Desarrollo de marcadores moleculares para la identificación de especies de *Eucalyptus*

Development of molecular markers for the *Eucalyptus* species identification

Hernando J. Rivera-Jiménez[1]*, Bruno C. Rossini[2], Vanusa do S. Leite[2], Paulo HM. Da Silva[3] and Celso L. Marino[4]

RESUMEN

Uno de los principales problemas que enfrentan los programas de mejoramiento genético en eucaliptos es la dificultad para identificar las especies e híbridos. El objetivo de este estudio fue encontrar marcadores moleculares asociados a cinco especies de *Eucalyptus* (*E. saligna, E. tereticornis, E. urophylla, E. grandis and E. brassiana*), mediante marcadores AFLP (Amplified Fragment Length Polymorphism) y BSA (Bulk Segregant Analysis), para su uso en programas de mejoramiento genético en Brasil. En 33 combinaciones de cebadores, se obtuvo un total de 868 fragmentos polimórficos, que representan un 91,65% del polimorfismo. Las mejores combinaciones que muestran potenciales marcadores para la identificación de especies, fueron encontradas en los cebadores M+GGT/E+ACC, que estuvo un 70% de los individuos asociados a la especie E. urophylla. Sin embargo, la combinación de cebadores compuesta de M+GGA/E+ACC identificó el 60% de individuos en la especie E. saligna; la combinación de los cebadores M+GTC/E +AAC, confirmó dos marcas, una en 60% y la otra en 50% para la identificación de individuos de la especie E. grandis. El tratamiento compuesto por los cebadores M+GGC/E+AAA, confirmó un 30% de los individuos perteneciente a la especie *E. brassiana*, siendo igual para la combinación de cebadores M+GGC/E+ACC, identificando el 30% de los individuos de la especie *E. tereticornis*. El análisis AFLP en asocio a BSA proporcionan una herramienta rápida para la identificación de cultivares en *Eucalyptus*, a la vez de que puede ser usados en los programas de mejoramiento genético forestal.

Palabras clave: AFLP- BSA, *Eucalyptus*, identificación de especies.

ABSTRACT

One of the main problems faced in several eucalypt breeding programs is the difficulty to identify the species and hybrids. This study aimed to find molecular markers associated with five species of *Eucalyptus* (*E. saligna, E. tereticornis, E. urophylla, E. grandis and E. brassiana*), by AFLP (Amplified Fragment Length Polymorphism) markers and BSA (Bulk Segregant Analysis), for their use in breeding programs in Brazil. In 33 primer combinations, a total of 868 polymorphic fragments was obtained, which represent a 91.65% of polymorphism. The best combinations that show potential markers for species identification were the primers M + GGT / E + ACC, which was linked to 70% of *E. urophylla* individuals. However, primer combination composed of M+GGA/ E+ACC identified 60% of individuals in the E. saligna species; combination by the primers M+GTC/E+AAC, confirmed two marks, one in 60% and the other in 50% of *E. grandis* individuals in the identification test. The treatment composed by the primers M+GGC/E+AAA, was confirmed in only 30% of *E. brassiana* individuals, being the same for the combination M+GGC/E+ACC primers, identifying 30% of *E. tereticornis* individuals. The AFLP analysis and BSA provide a quick tool for the identification of cultivars in Eucalyptus and can also be used to assist forest breeding programs.

Key words: AFLP, BSA, *Eucalyptus*, species identification

1* PhD. Biological Sciences-Genetics. Universidade Estadual Paulista. Departamento de Genética, Instituto de Biociências, Distrito de Rubião Junior, Caixa postal 510 – CEP: 18618-970 –, Botucatu – SP, Brasil. Corresponding author: E-mail: hriveraj@gmail.com
2 PhD. Biological Sciences-Genetics. Universidade Estadual Paulista. Departamento de Genética, Instituto de Biociências, Distrito de Rubião Junior, Caixa postal 510 – CEP: 18618-970 –, Botucatu – SP, Brasil. Corresponding author: E-mail: hriveraj@gmail.com
3. PhD. Forest resources. Instituto de Pesquisas e Estudos Florestais, Av. Pádua Dias 11, C. P. 530, CEP 13400-970, Piracicaba, SP, Brasil.
4. PhD. Biological Sciences-Genetics. Universidade Estadual Paulista. Instituto de Biotecnologia / IBB- UNESP/, Botucatu, SP, Brasil.

