. Trees were genotyped at 12 K polymorphic markers using the EUChip60K genotype array. Genomic best linear unbiased prediction, Bayesian lasso regression, Bayes B, and Bayes C models were used to predict genomic estimated breeding values (GEBV). For cross-validation, 260 individuals were sampled for model training and 50 individuals for model validation, using 2 folds and 10 replications each. The average predictive ability estimates for wood density and stem volume across the models were 0.58 and 0.75, respectively. The average rank correlations were 0.59 and 0.71, respectively. Models produced very similar bias for both traits. When clones were ranked based on their GEBV, models had similar phenotypic mean for the top 10% of the clones. The predicted ability of markers will likely decrease if the models are used to predict GEBV of new material coming from the breeding program, because of a different marker–trait phase introduced by recombination. The results should be validated with larger populations and across two generations before routine applications of GS in *E. globulus*. We suggest that GS is a viable strategy to accelerate clonal selection program of *E. globulus* in Chile.

Keywords *E. globulus*, Genomic selection, SNP, Predictive ability, Stem volume, Wood density

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3.2 INTRODUCTION

The *Myrtaceae* genus is generally diploid and has smaller genome than the *Pinaceae* genus (Neale and Kremer 2011). Eucalyptus species have been widely planted in tropical and subtropical regions of the world for production of biomass energy, wood and pulp, being one of the most widely planted species in the world because of its remarkable growth and adaptability (Eldridge et al. 1993; Doughty 2000; Potts et al. 2004). Since its introduction, the species has been subject to breeding and selection in many countries to develop locally adapted populations. The breeding programs aim to select genotypes for superior growth, stem form, and wood quality (Raymond et al. 1998); therefore, it is a target of genomic research, especially for the pulp and paper industry (Borralho et al. 1993; Grattapaglia 2004).

Breeding programs have relied on classical genetic evaluation based on pedigree and progeny testing to predict estimated breeding values (EBV). In *Eucalyptus* spp., the Brazilian breeding program is an example of cloning superior selections within full-sib families to improve the mean of the selected population (Rezende et al. 2013). Although traditional genetic evaluation system and cloned progeny testing have been successful to improve traits of interests in *Eucalyptus globulus* improvement programs, the process takes 12 years to complete and it is costly. Breeders look for new tools to select superior individuals at lower cost in a shorter time to make the breeding programs more efficient.

Marker assisted selection (MAS) was evaluated with the objective to increase the selection efficiency in tree breeding programs for decades. This methodology is based on the detection of QTLs (quantitative trait loci) controlling the phenotype and use them in breeding programs to increase the selection process (Muranty et al. 2014). However, marker assisted selection has not been a useful tool for complex traits due its inability to capture small-effect alleles (Isik 2014; Strauss et al. 1992) and most traits important to forest breeding are complex, controlled by many genes with small effects (Crosbie et al. 2006). It was suggested that compared to linkage mapping to detect QTLs, associative mapping could be more efficient to establish marker–trait associations in outbred species, such as forest trees (Neale and Savolainen 2004). However, it has been difficult to prove that one specific marker is related to a phenotype (Weigel and Nordborg 2005; Isik 2014). For example, genome-wide association studies have

not been successful to capture a large fraction of phenotypic variance (Thumma et al. 2005; Grattapaglia and Resende 2011; Cappa et al. 2013). Therefore, this strategy has failed to impact the improvement of polygenic characteristics of forest species (Zapata-Valenzuela and Hasbun 2011).

In contrast to MAS, genomic selection (GS) uses a dense marker coverage of genome and capture the effect of thousands of markers simultaneously (Meuwissen et al. 2001). Today, with advancements in high-throughput genotyping platforms, GS has been successfully implemented in cattle breeding and has been widely adapted for other major animal and crop breeding programs (Hayes et al. 2009; Heffner et al. 2009; Hayes and Goddard 2010; Jannink et al. 2010; Lin et al. 2014; Meuwissen et al. 2016). The success was mainly attributed to high-throughput DNA sequencing technologies and dramatic drop in DNA sequencing cost. GS assumes that, with a high density of markers, some markers would be in linkage disequilibrium (LD) with at least one QTL distributed on the genome (Goddard and Hayes 2007), so a high level of LD improves the marker-tagged QTL and marker–trait association. Unlike MAS, GS does not use a subset of markers, rather it analyzes the information of all markers in a population to explain the genetic variance from markers across the genome (Heffner et al. 2009; Isik 2014), thus precluding the search for significant marker–trait association. GS overcomes the problem of mapping association where a limited proportion of total genetic variation is captured by each individual marker and the marker effects do not need to exceed a significance threshold to be used to predict breeding values (Hayes et al. 2013; Isik 2014).

GS uses a “training set of individuals” that are phenotyped and genotyped by genome-wide panel of markers to estimate the marker effects. Then, the model is used to predict breeding values in validation set for which genotypes, but no phenotypic data are available (Zapata-Valenzuela et al. 2012). So, these genomic estimated breeding values (GEBV) are predicted only using the marker information (Goddard and Hayes 2009; Isik et al. 2015). To maximize the accuracy, the training population must be representative of selection candidates in the breeding program where GS applied (Heffner et al. 2009; Jonas and de Koning 2013). This approach can offer greater precision than traditional genetic evaluation, as long as the markers are in LD with some QTL that are controlling the phenotypic variation (Calus and Veerkamp

2007) or markers are capturing genetic relationships better than pedigree-based methods. This requires thousands of molecular markers to capture a significant proportion of the additive genetic variance (Heffner et al. 2009). The level of LD between marker and QTL, effective population size, number of individuals in the training population, marker density, and heritability of the traits are considered affecting the accuracy of GS (Grattapaglia and Resende 2011; Isik 2014).

Forest trees are among the species most likely to benefit from using genomic information in breeding programs. Today, sever- al forest tree genomes have been sequenced, including *Populus trichocarpa* (Tuskan et al. 2006), *Pinus taeda*, (Neale et al. 2014), and *Eucalyptus grandis* (Myburg et al. 2014). During the last several years, there have been numerous studies on GS in forest trees species (Zapata-Valenzuela et al. 2012; Resende et al. 2012a, b, c; Zapata-Valenzuela et al. 2013; Beaulieu et al. 2014a, b; Munoz et al. 2014; El-Dien et al. 2015; Ratcliffe et al. 2015; Bartholomé et al. 2016, Isik et al. 2016). For example, Isik et al. (2016) analyzed two generations of *Pinus pinaster* L. breeding population to test the predictive ability of markers. Bartholomé et al. (2016) analyzed three generations for the same species for assessment of the prediction accuracy of the GS model within and across generations. Ratcliffe et al. (2015) explored GS for interior spruce (*Picea engelmannii* × *glauca*). Resende et al. (2012c) reported encouraging results for GS for different species of *Eucalyptus*. Denis and Bouvet (2013) used simulated data for *Eucalyptus* breeding with different levels of heritability, dominance, and additive variance.

This study is the first to test the utility of GS in *E. globulus* for prediction of breeding values. The objectives in this study are to (1) evaluate LD levels between markers by each chromosome, (2) estimate the genomic relationship from markers, and (3) compare various statistical methods for genomic estimated breeding values of wood density and volume of *E. globulus* clones.

3.3 MATERIALS AND METHODS

3.3.1 Plant material and SNP genotyping by EUChip60K

This study was carried out with a breeding population of *E. globulus* owned by Forestal Arauco S.A., Chile. The breeding population consisted of a total of 310 clonal individuals

belonging to 40 full-sib families and 13 half-sib families, produced by crossing 23 parents. Two individuals did not have their parents identified (phenotypic selections). For the DNA extraction, diploid tissue bark or leaves from each individual were collected. DNA was purified using the Qiagen kit (Hilden, Germany) according to the manufacturer's protocol. The quality and quantity of the DNA were assessed using the Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). A genome-wide single nucleotide polymorphism (SNP) dataset was obtained using the EUChip60K system (GeneSeek, USA) proposed by Silva-Junior et al. (2015). Approximately 64 K SNP loci were filtered and processed to obtain about 12 K SNPs. These SNPs represent a group of polymorphic markers for E. globulus. SNPs were distributed across 11 chromosomes (Fig. S3.1). The average number of markers per chromosome was 1136 with a range of 800 to 1564 SNPs per chromosome. Larger chromosomes had more SNPs than smaller chromosomes. The polymorphic information content (PIC) values were in the range of 0.090 to 0.375, and heterozygosity values were in a range of 0.09 to 0.50 (Fig. S3.2).

3.3.2 Experimental design, data collection, and preliminary analysis

In 1990s, superior trees from plantations were grafted for controlled crosses. The clones were generated from the clonal breeding program of the company, after rooting evaluation. Data for the clones used in this study were obtained from the database of the breeding program, which was built as follows: phenotypic data for volume and wood density traits of 3150 eucalypt trees was assessed in 23 field genetic tests growing in one geographic coastal zone at ages ranging from 5 to 11 years. The experimental test design was randomized incomplete blocks with single-tree plots, with border rows. Spacing was 3 × 2 m in all tests. For each tree, the diameter at breast height (cm) using diameter tape and the total height (m) using a Vertex hypsometer were recorded. The data were stored in the field using a PSION data logger model Workabout Pro. Stem volume (m^3) was estimated from a general formula for juvenile trees as volume = 0.00003 × (diameter^2) × (height) (Ladrach 1986). The wood density was predicted by near-infrared (NIR) spectroscopy. A calibration model that included samples of *E. globulus* and *E. nitens*, between 4 and 20 years old, was developed using a partial least-squares algorithm. This method has been used as a fast method for the estimation of many wood properties using NIR spectra collected from milled wood chips (Schimleck 2008). For our

study, due to limited resources, the phenotypic data were available for 310 genotypes for volume trait and 231 genotypes were measured for wood density. Clonal EBVs were obtained by fitting the following linear mixed model:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zu} + \mathbf{e} \qquad (1)$$

where **y** is the vector of phenotypic observations (volume and density), **b** is the vector of the fixed effects intercept, site and block; **u** is the vector of random clone effect; **e** is the vector of random residuals; and **X** and **Z** are the incidence matrices, for the fixed and random effects, respectively. The random clone effect and the residuals have the expectations of $\mathrm{E}(\boldsymbol{u}) \sim \mathrm{iid}(0, \sigma_u^2)$ and $E(\mathbf{e}) \sim \mathrm{iid}(0, \sigma_e^2)$. Model to predict EBVs for density was used to estimate breeding values to samples without density measure. ASReml software (Gilmour et al. 2009) was used to estimated variance components and solve mixed mod- el equations to obtain the EBVs for wood density and volume, that what were not highly correlated (Fig. 3.1). The average broad sense heritability for these tests was 0.29 for volume and 0.46 for wood density.

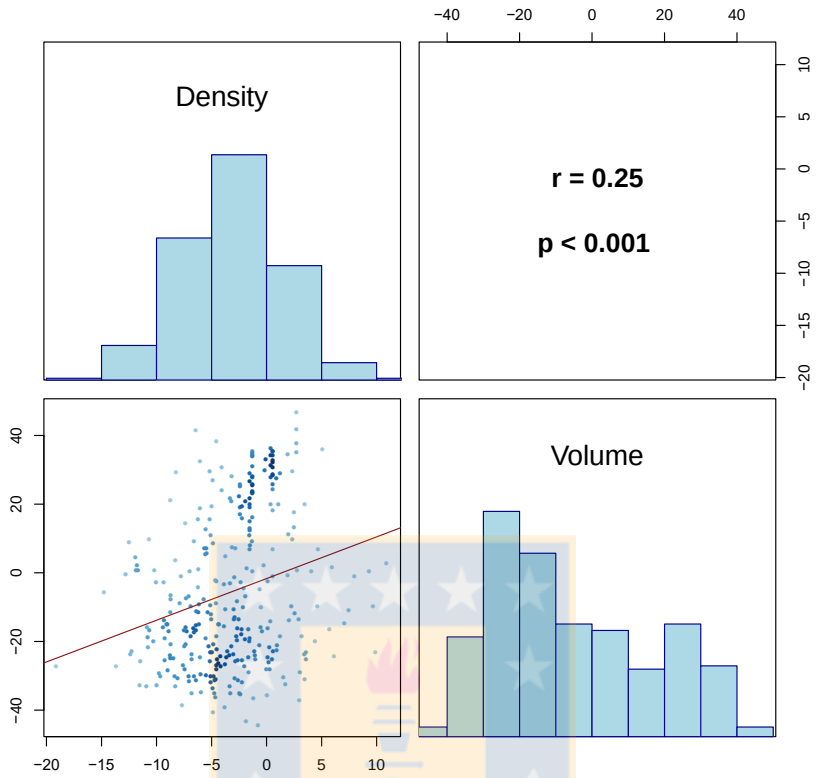


Fig. 3.1 Histograms(*diagonal*), scatter plots (*lower diagonal*), and correlation with p-value (*upper diagonal*) between wood density and volume (H_0: r = 0). *Fuente: Elaboración propia.*

3.3.3 Proccesing marker data

Synbreed (Wimmer et al. 2012) package in the R 3.0.2 envi- ronment was used (R Core Team 2013) to process various datasets. A total of 64,639 markers were evaluated. The genotypes were coded as zero, one, and two for the number of gene content. The frequencies of phased genotypes 0, 1, and 2 were 0.54, 0.40, and 0.06, respectively. SNPs with a minor allele frequency of 0.05 or less were discarded from the analysis. SNPs with >10% missing genotypes were also removed. No duplicated SNPs were found. A total of about 12 K markers remained after the filtering process. Missing genotypes were imputed using the random sampling from the marginal allele distribution of the markers. For two possible genotypes (zero and two values) missing, genotypes were sampled from distribution with probabilities $P(x = 0) = 1\text{-}p$ and $P(x = 2) = p$, where p is the minor allele frequency of marker j. In the case of three genotypes (two homozygous and one heterozygous), values were sampled from $P(x =$

0) = $(1 - p)^2$, $P(x = 1) = p(1 - p)$, and $P(x = 2) = p^2$ distribution assuming Hardy–Weinberg equilibrium for all loci (Wimmer et al. 2012).

Genetics (Warnes et al. 2013) package in R was used to estimate genotype frequency (the proportion of each genotype in the population) and allele frequency (proportion of all alleles that are of the specified type) (White et al. 2007). PIC and heterozygosity were estimated for each marker to evaluate the discriminatory power of a locus (Guo and Elston 1999). Intra-chromosomal LD measured as r^2 between pairs of loci was calculated using the Synbreed package (Wimmer et al. 2012). To analyze the LD decay, LD values versus the position of the markers was plotted for each chromosome. Using the LDheatmap package (Shin et al. 2006), a heat map with the r^2 values (from 0 to 1) between pairs of markers was generated. Physical distance was considered to indicate the total length of the region analyzed.

3.3.4 Statistical models for genomic prediction

Genomic best linear unbiased prediction (GBLUP), Bayesian Lasso (BLasso), Bayes B, and Bayes C models were used to predict GEBV. In the GBLUP approach, the additive genetic relationship matrix derived from pedigree is replaced with the realized genomic relationship matrix (**G**) obtained from shared alleles. A matrix of genomic relationship (**G**) between all pairs of individuals was computed as follows (VanRaden 2008).

$$G = \frac{ZZ'}{2\sum p_i(1 - p_i)} \qquad (2)$$

Where **Z** is the incidence matrix of markers. Considering the second allele frequency at the locus *i* as (p_i) and let **P** be a matrix with the allele frequencies expressed as $P = 2(p_i - 0.5)$. Genomic relationships could better estimate the proportion of chromosome segments shared by individuals, because high-density genotyping identifies genes identical in state that may be shared through common ancestors not recorded in the pedigree (Forni et al. 2011). Mixed model equations were solved to obtain GEBV values as:

$$\begin{bmatrix} XX' & X'Z \\ Z'X & Z'Z+G^{-1}a \end{bmatrix}+\begin{bmatrix} b \\ u \end{bmatrix}=\begin{bmatrix} X'y \\ Z'y \end{bmatrix} \qquad (3)$$

Where $\alpha=\sigma_e^2/\sigma_u^2$ is the sum across marker loci $2\sum p_i(1-p_i)$ times the ratio $\alpha=\sigma_e^2/\sigma_a^2$ where σ_a^2 is the total genetic variance and σ_u^2 is the additive genetic variance (VanRaden 2008).

Among the whole genome regression models, we used BLasso, Bayes B, and Bayes C. Lasso regression was initially developed by Tibshirani (1996) to overcome colinearity in multi-dimensional data. Park and Casella (2008) intro- duced the Bayesian approach to have a specific shrinkage for each marker effect. Lasso combines variable selection and shrinkage. Regression coefficients are shrunk towards zero; however, some of the coefficients are shrunk to exactly zero, which reduces the complexity of the model (Isik et al. 2016). The model has the form $y = x\beta + e$, and mathematically, Lasso estimates are:

$$\widehat{\beta_L}=\arg\min_{\beta}\left\{|y-X\beta|^2+\lambda\sum_{i=1}^{p}|\beta_i^2|\right\} \qquad (4)$$

Where y is the response (phenotype), μ is an intercept, X corresponds the $n \times p$ design matrix, β the $p \times 1$ vector of regression coefficients, **e** the $n \times 1$ error vector, and λ is reg- ularization parameter (Isik et al. 2016).

Meuwissen et al. (2001) proposed Bayes A and Bayes B whole genome regression models to genomic prediction. In Bayes B, the prior assumes the variance of markers equal to zero with probability π and the rest with probability (1 − π) follows an inverse X^2 distribution (Meuwissen et al. 2001). Bayes C is very similar to Bayes B, except for the estimation of the proportion of SNPs with zero effects (π), assuming a common variance for all fitted markers effects (Habier et al. 2011; Wolc et al. 2011) which follows a scaled inverse χ^2 prior with degrees of freedom v_a and scale parameter S^2_a (Habier et al. 2011). In Bayes C, the mixture probability π has a prior uniform distribution. The variable π is unknown and is estimated from the data. Synbreed package (Wimmer et al. 2012) and BGLR package (De los Campos and Perez Rodriguez 2014) in the R 3.0.2 environment were used to run the models. Results were visualized with the ggplot2 package (Wickham 2009).

3.3.5 Validation of the GS models

To test the predictive power of markers, several cross- validation scenarios were run. The population was sepa- rated into "training" and "validation" sets. A total of 260 random individuals were used as training set, and 50 individuals were used as validation set. For the cross-validation, 2 folds and 10 replications were used. The predictive ability of each fold in each replication was calculated as the correlation of EBV and GEBV. Spearman's rank correlation of each fold within each replication was calculated. Mean squared error (MSE) of each fold between the EBV and GEBV of validation set was calculated as MSE=$\frac{1}{n}\sum_{i=1}^{n}(\hat{Y}_i - Y_i)^2$. The bias of the model was obtained by regressing the EBV on the GEBV in the validation set. A regression coefficient of <1 implies inflation of genomic estimated breeding values and a coefficient of >1 suggests deflation of GEBVs (Wimmer et al. 2012). Finally, individuals in the validation set were ranked according with their GEBV and the mean EBV of the 10% best was calculat- ed for each replication.

3.4 RESULTS

3.4.1 LD between pair of markers

LD and descriptive statistics for each chromosome are presented in Table 3.1. The number of markers by chromosome ranged from 824 for chromosome one to 1564 for chromosome eight. The LD for each chromosome ranged from zero to one with a mean of 0.029. A scatter plot of LD between pairs of markers on chromosome eight suggests that LD decays rapidly according to the physical distance (Fig. 3.2a, b). LD decay scatter plots for all other 11 chromosomes are in Fig. S3.3. LD is decaying within the first 3 K. Heat maps were used to observe different levels of r^2 between pairs or markers by chromosome (Fig. 3.2c, Fig. S3.4). Heat maps suggest lack of LD between SNP markers on chromosomes, with some exceptions. The overall LD across genome suggests that only 0.09% of the LD values were greater than 0.50; therefore, most markers had lower levels of LD between 0 and 0.50 (Fig. 3.3).

Table 3.1 Descriptive statistics of linkage disequilibrium analysis for each chromosome. Total number of SNPs per chromosome, mean, minimum and maximum LD estimates (r^2) are presented. *Fuente: Elaboración propia.*

		Linkage Disequilibrium (r^2)		
Chromosome	**SNPs**	**Mean**	**Minimum**	**Maximum**
1	824	0.030	0.00	1
2	1,529	0.028	0.00	1
3	1,344	0.028	0.00	1
4	800	0.030	0.00	1
5	1,208	0.027	0.00	1
6	1,344	0.028	0.00	1
7	1,024	0.028	0.00	1
8	1,564	0.028	0.00	1
9	875	0.033	0.00	1
10	918	0.030	0.00	1
11	1,068	0.031	0.00	1
Mean	1,136	0.029	0.00	1

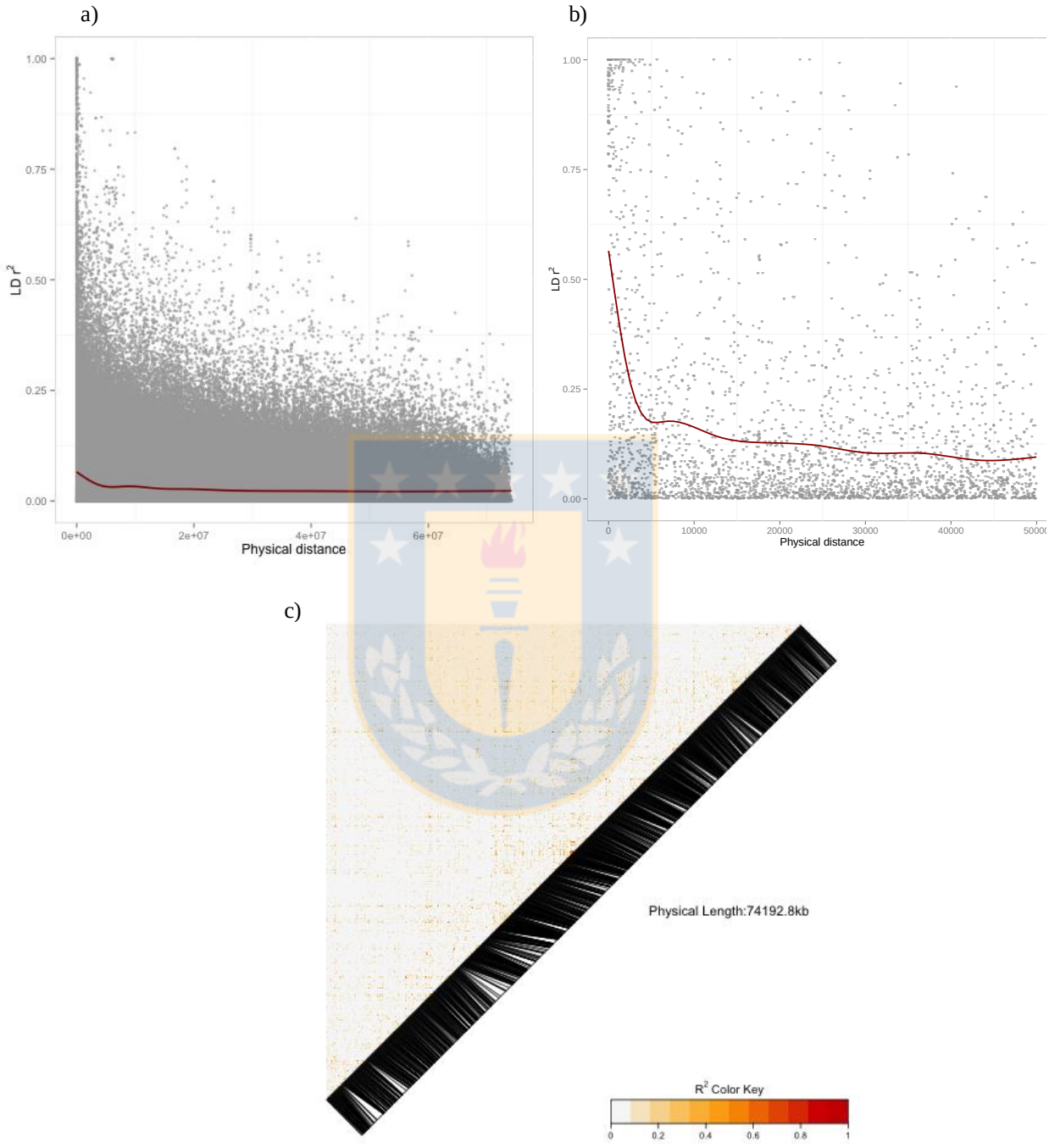


Fig. 3.2 a) Scatter of plot of LD level as coefficient of determination (r^2) between pair of SNPs against the physical distance (pair of bases) for chromosome 8. Smoothed spline represents the decline of LD. b) LD decay up to 50 K pairwise marker distance for chromosome 8. c) LD between pair of markers as heat map for the same chromosome. Lines in the diagonal

represent the position of the markers in the chromosome in pair of bases (pb). Color palette represents the LD level as coefficient of determination (r^2) between markers. *Fuente: Elaboración propia.*

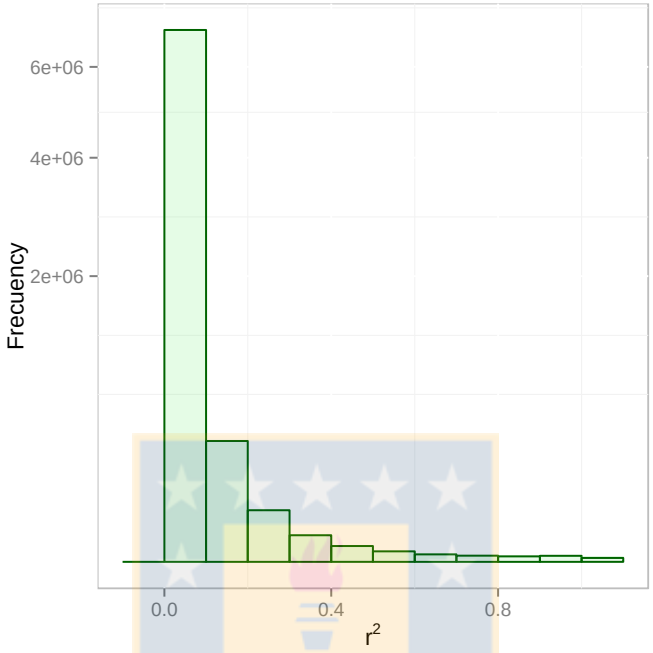


Fig. 3.3 Frequency of LD estimates (r^2) as measured across whole genome. LD between pairs of markers is largely zero with skewed distribution to the *right*. Large values of LD might be due to markers coming from the same loci or from the same contigs. *Fuente: Elaboración propia.*

3.4.2 Genetic relationships

Additive genetic relationship matrix from pedigree and genomic relationship matrix from SNPs are presented in Fig. 3.4. Individuals used in this study are clones belonging to half-sib and full-sib families, with the exception of two unrelated clones. According to the pedigree information, individuals had three levels of relationships within the population. Most individuals had zero coefficients (unrelated) with others within the study population. The remaining individuals had covariances of 0.25 and 0.50; expected coefficients for half-sibs and full-sib individuals (or parent–progeny coefficient), respectively. Relationships obtained from the SNPs markers varied between −0.22 and 0.98. The distribution of relationships between individuals estimated from markers was continuous rather than discrete. Regarding inbreeding levels, they were equal to zero from the pedigree-based calculation but they had a distribution on zero when estimated from shared SNP markers (Fig. S3.5).

