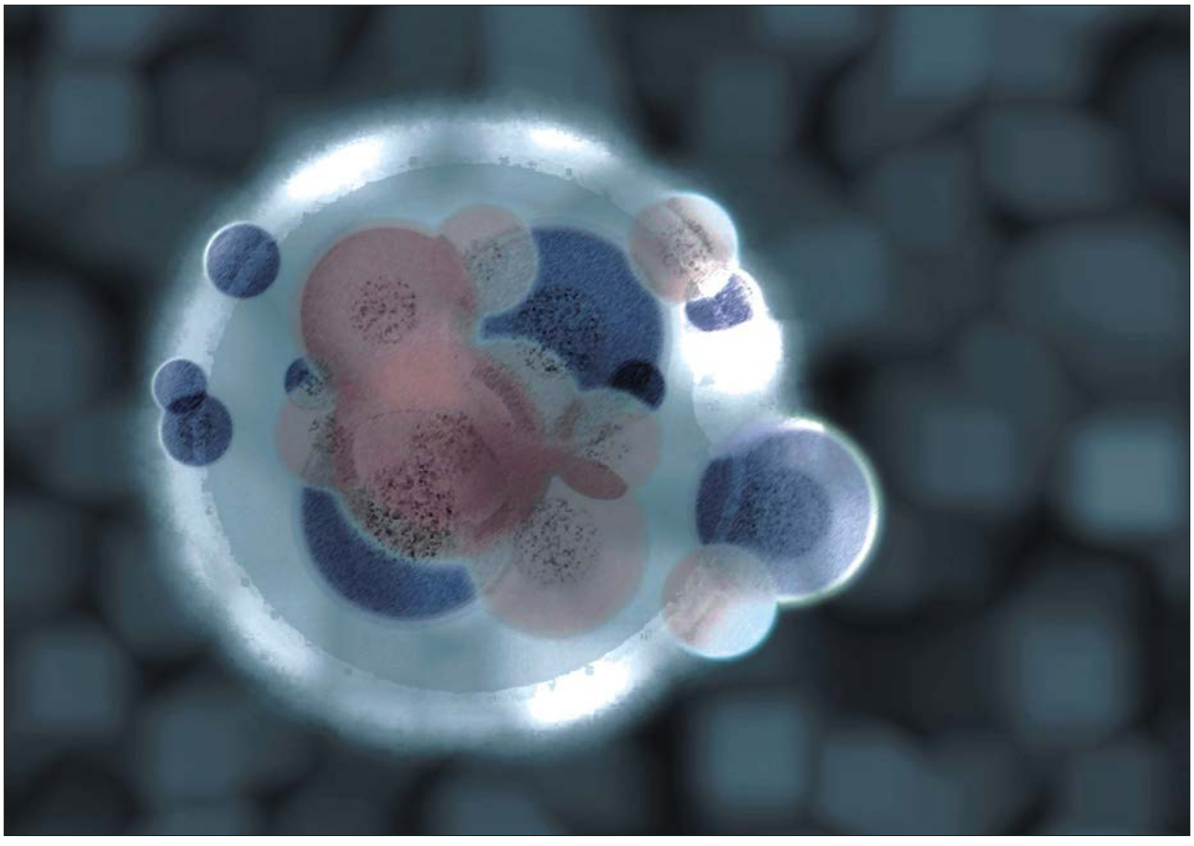

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 8 (4): 702-712, 2008
ISSN 1727-3048

Morphometric and Genetic Variation of Six Seed Sources of *Azadirachta excelsa* (Jack) Jacobs

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Abstract: Isozyme variability was examined in six seed sources representing the *Azadirachta excelsa* on two provenance trials in Batu Arang (Selangor, Malaysia) and Merchang (Terengganu, Malaysia). A concomitant study of morphometric variation revealed a slight variation in leaf morphology extending from quantitative to qualitative characteristics. The existence of this small variation presented an ideal opportunity to examine the genetic variation of these seed sources collected in Bukit Lagong and Manong (West Coast of P. Malaysia), Pengkalan Arang and Pasir Mas (East Coast of P. Malaysia), Semengoh (East Malaysia) and Narathiwat (Thailand). Nineteen enzyme systems were used to determine the genetic variation among seed sources using isozyme analysis. Allelic frequency data indicated little differentiation between seed sources. The mean values of observed heterozygosity (H_o) varied from 0.0229 (Pengkalan Arang) to 0.0451 (Bukit Lagong) whereas the mean values expected heterozygosity (H_e) varied from 0.0575 (Pengkalan Arang) to 0.0983 (Manong). The percentages of proportion polymorphic loci were found to vary between 31.43% (Pengkalan Arang and Pasir Mas) and 42.86% (Bukit Lagong and Manong). Genetic identities according to Nei ranged from 0.7727 to 0.9999. Despite these high levels of genetic similarity, the populations appeared to be highly inbred as indicated by positive mean of F_{IS} and F_{IT} values with the mean values of 0.5643 and 0.8038.

Key words: *Azadirachta excelsa*, isozyme, morphology, seed sources, observed heterozygosities, polymorphic loci

INTRODUCTION

Genetic studies have particularly been used to identify superior species populations or provenances and played an important role in the subsequent selection and breeding of the most desirable individuals within these populations or provenances. Biochemical techniques could provide an alternative approach for evaluating genetic diversity in tree species. Genetic analysis of isozyme or also called isoenzyme electrophoresis using starch gel has been extensively used over the past several decades in investigations of the genetics of a large number organisms from fruit flies and human to crop plants. With the fast technology advancements, several other reliable markers have been introduced such as RAPD, RFLP, AFLP and SSR. However, isozyme technique still stands by its own advantages such as cheap, fast and not affected by environmental changes.

Isozyme analyses have not been widely used in tropical tree species. The available studies were reported in *Acacia auriculiformis* and *A. crassicarpa* (Moran *et al*., 1989a; Wickneswari and Norwati, 1993), *A. mangium* (Moran *et al*., 1989b), *A. melanoxylon* (Playford *et al*., 1993), *Eucalyptus urophylla* and *E. grandis* (Martins-Corder and Lopes, 1997), *Hevea brasiliensis* (Paiva *et al*., 1994a, b), *Pterocarpus macrocarpus* (Liengsiri *et al*., 1995) and *Tectona grandis* (Kertadikara and Prat, 1995). Early works on *Azadirachta excelsa* were done by Norwati *et al*. (1997).

