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Inter- and intraspecific variation of chloroplast mini- and microsatellites DNA in the four closed related *Acacia* species

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Abstract

Mini- and microsatellites of four *Acacia* species, *A. aulacocarpa*, *A. auriculiformis*, *A. crassicarpa* and *A. mangium* were investigated on four non-coding regions of cpDNA, the intron of *trn*L, and the intergenic spacers of *trn*L - *trn*P, *trn*D - *trn*Y, and *trn*P – *trn*W. Nine single base substitutions and six informative mini- and microsatellites were detected in the the four cpDNA non-coding regions. Based on the substitutions and mini- and microsatellites, ten cpDNA haplotypes (A - J) could be distinguished. *Acacia auriculiformis* possessed five haplotypes, *A. aulacocarpa*, four haplotypes, and *A. crassicarpa*, three haplotypes. All samples of *A. mangium* possessed the same haplotype. Mini- and microsatellites recognized in this study can be used for species identification of the four *Acacia* species. The ten haplotypes could divided the four species into 2 groups, *A. aulacocarpa-A.crassicarpa* group and *A. auriculiformis-A. mangium* group. By developing the PCR-based markers based on the sequence information, many experiments can be carried out for the *Acacia* improvement programs.

Keywords: *Acacia*, microsatellite, variation, chloroplast DNA

Introduction

For this decade, microsatellite, also called simple sequence repeats (SSRs), has become a popular tool for investigating genetic variations (Filizi and Koc, 2012), genetic map (Butcher and Moran, 2000), and paternity diagnosis (Cato and Richardson, 1996). This popularity stems from its high informativeness, the codominance of alleles, unequivocalness and abundance in the genomes of almost all eukaryotes. SSR markers are assayed using polymerase chain reaction (PCR), which means that genotype data of numerous loci can be obtained relative quickly from small quantity of plant tissue.

Recently, genetic markers derived from organelle genomes are employed because of their simple and uniparental modes of inheritance. This makes organelle markers inevitable for population genetic and phylogenetical studies. Genetic markers based upon simple sequence repeats in chloroplast genomes (cpSSRs) have been shown to be high useful markers for the study of germplasm analysis in several plant species, such as Solanaceous (Bryan *et al*., 1999), pine (Powell *et al*., 1995), soybean (Xu *et al.,* 2002) and Olea (Filizi and Koc, 2012). Since chloroplast DNA (cpDNA) shows uniparental inheritance, cpDNA markers are used for the seed orchard management of Douglas-fir (Stoehr *et al*., 1998), lodgepole pine (Stoehr and Newton, 2002) and *Pinus pinaster* (Fernandes *et al*., 2008). Chloroplast SSRs are analogous to those in the nuclear genome, except that they are characteristically composed of mononucleotide repeats rather than di-, tri- or tetra-nucleotide repeats. Owing to the haploid nature and large numbers of the chloroplast genome copies, these markers

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are particularly easy to be analyzed by using PCR and electrophoresis.

Non-coding region has been used for elucidating phylogenetic relationship of different taxa (Olmstead and Palmer, 1994) and sequence variation (Ohsako and Ohnishi, 2000; Fujii *et al*., 1997, 1999; Dumolin-Lapègue *et al*., 1997). Compared with coding regions, non-coding regions may provide more informative characters in phylogenetic studies at species level because of their high variability due to the lack of functional constrains. Provan *et al.* (1996) and Bryan *et al.* (1999) found more cpSSR markers in non-coding regions compared with coding regions.