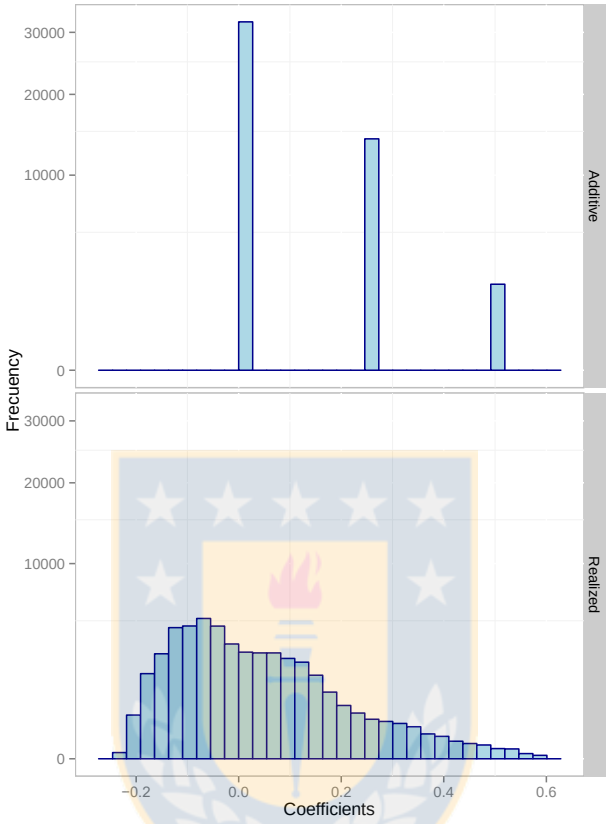


Fig. 3.4 Expected additive genetic relationship derived from pedigree (*top panel*) and realized genetic relationship estimated from SNP markers (*bottom panel*). Realized genetic relationships show a continuous distribution compared discrete distribution of relationships from pedigree. The scale of *y-axis* is the square root of the frequency. *Fuente: Elaboración propia.*

3.4.3 Genomic prediction models for volume and wood density

Statistical models were compared for different fit statistics such as predictive ability of markers, rank correlation, model bias, residual MSE, and the phenotypic mean of top 10% individuals based on ranking of GEBV. The summary statistics from cross-validation are presented in the box plots (Fig. 3.5). The horizontal lines in the box plots are the median of the prediction ability values from different sets of validation sets. The vertical lines extending from the boxes show the range of prediction abilities of the models (Fig. 3.5). For wood density, the predictive ability of GBLUP (mean 0.63) and BLasso (mean 0.61) was higher than Bayesian models (0.54) (Fig. 3.5). For volume, the predictive ability of GBLUP (mean 0.78)

and Bayes B (mean 0.76) was higher than BLasso (0.73) and Bayes C (0.72) (Fig. 3.5). For wood density, BLasso had a narrower distribution, while for volume, GBLUP had the narrowest distribution of predicted ability values.

Rank correlations from different statistical models followed similar patterns as predicted ability values (Fig. 3.5). The mean rank correlation for density (0.62) from GBLUP and BLasso models (0.61) was higher than the mean rank correlations from Bayesian models (0.57 and 0.56). Although symmetric, Bayes C validation samples produced a large variation of rank correlations for wood density. The mean rank correlation for volume (0.74) from GBLUP and Bayes B models (0.73) was higher than the mean rank correlations from BLasso (0.70) and Bayes C (0.67). In terms of the distribution, we observed mixed results. For example, for density, GBLUP and BLasso had a narrower distribution of rank correlations, while for volume, Bayes C had the narrowest distribution.

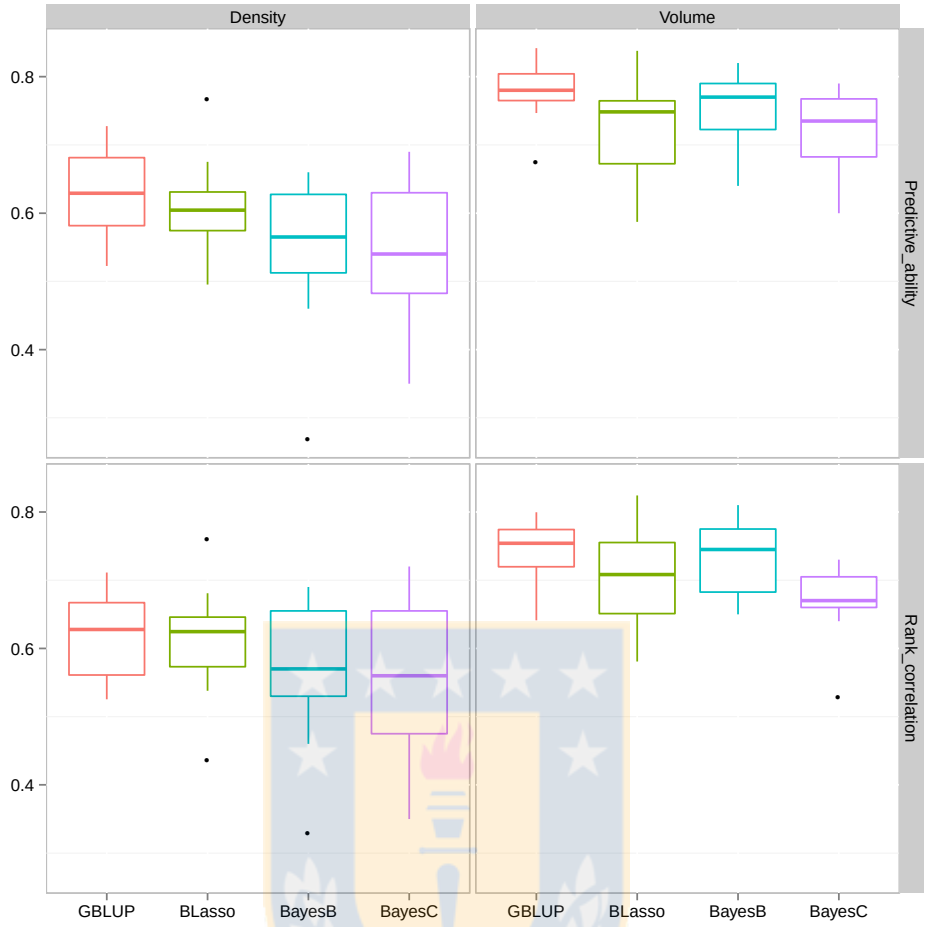


Fig. 3.5 Evaluation of statistical models (GLUP, BLasso, Bayes B, and Bayes C) using random sampling of 50 individuals as the validation set with 2 folds and 10 replications for wood density and volume. The box plots show the distribution of predictive ability of markers (*upper panel*) and rank correlation (*lower panel*) from validation sets. The *thick vertical lines* are the median. *Fuente: Elaboración propia.*

The relationships between GEBV and EBV for wood density and volume are illustrated in Fig. 3.6, where small blue dots are the relationships between direct GEBV and EBV in the training set, whereas the larger red dots are the relationships between GEBV and EBV for a one of the validation sets by GBLUP. The scatter plots show that the correlation between GEBV and EBV for wood density was 0.6 and for volume was 0.73, respectively, for both validation set. Correlation between direct GEBV and EBV (training set) for both traits was over 0.90.

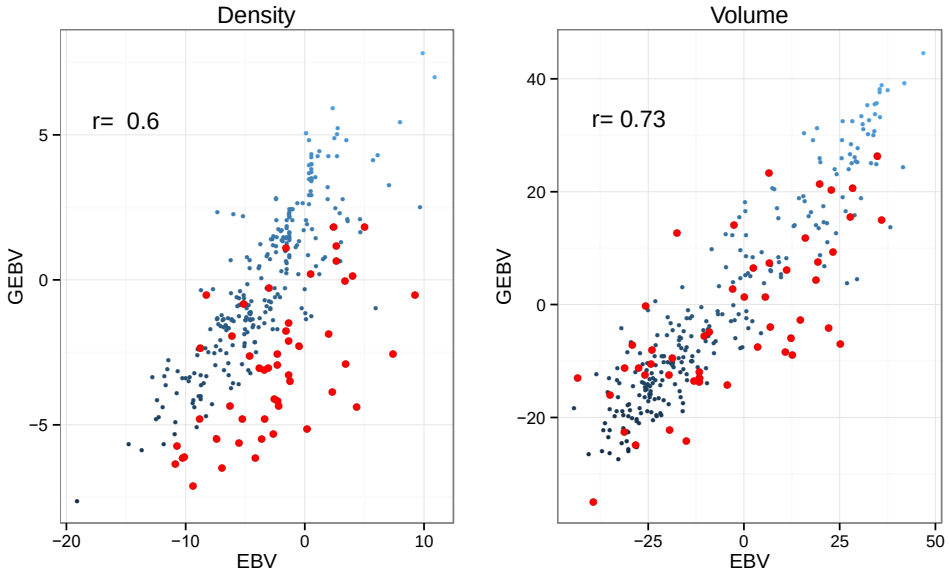


Fig. 3.6 Predictive ability of SNP markers for wood density and volume in a validation set (50 random samples) using the GLUP statistical model. The smaller blue dots are direct GEBV and EBV of the training set with a correlation of r = 0.98 for volume and r = 0.99 for density. The bigger red dots represent the relationship between GEBV (y- axis) and EBV (x-axis) of the validation set. *Fuente: Elaboración propia.*

Several other model fit statistics are given in Table S3.1. Bayes B and Bayes C models had higher density means (3.8) of the best 10% individuals when ranked for GEBV. GBLUP and BLasso both had a mean of 3.5 for wood density for the top 10% individuals. The bias from regressing the GEBV on EBV of density was >1 for the GBLUP and <1 for Bayes B. Other two models did not have bias (mean slope was one for both models). For volume, the mean of the best 10% individuals from models were the same. BLasso had a large bias (b>1) for volume, whereas others had the same smaller bias (1.1).

3.5 DISCUSSION

3.5.1 Linkage disequilibrium

This is the first empirical study for GS in a clonal breeding population of *E. globulus*. We detected an average LD of 0.029 when averaged across 11 chromosomes. LD is one of the factors that affect genomic selection accuracy (Hayes et al. 2009). In forest tree species, LD is low and decays rapidly according to increasing distance between pairs of markers (Neale and Savolainen 2004; Isik et al. 2016). Genome recombination and nucleotide diversity have a direct impact on the LD degree (Silva-Junior and Grattapaglia 2015). Higher LD decay rate

has been demonstrated in genes sequenced for some forest species; however, recent studies have described a lower LD decay than expected considering sequencing technologies for whole genome. For example in *E. globulus* (Thavamanikumar et al. 2011) and *E. grandis* (Faria and Grattapaglia unpublished from Grattapaglia and Kirst 2008), a few candidate genes were analyzed and the LD decay rate is higher than using genotyping whole genome in *E. grandis* (Silva-Junior and Grattapaglia 2015). In some cases, a few groups of SNPs in LD could be detected in some chromosomes. Probably, considering the small effective population size and the SNPs coverage on the genome, both factors increase the possibility to find some markers closer to each other in the same locus across the chromosome. Scatter plots by chromosome displayed that when the distance between markers is longer, LD levels are lower. Similar results were reported in *P. trichocarpa* from candidate genes and whole genome sequencing (Slavov et al. 2012). Isik et al. (2016) eported intra-chromosomal LD of 0.01 in maritime pine. When they corrected LD for the genomic relationships derived from markers, it was even smaller (0.006).

3.5.2 Genomic relationships

Genetic relationships based on shared markers between relatives show a continuous distribution and are more realistic compared to the expected genetic relationships derived from pedigree (Fig. 3.4). Genomic relationship can vary depending on the number of alleles shared between individuals. For example, full-sib trees share more genes that are identical by descent or identical by state than half-sib trees. When many markers are available and when there are large numbers of individuals in the population, they can be used to estimate the true relationships among relatives (Powell et al. 2010) and they can provide more realistic estimates about the shared genome between individuals. Lower diagonal elements in the matrix were centered at 0.5 and 0.25 for individuals from full-sib and half-sib families, respectively, corresponding with the expected values derived from pedigree (Simeone et al. 2011). Therefore, only two peaks should be expected with the genomic relationship frequency (Fig. 3.4) while other peaks closer to zero with positive and negative values are present as well. Those values represent individuals which do not have any relationship in the population, because they are not either full-sib or half-sib trees (Munoz et al. 2014). Depending on whether the values are close to zero, positive or negative, we can say that the individuals are more or less similar than expected or they are sharing fewer or larger number of alleles than

expected from the allele frequencies (Bartholomé et al. 2016). When the genomic relationship is closer to one (upper diagonal values), these individuals can be seen as biological replicates (ramets). They can also be attributed to errors in the sampling or genotyping errors.

3.5.3 Predicted ability of markers

We detected high predictive ability of markers, especially for stem volume (0.73) in our *E. globulus* population. Rank correlations between EBV and GEBV were also high. As in other previous studies (Munoz et al. 2014; Isik et al. 2016), the population was randomly split into training and validation sets for cross-validation. In such cross-validation scenarios, the marker–QTL phase does not change and thus the results in this study are proof of concept. The marker–QTL phase can drastically change once the population goes through a cycle of breeding due to recombination (Isik 2014). If the model developed in this study is used to predict GEBV of a new generation, the predicted ability values may go down. The small sample size for the training and validation set has caused a fold-to-fold variation represented as the difference between maximum and minimum predictive ability values for each model evaluated. Considering that we only have 310 samples for the analysis, splitting the total population in more and less samples for training and validation set, respectively, would be difficult. It is known that considering a large size for training population, the accuracy of the prediction increases; however, the relatedness between training and validation set is also important. Bartholomé et al. (2016) compared prediction accuracies across different sampling strategies showing more variation when the validation set was sampled between half-sib families comparing with random and within full-sib families as validation sets. Therefore, if we consider that in our study a random split was done, it is expected that the fold variation decreases with a high level of relatedness. However, relatedness between cross-validation sets is not just affecting the level of variation, but it is affecting the level of prediction as well. With a lower relatedness between training and validation set, predictive abilities of the models could decrease as well. Different studies reported in forest tree species have shown that both higher relatedness between training and validation set and a deep pedigree in the training set can increase the prediction accuracy. Isik et al. (2016) reported a marginal advantage when individuals from G0 and G1 generation were used as training set over when population was split randomly in two sets. Bartholomé et al. (2016) showed that when level of relatedness between training and validation was increased, the mean of the prediction accuracy was

increased as well. They also assessed the accuracy over generations using grandparents and parents as a training set to predict the descendants, showing high accuracy with a range of 0.70–0.85 depending of the trait evaluated. Beaulieu et al. (2014b) evaluated accuracy across different test sites and breeding groups by cross-validation analysis for wood quality and growth traits reporting that when there is a strong relatedness between training and validation set, prediction accuracy was high and moderately high depending of the traits, and it decrease when relatedness are removed between training and validation set. The underlying reasons for high accuracy of GEBV is attributed to better capturing genetic relationships rather than exploiting the LD between marker-tagged QTLs and traits (Zapata-Valenzuela et al. 2012; Isik 2014; Isik et al. 2016). With dense marker coverage and large population with deep pedigree, markers may be able to exploit LD between phenotype and trait loci in the future generations. In loblolly pine, an average accuracy of 0.56 and 0.36 from two random sampling methods were reported for volume (Zapata-Valenzuela et al. 2013). Resende et al. (2012b) reported a predicted accuracy from 0.67 to 0.77 for wood density in the same species with a heritability of 0.09 using four different methods. Similar prediction accuracies for growth and wood density were reported for white spruce (Beaulieu et al. 2014a, b). In *Eucalyptus* spp., predictive ability and accuracy of GS were from 0.38 to 0.60 and from 0.55 to 0.88, respectively, for four different traits in two elite breeding populations (Resende et al. 2012c).

It is true that EBVs of individuals predicted by BLUP are shrunk towards the parental average and towards the population mean. The amount of shrinkage is a function of heritability. Isik et al. (2016) suggested that if the experimental unit is a family or a clone with multiple data points, the EBVs are not regressed as much. Family mean and clone mean heritability estimates usually are much larger than individual tree heritabilities. They used the family EBV and family raw means in a maritime pine population as phenotype and did not find any noticeable difference for predictive abilities.

3.5.4 Effect of heritability

Wood density is one of the most important traits affecting quality of pulp. In *Eucalyptus* spp., studies reported higher heritability for wood density than growth traits (Borralho et al. 1992; Apiolaza et al. 2005; Hamilton and Potts 2008). Heritability is considered one of the important factors affecting the response to selection (Falconer and Mackay 1996). Therefore, breeders

consider wood density as a good trait for selection. Hayes et al. (2009) suggested that heritability affects accuracy of GS. For traits with low heritability, GS could enhance the selection process (Goddard 2009). In our study, predictive ability for wood density was lower than the predicted ability for volume. Wood density had a heritability of 0.46, whereas volume had a heritability of 0.29. Having a higher heritability but lower prediction accuracy in our study can be attributed to the small sample size used to calculate breeding values for wood density. Wood density measures were available for only 75% of the total samples. Resende et al. (2012c) evaluated marker–trait (growth and wood quality) associations in *Eucalyptus* spp., and they captured large fractions of trait heritability (≥80% h^2) using 300 largest marker effects for two populations. In a simulation study, Denis and Bouvet (2013) suggested that in some cases, the accuracy of GEBV can be improved for traits with low heritability than high heritability, controlling the relationship between the training and the validation set. Low heritability can be compensated by using a larger training set (Solberg et al. 2008). In a deterministic simulation study, Grattapaglia and Resende (2011) demonstrated insignificant effect of heritability on GS accuracy. Resende et al. (2012b) evaluated 17 traits including growth, development, and disease resistance properties with a range of heritability from 0.07 to 0.45 showing very consistent predictive ability between traits.

3.5.5 Comparison of models

Statistical models compared in various published results for forest tree species were not drastically different for prediction accuracy of GEBV (Resende et al. 2012b; Bartholomé et al. 2016; Isik et al. 2016). In our study, GBLUP had prediction accuracies comparable to Bayesian models employed. The Bayesian models assume that some markers have a large effect and many with small or no effect on the phenotype. In loblolly pine, BLasso and other Bayesian models achieved similar predictive ability for different traits across models but density predictive ability was lower than all other traits and similar across models (Resende et al. 2012b). Bartholomé et al. (2016) reported similar prediction accuracies for *P. pinaster* comparing both GBLUP and BLasso models. On the other hand, GBLUP assumes polygenic inheritance model with all the markers affecting the phenotype, with small contribution each. For complex traits, such as wood density and volume, the difference between models could be difficult to detect but for traits under oligogenic effect, the difference might be more

important. Results from our study and previous published results (Zapata-Valenzuela et al. 2013; Isik et al. 2016) suggest that GBLUP can be used for routine data analysis in a tree breeding program. As GBLUP models follow the traditional linear mixed model structure, they would account for heterogeneous covariance structures and genotype by environment interactions, especially in forestry where contrasting site types are commonly used and multiple traits are evaluated. Isik (2014) and Grattapaglia (2014) suggest that GS models are likely to be population specific.

3.6 CONCLUSIONS

This study is the first to test the predictive ability of markers in a clonal breeding population of *E. globulus* in Chile. The overall LD was low, but it was higher than LD estimates reported in P. pinaster. Predictive ability of markers was encouraging for both traits analyzed, wood density, and volume. The study is a first proof-of-concept experiment based on a small sample size of trees (310) composed of one generation. The results should be validated with a larger set of individuals, preferably across two generations. The predicted ability will likely decrease if the models are used to predict GEBV of new material coming from the breeding program, because of a different marker–trait phase introduced by recombination. The GBLUP model represents a good alternative, drawing the realized genetic relationships among relatives and use them in predicting their GEBVs as a complementary tool to assist the selection criteria of the best genotypes for propagation and deployment of the breeding program.

