RESEARCH ARTICLE

Mating system and seed variation of *Acacia* hybrid (*A. mangium* × *A. auriculiformis*)

CHIN-HONG NG[1]*, SOON-LEONG LEE[1], KEVIN KIT-SIONG NG[1], NORWATI MUHAMMAD[1] and WICKNESWARI RATNAM[2]

[1]*Genetic Unit, Forest Biotechnology and Pharmaceutical Programme, Forest Research Institute Malaysia, 52109 Kepong, Malaysia*

[2]*School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Malaysia*

Abstract

The mating system and seed variation of *Acacia* hybrid (*A. mangium* × *A. auriculiformis*) were studied using allozymes and random amplified polymorphic DNA (RAPD) markers, respectively. Multi-locus outcrossing rate estimations indicated that the hybrid was predominantly outcrossed (mean ± s.e. $t_m = 0.86 \pm 0.01$). Seed variation was investigated using 35 polymorphic RAPD fragments. An analysis of molecular variance (AMOVA) revealed the highest genetic variation among seeds within a pod (66%–70%), followed by among pods within inflorescence (29%–37%), and the least variation among inflorescences within tree (<1%). In addition, two to four RAPD profiles could be detected among seeds within pod. Therefore, the results suggest that a maximum of four seeds per pod could be sampled for the establishment of a mapping population for further studies.

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Introduction

Acacia is a widespread genus of about 1200 species of trees and shrubs that occur naturally in all continents except Antarctica and Europe (Maslin 2001). Tropical *Acacias* such as *A. auriculiformis* A. Cunn. ex Benth., *A. mangium* Willd., *A. aulacocarpa* and *A. richii* have been introduced into Malaysia since the early 1930s (Yap 1986). In the late 1970s, natural hybridization between *A. mangium* and *A. auriculiformis* was first reported in Sabah (FAO 1982). Synchronous flowering, common insect visitors, and the absence of fertility barriers between parental trees might be the reasons for the occurrence of interspecific hybridization between *A. mangium* and *A. auriculiformis* (Sedgley *et al.* 1992a).

Acacia hybrid (*A. mangium* × *A. auriculiformis*) shows greater resistance to diseases, higher growth rate and better adaptation to different soil types than the parental species (Pinso and Nasi 1992). In Malaysia, the incidence of heart rot disease has been frequently observed in *A. mangium* but it was never been reported in *A.* hybrid (Lee 2002). Martin (2004) reported that the growth rate of *A.* hybrid was almost double (22 m^3/ha/year) in comparison to that of the parental species (12 m^3/ha/year). Le (2001) demonstrated that the pulping productivity of *A.* hybrid (232 kg/m^3) was much higher than that of *A. mangium* (195 kg/m^3). Besides, *A.* hybrid also showed the ability to improve the physical and chemical properties of soil, which might create a favourable environment for soil microorganisms to enhance nitrogen fixation activities (Le 2001). The kind of hybrid vigour shown by *A.* hybrid was also found in the interspecific *Pinus* hybrids planted in USA, Korea and Australia (Dungey 2001). Of those hybrids that are used commercially, by far the most common are first generation crosses. These hybrids offer a new spectrum of genetic variation to the tree breeder and provide a premium source of wood for the forest industry.

*For correspondence. E-mail: chinhong@frim.gov.my.

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Australian *Acacias*, including *A. mangium* and *A. auriculformis* which are similar to many tropical tree species (Nason and Hamrick 1996), appear to be highly outcrossing (Moran *et al*. 1989a,b; Muona *et al*. 1991). However, the outcrossing habit of certain species may often maintained by inbreeding depression (Frankham *et al*. 2002). Inbreeding is usually avoided in forest tree breeding because of inbreeding depression of growth and fecundity. The mating system of *A*. hybrid has never been reported and the results from this study could enhance the understanding of the species in terms of inbreeding depression.