Acacia (Mimosoidea) has been introduced as multiple-purpose species for plantation in tropical and subtropical areas around the world, and becomes one of the most important species for a pulp production. Balodis and Clark (1988) reported that by the year 2000, tropical *Acacia* plantations in Southeast Asia and China might be more than one million hectares. Genus *Acacia* is a large genus of woody plant (more than 1200 species), and is occurring naturally in all continents except for Eurasia. About 650 species occur in Australia. *Acacia mangium* is one of the most important Australian *Acacia* species and has the largest plantations compared with the other *Acacia* species. Since a very low genetic variation has been reported for *A. mangium* (Moran *et al*., 1989a; Khasa *et al*., 1993, 1994), it is necessary to introduce other *Acacia* species especially for breeding program of *Acacia*. The genetic diversity of *A. mangium* has also been studied using RFLP (restriction fragment length polymorphism) markers (Butcher *et al*., 1998). *Acacia aulacocarpa, A. auriculiformis*, and *A. crassicarpa* are other important *Acacia* species that have a close relationship with *A. mangium*, and show a good property for pulp and paper production.

Aside from molecular data, there is few current knowledge of the phylogenetic relationship and genetic variation of the four *Acacia* species. Investigation of genetic variations and relationship of the four species were reported by Widyatmoko and Shiraishi (2010). Based on RAPD and SSCP analysis, the four species were grouped into two major strains, *A. aulacocarpa - A. crassicarpa* and *A. auriculiformis - A. mangium* groups. This result was also supported by the data of nucleotide sequencing of four cpDNA genes (Widyatmoko and Shiraishi, 2011). No sequence variation was found between the two species in the latter group. Among them, *A. aulacocarpa* possessed the highest genetic variation compared with the other three species. A very low genetic variability was revealed in *A. mangium*. Although interspecific relationship of these species has been clarified in previous study (Widyatmoko and Shiraishi, 2010 and 2011), the details on their intra- and interspecific relationships still are unsolved.

In the present study, we investigated mini- and microsatellites on four non-coding regions of cpDNA, the intron of *trn*L, and the intergenic spacers of *trn*L - *trn*P, *trn*D - *trn*Y, and *trn*P - *trn*W. The variation of mini- and microsatellite sequences in the four non-coding cpDNA regions, which provides sufficient information to clarify the intra- and interspecific variation of the four *Acacia* species, are described in this study.

Materials and methods

Seeds from different seed lots, which represent the natural distribution of each species, were used in order to investigate mini- and microsatellite variations of the four *Acacia* species (Figure 1). All the materials used in this study were obtained from the Australian Tree Seed Centre of CSIRO (Commonwealth Scientific and Industrial Research Organization), Australia and Research and Development Center for Biotechnology and Forest Tree Improvement, Indonesia. The detail of the materials is shown in Table 1.