3.7 REFERENCES

Apiolaza LA, Raymond CA, Yeo BJ (2005) Genetic variation of physical and chemical wood properties of Eucalyptus globulus. Silvae Genet 54:160–165

Bartholomé J, Van Heerwaarden J, Isik F, Boury C, Vidal M, Plomion C, Bouffier L (2016) Performance of genomic prediction within and across generations in maritime pine. BMC Genomics 17:604. doi: 10.1186/s12864-016-2879-8

Beaulieu J, Doerksen T, Clément S, MacKay J, Bousquet J (2014a) Accuracy of genomic selection models in a large population of open-pollinated families in white spruce. Heredity 113:343–352. doi:10.1038/hdy.2014.36

Beaulieu J, Doerksen TK, MacKay J, Rainville A, Bousquet J (2014b) Genomic selection accuracies within and between environments and small breeding groups in white spruce. BMC Genomics 15:1048. doi:10.1186/1471-2164-15-1048

Borralho NMG, Cotterill PP, Kanowski PJ (1992) Genetic parameters and gains expected from selection for dry weight in Eucalyptus globulus ssp. globulus in Portugal. For Sci 38:80–94

Borralho NMG, Cotterill PP, Kanowski PJ (1993) Breeding objectives for pulp production of Eucalyptus globulus under different industrial cost structures. Can J For Res 23:648–656

Calus MPL, Veerkamp RF (2007) Accuracy of breeding values when using and ignoring the polygenic effect in genomic breeding value estimation with a marker density of one SNP per cM. J Anim Breed Genet 124:362–368. doi:10.1111/j.1439-0388.2007.00691.x

Cappa EP, El-Kassaby YA, Garcia MN, Acuña C, Borralho NM, Grattapaglia D, Poltri SNM (2013) Impacts of population structure and analytical models in genome-wide association studies of com- plex traits in forest trees: a case study in Eucalyptus globulus. PLoS One 8:e81267. doi:10.1371/journal.pone.0081267

Core Team R (2013) R: a language and environment for statistical com- puting. R Foundation for Statistical Computing, Viena

Crosbie TM, Eathington SR, Johnson GR, Edwards M, Reiter R, Stark S, Mohanty RG, Oyervides M, Buehler RE, Walker AK, Dobert R, Delannay X, Pershing, JC, Hall MA, Lamkey KR (2006) Plant breeding: past, present, and future. In: Lamkey KR, Lee M (eds.) Plant breeding: the Arnel R. Hallauer International Symposium. Blackwell, pp 3–50

De los Campos G, Perez Rodriguez P (2014) BGLR: Bayesian general- ized linear regression. R package version 1.0.3

Denis M, Bouvet JM (2013) Efficiency of genomic selection with models including dominance effect in the context of Eucalyptus breeding. Tree Genet Genomes 9:37–51. doi:10.1007/s11295-012-0528-1

Doughty RW (2000) The eucalyptus: a natural and commercial history of the gum tree. Baltimore, Maryland

El-Dien OG, Ratcliffe B, Klápště J, Chen C, Porth I, El-Kassaby YA (2015) Prediction accuracies for growth and wood attributes of in- terior spruce in space using genotyping-by-sequencing. BMC Genomics 16:1. doi:10.1186/s12864-015-1597-y

Eldridge KG, Davidson J, Harwood CE, van Wyk G (1993) Eucalypt domestication and breeding. Clerendon Press, Oxford

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, Fourth edn. Longman Group, Ltd, Essex, p 464

Forni S, Aguilar I, Misztal I (2011) Different genomic relationship ma- trices for single-step analysis using phenotypic, pedigree and geno- mic information. Genet Sel Evol 43:1. doi:10.1186/1297-9686-43-1

Gilmour AR, Gogel BJ, Cullis BR, Thompson R (2009). ASReml user guide release 3.0. VSN International Ltd, Hemel Hempstead, HP1 1ES, United Kingdom

Goddard M (2009) Genomic selection: prediction of accuracy and maximisation of long term response. Genetica 136:245–257. doi: 10.1007/s10709-008-9308-0

Goddard ME, Hayes BJ (2007) Genomic selection. J Anim Breed Genet 124:323–330. doi:10.1111/j.1439-0388.2007.00702.x

Goddard ME, Hayes BJ (2009) Mapping genes for complex traits in domestic animals and their use in breeding programmes. Nat Rev Genet 10:381–391. doi:10.1038/nrg2575

Grattapaglia D (2004) Integrating genomics into Eucalyptus breeding. Genet Mol Res 3:369–379

Grattapaglia D (2014) Breeding Forest trees by genomic selection: current progress and the way forward. In: Tuberosa R, Graner A, Frison E (eds) Genomics of plant genetic resources. Springer, Dordrecht, pp 651–682

Grattapaglia D, Kirst M (2008) Eucalyptus applied genomics: from gene sequences to breeding tools. New Phytol 179:911–929. doi:10.1111/ j.1469-8137.2008.02503.x

Grattapaglia D, Resende MDV (2011) Genomic selection in forest tree breeding. Tree Genet Genomes 7:241–255. doi:10.1007/s11295-010-0328-4

Guo X, Elston RC (1999) Linkage information content of polymorphic genetic markers. Hum Hered 49:112–118. doi:10.1159/000022855

Habier D, Fernando RL, Kizilkaya K, Garrick DJ (2011) Extension of the Bayesian alphabet for genomic selection. BMC Bioinformatics 12:1. doi:10.1186/1471-2105-12-186

Hamilton MG, Potts BM (2008) Eucalyptus nitens genetic parameters. NZ J For Sci 38:102–119

Hayes B, Goddard M (2010) Genome-wide association and genomic selection in animal breeding. Genome 53:876–883. doi:10.1139/G10-076

Hayes BJ, Bowman PJ, Chamberlain AJ, Goddard ME (2009) Invited review: genomic selection in dairy cattle: progress and challenges. J Dairy Sci 92:433–443. doi:10.3168/jds.2008-1646

Hayes BJ, Cogan NO, Pembleton LW, Goddard ME, Wang J, Spangenberg GC, Forster JW (2013) Prospects for genomic selection in forage plant species. Plant Breed 132:133–143. doi:10.1111/pbr.12037

Heffner EL, Sorrells ME, Jannink JL (2009) Genomic selection for crop improvement. Crop Sci 49:1–12. doi:10.2135/cropsci2008.08.0512

Isik F (2014) Genomic selection in forest tree breeding: the concept and an outlook to the future. New For 45:379–401. doi:10.1007/s11056-014-9422-z

Isik F, Kumar S, Martínez-García PJ, Iwata H, Yamamoto T (2015) Chapter three—acceleration of forest and fruit tree domestication by genomic selection. In: Plomion C, Adam-Blondon AF (eds) Advances in botanical research. Academic Press, pp 93–124. doi: 10.1016/bs.abr.2015.05.002

Isik F, Bartholomé J, Farjat A, Chancerel E, Raffin A, Sanchez L, Plomion C, Bouffier L (2016) Genomic selection in maritime pine. Plant Sci 242:108–119. doi:10.1016/j.plantsci.2015.08.006

Jannink JL, Lorenz AJ, Iwata H (2010) Genomic selection in plant breed- ing: from theory to practice. Brief Funct Genomics 9:166–177. doi: 10.1093/bfgp/elq001

Jonas E, de Koning DJ (2013) Does genomic selection have a future in plant breeding? Trends Biotechnol 31:497–504. doi:10.1016/j. tibtech.2013.06.003

Ladrach WE (1986) Comparaciones entre procedencias de siete coníferas en la Zona Andina al finalizar ocho años. Informe de investigación Smurfit Carton Colombia 105:8

Lin Z, Hayes BJ, Daetwyler HD (2014) Genomic selection in crops, trees and forages: a review. Crop Pasture Sci 65:1177–1191

Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829

Meuwissen T, Hayes B, Goddard M (2016) Genomic selection: a para- digm shift in animal breeding. Anim Front 6:6–14. doi:10.2527/af. 2016-0002

Munoz PR, Resende MFR, Huber DA, Quesada T, Resende MDV, Neale DB, Wegrzyn JL, Kirst M, Peter GF (2014) Genomic relationship matrix for correcting pedigree errors in breeding populations: impact on genetic parameters and genomic selection accuracy. Crop Sci 54: 1115–1123. doi:10.2135/cropsci2012.12.0673

Muranty H, Jorge V, Bastien C, Lepoittevin C, Bouffier L, Sanchez L (2014) Potential for marker-assisted selection for forest tree breed- ing: lessons from 20 years of MAS in crops. Tree Genet Genomes 10:1491–1510. doi:10.1007/s11295-014-0790-5

Myburg AA, Grattapaglia D, Tuskan GA et al (2014) The genome of Eucalyptus grandis. Nature 510:356–362

Neale DB, Kremer A (2011) Forest tree genomics: growing resources and applications. Nat Rev Genet 12:111–122. doi:10.1038/nrg2931

Neale DB, Savolainen O (2004) Association genetics of complex traits in conifers. Trends Plant Sci 9:325–330. doi:10.1016/j.tplants.2004.05.006

Neale DB, Wegrzyn JL, Stevens KA et al (2014) Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. Genome Biol 15:R59. doi:10.1186/gb-2014-15-3-r59

Park T, Casella G (2008) The Bayesian lasso. J Am Stat Assoc 103:681– 686. doi:10.1198/016214508000000337

Potts BM, Vaillancourt RE, Jordan G et al (2004) Exploration of the Eucalyptus globulus gene pool. In: Borralho NMG, Pereira JS, Marques C, Coutinho J, Madeira M, Tomé M (eds) Eucalyptus in a changing world proceedings of IUFRO Conference, 11–15 October Aveiro, Portugal. RAIZ, Instituto Investigação de Floresta e Papel, pp 46–61

Powell JE, Visscher PM, Goddard ME (2010) Reconciling the analysis of IBD and IBS in complex trait studies. Nat Rev Genet 11:800–805. doi:10.1038/nrg2865

Ratcliffe B, El-Dien OG, Klápště J, Porth I, Chen C, Jaquish B, El- Kassaby YA (2015) A comparison of genomic selection models across time in interior spruce (Picea engelmannii × glauca) using unordered SNP imputation methods. Heredity 115:547–555. doi:10. 1038/hdy.2015.57

Raymond CA, Banham P, MacDonald AC (1998) Within tree variation and genetic control of basic density, fibre length and coarseness in Eucalyptus regnans in Tasmania. Appita J 51:299–305

Resende MFR, Muñoz P, Acosta JJ, Peter GF, Davis JM, Grattapaglia D, Resende MDV, Kirst M (2012a) Accelerating the domestication of trees using genomic selection: accuracy of prediction models across ages and environments. New Phytol 193:617–624. doi:10.1111/j. 1469-8137.2011.03895.x

Resende MFR, Muñoz P, Resende MDV, Garrick DJ, Fernando RL, Davis JM, Jokela EJ, Martin TA, Peter GF, Kirst M (2012b) Accuracy of genomic selection methods in a standard data set of loblolly pine (Pinus taeda L.) Genetics 190:1503–1510. doi:10. 1534/genetics.111.13702

Resende MDV, Resende MFR, Sansaloni CP, Petroli CD, Missiaggia AA, Aguiar AM, Abad JM, Takahashi EK, Rosado AM, Faria DA, Pappas GJ, Kilian A, Grattapaglia D (2012c) Genomic selection for growth and wood quality in Eucalyptus: capturing the missing heritability and accelerating breeding for complex traits in forest trees. New Phytol 194:116–128. doi:10.1111/j.1469-8137.2011.04038.x

Rezende GDSP, de Resende MDV, de Assis TF (2013) Eucalyptus breeding for clonal forestry In: Fenning T (ed). Challenges and opportunities for the world's forests in the 21st century. Netherlands, pp 393–424

Schimleck LR (2008) Near infrared spectroscopy: a rapid, non- destructive method for measuring wood properties and its applica- tion to tree breeding. N Z J For Sci 38:14–35

Shin JH, Blay S, McNeney B, Graham J (2006) LDheatmap: an R function for graphical display of pairwise linkage disequilibria between single nucleotide polymorphisms. J Stat Softw 16. doi:10.18637/jss.v016.c03\

Silva-Junior OB, Grattapaglia D (2015) Genome-wide patterns of recom- bination, linkage disequilibrium and nucleotide diversity from pooled resequencing and single nucleotide polymorphism genotyp- ing unlock the evolutionary history of Eucalyptus grandis. New Phytol 208:830–845. doi:10.1111/nph.13505

Silva-Junior OB, Faria DA, Grattapaglia D (2015) A flexible multi- species genome-wide 60K SNP chip developed from pooled resequencing of 240 Eucalyptus tree genomes across 12 species. New Phytol 206:1527–1540. doi:10.1111/nph.13322

Simeone R, Misztal I, Aguilar I, Legarra A (2011) Evaluation of the utility of diagonal elements of the genomic relationship matrix as a diagnostic tool to detect mislabelled genotyped animals in a broiler chicken population. J Anim Breed Genet 128:386–393. doi:10.1111/j.1439-0388.2011.00926.x

Slavov GT, DiFazio SP, Martin J, Schackwitz W, Muchero W et al (2012) Genome resequencing reveals multiscale geographic structure and ex- tensive linkage disequilibrium in the forest tree Populus trichocarpa. New Phytol 196:713–725. doi:10.1111/j.1469-8137.2012.04258.x

Solberg TR, Sonesson AK, Woolliams JA, Meuwissen THE (2008) Genomic selection using different marker types and densities. J Anim Sci 86:2447–2454. doi:10.2527/jas.2007-0010

Strauss SH, Lande R, Namkoong G (1992) Limitations of molecular-marker- aided selection in forest tree breeding. Can J For Res 22:1050–1061

Thavamanikumar S, McManus LJ, Tibbits JFG, Bossinger G (2011) The significance of single nucleotide polymorphisms (SNPs) in Eucalyptus globulus breeding programs. Aust For 74:23–29. doi: 10.1080/00049158.2011.10676342

Thumma BR, Nolan MF, Evans R, Moran GF (2005) Polymorphisms in cinnamoyl CoA Reductase (CCR) are associated with variation in microfibril angle in Eucalyptus spp. Genetics 171:1257–1265. doi: 10.1534/genetics.105.042028

Tibshirani R (1996) Regression shrinkage and selection via the lasso. J R Stat Soc B Methodol:267–288

Tuskan GA, DiFazio S, Jansson S et al (2006) The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). Science 313: 1596–1604. doi:10.1126/science.1128691

VanRaden PM (2008) Efficient methods to compute genomic predictions. J Dairy Sci 91:4414–4423. doi:10.3168/jds.2007-0980

Warnes G, Gorjanc G, Leisch F, Man M (2013) Genetics: population genetics. R Package, version 1.3.8.1.

Weigel D, Nordborg M (2005) Natural variation in Arabidopsis. How do we find the causal genes? Plant Physiol 138:567–568

White TL, Adams WT, Neale DB (2007) Forest genetics. CABI Publishing CAB International, Cambridge

Wickham H (2009) ggplot2: elegant graphics for data analysis. Springer, New York

Wimmer V, Albrecht T, Auinger HJ, Schön CC (2012) synbreed: a frame- work for the analysis of genomic prediction data using R. Bioinformatics 28:2086–2087. doi:10.1093/bioinformatics/bts335

Wolc A, Stricker C, Arango J et al (2011) Breeding value prediction for production traits in layer chickens using pedigree or genomic rela- tionships in a reduced animal model. Genet Sel Evol 43:1–9. doi:10. 1186/1297-9686-43-5

Zapata-Valenzuela J, Hasbun R (2011) Mejoramiento genético forestal acelerado mediante selección genómica. Bosque (Valdivia) 32:209– 213. doi:10.4067/S0717-92002011000300001

Zapata-Valenzuela J, Isik F, Maltecca C, Wegrzyn J, Neale D, McKeand S, Whetten R (2012) SNP markers trace familial linkages in a cloned population of Pinus taeda—prospects for genomic selection. Tree Genet Genomes 8:1307–1318. doi:10.1007/s11295-012-0516-5

Zapata-Valenzuela J, Whetten RW, Neale D, McKeand S, Isik F (2013) Genomic estimated breeding values using genomic relationship ma- trices in a cloned population of loblolly pine. G3 (Bethesda) 3:909– 916. doi:10.1534/g3.113.005975

3.8 SUPPLEMENTARY MATERIAL

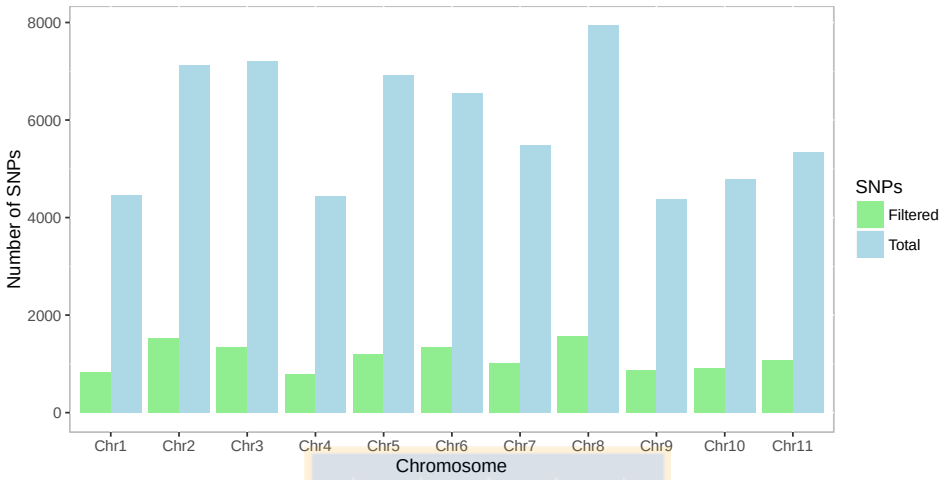


Fig. S3.1 Total SNPs by Euchip60k and filtered SNPs across 11 chromosomes (Chr1-Chr11).

Fuente: Elaboración propia.

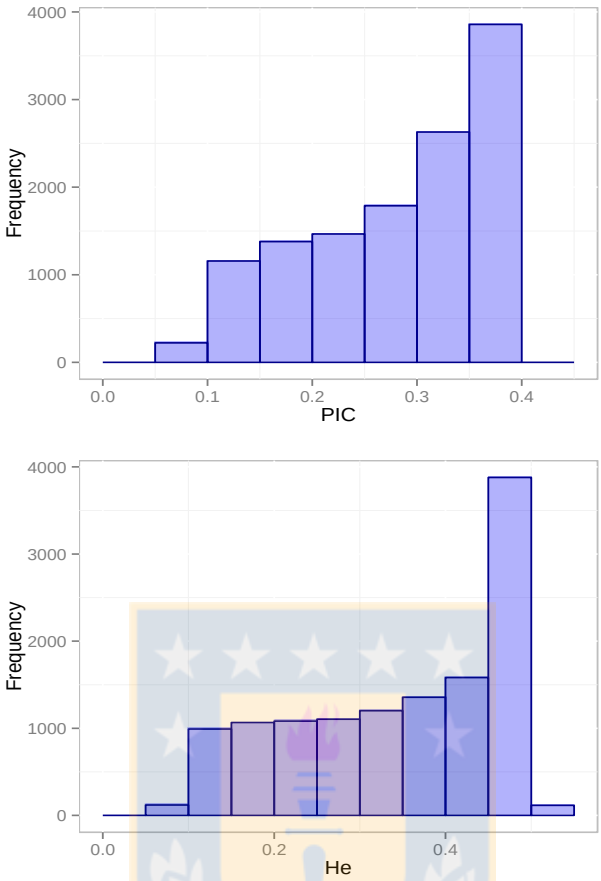


Fig. S3.2 Top panel represent PIC value frequencies derived from 12K of SNPs. Bottom panel represent He value frequencies derived from 12K of SNPs. Values are expressed in square root. *Fuente: Elaboración propia.*

