DISCRIMINATION OF TEAK (*TECTONA GRANDIS*) PLUS TREES USING SELECTED RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

A. Watanabe, A. Widyatmoko,

Laboratory of Silviculture, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

A. Rimbawanto

Forest Tree Improvement Research and Development Institute, Yogyakarta 555825, Indonesia

&

S. Shiraishi*

Laboratory of Silviculture, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

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WATANABE, A., WIDYATMOKO, A., RIMBAWANTO, A. & SHIRAISHI, S. 2004. Discrimination of teak (*Tectona grandis*) plus trees using selected random amplified polymorphic DNA (RAPD) markers. To achieve a highly reliable clone management of teak (*Tectona grandis*) plus trees, useful DNA molecular markers were surveyed using RAPD analysis and their ability to discriminate among plus tree clones was examined. A primary screening was performed for 120 arbitrary decamer primers, and 24 primers that generated 26 clear and unambiguous fragments were selected. In the secondary screening, the reproducibility of each fragment was investigated by six repetitions of polymerase chain reaction, and 13 fragments found to be most reproducible were finally selected. Evaluation of the discriminatory powers of these fragments suggested that the selected RAPD markers would be useful in the clone management of teak plus trees.

Key words: Clone management – clone identification – DNA marker

WATANABE, A., WIDYATMOKO, A., RIMBAWANTO, A. & SHIRAISHI, S. 2004. Pembezaan pokok terbaik jati (*Tectona grandis*) menggunakan penanda DNA polimorf ganda dan rawak (RAPD) yang terpilih. Untuk mencapai pengurusan klon pokok terbaik jati (*Tectona grandis*) yang boleh diharap, penanda molekul DNA yang penting ditinjau menggunakan analisis RAPD dan kebolehan penanda DNA untuk membezakan klon pokok terbaik dikaji. Penyaringan awal dibuat untuk 120 primer dekamer rawak dan 24 primer yang menghasilkan 26 fragmen yang jelas dipilih. Dalam penyaringan kedua, kebolehulangan setiap fragmen disiasat dengan enam ulangan tindak balas berantai polimerase. Sebanyak 13 fragmen yang menunjukkan kebolehulangan tertinggi dipilih. Penilaian kuasa perbezaan bagi fragmen tersebut mencadangkan bahawa penanda RAPD terpilih berguna dalam pengurusan klon pokok terbaik jati.

Author for correspondence. E-mail: sushi@agr.kyushu-u.ac.jp

Introduction

Teak *(Tectona grandis)* is classified under a monotypic genus (genus *Tectona*) of the family Verbenaceae. This species is a member of tropical deciduous forests and it grows in moist and dry monsoon climate zones throughout India, Myanmar, Laos and Thailand (Uetsuki 1989).

Teak, which is resistant to wood decay, acid soil, fire and drought, in addition to having excellent wood quality, has been utilised as a luxury timber around the world (Uetsuki 1989). As teak is a very important and valuable timber tree, it is widely planted not only in Southeast Asia, including Indonesia, Thailand and Malaysia, but also in tropical Africa and in South American countries. In Thailand, teak has played a role in attempts at a reforestation programme with important economic consequences (Niskanen 1998). In Indonesia, teak plantations occupy 617 000 ha (Palupi & Owens 1997) and many plus trees with excellent traits have been selected.

Accurate management of teak plus tree clones is essential from the point of view of tree improvement. Unfortunately, there is no guarantee that the ortets and ramets of plus tree clones have been correctly managed for long periods of time because it is difficult to identify misplanting or mislabelling of the plants by visual inspection (Harju & Muona 1989). In fact, such mistakes caused by conventional clone management have been revealed after DNA analyses were introduced to the practice of clone management (Keil & Griffin 1994, Wilhelmina & McNicol 1995, Scheepers *et al.* 1997).

RAPD (random amplified polymorphic DNA) analysis (Welsh & McClelland 1990, Williams *et al.* 1990) is one of the most effective tools for clone management (Takata & Shiraishi 1996, Goto 1998). RAPD analysis, a kind of PCR (polymerase chain reaction) with arbitrary decamer primers, is relatively easier than any other fingerprinting techniques and can be carried out with a simple instrument. This method has been used for the discrimination or identification of clones, varieties and cultivars of Sitka spruce (Wilhelmina & McNicol 1995), Japanese cedar (Takata & Shiraishi 1996, Goto *et al.* 1999), Japanese wax tree (Goto *et al.* 1997), Norway spruce (Scheepers *et al.* 1997), Japanese black pine (Goto 1998) and grape pine (Tessier *et al.* 1999).

In spite of the need for a highly reliable technique for managing teak plus trees, no useful method has been established for the discrimination or identification of these trees. In this study, we surveyed useful markers using RAPD analysis and estimated their discriminatory abilities.

