Identification of Species-Diagnostic ISSR Markers for Six *Eucalyptus* Species

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(Received 8th July 2005)

Abstract

Eucalyptus is planted worldwide for raw material in paper and rayon industry. It is a potential out-crosser and the natural populations are highly heterogeneous displaying strong inbreeding depression. *Eucalyptus* hybrids have been intensively utilized for their vigor, higher wood quality and resistance to diseases. Identification of species for hybridization is predominantly based on morphological characters and is not always reliable. Hence, DNA marker based species identification and hybrid validation is an important and efficient tool in breeding programs. In the present study, attempts were made to identify species – diagnostic markers for six eucalypt species (*E. camaldulensis* Dehnh, *E. citriodora* Hook, *E. grandis* W. Hill ex Maiden, *E. pellita* F. Muell, *E. tereticornis* Sm and *E. urophylla* S.T. Blake) using ISSR-PCR fingerprints. PCR amplification using seven ISSR primers resulted in significant polymorphism among the population from different species. *E. citriodora* and *E. tereticornis* showed monomorphic frequency of maximum 37.5% and minimum 14.3% respectively. Twenty species-diagnostic markers were identified for *E. camaldulensis*, *E. citriodora*, *E. grandis* and *E. urophylla* while no marker was detected for *E. pellita* and *E. tereticornis*. A maximum of eleven and a minimum of one species-diagnostic marker were recorded for *E. citriodora* and *E. camaldulensis* respectively. Among the twenty markers, nine were present in all the individuals of a particular species.

Key words: DNA, Inter-Simple-Sequence-Repeat markers, Eucalypt, Fingerprinting, Tree breeding.

Introduction

Eucalyptus, an exotic hardwood plantation tree is widely planted in tropical and subtropical regions of the world (Eldridge *et al.,* 1994). Although this genus consists of more than 800 species, only thirty species are commercially exploited due to their fast growth and other economical traits (Brooker and Kleinig, 2001). In particular, six species (*E. camaldulensis*, *E. citriodora*, *E. grandis*, *E. pellita*, *E. tereticornis* and *E. urophylla*) were found to be more suitable for Indian agro-climatic conditions and the wood is being extensively used as raw material for paper and rayon industry (Kallarackal and Somen, 1997; Kallarackal *et al.,* 2002). *Eucalyptus* is a potential cross pollinator (Gaiotto *et al.,* 1997) with out-crossing rate ranging from 0.69 to 0.86 (Moran and Bell, 1983) resulting in highly heterozygous populations. Selection of elite plants for clonal propagation and seed orchard have been important attempts carried out by *Eucalyptus* breeders for tree improvement. Earlier selections have been predominantly based on the phenotypic superiority and genetic stability through progeny trials. Recently, species-diagnostic markers are common among sytematicists and germplasm managers for identification, validation and their conservation. Phenotypic identification of species with foliar and floral characters remains difficult for non-specialists. In recent years, molecular markers are being widely used for identifying the genotypes. Plant breeders extensively use the DNA based species-specific markers to determine the parents and release reliable hybrids (Rossetto *et al.,* 1997; Kochieva, 1999; Scheef *et al.,* 2003; Kochieva *et al.,* 2004). It prevents the introgression of closer or distant taxa, which may lead to certain negative consequences like loss of genetic diversity, genetic assimilation and outbreeding depression on population viability. Hence, the objective of the present research was to identify species-diagnostic ISSR markers for six *Eucalyptus* species commonly used in breeding programmes.

Materials and Methods

Seeds of six eucalypts species were obtained from Australian tree seed center, CSIRO, Australia. The seedlings were raised and planted in Panampally Research Station, Kerala, India, and used for the present study. We examined 149 genotypes belonging to six *Eucalyptus* species (*E. camaldulensis*, *E. citriodora*, *E. grandis*, *E. pellita*, *E. tereticornis* and *E. urophylla*) for the development of species-diagnostic DNA markers (*Table 1*). DNA was isolated from the tissues of juvenile leaves using CTAB method (Doyle and Doyle, 1987). The resultant DNA was stored in TE buffer and concentrations were estimated by agarose gel (0.8%) electrophoresis using λ DNA (Bangalore Genei, India) as standard.

