STUDY ON GENETIC VARIATION AND RELATIONSHIPS AMONG FOUR ACACIA SPECIES USING RAPD AND SSCP MARKER

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ABSTRACT

Genetic diversity and relationship of four *Acacia* species, *Acacia aulacocarpa,* A. Cunn. ex Benth.*, A. auriculiformis* Cunn. ex Benth.*, A. crassicarpa* Cunn. ex Benth. and *A. mangium* Willd. were investigated using DNA molecular markers. Using RAPD analysis, a total of 20 arbitrary primers successfully gave 127 polymorphic fragments. These RAPD data were used to estimate genetic distance and construct dendrograms using the unweighted pair-group with the arithmetic mean average (UPGMA) method. The four species were divided into two major clusters. *A. auriculiformis* and *A. mangium* were in one cluster, and the other cluster contained both *A. aulacocarpa* and *A. crassicarpa*. The divergence time of the two species (*A. auriculiformis* and *A. mangium*) in the former cluster appeared to be relatively early in comparison to in the latter (*A. aulacocarpa* and *A. crassicarpa*) based on RAPD data. This result was also supported by Principal Component Analysis (PCA). Among the four species, *A. aulacocarpa* showed the highest divergence in nuclear DNA (ncDNA), followed by *A. auriculiformis*. SSCP analysis also revealed that these two species possessed a haplotypic variation of the *trn*L-*trn*F intergenic spacer region of chloroplast DNA. In *A. aulacocarpa*, a large difference in the composition of both nuclear and chloroplast genomes was observed between populations distributed in Queensland, Australia and those in New Guinea Island. In contrast, compared to the other species, *A. mangium* showed the lowest genetic diversity and less genetic differentiation among populations.

Keywords*: Acacia*, genetic variation, phylogeny, RAPD, SSCP

I. INTRODUCTION

The genus *Acacia* is the largest genus of subfamily Mimosoideae, family Leguminosae. It includes over 1,200 species widely distributed in the Southern hemisphere, extending from South America and Australia to Africa (Ross, 1981; Brain and Maslin, 1996; Harrier *et al*., 1997). The most species-divergence of this genus occurs in Australia where there are approximately 650 species distributed across the continent (Pettigrew and Watson, 1975; Ross, 1981; Boughton, 1986).

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Acacia auriculiformis A. Cunn. ex Benth. and *A. mangium* Willd., which belong to section *Juliflorae* (Benth.) Maiden & Betche in the subgenus *Phyllodineae* Benth., are naturally distributed throughout Australia, New Guinea Island, Aru Island, and Sula Island. These species have been introduced as multiple-purpose species for plantation in tropical and subtropical areas around the world. In Southeast Asia, more than 500,000 ha of *A. mangium* plantations have been established for pulp and paper production (Butcher *et al*., 1998). Likewise, in Zaire, the plantations of *A. auriculiformis* and *A. mangium* play an important role in providing sufficient fuel wood (Khasa, 1994). In Indonesia, *A. mangium* is the one of the major fast growing species used in plantation forestry program. *A. crassicarpa* is also become an important fast growing species for plantation in peat land.

Studies of the genetic diversity of the *Acacia* species have been reported by a number of authors. The genetic diversity of both *A. auriculiformis* and *A. mangium* has been assessed using isozyme analysis (Moran *et al*., 1988, 1989; Wickneswari and Norwati, 1993; Khasa, 1993, 1994). Moran *et al*. (1988) compared the genetic diversity of nine *Acacia* species based on isozyme analysis. The genetic diversity of *A. mangium* has also been studied using RFLP (restriction fragment length polymorphism) markers (Butcher *et al*., 1998).

Studies of the taxonomy and phylogeny of genus *Acacia* have been reported based on biochemistry and DNA molecular markers. Due to a serological investigation, Brain and Maslin (1996) have estimated the classification of 48 species of subgenus *Phyllodineae*, predominantly distributed in Australia. In addition, Harrier *et al*. (1997) have performed RAPD (random amplified polymorphic DNA) analysis to assess the phylogenetic relationship of African *Acacia* species.

Recently, various DNA molecular markers have been used to infer phylogenetic hypotheses at numerous taxonomic levels. One method that has been utilized for various applications employs the RAPD marker (Williams *et al*., 1990) and can simply and rapidly provide information regarding the nuclear genome. RAPD analysis has been used to elucidate phylogeny at lower taxonomic levels (Das *et al*., 2001) and population genetics and genetic diversity studies (Rimbawanto and Widyatmoko, 2006; Rimbawanto *et al*., 2006a; Rimbawanto *et al*., 2006b; Wang *et al*., 2006). PCR-SSCP is another rapid and sensitive marker especially for exploring point mutations and sequence polymorphisms. This marker is advantageous for detecting small DNA changes such as single base substitutions, deletions and insertions at various positions in DNA fragments (Orita *et al*., 1989). It has been applied for the investigation of inheritance and molecular variation (Bodenes *et al*., 1996; Caron *et al*., 2000; Shiraishi *et al*., 2001; Chen *et al*., 2002).