Materials and methods

Plant materials

Twenty-four teak plus trees selected from the Java Island of Indonesia were used in this study (Table 1). Total DNA was isolated using the modified CTAB method previously described by Shiraishi and Watanabe (1995). Total DNA was then

Table 1 A list of teak plus trees used in this study

Sample No.	No. plus tree	Regency	Subdistrict	Area
1	92	Blora	Ngapus	59
2	42	Bojonegoro	Bubulan	161
3	10	Cepu	Blungun	1005
4	14	Cepu	Cabak	7082
5	8	Cepu	Pasarsore	1048
6	50	Jatirogo	Bancar	27
7	105	Jatirogo	Bate	124
8	54	Jambang	Kroncong	86
9	26	Kebonharjo	Ngandang	112
10	28	Kebonharjo	Tawaran	70
11	102	Kendal	Pelen	74
12	87	Mantingan	Kalinanas	62
13	74	Mantingan	Ngiri	78
14	33	Nganjuk	Bagor	32
15	112	Ngawi	Watutinatah	57
16	47	Padangan	Mulyoagung	49
17	4	Parengan	Mulyoagung	46
18	84	Pati	Lungguh	56
19	81	Pati	Ngarengan	58
20	79	Purwodadi	Bandungsari	48
21	69	Randublatung	Boto	71
22	99	Randublatung	Ngliron	145
23	20	Randublatung	Temanjang	51
24	3	Saradan	Rejung	118

purified with GENECLEAN III (Bio101. INC.) and used as a template for the RAPD analysis.

RAPD analysis

PCR reactions were performed in a 10 µl volume containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 3.0 mM $MgCl_2$, 200 µM of each dNTP, 0.25 µM of primer, 0.25 units / 10 µl AmpliTaq DNA polymerase (Perkin-Elmer), and 10 ng / 10 µl of template DNA. After an initial denaturation process lasting 60 s at 94 °C, amplifications were carried out with an automatic DNA thermal cycler (Perkin-Elmer, GeneAmp 9600) for 45 cycles consisting of 30 s at 94 °C, 30 s at 37 °C, and 90 s at 72 °C, and finally 7 min extension at 72 °C. Amplification products were fractionated by electrophoresis on 1.0% agarose gel, detected by staining with ethidium bromide, and photographed with a 302 nm transilluminator. RAPD analysis was performed with 120 primers. A total of 111 of these primers were Operon primers (Operon Technologies Inc., California, USA) and the remaining 9 primers were FB01, FB02, FB03, FB09, FB10 (Watanabe *et al.* 1996), FB05 (Shiraishi *et al.* 1996), FB04 (GCCCTACGCG), FB06 (GCCGCCACCA) and FB07 (ACGTAGCGTC).

Results and discussion

Screening of highly reproducible RAPD markers

The reproducibility of RAPD fragments varied among the amplified fragments (Skroch & Nienhuis 1995). RAPD fragments utilised as markers for discrimination or identification should not only be polymorphic but also clear and unambiguous. To survey the RAPD primers that met these requirements, primary screenings were performed for 120 primers using 4 individuals randomly chosen from the 24 plus trees.

Of these 120 primers, 9 primers were not amplified and 34 primers were not variable within 4 plus trees. One to four polymorphic fragments were generated by the remaining 77 primers. Fifty-three of the primers yielding polymorphic bands were removed from this study because they did not produce clear fragments. The other 24 primers generated 26 clear and unambiguous fragments. A total of 22 of these primers yielded one polymorphic DNA fragment each, while OPI-18 and OPQ-09 yielded two polymorphic RAPD fragments each.

Some authors have pointed out that the reproducibility of RAPD fragments differs among the amplified fragments (Skroch & Nienhuis 1995, Wolff *et al.* 1995). Therefore, it is essential to estimate further which polymorphic RAPD fragments have an acceptable level of reproducibility in the primary screening. Goto *et al.* (1997) tested the reproducibility of polymorphic fragments by repeating the PCR amplification five times. As a result, only fragments clearly amplified in all PCR reactions were used for the discrimination or identification of *Pinus thunbergii* clones. Likewise, during the secondary screening of this study, the reproducibility of each fragment was investigated by repeating the PCR amplification six times (Figure 1), and we thus decided which primers would be useful for teak.

