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SHORT COMMUNICATION

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New validated *Eucalyptus* SSR markers located in candidate genes involved in growth and plant development

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Abstract

Aim of study: To validate and characterize new microsatellites or Simple Sequence Repeats (SSR) markers, located within genomic transcribed sequences related to growth and plant developmental traits, in *Eucalyptus* species.

Area of study: Eucalyptus species from different Australian origins planted in Argentina.

Material and methods: In total, 134 SSR in 129 candidate genes (CG-SSR) involved in plant development were selected and physically mapped to the *E. grandis* reference genome by bioinformatic tools. Experimental validation and polymorphism analysis were performed on 48 individuals from *E. grandis* and interspecific hybrids (*E. grandis* x *E. camaldulensis*; *E. grandis* x *E. tereticornis*), *E. globulus, E. maidenii, E. dunnii* and *E. benthamii.*

Main results: 131 out of 134 CG-SSR were mapped on the 11 chromosomes of *E. grandis* reference genome. Most of the 134 analyzed SSR (> 75%) were positively amplified and 39 were polymorphic in at least one species. A search of annotated genes within a 25 kbp up and downstream region of each SSR location retrieved 773 genes of interest.

Research highlights: The new validated and characterized CG-SSR are potentially suitable for comparative QTL mapping, molecular marker-assisted breeding (MAB) and population genetic studies across different species within *Symphyomyrtus subgenus.*

Keywords: CG-SSR; cross-transferability; EST; eucalypts; microsatellite.

Authors' contributions: Conceptualization: HEH, SNMP, CVA. Methodology: CVA, JGR, NCA, PVV. Formal analysis: CVA, JGR, NCA, PVV, MNG. Resources: SNMP, HEH. Writing original draft preparation: CVA. Writing-critical revision of the manuscript: MCM, MNG, NCA, PVV, SNMP, HEH. Project administration and funding acquisition: SNMP, MCM, HEH.

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Introduction

The *Eucalyptus* genus groups forest species and hybrids widely cultivated in the world as forestry plantations for the wood industry as renewable sources for timber, paper and pulp production (Govindan, 2005; Bauhus *et al.*, 2010). The high number of species within this genus, with different agroecological requirements and wood quality characteristics, makes *Eucalyptus* a very valuable resource for its adaptation to the numerous ecosystems worldwide. In this context, the *Eucalyptus* global production is estimated at 20 million hectares (Wingfield *et al.*, 2015).

Over the last 20 years, forest breeding programs, which require long developmental periods, have incorporated different molecular tools (Gudeta, 2018). Molecular markers detect differences between individuals directly from the genome and provide extensive discrete data that can be useful for statistical analyses. These tools are suitable to control the genetic traceability during multiplication processes, evaluate the genetic diversity and make predictions of more reliable breeding values (Cappa *et al.*, 2016; Gudeta, 2018).

SSR or microsatellites, short repetitive DNA sequences distributed throughout the genome showing a high level of polymorphism (first reviewed in plants by Powell

et al., 1996), are the most widely used markers for different applications (Hodel *et al.*, 2016).

The development of microsatellite markers in *Eucalyptus* has evolved over the years in parallel with the increased availability of sequence information. To date, different types of neutral microsatellites are being used in *Eucalyptus* and new SSR markers have been developed within the transcribed regions of the genome. In addition, researchers have designed SSR markers after mining the increasingly larger EST (Expressed Sequence Tags) collections deposited in sequence databases (Kirst *et al.*, 2005; Lehouque *et al.*, 2008; Faria *et al.*, 2010; Acuña *et al.*, 2012 (a, b); Zhou *et al.*, 2014; Grattapaglia *et al.*, 2015).