The polymerase chain reaction (PCR) mixture (10 µl) contained 1.0 µl 10x PCR buffer, 2.5 mM $MgCl_2$, 40 µM dNTP mix, and 0.3 U Taq DNA polymerase (Bangalore Genei, India). Ten ISSR primers used in earlier studies (Yasodha *et al.,* 2004) were custom synthesized (Sigma-Aldrich, USA) and used at the final concentration of 100 nM. The primers consisted of six "di" and four "tri" nucleotide repeat sequence either anchored at 5' or 3' end totaling 17 to 24 bases in length (*Table 2*). All the reactions were carried out with different *Eucalyptus* genomic DNA templates at the final concentration of 15 ng per reaction. PCR thermocycling was carried out on a MJ Research PTC-200 DNA engine with the initial predenaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds; the annealing tempera-

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DOI:10.1515/sg-2006-0017
edited by Thünen Institute of Forest Genetics

Table 1. – *Eucalyptus* populations used for the development of species-diagnostic markers.

S. No	Species	Common name	Seed lot No.	No. of individuals
1	*E. camaldulensis*	River red gum	18276	25
2	*E. citriodora*	Lemon scented gum	-	26
3	*E. grandis*	Rose gum/ flooded gum	19970	24
4	*E. pellita*	Large-fruited red mahogany	20574	23
5	*E. tereticornis*	Forest red gum	13660	25
6	*E. urophylla*	Timor white gum	18096	26

Table 2. – List of ISSR primers used for the determination of species-diagnostic markers in *Eucalyptus* species.

Primer code	Nucleotide sequence
5' anchored	
R $(CA)_7$	5' GRTRCYGRTRCACACACACACACA 3'
T $(GT)_9$	5' CRTAYGTGTGTGTGTGTGTGTGT 3'
TA $(CAG)_4$	5' ARRTYCAGCAGCAGCAG 3'
CRR $(ATT)_4$	5' AGCRRATTATTATTATT 3'
RA $(GCT)_6$	5' AYARAGCTGCTGCTGCTGCTGCT 3'
G $(ATT)_4$	5' YCYRRGATTATTATTATT 3'
$(GA)_8$R	5' GAGAGAGAGAGAGAGARGY 3'
3' anchored	
$(GT)_8$ R	5' GTGTGTGTGTGTGTGTRYRY 3'
UBC810	5' GAGAGAGAGAGAGAGAT 3'
UBC842	5' GAGAGAGAGAGAGAGAYG 3'

ture of 50°C for 30 seconds and the extension at 72°C for 1 minute. The cycles were completed by a final extension at 72°C for 10 minutes. DNA fragments amplified were separated by agarose gel (2%) electrophoresis using 1 X TAE buffer and run at 70V for two hours along with 1 Kb ladder (Gibco Brl) as molecular weight standard. The gel profiles were viewed under UV-transilluminator and documented using Kodak-DC290 digital camera.

During data analysis, only reproducible polymorphic bands in amplification reactions were considered as present. Each band was treated as a separate putative locus, and scored as present (1) or absent (0) in each genotype. The binary data developed by the manual scoring of the ISSR fingerprints were used for the further population genetic analyses. The number of monomorphic and polymorphic bands were derived from the binary data and their percentage was calculated. The presence of an ISSR fragment in a particular species population and its absence in all the other species was designated as species-diagnostic marker. Bands with frequency above 80% in a particular species and a frequency below 20% in the other species were considered as potential species-specific marker as described by GILI *et al.,* 2004.

Results

Among the ten primers tested, only seven of them produced unambiguous DNA fragments. All the individuals of six *Eucalyptus* species extensively amplified using the seven ISSR primers and produced 583 fragments ranging from 265 to 1535 bp (*Table 3*). The minimum size fragment of 265 bp was amplified by the primers R$(CA)_7$ and TA$(CAG)_4$ in *E. grandis* and the maximum of 1535 bp was in *E. citriodora* by TA$(CAG)_4$. Interestingly, in all the species, large sized fragments were amplified in presence of primer with CAG repeat. The total number of loci ranged from 70 (*E. pellita*) to 126 (*E. tereticornis*). A maximum of 32 monomorphic bands were recorded in the *E. grandis* but the highest percent monomorphism (37.5%) was calculated in the populations of *E. citriodora*. Similarly, the minimum number of 16 monomorphic bands were observed in *E. pellita* and the lowest ISSR monomorphism percentage (14.3%) was registered in *E. tereticornis.*