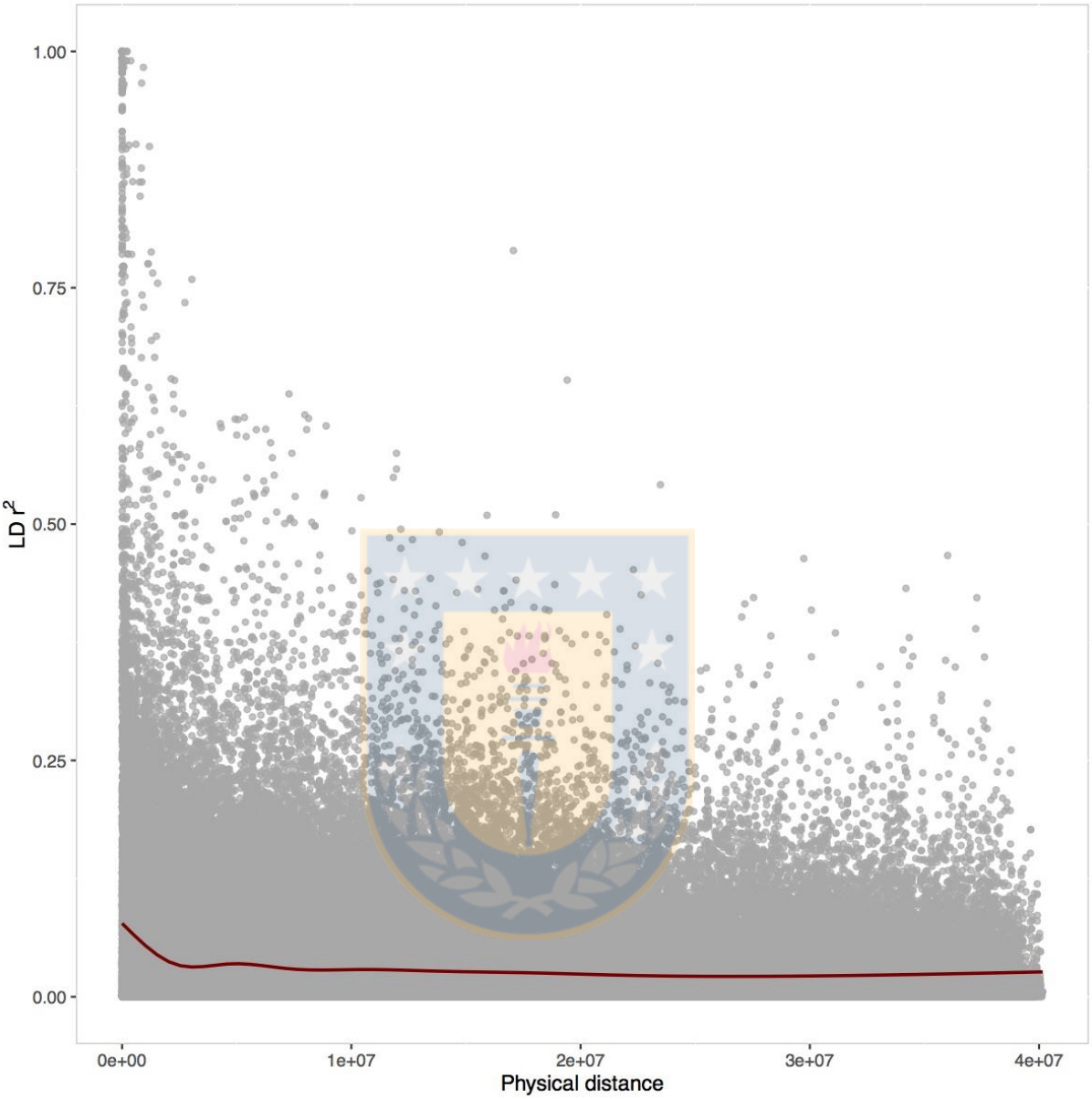


Fig. S3.3.1 LD-scatter plot for Chr1. *Fuente: Elaboración propia.*

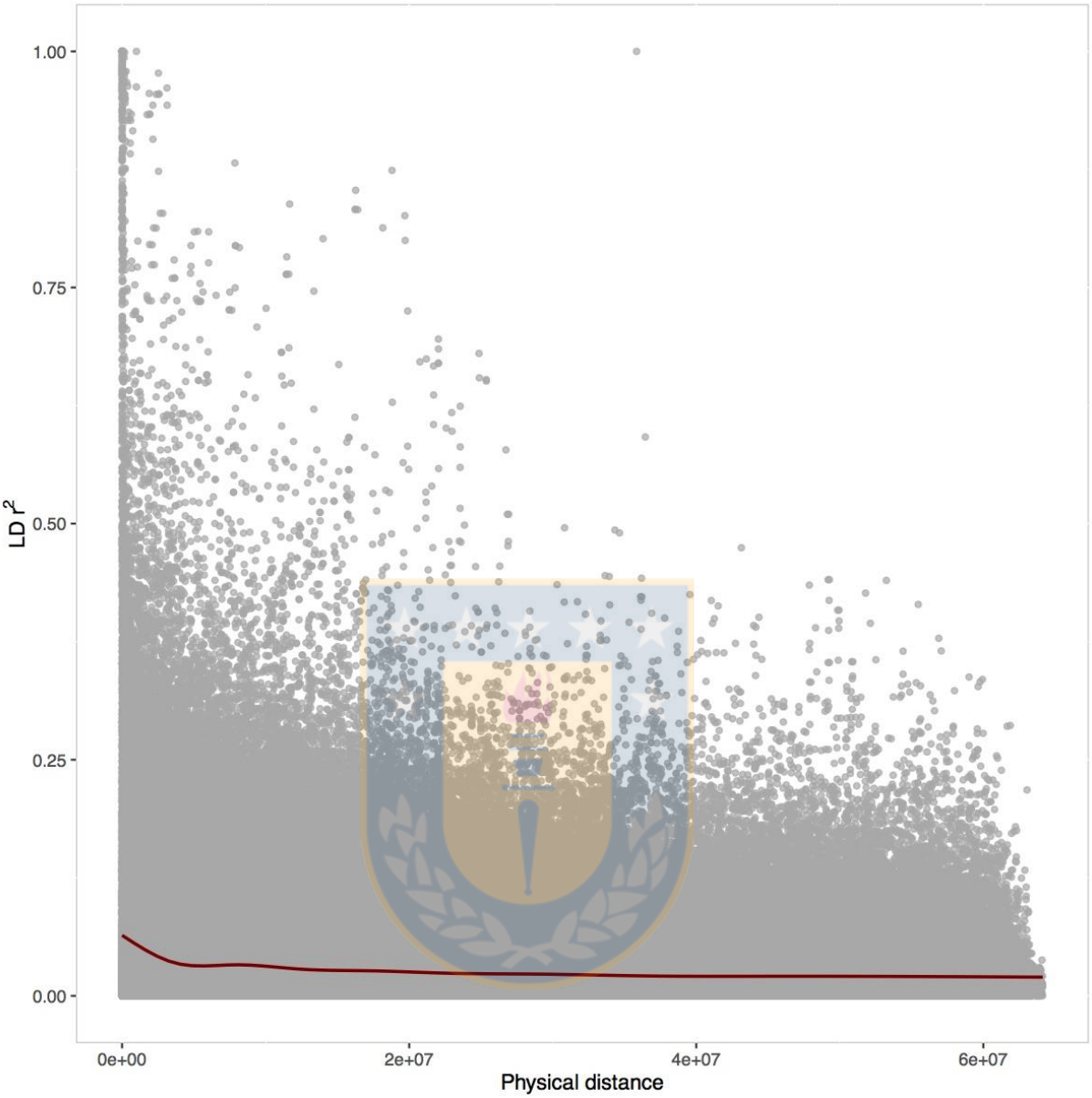


Fig. S3.3.2 LD-scatter plot for Chr2. *Fuente: Elaboración propia.*

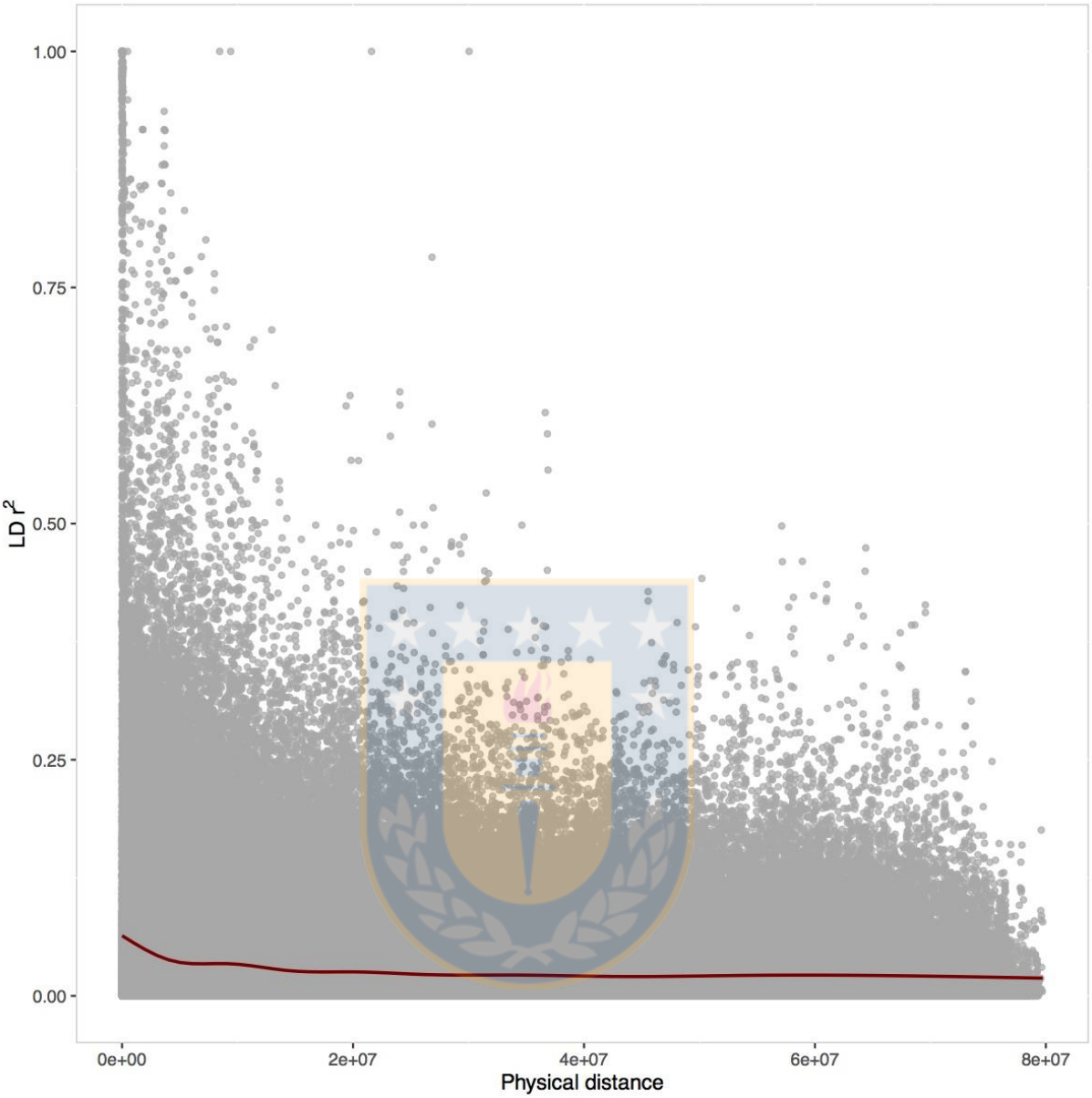


Fig. S3.3.3 LD-scatter plot for Chr3. *Fuente: Elaboración propia.*

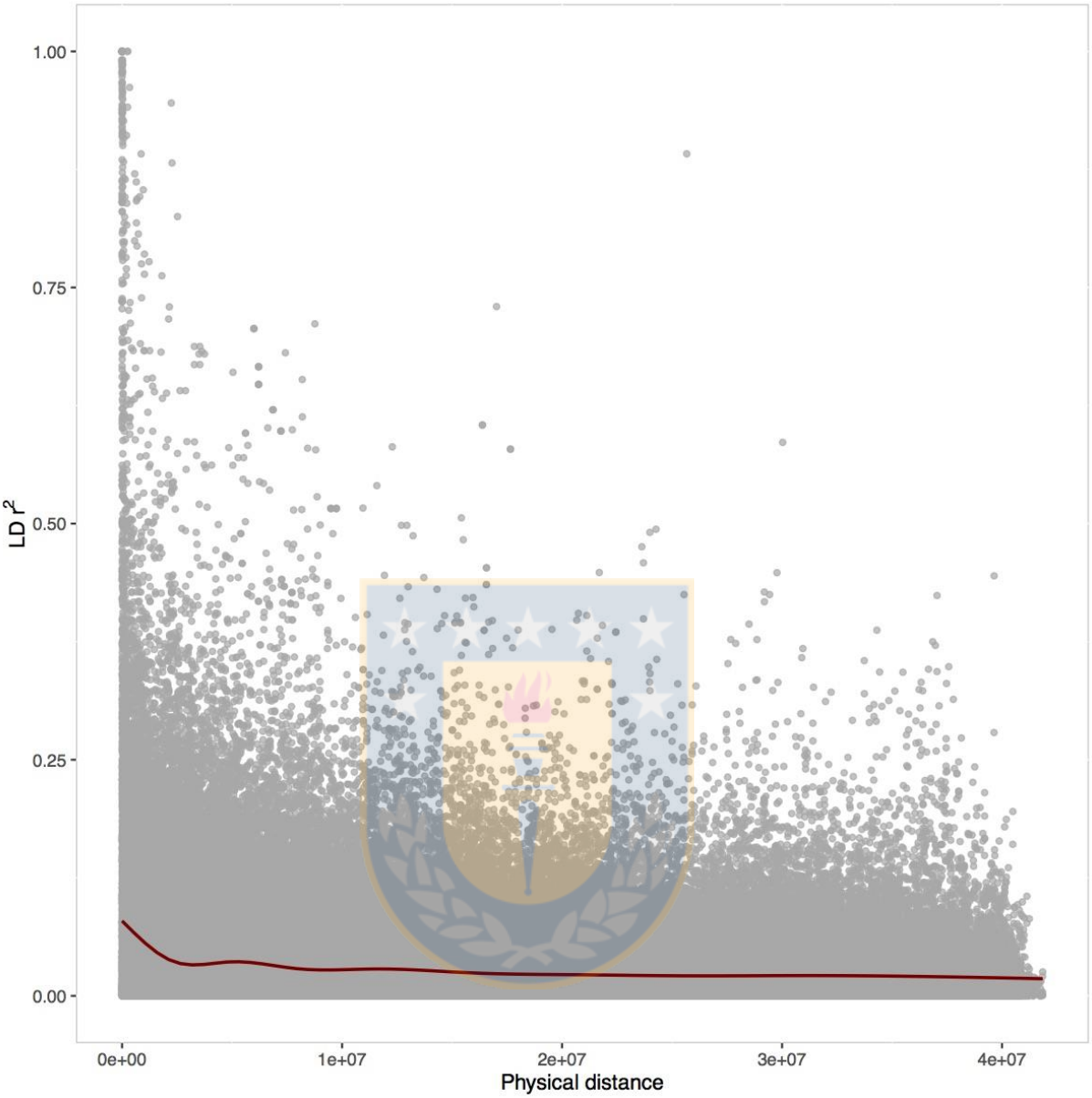


Fig. S3.3.4 LD-scatter plot for Chr4. *Fuente: Elaboración propia.*

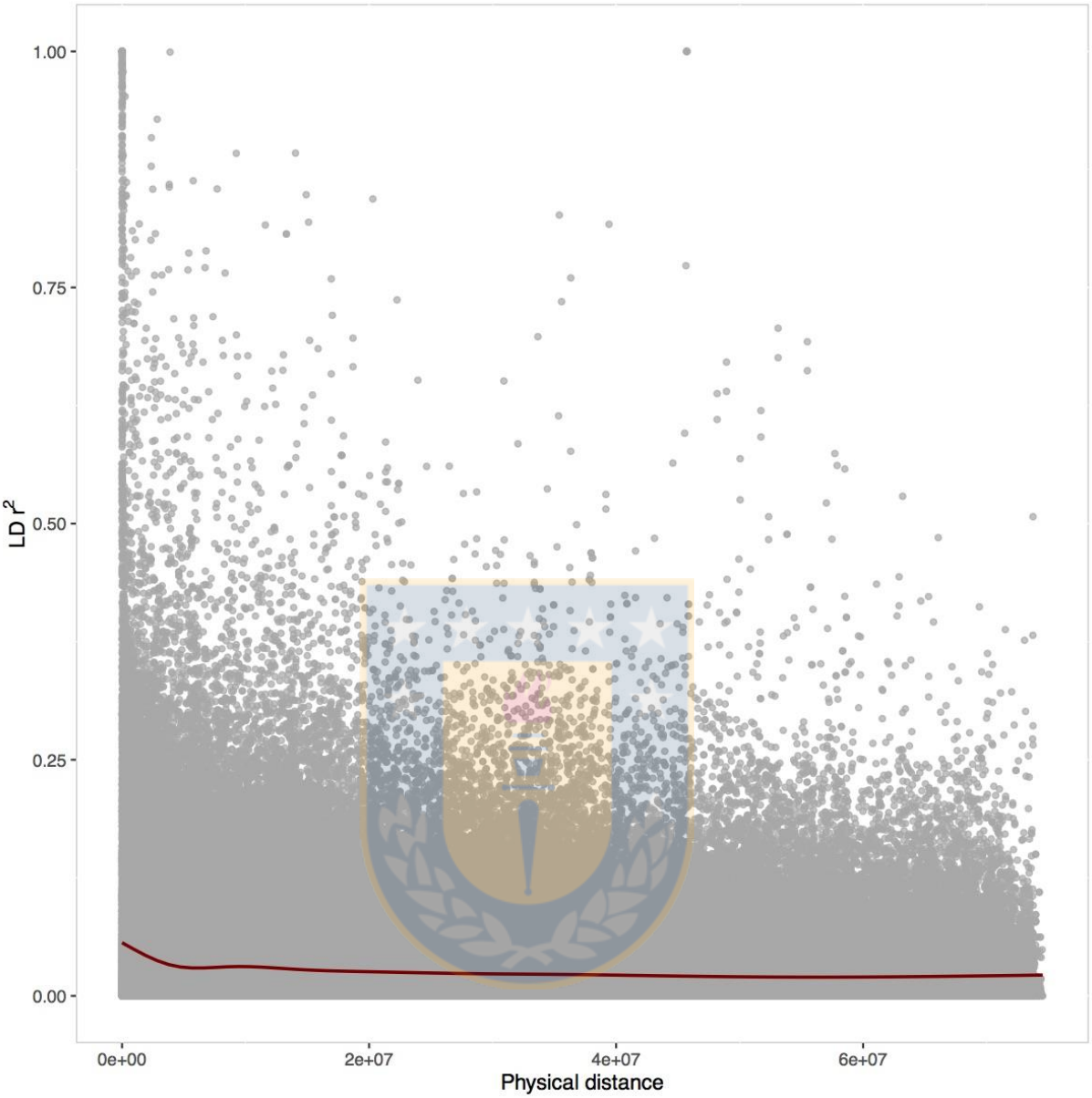


Fig. S3.3.5 LD-scatter plot for Chr5. *Fuente: Elaboración propia.*

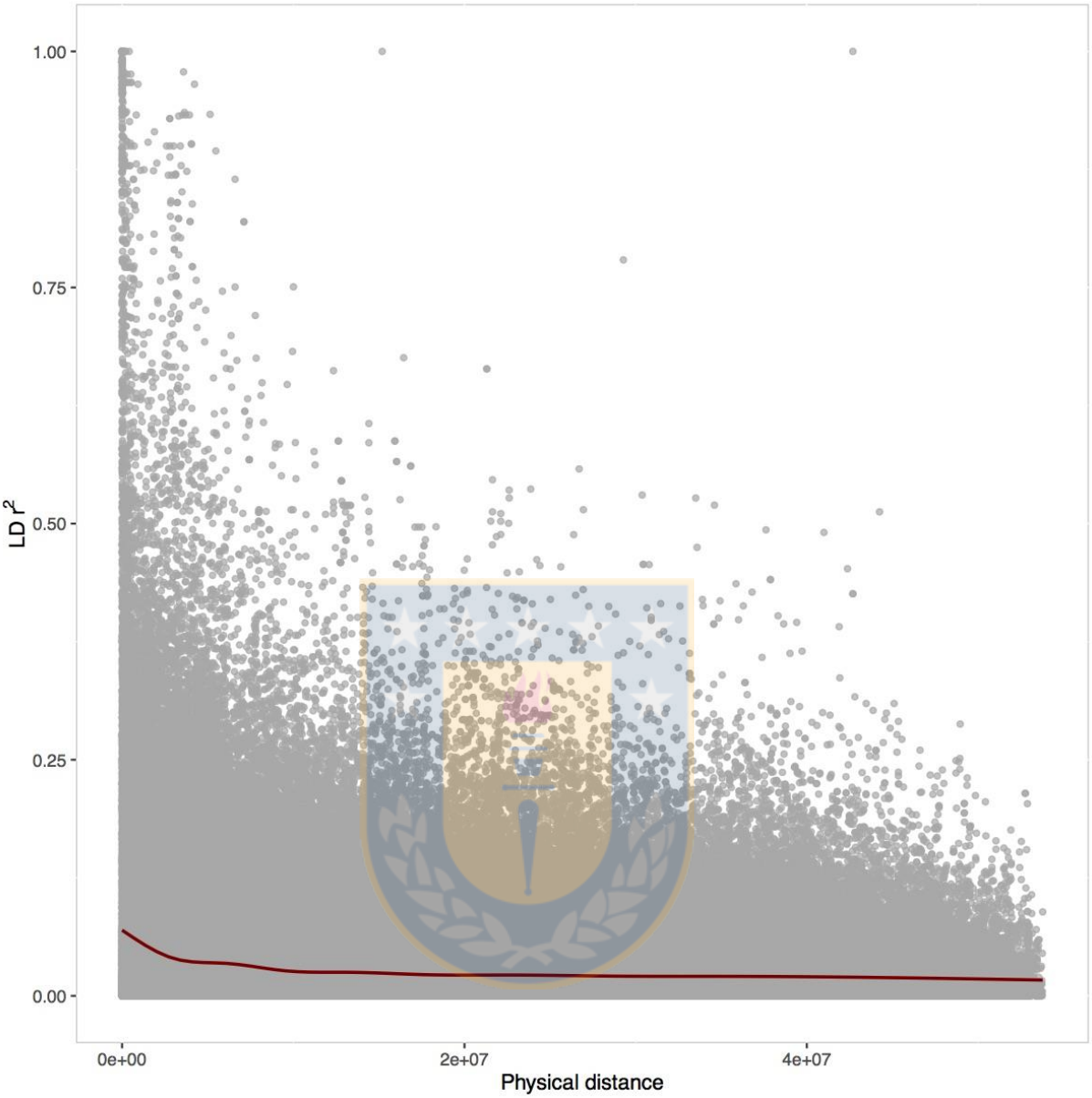


Fig. S3.3.6 LD-scatter plot for Chr6. *Fuente: Elaboración propia.*

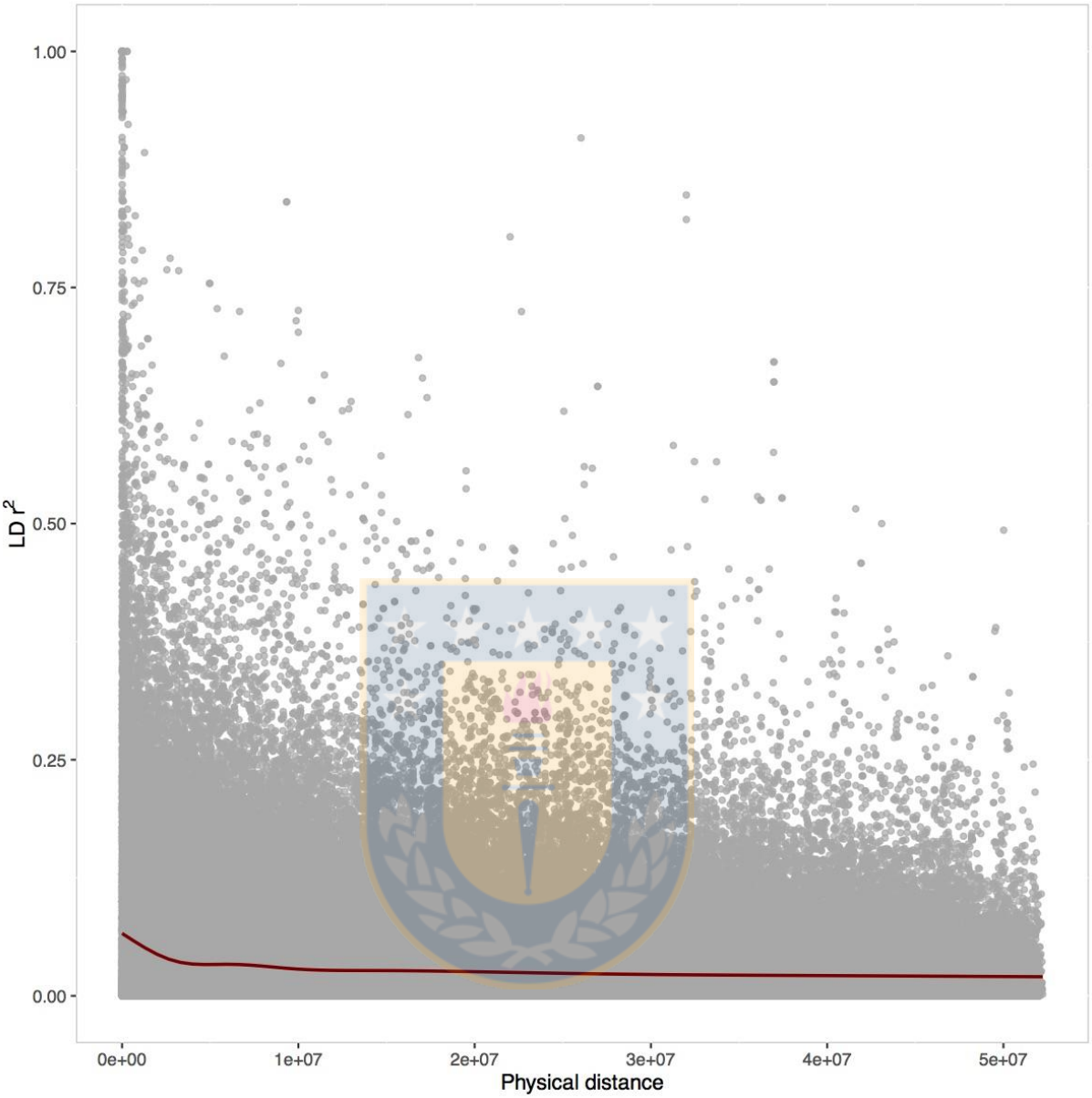


Fig. S3.3.7 LD-scatter plot for Chr7. *Fuente: Elaboración propia.*

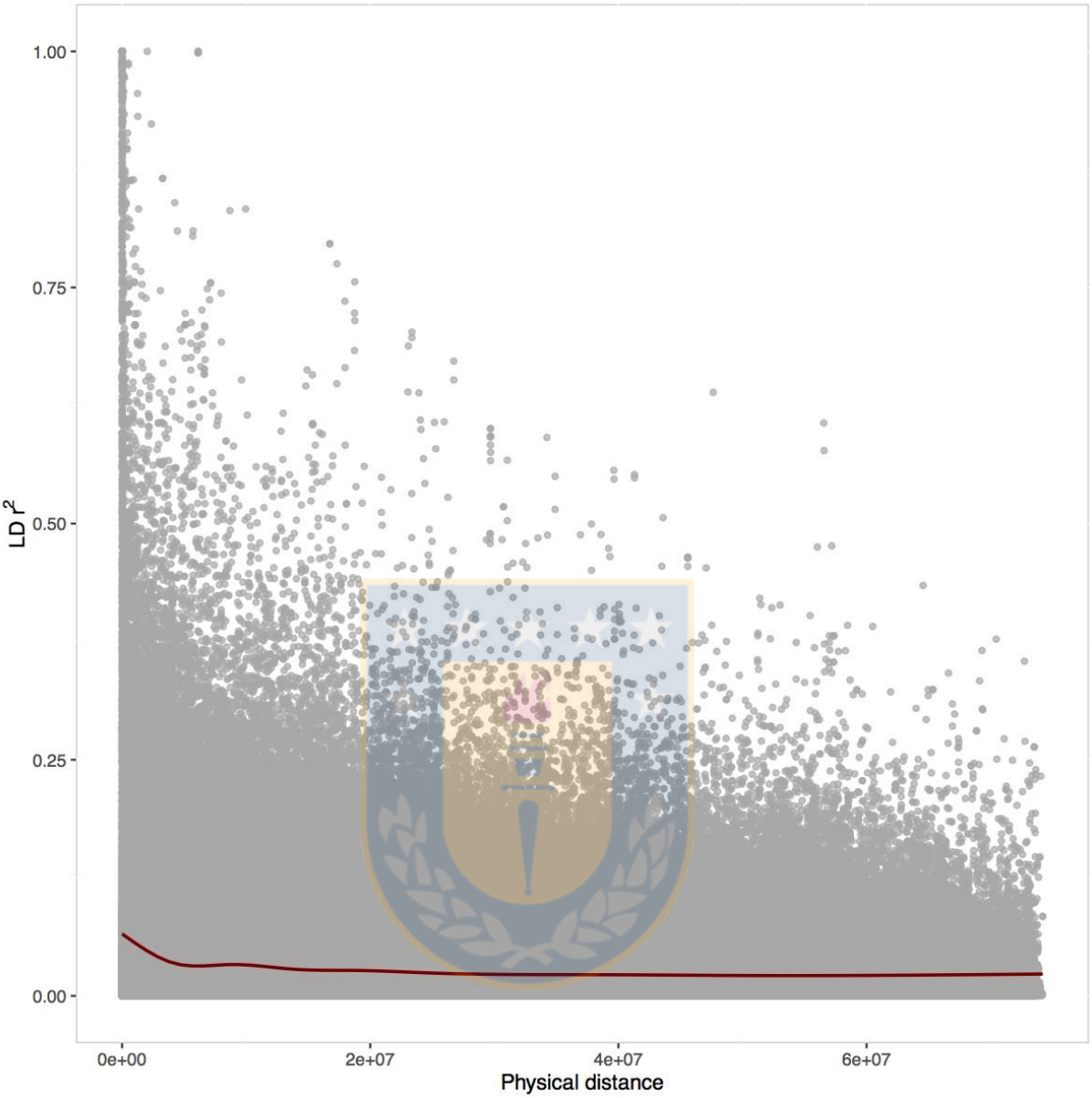


Fig. S3.3.8 LD-scatter plot for Chr8. *Fuente: Elaboración propia.*

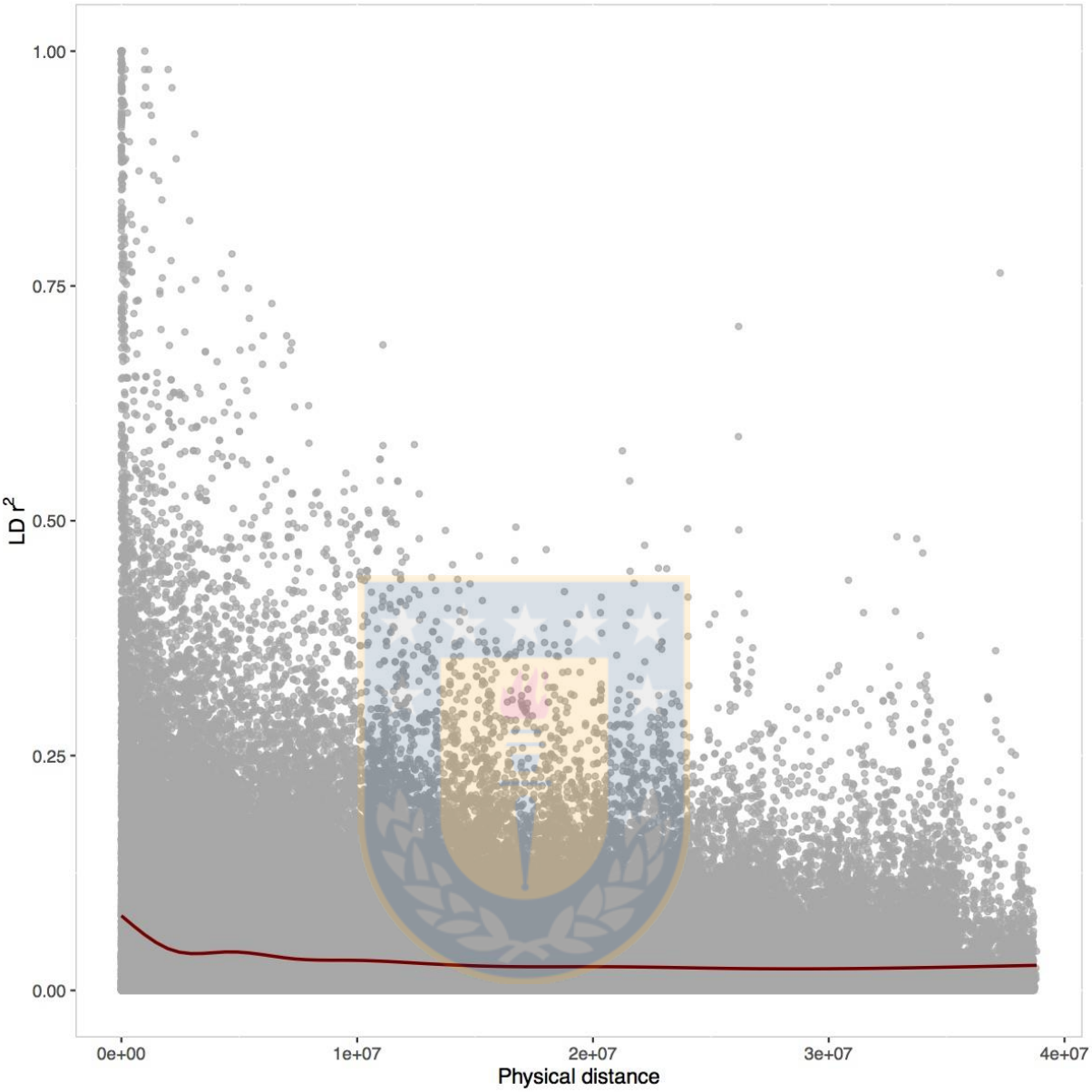


Fig. S3.3.9 LD-scatter plot for Chr9. *Fuente: Elaboración propia.*

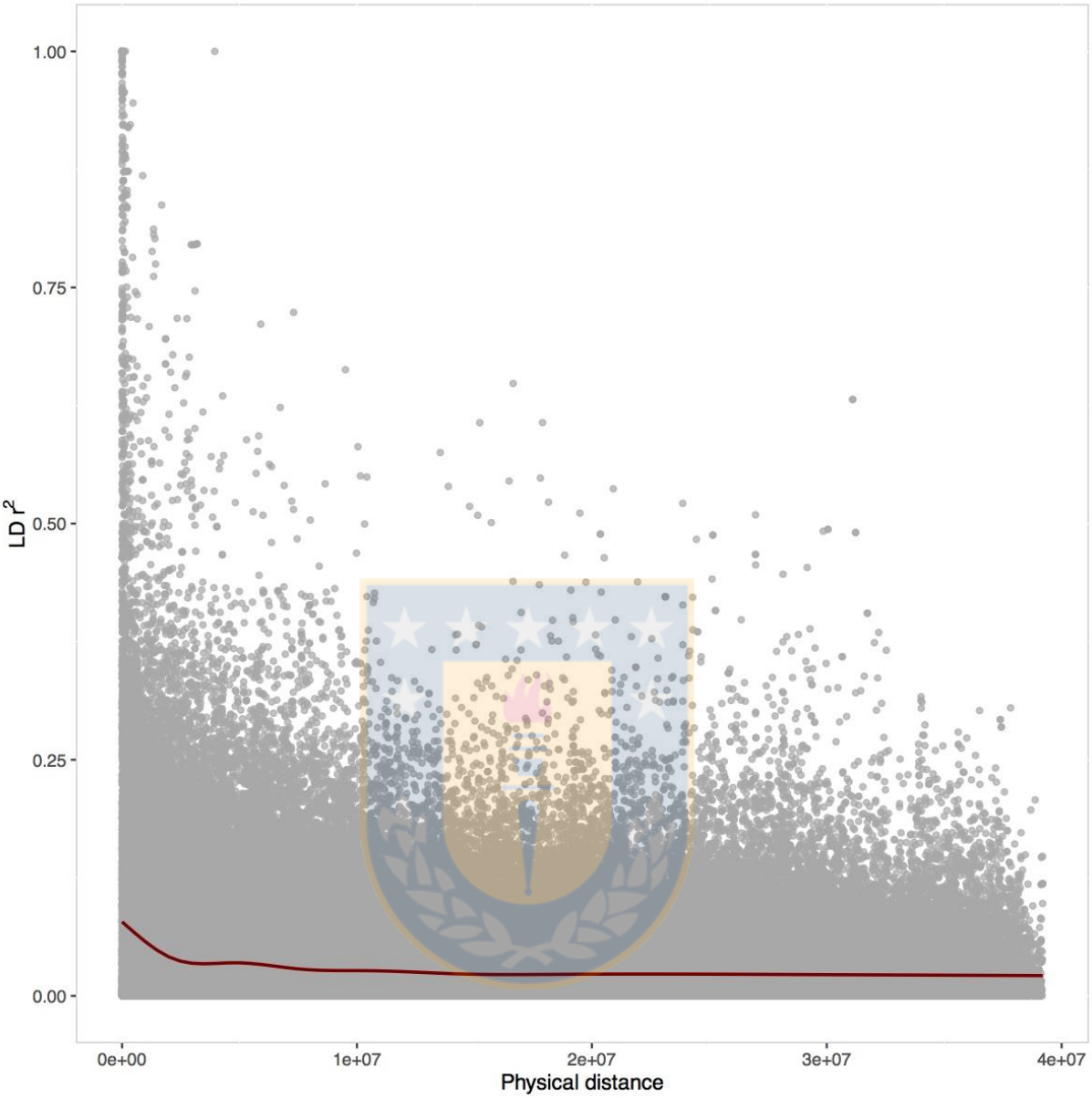


Fig. S3.3.10 LD-scatter plot for Chr10. *Fuente: Elaboración propia.*

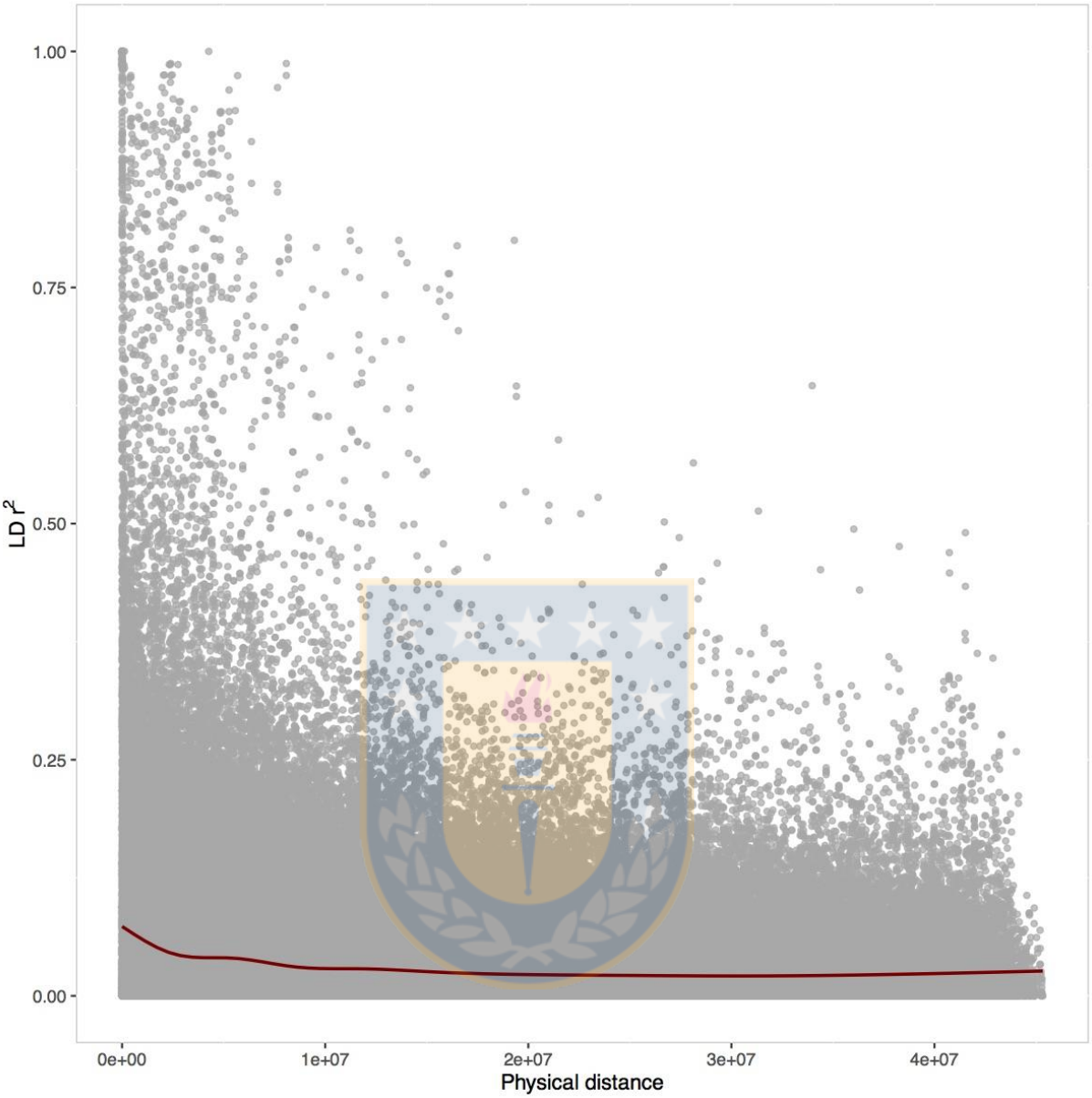


Fig. S3.3.11 LD-scatter plot for Chr11. *Fuente: Elaboración propia.*

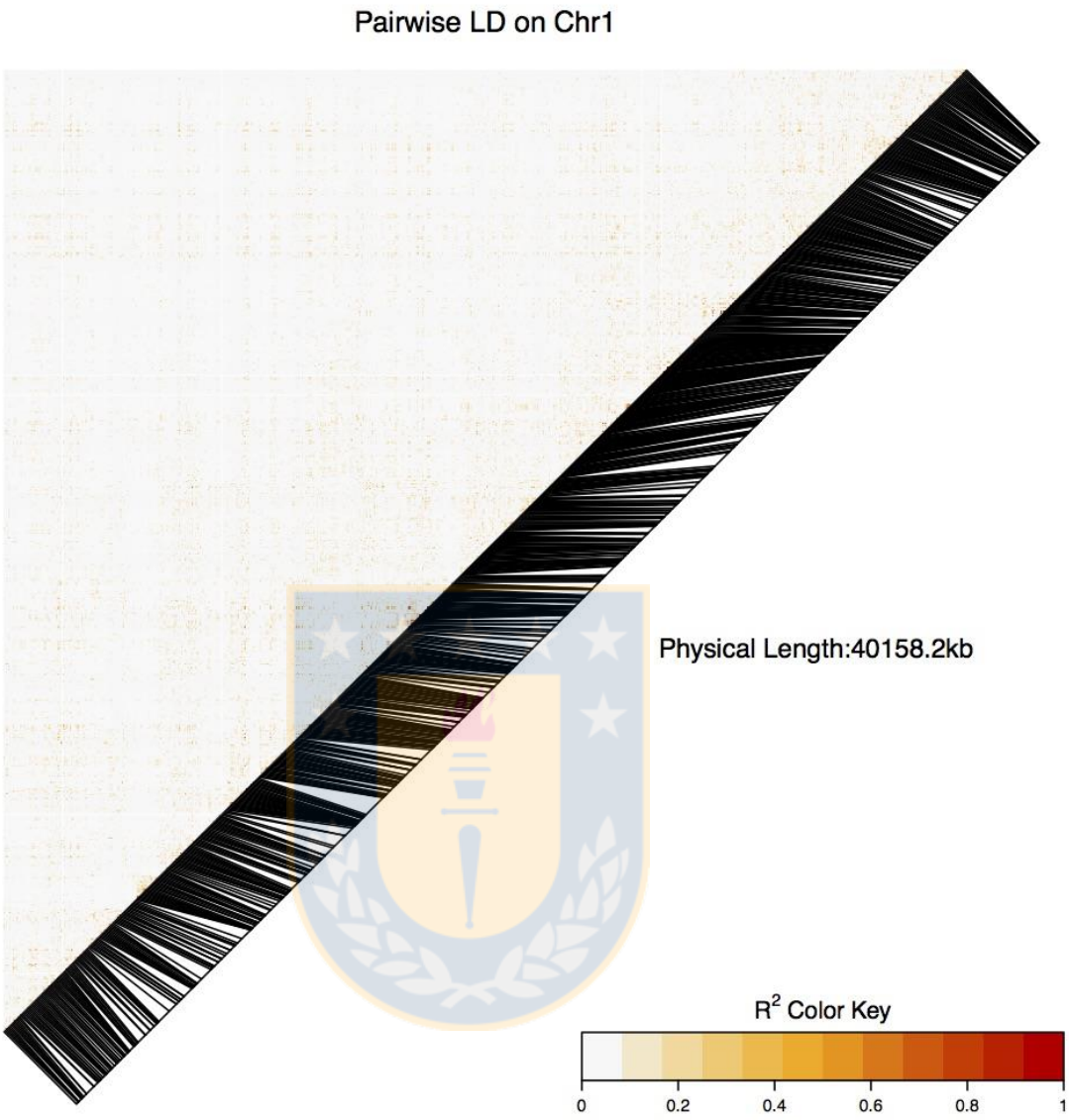


Fig. S3.4.1 Pairwise LD on Chr1. *Fuente: Elaboración propia.*

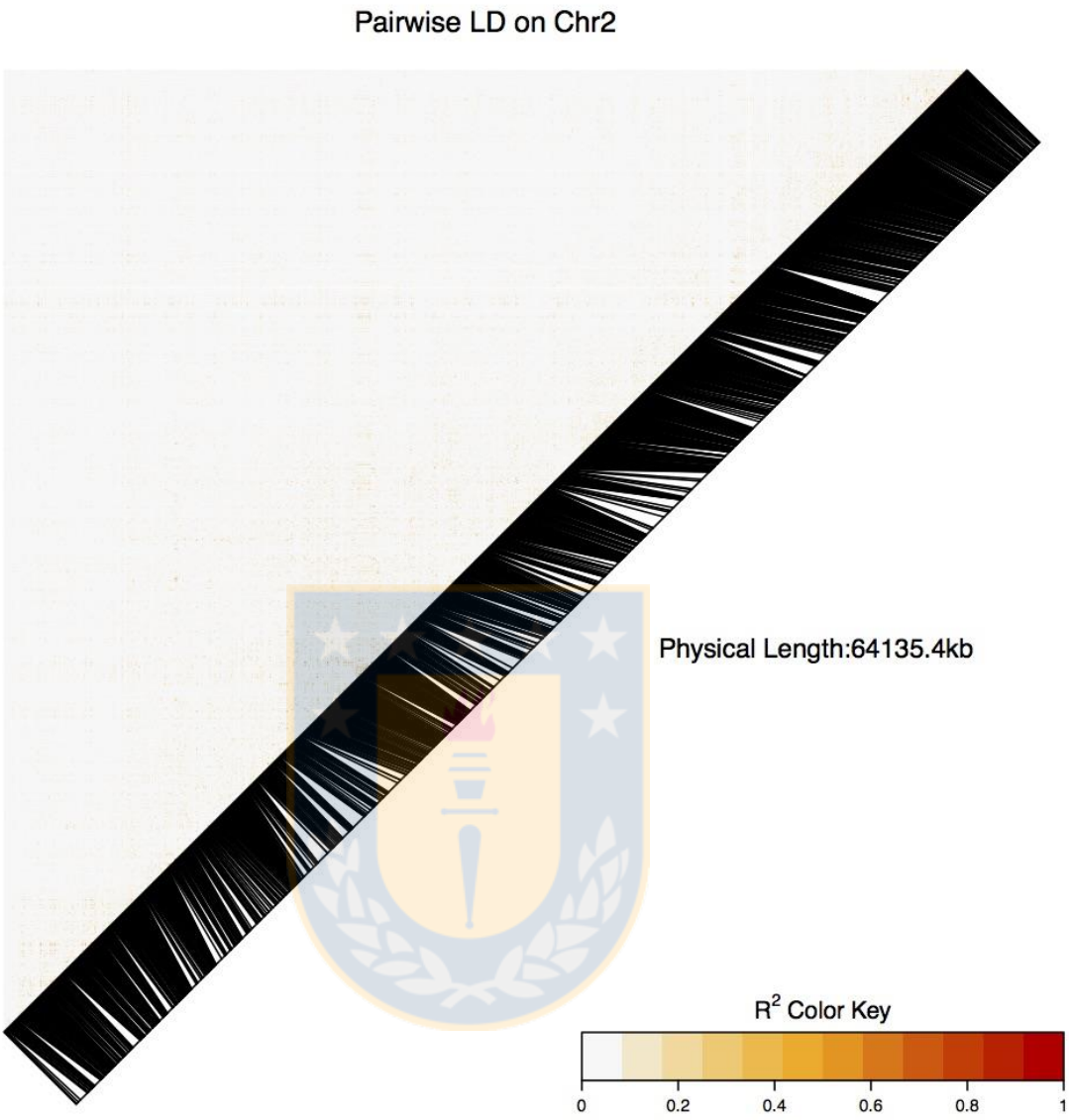


Fig. S3.4.2 Pairwise LD on Chr2. *Fuente: Elaboración propia.*

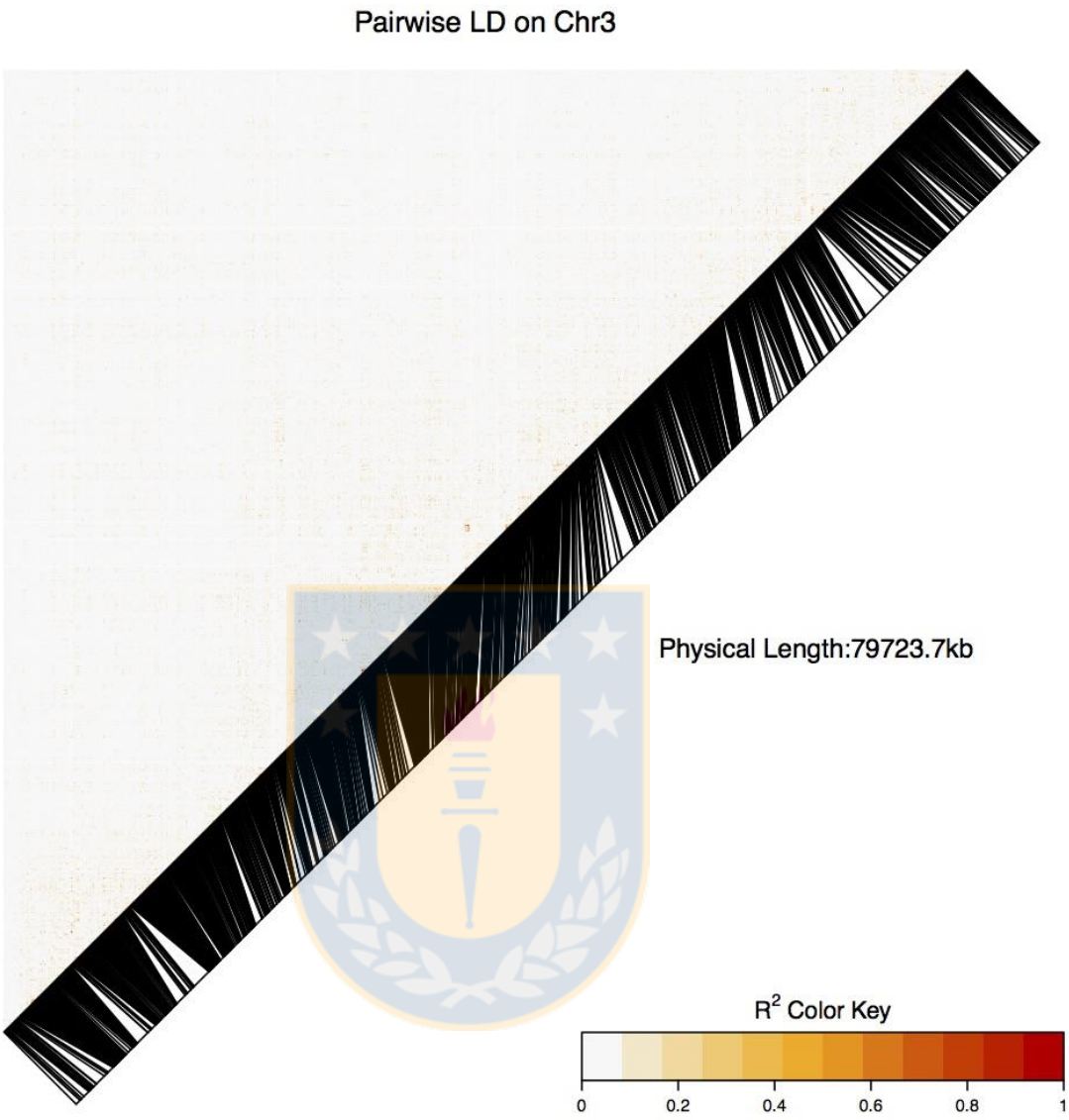


Fig. S3.4.3 Pairwise LD on Chr3. *Fuente: Elaboración propia.*

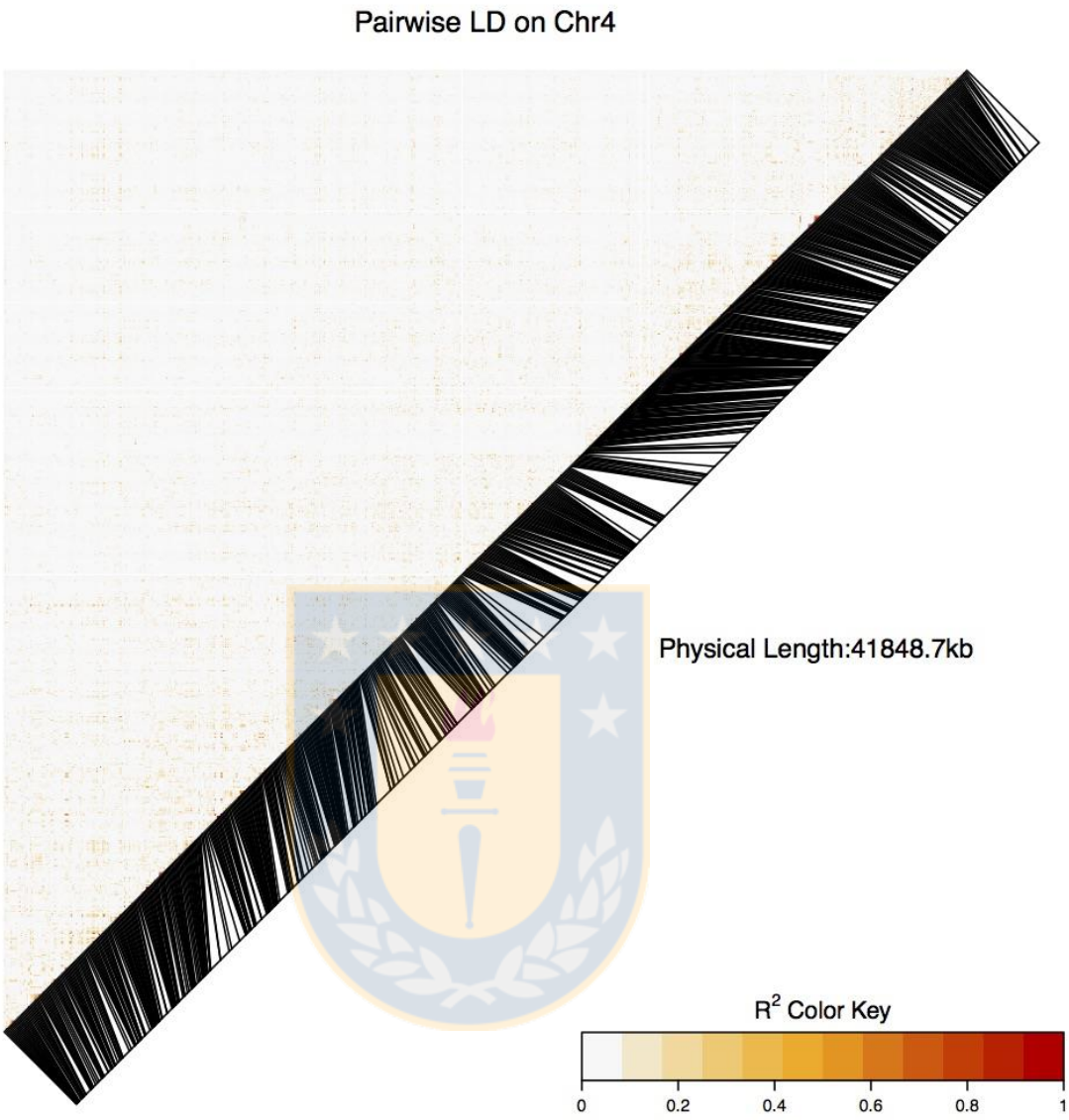


Fig. S3.4.4 Pairwise LD on Chr4. *Fuente: Elaboración propia.*

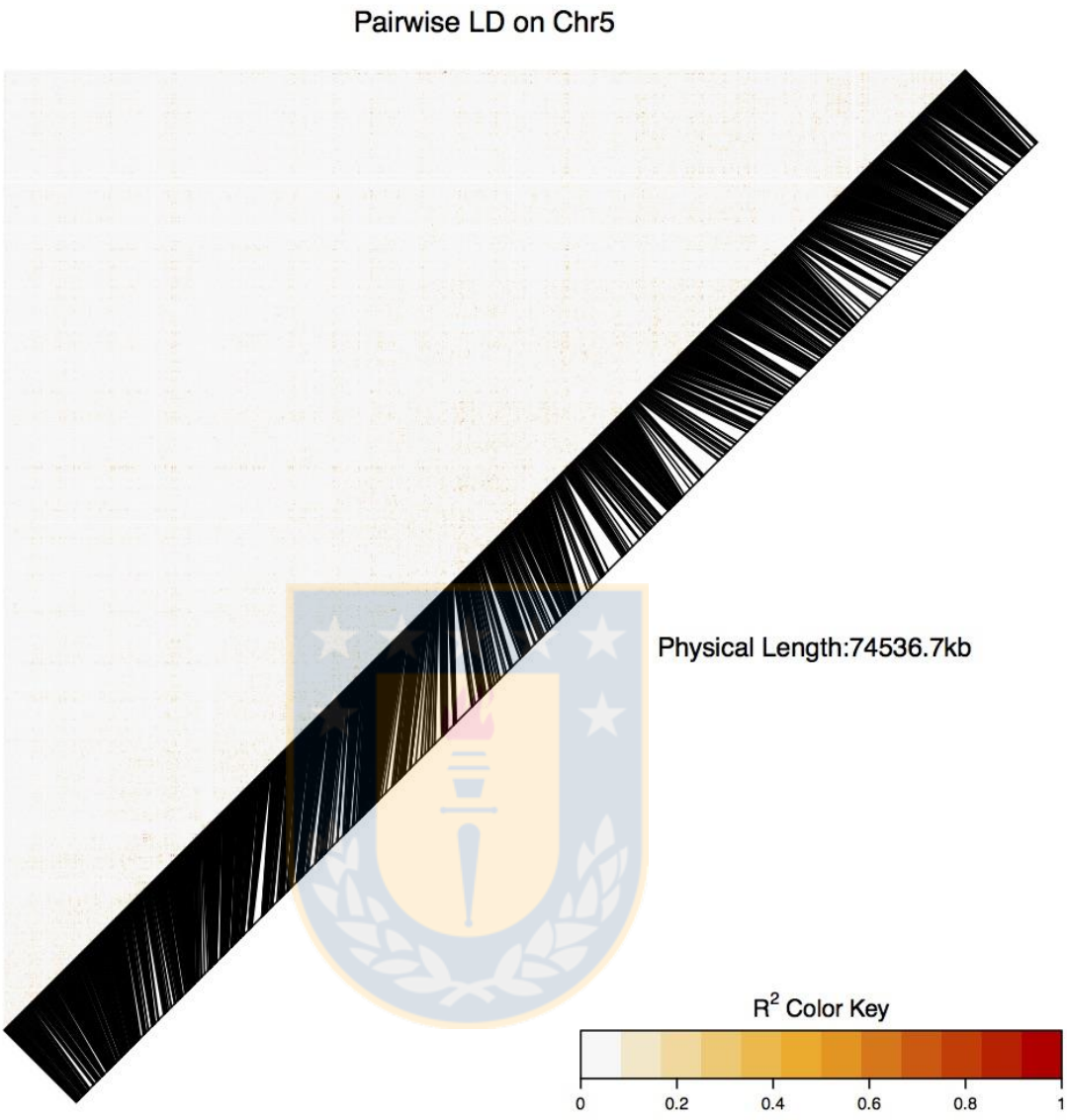


Fig. S3.4.5 Pairwise LD on Chr5. *Fuente: Elaboración propia.*

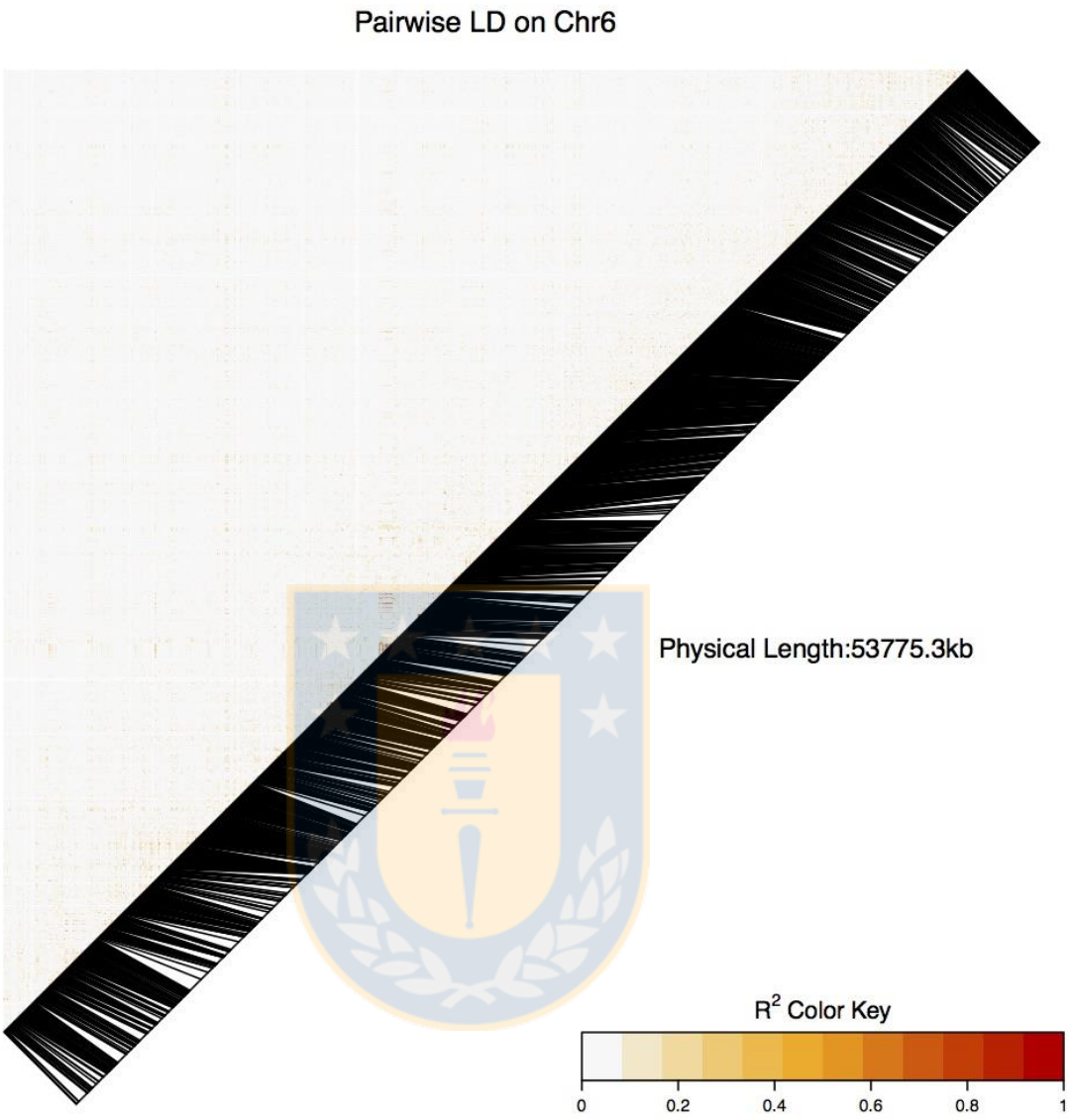


Fig. S3.4.6 Pairwise LD on Chr6. *Fuente: Elaboración propia.*

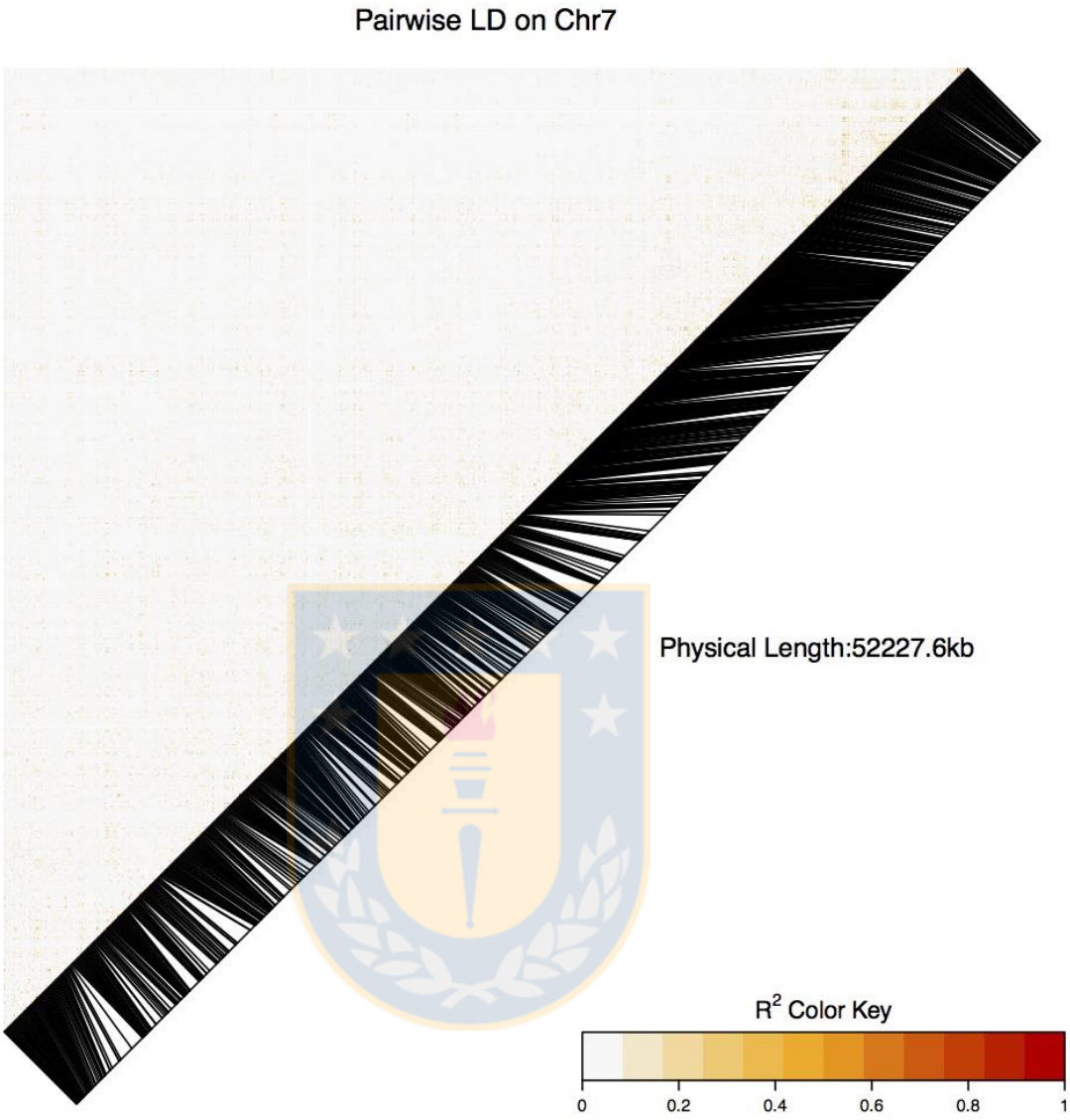


Fig. S3.4.7 Pairwise LD on Chr7. *Fuente: Elaboración propia.*

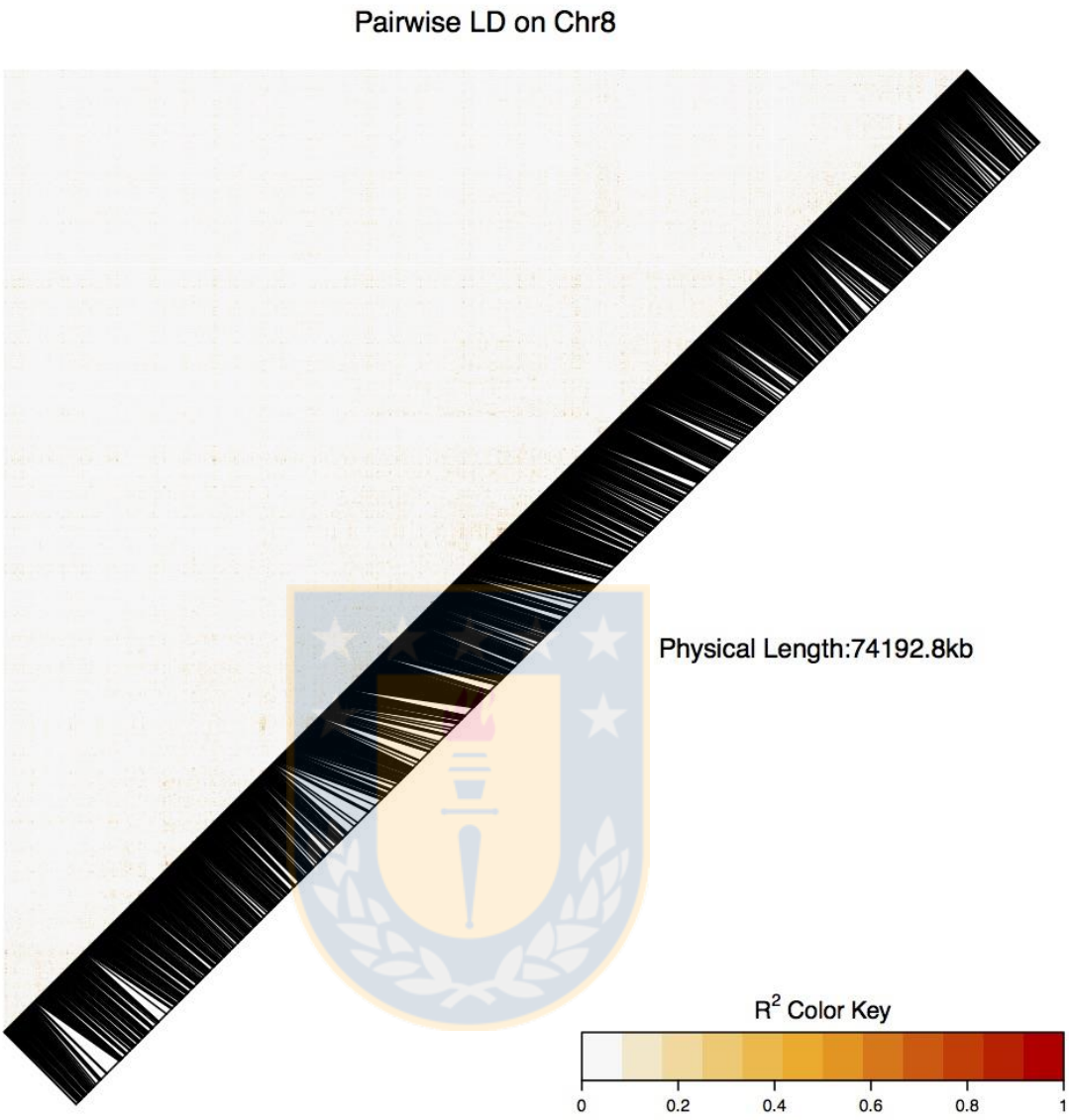


Fig. S3.4.8 Pairwise LD on Chr8. *Fuente: Elaboración propia.*

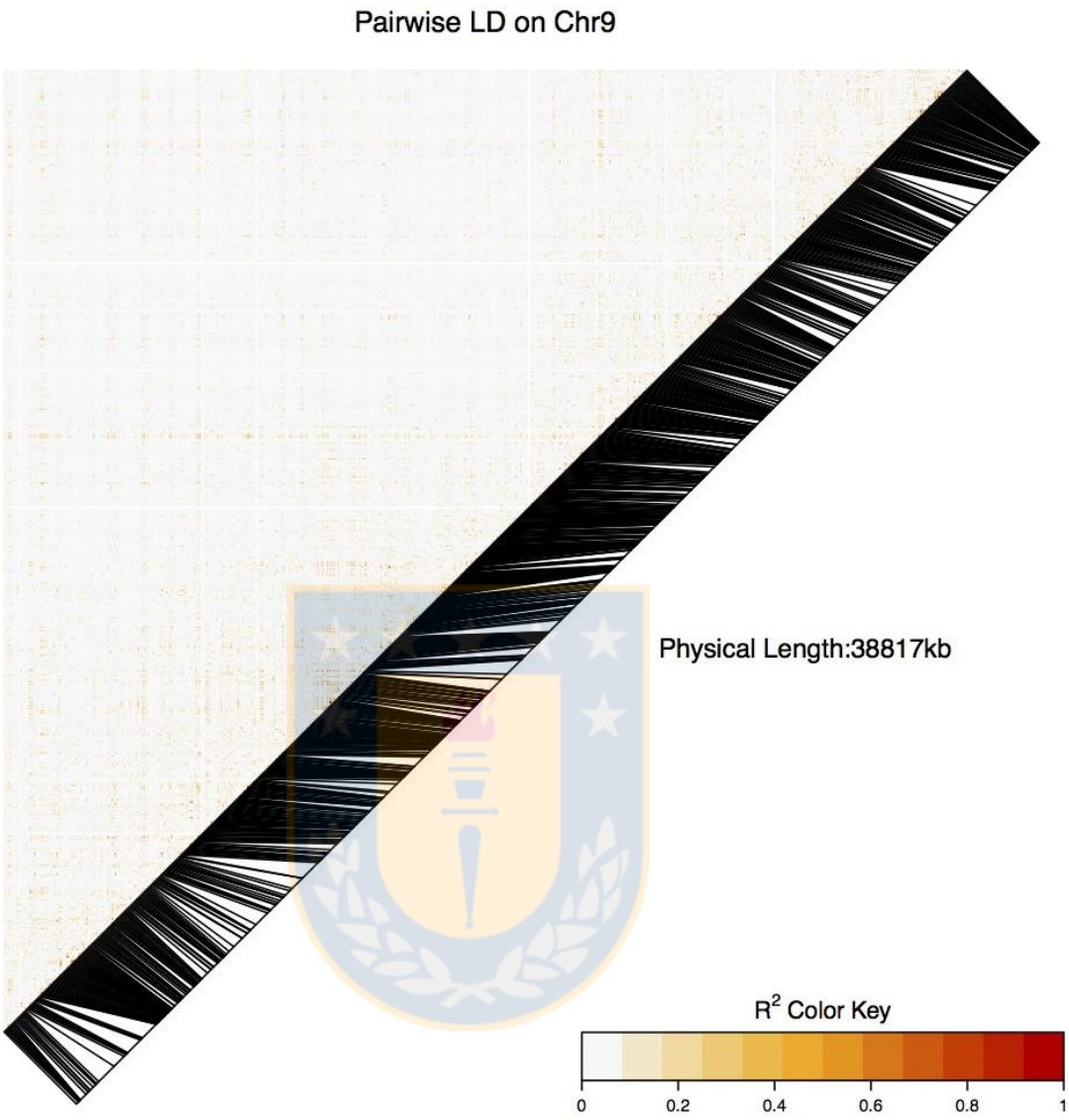


Fig. S3.4.9 Pairwise LD on Chr9. *Fuente: Elaboración propia.*

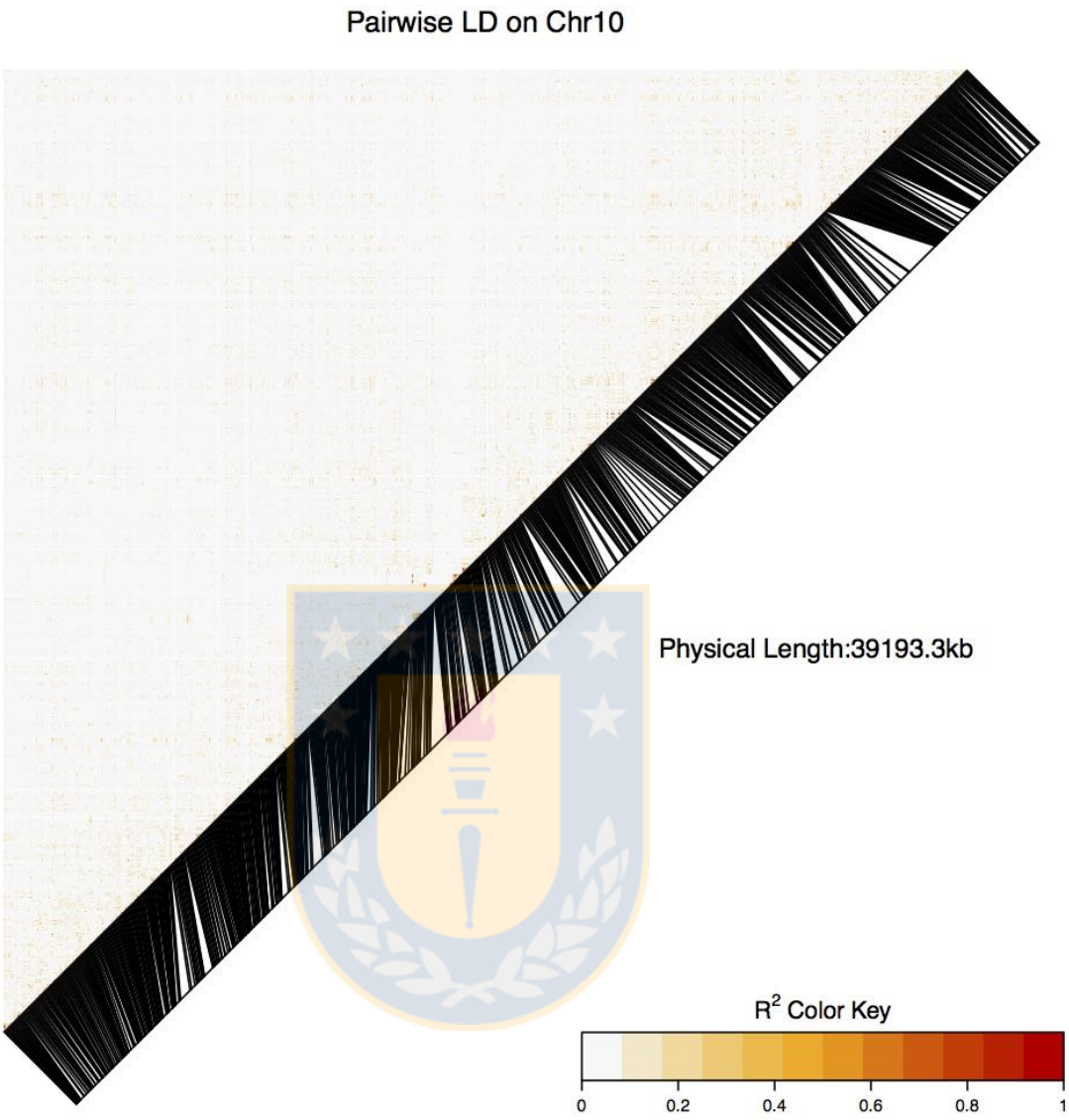


Fig. S3.4.10 Pairwise LD on Chr10. *Fuente: Elaboración propia.*

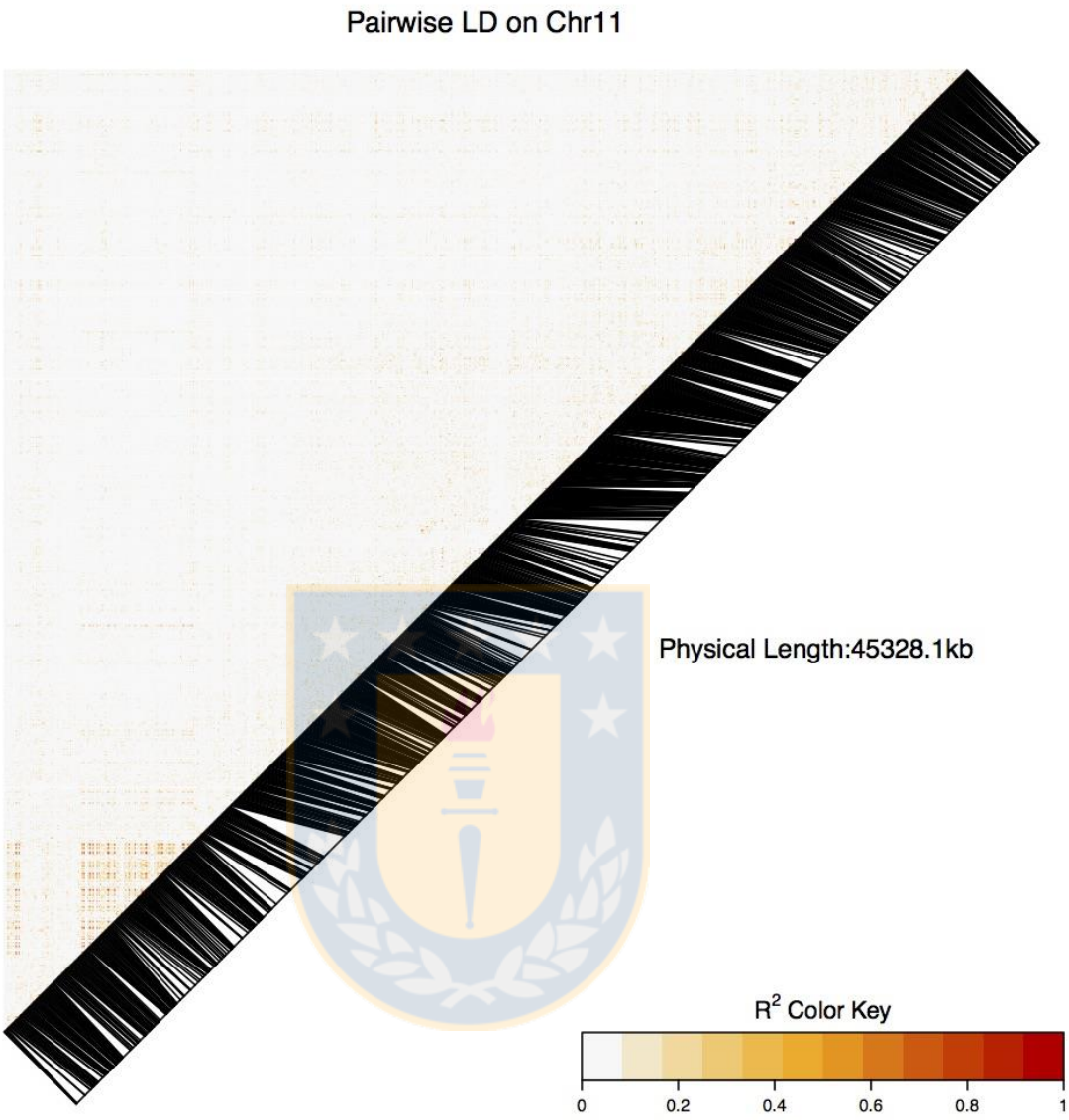


Fig. S3.4.11 Pairwise LD on Chr11. *Fuente: Elaboración propia.*

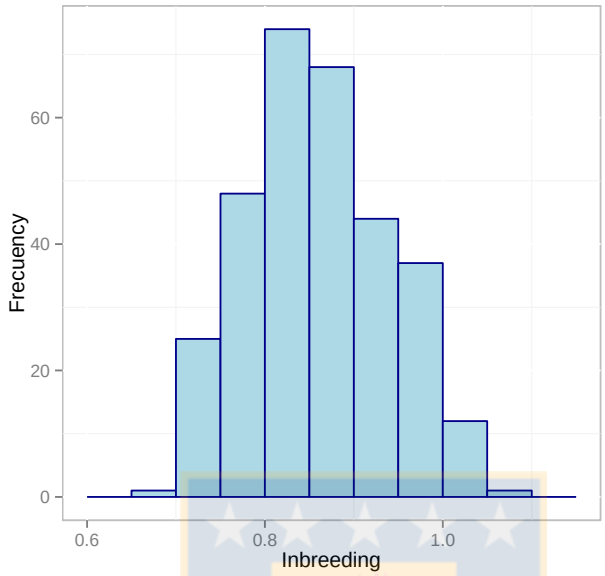


Fig. S3.5 Inbreeding values derived from shared SNP markers. *Fuente: Elaboración propia.*

Table S3.1 Evaluation of statistical models (GLUP, BLasso, Bayes B and Bayes C) by using random sampling of 50 individuals with 2 folds and 10 replications for wood density and volume. *Fuente: Elaboración propia.*

Trait/Statistics	**GBLUP** Mean (min-max)	**BLasso** Mean (min-max)	**Bayes B** Mean (min-max)	**Bayes C** Mean (min-max)
Wood density				
Mean best 10%	3.5 (1.9-5.6)	3.5 (2.8-5.1)	3.8 (2.0-5.8)	3.8 (1.8-6.8)
MSE	16.5 (15.7-17.3)	6.4 (5.1-8.0)	13.3 (10.4-20.0)	11.8 (7.2-17.2)
Bias	1.2 (0.8-1.5)	1.0 (0.8-1.4)	0.91 (0.5-1.2)	1.0 (0.5-1.3)
Volume				
Mean best 10%	34.6 (29.6-39.4)	34.87 (30.5-38.3)	34.9(32.1-37.3)	33.7 (27.6-37.7)
MSE	108.6 (96.0-124.6)	63.8 (52.4-79.3)	237.0 (165.2-313.9)	242.9 (202.4-296.0)
Bias	1.1 (0.9-1.2)	1.44 (0.9-4.6)	1.1 (0.9-1.4)	1.1 (0.9-1.4)

DISCUSIÓN GENERAL

Los SNPs corresponden a diferencias bi, tri y tetra-alélicas en la secuencia de ADN en sitios homólogos (Brookes 1999; Cho et al. 1999). Son el tipo de polimorfismos más abundante dentro de los genomas y, teóricamente, se pueden encontrar en cualquier lugar de una secuencia genómica (Rafalski 2002), hasta cercanos a algún locus de interés (Germano y Klein 1999; Picout-Newber et al. 1999). Hasta hace algunos años, su identificación era costosa, difícil y requería de mucho tiempo de análisis. Sin embargo, gracias a las tecnologías NGS, el descubrimiento de alto rendimiento de estos marcadores ha sido posible (van Tassel et al. 2008) y se ha vuelto más sencillo para especies que carecen de un genoma de referencia (Bachlava et al. 2012) o con poca información genómica disponible (Davey et al. 2011; Kim et al. 2016). Sin embargo, desafortunadamente, aún sigue siendo una tarea difícil para algunas especies que poseen tamaños de genomas mayores y que contienen una alta cantidad de secuencias repetidas (de Souza et al. 2016).

En el primer estudio presentado en este trabajo, se utilizó la tecnología de GBS para la identificación de SNPs, de la cual no existen reportes previos para *E. globulus* en Chile. Si bien se sabe que la secuenciación de todo el genoma por NGS es la herramienta ideal para la identificación de estos marcadores, en presencia de un genoma de referencia, no es necesario una mayor profundidad de secuenciación para fines de genotipificación. Por lo tanto, es posible implementar este tipo de enfoque, basado en la reducción de la complejidad del genoma por enzimas de restricción para el llamado de variantes (Gürcan et al. 2016). Hoy en día, el uso de herramientas bioinformáticas ha generado diferentes flujos de trabajo (Bradbury et al. 2014; Catchen et al. 2013) para extraer información sobre SNPs y genotipos desde una gran cantidad de secuencias (Kagale et al. 2016; McCormack et al. 2013). Uno de los grandes desafíos en GBS es el análisis de sus datos, el cual debe ser rápido y eficiente. Éste, considera las secuencias brutas a partir la secuenciación, la separación de las muestras multiplexadas, el filtrado de secuencias de baja calidad, el ordenamiento y alineamiento de secuencias y el llamado de los SNPs (Scheben et al. 2017), además de la disponibilidad de un genoma de referencia para la especie (Torkamaneh et al. 2016). En el estudio se presentó una alternativa de flujo de trabajo utilizando diferentes herramientas de análisis de libre acceso, donde en primer lugar, las librerías fueron secuenciadas en una sola dirección (single-read), siendo la