Our study approached the phylogenetic questions of four *Acacia* species, i.e. *A. aulacocarpa*, *A. crassicarpa*, *A. auriculiformis*, and *A. mangium*, based on the information derived from the RAPD analysis of nuclear DNA and SSCP (single strand conformation

polymorphism) analysis of chloroplast DNA. Further, we preliminarily estimated the genetic diversity of the four *Acacia* species.

II. MATERIALS AND METHODS

A. Plant materials and DNA extraction

Seeds of the four species were obtained from the Australian Tree Seed Centre of CSIRO (Commonwealth Scientific and Industrial Research Organization), Australia and Forest Tree Improvement Research and Development Institute, Indonesia.

In order to elucidate the phylogeny among the four species and their genetic diversity, six seeds per species were used (Table 1). For *A. aulacocarpa*, one seed from Indonesia (IND), two from Papua New Guinea (PNG), and three from Queensland (QLD) were used. For *A. auriculiformis*, two seeds from PNG, three from QLD, and one from the Northern Territory (NT) were used. For *A. crassicarpa* and *A. mangium*, two seeds were used for each region (IND, PNG and QLD). These samples represented the natural distribution of each species. The approximate location of each sample of the four species is shown in Figure 1.

To examine the genetic diversity of *A. mangium* in more detail, seed samples were further added from two populations in IND, PNG, and QLD, respectively (Table 2). Except for Piru of the Ceram Island, four seeds of different seedlots were used for each population. For Piru, four seeds from one bulk seedlot were used.

Total genomic DNA was extracted using a modified CTAB protocol reported by Shiraishi and Watanabe (1995). The DNA was purified using GENECLEAN III (BIO101) as a template for further analyses.

B. RAPD analysis

RAPD analysis was performed in a reaction containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.5 unit/10µl AmpliTaq DNA polymerase, a Stoffel fragment (Perkin-Elmer), 0.25 µM each of primers, and 10 ng/10µl template DNA. The condition of amplification was 94°C for 1 min., 45 cycles of 30 s at 94°C, 30 s at 37°C, and 90 s at 72°C, followed by 7 min. at 72°C. The amplification products were separated by electrophoresis in 1% agarose gel with ethidium bromide and detected with a 302-nm UV transilluminator.

Table 1. Details of samples of four *Acacia* species and their cpDNA haplotypes

Species	No.	Seed source (Territory /Nation)		Seedlot No.	CpDNA SSCP type
Acacia aulacocarpa	AL 1	Kuel, Irian Jaya	(IND)	AL-1001**	I
	AL 2	Makapa WP	(PNG)	16947-M 0000003	I
	AL 3	Balimo District	(PNG)	16946-AK 000001	I
	AL 4	Old Rock Hart Airstrip	(QLD)	18358-GJM 1377	III
	AL 5	10K NW. Mt. Molloy	(QLD)	17891-GB 000096	III
	AL 6	Samford	(QLD)	17905-TREE 1	II
Acacia auriculiformis	AR 1	Morehead R Rouku WP	(PNG)	16606-BVG 01220	IV
	AR 2	Bensbach WP	(PNG)	17553-KN 000011	V
	AR 3	(R) Orchard Melville Is.	(NT)	18601-6	VI
	AR 4	Lower Poscoe River	(QLD)	18359-MHL 20	IV
	AR 5	Boggy Creek	(QLD)	17966-BH 14061	IV
	AR 6	E Normamby River	(QLD)	16756-BG 004936	IV
Acacia crassicarpa	AC 1	Kuel, Irian Jaya	(IND)	AC-1107**	III
	AC 2	Wasur, Irian Jaya	(IND)	AC-1001**	III
	AC 3	Gubam Village WP	(PNG)	16597-BVG 01100	III
	AC 4	Wemenever Prov	(PNG)	13680-JC 001503	III
	AC 5	Claudie River	(QLD)	17944-MHL 04	III
	AC 6	Parish of Annan	(QLD)	16775-BH 013582	III
Acacia mangium	AM 1	Piru, Seram	(IND)	AM-570*	VI
	AM 2	Kuel, Irian Jaya	(IND)	AM-1001**	VI
	AM 3	Gubam Ne Morehead WP	(PNG)	16991-BVG 01547	VI
	AM 4	Wipim District WP	(PNG)	16971-BVG 01626	VI
	AM 5	Claudie River	(QLD)	17946-GJM 1110	VI
	AM 6	Tully-Mission Beach	(QLD)	17703-GLM 00920	VI