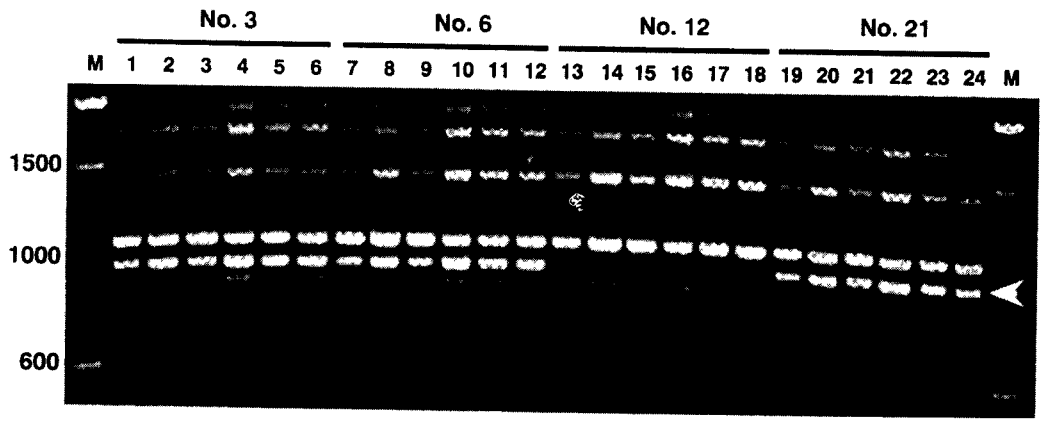

Figure 1 A RAPD profile (OPM–20) of the reproducibility test. Lanes 1–6, 7–12, 13–18 and 19–24 represent the amplification products of six consecutive PCRs for four teak plus trees, Nos. 3, 6, 12 and 21 respectively. M20 marker is indicated by an arrow. M represents a size marker.

Only 13 fragments showed successful amplification in the reproducibility test. Twelve primers produced these 13 polymorphic fragments. We concluded that these 13 RAPD markers were reliable for the discrimination and identification of teak plus trees (Table 2, Figure 2).

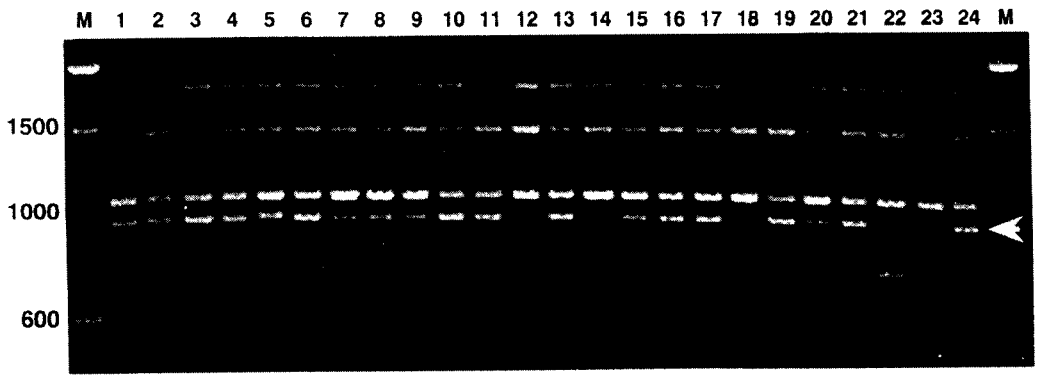

Figure 2 A polymorphic RAPD fragment (M20) among 24 plus trees in primer OPM-20. A discrimination marker (M20) is indicated by an arrow. M represents a size marker

Evaluation of the discriminatory power of 13 RAPD markers

Since a RAPD fragment is judged as either present (1) or absent (0), individual trees can be classified into two DNA types using a RAPD fragment. Assuming that the segregation in each fragment is at the same frequency [present (1) : absent (0) = 1 : 1], the discriminable DNA types combined with n fragments are calculated as 2^n. However, when the frequency of a fragment is distorted from the 1:1 segregation, the discriminatory power of the markers decreases (Tessier *et al.* 1999). The frequencies of the 13 RAPD markers were estimated with the 1/0 data of the 24 plus trees (Table 2). Among the 13 RAPD markers, the frequency of present (1) ranged from 0.17 to 0.88, and that of absent (0) from 0.13 to 0.83 (Table 2). The discriminatory power of the 13 markers calculated according to the formula of Tessier *et al.* (1999) ranged from 0.23 to 0.52. The maximum value of discriminatory power can be obtained in a condition of equal frequency, and for 24 samples this value was 0.52. In 8 (A03, C18, E14, E19, I18-1, I18-2, M10 and Z06) of the 13 markers, these values were ≥ 0.49 and they had high discriminatory powers.