forma más simple de preparación de librerías para secuenciación Illumina. Sin embargo, sería mejor considerar un tipo de secuenciación que permita leer desde ambos extremos de un fragmento (paired-end), ya que genera una mayor calidad de lecturas para alineamientos en contra de una referencia y una mejor identificación de zonas problemáticas dentro del genoma como re-arreglos genómicos y elementos repetitivos (Hillier et al. 2008).

Dado que *E. globulus* es una especie que carece de genoma de referencia, se utilizó el genoma de *E. grandis* para el mapeo de las librerías genómicas, obteniendo un alto número de SNPs catalogados como monomórficos o diferencias nucleotídicas intra-especie. Sin embargo, el alto número de marcadores monomórficos identificados puede atribuirse a productos de errores en la secuenciación, principalmente en la etapa de amplificación e intensidad de hibridación. Por otra parte, la mayoría de los SNPs se encontraban en regiones no codificantes, ya que pueden llegar a ser tres veces más frecuentes que aquellos en zonas codificantes (Ching et al. 2002).

Como se ha discutido anteriormente, el objetivo fue optimizar el proceso de identificación de SNPs, pero en general las características genómicas, la complejidad de los genoma, el nivel de heterozigosidad, la proporción de secuencias repetitivas y el nivel de polimorfismo, pueden contribuir positiva o negativamente a estos desafíos (Nielsen et al. 2011; Scheben et al. 2017). También se discutieron diferentes factores técnicos que podrían estar afectando el proceso de identificación y que han sido analizados en otros estudios para su mejora como la calidad del ADN, el grado de multiplexación de muestras secuenciadas (Poland et al. 2012), número de lecturas obtenidas por muestra (He et al. 2014), el largo de las secuencias (Melo et al. 2016) y errores de secuenciación como datos perdidos (Brouard et al. 2017). Por lo tanto, es necesario seleccionar parámetros apropiados, tales como, la profundidad de cobertura requerida, la calidad del mapeo de lectura o el grado de divergencia para un mapeo exitoso (Torkamaneh et al. 2017). Por otra parte, se hace necesario una comparación entre diferentes flujos de trabajo, principalmente para validar una mayor cantidad de SNPs comunes. Si bien se pensaba que es muy difícil comparar las estadísticas resultantes de diferentes estudio, principalmente debido a las metodologías utilizadas, técnicas de secuenciación, material base y que la detección de SNPs es independientes en cada análisis (da Souza et al. 2016), se ha visto que es posible

identificar marcadores comunes bajo diferentes flujos de trabajo, utilizando diferentes tecnologías de secuenciación (Mascher et al. 2013).

En este estudio, la utilización de GBS no fue enfocado en descubrir marcadores que pudieran ser posteriormente utilizados como una matriz física de genotipificación (SNP-Chip), si no más bien, para descubrir simultáneamente estos polimorfismos entre un grupo específico de individuos y utilizar esta información para posteriores estudios de selección asistida. Para poder evaluar el potencial de genotipificación de los SNPs descubiertos mediante GBS, un análisis de agrupamiento de individuos, de acuerdo a su estructura genética, fue realizado para la población "A" del estudio (datos no mostrados), donde los marcadores mostraron no ser capaces de discriminar los grupos genéticos (familias) que se presentaban dentro de la población (material suplementario MS1). Por lo tanto, considerando este resultado, el alto costo de análisis, tiempo y recurso informático, se hizo necesaria la evaluación de una siguiente alternativa que permitiera genotipificar de una manera rápida y fácil SNPs en *E. globulus.*

En el segundo estudio presentado, se evaluó la capacidad de genotipificación del EUChip60k para *E. globulus*, el primer chip generado a partir de la secuenciación de 12 especies diferentes de *Eucalyptus* (Silva-Junior et al. 2015a). En el capítulo II se discutió la utilización de esta herramienta como sistema de genotipificación clonal para la especie, pero es importante agregar algunas de las capacidades técnicas que acompañan a la genotipificación por chip. Aunque la tecnología se ha descrito como desfavorable para algunos enfoques de diversidad genética (Ganal et al. 2012), o búsqueda de SNPs en genes específicos, son muchas más sus ventajas las asociadas a su elección, principalmente por la gran cantidad de datos de salida, información adicional relevante, el hecho de que no necesita una etapa de pre-procesamiento y que además es mucho más rápido en análisis, eficiente y económico (Mason et al. 2017). Por ejemplo, a partir de los análisis realizados, al comparar ambas tecnologías (GBS v/s Chip), GBS permitió identificar un número previamente indeterminado de marcadores, que además resultaron ser de baja calidad para posteriores análisis de genotipificación (Informe S4). Por su parte, el EUChip60K entregó un alto número de SNPs de alta reproducibilidad, que pudieron

ser analizados simultáneamente y que fueron posteriormente utilizados para un análisis de identidad.

