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Sequence Characterized Amplified Region (SCAR) Markers in Sengon (*Paraseriathes falcataria* (L.) Nielsen

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Genotype identification of Sengon (*Paraseriathes falcataria* (L.) Nielsen needs accurate and reliable method for identification. Sixteen individuals of *P. falcataria* from Okinawa, Japan were used as materials for selecting polymorphic RAPD fragments and for developing SCAR marker whereas confirmation of polymorphisms of the SCARs was conducted using 24 materials from Candiroto Seed Orchard, Indonesia. Forty-eight polymorphic fragments were obtained from screening in 288 RAPD primers. Forty-six out of 48 SCAR primers were successfully developed. Examination of the newly-designed of SCAR primer using 24 *P. falcataria* from Candiroto Seed Orchard, Indonesia found 5 polymorphic SCAR primers. These five SCARs are considered as useful marker for genotype and clone identification within *P. falcataria*.

Key words: Sengon (*Paraserianthes falcataria*), genotype identification, SCAR, RAPD

INTRODUCTION

Genotype identification of Sengon (*Paraseriathes falcataria* (L.) Nielsen needs accurate and reliable method for identification. Random Amplified Polymorphic DNA (RAPD) markers (William *et al.* 1990) are the most common method for identification because it is a fast, cost effective and simple method. Nevertheless, RAPD markers are sensitive to minor changes in reaction condition during PCR amplification (Jones *et al.* 1997) and difficult to reproduce (McGregor *et al.* 2000). To improve the reliability of the RAPD markers, the marker can be converted into Sequence Characterized Amplified Regions (SCAR) marker (Paran & Michelmore 1993).

A SCAR is a genomic DNA fragment at a single genetically defined locus that is identified by PCR amplification using a pair of specific oligonucleotide primers (Paran & Michelmore 1993). SCAR markers are advantages over RAPD marker because their PCR amplification is less sensitive to reaction condition (Paran & Michelmore 1993; Bautista *et al.* 2002). Furthermore, PCR amplification of the SCARs is reproducible and can be easily scored (Weng *et al.* 1998). Therefore, analysis using SCAR markers are straightforward, rapid and easy to perform.

The objective of this study was to develop accurate and reliable SCAR marker for genotype identification in *P. falcataria*. Target fragments were selected from RAPD markers. The selected fragments were cloned and sequenced; the sequencing results were then used to design a pair of primers to amplify the target fragments.

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MATERIALS AND METHODS

Plant Materials. Sixteen individuals *P. falcataria* from Okinawa, Japan were randomly used for selecting the polymorphic fragments in the RAPD fragment screening and for developing SCAR markers, whereas 24 individuals *P. falcataria* that represents 4 provenances from Candiroto Seed Orchad, Indonesia were used for confirmation the polymorphisms of the newly-designed of SCAR markers. Total genomic DNA was isolated using CTAB Method modified from Shiraishi and Watanabe (1995) and purified with GENECLEAN III kit (Bio101.INC) following the procedure described by the supplier.

RAPD Marker Screening. Polymorphic fragments were screened using RAPD markers (William *et al.* 1990). A total of 288 RAPD primers from six series (D02, D18, E20, I02, S11, and Y16) were used in the screening. RAPD analysis was carried out in 10 µl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM each of each dNTPs, 0.025 unit/µl Platinum Taq DNA polymerase (Perkin-Elmer, USA), 1.5 µM primers, and 10 ng of template DNA. Amplification was performed using a Gene Amp 9600 (Perkin Elmer, USA) for 1 min at 94 °C, amplification for 45 cycles of 30 sec at 94 °C, 30 sec at 37 °C and 90 sec at 72 °C; and followed 7 min at 72 °C. Amplification products of RAPD analyses were verified using1.2% agarose gel in 1 x TBE of electrophoresis buffer.

SCAR Marker Development. The selected fragment was excised from the gel using a pipette and used as a template. Re-amplification was done in a 20 µl reaction of PCR amplification above but with 0.5 µM of primer and 4 µl template DNA. Amplification was performed using a Gene Amp 9600 (Perkin Elmer, USA) using the same annealing described above except for 30 cycles at 94 °C

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for 30 sec, 55 °C for 30 s and 72 °C for 90 s. Verification of the PCR product was conducted using 1.2% agarose gel in 1 x TBE of electrophoresis buffer.

