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Development of simple sequence repeat (SSR) markers in *Eucalyptus* from amplified inter-simple sequence repeats (ISSR)

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Abstract

Eucalyptus spp. are widely used in exotic plantations. Since many of these trees are derived from vegetative propagation, the routine identification of clones has become increasingly important. The most widely used molecular based method for fingerprinting these clones is by random amplified polymorphic DNAs (RAPDs). Although this technique is useful, its results are not very repeatable, especially between laboratories. The aim of this study was to develop microsatellite markers that are highly repeatable, and to investigate their value in *Eucalyptus* fingerprinting. Typically, this process involves the expensive procedure of constructing an enriched genomic library. However, we used an inter-simple sequence repeat (ISSR) polymerase chain reaction (PCR)-based enrichment technique for microsatellite-rich regions. With this relatively inexpensive method, microsatellite-rich regions were amplified directly from genomic DNA, after which PCR products were cloned and sequenced. From these microsatellite-rich sequences, primer sets were constructed to amplify mono-, di-, tri-, hexa-and nona-nucleotide repeats. These markers were all inherited in a Mendelian fashion in the progeny of a test cross between two *Eucalyptus grandis* trees. The primer sets developed were also able to amplify the corresponding microsatellite loci from five different *Eucalyptus* spp., namely *E. grandis*, *E. nitens*, *E. globulus*, *E. camaldulensis* and *E. urophylla*.

Key words: *Eucalyptus* spp. — fingerprinting — molecular markers — inter-simple sequence repeat (ISSR) — microsatellite

Eucalyptus spp. are extensively grown in commercial forest plantations world-wide (Eldridge et al. 1993). These plantations are commonly established through vegetative propagation based on rooted cuttings (Campinhos and Ikemori 1980). The routine identification of individual clones, as well as parental identification is consequently becoming increasingly important. Since a high degree of reproducibility is required for these tests, traditional molecular techniques such as random amplified polymorphic DNAs (RAPDs) are unsuitable (Welsh and McClelland 1990, Williams et al. 1990, Rafalski et al. 1996). Alternative techniques to RAPDs that are more reproducible include amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995), restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1980), isozymes (Smithies 1955, Markert and Moller 1959) and microsatellites or simple sequence repeats (SSRs) (Litt and Luty 1989, Tautz 1989, Weber and May 1989). However, microsatellite markers is the method of choice for use in forestry industries, because it is a fast and simple technique compared with AFLPs, RFLPs or isozymes (Litt and Luty 1989, Tautz 1989, Weber and May 1989, Rafalski et al. 1996).

The development of SSR primers for the amplification of microsatellites typically involves the screening of enriched or non-enriched genomic libraries for microsatellite sequences (Rafalski et al. 1996). This is the most widely, but also the most expensive approach used. Previously, a relatively inexpensive enrichment technique using RAPD–polymerase chain reaction (PCR) of genomic DNA, has been reported (Ender et al. 1996, Lunt et al. 1999). The PCR fragments generated in this way are cloned into commercially available T-vectors, after which the clones are screened for SSR-containing inserts. Screening is required, since not all the cloned fragments contain microsatellite regions. The polymorphisms generated with RAPDs reflect both variation in microsatellite regions (Cifarelli et al. 1995, Richardson et al. 1995, Ender et al. 1996, Davis et al. 1997), and point mutations, insertions or deletions of sequences other than SSRs (Paran and Michelmore 1993). However, a variation of RAPD-PCR, known as inter-simple sequence repeat PCR (ISSR-PCR), targets only those regions of the genome that are rich in microsatellite motifs (Zietkiewicz et al. 1994).

The aim of this study was to develop primer pairs that will amplify specific microsatellite loci in *Eucalyptus* spp., using ISSR-PCR. ISSR-PCR products were cloned and sequenced, after which specific primers were constructed for the amplification of SSR-containing fragments. Amplified SSR alleles were tested for Mendelian inheritance on the progeny from a cross between two *E. grandis* parents. The SSR primer sets developed here were also tested for interspecies amplification on *Eucalyptus urophylla*, *Eucalyptus nitens*, *Eucalyptus globulus* and *Eucalyptus camaldulensis*.