A. excelsa is one of the indigenous potential plantation species being selected to be investigated in this study in order to gain more information especially about provenance variation to ensure the suitability for a large scale plantation programmes in the future. This study considered six seed sources (genotypes) of *A. excelsa* in the assessment of its variation. The objective of this study is to determine the variation of *A. excelsa* seed sources using morphological and genetic markers.

MATERIALS AND METHODS

Seed sources and study sites: The study was done using six seed sources of *A. excelsa*. The seeds originated from

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Table 1: Details of the six seed sources of *A. excelsa*

Source		Latitude (S)	Longitude (E)	Altitude (m)
Bukit Lagong	Selangor	3° 12'	101° 35'	60
Pengkalan Arang	Terengganu	5° 20'	103° 07'	25-50
Narathiwat	Thailand	6° 26'	101° 42'	30-60
Manong	Perak	4° 35'	100° 52'	75-150
Semengoh	Sarawak	1° 25'	110° 18'	25-60
Pasir Mas	Kelantan	6° 03'	102° 07'	30-55

Bukit Lagong Forest Reserve (FRIM, Selangor), Pengkalan Arang (Terengganu), Narathiwat (Thailand), Manong Forest Reserve (Perak), Semengoh (Sarawak) and Pasir Mas (Kelantan). Table 1 shows all of seed origin and the location of the sources. The seeds were sown and germinated immediately in seed tray and later the seedlings were transplanted into polythene bags. They were about 3 months-old before being transplanted to the field in Merchang (Terengganu) and Batu Arang (Selangor) in year 2002.

Evaluation of genetic variation: The selection of 30 trees from each seed source in both provenance trials was done randomly and leaves which were still attached to the twigs were preferred, in order to avoid wilting. The leaf samples were kept in plastic bags and placed in a cool container containing crushed ices. The samples were then ground immediately, if possible or preserved fresh in refrigerator at -20°C to avoid the protein denaturation. An amount of 0.6 g of leaf was homogenised with liquid nitrogen using mortar and pestle. Then 1200 μL of leaf extraction buffer was added into the powdered form of leaf material with a ratio of 1:2. This is to create a slurry homogenate. The sample was filled into Eppendorf tube and labelled and later was kept in crushed ices before centrifugation at -10°C, 1000 rpm for 3 min. The clear filterate was stored in a freezer at -70°C.

Three types of gel and electrode buffers were used to detect the various enzymes. These include Histidine (H), Lithium (L) and Morpholine Citrate (MC). The mould frame was made of perspex, with internal dimensions of 18.8×15.0×0.6 cm and a glass plate of equal area. Both the frame and the glass plate were cleaned and dried properly by placing them in the oven at 40°C to prevent breakage when pouring the hot gel solution. A 10.5% of potato hydrolysed starch (SIGMA-ALDRICH Inc.) was prepared by adding 31.5 g of starch into 100 mL of cold buffer in a dry Buchner flask. The remainder 200 mL of boiled buffer was then added into the flask and the buffer was swirled thoroughly to create an even suspension. The buffer was once again cooked together with the starch in the microwave oven until it boiled or big bubbles started to form. When the starch was fully cooked, the flask that contained the gel mixture was connected to the suction pump for a degassing process. The starch gel solution was then poured into the perspex mould and was left overnight at room temperature for hardening.

Table 2: Enzymes assayed, their abbreviations and Enzymes Commission (EC) designations

Enzyme	Abbreviation	EC designation
Aconitase	ACO	4.2.1.3
Adenylate kinase	ADK	2.7.4.3
Alcohol dehydrogenase	ADH	1.1.1.1
Esterase	EST	3.1.1.1
Glutamate dehydrogenase	GDH	1.4.1.2
Glycerol dehydrogenase	GLD	1.1.1.29
Glutamate-oxaloacetate transaminase	GOT	2.6.1.1
Glucose-6-phosphate dehydrogenase	GPDH	1.1.1.49
Isocitrate dehydrogenase	IDH	1.1.1.42
Leucine amino peptidase	LAP	3.4.11.1
Malate dehydrogenase	MDH	1.1.1.37
Malic enzyme	ME	1.1.1.40
Peroxidase	PER	1.11.1.7
6-phosphogluconic dehydrogenase	6PGD	1.1.1.44
Phosphoglucose isomerase	PGI	5.3.1.9
Phosphoglucomutase	PGM	2.7.5.1
Shikimate dehydrogenase	ShDH	1.1.1.25
Sorbitol dehydrogenase	SDH	1.1.1.14
Tetrazolium oxidase	TO	1.15.1.1

Source: Weeden (1989)

Each resulting homogenate was absorbed onto a filter paper wick (i.e., 0.6×0.4 cm Whatman No. 3). This saturated wick and a tracking marker were inserted into the sliced gel that was tightened up by space bars before the electrophoretic run. The prepared gel (with wicks) was placed between the 2 electrode tanks containing 500 mL of buffer. The gel and the buffers were connected by two sets of bridge wicks (J-cloth or sponge) that were placed with one end overlapping about 1.0 to 3.0 cm on the gel and the other end dipping into the buffer. The set-up was covered with a thin polythene sheet to prevent evaporation of the buffer. A bag or plastic container containing ice was placed on the polythene sheet to avoid the drying up of gel during electrophoretic run due to high voltage or current. The electrophoretic run was carried out in a 4°C refrigerator with an appropriate electric voltage depending on the buffer used. The wicks were taken out from the gel after 30 min of running.

After the electrophoretic run, the gel was removed from the mould in the refrigerator and cut into a smaller size by discarding only the sides. A mark was made to determine the origin of the sample arrangement by slashing the side. Later, the gel was sliced into three or four sheets with a thickness of approximately 2 mm per slice. The slices were stained for 19 different enzyme systems. The different enzyme systems used are as listed in Table 2. The stained gels were incubated in an oven at 37°C for 30 to 60 min or until coloured bands developed. Then the gel was fixed using a fixing solution. Observation on the pattern of the bands was made and a

clear zymogram was photographed whenever necessary. The banding of phenotypes was recorded in the prepared data sheets and followed with the interpretation.