Like the majority of *Acacias, A*. hybrid produces a substantial number of seeds within a pod. Butcher and Moran (2000) reported incidence of genetic variation among seeds within pod in *A. mangium*. Similar findings were observed in other legumes, e.g. the seeds within pods of *Pithecellobium elegans* were not clonal materials as the seeds were sired by different paternal trees (Chase *et al*. 1996). Cytological studies of *Acacias* showed that the ovules within the ovary can be derived from independent meiotic events (Buttrose *et al*. 1981). However, due to the relatively small stigma size in relation to polyad, the majority of the pollinated flowers of *A*. hybrid receive only one polyad (Sornsathapornkul and Owens 1998). A polyad is a single pollen unit of composite pollen grains and the number varies with four, eight, 12 or 16 grain polyads in Australian *Acacia* species (Kenrick and Knox 1979). The 16-grain polyads in *Acacia* hybrid are derived from two mitoses of the sporogenous cells preceding meiosis. The possession of polyads functions as a mass of pollen grains, presumed to confer a selective advantage in reproduction.

The seeds of a pod resulting from a polyad reflect the genetic composition of only one paternal tree and the opportunity for variability between progeny from a single pod is thus reduced. It is necessary to avoid the clonal seeds within a pod in order to maximize the variation found in the mapping population used for genetic linkage studies. Therefore, the extent of variations among seeds within a seed pod should be determined to formulate a proper sampling strategy of the controlled-pollinated seeds for the establishment of mapping populations. If 100% variation was found in the seed pod, all the seeds could be used for mapping population. Hence, the specific objectives of this study were to determine the outcrossing rate, using allozyme markers, and to determine the seed-pod variations (among inflorescences within a tree, among pods within an inflorescence and among seeds within a seed pod) of *A*. hybrid using random amplified polymorphic DNA (RAPD) markers.

Materials and methods

Seed collections

Seed collections were conducted within an eight-year-old *A*. hybrid (*A. mangium* × *A. auriculiformis*) experimental plot (line-planted at a spacing of 3 m × 3 m) in Bukit Hari, Forest Research Institute, Malaysia (3°14′ N, 101°38′ E). The plants originated from five single clones, derived from a single crossing event followed by tissue culture micropropagation. Approximately 50 *Acacia* hybrid trees were planted in the plot.

During the fruiting season in the year 2004, almost 85% of the trees within the plot flowered. For the mating system study, open-pollinated seeds (40) were collected from 12 single individual trees. Seeds were also collected from two single individual trees (family 171 and family 173) for the study of seed-pod variations. For each of these two individuals, four inflorescences from different branches were sampled to determine the variation among inflorescences within a tree (figure 1,a). In addition, two pods from each inflorescence were sampled to determine the variation among pods within an inflorescence (figure 1,b). Finally, all the seeds within a pod were sampled to determine the variation among seeds within a pod (figure 1,c).

Allozyme analysis

The seeds were treated according to Doran and Gunn (1987) to promote germination. Enzyme extraction and starch gel electrophoresis of six-day-old germinating embryo tissues were carried out following Lee *et al*. (2000). Initially, nine allozyme systems were selected for consistent resolution and enzymatic activity. However, isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), and phosphogluconate dehydrogenase (PGD) staining zones were uninterpretable, and were omitted from the analysis. The other six allozymes systems were assayed on two types of gel and electrode buffer systems. Alcohol dehydrogenase (ADH), uridine diphosphogluconate pyrophosphatase (UGP), aspartate aminotransferase (AAT), and shikimic dehydrogenase (SDH) were resolved on a tris-citric buffer (Kahler and Allard 1970); phosphoglucomutase (PGM) and glucose phosphate isomerase (GPI) were resolved on a morpholine citrate buffer system at pH 6.1 (Clayton and Tretiak 1972).

RAPD analysis

Total genomic DNA was extracted from the germinating embryo tissues using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Three kits (OP-A, OP-C and OP-U; Operon Technologies, Alabama, USA), each of 20 decamer primers of random sequences, were initially screened on four samples for specific amplifications and polymorphic fragments. Subsequently, 10 primers were selected. Many studies have pointed out that the reproducibility of RAPD fragments differs among the amplified fragments (Skroch and Nienhuis 1995; Wolff *et al*. 1995). Therefore, it is crucial to estimate further which polymorphic RAPD fragments have an acceptable level of reproducibility. In the secondary screening, the reproducibility of fragments was investigated by repeating the PCR amplification four times so as to decide which primers would be useful for this study. Consequently, six primers (OP-A13, OP-C02, OP-C05, OP-C08, OP-U15 and