Figure 1. Approximate location of four species samples

Twenty useful primers were selected in a preliminary primer screening and used in the RAPD analysis of this study (Table 3). Among the 20 primers, 15 primers were from Operon Technologies. The sequences of the other five primers were, FB-02: 5'-CAGTTCTGGC-3', FB-05: 5'-ACTCGTAGCC-3' (Shiraishi *et al*., 1996), FB-03: 5'-CCTGGCGAGC-3' (Narazaki *et al*., 1996), FB-08: 5'-CGACAAGCTC-3', and FB-09: 5'-CTCGGTGATC-3'. In order to produce reliable data, DNA samples for the RAPD analysis were randomized (Figure 2) and a sample-blind analysis was carried out.

Table 2. Details of samples of *Acacia mangium* (AM)

Seed source (population)	Code	Seedlot No.
Piru, Seram (IND)	PS	AM-570* AM-570* AM-570* AM-570*
Kuel, Irian Jaya (IND)	KI	AM-1001** AM-1002** AM-1003** AM-1004**
Gubam Ne Morehead WP (PNG)	GM	16991-BVG 01547 16991-GUB 00012 16991-GUB 00004 16991-BVG 01541
Wipim District WP (PNG)	WD	16971-BVG 01626 16971-BVG 01595 16971-BVG 01588 16971-BVG 01625
Claudie River (QLD)	CR	17946-GJM 1110 17946-GJM 1373 17946-GJM 1097 17946-GJM 1120
Tully-Mission Beach (QLD)	TB	17703-GJM 00920 17703-GJM 00926 17703-GJM 00933 17703-GJM 00939

Notes: *, ** The seeds were collected by the Forest Tree Improvement Research and Development Institute, Yogyakarta, Indonesia (* bulked; ** individual)
The other seeds were collected by Australia Tree Seed Centre, CSIRO, Australia
IND, Indonesia; PNG, Papua New Guinea; QLD, Queensland, Australia

Table 3. RAPD primers used in this study

No.	Primer	No.	Primer
1	OPA-06	11	OPK-16
2	OPA-08	12	OPN-07
3	OPA-17	13	OPO-13
4	OPA-18	14	OPS-10
5	OPB-06	15	OPS-18
6	OPC-02	16	FB-2*
7	OPC-11	17	FB-3*
8	OPJ-13	18	FB-5*
9	OPJ-18	19	FB-9*
10	OPK-02	20	FB-8*

Note: * These primers were synthesized by our laboratory.

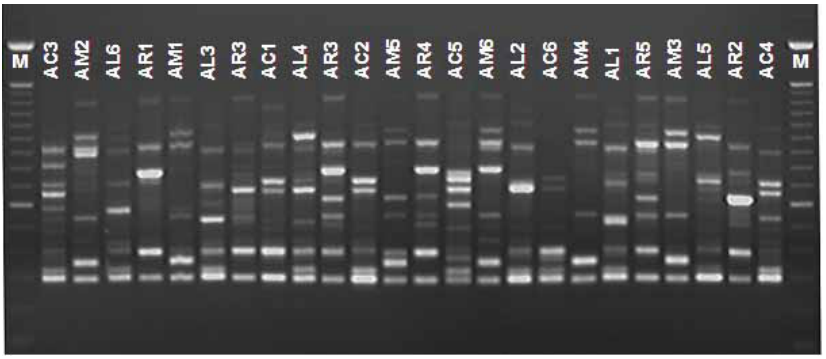

Figure 2. Example of RAPD profile of four *Acacia* species using primer FB-02. Number of each lane according to Table 1

The presence (1) or absence (0) of the polymorphic fragments attained from electrophoresis was noted as 1/0 data. Based on this data, the genetic similarity (S) and genetic distance (D = 1 - S) among all individuals were calculated using a simple matching coefficient (Sokal and Michener, 1958). A dendrogram was constructed using the UPGMA method from the matrix of genetic distance among individuals (using POPGEN software program). A Principal Component Analysis was also performed to compare genome information between the species.

C. SSCP analysis

In order to investigate a chloroplast DNA haplotype variation, an SSCP analysis of the intergenic spacer region between the *trn*P and *trn*W genes was carried out using the newly designed primers (5'-TTGGTAGCGTGTTTGTTTTGGG-3', 5'-TACGGCATCGGTTTTGGAGAC-3'). Except for the primers, the reaction solution and PCR conditions followed previously reported guidelines (Watanabe *et al*., 1997).