Data analysis: The soluble enzymatic proteins from the leaves were assayed for each enzyme. The locus alleles were labeled as fast (F), medium (M) and slow (S) according to the decreasing anodal mobility. Standard measures of allozyme diversity within species and seed sources were calculated as described in Hamrick and Godt (1989). These measures included the percentage polymorphic loci (P), the mean number of alleles per locus (A), the effective alleles per locus (Ae), observed (H_o) and expected (H_e) heterozygosity, corrected for sample size (Nei, 1978). Other analyses done on the isozyme data also included Shannon's information index (I) (Shannon and Weaver, 1949) and population genetic structure was measured using F-Statistics and, fixation index (Wright, 1978), gene flow estimation (Slatkin and Barton, 1989) and gene diversity among provenances (Nei, 1973). F_{IS} compares the observed and expected levels of heterozygosity within provenances. A positive F_{IS} indicates a lower than expected level of heterozygosity, possibly due to nonrandom mating (Wright, 1969). F_{IT} measures the heterozygosity of an individual relative to the entire population. It is an indication of inbreeding due to nonrandom mating and random genetic drift within the subpopulations. G_{ST} is a measure of the level of variation that resides within, rather than between, populations (Nei, 1973). Finally, the construction of a dendrogram was made based on Nei's genetic distance using UPGMA (Nei, 1978). The programme is actually an adoption programme of NEIGHBOR and PHYLIP version 3.5c. The data were analysed using POPGENE version 1.21. The banding patterns were first encoded using Microsoft Excel for easier editing before being transformed into a POPGENE data file. The formulas for calculating those analyses are as follows:

The formula was used for allelic frequency are:

$$\frac{2NH_o + NH_e}{2N} \quad (1)$$

Where:

Nh_o = No. of homozygous for that allele
Nh_o = No. of heterozygotes for that allele
N = No. of individuals screened or examined

The observed heterozygosity (H_o) is simply the proportion of all genotypes that was heterozygote and may be expressed on a per-locus basis or averaged over all loci. Meanwhile, the expected heterozygosity was estimated using formula:

$$H_e = (1 - \Sigma X_{ik}^2) \text{ (Nei, 1972)} \quad (2)$$

where, X_{ik} is the frequency of the k^{th} allele at a locus in the i^{th} population.

The gene diversity in this populations is defined as $H_e = (1 - {}_k\Sigma X_{ik}^2)$. The average of H_e over loci is H_S. The gene diversity in the total population is:

$$H_T = (1 - {}_k\Sigma X_k^2) \quad (3)$$

where, $X_k = {}_i\Sigma X_{ik}/n$, n being the number of populations. Then H_T-H_S, the diversity due to interpopulational gene differences, is denoted as D_{ST} and the coefficient of gene differentiation is D_{ST}/H_T denoted as G_{ST}.

The genetic identity (GI) between the two population or operational taxonomic unit (OTUs) was estimated according to Nei's coefficient using this formula:

$$GI = \frac{\sum X_i Y_i}{\sqrt{\sum X_i^2 \sum Y_i^2}} \quad (4)$$

where, X_i and Y_i = Frequencies of the i^{th} allele in the two OTUs or populations.

Morphological traits: A total of 40 trees were selected randomly from each seed source in both provenance trials to determine the variability of morphological characteristics when the trees were about two years old. Morphological traits such as leaf shape, leaf margin, leaf base, leaf angle and internode were also determined. The leaf angle was determined using the triangulation method. The length between nodes (inter-node) of two leaves (a) was measured. Then the length from the base of the node to the centre length of the leaf (b) was measured. The distance from the centre of the leaf to the base of the next leaf was then measured as (c). The value of the distances (c) was obtained using the following formula:

$$c = a + b - 2ab \cos\theta$$
$$\cos = \frac{a + b - c}{2ab} \quad (5)$$

The general analysis for morphological traits was done according to one-way ANOVA. In addition, similarity coefficients based on these morphological characteristics were estimated using the formula given below;

$$\text{Similarity coeficient} = S_{RT} = \frac{m}{m + 2(u)} \text{ (Rogers and Tanimoto, 1960)} \quad (6)$$

Where:

m = No. of matches
u = No. of mismatches

RESULTS

Isozyme analysis

Intra-population variation: Table 3 shows the summary of genetic diversity from 35 loci scored including observed (H_o) and expected (H_e) heterozygosities, Shannon's information index (I), effective number of alleles (A_e), proportion of polymorphic loci and the average number of alleles per locus per seed source.

The values of the observed heterozygosity varied from 0.0000 to 0.3333 with mean values ranging from 0.0229 (Pengkalan Arang) to 0.0451 (Bukit Lagong). Meanwhile, the values of expected heterozygosity ranged from 0.0000 to 0.5161. The mean values of H_e were found to vary from 0.0575 (Pengkalan Arang) to 0.0983 (Manong). Moreover, Shannon's information index showed the diversity within the seed source to vary from 0.0000 to 0.8897 with mean values ranging from 0.1098 in the Pengkalan Arang seed source to 0.1852 in the Manong seed source. The effective number of alleles was found to range between 1.0000 and 2.0666 with mean values found to be within 1.0808 to 1.1468.

Ten loci were found to be monomorphic for all seed sources whereas the other 25 loci were polymorphic. The percentages of proportion polymorphic loci were found to vary between 31.43% (Pengkalan Arang and Pasir Mas) and 42.86% (Bukit Lagong and Manong). Meanwhile, the mean number of alleles per locus ranged from 1.48 to 1.69.

Genetic structure: F-statistics revealed varying fixation indices among the loci (Table 4). The estimates of F_{IS} revealed one locus (Pgi-3) with excess of heterozygotes, as revealed by the negative mean fixation index value. The 34 remaining loci exhibited heterozygote deficiencies. The F_{IS} values calculated ranged from -0.0265 at Pgi-3 to 1.0000 at Pgm-2 with the average of 0.5643. The F_{IT} values for all loci ranged from -0.0043 at Pgi-3 to 1.0000 at Pgm-2 with the mean value being 0.8038.

Genetic differentiation among seed sources as measured by F_{ST} showed that 21.32% of the total genetic variation was due to differences among seed sources; 78.68% of the isozyme variation resided within seed sources. The F_{ST} values ranged from 0.0000 to 0.9681 among the polymorphic loci. The estimated gene flow (Nm) based on the F_{ST} values was 2.9124.

