

DEVELOPMENT OF SEQUENCE CHARACTERISED AMPLIFIED REGION (SCAR) MARKERS IN *ACACIA AURICULIFORMIS*

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YUSKIANTI V, XIANG ZB & SHIRAISHI S. 2011. Development of sequence characterised amplified region (SCAR) markers in *Acacia auriculiformis*. This paper reports the development of sequence characterised amplified region (SCAR) markers and their multiplexing for easy, fast and robust identification in *Acacia auriculiformis*. A total of 44 of 48 fragments screened using random amplified polymorphic DNA (RAPD) markers were successfully converted and developed into SCAR markers. Six SCAR markers that had high discrimination ability and good fragment quality were selected and multiplexed into one set called a multiplex PCR (polymerase chain reaction) of SCAR (MuPS) marker. Results using 40 individuals of *A. auriculiformis* showed that the discriminatory power of each marker ranged between 0.385 and 0.512. However, when MuPS marker was used the discriminatory power increased to 0.974. When the MuPS was applied to 40 *A. mangium* individuals and 16 different *Acacia* hybrids, the discriminatory power decreased to 0.652 and 0.483 respectively.

Keywords: Multiplex PCR of SCAR marker, MuPS, genotype identification, DNA marker

YUSKIANTI V, XIANG ZB & SHIRAISHI S. 2011. Pembangunan penanda SCAR (sequence characterised amplified region) bagi *Acacia auriculiformis*. Artikel ini melaporkan pembangunan penanda SCAR (sequence characterised amplified region) dan pemultipleksan jujukannya untuk pengecaman *Acacia auriculiformis* yang mudah, cepat dan berkesan. Sebanyak 44 daripada 48 fragmen disaring menggunakan penanda RAPD (random amplified polymorphic DNA) berjaya diubah menjadi penanda SCAR. Enam penanda SCAR yang mempunyai keupayaan diskriminasi yang tinggi serta kualiti fragmen yang baik dipilih lalu dimultiplekskan menjadi satu set yang dinamai multipleks reaksi rantai polimerase (PCR) SCAR (MuPS). Uji kaji menggunakan 40 individu *A. auriculiformis* menunjukkan bahawa kuasa diskriminasi setiap penanda berjalat antara 0.385 dengan 0.512. Bagaimanapun apabila penanda MuPS digunakan, kuasa diskriminasi meningkat kepada 0.974. Apabila MuPS diaplikasikan kepada 40 individu *A. mangium* dan 16 hibrid *Acacia* yang berbeza, kuasa diskriminasi berkurangan masing-masing kepada 0.652 dan 0.483.

INTRODUCTION

Acacia auriculiformis is native to Cape York Peninsula, Queensland and northern areas of the Northern Territory in Australia; western and southern Papua New Guinea; and Irian Jaya and the Kei Islands in Indonesia (Turnbull 1986). It is extensively planted for pulpwood and firewood, often as part of reforestation programmes and rehabilitation of degraded land. The species is also used for production of timber and as substitute for traditional timber species (Kojima et al. 2009). Tree breeding programmes are expected to lead to improvements in wood quality of *A. auriculiformis* (Chowdhury et al.

2009). Selection programmes of *A. auriculiformis* in Vietnam showed that individuals from seedling seed orchard and seed production area seedlots had straighter stems and reduced incidence of stem forking compared with those selected from natural provenance controls (Hai et al. 2008).

A number of studies using molecular markers have been conducted on *Acacia* species but mostly on *Acacia mangium* (Butcher et al. 2000). With the increasing use of *A. auriculiformis* in plantation forestry, there is a need for an easy, fast and robust molecular marker that can discriminate individuals of this species. This

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study was thus conducted to develop specific molecular markers for *A. auriculiformis*. The developed marker can be used for genotype identification within *A. auriculiformis* and also to support clonal identification in the clonally-based tree improvement programmes for the species (Zobel & Talbert 1984, Park 2002).

In this study, RAPD fragments were screened for markers that discriminate genotypes and converted into stable and reliable SCAR markers, following Paran and Michelmore (1993). A marker based on a multiplex PCR of SCAR (MuPS) using six selected SCAR markers was then developed. Due to the close relationship between *A. mangium* and *A. auriculiformis*, the effectiveness of the newly-designed SCAR markers was examined in *A. auriculiformis*, *A. mangium* and *A. mangium* × *A. auriculiformis* hybrid.