PCR product (2.5 μl) was mixed with 12 μl of loading buffer (1xTBE, 0.25% bromophenol blue, 77% formamide). After the solution was concentrated for 15 min. using the vacuum evaporator, it was denatured for 5 min. at 94°C and then immediately cooled on ice. For electrophoresis, this sample was loaded into 7% polyacrylamide gel (39 : 1 acrylamide to bis acrylamide) with 1x TBE buffer. The electrophoresis was run at 1.5 V/cm, 10°C. After electrophoresis, the gel was stained for 15 min. in a staining solution (1xTBE, 0.5 μg/ml ethidium bromide) and visualized using a 302-nm UV transilluminator.

III. RESULTS

A. RAPD analysis of four Acacia species

In the RAPD analysis, the informative polymorphic fragments were amplified in a range from 200 to 1,400 bps, with most being located between 400 and 800 bps. A total of 127 polymorphic fragments were scored for the 20 primers in the 24 individuals of the four species. The FB-02 primer amplified the maximum number of polymorphic fragments (10 fragments). The mean number per primer was 6.4.

The polymorphic fragment rate, which differed widely among the species, is shown in Table 3. Of 127 fragments, *A. aulacocarpa* had 55 polymorphic fragments, and its polymorphic rate was the highest (0.43); that of *A. auriculiformis* was the second highest (0.31). In contrast, *A. mangium* had only 24 polymorphic fragments out of all fragments. This value was less than the half that of *A. aulacocarpa*.

Furthermore, based on the 1/0 data of 127 polymorphic fragments, the genetic distances between the individuals were derived from a simple matching coefficient. The mean genetic distances within and between species are given in Table 5. The mean genetic distance between individuals of *A. aulacocarpa* was 0.20 ± 0.09. For the other species, *A. auriculiformis*, *A. crassicarpa*, and *A. mangium*, the distances were 0.13 ± 0.05, 0.10 ± 0.05, and 0.08 ± 0.02, respectively.

In order to clarify the relationship among species, a UPGMA dendrogram was constructed from these genetic distances (Figure 3), and two distinct clusters were identified. The first cluster comprised *A. auriculiformis* and *A. mangium*, and the remaining two species, *A. aulacocarpa* and *A. crassicarpa* formed the other cluster. The

genetic distances between the two clusters were 0.49 – 0.63 (0.56 ± 0.10). These results suggest that the two clusters can be phylogenetically separated.

Table 4. Number of RAPD fragments obtained in each species and the ratio of polymorphic fragments

Species	Number of polymorphic fragments	Number of monomorphic fragments	Ratio of polymorphic fragments
A. aulacocarpa	55	74	0.43
A. auriculiformis	39	88	0.31
A. crassicarpa	30	97	0.24
A. mangium	24	103	0.19

A. auriculiformis and *A. mangium* were grouped into a monophyletic group. The divergence between both species is represented by a long branch, and they diverged in relatively earlier time. The genetic distance between the two species was 0.4 ± 0.02, and the difference in the genome formation of each species can be clearly identified.

Table 5. Mean genetic distances between and within four *Acacia* species

Species	AL	AR	AC	AM
A. aulacocarpa (AL)	0.20±0.09*			
A. auriculiformis (AR)	0.49±0.06	0.13±0.05		
A. crassicarpa (AC	0.23±0.05	0.52±0.05	0.10±0.05	
A. mangium (AM)	0.59±0.05	0.40±0.05	0.63±0.07	0.08±0.02

Notes: * mean ± standard deviation. Diagonal was genetic diversity within species. Below diagonal was genetic distance between species

A. aulacocarpa and *A. crassicarpa* were found to be closely related phylogenetically. The difference in genome formation between them was not clear compared with that between *A. auriculiformis* and *A. mangium*. Six samples of *A. crassicarpa* formed a monophyletic group, with the mean genetic distances among them suggesting that the genetic difference within the species was small. In *A. aulacocarpa*, two clusters were observed in Figure 2. One was formed with three individuals from New Guinea Island (NGI: PNG and IND), and the other was represented by three individuals from QLD.

The mean genetic distance within NGI was 0.11 ± 0.01, which was almost the same as the mean genetic distance within *A. crassicarpa* and *A. mangium*. In contrast, a higher value was obtained within QLD (0.18 ± 0.01). The mean genetic distance between NGI and QLD was 0.24 ± 0.028. This species was clearly split into two phylogenetically different groups between New Guinea Island and Australia.