The PCR product of the selected fragment was then ligated with a plasmid vector (pGEM-T Vector System), and transformed into *E. coli* (JM109 High efficiency Competent Cell) according to a half-scale method recommended by the supplier. A colony PCR was amplified with the same components described above using two primers (5'-TCCGGCTCGTATGTTGTGTGGA-3' and 5'-GTGCTGCAAGGCGATTAAGTTGG-3') (Hattori *et al.* 1997) for 29 cycles at 96 °C for 15 s and 70 °C for 2 min followed by one cycle at 70 °C for 10 min in a Gene Amp 9600 (Perkin Elmer, USA). Five µl of PCR product were electrophoresed to check the target fragment. If the amplification was successful, the other PCR product will be purified using AMpure (Agencourt Bioscience, Beverly, MA, USA) for sequencing.

Sequencing reactions were performed using direct sequencing of PCR products. The sequence reaction in 12 µl reactions containing 1 x Big Dye terminator v 1.1 v.3.1 5x Sequencing buffer, 0.21 M Betaine, 0.5 µl reaction premix, 2 ñmol forward/reverse primer and 4 µl of PCR products as template DNA. The reaction was conducted in a Thermocycler (Biometra, Germany) at 98 °C for 60 sec; 30 cycles at 98 °C for 10 sec, 50 °C for 10 sec and 60 °C for 4 min; followed by 72 °C for 1 min. PCR products were purified using CleanSeq Sequencing Reaction Clean-up System (Agencourt Bioscience, Beverly, MA, USA) and electrophoresed using 3100-Avant Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). DNA fragments were separated using capillary array filled with POP7 polymer (Applied Biosystems).

SCAR primer pairs were designed by using the OLIGO software program (Version 6.8; National Bioscience Inc., Plymouth, MN, USA). The newly-designed of SCAR marker were amplified using a Thermocycler (Biometra, USA) in 10 µl reaction according to the method above except for the primer concentration (0.2 µM of each primer pairs) and the extension time (30 cycles at 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 60 sec). Verification the amplified products of SCARs were conducted using 1.2% agarose gel in 1 x TBE of electrophoresis buffer.

Polymorphisms of the newly-designed of SCAR markers were examined using 24 materials that represent 4 provenances in Candiroto Seed Orchard, Indonesia. First screening was conducted using eight individuals *P. falcataria* whereas the second screening was conducted using others 16 individuals *P. falcataria*. The value of Discrimination Power (DP) of the developed SCAR marker to discriminate genotype of *P. falcataria* was estimated. Estimation of DP was based on frequency of present or absent fragment at each marker. The calculation was derived from the formula of Tessier *et al.* (1999).

RESULTS

The first screening using 4 individual's *P. falcataria* from Okinawa, Japan showed that 285 out of 288 primers were successfully amplified fragments. A hundred and seven primers were suitable and continued to the second screening. In the second screening using 16 individuals of *P. falcataria*, 59 out of 107 primers were not counted as genetic marker because they produced ambiguous and less polymorphic fragments. As a result, forty-eight polymorphic RAPD fragments were obtained in the RAPD screening (data not shown).

From 48 RAPD cloned, 46 pairs of SCAR primer were successfully developed. First SCAR screening using 8 individuals of *P. falcataria* in Candiroto Seed Orchard, Indonesia showed that 35 SCAR primers amplified monomorphic fragment, 6 primers did not amplify any fragment and 5 primers produced polymorphic fragments. These primers were continued to the second screening using others 16 individuals from Candiroto Seed Orchard and all the primers (F01, F02, F03, F04, and F05) consistently showed their polymorphisms (Table 1 & Figure 1). These five SCAR primers were finally selected and it considered as useful marker for genotype identification in *P. falcataria* (Table 1 & Figure 1).

SCAR markers were generally a dominant marker. Each marker amplified one fragment; however, there was other cases that one marker produce more than one fragment. In our research, these five SCAR markers produced both types of polymorphisms. Three markers i.e. F01, F02, and F04 amplified only one fragment in each marker; whereas, two other markers (F03 and F05) amplified 2 and 3 fragments, respectively (Table 1 & Figure 1).

Further analysis of the condition of the three fragments generated from one marker was conducted. Three samples that amplified different loci in F05 marker were sequenced to reveal the cause of its polymorphisms.