Several strategies have been explored to find loci controlling traits of interest in woody species. Thus, many genomic studies have reported the analysis of genes and transcription factors expressed during wood formation and xylogenesis in *Eucalyptus* (reviewed by Foucart *et al.*, 2006; Paux *et al.*, 2004; Rengel *et al.*, 2009). Besides, for genes controlling plant growth traits several QTL (Quantitative Trait Loci) approaches have been carried out (revised in Gion *et al.*, 2015; Li *et al.*, 2015; Du *et al.*, 2018; Müller *et al.*, 2019; Kainer *et al.*, 2019).

Therefore, the use of already well-established polymorphic markers located in CG for plant traits is an interesting approach for mapping purposes and population genetic studies with an emphasis in non-model species (Acuña *et al.*, 2012b, 2014; Pomponio *et al.*, 2015; Azpilicueta *et al.*, 2016). Also, the availability of *E. grandis* genome sequence, with annotated and classified genes, is an important information source to study and characterize different traits of interest (Myburg *et al.*, 2014).

Although high-throughput sequence-based SNP marker assays are increasingly becoming available (Silva-Junior *et al.*, 2015, Aguirre *et al.*, 2019), microsatellites still constitute a very useful and accessible tool for fast and precise genetic analysis in *Eucalyptus* (Grattapaglia *et al.*, 2015). Besides genetic diversity studies, SSR have numerous uses, including cultivar or clone fingerprinting, population structure, marker-assisted selection, linkage map development and QTL mapping, among others, thus showing an important role in this genomic age (Hodel *et al.*, 2016).

In this study, we in silico characterized and in vitro validated new microsatellite markers located in CG (structural genes and transcriptional factors) related to plant growth and development. The selection of SSR located on these genes was based on data from a previous study of our group (Acuña et al, 2012a), with a focus on genome regions potentially involved in these important characteristics for tree breeding. The identified SSR markers were wet-lab validated in five different *Eucalyptus* species and hybrids, and physically mapped on the *E. grandis* reference genome. Furthermore, we also identified and analyzed known predicted genes contiguous (<25kbp) to these SSR markers.

Materials and Methods

Leaves from 48 individuals of *Eucalyptus* sp. were analyzed: 12 individuals of *Eucalyptus grandis* (4 individuals from a clonal population, 4 individuals from two controlled crosses and 4 individuals from their offspring), *E. globulus* (6 individuals), *E. maidenii* (8 individuals), *E. dunnii* (8 individuals), *E. benthamii* (8 individuals) and the hybrids *E. grandis* x *E. camaldulensis* (3 individuals) and *E. grandis* x *E. tereticornis* (3 individuals). Trees were planted in EEA INTA Concordia (31°22'28.9"S 58°07'01.0"W) and IRB-CIRN-CNIA-INTA (34°36'58.6"S 58°40'06.2"W), Argentina. Total DNA was extracted from young leaves using the CTAB method with modifications, as described in Marcucci Poltri *et al.* (2003).

We selected SSR markers on CG from a previous study (Acuña *et al.*, 2012a), 1,140 SSR within 952 CG were in silico characterized. These CG had been selected for their possible biological function predicted according to Gene Ontology (GO) (Ashburner *et al.*, 2000; http://www.geneontology.org/) using Blast2GO (Conesa *et al.*, 2005). From those genes, in the present study, 129 CG with 134 SSR sequences were selected based on their correspondence to genes and transcriptional factors involved in different plant growth and developmental features.

Validation was carried out using PCR reactions in a final volume of 12 µl with 20 ng of genomic DNA, 0.25 µM of each primer (Alpha DNA, Canada), 2mM MgCl2, 0.2 mM of each dNTP, 1X reaction buffer and 1U Platinum Taq polymerase (Invitrogen, Waltham, USA). Amplifications were performed following a denaturation step of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at annealing temperature and 1 min at 72 °C. The final extension step was for 10 min at 72 °C. The SSR amplification products were denatured for 5 min in denaturing loading buffer at 95 °C and separated by a 6% polyacrylamide gel electrophoresis (6% acrylamide/bisacrylamide 20:1, 7.5 M urea, 0.5 × TBE) along with a 25 bp DNA ladder standard (Invitrogen, Waltham, USA). The DNA silver-staining procedure of Promega (Madison, WI, USA) was used for visualization. Details on primer sequences, SSR location, annealing temperature and product sizes are described in Table S1 [supplementary].