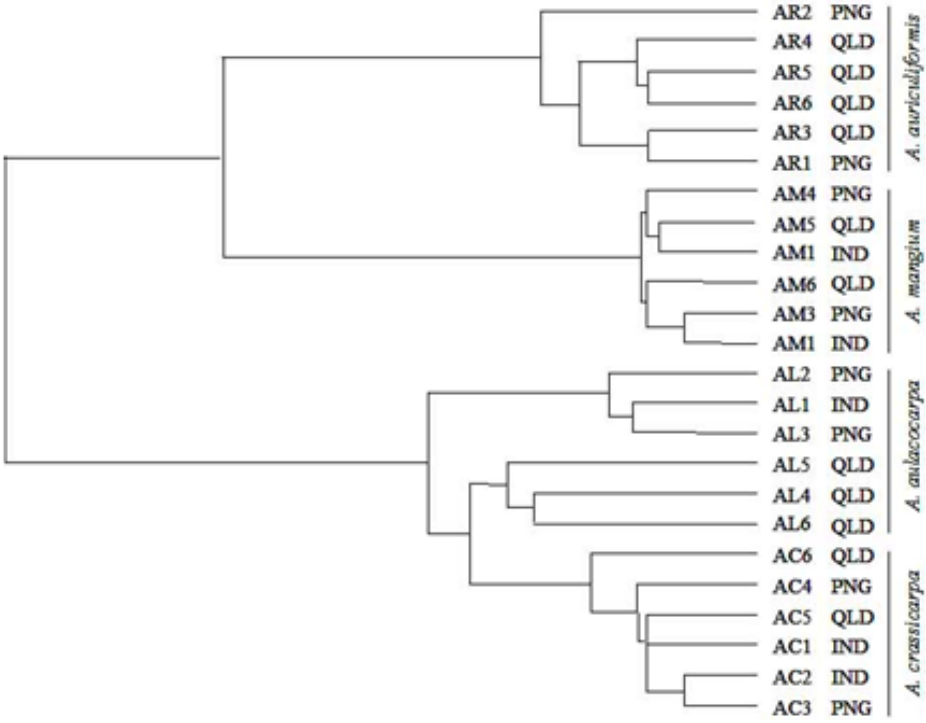

Figure 3. The relationships between four *Acacia* species. Number of each seed source according to Table 1

The results of the Principal Component Analysis (PCA) are shown in Figure 4. The first three principal components from PCA accounted for 69.6% of the total variation among samples. The proportions of the principal components one (PC1), two (PC2), and three (PC3) were 41.3%, 19.5% and 8.8%, respectively. In the plot of PC1 and PC2 (Figure 4(A)), 24 samples of the four species are separated into three groups. *A. auriculiformis* and *A. mangium* are clearly separated into different groups, but *A. aulacocarpa* and *A. crassicarpa* are not. In the plot of PC1 and PC3 (Figure 4(B)), *A. aulacocarpa* and *A. crassicarpa*, which are grouped together in Figure 4 (A), are separated into three groups: *A. aulacocarpa* of NGI, *A. aulacocarpa* of QLD, and *A. crassicarpa*. The result of the PCA analysis was similar to that of the UPGMA analysis.

B. SSCP analysis of cpDNA in four *Acacia* species

An SSCP analysis of the intergenic spacer region between the *trn*P and *trn*W genes was carried out in order to investigate the chloroplast DNA variation of the four *Acacia* species (Figure 5 and Table 1). In *A. crassicarpa* and *A. mangium*, no variation within species was observed in the six samples. In contrast, an intraspecific cpDNA haplotype polymorphism was recognized in *A. auriculiformis* and *A. aulacocarpa*. In *A. aulacocarpa*, three samples from NGI (AL1, AL2, AL3) confirmed two PCR products (ca. 320 bps and 420 bps), and three samples from QLD (AL4, AL5, AL6) also confirmed two PCR products (320 bps and 360 bps), one of which showed a length variation (Figure 5(A)). The SSCP analysis of the PCR products is shown in Figure 5 (B). Three NGI samples (type I) and three QLD samples possessed four bands of single- strand DNA, of which two bands were common and the other two showed a different electrophoretic mobility. Furthermore, of the three QLD samples, there were two different single-strand DNA profiles, one for AL6, type II, and the other for the two type III samples. *A. aulacocarpa* from QLD possessed at least two cpDNA haplotypes. In *A. auriculiformis*, three haplotypes (type IV, V, and VI) were observed. No common haplotype existed between the three types of *A. aulacocarpa* and the three types of *A. auriculiformis*. The chloroplast DNA haplotype appearing in *A. crassicarpa* and *A. mangium* was one of the haplotypes observed in *A. aulacocarpa* and *A. auriculiformis*, respectively.

The SSCP analysis of cpDNA also showed that the genetic diversity of *A. aulacocarpa* is high. A differentiation in cpDNA haplotypes could be recognized between QLD and NGI. The cpDNA information also supported the results of the cluster analysis and PCA of ncDNA. *A. auriculiformis*, which had high diversity in its ncDNA, also showed an intraspecific polymorphism in its cpDNA.