INTRODUCCIÓN

The area of trees planted for industrial purposes in Brazil totaled 7.74 million hectares in 2014, an increase of 1.8% over 2013. This total corresponds to only 0.9% of Brazilian territory. The overall success of the Brazilian forestry industry is a result of the high productivity of trees planted in the country. For example, in Brazil, the forest area required for the production of 1.5 million tons of pulp per year is 140 thousand hectares (IBA 2015). The Brazilian genetic improvement program has the general objective of making available to the different Brazilian regions with sufficient genetic variability in such a way that it obtains plants more adapted to the different environments to meet the demands of wood for multiple uses (Santos 2011). *Eucalyptus* are planted on a large scale due to their high productivity obtained by breeding program that explored the adaptability of genus species to different environmental conditions (Gonçalves et al. 2013). Know the species that are being worked in the breeding program is important for the strategic planning, especially to identify interspecific hybrids and germplasm conservation (Poltri et al. 2003; Ballesta et al. 2015). Overall, a good breeding program selects the best individuals in intrafamily level, increasing the plantations operating performance and industrial products quality (Grattapaglia and Kirst, 2008). Brazilian forest breeding programs aim to provide for all Brazilian regions enough genetic variability in order to obtain plants adapted to different environments to attend the demand for wood (Forrester and Smith 2012). However, one of the problems is the difficulty to identify the species, especially in hybrid combinations (Grattapaglia et al. 2004). In this context, the incorporation of molecular biology techniques in plant breeding programs is allowing the optimization of time and the conduction of these programs, being the hybrid plants, selected for planting most of the time (Pongitory et al. 2004; Soares et al. 2010). In *Eucalyptus* sp., the hybridization strategy can significantly increase production and adaptability in the resulting progeny, while the hybrid plants are commonly selected to be part of the commercial plantation, selection based on phenotypic superiority and genetic stability (Potts and Dungey 2004). According to Silva et al. (2016), it is possible that the number of commercially unused hybrid species increases, this is due to the new biotic and abiotic stresses that can occur with climate change. This increase in the number of species involved will make it even more difficult to identify, if there is no reliable and practical tool, it will be difficult to use the hybridization intensively, whose use has been expanding for years (Denison and Kietzka 1993). A tool to assist the breeding programs are the molecular markers. Bulked segregant analysis (BSA) is a method introduced by Michelmore et al. (1991), which has been used to identify molecular markers linked to a monogenic, dominant or recessive trait. The technique consists in comparing two sets of DNA samples from a segregating population, where each bulk is composed of individuals from the same species that have the same trait or gene of interest (Blanco and Valverde 2005; Fuchs et al. 2011). The AFLP (Amplified Fragment Length Polymorphism) markers are high efficient for detecting polymorphism of genomic restriction fragments by PCR amplification (Polymerase Chain Reaction) (Vos et al. 1995). These markers are applied in genetic diversity studies in germplasm banks (Rivera-Jiménez et al. 2011) and allow to obtain a large number of tags randomly distributed in the genome (Vos et al. 1995). The use of molecular markers in eucalypt breeding programs already occurs for many years, the markers are used with good results for genetic diversity studies pedigree reconstruction, crossing rate, endogamy study, population genetic structure, genetic flow study , contamination of natural areas or seed orchards (Brondani et al. 1998; Barbour et al. 2005; Jones et al. 2005; Jones et al. 2008; Payn et al, 2008; Silva et al. 2014; Mora et al. 2016; Randal et al. 2016; Silva et al. 2017).