This study revealed twenty species-diagnostic markers for four species (*E. camaldulensis*, *E. citriodora*, *E. grandis* and *E. urophylla*), out of six species (*Table 4*). However, it failed to detect even a single marker for *E. pellita* and *E. tereticornis*. For example, *figure 1* shows an ISSR profile of *E. citriodora* amplified with UBC842 primer with species-diagnostic marker at 1088 bp. A maximum of eleven species-diagnostic markers were identified for *E. citriodora* and only one was available for *E. camaldulensis* at 813 bp.

Among the diagnostic markers identified, nine markers belonging to *E. citriodora*, *E. grandis* and *E. urophylla* had a frequency of 100% (*Table 4*). T$(GT)_9$-813 generated the only marker for *E. camaldulensis* and was

Table 3. – Level of polymorphisms among different *Eucalyptus* species.

Eucalyptus sp.	MW range	Total bands	No.of bands		Percent	
			Mono morphic	Poly morphic	Mono morphism	Poly morphism
E. camaldulensis	295-1318	124	23	101	18.5	81.5
E. citriodora	280-1535	80	30	50	37.5	62.5
E. grandis	265-1250	92	32	60	34.8	65.2
E. pellita	330-1225	70	16	54	22.9	77.1
E. tereticornis	270-1305	126	18	108	14.3	85.7
E. urophylla	276-1440	91	18	73	19.8	80.2

DOI:10.1515/sg-2006-0017
edited by Thünen Institute of Forest Genetics

Table 4. – Species-diagnostic ISSR markers in *Eucalyptus* species.

Taxon	ISSR Marker	Same Species		Other Species	
		No. of Individuals	Presence (%)	No. of Individuals	Presence (%)
E. camaldulensis	$T(GT)_9$-813	25	80.00	124	0
E. citriodora	$R(CA)_7$-950	26	92.31	123	0.81
	$T(GT)_9$-1150	26	100.00	123	0
	$T(GT)_9$-1018	26	100.00	123	6.50
	$T(GT)_9$-405	26	100.00	123	3.25
	$T(GT)_9$-345	26	100.00	123	6.50
	$TA(CAG)_4$-1535	26	100.00	123	0
	$TA(CAG)_4$-1000	26	100.00	123	0
	$TA(CAG)_4$-625	26	100.00	123	8.13
	$R(GCT)_6$-635	26	100.00	123	0
	$R(GCT)_6$-490	26	100.00	123	0
	UBC842-1088	26	100.00	123	0
E. grandis	$R(CA)_7$-528	24	83.33	125	4.80
	$T(GT)_9$-631	24	100.00	125	12.80
	$TA(CAG)_4$-1150	24	87.50	125	0
	$TA(CAG)_4$-577	24	100.00	125	1.60
	$TA(CAG)_4$-460	24	100.00	125	0
E. urophylla	$R(CA)_7$-390	26	100.00	123	7.32
	$TA(CAG)_4$-1440	26	100.00	123	0
	$TA(CAG)_4$-740	26	100.00	123	0

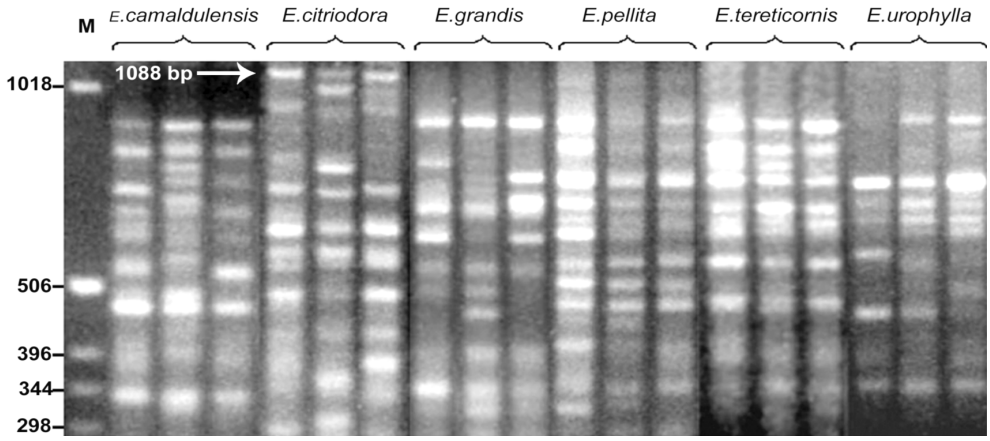

Figure 1. – ISSR profile of *Eucalyptus* species populations amplified using UBC842 primer showing a species-diagnostic marker (MW–1088) for *E. citriodora.*