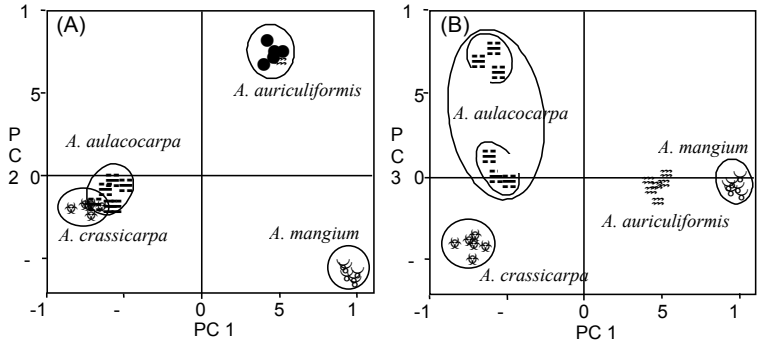

Figure 4. Principal components analysis of RAPD fragments. (A) Plot of the first two principal components. (B) Plot of the first and third principal components. NGI, New Guinea Island; QLD, Queensland

C. RAPD analysis of A. mangium

The RAPD analysis suggested that *A. mangium* possesses the least genetic diversity among the four *Acacia* species. The genetic diversity of *A. mangium* was confirmed using 24 samples from six populations (four samples per population).

In addition to the experiment to elucidate the phylogeny relationship of the four species, samples for RAPD analysis were also randomized and analyzed with a sample-blind in this experiment. The same primers as those of the former experiment were used in this analysis. The 1/0 of the 127 polymorphic fragments found in the previous experiment was scored in all 24 samples. A RAPD analysis of 24 samples from six populations showed that 24 fragments of 127 fragments were polymorphic and the other 103 were monomorphic, which was in agreement with previous results (Table 4).

The mean genetic distances within and between populations calculated using a simple matching coefficient are shown in Table 6. The genetic distances between samples were from 0.03 to 0.09 (0.07 ± 0.02), a result that is not significantly different from that estimated from the six samples in the previous experiment (0.08 ± 0.02).

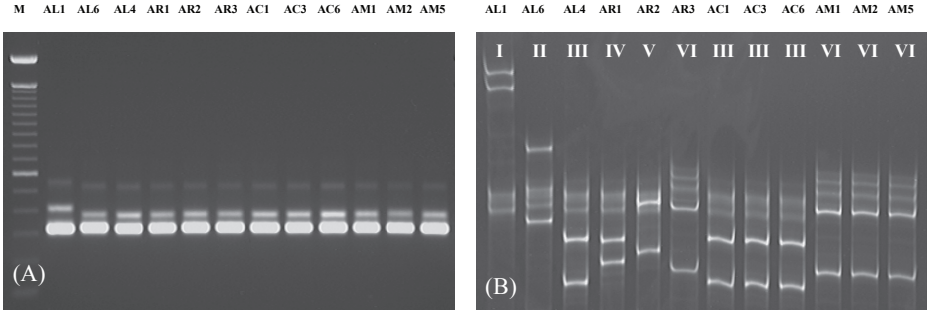

Figure 5. PCR (A) and Cold SSCP (B) profiles of the chloroplast intergenic spacer between *trn*P and *trn*W of *A. aulacocarpa*. The arrows show the differences in the band migration between the two PCR products. I-VI denote the SSCP types. Number of each lane according to Table 1. M: Size marker

The smallest mean genetic distances within populations were found in the Wipim District WP (WD), PNG (0.03 ± 0.01), and Tully-Mission Beach (TB), QLD (0.03 ± 0.01). The highest value was in Kuel (KI), IND (0.07 ± 0.012) and Gubam Ne Morehead WP (GM), PNG (0.07 ± 0.030). The highest mean genetic distance between populations was recognized between Piru (PS) and Kuel (KI), IND (0.09 ± 0.02). The mean genetic distances between and within populations were 0.05 – 0.09 (0.07 ± 0.01) and 0.03 - 0.07 (0.06 ± 0.02), respectively, suggesting that there is little difference between the mean genetic distances of inter- and intra- populations, and that there has been no remarkable geographical differentiation in *A. mangium*.