Total genomic DNA was extracted from the seeds with a mortar and pestle by

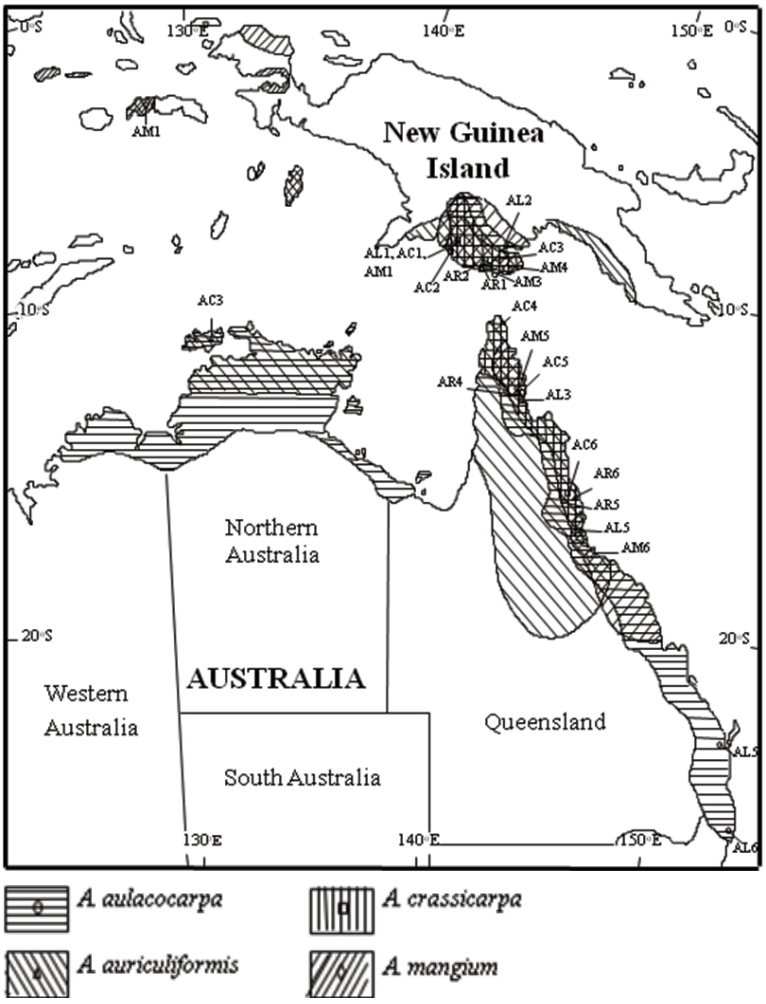

Figure 1. Samples used in this study and the natural distribution of the four *Acacia* species.

an SDS isolation method. Each seed was ground using 400 µl SDS extraction buffer, which contained 50 mM Tris-HCl (pH 9.0), 1% (w/v) SDS, 10 mM EDTA, and 0.5% (v/v) 2-Mercaptoethanol. After incubation at 65°C for 60 min, 200 µl of 7.5 M ammonium acetate was added. The solution was kept on ice for 30 min, and was then centrifuged at 0°C at 15,000 rpm for 40 min. The sample in the aqueous phase (400 µl), was transferred to a new tube, and the DNA was precipitated by the addition of 400 µl isopropanol. After circa 10 min, the precipitate was collected by centrifugation at 15,000 rpm for 10 min. The supernatant was completely removed, and the pellet was washed twice with 1.0 ml of 70% Ethanol. After the pellet was washed using a vacuum evaporator for 2 min, it was resuspended in 100 µl purified H_2O. The crude DNA was purified with GENECLEAN III (BIO 101), and the purified DNA was utilized as a template for PCR.

For PCR amplification of the four non-coding cpDNA regions (the *trn*L intron, the intergenic spacer between *trn*L - *trn*P, *trn*D - *trn*Y, and *trn*P - *trn*W), four primer pairs described in Watanabe *et al*. (1997) were used. PCR was performed in a total volume of 20 µl containing 4 ng of genomic DNA, 0.25 µM of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 200 mM of each dNTP, and 0.25 unit/10µl *Ex Taq* DNA polymerase.

Table 1. Details of samples of the four *Acacia* species

Species	No.	Seed source		Seedlot No.
Acacia aulacocarpa A. Cunn. *ex* Benth.	AL1	Kuel, Irian Jaya	NGI	AL-1001**
	AL2	Makapa WP	NGI	16947-M 0000003
	AL3	Old Rock Hart Airstrip	QLD	18358-GJM 1377
	AL4	10K NW. Mt. Molloy	QLD	17905-TREE 1
	AL5	3K S Mt. Larcom	QLD	17739-GB 000022
	AL6	Samford	QLD	17891-GB 000096
Acacia auriculiformis Cunn. *ex* Benth.	AR1	Morehead R Rouku WP	NGI	16606-BVG 01220
	AR2	Bensbach WP	NGI	17553-KN 000011
	AR3	(R) Orchard Melville INT	NT	18601-6
	AR4	Lower Poscoe River	QLD	18359-MHL 20
	AR5	Boggy Creek	QLD	17966-BH 14061
	AR6	E Normamby River	QLD	16756-BG 004936
Acacia crassicarpa Cunn. *ex* Benth.	AC1	Kuel, Irian Jaya	NGI	AC-1107**
	AC2	Wasur, Irian Jaya	NGI	AC-1001**
	AC3	Gubam Village WP	NGI	16597-BCG 01100
	AC4	Jardine River-Bamaga.	QLD	16128-JM 001440
	AC5	Claudie River	QLD	17944-MHL 04
	AC6	Parich of Annan	QLD	16775-BH 013582
Acacia mangium Willd.	AM1	Piru, Seram	Seram	570*
	AM2	Kuel, Irian Jaya	NGI	AM-1001**
	AM3	Gubam Ne Morehead WP	NGI	16991-BVG 01574
	AM4	Wipim District WP	NGI	16971-BG 01626
	AM5	Claudie River	QLD	17946-GJM 1110
	AM6	Tully-Mission Beach	QLD	17703-GLM 00920