MATERIALS AND METHODS

Plant materials

Leaves from 48 individuals each *A. auriculiformis* and *A. mangium* from a provenance trial of the Center for Forest Biotechnology and Tree Improvement (CFBTI) in Wonogiri, Central Java, Indonesia and 16 individuals of *Acacia* hybrid from Quy Nhon Plantation Forest Company of Vietnam Ltd (QPFL) were used in this experiment.

DNA extraction and purification

Total genomic DNA was isolated using a modified CTAB method (Shiraishi & Watanabe 1995). Crude DNA was purified using MagneSil™ Red particles.

Screening RAPD fragments

Sixty-four 13-mer RAPD primers (William et al. 1990) were used for RAPD fragment screening. PCR amplification was carried out in a 10 µl reaction medium containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 3.0 mM MgCl₂, 0.2 mM of each dNTP, 0.025 unit µl⁻¹ Platinum Taq DNA polymerase, 1.5 µM of primer, and 10 ng template DNA. Amplification was performed using a thermocycler at 94 °C for 1 min, followed by 45 cycles of 94 °C for 30 s, 37 °C for 30 s and 72 °C for 90 s, and a final extension step at 72 °C for 7 min. The amplified products were electrophoresed on a 1.2% agarose gel in 1 ×

Tris Borac-EDTA and 0.5 µg ml⁻¹ EtBr buffer and visualised under UV light.

SCAR marker development

The selected RAPD fragment was excised from the gel using a pipette. The gel slice was soaked in 50 µl of 1 × TE buffer at pH 8.0 and the leachate used as template DNA. Verification of the target fragment was done in a 20 µl reaction as in the first PCR amplification above but with 0.5 µM primer and 4 µl template DNA. Amplification was performed using a Gene Amp 9600 using the same annealing described above except for 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s. Electrophoresis of the PCR product was done on a 1.2% agarose gel as described above.

The PCR product of the selected fragment was then ligated with a plasmid vector (pGEM-T Vector System) and transformed into *Escherichia coli* according to a half-scale method recommended by the supplier. PCR using transformed *E. coli* colonies as template was performed with the same components described above and primers LR (5' TCCGGCTCGTATGTTGTGTGGA-3') and LL (5' GTGCTGCAAGGCGATTAAGTTGG-3') that flanked the cloning site (Hattori et al. 1997) in a 25 µl reaction. The thermocycler program consisted of 29 cycles of 96 °C for 15 s and 70 °C for 2 min, followed by one cycle at 96 °C for 15 s and 72 °C for 10 min in the Gene Amp 9600. A total of 5 µl of PCR reaction were electrophoresed to check the target fragment. If the amplification was successful, the remaining PCR product was purified using AMPure for sequencing.

Sequencing reactions contained BigDye Terminator v 1.1/3.1 Sequencing Buffer, 0.21 M betaine, 0.5 µl reaction premix, 2 µmol primer (LR or LL) and 4 µl of PCR products as template DNA in a final volume of 12 µl. The thermocycler program for sequencing reactions was 98 °C for 60 s, 30 cycles at 98 °C for 10 s, 50 °C for 10 s and 60 °C for 4 min, followed by 72 °C for 1 min. The products were then purified using CleanSeq Sequencing Reaction Clean-up System and electrophoresed using 3100-Avant Genetic Analyzer. DNA fragments were separated using capillary array filled with POP7 polymer.

SCAR primer pairs were designed using the OLIGO software program version 6.8. Amplification with the newly-designed SCAR primers either in single or in multiplexing analysis was conducted in a 10 µl reaction medium as in the first PCR amplification but containing

0.2 µM of each primer pair for single analysis and adjusted primer concentration (Table 1) for multiplex analysis. The thermocycler program consisted of an initial denaturation at 94 °C for 30 s; 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, followed by 72 °C for 5 min. Analysis of the amplification products was conducted using 1.2% agarose gel for a SCAR and 1.5% agarose gel for its multiplex using the same procedure above.

To enhance the prospects of applying the marker not only in *A. auriculiformis* but also in *A. mangium*, screening was conducted using equal numbers of individuals of the two species. In the first screening, four samples of each *Acacia* species were used, and eight of each in the second screening. In the third screening, 48 *A. auriculiformis* individuals were used. Based on results of this third screening, useful SCAR markers for genotype identification were selected and then multiplexed into one set.