Table 1. Five developed SCAR markers for genotype and clone identification within *P. falcataria*

Marker	RAPD		SCAR		
	Sequence(5' to 3')	No nucleotide (nt)	Sequence (5' to 3')	No nucleotide (nt)	Fragment size (bp)
F01	GGACCCAACCTCA	13	F:TATCACCATCGGCGTCAACC	20	500
			R:TCGACCAACTACCGAACAACG	21	
F02	GAGAGCCAACATC	13	F:ATCGATGAAACTTTGTAAGAACCCC	25	500
			R:GGCCTCAAACCAATCCAAATCC	22	
F03	AACGGTGACCTAG	13	F:ACCAATCGGACCTTCGTTCG	20	340
			R:CGGTAACCAAAACTCTAACGGCC	23	300
F04	AACGGTGACCTGT	13	F:CTTAGGTACTCCCAGAGCACCAGC	24	300
			R:CAAACCTCTCTACCACATCCATCC	24	
F05	AGTCGGGTGGGAC	13	F:CTCGAGTACGTTTGTGGAGAATCC	24	490
			R:TTGATGCACAGAGTTGGAGGC	21	460
					420

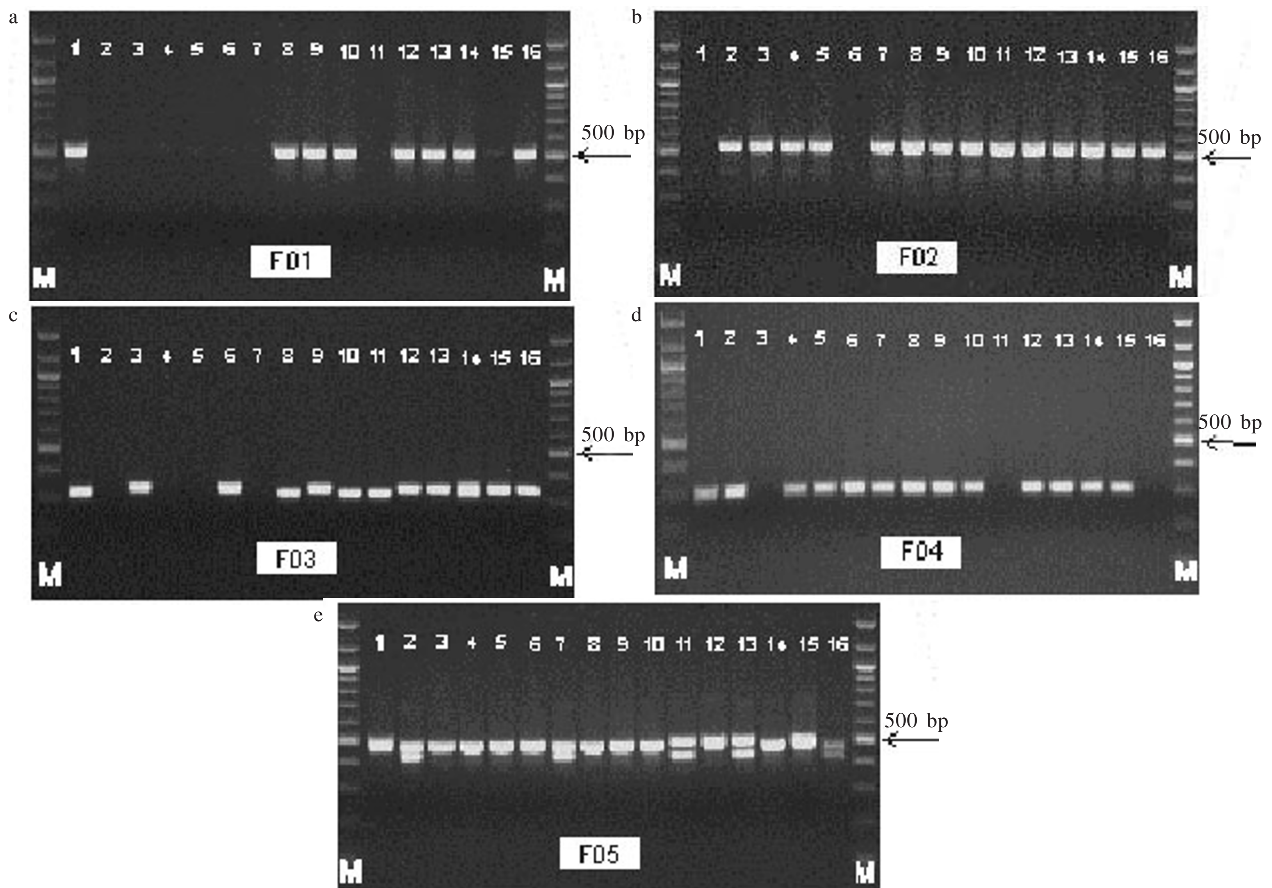

Figure 1. Amplification products of 16 individuals' *P. falcataria* from Candiroto Seed Orchard Indonesia in the five polymorphic SCAR markers. M represents 100 bp of DNA ladder. Number in the lanes 1-16 represents individual *P. falcataria*. Arrow indicates 500 bp.