* The seedlots were collected by Forest Tree Improvement Research and Development Institute, Yogyakarta, Indonesia (*: bulk seedlot; **: individual seedlot)
The other seedlots were collected by Australia Tree Seed Centre, CSIRO, Australia
NGI: New Guinea Island; QLD: Queensland; NT: Northern Terrritory

DNA amplification was performed with a Gene Amp PCR System Model 9600 (Perkin-Elmer) programmed as follows: 95°C for 90 s, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C, followed by 60 s at 72°C. The PCR product was separated by electrophoresis in 1.5% agarose gel and the target fractions were excised from the gel. DNA was recovered from the gel particles and was purified using QIAEX II Gel Extraction (QIAGEN). The sequence reaction was carried out using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech), the template DNA, and -21M13 (TGTAAAACGACGGCCAGT) / M13Rev (CAGGAAACAGCTATGA-CC) sequence primer 5'-labeled with Texas Red fluorescent dye (Amersham Pharmacia Biotech). The sequence was analyzed with a Hitachi SQ5500 DNA Sequencer.

Results

Sequence variations in the four chloroplast non-coding regions

Length polymorphisms were revealed in the four chloroplast non-coding regions (Tables 2 and 3). For the four *Acacia* species, the length of the *trn*L intron ranged from 578 up to 596 bps. This region was the highest length polymorphic compared with the other regions. The lengths of the *trn*L - *trn*F, *trn*D – *trn*Y, and *trn*P - *trn*W spacer regions were 456 - 459, 530 - 531, and 275 - 280 bps, respectively (Table 3). The length variations of the regions were caused by SSRs and indels (insert/deletion). Compared with another

Table 2. Substitution and insertion/deletion in the four intergenic spacer regions of chloroplast DNA