Como se ha señalado anteriormente, los SNPs constituyen una importante fuente de MMs que pueden tener variadas aplicaciones. Uno de ellos es la genotipificación de material genético, tarea que hasta la fecha ha sido realizada satisfactoriamente por los marcadores SSR. En el proceso de genotipado, para un buen análisis de marcadores desde un chip, uno de los puntos críticos descritos ha sido siempre la calidad de ADN utilizado, por su influencia directa sobre la calidad del llamado de cada variante (Chagné et al. 2015). Sin embargo, de acuerdo a los resultados obtenidos en este estudio, este factor no afectaría la genotipificación. Dado lo anterior, estrictos parámetros de calidad han sido ajustados para optimizar el proceso de *clustering* de los marcadores (Myles et al. 2010; Verde et al. 2012), los que tampoco fueron necesarios de calibrar cuando el llamado de SNPs desde el chip fue reportado. La presencia de falsos positivos dentro del genotipado de SNPs por Chips, en muchos casos aumenta en muestras de mala calidad, por lo que estrategias de depuración de aquellos SNPs que deben ser excluidos para posteriores análisis se hace recurrente. Para mayor información, un completo reporte sobre la generación de estas plataformas físicas de genotipado, posteriores análisis y aplicaciones han sido descritos por Rasheed et al. (2017) y Nicolazzi et al. (2015). Ganal et al. (2014) destacan además, que aún no está claro hasta qué punto la tecnología de GBS podría reemplazar el genotipado por chips, considerando su falta de estandarización y dificultad para comparar datos desde diferentes proyectos y laboratorios, mientras que los datos desde chips resultan más comparables. Sin embargo, para otros autores, como Bajgain et al. (2016), los estudios comparativos entre ambas tecnologías, demostraron que GBS puede ser un mejor método de genotipado de SNPs, ya que es capaz de proporcionar una cobertura más amplia. Sin embargo, el genotipado por SNP-chip requiere menos conocimiento computacional y recursos para el procesamiento de datos.

Si bien los costos de genotipado por secuenciación y chips han ido reduciéndose con los años, no es fácil predecir qué tecnología será más barata en un futuro próximo. Por ejemplo en nuestros estudios, los costos asociados la genotipificación mediante GBS y chip tuvieron un valor aproximado de 6 dólares por kilobase de SNPs, para ambas tecnologías, y de 60 dólares

por muestra, lo que grandes volúmenes, significaría considerables costos asociados solo al sistema de genotipado.

Validada la estrategia de genotipado mediante el EUChip60k, esta plataforma fue utilizada para genotipificar un nuevo set de muestras de *E. globulus*, donde un total de 12 K de SNPs resultaron ser polimórficos y fueron utilizados para ajustar un modelo de predicción genómico. La SG es una alternativa al mejoramiento asistido por marcadores que ha revolucionado la genética animal (Hayes y Goddard 2010) y de plantas (Heslot et al. 2015) para la predicción de valores genéticos. Ello ha generado en los últimos años grandes expectativas para su aplicación en especies forestales, donde ha existido un aumento significativo de trabajos publicados discutiendo la respuesta de los modelos de SG para diferentes características de interés, comparando modelos estadísticos, métodos de validación y densidad de marcadores, entre otros parámetros.

En el estudio presentado en el capítulo III, la capacidad predictiva de la SG, para estimar valores genómicos para dos rasgos de interés (densidad de la madera y volumen del fuste), fue evaluada bajo un modelo de validación cruzada, con una densidad media de SNPs y bajo diferentes modelos estadísticos. En general, el modelo ajustado en este pequeño grupo de clones de *E. globulus* reportó resultados bastante consistentes y prometedores, muy similares a los ya publicados para especies de coníferas y eucaliptos por otros autores mencionados anteriormente. Por ejemplo, no existieron diferencias en las habilidades de predicción de los modelos GBLUP y aquellos basados en Bayes, lo que ha sido ampliamente validado para otras especies, sugiriendo que, si bien la arquitectura genética de las características son complejas, el modelo de GBLUP posee un buen ajuste, lo cual favorece su aplicación, principalmente debido a la simplicidad de este modelo en comparación a aquellos no paramétricos y el bajo consumo de análisis (Ratcliffe et al. 2015; Resende et al. 2017).

Entre los factores que más estarían influyendo en la habilidad de predicción de los modelos, estaría el grado de relación genética capturada por los marcadores entre las poblaciones de entrenamiento y validación, así como también el grado de DL favorecido por el tamaño efectivo de la población. En el estudio, los individuos divididos entre población de

entrenamiento y validación fueron seleccionados aleatoriamente dentro del grupo total de muestras, por lo tanto, si se aumentara el grado de relación entre ambas, las habilidades predictivas podrían mejorar para ambas características ó disminuirían si la relación entre ambas fuera inferior. En el estudio, no se presentaron altos grados de DL entre pares de marcadores, resultado bastante similar a lo anteriormente propuesto por Silva-Junior y Grattapaglia (2015b) para *Eucalyptus*. Por lo tanto, la capacidad de los modelos es atribuible principalmente al grado de estrechez que existía en la población y que permitía estimar de mejor manera los distintos grados de relaciones genéticas.