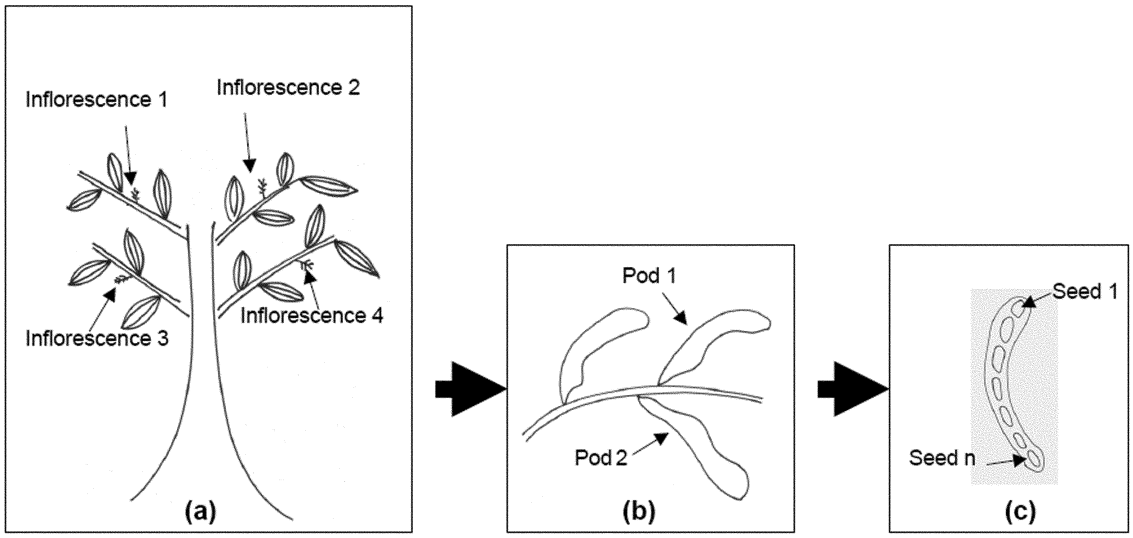

Figure 1. The sampling strategy for seed variations study. (a) Four inflorescences from different branches of a tree were sampled to determine the variation among inflorescences within a tree. (b) Two pods from each inflorescence were sampled to determine the variation among pods within an inflorescence. (c) All the seeds within a pod were sampled to determine the variation among seeds within a pod.

OP-U19) that yielded the highest number of reproducible and unambiguous polymorphic fragments were selected for analysis of the remaining samples.

A total of 65 individuals from the family 171, and 62 individuals from family 173 were analysed. PCR was carried out using GeneAmp 9700 thermal cycler (Applied Biosystems, California, USA) in 10 μl reaction mixture consisting of 30 ng DNA, 0.3 μM primer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.5 U *Taq* polymerase (Promega, Madison, USA), 200 mM Tris-HCl (pH 8.4), and 500 mM KCl. The following programmes were used for the amplifications: 2 min at 94°C, followed by 40 cycles of 45 sec at 94°C, 45 sec at 34°C and 2 min at 72°C. A final cycle of 10 min at 72°C was used to complete the extension of any remaining products. Amplification fragments were separated on 2% agarose gels in 1× TAE (tris-acetate-EDTA) buffer, stained with ethidium bromide, visualized by illumination with ultraviolet light, and recorded using image analyser (Alpha Innotech, California, USA). The amplification fragments were scored as discrete character states, (1) if present or (0) if absent, against a 1 kb base-pair-ladder marker (New England Biolabs, Hertfordshire, UK). Only reproducible amplified DNA products ranging in size from 450 to 2500 bp were scored because they were the clearest and most unambiguous.

Statistical analysis

Outcrossing rate was estimated using the procedure of Ritland (1994), based on the mixed mating model of Brown and Allard (1970). The programme (MLTR; Ritland 1994) estimated multi-locus outcrossing rate from the progeny array data, using maximum likelihood procedures, (t_m), implemented with the Newton–Raphson method. Standard errors of the estimates were based on 250 bootstraps with the progeny array within families as the unit of resampling. The seed variation was determined at three different hierarchical levels: (i) among inflorescences within a tree; (ii) among pods within an inflorescence; and (iii) among seeds within a pod. The binomial matrix of RAPD data was subjected to an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) to partition the genetic variability along the three hierarchical levels. Variance components and their significant levels of variation were determined using 2000 permutations. For RAPD profiling, the RAPD bands for each individual were treated as single phenotype. The RAPD markers are not able to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. It was assumed that RAPD fragments as dominant markers will represent the phenotype at a single bi-allelic locus (William *et al.* 1990). Therefore, each RAPD profile represents the genotype of the respective individual.

