

# SEQUENCE POLYMORPHISMS OF FOUR CHLOROPLAST GENES IN FOUR ACACIA SPECIES

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## ABSTRACT

Sequence polymorphisms among and within four *Acacia* species, *A. aulacocarpa*, *A. auriculiformis*, *A. crassicarpa*, and *A. mangium*, were investigated using four chloroplast DNA genes (*atpA*, *petA*, *rbcL*, and *rpoA*). The phylogenetic relationship among these species is discussed in light of the results of the sequence information. No intraspecific sequence variation was found in the four genes of the four species, and a conservative rate of mutation of the chloroplast DNA genes was also confirmed in the *Acacia* species. In the *atpA* and *petA* of the four genes, all four species possessed identical sequences, and no sequence variation was found among the four *Acacia* species. In the *rbcL* and *rpoA* genes, however, sequence polymorphisms were revealed among these species. *Acacia aulacocarpa* and *A. crassicarpa* shared an identical sequence, and *A. auriculiformis* and *A. mangium* also showed no sequence variation. The fact that *A. mangium* and *A. auriculiformis* shared identical sequences as did *A. aulacocarpa* and *A. crassicarpa* indicated that the two respective species were extremely closely related. Although a putative natural hybrid of *A. aulacocarpa* and *A. auriculiformis* has been reported, our results suggested that natural hybridization should be further verified using molecular markers.

Keywords: *Acacia*, sequencing, chloroplast DNA, phylogeny

## I. INTRODUCTION

There are more than one thousand documented species of *Acacia*, of which about 650 species occur in Australia. *Acacia auriculiformis*, *A. mangium*, *A. aulacocarpa*, and *A. crassicarpa* are four of only nine Australian *Acacia* species, whose distributions extend northward into Papua New Guinea and Indonesia (Moran *et al.*, 1989). The four species are multiple-purpose plantation species, and in the last decade, they have become a major plantation species used for pulp production in Southeast Asia. *Acacia* species have been introduced in commercial plantations in Southeast Asia. The total area of tree plantations is now approaching two million ha and the largest of these plantations (about 1.2 million ha) is located in Indonesia, where the major planted species is fast-growing *Acacia mangium* Wild. (Arisman and Hardiyanto, 2006; Potter *et al.*, 2006). In industrial pulpwood plantations, these four *Acacia* species are newcomers compared

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to *Eucalyptus*. However, these species are suitable for kraft pulp production based on criteria such as basic density, bleaching properties, and pulp quality.

The genetic relationship among the four species is important for evolutionary study. In breeding programs, this information is useful for predicting hybridization activities. Until now there has been little discussion of the relationship among these species (Pettigrew and Watson, 1975; Boland *et al.*, 1990; Brain and Maslin, 1996). Moreover, the classification of the four species is not well elucidated. Some studies have been conducted in order to investigate the relationship among the four species (Widyatmoko *et al.*, 2010); however, no phylogenetic analysis among the four species has been reported using the sequence information of chloroplast DNA (cpDNA) genes. Phylogenetic relationship among *Acacia* species have been reported by Clarke *et al.* (2000), Byrne *et al.* (2001; 2002) and Brown *et al.* (2008). Both of the research were using chloroplast DNA. *Acacia* species which have been used for phylogenetic relationship study revealed the significant association between phylogenetic position of many haplotypes and their geographical distribution. Hamrick *et al.* (1992) reported the effect of pollination to genetic diversity of species. Long-lived, outcrossing and wind-pollinated species has higher levels of allozyme diversity within population and less among population.

DNA sequences of cpDNA genes have been utilized for estimating the phylogeny of many taxa of plants. In particular, the chloroplast gene (*rbcL*) that encodes a large subunit of the enzyme ribulose-1,5-biphosphate carboxylase has been used to elucidate the relationships of Betulaceae (Chen *et al.*, 1999), Rutaceae (Chase *et al.*, 1999), *Salix* (Azuma *et al.*, 2000) and *Solanum* (Bohs, 2004). Intraspecific sequence polymorphism of cpDNA has also been investigated (Fujii *et al.*, 1999; Amane *et al.*, 2000; Zimmer *et al.*, 2002).

In this study, sequence polymorphisms among the four *Acacia* species were investigated using four cpDNA genes (*atpA*, *petA*, *rbcL*, and *rpoA*). Furthermore, a phylogenetic relationship among these species was discovered and is discussed with the results of the sequence polymorphism.