Dentro de las nuevas consideraciones para estudios en SG, se aconseja que los modelos deban integrar relaciones más realistas y representativas de la población donde serán aplicados, principalmente disminuyendo el grado de relación que existe entre la población de entrenamiento y validación al momento ajustar la ecuación de predicción. Por ejemplo, en *Eucalyptus*, los últimos reportes de SG han estimado la precisión de los modelos para clones y familias de híbridos (*E. grandis* x *E. urophylla*), con pérdida no significativa de la habilidad predictiva, en ausencia de relaciones entre los sets de entrenamiento y validación (Resende et al. 2017). En el estudio presentado en este trabajo, al utilizar un set de muestras inferior a lo reportado para estimación de valores genéticos por SG, se debe considerar en primer lugar, un aumento en el número de individuos para reajustar las ecuaciones de predicción. Si bien teóricamente la habilidad de predicción disminuiría en este nuevo set, creemos que la buena representatividad de la población objetivo, dentro del primer grupo de entrenamiento utilizado en el estudio, permitirá de igualmente perdidas no considerables de habilidad de predicción.

En el capítulo, adicionalmente se utilizó una densidad media de marcadores de 12 K para ajustar el modelo. Comparado con estudios en *Eucalyptus* y *Picea*, se ha visto que con una baja densidad de SNPs (~5.000), las habilidades predictivas son bastante estables, independiente de la posición genómica de los marcadores (Müller et al. 2017; Lenz et al. 2017). Se debe considerar que la principal ventaja del uso de paneles de SNPs reducidos podría ser la rentabilidad de la genotipificación, aunque se espera que a una mayor cantidad de marcadores, estos tengan mejor capacidad de predicción a lo largo de diferentes generaciones, principalmente debido a la recombinación y el DL (Solverg et al. 2008). GS se puede aplicar

de manera más eficiente en poblaciones más estructuradas donde las relaciones y el DL son mayores, lo que requiere una menor cobertura del genoma para alcanzar una alta precisión de predicción (Lenz et al. 2017).

La SG busca considerar la aplicabilidad de los modelos en futuras generaciones de mejoramiento de diferentes especies, análisis que durante el último tiempo también han sido reportado por Isik et al. (2016). En general los estudios plantean que, para estas estimaciones, una buena estrategia para obtener mejores habilidades de predicción es considerar a los padres dentro del set de entrenamiento donde el modelo estadístico es ajustado para la predicción; lo mismo para el caso de híbridos que quieran ser predichos, donde sus padres debe ser considerados para el ajuste. De acuerdo a lo propuesto por Tan et al. (2017a), los modelos ajustados, exclusivamente con padres de especies puras, no permitirían buenas predicciones en sus híbridos, lo que probablemente podría atribuirse a la divergencia genética entre las especies y la falta consistente de DL entre las especies y sus híbridos. Actualmente, *E. globulus* solo posee una generación de mejoramiento en Chile, sin embargo, esta consideración debe ser evaluada para futuras proyecciones de nuevas cruzas, donde una nueva generación de padres puede dar origen a nuevas poblaciones.

De acuerdo a lo descrito por Resende et al. (2012), los modelos genómicos resultan ser sitio-específicos y su habilidad de predicción disminuye al ser evaluados en sets de validación provenientes de sitios geográficamente más lejanos al sitio donde el modelo fue ajustado (Gamal et al. 2015). Sin embargo, esto ha sido discutido y se ha visto que la precisión de los modelos desarrollados con los datos desde un sitio, y validados en un segundo sitio, puede seguir siendo alta o marginalmente inferior a las realizada en el mismo sitio. Estudios en *Piceas* (Lenz et al. 2017; Beaulieu et al. 2014) indican que la interacción genotipo-ambiente podría ser baja o con condiciones medio ambientales constantes, y que los modelos de SG podrían ser aplicados en una amplia gama de sitios, sin necesidad de modelos independientes. En éste estudio de SG, los clones utilizados están distribuidos en sitios muy cercanos, por lo tanto, se espera que la capacidad de predicción de los modelos no se vea influenciada por el efecto ambiental ó que su incidencia sea poco representativa de la variabilidad fenotípica que pueda existir entre los sitios.

Finalmente, se debe discutir el hecho que los modelos de SG deberían considerar la contribución no aditiva para las habilidades predictivas y así evaluar si la incorporación de dominancia y/o epistasis mejora la efectividad de predecir valores genéticos de individuos o familias. Esto ya ha sido evaluado en híbridos de *Eucalyptus* en donde el componente no aditivo aumenta la eficiencia de los modelos de predicción para características de crecimiento (Tan et al. 2017b). En el estudio de *E. globulus*, solo se consideró el efecto aditivo para las predicciones genómicas, por lo tanto, este punto puede ser considerado para futuras validaciones de precisión y habilidad de los modelos, posiblemente para estudios en híbridos.

MATERIAL SUPLEMENTARIO

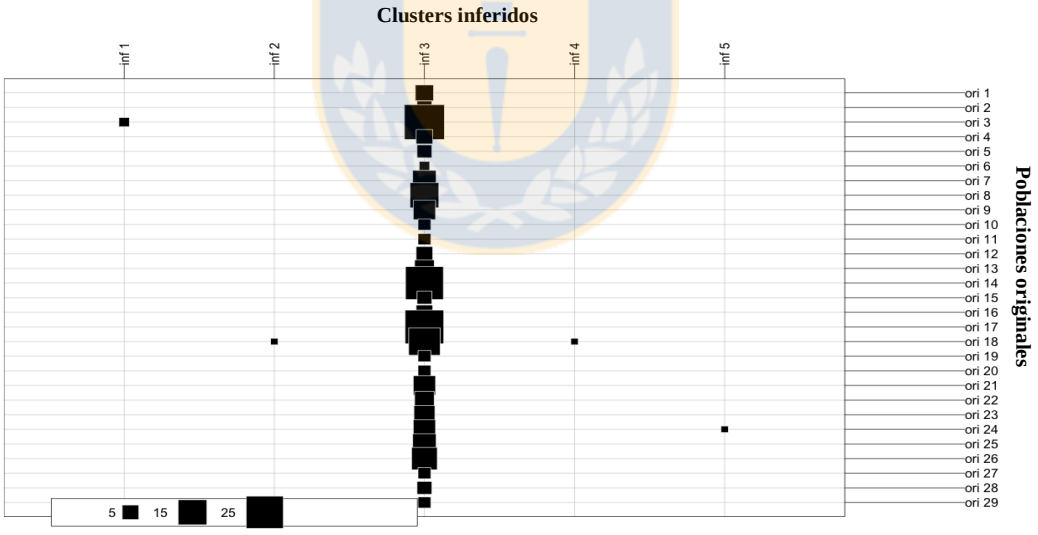
MS1. Resultados del estudio de calidad de genotipificación de SNPs descubiertos mediantes GBS, para una población clonal de *E. globulus.*

a) Clasificación de muestras de acuerdo al análisis exploratorio de PCs. Clusters inferidos: Numero de grupos que fueron seleccionados de acuerdo a análisis de BIC. Clusters originales: Familias originales a las que pertenece cada muestra.