We carried out the in silico characterization of the CG-SSR through physical mapping and nearby gene search. The 134 SSR obtained sequences were mapped to the *E. grandis* reference genome (Myburg *et al.*, 2014) (http://phytozome.jgi.doe.gov, version 2.0). Mapping was performed using the Bowtie2 alignment tool with default settings (Langmead & Salzberg, 2012). A custom Perl

script was used to determine the annotated genes of the *E. grandis* genome within a flanking region of 50 kbp (up to 25 kbp from each SSR locus). The predicted genes classified and reported by Myburg *et al.* (2014) were used to describe some of the genes found within the window surrounding each SSR.

Results and Discussion

Selected CG-SSR markers

Based on the results from a previous study (Acuña *et al.*, 2012a), we selected sequences similar to structural genes and transcriptional factors involved in plant developmental features and obtained 129 CG with 134 SSR sequences (details of SSR markers in Table S1 [suppl.]). This study revealed the following distribution of the selected 129 GC within the GO terms: most of them belonged to the "Biological Process" category and within this category, to the subcategories "Metabolic Process of Organic Substances" (17%), "Cellular Metabolic Process" (15%) and "Primary Metabolic Process" (15%), among other less represented subcategories. Also, most of the GO terms within the "Molecular Function" class belonged to the subcategories "Binding to Heterocyclic Compounds" (15%), "Binding to Organic Cyclic Compounds" (15%) and "Ion Binding" (14%). Among them, we detected SSR in transcriptional factor genes involved in xylogenesis (MYB, bZIP, WRKY, SWI/SNF, ARF) (Rengel *et al.*, 2009) and responses to abiotic stress (BES, bZIP) (Bechtold & Field, 2018) (Table S1 [suppl.]).

Marker validation in different *Eucalyptus* spp.

Most of the SSR (75.4%) analyzed in the laboratory were positive PCR-amplified according to similar studies in *Eucalyptus* (Acuña *et al.*, 2012a; He *et al.*, 2012; Zhou *et al.*, 2014). Nonspecific amplicons (17 SSR) or amplicons with sizes above 500 bp (20 SSR) were discarded. Thus, 64 markers resulted in amplification products of the expected size according to bioinformatic analysis. Among them, 39 (29%) were polymorphic (*i.e.*, they had at least 2 alleles in at least one species) and 25 were monomorphic (Table S1 [suppl.]). The polymorphism rate of EST-SSR was similar to that described by Faria *et al.* (2011) (25%) and Acuña *et al.* (2012a) (30%), but lower than that observed by Faria *et al.* (2010) (39%) and Grattapaglia *et al.* (2015) (65%) in *Eucalyptus.*

The relative proportions of repeated motifs in polymorphic SSR were 28.2% for di-, 56.4% for tri-, 10.2% for tetra-, and 5.2% for pentanucleotides. Our results are similar to those reported by other authors, in which trinucleotide repeats were the most common, followed by di- and tetranucleotide repeats (Varshney *et al.*, 2005, Grattapaglia *et al.*, 2015).

The number of alleles per marker (between 2 and 7) (Table S1 [suppl.]) is equivalent to that reported by other authors for EST-SSR markers validated on a small number of samples (about 8 individuals per species) (Zhou *et al.*, 2014). On the other hand, the values found in the present study are lower than those described by Faria *et al.* (2010, 2011) and Grattapaglia *et al.* (2015) in *Eucalyptus*. These results could be explained by the marker selection criteria used. While these studies based their selection on the polymorphism level, we selected SSR markers focusing on their putative function in growth and plant development. Nevertheless, the number of alleles per marker may increase with a larger sample size.