The results obtained for genetic diversity in the seed source, measured by Nei's (1973) index, through the partition of total genetic diversity (H_T) in the components of diversity within populations (H_S) and between populations (D_{ST}) showed relatively low values for total diversity (Table 5). The average G_{ST} (coefficient of differentiation) value indicated that approximately 29.84% of the observed variability was found among seed sources, more than that estimated by F_{ST}.

Genetic distance: Nei's genetic distance (Nei, 1978) and the UPGMA dendrogram revealed low levels of genetic distance among seed sources. Most of the seed sources produced high similarities to each other with mean identity values ranging between 0.7727 and 0.9999 (Table 6). The most related seed sources were Pengkalan Arang and Pasir Mas with a genetic identity of 0.9999 between them. There were two clusters formed where the first cluster was made up of Pengkalan Arang, Pasir Mas, Narathiwat and Semengoh seed sources while the second

Table 3: Summary of genetic diversity for six seed sources of *A. excelsa* using isozymes

		Seed source					
Statistic	Range	Bukit lagong	Pengkalan arang	Narathiwat	Manong	Semengoh	Pasir mas
H_o	Min	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	Max	0.3333	0.1667	0.1667	0.2759	0.2000	0.2333
	Mean	**0.0451**	**0.0229**	0.0288	0.0446	0.0355	0.0239
	SE	0.0144	0.0076	0.0086	0.0128	0.0096	0.0086
H_e	Min	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	Max	0.5161	0.4350	0.4050	0.4501	0.3844	0.4178
	Mean	0.0960	**0.0575**	0.0671	**0.0983**	0.0797	0.0624
	SE	0.0249	0.0188	0.0193	0.0247	0.0206	0.0201
I	Min	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	Max	0.8897	0.7507	0.7306	0.7825	0.7026	0.7299
	Mean	0.1800	**0.1098**	0.1274	**0.1852**	0.1515	0.1184
	SE	0.0444	0.0335	0.0348	0.0447	0.0378	0.0360
Ne	Min	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
	Max	2.0666	1.7699	1.6807	1.8184	1.6245	1.7176
	Mean	**1.1468**	**1.0808**	1.0919	1.1467	1.1092	1.0894
	SE	0.0442	0.0297	0.0287	0.0409	0.0298	0.0315
Loci scored		35.00	35.00	35.00	35.00	35.00	35.00
Polymorphic loci (%)		**42.86**	**31.43**	34.29	**42.86**	37.14	**31.43**
Mean allele per locus (A)		1.66	1.49	1.57	**1.69**	1.66	**1.48**

H_o: Heterozygosity observed, H_e: Heterozygosity expected, I: Shannon's information index, Ne: Effective number of alleles

Table 4: F-statistics and gene flow for all loci

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
Aco-1	0.7158	0.8583	0.5013	0.2488
Aco-2	-	-	0.0000	-
Adh-1	-	-	0.0000	-
Adh-2	0.6657	0.6858	0.0601	3.9116
Adk-1	0.3148	0.9133	0.8735	0.0362
Est-1	0.6571	0.6639	0.0196	12.5000
Est-2	0.4783	0.4944	0.0309	7.8409
Gdh-1	0.8478	0.9649	0.7690	0.0751
Got-1	0.4995	0.8976	0.7954	0.0643
Got-2	-	-	0.0000	-
Gld-1	0.5332	0.5701	0.0791	2.9124
Gpdh-1	0.6532	0.9889	0.9681	0.0082
Gpdh-2	0.4880	0.5087	0.0405	5.9243
Idh-1	-	-	0.0000	-
Idh-2	0.3339	0.7582	0.6371	0.1424
Lsp-1	0.3932	0.7835	0.6432	0.1387
Mdh-1	-	-	0.0000	-
Mdh-2	0.4402	0.4891	0.0873	2.6123
Me-1	0.7447	0.7729	0.1104	2.0143
Per-1	-	-	0.0000	-
Per-2	0.5496	0.6052	0.1234	1.7754
6Pgd-1	-	-	0.0000	-
6Pgd-2	0.4210	0.5194	0.1699	1.2215
Pgi-1	0.8582	0.8698	0.0824	2.7829
Pgi-2	0.6004	0.9100	0.7748	0.0726
Pgi-3	-0.0265	-0.0043	0.0216	11.3000
Pgm-1	0.7875	0.8999	0.5291	0.2225
Pgm-2	1.0000	1.0000	0.0409	5.8571
$S_h$dh-1	0.6491	0.6610	0.0339	7.1250
$S_h$dh-2	0.4783	0.4944	0.0309	7.8409
Sdh-1	-	-	0.0000	-
Sdh-2	0.5536	0.5638	0.0229	10.6590
To-1	-	-	0.0000	-
To-2	0.4324	0.4419	0.0168	14.6467
To-3	-	-	0.0000	-
Min	-0.0265	-0.0043	0.0000	0.0000
Max	1.0000	1.0000	0.9681	14.6467
Mean	0.5643	0.8038	0.2132	0.2048
SE	0.0529	0.0629	0.0532	0.7134

F_{IS}, F_{IT} and F_{ST}: Inbreeding coefficients, Nm: Gene flow

Table 5: Nei's (1973) statistics of genetic diversity for 18 polymorphic loci in six seed sources of *A. excelsa*

	H_T	H_S	D_{ST}	G_{ST}
Minimum	0.0115	0.0112	0.0003	0.0118
Maximum	0.6666	0.2782	0.6505	0.9760
Mean[1]	0.2461	0.1076	0.1385	0.2984
Standard Error	0.0432	0.0158	0.0383	0.0679

[1]: Mean values do not include monomorphic loci

cluster consisted of Bukit Lagong and Manong seed sources (Fig. 1). This clusteration is comparatively similar to the ones based on morphological similarities with a few notable exceptions. Four seed sources (Pengkalan Arang, Pasir Mas, Narathiwat and Semengoh) were assigned to CLUSTER I on the basis of isozyme and morphological data.