Estimation of discriminatory power was calculated based on frequency of present or absent fragment at each marker (Tessier et al. 1999). Discriminatory power was examined using 40 of 48 *A. auriculiformis* samples. Due to the close relationship between *A. auriculiformis*, *A. mangium* and their hybrids, the effectiveness of the new marker was also tested on 40 individuals of *A. mangium* and 16 individuals of the hybrid.

RESULTS AND DISCUSSION

To overcome low reproducibility of RAPD fragments, only clear fragments with strong and reliable amplification were selected. Forty-eight RAPD fragments were obtained based on these criteria. For each cloned RAPD amplification product, two oligonucleotides were developed into SCAR primer pairs. Forty-four SCAR primer pairs were successfully developed (data not shown).

In order to increase the discriminatory power of these markers for the identification of individual genotypes within a species, not only reliable and accurate SCAR markers were developed, but these were also multiplexed (Schoske et al. 2003). The first and second screening of the 44 SCAR primers against subsets of individuals of *A. auriculiformis* and *A. mangium* resulted in 16 polymorphic SCARs. Based on the allele frequency and amplification quality of fragments, six polymorphic SCAR primers were selected in the final screening using 48 *A. auriculiformis* individuals (Table 1). The six SCAR primer pairs were then multiplexed into one set of MuPS marker (Table 1, Figure 1).

The ability to discriminate between individuals of *A. auriculiformis*, *A. mangium* and the hybrid was significantly increased when all markers were used together in a multiplexed PCR compared with using a single primer. Analysis

Table 1 RAPD and SCAR primer sequences and lengths and concentrations of SCAR primer in the MuPS marker

Loci	RAPD	SCAR		
	Sequence (5' to 3')	Sequence (5' to 3')	Fragment length (bp)	Concentration (µM)
aR1	GGAGGAGAGGGGA	F: GTGGTAAAGCAAACGCTTATAAATAC R: GGAGGAGAGCGGACGGTTAATAG	230	0.11 0.11
aR2	AACGGTGACCCAA	F: AACGGTGACCCAAATGCACA R: GCTCACAGCTTTGCGTCACT	260	0.10 0.10
aR3	AGTCGGGTGGACA	F: AGTCGGGTGGACAGACGGCG R: AGTCGGGTGGACACTGGTGAC	360	0.07 0.07
aR4	GGAGGAGAGGATG	F: GGAGGAGAGGATGAGGGAAGAGTAAC R: GAGGAGAGGATGGCCAACATG	400	0.12 0.12
aR5	GGAGGAGAGGTGC	F: GTGCTAATCCTTCTGCCTCAAGA R: GGAGGAGAGGTGCTAAGTTCACAATC	500	0.10 0.10
aR6	AACGGTGACCTCA	F: AGTTGTGTCTTTGGGTGGGTG R: ACGGTGACCTCATACGTACATATAGTTG	650	0.14 0.14

F = forward, R = reverse, describing the position of the primer

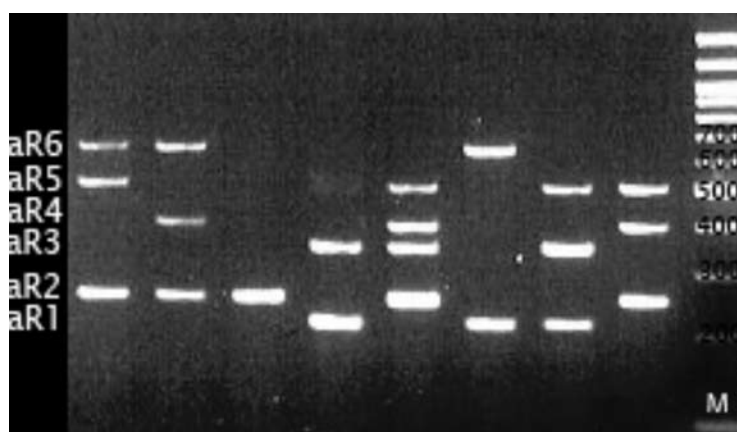


Figure 1 Amplification products of the six polymorphic SCAR markers. Lanes 1–8 contain different *Acacia auriculiformis* individuals. M is the DNA ladder.

using 40 *A. auriculiformis* individuals showed that the discrimination power of each of the six markers ranged from 0.385 to 0.512 in a single primer and the value increased to 0.974 when all markers were analysed in a MuPS (Table 2). A similar result was also obtained when the marker was tested with the other *Acacia* species. The discrimination power of 40 individuals of *A. mangium* and 16 individuals of the hybrids increased from 0.00 to 0.328 and 0.00 to 0.325 in a single primer to 0.652 and 0.483 in a MuPS respectively (Table 2). Multiplex PCRs have been used in many studies (Bezuidenhout et al. 2006, Xu et al. 2006) because they provide a rapid and convenient screening assay. The MuPS marker developed in this study will enable better discrimination between individual genotypes of *A. auriculiformis*, *A. mangium* and *A. auriculiformis* × *A. mangium* hybrid, while reducing the overall cost and time for such analysis.