Results

The mean multi-locus outcrossing rate (t_m) was 0.86 ± 0.01 and the family multi-locus outcrossing rates ranged from 0.49 (family 178) to 1.00 (families 002, 171, 172, 174, and 176; table 1). Table 1 also shows that 10 out of the 12 families exhibited t_m values > 0.70. This might indicate that the *A*. hybrid from Bukit Hari was predominantly outcrossed. The number of alleles found at each of the six loci was: ADH (4), UGP (11), AAT (8), SDH (11), PGM (6) and GPI (7).

The six RAPD primers generated 44 clear and unambiguous fragments ranging from 450 to 2500 bp. Out of these 44 fragments, 35 were polymorphic: OP-A13, OP-C02, and OP-U19 each produced seven polymorphic fragments;

Table 1. Multi-locus outcrossing rates (t_m) of 12 single individual families of *Acacia* hybrid. The mean of $t_m = 0.86 \pm 0.01$ and the standard errors (s.e.) are given in parentheses.

Family	Number of seeds	t_m (s.e.)
001	40	0.81 (0.22)
002	40	1.00 (0.10)
171	40	1.00 (0.04)
172	40	1.00 (0.00)
173	40	0.95 (0.21)
174	40	1.00 (0.02)
175	40	0.95 (0.18)
176	40	1.00 (0.08)
177	40	0.88 (0.21)
178	40	0.49 (0.24)
179	40	0.53 (0.23)
180	40	0.74 (0.13)

OP-C05 and OP-C08 each produced five polymorphic fragments; and OP-U15 produced four polymorphic fragments (table 2). The number of pods within an inflorescence ranged from 2 to 12, whereas the number of seeds within a pod ranged from 4 to 13. The partitioning of genetic variation by AMOVA showed highest variation (66%–70%) among seeds within pod (table 3). This was followed by variation among pods within inflorescence (29%–37%). However, no significant variation was found among inflorescences within tree ($P > 0.05$).

The 35 polymorphic fragments also generated 23 different RAPD profiles for family 171, and 21 different profiles for family 173 (table 4). The pods were assigned alphabetically (A to H for family 171, and I to P for family 173), whereas the profiles were designated numerically (beginning with 01) with 'P' as the initial. The results showed that in all the pods analysed, a minimum of two different profiles were observed within a seed pod (A, B, D, I and P). Nine pods (C, E, F, J, K, L, M, N and O) possessed three different profiles and two pods (G and H) had four different profiles (table 4). In addition, all the seeds displayed different profiles, except profile P40, which appeared twice in pods N and O of family 173.

Discussion

The predominantly high outcrossing category of *A.* hybrid (mean $t_m = 0.86$) revealed in this study was comparable with the outcrossing rate reported in *A. auriculiformis* (mean $t_m = 0.93$, Moran *et al.* 1989a). Bertin (1988) noted that hybrid plants may produce staminate flowers which can be functional as pollen donors to increase the outcrossing rate. In addition, some hybrid individuals may also have the mechanism of self-incompatibility due to prezygotic selection as reported in *A. retinodes* (Kenrick *et al.* 1986). Nevertheless, in this study, despite the high value of mean multi-locus outcrossing rate, variability in outcrossing rates among families of *A.* hybrid is prevalent. Specifically, low outcrossing rates were observed in families 178 and 179.

Table 2. Primers, sequence, size and number of fragments used in RAPD analysis.

			Number of fragments		
Primer	Sequence (5′–3′)	Size (bp)	Polymorphic	Monomorphic	Total
OP-A13	CAGCACCCAC	450–2000	7	2	9
OP-C02	GTGAGGCGTC	500–2000	7	1	8
OP-C05	GATGACCGCC	500–2500	5	2	7
OP-C08	TGGACCGGTG	500–2500	5	1	6
OP-U15	ACGGGCCAGT	500–2000	4	1	5
OP-U19	GTCAGTGCGG	450–2500	7	2	9

Table 3. Analysis of molecular variance (AMOVA) in families 171 and 173.