Table 6. Mean genetic distances within and between the *A. mangium* population

Population	PS	KI	GM	WD	CR	TB
Piru, Seram, IND (PS)	0.06 (0.03)					
Kuel, Irian Jaya, IND (KI)	0.09 (0.02)	0.07 (0.01)				
Gubam NE Morehead WP, PNG (GM)	0.08 (0.02)	0.07 (0.02)	0.07 (0.03)			
Wipim District WP, PNG (WD)	0.07 (0.01)	0.06 (0.02)	0.07 (0.02)	0.03 (0.01)		
Claudie River, QLD (CR)	0.07 (0.01)	0.08 (0.02)	0.07 (0.02)	0.06 (0.01)	0.06 (0.01)	
Tully-Mission Beach, QLD (TB)	0.07 (0.01)	0.07 (0.02)	0.07 (0.02)	0.05 (0.01)	0.06 (0.01)	0.03 (0.01)

Note: The standard deviation of each genetic distance is given in parenthesis

IV. DISCUSSION

A. Phylogenetic relationship among the four Acacia species

Genetic diversity in the *Acacia* species, especially in *A. auriculiformis*, *A. aulacocarpa*, *A. crassicarpa,* and *A. mangium*, has been evaluated based on molecular data (Moran *et al*., 1988; Khasa *et al*., 1993, 1994; Butcher *et al*., 1998). According to these studies, the four species are closely related, and their ecological characteristics and distribution area are extremely similar, whereas the classification under the subsection level is not yet well understood.

Pettigrew and Watson (1975) have indicated that the section *Juliflorae* can be classified into three groups based on their numerous morphological characteristics. In this classification, the four species investigated in this study were placed in the same group, suggesting their close relationship. However, using RAPD analysis, which is effective in examining relationships between closely related species, the four species could clearly be separated into two distinct groups (*A. auriculiformis* and *A. mangium*, *A. aulacocarpa* and *A. crassicarpa*). This result indicates that the taxon of Pettigrew and Watson's classification can be subdivided into some lower taxa. In the SSCP of cpDNA, the intergenic spacer between the *trn*P and *trn*W genes, a cpDNA haplotype was shared

between *A. auriculiformis* and *A. mangium*, and another haplotype was shared between *A. aulacocarpa* and *A. crassicarpa*. The chloroplast DNA information supported the phylogenetic relationships revealed by the RAPD analysis.

Inter-specific hybrid between *A. mangium* and *A. auriculiformis* has been found in both natural distribution and also plantation (Awang and Taylor, 1993; Sedgley *et al*., 1992). Putative natural hybrid between *A. aulacocarpa* and *A. crassicarpa*, and also between *A. aulacocarpa* and *A. auriculiformis* has been reported (Thomson, 1994). These conditions revealed that hybridization and introgression maybe happened among the four Acacia species. By hybridization and introgression, genomic regions of one species can be transferred into the genome of another. If introgression is extensive, genes can move across species boundaries by using hybrid individuals and hybrid populations as bridges for gene flow which can have eventually different consequences. At one extreme, it may cause a merging of the hybridizing species which in turn leads to a decrease in biodiversity (Arnold, 1997).

These results suggest that natural hybridization should be further verified using molecular markers. Molecular markers have proved to be extremely useful in determining whether a species is introgressed with genes from another species (Martinsen *et al.*, 2001).

In Figure 3, it can be seen that the branching in each cluster is essentially different. *A. auriculiformis* and *A. mangium* diverged earlier, and each formed a subcluster, indicating that these two species speciate each other completely. Since both species are closely related and easily hybridize both naturally and artificially, Sedgley *et al*. (1992) have proposed that the two *Acacia* species should not be classified as two independent species but as two subspecies of the same species. Khasa *et al*. (1994) has demonstrated that *A. auriculiformis* can be placed as a direct ancestral species of *A. mangium* based on the results of isozyme analysis. In an artificial cross between the two species, the fertility when *A. mangium* is used as the female parent is higher than that when *A. auriculiformis* is used (Sedgley *et al*., 1992), possibly suggesting an imperfect reproductive isolation between the species. Although a similar phenomenon has been observed between *Pinus densiflora* Sieb. et Zucc. and *P. thunbergii* Parl. distributed in Japan, the phylogenic relationship between these two species is not as close (Watanabe *et al*., 1996). It seems that *A. auriculiformis* and *A. mangium* are quite different species and that they are not closely related. Boland *et al*. (1990) have considered *A. mangium* to be an ancestor of the *Acacia* species. However, the results of Khasa *et al*. (1994) and this study are in conflict with this hypothesis.

On the other hand, *A. aulacocarpa* and *A. crassicarpa* are closely related. As their distributions overlap on New Guinea Island and in north Queensland, they are hybridized naturally, and they are morphologically similar, Thomson (1994) has pointed out that *A. crassicarpa* should be classified as a subspecies in *A. aulacocarpa*. McDonald and Maslin (2000) reported the closed relationship between *A. aulacocarpa* (that taxonomically

have been revised into 7 species) and *A. crassicarpa*. Our principal component analysis (Figure 4 (A)) also showed that the genome compositions of both species are similar. The separation between the two species in the dendrogram (Figure 3) is relatively imprecise. In addition, from the molecular information, *A. crassicarpa* should be regarded as a subspecies of *A. aulacocarpa*.