present in 80% individuals. *E. citriodora* had a maximum of six diagnostic markers at [$T(GT)_9$-1150, $TA(CAG)_4$-1535, $TA(CAG)_4$-1000, $R(GCT)_6$-635, $R(GCT)_6$-490 and UBC842-1088] which were absent in all other populations. In *E. urophylla*, two diagnostic markers at [$TA(CAG)_4$-1440, $TA(CAG)_4$-740] was identified while in *E. grandis*, one diagnostic marker at [$TA(CAG)_4$-460] was identified. All the other markers were not exclusive due to their occurrence in individuals of other species.

Discussion

Due to the advancements in the DNA fingerprinting techniques, species-specific markers are widely used in the field of molecular taxonomy, particularly in animals (GILI *et al.,* 2004; MAIER *et al.,* 2001). However, only a few reports on species-specific markers are available in plants (SCHEEF *et al.,* 2003; KOCHIEVA, 1999; KOCHIEVA *et al.,* 2004). SCHEEF *et al.* (2003) identified two RAPD based species-specific markers for two *Agrostis* species (*A. capillaries* – colonial type and *A. palustris* – creeping type) and subsequently developed SCAR markers, which were tested in 17 cultivars belonging to four species, consisting of 140 individuals of colonial and dry-land bent grasses. Similarly, species-specific RAPD markers were generated to distinguish two kinds of spruce, *Picea glauca* and *P. engelmannii* (KHASA and DANCIK, 1996). The molecular identification using RAPD markers was employed to discriminate eight kinds of *Coptidis rhizomes* (a Chinese herbal medicine) from one another (CHENG *et al.,* 1997). Genus and species-specific markers were also developed for certain solanaceae members using RAPD fingerprints (KOCHIEVA, 1999). In another study, KOCHIEVA *et al.* (2004) revealed both species and cultivar-specific markers in the genus, *Syringa*. In eucalypts, exclusive RAPD markers were reported in *E. rudis* and *E. drummondii* (ROSSETTO *et al.,* 1997). Similarly, RAPD markers were used to individually discriminate ten clones from different eucalypt species including *E. grandis*, *E. urophylla*, *E. grandis* X *E. urophylla*, *E. pellita*, *E. resinifera*, *E. tereticornis*, *E. paniculata*, *E. dunnii*, *E. torelliana* and *E. citriodora* (GRATTAPAGLIA *et al.,* 1992). However, ISSR being a highly informative and reproducible marker has not been employed in eucalypts for identification of species except for develop-

DOI:10.1515/sg-2006-0017
edited by Thünen Institute of Forest Genetics

ment of SSRs (VAN DER NEST *et al.,* 2000). In this study, species-diagnostic ISSR markers were identified in *E. camaldulensis*, *E. citriodora*, *E. grandis* and *E. urophylla* while no diagnostic markers were observed in *E. tereticornis* and *E. pellita*. Similar problem was experienced while developing species-specific markers using RAPDs (SCHEEF *et al.,* 2003). This could be due to the occurrence of high divergence among the particular species populations. It is inferred that more primers should be used in future to develop species-diagnostic markers for these two species.

The developed species-diagnostic markers can be used for the precise and rapid identification of species and putative hybrids during selection and hybridization programmes of *Eucalyptus.* This is also extremely important to accelerate tree breeding programs and for protection of breeder's rights. Sequencing of these markers and developing PCR based primers from the sequencing data will be used as ready reckonors to satisfy the above requirements.

Acknowledgement

This research was funded by the Department of Biotechnology, Ministry of Science and Technology, Government of India.

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DOI:10.1515/sg-2006-0017
edited by Thünen Institute of Forest Genetics