Also provides early identification of the individuals used and maximizes genetic gains (Silva et al. 2015). in the same way is used in industrial timber planning, by identifying genetic hybrids and early identification of species of eucalyptus (Ishii 2009). This study aimed to identify molecular markers associated with five species of *Eucalyptus* sp., through AFLP molecular markers and bulked segregant analysis (BSA).

MATERIAL AND METHODS

A total of 10 individuals from five *Eucalyptus* species was provided by Suzano Paper and Cellulose Company (Suzano Papel e Celulose SA). The three main species (*E. grandis, E. saligna and E. urophylla*) of Brazilian forestry were among the nine most cultivated *Eucalyptus* species in the world (Harwood 2011). Also evaluated were two species of importance for plantations with abiotic stress and that are used to obtain hybrids in commercial crops. table 1. Genomic DNA was extracted according to the CTAB protocol described by Doyle and Doyle (1990), with some modifications as follow: 5% CTAB, removing the proteinase K extraction buffer; CIA step (chloroform: isoamyl alcohol 24:1), was carried out only once; and finally, it was removed the cleaning step with NaCl to extract the DNA from fresh leaves. For each sample, approximately 50 mg of fresh leaf tissues was macerated without main vein. Quantification was performed in spectrophotometer Nano Drop®- ND1000. The DNA used in the amplification reactions was absent of impurities and phenolic compounds, diluted to a concentration of 50 ng/µl in autoclaved ultra-pure water.

Table 1. Proceedings of Eucalyptus species used for identification with AFLP-BSA markers.

Especies	Origin
Eucalyptus brassiana	Embrapa – CSIRO 10972 (North Moreton, QLD, Austrália)
Eucalyptus saligna	Coffs Harbour (Austrália)
Eucalyptus grandis	Coffs Harbour (Austrália)
Eucalyptus urophylla	IPEF – Timor
Eucalyptus tereticornis	Embrapa – CSIRO 10975-8140 (Cooktown e Laura, QLD, Austrália)

Five species of *Eucalyptus* identifcated as *E. saligna, E. tereticornis, E. urophylla, E. grandis and E. brassiana* were analizated, building a bulk for each specie composed of 10 individuals, in a DNA concentration of 10 ng/µL per individual and the final concentration of each bulk were 100 ng/µL. The DNA bulks were screened for polymorphic markers using AFLP markers in order to identify polymorphisms associated with each specie. AFLP protocol was adapted from Vos et al. (1995). Genomic DNA was digested with a combination of two enzymes, EcoRI + MseI. 700 ng of DNA were digested with 5U per enzyme, 5 uL of One Phor All buffer (OPA, Amersham), 0.5 uL of BSA (10 ug / uL) in a final volume 50 uL. The reaction was incubated at 37°C for 16 hours. Amplified products were visualized after electrophoresis in 0.8 % agarose gel in 1× TBE (Tris-Borate-EDTA) stained with ethidium bromide and visualized under UV light transilluminator. The EcoRI adapter was diluted to 5 pM solution containing 0.5 x One Phor All buffer 10x (OPA). The MseI adapter was diluted to 50 pM solution 0.5 x One Phor All buffer 10x (OPA). The hybridization of adapters was performed in a thermocycler model PTC-100 (MJ Research ®) in a reaction consisted of 10 min 65°C, 10 min at 37 °C and 10 min at 25 °C. The adapters were ligated to the DNA fragments in a reaction containing 1 uL of the enzyme T4 DNA ligase buffer (10x), 1 uL of each adapter (5 or 50 pM), 3U T4 DNA ligase (Invitrogen, Carlsbad CA, USA), 6.67 uL of ultrapure water and 45 uL digested DNA solution. Ligation was performed at 17 °C for 17 hours. There were four combinations of primers in the pre-amplification reactions (Ea / Mg and Ea / Mc), primers with complementary sequences to each of the adapters plus one selective nucleotide at the 3 'end (Table 2). The reactions were composed with 1 uL of each primer (25 ng / uL), 10 uL PCR Master Mix (Promega ®), 6 uL Nuclease-Free Water and 2 uL digested and ligated DNA in a final amount of 20 uL. The program for preamplification was: 94 °C for 2 min, 26 cycles of 94 °C for 1 minute, 56 °C for 1

minute, 72 °C for 1 minute and a final extension at 72°C for 5 minutes. In the product of this reaction was added 80 uL of ultrapure water. A