Morphological variability and similarity: Most seed sources have the same pattern of qualitative characteristics for all leaf parameters except for Narathiwat and Semengoh seed sources which had one extra pattern in leaf margin (Table 7). Quantitative characteristics of leaf

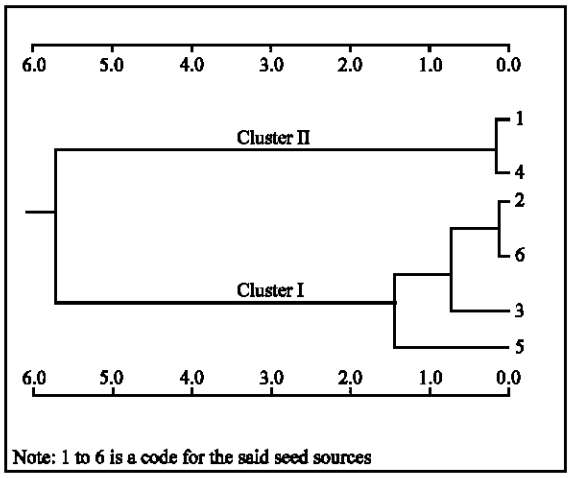

Fig. 1: Dendrogram of six seed sources of *A. excelsa* using Un-Weight Pair Group Cluster Analysis of Identity Coefficients (Nei's, 1972)

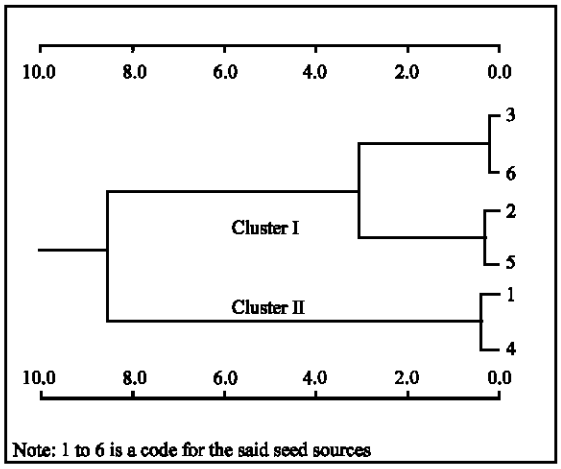

Fig. 2: Dendrogram of six seed sources of *A. excelsa* based on morphological characteristics (qualitative)

angle and internode showed significant differences among seed sources for both positions but no significant difference was detected on other parameters (Table 8).

The mean morphological similarities for the qualitative characteristics ranged from 0.7930 between Pasir Mas and Bukit Lagong seed sources to 0.9952 between Semengoh and Bukit Lagong seed sources (Table 9). The dendrogram for six seed sources of *A. excelsa* based on these similarities is given in Fig. 2. Narathiwat-Pasir Mas seed sources and Pengkalan Arang-Semengoh seed sources formed one cluster while Bukit Lagong and Manong seed sources formed the other cluster.

Table 6: Nei's (1972) Coefficients of genetic identity (above diagonal) and genetic distance (below diagonal) among six seed sources of *Azadirachta excelsa*

Seed source[C]	Bukit lagong[1]	Pengkalan arang[2]	Narathiwat[3]	Manong[4]	Semengoh[5]	Pasir Mas[6]
Bkt.Lagong[1]	-	0.7727	0.7990	0.9989	0.8528	0.7756
Pgkl. Arang[2]	0.2579	-	0.9716	0.7777	0.9296	0.9999
Narathiwat[3]	0.2243	0.0288	-	0.8044	0.9555	0.9715
Manong[4]	0.0011	0.2514	0.2176	-	0.8585	0.7808
Semengoh[5]	0.1592	0.0730	0.0455	0.1526	-	0.9313
Pasir Mas[6]	0.2542	0.0001	0.0289	0.2475	0.0712	-

[C]: Seed source code

Table 7: Leaf characteristics of six *A. excelsa* seed sources

	Leaf characteristics		
Seed source	Leaf shape	Leaf base	Leaf margin
Bukit Lagong	Lanceolate	Asymmetric	Entire
	Ovate	Cuneate	Wavy
Pengkalan Arang	Lanceolate	Asymmetric	Toothed
	Ovate	Cuneate	Wavy
Narathiwat	Lanceolate	Asymmetric	Toothed
	Ovate	Cuneate	Entire
			Wavy
Manong	Lanceolate	Asymmetric	Entire
	Ovate	Cuneate	Wavy
Semengoh	Lanceolate	Asymmetric	Toothed
	Ovate	Cuneate	Entire
			Wavy
Pasir Mas	Lanceolate	Asymmetric	Toothed
	Ovate	Cuneate	Wavy

Table 8: Analysis of variance for qualitative and quantitative traits of morphological variants

Parameter	Mean square	F
Leaf shape	1.760	8.507[ns]
Leaf base	0.129	0.522[ns]
Leaf margin	2.289	3.688[ns]
Leaf angel[1]	0.032	5.481*
Leaf angle[2]	0.101	17.208*
Internode[1]	1.174	5.288*
Internode[2]	2.570	9.452*

[1]Basal position, [2]middle position, *Significant at $p<0.05$, [ns]: Not significant

DISCUSSION

Intra- and inter-provenance variation: Genetic variation within populations is the basis for evolutionary change to occur. The amount of such variation is dependent on the species level. According to Hopper and Coates (1990), this is necessary to occur in order for plants to evolve and adapt under different conditions and numerous environments that they encounter during a single or many generations. There are five evolutionary processes that affect the level of genetic variation such as random genetic drift, selection, migration, mating and mutation. All these evolutionary processes affect the levels and distribution of genetic variation and the present state of the genetic resource is a result of their joint effects (Wickneswari, 1999).

The genetic variation among provenances is quantified by measuring the mean heterozygosity, the percentage of polymorphic loci and the number of alleles per locus. The range of expected heterozygosity calculated in the present study was from 0.0575 (Pengkalan Arang) to 0.0983 (Manong) and found to be within the range recorded by Norwati *et al.* (1997) on the same species but in different populations. These values were also generally consistent with those reported on tropical species by Hamrick and Loveless (1986).

The range of the proportion of polymorphic loci obtained was from 31.43% (Pengkalan Arang and Pasir Mas) to 42.86% (Bukit Lagong and Manong) and found to be within the range recorded on tropical species by Hamrick and Loveless (1986) in general. This range was also found to be similar to the ones reported by Norwati *et al.* (1997) on the same species.

The range of the average number of allele per locus for the six seed sources of *A. excelsa* obtained in the present study was found to be within the range recorded on tropical species. This range was also about similar than that recorded by Norwati *et al.* (1997) but lower than those recorded on other popular tropical tree species for plantation such as *H. brasiliensis* (Paiva *et al*., 1994a, b), *P. macrocarpus* (Liengsiri *et al*., 1995) and *T. grandis* (Kertadikara and Prat, 1995).