Materials and Methods

Plant materials and DNA extraction: Two *Eucalyptus grandis* trees known as TAG 5 and ZG 14 were used in this study. They were also used as parents in a controlled cross, from which 36 progeny were included to test Mendelian inheritance of developed SSR markers. Two individuals of each of *E. urophylla*, *E. nitens*, *E. globulus* and *E. camaldulensis* were also included to assess the use of the SSR primer sets constructed in cross-species amplification. DNA was isolated from young leaves of each tree using a method described by Murray and Thompson (1980).

Amplification, cloning and sequencing of ISSR fragments: Microsatellite regions were randomly amplified from TAG 5 genomic DNA, using primer $(ACA)_5$, which was previously described by Hantula et al. (1996).

The sequence of primer $(ACA)_5$ is 5′-BDB$(ACA)_5$–3′. Each PCR reaction contained 1 mM dNTPs (0.25 mM of each), 2.5 mM $MgCl_2$, 0.2 μM primer, 0.25 ng/μl DNA, 0.05 U/μl Super-Therm DNA polymerase and 1 × reaction buffer (Southern Cross Biotechnology (Pty) Ltd, Cape Town, South Africa). Reactions were overlaid with mineral oil to prevent evaporation and performed on a Hybaid Omnigene thermocycler (Hybaid, Teddington, Middlesex, UK). The PCR programme consisted of an initial denaturation step of 1 min at 92°C, followed by 30 cycles of 1 min at 92°C, 1 min at 58°C, 1 min at 72°C and a final extension at 72°C for 10 min. PCR products were purified (QIAquick PCR Purification Kit, Qiagen GmbH, Hilden, Germany) and cloned (pGEM-T Easy Vector System, Promega Corporation, Madison, WI, USA). Plasmids were harvested with alkaline lysis (Sambrook et al. 1989) from 20 randomly selected clones. Approximately 500–1000 bp of each insert was sequenced using plasmid specific primers T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-TATTTAGGTGACACTATAG-3′). Sequencing reactions were performed on an ABI PRISM™ 377 automated DNA sequencer with an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer, Warrington, UK). From the sequence data, 13 primer sets for amplifying *Eucalyptus* microsatellite regions or SSRs were constructed. These primer sets were designed to flank microsatellites and amplified 100–500 bp fragments.

Specific amplification of SSRs: SSR amplification reaction conditions were similar to those described for the random amplification of microsatellite regions, except that the specific annealing temperature for each primer set was used. The primer sets FMRSA 1 (5′-GCC TTGAAAGAGAGGGAGAG-3′ and CCACACGGAAGAGCTT CAGC), and FMRSA 2 (5′-CGTCGTACTCTAGTCAATGC-3′ and 5′-ATCCTCCGCTTAAGAGGCTC-3′) had an annealing temperature of 55°C. The annealing temperature for the primer sets FMRSA 3 (5′-TTATGGAAGAGAAAGACCAGCC-3′ and 5′-TTCGTCCGCG AATAGAGAAT-3′), FMRSA 4 (5′-GACGATGAAGATGAGG ATGG-3′ and 5-GCAACAGCGAAACTGAAAAT-3′) and FMRSA 5 (5′-GCAGCAACAGATGAAGGACA-3′ and 5′-GTCTTTT AGGGGGTGCATGA-3′) were 57°C. One primer in each of the sets was labelled with a phosphoramidite fluorescent label (HEX, TET or FAM). PCR products were separated with an ABI™ PRISM 377 automated DNA sequencer using Genescan-500 Tamra (Perkin Elmer) as an internal standard. Separated fragments were analysed with the ABI PRISM Genescan®2.1 software (Perkin Elmer).