Although, six single nucleotide polymorphisms (SNPs) in term of mutation or insertions/deletions (indels) were detected along the sequences, the presence of long indels revealed in the three samples seemed to be the source of the SCAR polymorphisms. The longest and the shortest loci were detected having 64 nucleotides differences in sequence whereas 44 nucleotides differences were revealed from the shortest loci and middle loci (Figure 2).

These five SCAR markers have low to moderate level of discrimination power with average DP was 0.425. DP of each marker was ranging from 0.233-0.533. Most of the markers produce moderate level of DP except in F02 and F04, respectively (Table 2).

DISCUSSION

Forty-eight RAPD fragments were selected and 46 SCAR primers were successfully developed. Five SCAR markers were finally selected. These markers have low to moderate level of DP (Table 2). The five SCAR markers were considered as useful markers for genotype identification within *P. falcataria* (Table 1 & Figure 1). SCAR markers are powerful tool for identification. It has been demonstrated for identification of hybrid (Weng *et al*. 1998), genotype (Bautista *et al*. 2002), specific genes (Arnedo-Andres *et al*. 2002) as well as for sex diagnosis (Deputy *et al*. 2002), and marker assisted selection (MAS) (Cairo *et al*. 2002; Lecouls *et al*. 2004). SCARs proved useful in genotype identification, although SCAR is less polymorphic in comparison with other markers like Simple Sequence Repeat (SSR) (Busconi *et al*. 2006).

In this study most of the SCARs produced monomorphic fragments, only 10.9% (5 out of 46 SCAR markers) were polymorphic. The inability to convert the RAPDs to SCARs has been reported in other studies where a high number of polymorphisms were identified through RAPD analysis and only a small subset of these was convertible to SCAR markers (Masuelli *et al*. 1995; Simon *et al*. 1999; Venter & Botha 2000; Bautista *et al*. 2002). It has been argued that RAPD polymorphisms might be the result of mismatch at the priming sites, rather than sequence divergence or structural rearrangements (Hernandez *et al*. 1999). Thus, loss of polymorphism when a short arbitrary primer is converted to a more specific and longer primer may be due to the loss of the uniqueness of the primer-binding site (Venter & Botha 2000).

The number of the SCAR markers developed in this study was five markers, however, because of two markers generated more than one polymorphic fragment so a possibility of the markers to discriminate individuals within *P. falcataria* was not based on only these five markers but become eight markers. The fact can be used to compensate the low level of DP in the two markers (F02 and F04) (Table 2).

```
     1                                                              63
01:  CTCGAGTACGTTTGTGGAGAATCCTGCTGGGAAGGCACTAGCGAGTGAACCAGGAAGCGAACA
02:  CTCGAGTACGTTTGTGGAGAATCCTGCTGGGAAGGCACTAGCGAGTGAACCAGGAAGCGAACA
03:  CTCGAGTACGTTTGTGGAGAATCCTGCTGGGAAGGCACTAGCGAGTGAACCAGGAAGCGAACA

64                                                                 130
ATGAGTAAAAAATAGAGCCTAGTGTCACGTGCGAATCCTAAAAGCCTAATTCATGCATACAAAAGCA
ATGAGTAAAAAATAGAGCCTAGTGTCACGTGCGAATCCTAAAAGCCTAATTCATGCATACAAAAGCA
ATGAGTAAAAAATAGAGCCTAGTGTCACGTGCGAATCCTAAAAGSCTAATTCATGCATACAAAAGCA

131                                                                197
GACCATGTTAATGGATATTGGATATTTACCCAACGAACKAATAAAGCGTGTAGGTAGGGCRAATATT
GACCATGTTAATGGATATTGGATATTTACCCAACGAACTAATAAAGCGTGTAGGTAGGGCGAATATT
GACCATGTTAATGGATATTGGATATTTACCCAACGAACTAATAAAGCGTGTAGGTAGGGCGAATATT

198                                                                264
TTGCCAGTACTGCACTTTCGCAACTTTAATTATCGTATAACTCTAAACCAGCATCTTTAGCGGATGA
TTGCCAGTACTGCACTTTC::::::::::::::::::::::::::::::::::::::::::::::::
TTGCCAGTACTGCACTTTCGCAACTTTAATTATCGTATA::::::::::::::::::::GCGGATGA

265                                                                331
CTTTTATTCCACTTTCTGGGCATTTTGGACCCCAAACGGCATCTGCCCTTTTGTTGTTTTACAGTCT
:::::::::::::::TGGGCATTTTGGACCCCAAACGGCATCTGCCCTTTTGTTGTTTTACAGTCT
CTTTTATTCCACTTTCTGGGCATTTTGGACCCCAAACGGCATCTGCCCTTTTGTTGTTTTACAGTCT

332                                                                398
CTAAAATCATAGATAATAGCGATATCAACGGAAAACAGAATAAAC:TCACTCTTTGTGAATCCCGTC
CTAAAATCATAGATAATAGCGATATCAACGGAAAACAGAATAAACCTCACTCTTTGTGAATCCCGTC
CTAAAATCATAGATAATAGCGATATCAACGGAAAACAGAATAAACCTCACTCTTTGTGAATCCCGTC

399                                                                465
TCTTTGATTCTGCCACCTTTTCGATGGGGAATAAAAAGAAAGAAAAATTAATTATCCCACCCTTTCT
TCTTTGATTCTGCCACCTTTTCGATGGGGAATAAAAAGAAAGAAAAATTAATTATCCCACCCTTTCT
TCTTTGATTCTGCCACCTYTTCGATGGGGAAAAAAAAGAAAGAAAAATTAATTATCCCACCCTTTCT

466                    489
CTTGCCTCCAACTCTGTGCATCAA
CTTGCCTCCAACTCTGTGCATCAA
CTTGCCTCCAACTCTGTGCATCAA
```