Family	Level of variation	SS	MS	%	*P*
171	Among inflorescences within tree	40.88	13.63	0.95	0.2340
	Among pods within inflorescences	50.12	12.53	28.89	< 0.001
	Among seeds within pod	169.62	2.98	70.16	< 0.001
173	Among inflorescences within tree	28.74	9.58	–2.79	0.6027
	Among pods within inflorescences	38.45	9.61	37.06	< 0.001
	Among seeds within pod	103.28	1.91	65.73	< 0.001

Levels of significance were based on 2000 permutations (SS, sums of squares; MS, mean squares; %, proportion of genetic variability; *P*, level of significance).

Table 4. The seed genotypes represented by RAPD profiles.

Family	Inflorescence	Pod	No. of seed	No. of RAPD profile	RAPD profile	Symbol
171	1	A	5	2	11110001111000111111110111111111111	P01
					11110001111111111011111111101011111	P02
		B	5	2	11110011111111111111111111101001111	P03
					01110011111111111111101111111111111	P04
	2	C	13	3	01111101111111110011100110111111110	P05
					11110011111011111111101111111111111	P06
					11100001111100001111011111111011111	P07
		D	7	2	11111101111111111111111111111111111	P08
					11111101111001011110011111101111000	P09
	3	E	7	3	11011001111100100111001101011111110	P10
					11111001111110111111111110111111111	P11
					11111001111110011111011110111111111	P12
		F	6	3	01111000011110011111001111111110000	P13
					10100000001111101111111111111111111	P14
					10100001111111111111110111101011111	P15
	4	G	13	4	10110001111110011111011111111001111	P16
					01110001111111011111011111111001111	P17
					11111111110111111111110111111111111	P18
					11111111111100100111111111111111111	P19
		H	9	4	11111111111111111111110111111111111	P20
					01111111111111011111111101110111111	P21
					11111001111101011110011111111100111	P22
					11111111111101111111111111101111111	P23
173	1	I	5	2	11110001111000111111110111111111011	P24
					11100001110000111111111110000011001	P25
		J	6	3	11110001111100011111111111100011111	P26
					11111011111111111111011111111111111	P27
					11111111111101111110111111101111110	P28
	2	K	6	3	11111001111101111101111111101111110	P29
					11111001111101111111110111101111111	P30
					11111011111111011110111111101111110	P31
		L	11	3	11111001111100111111111111111111111	P32
					11111011111111001111111111111110001	P33
					01111001111100011111000111111110000	P34
	3	M	10	3	01111001111100011111011101111111111	P35
					11111001111111111111111111110011111	P36
					01111001111100011111111111101111111	P37
		N	9	3	11111001111111111111111101111111111	P38
					11111001111100111111011111101110011	P39
					11111001111111111111111111101111111	P40
	4	O	11	3	01111001111100101111011111111110111	P41
					01111001111111111110111111101111111	P42
					11111001111111111111111111101111111	P40
		P	4	2	11111001111000000010101111110000111	P43
					11111001111111111110111111101111111	P44

The cause of variability in outcrossing rates among families is not readily comprehensible in the present study but it may reflect heterogeneity in the pollen pool, differences in the mating neighbourhood of individuals, differences in flowering densities, differences in spatial positioning of flowers, or differences in self-compatibility. Outcrossing in *A*. hybrids could be facilitated by various insects but *Ceratina* sp. and *Apis mellifera* have been reported to be the two most common visitors and carried a heavy load of hybrid polyads (Sornsathapornkul and Owens 1998). However, *A*. hybrid produces hermaphroditic flowers (Sedgley *et al*. 1992b) that are adapted for cross-pollination as well as selfing. If the supply of outcross pollens is inconsistent within a period of time, the conditions will favour self-pollination, resulting in a mixture of self and outcross seed production.