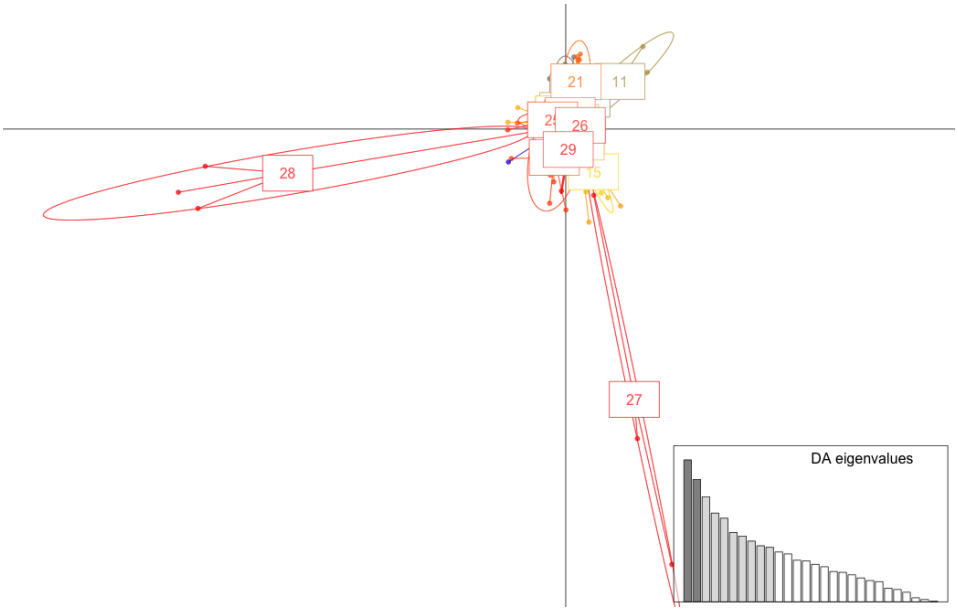
		Clusters inferidos				
		1	**2**	**3**	**4**	**5**
Clusters originales	**1**	0	0	6	0	0
	2	0	0	4	0	0
	3	2	0	29	0	0
	4	0	0	5	0	0
	5	0	0	4	0	0
	6	0	0	2	0	0
	7	0	0	10	0	0
	8	0	0	15	0	0
	9	0	0	9	0	0
	10	0	0	3	0	0
	11	0	0	3	0	0
	12	0	0	5	0	0
	13	0	0	7	0	0
	14	0	0	26	0	0
	15	0	0	4	0	0
	16	0	0	5	0	0
	17	0	0	27	0	0
	18	0	1	18	1	0
	19	0	0	3	0	0
	20	0	0	3	0	0
	21	0	0	9	0	0

	22	0	0	7	0	0
	23	0	0	8	0	0
	24	0	0	9	0	1
	25	0	0	10	0	0
	26	0	0	12	0	0
	27	0	0	3	0	0
	28	0	0	4	0	0
	29	0	0	3	0	0

b) Plot 2D para clasificación de las muestras de acuerdo al *k* óptimo y el numero de familias originales. Inf: Grupos inferidos a partir de análisis de BIC. Ori: Grupos originales (familias). Las cajas en negro representan la cantidad de individuos presentes en cada una de grupos inferidos de acuerdo a su origen familiar. El tamaño de las cajas es proporcional al número de individuos agrupados.



c) Plot 2D. Análisis discriminante de componentes principales. Los numero representan las familias a las que pertenecen las muestras. Los puntos presentan a las muestras de cada familia. El gráfico DA eigenvalues muestra la distribución de la matriz de peso para el análisis discriminante de PCs.


21
11
26
29
28
27
DA eigenvalues

CONCLUSIONES GENERALES

Utilizando la tecnología de GBS, alrededor de 1.200 SNPs polimórficos, en cada una de las poblaciones de clones de *E. globulus* estudiadas, pudieron ser descubiertos. Si bien estos marcadores estaban distribuidos a lo largo del genoma, poseían un bajo nivel de polimorfismo producto de la baja calidad de secuenciación obtenida por esta técnica y no fueron capaces de estimar la estructura genéticas entre muestras analizadas.

La plataforma de genotipificación EUChip60K permitió identificar un set de SNPs con capacidad de resolución de genotipos molculares a nivel intra-clonal y familiar. La genotipificación mostró un alto porcentaje de marcadores correctamente asignados entre duplicados de réplicas biológicas y técnicas. Por otra parte, la genotipificación mostró ser independiente de la calidad del ADN utilizado.

Un set de 12 K de SNPs polimórficos identificados entre clones de *E. globulus*, utilizando el EUChip60K, permitió estimar las relaciones genéticas entre individuos, con una distribución continua y con modas cercanas a lo esperado para individuos no relacionados (0), medios hermanos (0,25) y hermanos completos (0,50). Esta metodología representa una manera más precisa las relaciones genéticas alcanzadas utilizando la información genealógica de la población, donde se asume que los individuos dentro de un mismo grupo de parientes comparten el mismo genoma. Sin embargo, utilizando esta densidad media de 12 K marcadores, solo un bajo porcentaje de pares SNPs mostró estar en niveles de DL superior a 0,4 a nivel intra-cromosomal para los 11 cromosomas representados para Eucalyptus, lo que se vió probablemente favorecido por la distancia entre ellos.

Los modelos de selección genómica, tuvieron correlaciones entre los valores genéticos estimados y reales de 0,58 y 0,75, para densidad de la madera y volumen del fuste respectivamente. Considerando que el análisis fue realizado en un set de muestras de solo 310 individuos provenientes de una sola generación de mejoramiento, por lo que debe ser validado para un mayor número de individuos desde la población de mejoramiento.

BIBLIOGRAFÍA GENERAL

Bachlava E, Taylor CA, Tang S, Bowers JE, Mandel JR, Burke JM, Knapp SJ (2012) SNP discovery and development of a high-density genotyping array for sunflower. PLoS One 7:e29814.

Bajgain P, Rouse MN y Anderson JA (2016) Comparing genotyping-by-sequencing and single nucleotide polymorphism chip genotyping for quantitative trait loci mapping in wheat. Crop Science 56:232-248.

Beaulieu J, Doerksen TK, MacKay J, Rainville A, Bousquet J (2014) Genomic selection accuracies within and between environments and small breeding groups in white spruce. BMC Genomics 15:1048. doi:10.1186/1471-2164-15-1048

Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES (2007) TASSEL: Software for association mapping of complex traits in diverse samples. Bioinformatics. 23:2633–2635.

Brookes AJ (1999) The essence of SNPs. Gene 234:177-186

Brouard J S, Boyle B, Ibeagha-Awemu EM y Bissonnette N (2017) Low-depth genotyping-by-sequencing (GBS) in a bovine population: strategies to maximize the selection of high quality genotypes and the accuracy of imputation. BMC genetics 18: 32.

Brown GR, Bassoni DL, Gill GP, Fontana JR, Wheeler NC, Megraw RA, Davis MF, Sewell MM, Tuskan GA, Neale DB (2003) Identification of quantitative trait loci influencing wood property traits in loblolly pine (*Pinus taeda* L.). III. QTL Verification and candidate gene mapping. Genetics 164:1537–1546.

Butcher P, Southerton S (2007) Marker-assisted selection in forestry species. In Marker-Assisted Selection, Current Status and Future Perspectives in Crops, Livestock, Forestry and Fish pp 283-305.

Calus MPL, Veerkamp RF (2007) Accuracy of breeding values when using and ignoring the polygenic effect in genomic breeding value estimation with a marker density of one SNP per cM. J Anim Breed Genet 124:362–368.

Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA (2013) Stacks: an analysis tool set for population genomics. Mol Ecol. 22:3124–40.

Chagné D, Bianco L, Lawley C, Micheletti D, Jacobs JME (2015) Methods for the design, implementation, and analysis of Illumina InfiniumTM SNP assays in plants. In: *Plant genotyping: methods and protocols*. Springer, New York, pp 281–298.

Ching A, Caldwell KS, Jung M, Dolan M, Smith OS, Tingey S, Morgante M y Rafalski AJ (2002) SNP frequency, haplotype structure and linkage disequilibrium in elite maize inbred lines. BMC Genet 3:19–32.

Cho RJ, Mindrinos M, Richards DR (1999) Genome wide mapping with biallelic markers in *Arabidopsis thaliana*. Nat Genet 23:203–207.

Corporación Nacional Forestal. (2013) Por un Chile forestal sustentable. Santiago, Chile. p 83.

Davey JW, Hohenlohe PA, Etter PD et al (2011) Genome-wide genetic marker discovery and genotyping using next generation sequencing. Nat Rev Genet, 12:499–510.

de Souza LM, Toledo-Silva G, Cardoso-Silva CB, Da Silva CC, de Araujo Andreotti IA et al (2016) Development of single nucleotide polymorphism markers in the large and complex rubber tree genome using next-generation sequence data. Molecular breeding, 36:115.

Devey ME, Carson SD, Nolan MF, Matheson AC, Te Riini C, Hohepa J (2004) QTL associations for density and diameter in *Pinus radiata* and the potential for marker-aided selection. Theor Appl Genet 108:516–524.

Doughty RW (2000) The eucalyptus: a natural and commercial history of the gum tree. Baltimore, Maryland

Eldridge KG, Davidson J, Harwood CE, van Wyk G (1993) Eucalypt domestication and breeding. Clerendon Press, Oxford

El-Kassaby YA, Isik F, Whetten R (2014) Modern advances in tree breeding. In *Challenges and Opportunities for the World's Forests in the 21st Century.* Springer, Netherlands, pp 441-459

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchel SE (2011) A robust, simple genotyping-by- sequencing (GBS) approach for high diversity species. PLoS One 6:e19379

Gamal El-Dien O, Ratcliffe B, Klápště J, Chen C, Porth I, El-Kassaby YA (2015) Prediction accuracies for growth and wood attributes of interior spruce in space using genotyping-by-sequencing. BMC Genomics 16:370.

Ganal MW, Polley A, Graner EM, Plieske J, Wieseke R, Luerssen H y Durstewitz G (2012) Large SNP arrays for genotyping in crop plants. J Biosci 37:821–828.

Ganal MW, Wieseke R, Luerssen H, Durstewitz G, Graner EM, Plieske J y Polley A (2014) High-throughput SNP profiling of genetic resources in crop plants using genotyping arrays. In *Genomics of Plant Genetic Resources*. Springer, Netherlands, pp 113-130.

Grattapaglia D (2014) Breeding forest trees by genomic selection: current progress and the way forward. In *Genomics of plant genetic resources*. Springer, Netherlands, pp 651–682.

Grattapaglia D, Resende MDV (2011) Genomic selection in forest tree breeding. Tree Genet Genomes 7:241–255.

Germano J, Klein AS (1999) Species-specific nuclear and chloroplast single nucleotide polymorphisms to distinguish *Picea glauca, P. mariana* and *P. rubens*. Theor Appl Genet 99:37–49.

González-Martínez SC, Krutovsky KV, Neale DB (2006) Forest-tree population genomics and adaptive evolution. New Phytologist, 170(2), 227-238.

Gürcan K, Teber S, Ercisli S y Yilmaz KU (2016) Genotyping by Sequencing (GBS) in Apricots and Genetic Diversity Assessment with GBS-Derived Single-Nucleotide Polymorphisms (SNPs). Biochemical genetics, 54:854-885.

Habier D, Fernando RL, Dekkers JCM (2009) Genomic selection using low-density marker panels. Genetics society of America 182:343-353.

Hamilton HP, Hansey CN, Whitty BR, Stoffel K, Massa AN, Van Deynze A, De Jong WS, Douches DS, Buell CR (2011) Single nucleotide polymorphism discovery in elite north american potato germplasm. BMC Genomics 12:302.

Hayes BJ, Bowman PJ, Chamberlain AJ, Goddard ME (2009) Invited review: Genomic selection in dairy cattle: Progress and challenges. J. Dairy Sci. 92:433–443.

Hayes B, Goddard M (2010) Genome-wide association and genomic selection in animal breeding. Genome 53:876–883. doi:10.1139/ G10-076.

He J, Zhao X, Laroche A, Lu ZX, Liu H y Li Z (2014) Genotyping-by-sequencing (GBS), an ultimate marker-assisted selection (MAS) tool to accelerate plant breeding. Front Plant Sci 5:484.

Heffner EL, Sorrell ME, Jannink JL (2009) Genomic selection for crop improvement. Crop Sci. 49:1–12.

Heslot N, Jannink JL, Sorrells ME (2015). Perspectives for genomic selection applications and research in plants. Crop Sci 55: 1–12.

Hillier LW, Marth GT, Quinlan AR, Dooling D, Fewell G, Barnett D, Fox P et al (2008) Whole-genome sequencing and variant discovery in *C. elegans*. Nat Methods 5:183–188.

Instituto Forestal (2014) Mejoramiento genético de Eucalyptos en Chile. Santiago, Chile. p 488.

Instituto Forestal (2016) Anuario Forestal. Boletín estadístico Nº 154. Santiago, Chile. p 171.

Ipinza R (2000) Modelo Básico de Mejora Genética. En: Domesticación y Mejora Genética de Raulí y Roble. Editado por Roberto Ipinza, Braulio Gutiérrez y Verónica Emhart; Editora e Imprenta Maval Ltda, pp 197-213.

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

Isik F, Bartholomé J, Farjat A, Chancerel E, Raffin A, Sanchez L, Plomion C, Bouffier L (2016) Genomic selection in maritime pine. Plant Sci 242:108–119.

Jehan T, Lakhanpaul S (2006) Single nucleotide polymorphism (SNP)–methods and applications in plant genetics: a review.

Jones E, Chu WC, Ayele M, Ho J, Bruggeman E, Yourstone K, Rafalski A et al (2009). Development of single nucleotide polymorphism (SNP) markers for use in commercial maize (*Zea mays* L.) germplasm. Mol Breeding. 24:165–176

Kagale S, Koh C, Clarke WE, Bollina V, Parkin IA y Sharpe AG (2016) Analysis of genotyping-by-sequencing (GBS) data. Plant Bioinformatics: Methods and Protocols 269-284.

Kim C, Guo H, Kong W, Chandnani R, Shuang LS y Paterson AH (2016) Application of genotyping by sequencing technology to a variety of crop breeding programs. Plant Sci 242:14–22.

Lande R, Thompson R (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743–756.

Lenz PR, Beaulieu J, Mansfield SD, Clément S, Desponts M, Bousquet J (2017) Factors affecting the accuracy of genomic selection for growth and wood quality traits in an advanced-breeding population of black spruce (*Picea mariana*). BMC genomics 18:335.

Mascher M, Wu S, Amand PS, Stein N, Poland J (2013) Application of Genotyping-by-Sequencing on Semiconductor Sequencing Platforms: A Comparison of Genetic and Reference-Based Marker Ordering in Barley. PLoS ONE 8:e76925.

Mason AS, Higgins EE, Snowdon RJ, Batley J, Stein A, Werner C y Parkin IA (2017) A user guide to theBrassica60K Illumina Infinium™ SNP genotyping array. Theoretical and Applied Genetics 1-13.

McCormack JE, Hird SM, Zellmer AJ, Carstens BC y Brumfield RT (2013) Applications of next-generation sequencing to phylogeography and phylogenetics. Mol. Phylogenet. Evol. 66:526–538.

Melo AT, Bartaula R y Hale I (2016) GBS-SNP-CROP: a reference-optional pipeline for SNP discovery and plant germplasm characterization using variable length, paired-end genotyping-by-sequencing data. BMC bioinformatics 17:29.

Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of Total Genetic Value Using Genome-Wide Dense Marker Maps. Genetics 157:1819–1829.

Misztal I (2011) FAQ for genomic selection. Editorial. J. Anim. Breed. Genet. 128:245–246.

Muranty H, Jorge V, Bastien C, Lepoittevin C, Bouffier L, Sanchez L (2014) Potential for marker-assisted selection for forest tree breeding: lessons from 20 years of MAS in crops, Tree Genet. Genomes 10:1491-1510.

Myles S, Chia JM, Hurwitz B et al (2010) Rapid genomic characterization of the genus *Vitis*. PLoS One 5:e8219.

Müller BS, Neves LG, de Almeida Filho JE, Resende MF, Muñoz PR, dos Santos PE et al (2017) Genomic prediction in contrast to a genome-wide association study in explaining heritable variation of complex growth traits in breeding populations of *Eucalyptus*. BMC genomics 18:524.

Neale DB, Savolainen O (2004) Association genetics of complex traits in conifers. Trends Plant Sci 9:325–330.

Neale DB, Sewell MM, Brown GR (2002) Molecular dissection of the quantitative inheritance of wood property traits in loblolly pine. Ann. For. Sci. 59:595–605.

Nelson JC, Wang S, Wu Y, Li X, Antony G, White FF, Yu J (2011) BMC Genomics. 12:352

Nicolazzi E L, Biffani S, Biscarini F, Orozco ter Wengel P, Caprera A, Nazzicari N y Stella A (2015) Software solutions for the livestock genomics SNP array revolution. Animal genetics 46:343-353.

Nielsen R, Paul JS, Albrechtsen A y Song YS (2011) Genotype and SNP calling from next-generation sequencing data. Nat Rev Genet. 12:443–51.

Novaes E, Drost DR, Farmerie WG, Pappas JR GJ, Grattapaglia D, Sederoff RR, Kirst M (2008) High-throughput gene and SNP discovery in *Eucalyptus grandis*, an uncharacterized genome. BMC Genomics 9:312.

Ona T, Sonoda T, Ito K, Shibata M, Tamai Y, Kojima K, Ohshima J, Yokota S, Yoshizawa N (2001) Investigation of relationships between cell and pulp properties in *Eucalyptus* by examination of within-tree property variations. Wood Science and Technology 35:229-243.

Picoult-Newberg L, Ideker TE, Pohl MG, Taylor SL, Donaldson MA, Nickerson DA, Boyce-Jacino M (1999) Milling SNPs from EST databases. Genome Res 9:167–174.

Plomion C, Bastien C, Bogeat-Triboulot MB, Bouffier L, Déjardin A, Duplessis S et al (2016) Forest tree genomics: 10 achievements from the past 10 years and future prospects. Annals of forest science 73:77-103.

Poland JA y Rife TW (2012) Genotyping-by-sequencing for plant breeding and genetics. Plant Genome 5:92–102.

Rafalski A (2002) Applications of single nucleotide polymorphisms in crop genetics. Curr Opin Plant Biol 5:94–100.

Ramírez M, Rodríguez J, Balocchi C, Peredo M, Elissetche JP, Mendonça R, Valenzuela S (2009) Chemical composition and wood anatomy of *Eucalyptus globulus* clones: Variations and relationships with pulpability and handsheet properties. J. Wood Chem. Tech 29:43–58.

Rasheed A, Hao Y, Xia X, Khan A, Xu Y, Varshney RK y He Z (2017) Crop Breeding Chips and Genotyping Platforms: Progress, Challenges, and Perspectives. Molecular Plant, 10:047-1064.

Ratcliffe B, El-Dien OG, Klapste J, Porth I, Chen C, Jaquish B et al. (2015).A comparison of genomic selection models across time in interior spruce (*Picea engelmannii x glauca*) using unordered SNP imputation methods. Heredity 115: 547–555.

Raymond CA, Banham P, MacDonald AC (1998) Within tree variation and genetic control of basic density, fibre length and coarseness in *Eucalyptus regnans* in Tasmania. Appita J 51:299–305.

Resende MFR, Muñoz P, Acosta JJ, Peter GF, Davis JM, Grattapaglia D, Resende MDV, Kirst M (2012) Accelerating the domestication of trees using genomic selection: accuracy of prediction models across ages and environments. New Phytol 193:617–624.

Resende RT, Resende MDV, Silva FF, Azevedo CF, Takahashi EK, Silva-Junior OB, Grattapaglia D (2017) Assessing the expected response to genomic selection of individuals and families in *Eucalyptus* breeding with an additive-dominant model. Heredity 119:245.

Scheben A, Batley J y Edwards D (2017) Genotyping by sequencing approaches to characterise crop genomes: choosing the right tool for the right application. Plant biotechnology journal 15:149-161.

Sewell MM, Neale DB (2000) Mapping quantitative traits in forest trees. In *Molecular biology of woody plants* Dordrecht, Netherlands, Kluwer, Vol. 1, pp 407–423.

Silva-Junior OB, Faria DA, Grattapaglia D (2015a) A flexible multi- species genome-wide 60K SNP chip developed from pooled resequencing of 240 *Eucalyptus* tree genomes across 12 species. New Phytol 206:1527–1540. doi:10.1111/nph.13322.

Silva-Junior OB, Grattapaglia D (2015b) Genome-wide patterns of recombination, linkage disequilibrium and nucleotide diversity from pooled resequencing and single nucleotide polymorphism genotyping unlock the evolutionary history of *Eucalyptus grandis*. New Phytol 208:830–845. doi:10.1111/nph.13505.

Solberg TR, Sonesson AK, Woolliams JA, Meuwissen THE (2008) Genomic selection using different marker types and densities. Journal of animal science 86: 2447-2454.

Strauss S, Lande R, Namkoong G (1992) Limitations of molecular-marker-aided selection in forest tree breeding. Can J For Res 22:1051–1061.

Tan B, Grattapaglia D, Martins GS, Ferreira KZ, Sundberg B, Ingvarsson PK (2017a) Evaluating the accuracy of genomic prediction of growth and wood traits in two *Eucalyptus* species and their F 1 hybrids. BMC plant biology, 17(1), 110.

Tan B, Grattapaglia D, Wu HX, Ingvarsson K (2017b) Genomic prediction reveals significant non-additive effects for growth in hybrid *Eucalyptus*. bioRxiv 178160.

Thomson MJ, Zhao K, Wright M, McNally KL, Rey J, Tung CW, Reynolds A et al (2012) High-throughput single nucleotide polymorphism genotyping for breeding applications in rice using the BeadXpress platform. Mol Breeding 29:875–886.

Thumma RF, Nolan MF, Evans R, Moran GF (2005) Polymorphisms in cinnamoyl CoA reductase (CCR) are associated with variation in microfibril angle in *Eucalyptus* spp. Genetics 171:1257–1265.

Trebbi T, Maccaferri M, De Heer P, Sørensen A, Giuliani S, Salvi S, Sanguineti MC, Massi A, Gerard van der Vossen EA, Tuberosa R (2011) High-throughput SNP discovery and genotyping in durum wheat (*Triticum durum* Desf.). Theor Appl Genet 123:555–569.

Torkamaneh D, Laroche J y Belzile F (2016) Genome-wide SNP calling from genotyping by sequencing (GBS) data: a comparison of seven pipelines and two sequencing technologies. PloS one 11:e0161333.

Torkamaneh D, Laroche J, Bastien M, Abed A y Belzile F (2017) Fast-GBS: a new pipeline for the efficient and highly accurate calling of SNPs from genotyping-by-sequencing data. BMC bioinformatics 18:5.

Vallejos J, Badilla Y, Picado F, Murillo O (2010) Metodología para la selección e incorporación de árboles plus en programas de mejoramiento genético forestal. Agronomía Costarricense 34:105-119.

van Tassell CP, Smith TPL, Matukumalli LK et al (2008) SNP discovery and allele frequency estimation by deep sequencing of reduced representation libraries. Nat Methods, 5:247–52.

Verde I, Bassil N, Scalabrin S, Gilmore B, Lawley CT, Gasic K et al (2012) Development and evaluation of a 9K SNP array for peach by internationally coordinated SNP detection and validation in breeding germplasm. Plos one *7*:e35668.

White TL, Adams WT, Neale DB (2007). Forest genetics. CABI Publishing CAB International, Cambridge.

Wimmer R, Downes GM, Evans R, Rasmussen G, French J (2002) Direct effects of wood characteristics on pulp and paper handsheet properties of *Eucalyptus globulus*. Holzforschung 56:244–252.

Wong C, Bernardo R (2008) Genome-wide selection in oil palm: increasing selection gain per unit time and cost with small populations. TAG Theoretical and Applied Genetics 116:815-824.

You FM, Huo N, Deal KR, Gu YQ, Luo MC, McGuire PE, Dvorak J, Anderson OD (2011) Annotation-based genome- wide SNP discovery in the large and complex *Aegilops tauschii* genome using next-generation sequencing without a reference genome sequence. BMC Genomics 12:59.

Zapata-Valenzuela J, Hasbun R (2011) Mejoramiento genético forestal acelerado mediante selección genómica. Bosque (Valdivia) 32:209–213.

Zhao Y, Gowda M, Liu W, Würschum T, Maurer HP, Longin FH, Ranc N, Reif JC (2012) Accuracy of genomic selection in European maize elite breeding populations. Theor. Appl. Genet. 124: 769-776.