A combination of the 13 RAPD markers made the 24 plus trees classifiable into 23 DNA types (Table 2, type A–W). The probability of the each DNA type varied from 1.61×10^{-6} to 4.90×10^{-3} and the average was 1.54×10^{-3}. Of these, type D was represented by two individuals (#4 and #6). These two individuals showed the same DNA type and were indistinguishable also in the RAPD fingerprinting using all fragment profiles of the 12 primers, though there were slight differences in the extremely weak fragments (Figure 2). Some authors have noted a high possibility

Table 2 DNA types of 24 plus trees and each level of discriminatory power derived from 13 RAPD markers

Sample No.	RAPD marker A03	C18	E14	E19	G19	I18-1	I18-2	K02	M10	M20	N04	Z05	Z06	DNA type	Probability [a]
1	1	1	0	1	1	1	1	1	0	1	1	1	0	A	1.67×10^{-4}
2	0	1	1	0	1	1	1	0	1	1	1	0	1	B	2.53×10^{-4}
3	0	0	1	1	1	0	0	0	1	1	1	1	0	C	2.28×10^{-3}
4	0	1	1	0	1	0	0	0	0	1	1	1	0	D	4.13×10^{-3}
5	1	1	0	0	0	1	0	1	0	1	1	1	1	E	5.06×10^{-5}
6	0	1	1	0	1	0	0	0	0	1	1	1	0	D	4.13×10^{-3}
7	1	1	0	0	1	0	0	0	0	1	1	1	1	F	1.76×10^{-3}
8	0	1	1	0	1	0	0	0	0	1	1	1	1	G	4.90×10^{-3}
9	0	1	0	1	1	1	1	0	0	1	1	1	1	H	1.64×10^{-3}
10	1	1	1	0	1	0	0	0	0	1	1	1	0	I	2.48×10^{-3}
11	0	0	0	1	1	0	0	0	0	1	1	1	1	J	2.70×10^{-3}
12	1	1	0	1	1	1	1	1	1	0	1	0	0	K	5.26×10^{-6}
13	1	1	0	1	0	0	1	0	1	1	1	1	1	L	1.65×10^{-4}
14	0	0	1	0	1	0	1	0	1	0	1	1	0	M	2.77×10^{-4}
15	0	1	1	1	1	0	1	0	1	1	1	1	0	N	1.94×10^{-3}
16	1	0	1	0	1	0	1	0	0	1	1	1	1	O	1.25×10^{-3}
17	0	0	1	1	1	0	0	0	0	1	1	1	0	P	3.81×10^{-3}
18	0	1	1	1	1	1	1	0	1	0	1	1	1	Q	4.30×10^{-4}
19	0	0	0	1	0	1	0	0	0	1	1	1	0	R	3.28×10^{-4}
20	1	0	0	0	1	1	0	0	0	1	0	1	1	S	1.28×10^{-4}
21	0	0	1	1	1	1	0	1	1	1	0	1	1	T	5.54×10^{-5}
22	0	0	1	1	1	0	0	0	0	0	1	0	1	U	2.37×10^{-4}
23	1	1	1	0	0	1	0	0	1	0	0	0	0	V	1.61×10^{-6}
24	0	0	1	1	1	0	0	0	0	1	1	1	1	W	4.51×10^{-3}
Frequency of Present (1)	0.38	0.58	0.63	0.54	0.83	0.42	0.38	0.17	0.38	0.79	0.88	0.83	0.54		
Absent (0)	0.63	0.42	0.38	0.46	0.17	0.58	0.63	0.83	0.63	0.21	0.13	0.17	0.46		Average
DP[b]	0.49	0.51	0.49	0.52	0.29	0.51	0.49	0.29	0.49	0.34	0.23	0.29	0.52		1.54×10^{-3}

[a]Probability was calculated using an equation based on the frequency of present or absent at each marker ; Probability = π Pn · Pn+1

[b]Estimation of discriminatory power was derived from the formula of Tessier *et al.* (1999)

that two individuals identical according to numerous markers were clones (Keil & Griffin 1994, Wilhelmina & McNicol 1995, Goto *et al.* 1999). Tessier *et al.* (1999) reported that confusion like this might happen between varieties from the same origin or between varieties from diverse geographical origins. In Indonesia, especially Java Island, teak plantation projects have been progressing steadily. We suspect that the two plus trees of type D were the same clone or descendants from an identical line planted in separate areas.

The reproducibility (Keil & Griffin 1994, Wilhelmina & McNicol 1995, Goto *et al.* 1997) and discriminatory power (Tessier *et al.* 1999) of markers are the most important factors in the application of RAPD analysis as a fingerprinting technique. In this study, we avoided utilising RAPD analysis as a fingerprinting technique and tried to establish a reliable discrimination method in which only highly reproducible RAPD fragments were used. More than half of the 13 highly reproducible markers showed high powers of discrimination. These markers contributed to the discrimination of teak plus trees.

Tessier *et al.* (1999) emphasised the necessity of fundamental information on the genetic diversity of population in order to efficiently develop markers with high discriminatory power. Kertadikara and Prat (1995) had reported that teak possesses high genetic diversity, while Kjaer *et al.* (1996) reported low diversity. The genetic diversity of teak has not been definitely estimated yet. The reproducible RAPD markers in this study can be used to estimate the genetic diversity of this species.

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