B. Genetic diversities in four *Acacia* species

Acacia aulacocarpa has been considered to be a species with high genetic diversity (Thomson, 1994) and has been classified into five subspecies. Four subspecies are distributed in Australia (subspecies A, C, D, E) and the other subspecies (subspecies B) is distributed on New Guinea Island. The results of our DNA analysis, which suggest that *A. aulacocarpa* possesses the highest genetic diversity of the four species examined (Tables 4 and 5), support the previous morphological classification. Of the four subspecies distributed in Australia, three subspecies (subspecies C, D, E) are distributed in QLD. Some of these subspecies are thought to have been contained in the three samples from QLD used in this study. The RAPD data showed high genetic distances among the three samples of *A. aulacocarpa* from QLD. Two types of the cpDNA haplotype were observed in the SSCP analysis of cpDNA. These results suggest the advance of genetic differentiation among QLD subspecies. In addition, a high genetic diversity in *A. aulacocarpa* was also observed between NGI and QLD. The three samples from NGI used in this experiment might have been the *A. aulacocarpa* subspecies B distributed in NGI. The RAPD analysis showed a separation of the samples from the two regions into two different clusters (Figure 3), and as for the cpDNA, different haplotypes were observed between NGI and Australia (Figure 5). In summary, we suggest that the genome compositions between NGI and Australia are distinctly different based on ncDNA and cpDNA information. As New Guinea Island was isolated from the Australian continent relatively early, interfering in the gene flow between the two regions, genetic differentiation between the regions appears to have been promoted. McDonald and Maslin (2000) have done taxonomic revision of *A. aulacocarpa*. The species have revised into 7 species and geographic distribution of those species is related with 5 subspecies revealed in this study. The revision also recognized the high genetic diversity of *A. aulacocarpa*.

Moran *et al*. (1988) have evaluated the genetic diversity of nine *Acacia* species distributed from Australia to Papua New Guinea and Indonesia, showing that the heterozygosity of *A. mangium* is extremely low among these species. The same result using isozyme analysis has also been reported by Khasa *et al*. (1993). Although the RAPD and isozyme markers are distinct, the RAPD in this study gave similar results to those described above. Recently, Butcher *et al*. (1998) have estimated the genetic diversity of *A. mangium* using RFLP (restriction fragments length polymorphism) analysis. This estimated value was eight times higher than that obtained from isozyme analysis. They concluded that the number of loci used and the different marker characteristics between

the isozymes and the RFLPs caused the different results. Certainly, isozyme information is related to the coding regions, whereas RFLP detects variations in the non-coding regions, which are more variable than the coding regions.

A. crassicarpa appears to have a low level of genetic diversity based on the results of the RAPD analysis. This finding corresponds to that obtained from an isozyme analysis reported by Moran *et al.* (1988, 1989). In this study, a cpDNA haplotype polymorphism within species was observed in *A. aulacocarpa* and *A. auriculiformis*, whereas no intraspecific variations were observed in *A. mangium* and *A. crassicarpa*. Although the sample size used in this study was small (six samples for each species), these different molecular markers provided similar results.

C. Micro-evolutional processes of *A. mangium* and *A. aulacocarpa*

Acacia species commonly distributed on New Guinea Island and its vicinities were considered to have migrated from the Australian continent. The land bridge connecting northeastern Australia to its vicinal islands in the past played an important role in the migration and radiation of the *Acacia* species in this region (Moran *et al*., 1988, 1989; Khasa *et al*., 1994; Butcher *et al*., 1998). All four species investigated in this study were distributed in this area. Because of the small sample size used in this study, we must abstain from a more detailed discussion. However, for *A. aulacocarpa*, an appreciable difference was recognized between Australia and NGI. On the other hand, in *A. mangium*, which was investigated using six populations (24 individuals), less variation was recognized among populations. These results seem to suggest that *A. aulacocarpa* and *A. mangium* spread at significantly different times. In order to infer in detail the micro-evolution of the *Acacia* species from Australia to circumference islands, an accumulation of biogeographic information will be needed in addition to the molecular phylogeny.

V. CONCLUSSION

Based on DNA molecular markers, the four *Acacia* species were divided into 2 groups. *A. auriculiformis* and *A. mangium* formed one group, and the other group comprised *A. crassicarpa* and *A. aulacocarpa*.

The highest genetic diversity between and within species was revealed by *A. aulacocarpa*. However, *A. mangium* showed the lowest genetic diversity.

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