Evaluation of SSRs: The SSR primer sets were tested for interspecies amplification on all of the *Eucalyptus* spp. included in this study. For this purpose, the PCR reaction and cycling conditions were similar to these described above. The amplified alleles were also tested for Mendelian segregation based on the 36 F_1 *E. grandis* progeny using JOINMAP® version 2.0 (Stam and Van Ooijen 1995). A series of χ^2 tests were performed to determine whether the segregation ratios obtained of the amplified alleles at the five loci tested corresponded with the expected ratios. Furthermore, these fragments were subjected to linkage analyses with JOINMAP®, set to test a range of LOD values (1.0–14.0) and recombination frequencies (45–55%).

Results

The ISSR primer $(ACA)_5$ amplified a variety of fragments from the *E. grandis* tree, TAG 5, ranging from 100 to 20 000 bp. The use of this primer in PCR on the other *E. grandis* tree, ZG 14, also generated a range of fragments, but the profiles for the two clones were different. For this reason, the total ISSR-PCR product from TAG 5 was cloned. All 20 of the randomly selected cloned fragments had a stretch of $(ACA)_n$ at the one end and a stretch of $(TGT)_n$ at the opposite end. These motifs flanked various SSR motifs in all 20 of the selected clones. They included polynucleotide SSRs such as $(GAA)_n$, $(GA)_n$, $(CAG)_n$, $(TC)_n$ and many more.

Thirteen of the 20 randomly selected cloned fragments harboured SSR motifs that were flanked by regions suitable for constructing PCR primers. After construction of these primer sets, only 10 generated fragments that were polymorphic in either of the two *E. grandis* parent trees (TAG 5 and ZG 14), or their progeny. For the purposes of this study, five of these primer sets, namely FMRSA 1–5, are reported. FMRSA 1 amplified a perfect $(GAGCAC)_4$ hexanucleotide repeat and an imperfect $(GA)_{25}$ dinucleotide repeat in the *E. grandis* tree TAG 5, whereas FMRSA 2 targeted perfect $(TC)_7$ dinucleotide and $(TCCGCT)_3$ hexanucleotide repeats. FMRSA 3 amplified a perfect $(CACCATCAT)_4$ nonanucleotide and an imperfect $(CAG)_8$ trinucleotide repeat, while FMRSA 4 amplified perfect $(GAG)_5$ and imperfect $(GAA)_{11}$ trinucleotide repeats. Only FMRSA 5 amplified compound $(A)_n$ and $(T)_n$ mononucleotide repeats.

Testing these primer sets on the 36 progeny of the TAG5 × ZG 14 cross, indicated that the amplified fragments were inherited in a Mendelian fashion as codominant alleles at a significance level of $P = 0.05$ (Table 1). Although a limited number of cloned fragments were randomly selected for sequence analysis, these results suggest that the ISSR-PCR enrichment technique described here, preferentially targets different SSR motifs from nuclear DNA and not chloroplast and mitochondrial DNAs. These loci also appear to be scattered across the genome, since none of the loci were linked.

The use of the SSR primer sets developed on species other than *E. grandis* was also tested and amplification using the five primer sets generated fragments from each of the different *Eucalyptus* spp. tested (Table 2). Although only two individuals (A and B) from each *Eucalyptus* sp. were tested, the majority of the trees were polymorphic or heterozygous at these loci. The exceptions were FMRSA 2, which amplified only a 115-bp fragment from the *E. urophylla* individuals and FMRSA 5, which amplified only a 256-bp fragment from *E. urophylla* and *E. globulus*. The fact that these primer sets can easily be used on species other than the one used to construct them is a great

Table 1: Segregation ratios of the FMRSA 1–5 amplified alleles at the five simple sequence repeat (SSR) loci, in the F_1 progeny from the cross between TAG 5 and ZG 14