Figure 2. Sequence alignment of three *P. falcataria* amplified using F05 SCAR primer. Bold letter in the beginning of the sequences represent the name of sequenced individual. Underlined letter represents upper and lower SCAR primer sequences. Bold letter in the middle of the sequence represent single nucleotide polymorphisms (SNPs where S = C or G, K = G or T, R = A or G, Y = C or T, and: = insertions and deletions (indels).

Table 2. DNA fragments and each level of discriminatory power (DP) of 16 individuals *P. falctaria* in the five SCAR markers

Sample No.	SCAR marker							
	F01	F02	F03a	F03b	F04	F05a	F05b	F05c
1	1	0	0	1	1	1	0	0
2	0	1	0	0	1	0	1	1
3	0	1	1	0	0	0	1	0
4	0	1	0	0	1	0	1	0
5	0	1	0	0	1	0	1	0
6	0	0	1	0	1	0	1	0
7	0	1	0	0	1	0	1	1
8	1	1	0	1	1	0	1	0
9	1	1	1	0	1	0	1	0
10	1	1	0	1	1	0	1	0
11	0	1	0	1	0	1	0	1
12	1	1	1	0	1	1	0	0
13	1	1	1	0	1	1	0	1
14	1	1	1	0	1	0	1	0
15	0	1	1	0	1	1	0	0
16	1	1	1	0	0	0	1	1
Frequency of								
Present (1)	0.50	0.88	0.50	0.25	0.81	0.31	0.69	0.31
Absent (0)	0.50	0.13	0.50	0.75	0.19	0.69	0.31	0.69
DP*	0.53	0.23	0.53	0.40	0.33	0.46	0.46	0.46

*Discrimination power was calculated based on the formula of Tessier *et al*. (1999).

SCAR markers developed in this study can be used to support tree improvement program of *P. falcataria*. The markers can be used for genotype identification within *P. falcataria*. To date, development of SSR markers, with no specific sequence primer published (Saito *et al*. 2005) and multiplex Single Nucleotide Primer Extension (SNuPE) (Yuskianti & Shiraishi 2010) have been reported. The markers although proved useful for high-throughput identification of *P. falcataria*, these applications need high cost and skills. Identification using SCAR markers offers simple and cost-efficient procedure than analysis in SSR and multiplex SNuPE markers. The SCAR markers can be used for small scale laboratory with limited equipments as preliminary analysis for genotype identification within *P. falcataria,* whereas, further analysis can be conducted using SSR markers and multiplex SNuPE marker.

The SCAR markers have ability to discriminate between genotype of *P. falcataria*, they also have potential to discriminate between *P. falcataria* clones. Confirmation of identity of seedling generated from vegetative propagation can be conducted using these SCAR markers, therefore, errors resulted from the propagation can be avoided. Application of the SCARs can also facilitate a development of clonal data base or clonal bank of *P. falcataria* clones.

As the markers have only 5 markers the maximum number of individuals that can be discriminated is 32, however, because of the markers generated 8 polymorphic SCAR markers, so the possibility to discriminate individuals within *P. falcataria* was increased to be 256. The next step to increase the ability of the markers for identification is by multiplexing the developed SCAR markers into one set (Schoske *et al*. 2003). The approach can significantly increase the ability to discriminate individuals within the species while reduce cost and time of analysis (Yuskianti & Shiraishi 2010).

In conclusion, five polymorphic SCAR markers in *P. falcataria* have been developed. These five SCAR markers can be used for identification of genotype and clone within *P. falcataria*.

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