Samples	*trn*L intron	*trn*L - *trn*F spacer	*trn*D - *trn*Y spacer	*trn*P - *trn*W spacer	haplotype
	1111 11111111 1 2 33333333333333	1	2222222 3 3	1 11111 1 2	
	1112 44555555 8 3 77777778888888	444444555555 6 8	5666666 4 8	1 22222 4 3	
	7890 89012345 1 2 34567890123456	456789012345 5 3	9012345 8 9	6 23456 7 4	
AL1 (NGI)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTT- A C	AAAAAAA A T	G ----- A A	A
AL2 (NGI)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTT- A C	AAAAAAA A T	G ----- A A	A
AL3 (QLD)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTT- A C	AAAAAAA A T	G ----- A A	A
AL4 (QLD)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTTT A C	AAAAAAA A T	G ----- A A	B
AL5 (QLD)	AA-- AAAAAA-- G T --------------	TTTTTTTTTTT- A C	AAAAAAA A T	G ----- A A	C
AL6 (QLD)	AA-- AAAAAA-- A C --------------	TTTTTTTTTTT- A C	AAAAAAA A G	T AAATA A A	D
AR1 (NGI)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTT--- A A	AAAAAA- A T	G ----- A C	F
AR2 (NGI)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTT-- G A	AAAAAA- A T	G ----- A C	G
AR3 (NT)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTT-- A A	AAAAAA- A T	G ----- C C	H
AR4 (QLD)	AAAA AAAAAAA- G C TTATTTTAATATTT	TTTTTTTTT--- A A	AAAAAA- G T	G ----- A C	I
AR5 (QLD)	AAAA AAAAAAAA G C --------------	TTTTTTTTT-- A A	AAAAAA- A T	G ----- A C	J
AR6 (QLD)	AAAA AAAAAAAA G C --------------	TTTTTTTTT-- A A	AAAAAA- A T	G ----- A C	J
AC1 (NGI)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTTT A C	AAAAAAA A T	G ----- A A	B
AC2 (NGI)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTTT A C	AAAAAAA A T	G ----- A A	B
AC3 (NGI)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTTT A C	AAAAAAA A T	G ----- A A	B
AC4 (QLD)	AA-- AAAAAA-- G C --------------	TTTTTTTTTT-- A C	AAAAAAA A T	G ----- A A	E
AC5 (QLD)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTT- A C	AAAAAAA A T	G ----- A A	A
AC6 (QLD)	AA-- AAAAAA-- G C --------------	TTTTTTTTTTT- A C	AAAAAAA A T	G ----- A A	A
AM1 (Seram)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTTT-- A A	AAAAAA- A T	G ----- C C	H
AM2 (NGI)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTTT-- A A	AAAAAA- A T	G ----- C C	H
AM3 (NGI)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTTT-- A A	AAAAAA- A T	G ----- C C	H
AM4 (NGI)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTTT-- A A	AAAAAA- A T	G ----- C C	H
AM5 (QLD)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTTT-- A A	AAAAAA- A T	G ----- C C	H
AM6 (QLD)	AAAA AAAAAAAA G C TTATTTTAATATTT	TTTTTTTTTT-- A A	AAAAAA- A T	G ----- C C	H

Table 3. Number of substitution and insertion/deletion within the four *Acacia* species in four intergenic spacer regions of chloroplast DNA

Region	Length	Number of substitution					Number of insertion/deletion*				
		among species	within species				among species	within species			
			AL	AR	AC	AM		AL	AR	AC	AM
*trn*L intron	578-596	2	2	0	0	0	5	0	2	0	0
*trn*L - *trn*F	456-459	2	0	1	0	0	3	1	1	2	0
*trn*D - *trn*Y	530-531	2	1	1	0	0	1	0	0	0	0
*trn*P - *trn*W	275-280	3	1	1	0	0	1	1	0	0	0
Total		9	4	3	0	0	10	2	3	2	0

* The number of insertion/deletion for simple sequence repeat (SSR), 1 base is counted as 1 indel (see Table 2 for details)
AL: *A. aulacocarpa*; AR: *A. auriculiformis*; AC: *A. crassicarpa*; AM: *A. mangium*

Acacia sp. (Murphy *et al*., 2000), the lengths of the *trn*L intron and the *trn*L - *trn*F spacer regions of the four *Acacia* species used in this study were longer. These spacer regions were also longer than those in soybean (Xu *et al*., 2000).

Intra- and interspecies variations among the four Acacia species

Nine single base substitutions were detected in the four cpDNA non-coding regions (Tables 2 and 3). These substitutions included four transitions and five transversions. A numerous numbers of transversions were also detected in soybean chloroplast genome (Xu *et al*., 2000).

Of the nine substitutions detected in the four non-coding regions, two substitutions detected in the *trn*D - *trn*Y and *trn*P - *trn*W were interspesific, and were A/C transversions (Table 3). In the transversion at the 183rd position of the *trn*L - *trn*F spacer region, *A. aulacocarpa* and *A. crassicarpa* showed C-base, and *A. auriculiformis* and *A. mangium*, A-base. Also at the 234th position of the *trn*P - *trn*W, *A. aulacocarpa* and *A. crassicarpa*, showed A-base, and *A. auriculiformis* and *A. mangium*, C-base. Based on these substitutions, *A. aulacocarpa* and *A. crassicarpa* could be distinguished from *A. auriculiformis* and *A. mangium.*

As intraspecific variations, seven substitutions were revealed in the four non-coding regions, and they were recognized only in *A. aulacocarpa* and *A. auriculiformis*. No intraspesific substitutions were in *A. crassicarpa* and *A. mangium* (Table 3). In the *trn*L intron, only *A. aulacocarpa* possessed variations within species. Otherwise, in the *trn*L - *trn*F, only *A. auriculiformis* showed an intraspecific variation. In the *trn*D - *trn*Y and *trn*P – *trn*W regions, *A. aulacocarpa* and *A. auriculiformis* showed within-species polymorphisms.