Table 2. Sequence of the adapters and primers used in the binding reactions, pre-amplification

Adaptor or primer	Oligonucleotide
EcoRI adaptors	5´ CTCGTAGACTGCTACC 3´ 5´ AATTGGTACGCAGTCTAC 3´
Pre-selective amplification primer N: T ou A	5´ GACTGCGTACCAATTCN 3´
Selective amplification primer NNN: AAA,	5´ GACTGCGTACCAATTCNNN 3´
MseI adaptors	5´ GACGATGAGTCCTGAG 3´ 5´ TACTCAGGAACTCAT 3´
Pre-selective amplification primer N: T ou A	5´ GATGAGTCCTGAGTAAN 3´
Selective amplification primer NNN: AAA,	5´ GATGAGTCCTGAGTAANNN 3´

A total of 33 primers combinations for selective amplification were tested (Table 3). In these reactions were used primers with sequences containing more three selective nucleotides at the 3 'end (Table 2), composed of 1 uL of each primer (25 ng / uL), 10 uL PCR Master Mix (Promega ®), 6 uL Nuclease-Free Water and 2 uL of pre-diluted reaction, in a final volume of 20 uL. The conditions for selective amplification was: 94 ° C for 2 min, 12 cycles of 94 °C for 30 seconds, 65 °C for 30 seconds and 72 °C for 1 min, 23 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 1 min, with final extension at 72 °C for 2 minutes. After that, were added 8 uL of loading buffer. The samples were denatured for 5 minutes at 94 °C and visualized after electrophoresis in a 6% polyacrylamide gel, 0.5 mm thick with the System "Sequi-Gen GT" (BioRad ®), stained with silver nitrate according to the protocol proposed by Creste et al. (2001). In cases of polymorphic fragments, their size was estimated by comparison with standard molecular weight of 100 bp (Promega ®).

In the band analysis, we considered especially those located between 200 and 700 bp. The bands located in the associated group in each species were taken as the model for the species molecular characterization. For each combination of primers, the amount of polymorphic bands and the percentage of each combination was established in the five bulk. In each of the combinations tested was taken as polymorphism any band that was different compared to the species group.

Table 3. Combinations of primers used to obtain Eucalyptus AFLP markers.

Treatment	Primer combinatons		Treatment	Primer combinatons		Treatment	Primer combinatons	
	MSel	*Eco RI*		*MSel*	*Eco RI*		*MSel*	*Eco RI*
1	GTG	AGA	12	CAA	AAC	23	GAA	AAA
2	GTG	AGC	13	CAA	AAA	24	GAA	AAC
3	GTG	AGG	14	CAT	AAA	25	GAA	ACC
4	GTG	ACA	15	CAT	AAC	26	GGC	AAA
5	GTT	AGC	16	CAT	ACC	27	GGC	AAC
6	CAG	AAC	17	CCT	AAA	28	GGC	ACC
7	CAG	AAA	18	CCT	AAC	29	GGT	AAA
8	CAG	ACC	19	CCT	ACC	30	GGT	ACC
9	CAC	AAC	20	GTC	AAA	31	GGA	AAA
10	CAC	AAA	21	GTC	AAC	32	GGA	AAC
11	CAC	ACC	22	GTC	ACC	33	GGA	ACC

RESULTS AND DISCUSSION

Based on the DNA mixture of individuals of each specie was generated an AFLP profile of the five bulks by 33 primer combinations, resulting in 803 polymorphic fragments with 91.65% of polymorphism, the minimum and maximum number of fragments per primer was 9 and 62, respectively. Figure 1 shows the pattern of fragments formed by the DNA bulk and polymorphism found by BSA method. Considering each AFLP fragment as an independent locus, 868 different fragments were analyzed. The standard AFLP bands proved to be consistent and highly reproducible.