The mean average of expected heterozygosities was 0.077 and this value was found to be lower than those reported on tropical species by Hamrick and Loveless (1986) in general. This value is comparable to those reported by Mohamad *et al*. (1997) on the same species and Wickneswari and Norwati (1993) on *A. auriculiformis*. The value was again found to be lower than those reported on *H. brasiliensis* (Paiva *et al*., 1994a, b), *P. macrocarpus* (Liengsiri *et al*., 1995) and *T. grandis* (Kertadikara and Prat, 1995).

The mean value of the proportion of polymorphic loci of six *A. excelsa* seed sources was 36.67. This value was found to lower than those reported by Hamrick and Loveless (1986) on tropical species. The value was comparatively similar to those reported by Norwati *et al.* (1997) on the same species as well as *A. auriculiformis* (Wickneswari and Norwati, 1993), *A. mangium* (Moran *et al*. 1989b) and *S. macrophylla* (Kanzaki *et al*., 1996).

Meanwhile, the value of the average mean number of alleles per locus for six *A. excelsa* seed sources was found to be within those reported by Hamrick and Loveless

Table 9: Morphological identity (above diagonal) and morphological distance (below diagonal) based on the morphological characteristics

Seed source[C]	Bukit Lagong[1]	Pengkalan Arang[2]	Narathiwat[3]	Manong[4]	Semengoh[5]	Pasir Mas[6]
Bkt. Lagong[1]	-	0.8975	0.8152	0.9889	0.8603	0.8536
Pgkl. Arang[2]	0.1082	-	0.9247	0.8884	0.9895	0.9414
Narathiwat[3]	0.2043	0.0782	-	**0.7930**	0.9291	**0.9952**
Manong[4]	0.0112	0.1184	**0.2319**	-	0.8551	0.9416
Semengoh[5]	0.1505	0.0106	0.0735	0.1565	-	0.8347
Pasir Mas[6]	0.1583	0.0604	**0.0048**	0.0602	0.1807	

[C]: Seed source code

(1986) for tropical species in general. The mean value obtained was again comparable to those reported by Norwati *et al.* (1997) on the same species as well as *A. auriculiformis* (Wickneswari and Norwati, 1993) and *A. mangium* (Moran *et al.*, 1989b).

Species which are widespread, long-lived woody and primarily outcrossed by wind or insect pollination have been reported to produce high levels of genetic diversity (Brown, 1978; Loveless and Hamrick, 1984; Hamrick and Godt, 1989; Hamrick *et al.*, 1992). *A. excelsa* is also expected to produce higher values of heterozygosities based on mode of pollen and seed dispersal mechanisms. *A. excelsa* can be classified as one of the entomophilus species pollinated by insects such as bees and moth while the fruit is transported by bats and birds. In contrast, lower level of heterozygosity was reported on all seed sources, might be assumed that the seed sources consist of small population size. This could be due to the species was confined to scattered distribution along farm or plantation boundaries, roadsides or sparsely grown which could lead to the reduction in population size. According to Kijkar and Boontawee (1995), this species is thought to be an introduced species in Peninsular Malaysia and not indigenous and may established from a very restricted genetic base. Species with small population densities often increased the possibility of genetic drift, which will reduce the genetic variability as a result of bottlenecking and inbreeding (Moran and Hopper, 1987). This phenomena is well discussed by other researchers such as Hamrick (1983), Chamberlain *et al.* (1996a, b) and Liengsiri *et al.* (1995).

Although the information gathered was inadequate to relate concisely on the actual level of genetic variation especially on the number of mother trees but the information gathered from Pengkalan Arang seed source could be used to explain the lower level of genetic variation. Lower values of heterozygosities would suggest that the seeds were collected from a few mother trees. For instance, ground truthing at Pengkalan Arang revealed that seed sources might originated from only two to three mother trees separated about eight to 10 m away. Also, there is a possibility that the seeds may be from the same maternal tree and thus leading to genetic heterogeneity reduction and limited gene exchange.

In addition, seed sources from Bukit Lagong and Semengoh were obtained from research plot which is an artificial stand where the genetic base of the planted population is usually be rather narrow. This postulation is supported by Dusan (1992) who found a lower value of mean heterozygosity of 0.275 from artificial stand of *Picea abies* when compared to the value (0.322) obtained from virgin forest. This is because the artificial stand normally receives uniform treatment levels, which explain for unnecessary increased for heterozygosity.

The percentage of proportion polymorphic loci among provenances was 36.67%, which is lower than those reported for other tropical trees. The low value of polymorphism suggests that the seed sources in the present study had lesser opportunities to evolve. Normally, polymorphism is required as part of the adaptive strategies of populations in a heterogenous environment of the forest (Feret and Bergman, 1976). Consequently, a species has to evolve to compete for optimum growth against other species for growth resources such as space, light, nutrient and water. A concrete discussion cannot be done further since information on the actual details of their history, origin and the ecological background of source materials are not available.

Genetic distance: Isozyme analysis in *A. excelsa* revealed 35 loci where 25 of them being polymorphic. One (Pgi-3) out of 25 polymorphic loci showed significant excess of heterozygosity (Table 4). Meanwhile, there was a general excess of homozygosity within seed sources, as revealed by the positive mean fixation index value. According to Gregorius and Namkoong (1983), a lack of heterozygosity can be observed in a population because of restriction of gene flow within the whole provenance and of increased of relatedness between neighbour individuals. Similarly, the results noted in an isozyme analysis of population structure of *Q. rubra* (Schwarzmann and Gerhold, 1991), in which observed heterozygosities did not differ significantly from those expected under random mating and in a population study of *F. sylvatica* (Cuguen *et al.*, 1988; Comps *et al.*, 1990) in which F_{IS} and F_{IT} values were generally positive indicating heterozygote deficit relative to panmixia. Houston and Houston (1993) stated that the

differences between two species in population structure might reflect differences in their reproductive strategies which result in differing mating patterns and existing stand structures. The positive mean of F_{IT} and F_{IS} values observed for this species implies an overall deficit of heterozygotes, perhaps caused by assortative mating between near neighbours related descent.