## II. MATERIALS AND METHODS

Plant materials for the study were obtained from the Australian Tree Seed Centre of CSIRO (Commonwealth Scientific and Industrial Research Organization), Australia, and from the Forest Tree Improvement Research and Development Institute, Indonesia. For each species, four seedlots (represented by one seed) were used for sequencing. Details of each sample are shown in Table 1.

Total genomic DNA was extracted from the seeds with a mortar and pestle by an SDS isolation. Each seed was ground using 400  $\mu$ l 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  $\mu$ l 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<sub>2</sub>O. Finally, the crude solution was purified using a GeneClean III Kit (BIO 101), and the purified DNA was utilized as a template for PCR.

Table 1. List of sample materials for the four cpDNA genes

Species	Seed source	
	Seedlot No.	Location*
<i>A. aulacocarpa</i>	16946-AK 000012**	Balimo District, PNG
	13866-BH 012313**	Garioch, QLD
	17905-TREE**	10K NW Mt. Molloy, QLD
	17739-BG 000022**	3K S Mt. Larcom, QLD
<i>A. auriculiformis</i>	16606-BVG 01220**	Morehead R Rouku WP, PNG
	18359-MHL 20**	Lower Poscoe River, QLD
	16756-BG 004936**	E Normamby River, QLD
	18601-6**	(R) Orchard Melville Is., NT
<i>A. crassicarpa</i>	AC-1107**	Kuel, Irian Jaya, IND
	13680-JC 001503**	Wemenever Prov., PNG
	17944-MHL 04**	Claudie River, QLD
	16775-BH 013582**	Parish of Annan, QLD
<i>A. mangium</i>	570***	Piru, Seram, IND
	16971-BVG 01626**	Wipim District WP, PNG
	17946-GJM 1110**	Claudie River, QLD
	17703-GLM 00920**	Tully-Mission Beach, QLD

Notes: \* PNG, Papua New Guinea; QLD, Queensland, Australia; NT, Northern Territory, Australia; IND, Indonesia

\*\* Seedlot No. of CSIRO, Australia

\*\*\* Seedlot No. of FTIRDI, Indonesia

Seven pairs of PCR primers shown in Table 2 (Shiraishi *et al.*, unpublished) were used for amplifying four genes. 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<sub>2</sub>, 200 mM of each dNTP, and 0.25 unit/10µl *Ex Taq* DNA polymerase. 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 (QUIAGEN). The sequence reaction was carried out using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech), the template DNA, and -21M13 (TGTA AACGACGGCCAGT) / M13Rev (CAGGAAACAGCTATGACC) sequence primer 5'-labeled with Texas Red fluorescent dye (Amersham Pharmacia Biotech). The sequence was analyzed with a Hitachi SQ5500 DNA Sequencer.

Table 2. Primer sequences for amplifying the four cpDNA genes

Gene	Zone	Primer	Sequences (5' → 3')
<i>atpA</i>	a	FO-021	(TGTA AACGACGGCCAGT)GAGGCTTATTTGGGTCGTGT
		FO-124	(CAGGAAACAGCTATGACC)GAGA ACTTGATTTAGCGGCTC
	b	FO-122	(TGTA AACGACGGCCAGT)GCTTATCGCCAAATGTCTCTT
		FO-026	(CAGGAAACAGCTATGACC)ATATGATTTCTTGAACTGAGG
<i>petA</i>		FO-055	(TGTA AACGACGGCCAGT)CTTCCCGATACCGTATTT-GAAGCA
		FO-056	(CAGGAAACAGCTATGACC)TCTGCATCTCCTTGACCAAATCC
<i>rbcL</i>	a	FO-001	(TGTA AACGACGGCCAGT)GTCGGATTCAAAGCTGGTGT
		FO-104	(CAGGAAACAGCTATGACC)TCGCATGTACCCGCAGTAGC
	b	FO-102	(TGTA AACGACGGCCAGT)TGAGAATGTGAACTCCCAACC
		FO-006	(CAGGAAACAGCTATGACC)TCACAAGCAGCAGCTAGTTC
<i>rpoA</i>	a	FO-038	(TGTA AACGACGGCCAGT)GACCTTTTGAGGCAATTATACATCC
		FO-040	(CAGGAAAGAGCTATGACC)CCAAATAACTCTCAAGACGGAA
	b	FO-032	(TGTA AACGACGGCCAGT)CGAACAGGCATGAATACAGC
		FO-034	(CAGGAAACAGCTATGACC)TGGAAGTGTGTTGAATCAAG

Remarks: - upper primer was forward primers, below primers was reverse primers  
 - The sequence in parentheses represents the M13/M13Rev universal primer

Raw data of sequences was analyzed using Sequencer 4.7 (Gene Codes Corporation). Both forward and reverse sequences of each samples of each region were assembled automatically using the program. Chromatogram of both sequences was used when incompatibility was found in order to decide the correct sequence of each sample for each region. Finally, all samples for each region were assembled automatically in order to recognize insertion-deletion and base substitution between the samples.