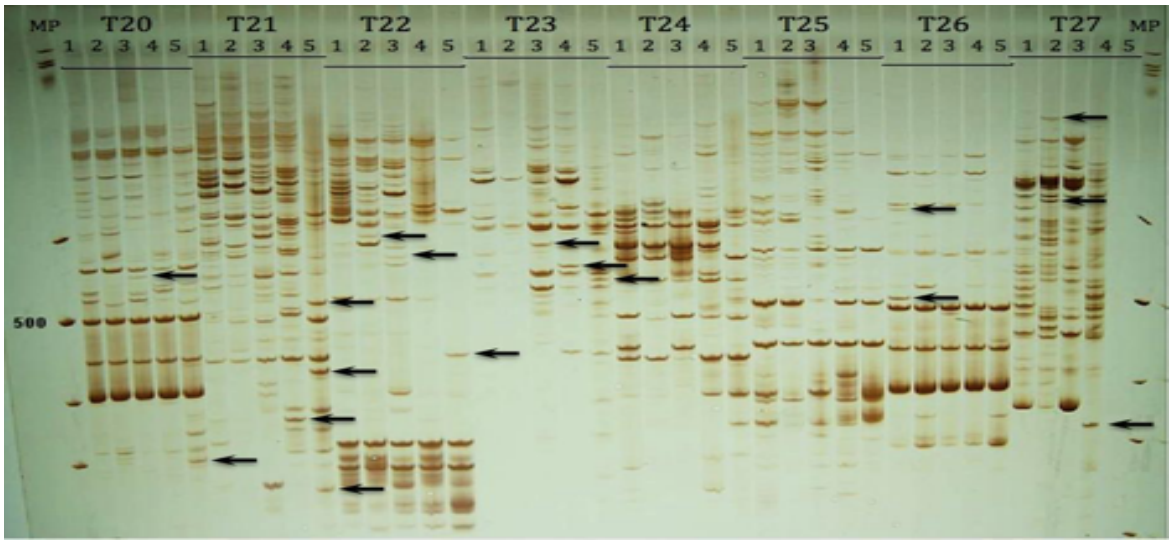

Figure 1. Banding pattern formed by bulk DNA. They are suitable candidate markers associated with specific species in eucalypts. *Rail 1= *E. brassiana*, rail 2= *E. saligna*, rail 3= *E. tereticornis*, rail 4= *E. urophylla*, rail 5=*E. grandis*. MP= DNA Ladder 1 Kb.

A total of 67 markers was identified to be Eucalyptus species specific, ranging between 200 and 700 bp, according to each primer set.

It was found seven candidate fragments for *E. brassiana*, 18 fragments for *E. saligna*, 12 for *E. tereticornis*, 13 fragments for *E. urophylla* and 17 fragments for *E. grandis*, as shown in Table 4. In figure 1 shows treatments from 21 to 27, candidate tags were identified by Bulk-DNA to identify the species under study.

Table 3. Primer combinations used for obtaining bands associated with the identification of Eucalyptus species.