Physical mapping and nearby gene search

The alignment of the 134 CG-SSR sequences against the *E. grandis* public reference genome revealed that 131 SSR were mapped on the 11 chromosomes, while three of the markers were located on scaffolds. The number of SSR by chromosome ranged between 4 (Chromosome 5) and 17 (chromosomes 3, 8 and 11), thus showing a good distribution in the genome (Table S2 [suppl.]).

According to an exhaustive bibliographic revision of the available publications that developed this kind of markers in *Eucalyptus*, only 16 of the 134 SSR validated here coincide with those of other studies (2 in Yasodha *et al.*, 2008; 8 in Rengel *et al.*, 2009; 4 in He *et al.*, 2012; 4 in Zhou *et al.*, 2014 and 1 in Grattapaglia *et al.*, 2015, where some markers were shared between studies). Nonetheless, none of these studies involved the characterization related to plant growth and developmental traits. Moreover, only in this study and in that by Grattapaglia *et al.* (2015), EST-SSR were aligned to the *E. grandis* genome sequence, thus providing information on their distribution and physical position (Table S1 [suppl.]).

Interestingly, the 39 polymorphic SSR markers are located in protein-coding CG, *e.g.* serine-threonine kinase (which is involved in the completion of embryonic development in dormant seeds), F-box type (signal transduction and cell cycle) (Jia *et al.*, 2020) and various transcription factors that regulate processes of cellular development, seed maturation, floral development, among others (bZIP, GATA, BES1) (Bechtold and Field, 2018) (Table S1 [suppl.]).

Additionally, we performed a search for genes of interest that could be linked to the identified SSR within a flanking window of 50 kpb (25kpb up- and downstream regions). This window size was selected based on Linkage Disequilibrium (LD) in *E. grandis* reported by Silva-Junior *et al.*, 2015). This analysis yielded 773 *E. grandis* predicted genes (named Eucgr. in Myburg *et al.*, 2014)

neighbouring these SSR. Among them, 394 were within a Gene Ontology (GO) category (Table S2 [suppl.]).

Based on Myburg *et al.* (2014), who classified predicted genes according to different classes related to wood quality, 30 of the 773 genes belong to the following categories: 3 into "MYB Transcription Factors", 1 into "Genes Encoding Laccases and Peroxidases", 8 into "Lignin Biosynthesis", 7 into "predicted cellulose and xylan genes" and 11 into "Interpro Domain of 968 Unique *Eucalyptus* Genes". This categorization gives these markers an added value, since we detected genes related not only to plant growth and development, but also to wood quality (Table S2 [suppl.]). Examples of genes related to wood quality are cinnamoyl CoA reductase (CCR), phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL) and Caffeic Acid O-Methyl Transferase (COMT) genes, which code for the key enzymes in lignin biosynthesis (Boerjan *et al.*, 2003). Other of these identified genes are PARVUS, cellulose synthase (CESA) and sucrose synthase (SUSY), which are involved in cellulose and xylan biosynthesis (Myburg *et al.*, 2014).

In this work, the evaluated candidate genes sequences were up to 25 kbp distance from the validated SSR markers. Therefore, linkage between them seems to be high enough to make this panel of SSR markers useful in future association mapping studies for *Eucalyptus* breeding purposes.

Conclusions

In the present study, a new set of SSR especially located in candidate genes for growth and plant development is proposed as a tool for *Eucalyptus* genetic analysis. Additionally, some of the SSR are particularly interesting, because they are close to candidate genes for wood quality.

These new CG-SSR markers, in addition to those already publicly available, could be included in studies for the identification of different *Eucalyptus* genetic materials, in population genetics, taxonomy, verification of synteny and collinearity between different *Eucalyptus* maps. Furthermore, they could be implemented in QTL and association mapping studies and genomic selection through relatedness correction in breeding value predictions.

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