### III. RESULTS AND DISCUSSION

#### A. Intraspecific Variation of the Four Chloroplast Genes

In order to investigate sequence variations within species, four samples from four separate populations were used in each species (Table 1). Of the four *Acacia* species, *A. aulacocarpa* was thought to have the largest genetic diversity (Widyatmoko *et al.*, 2010). The samples of this species from New Guinea Island, North Queensland, and South Queensland corresponding to the different subspecies were separated morphologically by Thomson (1994). The length of *atpA*, *petA*, *rbcL*, and *rpoA* were 1084 bp, 561 bp, 1309 bp, and 782 bp, respectively, and no sequence variation was found in the four genes among the four samples. Eventhough McDonald and Maslin (2000) divided *A. aulacocarpa* into 6 species, no sequence variation was found among those species. In the remaining three species, exactly the same results were shown. A low rate of cpDNA mutation has been reported in *Acacia acuminata* complex in Western Australia (Byrne *et al.*, 2002). the time of divergence between the two main lineages within *A. acuminata* is in the order of 800,000 years ago, in the middle of the Pleistocene. Parfit and Badenness (1997) and Provan *et al.* (1999) reported a low cpDNA mutation rate for the genus *Pistacia* and *Pinus torreyana* respectively. Restriction site mapping of chloroplast DNA was chosen for phylogenetic analysis because of its ability to provide many information characters, even in comparison to DNA sequence from any particular gene (Jansen *et al.*, 1998)

#### B. Interspecific Variation Among the Four *Acacia* species

In the *atpA* and *petA* genes, no sequence variation was found among the four *Acacia* species. All four species possessed identical sequences. Sequences of the *rbcL* and *rpoA* genes are shown in Figures 1 and 2. Although no length variation among species was observed in either gene, sequence polymorphisms were revealed among these species. Within the four species, *A. aulacocarpa* and *A. crassicarpa* shared an identical sequence, and *A. auriculiformis* and *A. mangium* also showed no sequence variation.

Number of base substitutions and amino acid changes among species are shown in Table 3. In the *rbcL* sequence, six transitions and three transversions were found between the two groups mentioned above. In *rpoA*, two transitions between the two groups were identified. Five amino acid changes were caused by these substitutions between the groups. The amino acid changes were observed only in the *rbcL* gene.

An inference concerning the genetic relationship among the four *Acacia* species using RAPD analysis has been reported (Widyatmoko *et al.*, 2010). We indicated that the four species were separated into two clades: *A. auriculiformis* and *A. mangium* were grouped into one clade, and the other clade contained *A. aulacocarpa* and *A. crassicarpa* (Figure 3). In the RAPD study, moreover, genetic variations were observed within and among the species, because a RAPD marker is a more effective means of examining the

relationship among closely related species. *A. auriculiformis* and *A. mangium*, which were grouped into the same clade, were separated into different respective subclades. In the latter clade, however, which contained *A. aulacocarpa* and *A. crassicarpa*, each species could not clearly form a clade. As a result, *A. crassicarpa* is considered a subspecies of *A. aulacocarpa*. McDonald and Maslin (2000) also mentioned closed relationship between those species. A result similar to this has also been reported by Thomson (1994) whose study was based on morphological observations.

The present study clarified a phylogenetic relationship among the four species. The four *Acacia* species were classified into two groups. One group contained *A. auriculiformis* and *A. mangium*, and the other consisted of the remaining two species. Within each group, two species had exactly the same sequences in the four cpDNA genes. Between the two groups, 11 substitutions were found in the *rbcL* and *rpoA* genes. Putative natural hybrids of *A. aulacocarpa* and *A. auriculiformis* have been found (Thomson, 1994). However, our results suggested that *A. aulacocarpa* and *A. auriculiformis* were distantly related. Therefore, natural hybridization between these two species might be further verified using molecular markers.

Interspecific variation among *Acacia* species have been reported by and Clarke *et al.* (2000) and Byrne *et al.* (2002). Byrne *et al.* (2002) reported a significant association between phylogenetic position of many haplotypes and their geographical distribution. The cpDNA analysis clearly identified *A. oldfieldii* as distinct from the rest of the *A. acuminata* complex. Clarke *et al.* (2000) reported the phylogenetic relationship between 4 *Acacia* sub genus *Acacia* in Caribbean, Africa, South America and North America. A group of Carribean species was found to be ancestral in *Acacia* subgenus *Acacia*, and African and South American species were found to relatively derive with respect to North American species.