Treatment	Primer combinatons		DNA Bulk					Candidate fragments
	MSel primer	*EçoRI primer*	*sp1/ E. brassiana*	*sp2/ E. brassiana*	*sp1/ E. brassiana*	*sp2/ E. brassiana*	*sp2/ E. brassiana*	
1	GTG	AGA	0	1	1	0	0	2
2	GTG	AGC	0	2	0	0	0	2
3	GTG	AGG	0	0	0	0	1	1
4	GTG	ACA	0	2	3	0	0	5
5	GTT	AGC	0	2	0	0	0	2
6	CAG	AAC	0	1	0	0	5	6
7	CAG	AAA	0	0	0	2	0	2
8	CAG	ACC	0	0	1	1	1	3
9	CAC	AAC	0	0	0	0	1	1
10	CAC	AAA	0	0	0	0	0	0
11	CAC	ACC	0	0	0	0	0	0
12	CAA	AAC	0	0	0	0	1	1
13	CAA	AAA	0	0	1	0	0	1
14	CAT	AAA	0	0	0	1	0	1
15	CAT	AAC	0	1	0	0	0	1
16	CAT	ACC	0	0	0	0	0	0
17	CCT	AAA	0	1	1	0	1	3
18	CCT	AAC	0	0	0	0	2	2
19	CCT	ACC	0	0	0	0	0	0
20	GTC	AAA	0	0	1	0	1	2
21	GTC	AAC	0	0	0	1	3	4
22	GTC	ACC	0	1	1	1	0	3
23	GAA	AAA	0	0	1	1	1	3
24	GAA	AAC	0	0	0	0	0	0
25	GAA	ACC	0	0	0	0	0	0
26	GGC	AAA	2	0	0	0	0	2
27	GGC	AAC	0	2	0	1	0	3
28	GGC	ACC	2	0	0	4	0	6
29	GGT	AAA	0	0	1	0	0	1
30	GGT	ACC	1	0	0	1	0	2
31	GGA	AAA	0	1	0	0	0	1
32	GGA	AAC	1	2	0	0	0	3
33	GGA	ACC	1	2	1	0	0	4
Total			**7**	**18**	**12**	**13**	**17**	**67**

Of the 33 primers tested, the best combinations for species identification was T30 treatment, composed of M + GGT / E + ACC primer, which detected 70% of *E. urophylla* individuals. The treatment T33 (M + GGA / E + ACC primer) identify 60% of *E. saligna* individuals; the T21 treatment (M+GTC/ E+AAC primer), had two markers, one 60% and the other 50% in the identification of *E. grandis* individuals. In T26 treatment (M+GGC/ E+AAA primer), there was only confirmed 30% of *E. brassiana* individuals, and also for T28 treatment (M + GGC / E + ACC primer), was detected 30% of *E. tereticornis* individuals, as shown in figure 2. The combinations presented in this study show a high degree of polymorphism identifying candidate fragments for the species identification, with an average of 95.65%. However, once the bulk was opened, DNA detection power has decreased, from 30 to 70% of fragment frequency. Our choice of restriction enzymes and primer sequences was