Generally, F_{ST} values with average 0.213 obtained in this study indicated moderate differentiation (Wright, 1978) between the two provenances which approximately 78.7% of the observed variation residing within seed source. This value was comparative to the one reported on *G. sepium* (0.172) by Chamberlain *et al.* (1996a). However, this value was higher than those reported on *P. macrocarpus* (0.121) by Liengsiri *et al.* (1995) and other tropical trees (0.119) by Hamrick *et al.* (1992). The value was also higher than those reported on *F. sylvatica* (0.060) by Cuguen *et al.* (1988), *Populus tremuloides* (0.030) by Jelinski and Cheliak (1992) as well as on European beech (0.054) by Comps *et al.* (1990). This level of population subdivision is high when compared with that of temperate tree species and this supports the notion that there is greater population differentiation in the tropics than in the temperate zone (Hamrick *et al.*, 1992). Possible reasons for higher levels of population differentiation in the tropical species have been reviewed by Bawa (1983). They include lower population densities, more widely scattered populations that reduce gene flow and increase genetic drift and greater spatial variation in the natural selection pressure. However, higher levels of population differentiation in present study might also be due to bottleneck effects where the sources of *A. excelsa* are mostly not from natural population (Nei, 1975).

Nei's (1978) statistics of genetic diversity was also calculated to analyse the distribution of allelic diversity. The average G_{ST} value indicated that approximately 29.84% of the observed variability was found among seed sources, more than the one estimated by F_{ST} and was higher than the average value found in other tropical tree species by Loveless (1992), i.e., G_{ST} = 10.9%. This value was also found to be higher than those reported on *F. sylvatica* (3.6%) by Comps *et al.* (1990), *Pinus kesiya* (3.9%), a wind-pollinated tropical pine by Boyle *et al.* (1991), *Q. macrocarpa* (7.6%) by Schnabel and Hamrick (1990) and *Rugelia nudicaulis* (1.5%) by Godt and Hamrick (1995). This result once again showed that most of the variation was found within seed sources.

The overall result corresponded to long-lived woody plants which acquired relatively high genetic diversity within species but most of the genetic diversity was found within populations and little existed among populations. The distribution of allozyme variation among populations is the product of interactions among several evolutionary factors. Of primary importance are selection, effective population size and the ability of the species to disperse pollen and seeds. On the other hand, species with more pollen and seed movement should have less differentiation among population than species with restricted gene flow (Hamrick, 1989). However, low level of diversity among seed sources and the high level of diversity within seed sources of *A. excelsa* in this study could be due to the certain relatedness among the seed sources or land races of this species whereby the seed sources might come from similar source in terms of ancestry genetic constituent. The reason is simply that *A. excelsa* is not usually found in the jungle or natural forests. It occurs mostly on farmland or marginal lands through shifting cultivation (Kijkar and Boontawee, 1995) and therefore likely to be naturalised in this area.

Cluster analyses: The dendrograms produced by the UPGMA clustering technique are presented in Fig. 1 and 2 for morphological and genetic values, respectively. The morphological and genetic identity values were found to range between 0.7930 and 0.9952 and 0.7727 and 0.9999, respectively. Such high ranges revealed that they were very much related both in terms of morphological and genetical aspects.

Two groups of clustering were formed using both techniques. Both dendrograms showed seed source of Pengkalan Arang, Narathiwat, Semengoh and Pasir Mas formed one cluster whereas Bukit Lagong and Manong seed sources formed the other group. Such relatedness among the seed sources are as expected based on their geographic distances among them. Narathiwat, Pasir Mas and Pengkalan Arang seed sources were located at a closer latitudes and longitudes. Similar clusteration based on latitudes, longitudes and altitudes have been reported for *Casuarina cunninghamiana* (Moore and Moran, 1989) and *A. crassicarpa* (Nor Aini *et al.*, 2006). Normally, as geographic distance between population is small or decreases, then the genetic similarity will increase.

The pattern of such clusteration is also found to be associated with historical factors. The Bukit Lagong seed source was once thought to be originating from Perak while the source from Thailand was believed to have some relationships with Borneon sources as a result from the scientific expedition made earlier. This is because, (Roland Kueh, unpublished) reported that a group of scientists from Thailand made an expedition for seed collection of *A. excelsa* to Borneo Island (i.e., Sabah and Sarawak). Perhaps, through such activity could possibly caused such relatedness between Sarawak and Thailand.

The general pattern exhibited by the dendrogram based on morphological similarity values is similar to that based upon genetic identity values with a few notable exceptions. The uncertainty regarding the phenetic-phylogenetic correspondence is due primarily to variation caused by the confounding selective forces as a result of changes in environment. The majority of allozyme loci are neutral with respect to natural selection and their distribution is largely dependent on the stochastic events of mutation, migration, drift and founder effect compared to morphological characteristics. If so, UPGMA clustering of genetic identity values could provide a less biased view of the evolutionary relationships of the species than the clustering of morphological similarity values.

CONCLUSION

Genetic parameters were found to produce low levels of genetic variability for all *A. excelsa* seed sources. The Manong seed source was found to possess higher level of diversity followed by Bukit Lagong, Semengoh, Narathiwat, Pasir Mas and Pengkalan Arang seed sources. Moreover, low level of polymorphism might indicate that this species has lesser opportunities to evolve in different environment pressures.

The overall result corresponded that most of the genetic diversity found within seed sources with little existing among seed sources where it is generalised the genetic diversity of woody plants. The founder effect could help to explain such phenomenon for this species. High values of morphological and genetic in cluster analyses suggests that the close relatedness of the seed sources. This argument is made based on the historical background and geographical distance among those seed sources. Seed sources having similar latitudes and longitudes were found to give smaller distance. Moreover, possible explanation on the relatedness between groups was also due to the source of the land races.

ACKNOWLEDGMENTS

We wish to express our deepest gratitude and thanks to Associate Professor Dr. Kamis Awang for his invaluable suggestions and comments. Our special thanks also extended to Mr. Salim Ahmad and Mr. Zakaria Taha for their assistance and co-operation during sample and information collection of the project. We would also like to thanks to Forestry Department of Peninsular Malaysia for providing us the research sites.

REFERENCES

Bawa, K.S., 1983. Pattern of Flowering in Tropical Plants. In: Handbook of Experimental Pollination Biology, Jones, C.E. and R.J. Little (Eds.). Van Nostrand and Reinhold Company, New York, pp: 394-410.