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1      TAAAGATTAT AAATTGACTT ATTATACTCC TGACTATGAA ACCAAAGATG GTGATATCTT 60
.....
.....A.....

61     GGCAGCATTC CGAGTAACTC CTCAACAGTT CCGAATCTGG GAAGAAGCAG GTGCCGCGGT 120
.....

121    AGCTGCTGAA TCTTCTACTG GTACATGGAC AACTGTGTGG ACCGATGGGC TTACCAGTGT 180
.....

181    GATCGTTACA AAGGACGATT GCTACCACAT CGAGTCCGTT GCTGGAGAAG AAAATCAATA 240
.....
Notes: Upper row: A. aulacocarpa-A. crassicarpa
      Under row: A. mangium-A. auriculiformis

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Figure 1. Sequences of *rbcL* of the four *Acacia* species

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TATTGCTTAT GTAGCTTATC CCTTAGACCT TTTTGAAGAA GTTTCTGTTA CTAACATGGT
.....

301
TACTTCGATT GTGGGTAATG TATTTGGGTT CAAGGCCCTG CGCGCTCTAC GTCTGGAAGA
.....

361
TTTGCGAATC CCTCCTTCTT ATTCTAAAAC TTTCCAAGGT CCGCCTCACG GCATCCAAGT
.....

421
TGAGAGAGAT AAATTGAACA AGTACGGCCG TCCCCTATTG GGATGTACTA TTAAACCAAA
.....

481
ATTGGGGTTA TCCGCGAAGA ATTACGGTAG AGCGGTTTAT GAATGTCTCC GTGGTGGACT
.....

541
TGATTTTACC AAAGATGATG AGAATGTGAA TTCCAACCA TTTATGCGTT GGAGAGACCG
.....

601
TTTCTTATTT TGTGCCGAAG CAGTTTTTAA AGCACAGGCC GAAACAGGTG AAATCAAAGG
.....GC.....

661
GCATTACTTG AATGCTACTG CAGGTACATG CGAAGAAATG ATCAAAAGAG CTGTATTTGC
.....G.....

721
CCGAGAATTA GCGGTCCTA TCGTAATGCA TGACTACTTA ACAGGGGGAT TCACTGCAAA
.....G.....AC.....

781
TCATAGCTTG GCTCATTATT GCCGAGATAA TGGTCTACTT CTTCATATCC ATCGTGCAAT
.....

841
GCATGCAGTT ATCGATAGAC ACAAGAATCA TGGTATGCAC TTTCGTGTAC TAGCTAAAGC
.....

901
GTTACGTATG TCTGGTGGAG ATCATATTCA CGCTGGTACC GTAGTAGGTA AACTTGAAGG
.....T.....

961
TGAAAAGAGAA ATCACTTTAG GTTTTGTGTT TACTACGTA GATGATTATA TTGAGAAAAGA
.....

1021
TCGAAGCCGC GGTATTTATT TCACTCAGGA TTGGGTCTCT ATGCCGGGTG TTCTGCCCTG
.....A.....

1081
CTTCGGGGGG TATTCACGGT TTTGGCATAT GCCTGCTCTT ACCGAGATCT TTGGAGATGA
.....

1141
TTCCGTACTA CAATTCGGGG GGGGAACTTT AGGCACCCT TGGGGAAATG CACCCGGTGC
.....

1201
CGTAGCTAAC CGAGTAGCTC TAGAAGCATG TGTACAGGCT CGTAATGAGG GACGTGATCT
.....

1261
TGCTCGTGAG GGTAATGAAA TTATTCGTCA GGCTAGCAAA TGGAGTCCT
.....

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Notes: Upper row: *A. aulacocarpa*-*A. crassicarpa*  
Under row: *A. mangium*-*A. auriculiformis*

Figure 1. (continued)

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661                                     720
GCATTACTTG AATGCTACTG CAGGTACATG CGAAGAAATG ATCAAAAGAG CTGTATTTGC
.....G.....

721                                     780
CCGAGAATTA GCGGTTCTTA TCGTAATGCA TGACTACTTA ACAGGGGGAT TCACTGCAAA
.....G.....AC.....

781                                     840
TCATAGCTTG GCTCATTATT GCCGAGATAA TGGTCTACTT CTTCATATCC ATCGTGCAAT
.....

841                                     900
GCATGCAGTT ATCGATAGAC ACAAGAATCA TGGTATGCAC TTTTCGTGTAC TAGCTAAAGC
.....