The SCAR markers from MuPS marker had a high level of discrimination power for genotype identification within *A. auriculiformis* (0.974) but moderate for *A. mangium* (0.652) and *Acacia* hybrid (0.483) (Table 2). SCAR markers were able to discriminate among genotypes with satisfactory results. The use of SCAR markers had facilitated the identification of genotypes in two closely related oak species, i.e. *Quercus petraea* and *Q. robur* (Bodenes et al. 1997), and cultivar identification in grapevine (*Vitis vinifera*) (Vidal et al. 2000), *Prunus armeniaca* (Mariniello et al. 2002), and *Pyrus pyrifolia* and *P. communis* (Lee et al. 2004). In addition, these markers were also used for the detection of resistant genes (Venter & Botha 2000, Giovannelli et al. 2002, Huaracha

et al. 2004) and sex determination (Parasnis et al. 2000, Deputy et al. 2002).

The SCAR markers developed in this study provided the first markers able to identify genotypes of *A. auriculiformis*. The markers can be used in small laboratories with limited equipments for preliminary analysis because of its simplicity in applications. SCAR markers detect only single genetically defined loci. Their amplification is less sensitive to reaction conditions and scoring systems are more straightforward than for other PCR-based markers such as RAPD markers (Bautista et al. 2002). PCR amplification of SCARs is also reproducible (Weng et al. 1998).

This MuPS marker can also be used in clonal propagation systems for confirming the identity of clones and for the construction of a clonal data base/clonal bank for these species. Another application of the MuPS marker developed here is its combination with other markers such as RFLP (Butcher et al. 1998) and SSR (Butcher et al. 2000) markers in *A. mangium* and *Acacia* hybrid (Ng et al. 2005) to create a genetic map and do linkage analyses with traits of interest.

Although the ability of the MuPS marker to distinguish between genotypes is moderate to high depending on species, combining this marker with a previously developed MuPS marker in *A. mangium* (Widyatmoko 2003) will be able to increase the discrimination power and number of individuals that can be distinguished. By combining both markers in the analyses, high discrimination power (e.g. 1.000 as shown by Yuskianti and Shiraishi (2010) in *Paraserianthes falcataria*) for discrimination between genotypes of *A. mangium*, *A. auriculiformis* and the *A.*

Table 2 Discrimination power (DP) of developed SCAR markers in *Acacia auriculiformis*, *A. mangium* and *Acacia* hybrids

Loci	No. of observed genotype		Observed genotype frequency (pi)		DP
	Present	Absent	Present	Absent	
<i>A. auriculiformis</i> (n = 40)					
aR1	21	19	0.53	0.48	0.512
aR2	15	25	0.38	0.63	0.481
aR3	24	16	0.60	0.40	0.492
aR4	10	30	0.25	0.75	0.385
aR5	28	12	0.70	0.30	0.431
aR6	12	28	0.30	0.70	0.431
DP in all markers					0.974
<i>A. mangium</i> (n = 40)					
aR1	32	8	0.80	0.20	0.328
aR2	1	39	0.03	0.98	0.050
aR3	40	0	1.00	0.00	0.000
aR4	0	40	0.00	1.00	0.000
aR5	7	33	0.18	0.83	0.296
aR6	5	35	0.13	0.88	0.224
DP in all markers					0.652
<i>Acacia</i> hybrid (n = 16)					
aR1	16	0	1.00	0.00	0.000
aR2	13	3	0.81	0.19	0.325
aR3	16	0	1.00	0.00	0.000
aR4	0	16	0.00	1.00	0.000
aR5	2	14	0.13	0.88	0.233
aR6	0	16	0.00	1.00	0.000
DP in all markers					0.483

auriculiformis × *A. mangium* hybrid can be obtained.

In conclusion, we have developed a MuPS marker containing six polymorphic SCAR markers. The marker can be used to identify genotypes in *A. auriculiformis*, *A. mangium* and their hybrids with high discrimination power.

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