Six informative mini- and microsatellites were detected in the four non-coding regions (Table 2). In the *trn*L intron, two single-base SSRs of A and a 14-base repeat (TTATTTTAATATTT) were detected. In the *trn*L - *trn*F and the *trn*D - *trn*Y, one single-base SSR of T and A was detected, respectively. In *trn*P - *trn*W, a 5-base repeat (AAATA) was detected. Three of the four single-base SSRs revealed in this study can be used for distinguishing species. Two SSRs were detected in the *trn*L intron, and one SSR was found in the *trn*D - *trn*Y. A single-base repeat that consisted of two to four A-bases was detected at the 117 - 120th position of the *trn*L intron region. *Acacia aulacocarpa* and *A. crassicarpa* showed two A-bases. On the other hand, *A. auriculiformis* and *A. mangium* showed four A-bases. Also at 148 - 155th position, another A-repeat SSR was revealed. *A. aulacocarpa* and *A. crassicarpa* showed six A-bases, and *A. mangium*, eight A-bases. Five samples of *A. auriculiformis* showed eight A-bases, and only one sample showed seven A-bases. In the *trn*D - *trn*Y, an informative A-repeat was detected at the 259 - 265th position. *A. aulacocarpa* and

Table 4. Number of cpDNA haplotype in the four *Acacia* sp

Species	Number of haplotype	Haplotype frequencies*									
		A	B	C	D	E	F	G	H	I	J
A. aulacocarpa	4	3	1	1	1	0	0	0	0	0	0
A. auriculiformis	5	0	0	0	0	0	1	1	1	1	2
A. crassicarpa	3	2	3	0	0	1	0	0	0	0	0
A. mangium	1	0	0	0	0	0	0	0	6	0	0
Total		5	4	1	1	1	1	1	7	1	2

* See Table 2 for details of sequences of each haplotype

A. crassicarpa showed six A-bases, and *A. auriculiformis* and *A. mangium* showed seven A-bases. Based on these single-base SSRs, the four *Acacia* species could be grouped into two strains, *A. aulacocarpa - A. crassicarpa* and *A. auriculiformis - A. mangium*. This supports the result mentioned in Widyatmoko and Shiraishi (2010).

Of these three SSRs and one minisatellite recognized in the four *Acacia* species, a variation within species was detected only in the *trn*L intron (148 - 155th position) of *A. auriculiformis* (Tables 2). In addition to the intraspecific variation, SSR variation of the *trn*P - *trn*W (122nd - 126th position) was detected only in *A. aulacocarpa*. Moreover, a single-base repeat of T appeared in the *trn*L - *trn*F (44 - 55th position) was polymorphic in the three *Acacia* species except for *A. mangium*. *Acacia aulacocarpa* and *A. auriculiformis* included two variations, and *A. crassicarpa*, three variations at this position.

Distribution of cpDNA haplotypes

Ten cpDNA haplotypes (A - J) could be distinguished based on base substitutions and mini- and microsatellites detected in the four non-coding regions of cpDNA (Table 4). *Acacia auriculiformis* possessed five haplotypes (haplotypes F - J), *A. aulacocarpa*, four haplotypes (haplotypes A - D), and *A. crassicarpa*, three haplotypes (haplotypes A, B and E), respectively. All samples of *A. mangium* possessed the same haplotype (haplotype H).