SSR primer set	Parental genotypes (bp) TAG 5	ZG 14	Progeny genotypes (bp) A	B	C	Observed ratio A:B:C	Expected ratio A:B:C	χ^2	P
FMRSA 1	239; 245	235; 235	235; 245	235; 239	—	21 : 15	1 : 1	1.00	0.317
FMRSA 2	109; 115	109; 115	109; 109	109; 115	115; 115	11 : 15 : 10	1 : 2 : 1	1.06	0.589
FMRSA 3	163; 163	163; 171	163; 163	163; 171	—	16 : 20	1 : 1	0.44	0.507
FMRSA 4	295; 302	302; 302	302; 302	295; 302	—	15 : 21	1 : 1	1.00	0.317
FMRSA 5	246; 246	246; 250	246; 246	246; 250	—	14 : 22	1 : 1	1.78	0.182

Table 2: The sizes (bp) of the different simple sequence repeat (SSR) alleles amplified with the primer sets FMRSA 1–5, from the *Eucaluptus grandis* trees ZG 14 and TAG 5, as well as from the two trees (A and B) representing each of the other species used in the study

	SSR allele sizes (bp) in the different *Eucalyptus* spp.									
	E. grandis		*E. nitens*		*E. globulus*		*E. camaldulensis*		*E. urophylla*	
Primer set	TAG 5	ZG 14	A	B	A	B	A	B	A	B
FMRSA 1	239; 245	235	219; 235	230; 255	230; 249	230	239; 249	245	233	233; 255
FMRSA 2	109; 115	109; 115	120	109	115; 120	115	115	109; 115	115	115
FMRSA 3	163	163; 171	179; 184	196; 202	188; 190	188	171; 173	167; 173	163; 176	163
FMRSA 4	295; 302	302	311	311; 313	315; 327	313	325	297; 325	297; 325	297; 305
FMRSA 5	246	246; 250	250; 256	256	256	256	246; 256	256	256	256

advantage, because construction of unique SSR-primer sets for every *Eucalyptus* sp. cultivated by forestry companies would not be economically viable.

Discussion

The simple and efficient method for constructing primers for the amplification of SSRs from *Eucalyptus* spp. described here, is of great value for several reasons. First, by using this technique it is now possible to construct SSR primer sets in cases where little or no sequence data are available from public domain databases, such as GenBank. Second, this technique allows for the construction of SSR primer sets in cases where there is no immediate access to genomic libraries. Third, the SSR primer sets developed using our ISSR-PCR enrichment method appear to target only nuclear DNA and not the mitochondrial and chloroplast genomes, which make them extremely useful for determining clonal, as well as paternal and maternal identities. A fourth major advantage of using our ISSR-PCR-based technique for constructing SSR primers, rather than those based on RAPD technology (Ender et al. 1996, Lunt et al. 1999), is that all cloned fragments harbour microsatellite motifs. However, where there is a need for selecting only a specific class of repeat, an anchored PCR procedure (Taylor et al. 1992, Rafalski et al. 1996) could be used to screen plasmid inserts.

The use of the SSR primer sets developed here, as well as those constructed by Brondani et al. (1998), have been successfully incorporated into the research and diagnostic services provided by the Forestry Molecular Biology Co-operative programme (FMBC). These primers work remarkably well for identification of parent trees, as well as for the routine identification of *Eucalyptus* clones, especially where clones are being licensed to other groups for vegetative propagation. The long-term objective is to saturate the existing genetic *Eucalyptus* linkage maps. The fact that a single marker can be used on more than one species also suggests that it would be possible to superimpose the genetic information or a genetic linkage map from one *Eucalyptus* sp. on another. It is also intended to use selected markers from these maps for marker-assisted selection in breeding programmes. Furthermore, selected SSR primer sets from this study, as well as from those generated using enriched genomic libraries (Brondani et al. 1998), will be used for *Eucalyptus* sp. identification. Using SSR primer sets, large-scale population diversity studies of the different *Eucalyptus* species in commercial plantations is proposed, as well as monitoring the diversity in *Eucalyptus* clone banks to ensure appropriate genetic conservation.

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