Pollination is a reproductive process resulting from a complex series of interaction between the plant and vector agent, conditioned by the environment experienced prior to

and during anthesis. Hence, variability in outcrossing rates among families also could be due to pollinator behaviour, the relative number of pollinators, or pollen movement within and among trees. The hybrid flower has features where the style is slightly longer than the surrounding stamens (Sornsathapornkul and Owens 1998). During foraging activities, pollens might easily be transferred onto the stigma to promote self-pollination. The pollinators may also have visited different inflorescences in the same tree prior moving to another tree. Therefore, self-pollination can occur if pollinators transport pollens within a flower, or among flowers on the same plant (Lloyd 1992). Besides, selfing might also occur in the context of decreased pollinator visits since the plant has a mechanism to evolve autonomous self-fertilization that provides reproductive assurance (Kalisz *et al*. 2004).

The superior characteristics of *A*. hybrid have made the species one of the promising candidates for forest plantation (Bueren 2004). The planting materials of *A*. hybrid F_1 can be produced through various methods, i.e., controlled pollination (Sedgley *et al*. 1992a), biclonal seed orchards of *A. mangium* and *A. auriculiformis* (Griffin *et al*. 1992), cutting (Wong and Haines 1992), and tissue culture (Darus 1992). However, pure *A*. hybrid could not be generated by successive generations of crossing among F_1 hybrids due to recombination effects of the parental alleles. Nevertheless, the seeds produced by the F_1 hybrid enable the performance of the F_2 materials to be evaluated. The study of the straightness and basic density of F_2 hybrid population compared to pure *A. mangium*, *A. auriculiformis* and F_1 hybrid would indicate whether heterosis and the possibility of fixing deleterious alleles takes place in F_2 generation. Subsequently, the selected superior F_2 hybrid could be micropropagated and used in commercial breeding programme.

The F_1 hybrid inviability of seed set, which can become a hindrance to *A*. hybrid as seed source, was not observed in the present study. Lethality or semi-lethality of F_1 hybrids after germination has been reported in many genera of plants, with hybrid weakness and dwarfs a common feature (Levin 1978). A study of *Eucalyptus ovata* × *E. globulus* F_1 hybrid (Lopez *et al*. 2000) showed that the F_1 hybrids exhibited reduced viability compared with intraspecific cross-types at all stages of the life cycle and are at a selective disadvantage. Several genetic causes of inviability of the F_1 hybrids include genome disharmony and incompatible development cues (Levin 1978). Besides, deleterious interactions between alleles from the same locus have been implicated in F_1 hybrid inviability and sterility in several cases (Abbo and Ladizinsky 1994). Such genes have no deleterious effects within a species and probably accumulate as a by-product of divergence, but may cause inviability or sterility in combination with genes from another species. The *A*. hybrid did not suffer from the fitness disadvantages mentioned and most probably they are closely related as increased hybrid inviability will only be observed with relatively large taxonomic distance between parental taxa (Griffin *et al*. 1988).

The present study shows that the number of pods within an inflorescence of *A*. hybrid ranged from 2 to 12, while the number of seeds within a pod ranged from 4 to 13. The number of seeds within a pod depends on the availability of ovules and pollens. *Acacia* hybrid ovary consists of an average of 15 ovules (Sornsathapornkul and Owens 1998) and the majority of the pollinated flowers of *A*. hybrid receive only one polyad. The presence of polyad enables the full pod-set following a single pollination event. The seeds produced would not exceed the number of pollens and this was reflected in the present study as the number of seeds within a pod was observed to be less than 16.

The AMOVA analysis revealed that the genetic variation among inflorescences within tree was not significant. This might be due to the pollinator in foraging for pollen in different inflorescences within the same tree rather than among trees (Sornsathapornkul and Owens 1998). Therefore, in order to capture most of the variation on a given mother tree, sampling all the seeds within a pod should be carried out instead of sampling as many pods per inflorescence as possible. Besides, the genetic variation of *Acacia* hybrids within a population should be measured by seed sampling from many mother trees. The observation of more than one RAPD profile indicates that there are seed variations within the pod of *A*. hybrid which is comparable to seed variation within the pod of *A. mangium* (Butcher and Moran 2000). A maximum of four different RAPD profiles were observed within a seed pod and the variations of RAPD profiles within a seed pod could be due to random combination during the process of pollination. Two pods possessed four different profiles and other pods possessed either two or three profiles. These results show that a maximum of four seeds per pod could be sampled for the establishment of mapping population. Future work may include some studies to see if the superior characteristics of *A*. hybrid could be maintained in F_2 generation and the results will be useful for a large scale breeding programme of *A*. hybrid.

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