Of the ten cpDNA haplotypes, five haplotypes were recognized in *A. aulacocarpa* and *A. crassicarpa*, while, remaining five haplotypes in *A. auriculiformis* and *A. mangium*. Of three haplotypes detected in *A. crassicarpa*, two haplotypes except for haplotype E were recognized also in *A. aulacocarpa*. The haplotype appeared in *A. mangium* was also detected in one sample of Northern Territory (NT) in *A. auriculiformis*. In this study, six samples of *A. aulacocarpa* can be separated into three groups, New Guinea Island (NGI) (haplotype A), North Queensland (NQ) (haplotypes A and B), and South Queensland (SQ) (haplotypes C and D). For *A. crassicarpa*, NGI and Queensland (QLD) samples showed different haplotypes: haplotype B for NGI, and haplotypes A and E for QLD. Geographical variations of cpDNA haplotypes were also revealed in *A. auriculiformis*. Six samples from three different areas showed regionally specific haplotypes, haplotypes F and G for NGI, haplotype H for NT, and haplotypes I and J for QLD.

Discussion

Variations of the four non-coding regions of cpDNA in the four Acacia species

In this study, inter- and intraspecific polymorphism was shown in the four non-coding regions of cpDNA. These polymorphisms were higher than those in the four genes of chloroplast genome (Widyatmoko and Shiraishi, 2011). In the four chloroplast genes, a sole polymorphism between the two groups ((*A. aulacocarpa - A. crassicarpa* (group I) and *A. auriculiformis - A. mangium* (group II)) was revealed, and no

polymorphism was recognized between the species of the same group. The sequence of the non-coding regions of cpDNA demonstrated that group I was distinguished from group II. There were two A/C substitutions and two indels between the two groups. Xu *et al*. (2000) distinguished three types of soybean based on sequences of non-coding regions of cpDNA. These results suggested that two *Acacia* species that belong to the same group were closely related.

In the three species except for *A. mangium*, intraspecific polymorphisms were detected in the four non-coding regions. Of the four *Acacia* species, only *A. mangium* showed no polymorphism in the six regions. *Acacia aulacocarpa* and *A. auriculiformis* showed intraspecific polymorphism. Otherwise, *A. crassicarpa* showed polymorphism only in the intergenic spacer region between *trn*L - *trn*F. This supports the result in Widyatmoko and Shiraishi (2010) that *A. aulacocarpa* and *A. auriculiformis* possessed higher genetic variation compared with the other two species.

Mini- and microsatellites of cpDNA in the four Acacia species

Recently, there has been reported the presence of minisatellite sequence of chloroplast genome in several plant species. Blasko *et al*. (1988) reported that repeated sequence of *Oenothera* was recognized in an unsigned open reading frame of the inverted region. In the two European *Alnus* species (King and Ferris, 2000) and Italian populations of *Orchis palustris* (Cafasso *et al*., 2001), minisatellites were found in an intergenic spacer and an intron, respectively. Isoda *et al*. (2000) reported a tandem repeat in *Abies*. Filizi and Koc (2012) reported that all types of repeat motif of SSR (mono-, di-, tri-, tetra-and pentanucleotide) were detected in genus *Olea* except hexanucleotide motifs. In this study, minisatellites were revealed in the three non-coding regions.

The four non-coding regions of cpDNA used in this study revealed a mini- and four microsatellites. The *trn*L intron possessed two minisatellites and a microsatellite, and the other two regions had only microsatellite, respectively. Most of the SSRs were single-base repeats. Mini- and microsatellites were also recognized in cpDNA of Douglas-fir (Hipkins *et al*., 1995). Powell *et al*. (1995) demonstrated that chloroplast microsatellites are predominantly mononucleotide repeats.