901                                     960
GTTACGTATG TCTGGTGGAG ATCATATTCA CGCTGGTACC GTAGTAGGTA AACTTGAAGG
.....T.....

961                                     1020
TGAAAGAGAA ATCACTTTAG GTTTTGTTGT TTACTACGTA GATGATTATA TTGAGAAAGA
.....

1021                                    1080
TCGAAGCCGC GGTATTTATT TCACTCAGGA TTGGGTCTCT ATGCCGGGTG TTCTGCCCTG
.....A.....

1081                                    1140
CTTCGGGGGG TATTCACGGT TTTGGCATAT GCCTGCTCTT ACCGAGATCT TTGGAGATGA
.....

1141                                    1200
TTCCGTACTA CAATTCGGGG GGGGAAC TTT AGGGCACCCT TGGGGAAATG CACCCGGTGC
.....

1201                                    1260
CGTAGCTAAC CGAGTAGCTC TAGAAGCATG TGTACAGGCT CGTAATGAGG GACGTGATCT
.....

1261
TGCTCGTGAG GGTAATGAAA TTATTCGTCA GGCTAGCAAA TGGAGTCCT
.....

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Notes: Upper row: *A. aulacocarpa*-*A. crassicarpa*  
 Under row: *A. mangium*-*A. auriculiformis*

Figure 2. Sequences of *rpoA* of the four *Acacia* species

Table 3. Number of substitutions and amino acid changes in the four cpDNA genes of *A. aulacocarpa*-*A. crassicarpa* and *A. auriculiformis*-*A. mangium*

Gene	Length (bp)	Substitution			Amino acid change
		Transition	Transversion	Total	
<i>atpA</i>	1084	0	0	0	0
<i>petA</i>	561	0	0	0	0
<i>rbcL</i>	1309	6	3	9	5
<i>rpoA</i>	782	2	0	2	0
Total	3746	8	3	11	5



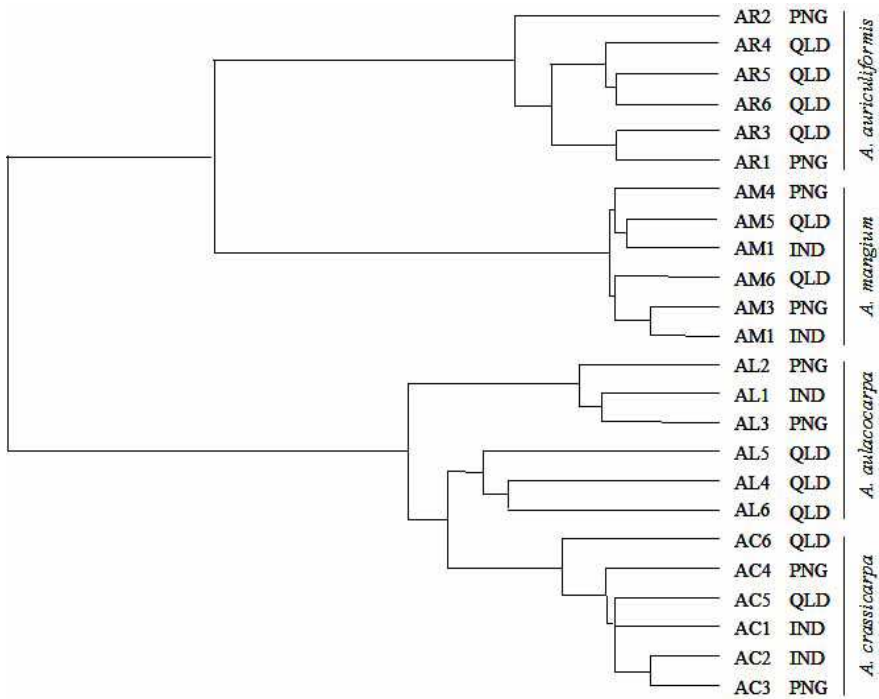


Figure 3. The relationships between four *Acacia* species (Widyatmoko *et al.*, 2010)

#### IV. CONCLUSION

Sequence polymorphisms were revealed among these species for *rbcL* and *rpoA* genes. No sequence variation was found for *Acacia aulacocarpa* and *A. crassicarpa*, and also for *A. auriculiformis* and *A. mangium*. Both groups were differentiated by 11 bases.

The phylogenetic results of this and previous studies may be useful in planning, especially in breeding programs. For *A. mangium* and *A. auriculiformis*, which are extremely closely related, it might be necessary that interspecific hybridization breeding be carried out on a larger scale in the breeding programs. A similar breeding strategy is also worth discussing for *A. aulacocarpa* and *A. crassicarpa*.

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