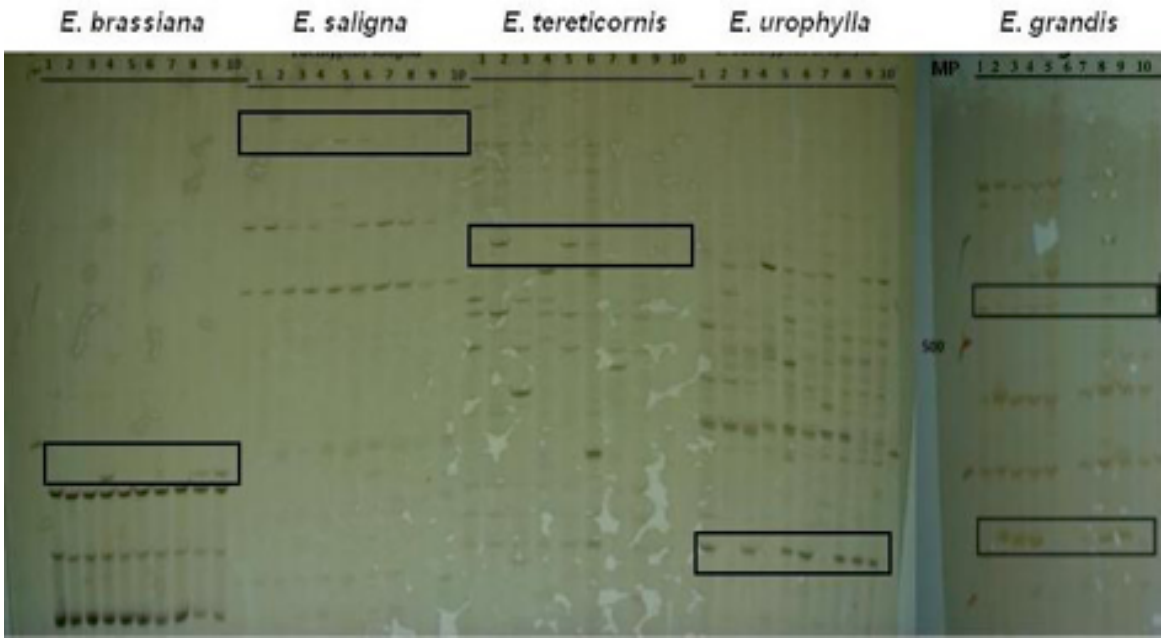

Figure 2. Standard bands formed by DNA bulk open. For confirmation marks associated with specific species in Eucalyptus. *sp1= E. brassiana, sp2= E. saligna, sp3= E. tereticornis, sp4= E. urophylla, sp5=E. grandis. MP= DNA Ladder 1 Kb.

based on studies performed in *Eucalyptus* by Leite et al, 2011, which detected the different primers exhibiting a maximum number of bands on acrylamide gels (results not showed). Leite et al. (2011) considers these primer combinations highly informative, being common and polymorphic in the eucalyptus genome, which makes these sequences valuable for the design of the other primer to generate polymorphisms, both for linkage maps or physical maps. This AFLP technique has been used to identify markers linked to plagues resistance genes (Najimi et al. 2002) and assess genetic diversity in several important forest plantations including *Eucalyptus* (Steane et al. 2011). BSA efficiency has been witnessed in other studies on the identification of markers linked to the genes that control resistance to rust on Eucalyptus sp. identifying polymorphism genetically linked to the characteristic resistance groups (Zamprogno et al. 2008). According to Domingues et al. (2006), BSA technique allowed the identification of molecular markers linked to early flowering in E. grandis by RAPD (Random amplified polymorphic DNA) and SCAR (Sequence Characterized

Amplified Region) with 60% of efficiency, confirming the usefulness of this technique as a molecular tool. These studies show that for an effective exploitation of bulked segregant analysis method, the only requirement is the existence of a segregating population of a gene of interest (Michelmore et al. 1991). Mellish et al. (2002), exploring the genetic diversity of the forage populations from the genus *Agropyron* spp. opted to use bulk and AFLP markers in identifying intra-population variation.

Likewise, Herrmann et al. (2005) worked with bulk by AFLP analysis to determine the genetic diversity and relationships within and among red clover populations (*Trifolium pratense* L.). The bulk analysis in white clover plants (*Trifolium repens* L.) by AFLP markers proved to be a powerful tool for the fast screening of genetic variability to identify cultivars (Kolliker et al. 2001).

According to Michelmore et al. (1991), BSA technique is a method that allows the identification of markers in specific genomic regions linked to any specific gene or genomic region. Zhu et al. (1998) raised some questions about the effectiveness of DNA bulks in relation to the low sensitivity to detect polymorphism using AFLP markers. Fuchs et al. (2011), successfully identified genes associated with abnormal seedlings of *Eucalyptus* who died in a few months by BSA technique. However, when analyzed the bulks in studies with onion, barley, potato, lettuce, cabbage, and linen, the frequency of fragments was reduced below 50%, concluding that by using this method was not found a practical approach to detect genetic polymorphisms (Van Treuren 2001). Guthridge et al. (2001), showed that profiles generated from individual samples could be or not present in the bulk samples, showing a high relationship between the frequency of occurrence and the presence of fragments in bulk samples.

CONCLUSIONS

In this investigation, we show that the AFLP technique combined with BSA can play an important role in identification of specific species *Eucalyptus* as it allowed to select a series of polymorphic markers associated with different *Eucalyptus* species, confirming 70% of *E. urophylla* and 60% of *E. saligna and E. grandis* individuals. These molecular markers can be used as auxiliary tool to the rapidly and efficiently identification of some brands associated with the identification of species of *Eucalyptus*, in a faster and economical way, thus aiding breeding programs that do not have access to more expensive technologies.

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