Mini- and microsatellites revealed in this study could be used for identifying species. The minisatellite in the *trn*L intron could distinguish *A. aulacocarpa* and *A. crassicarpa* from *A. mangium*. However, since *A. auriculiformis* possessed the polymorphism within species, the minisatellite could not be used for distinguishing *A. auriculiformis* from the other species. The two microsatellites could distinguish the two groups of *Acacia* species. Thus, mini- and microsatellites recognized in this study can be used for species identification of the four *Acacia* species. Morand-Prieur *et al*. (2002) reported that *Fraxinus excelsior* could be distinguished from *F. angustifolia* by cpDNA microsatellite.

Distribution of cpDNA haplotypes in the four Acacia species

Ten cpDNA haplotypes based on the substitution and indel (mini- and microsatellite) were recognized. Half of them (A - E) were observed in *A. aulacocarpa* and *A. crassicarpa*, and the other half in *A. auriculiformis* and *A. mangium*. These two groups had totally different cpDNA haplotypes.

Of the four species, only *A. mangium* revealed no haplotype variation within species. The other three species showed a geographically different distribution of cpDNA haplotypes. By the haplotype distribution, *A. aulacocarpa* and *A. auriculiformis* show the similar geographic separation as has been reported previously. Thomson (1994) and McGranahan *et al*. (1997) distinguished *A. aulacocarpa* into five distinct groups, that is, New Guinea, Cairns, Northern Territory/

Western Australia, North Queensland and South Queensland/North New South Wales. In this study, the six samples were distinguished into three groups, New Guinea, North Queensland and South Queensland. No samples from Northern Territory and Cairns were used in this study. In case of *A. auriculiformis*, the six samples were separated into three areas, New Guinea, Northern Territory and Queensland. This result is similar to Wickneswary and Norwati (1993). The genetical separation of *A. crassicarpa* has not been reported. However, this study showed that New Guinea and Queensland could be separated based on the sequence of four non-coding regions of cpDNA.

Application of the result for the breeding program and the future study

Many experiments using cpDNA markers have been reported. Microsatellite markers have become popular for many genetic studies because they are highly polymorphic in many species. Chloroplast microsatellite has been used for analyzing genetic diversity in genus *Olea* (Filizi and Koc, 2012), maritime pine (Ribeiro *et al*., 2001), Solanaceous plants (Bryan *et al*., 1999), *Pinus radiata* (Cato and Richardson, 1996), soybean (Xu *et al*., 2002) and *Sorbus* L. (King and Ferris, 2000). Studies of the inheritance using chloroplast microsatellite also have been reported in *Fraxinus* (Morand-Prieur *et al*., 2002), *Abies alba* (Ziegenhagen *et al*., 1998) and *Helianthus annuus* (Wills *et al*., 2005). Stoehr *et al*. (1998) and Fernandes *et al*. (2008) applied cpDNA marker to the seed orchard management.

In this study, base substitutions, mini- and microsatellites have been recognized in non-coding regions of cpDNA. This information can be used to develop the cpDNA markers for the *Acacia* species. Bukhari *et al*. (1999) reported phylogenetic relationships of several *Acacia* species based on the chloroplast RFLP data. Although chloroplast RFLPs are more variable than most of the phenotypic characteristics, RFLPs are time- and cost-consuming, and need a large quantity of DNA. Genetic analysis using the polymerase chain reaction (PCR) is less time consuming, technically simple and needs small amount of DNA. Microsatellites have become the available markers for many genetic studies because they are easy to analyze by using PCR and polyacrylamide gel electrophoresis (Powel *et al*., 1995). By developing the PCR-based markers based on the sequence information, many experiments can be carried out for the *Acacia* improvement programs.

Conclusion

Many information could be obtained by developing the PCR-based markers based on the sequence information. Not only substitutions, but also mini- and microsatellite could be detected. This information can be used for carrying out another experiment for conservation and tree improvement programs. In this study, for example, substutitions and mini-and microsatellite detected in four non-coding regions of chloroplast DNA can distinguished ten haplotypes. The haplotypes can be used for identifying 2 groups of four *Acacia* species. In future, many experiment using the substitutions, mini- and microsatellite and haplotypes can be carried out to support conservation and breeding programs of the *Acacia* species.

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