Boyle, T.J.B., C. Liengsiri and C. Piewluang, 1991. Genetic studies in a tropical pine-*Pinus kesiya* II. Genetic variation among four populations in Northern Thailand. J. Trop. For. Sci., 3: 308-317.

Brown, A.H.D., 1978. Isozyme, plant population genetic conservation. Theory Applied Genetic, 52: 55-57.

Chamberlain, J.R., N.W. Galwey and A.J. Simons, 1996. Population structure in *Gliricidia sepium* (Leguminosae) as revealed by isozyme variation. Silvae Genetica , 45: 112-118.

Chamberlain, J.R., C.E. Hughes and N.W. Galwey, 1996. Patterns of ioszyme variation in the *Leucaena shannonii* alliance (Leguminosae: Mimosoideae). Silvae Genetica, 45: 1-7.

Comps, B., B. Thiebaut, L. Paule, D. Merzdau and J. Letouzey, 1990. Allozymic variability in beechwoods (*Fagus sylvatica* L.) over central Europe: Spatial differentiation among and within populations. Heredity, 65: 407-417.

Cuguen, J., D. Merzeau and B. Thiebaut, 1988. Genetic structure of European beech stands (*Fagus sylvatica* L.): F-statistics and importance of mating system characteristics in their evolution Heredity, 60: 91-100.

Dusan, G., 1992. Effect of stand origin on the genetic diversity of Norway spruce (*Picea abies*) populations. For. Ecol. Manage., 54: 215-223.

Feret, P.P and F. Bergmann, 1976. Gel Electrophoresis of Proteins and Enzymes. In: Moderns Methods in Forest Genetics, Miksche, J.P. (Ed.). Springer, Berlin Heidelberg, New York, pp: 49-77.

Godt, M.J.W. and J.L. Hamrick, 1995. Allozyme variation in two Great Smoky Mountain endemics: *Glyceria nubigena* and *Rugelia nudicaulis* Heredity, 86: 194-198.

Gregorius, H.R. and G. Namkoong, 1983. Conditions for protected polymorphisms in subdivided plant populations. I. Uniform pollen dispersal. Theor. Population Biol., 24: 252-267.

Hamrick, J.L., 1983. The Distribution of Genetic Variations Within and Among Natural Plant Populations. In: Genetics and Conservation, Schonewald-Cox, C.M., S.M. Chambers, B. Macbryde and L. Thomas (Eds.). Benjamin-Cunnings, Menlo Park, New Jersey, pp: 335-348.

Hamrick, J.L. and M.D. Loveless, 1986. Isozyme variation in tropical trees: Procedures and preliminary results. Biotropica, 18: 201-207.

Hamrick, J.L., 1989. Isozymes and the Analysis of Genetic Structure in Plant Populations. In: Isozymes in Plant Biology, Soltis, D.E. and P.S. Soltis (Eds.). Dioscorides Press, Portland, Oregon, pp: 87-105.

Hamrick, J.L. and M.J.W. Godt, 1989. Allozyme Diversity in Plant Species. In: Plant Populations Genetics, Breeding and Genetic Resources, Brown, A.H.D., M.T. Clegg, A.L. Kahler and B.S. Weir (Eds.). Sinauer Associates, Massachusetts, pp: 43-63.

Hamrick, J.L., M.J. Godt and S.L. Sherman-Broyles, 1992. Factors influencing levels of genetic diversity in woody plant species. New Forests, 6: 95-124.

Hopper, S.D. and D.J. Coates, 1990. Conservation of genetic resources in Australia's flora and fauna. Proceedings of the Ecology Society of Australia, 16: 567-577.

Houston, D.B. and D.R. Houston, 1993. Spatial distribution of cohorts and clones in American beech stands. Proceeding 1st Northern Forest Genetics Conference, July 23-26, 1991, University of Vermont, Burlington,. pp: 24-24.

Jelinski, D.E. and W.M. Cheliak, 1992. Genetic diversity and spatial subdivision of *Populus trimulodes* (Salicaceae) in a heterogeneous landscape. Am. J. Bot., 79: 728-736.

Kanzaki, M., M. Watanabe, J. Kuwahara, J.J. Kendawang, H.S. Lee, S. Kunsuke and T. Yamakura, 1996. Genetic structure of *Shorea macrophylla* in Sarawak, Malaysia. Tropics, 6: 153-160.

Kertadikara, A.W.S. and D. Prat, 1995. Genetic structure and mating system in teak (*Tectona grandis* L.f.) provenances. Silvae Genetica, 44: 104-110.

Kijkar, S. and B. Boontawee, 1995. *Azadirachta excelsa* (Jack) Jacobs: A lesser known species. Review Paper No. 3, Asean Forest Tree Seed Centre Project Paper No. 3. Mual-Lek, Saraburi, Thailand pp: 33.

Liengsiri, C., F.C. Yeh and T.J.B. Boyle, 1995. Isozyme analysis of a tropical forest tree, *Pterocarpus macrocarpus* Kurz. in Thailand. For. Ecol. Manage., 74: 13-22.

Loveless, M.D. and J.L. Hamrick, 1984. Ecological determinants of genetic structure in plant populations. Ann. Rev. Ecol. Syst., 15: 65-95.

Loveless, M.D., 1992. Isozyme variation in tropical trees: Patterns of genetic organization. New Forest, 6: 67-64.

Martins-Corder, M.P. and C.R. Lopes, 1997. Isozyme characterisation of *Eucalyptus urophylla* (S.T. Blake) and *E. grandis* (Hill ex Maiden) populations in Brazil. Silvae Genetica, 45: 192-197.

Moore, N.J. and G.F. Moran, 1989. Microgeographical patterns of allozyme variation in *Casuarina cunninghamiana* Miq. within and between the Murrumbidgee and coastal drainage systems. Aust. J. Bot., 37: 181-192.

Moran, G.F. and S.D. Hopper, 1987. Conservation of the Genetic Resources of Rare and Widespread Eucalyptus in Remnant Vegetation. In: Nature Conservation: The Role of Remnants of Native Vegetation, Saunders, D.A., G.W. Arnold, A.A. Burbidge and A.J.M. Hopkins (Eds.). Surrey, Beatty and Sons, Chipping Norton, pp: 151-162.

Moran, G.F., O. Muona and J.C. Bell, 1989a. Breeding system and genetic diversity in *Acacia auriculiformis* and *A. crassicarpa*. Biotropica, 21: 250-256.

Moran, G.F., O. Muona and J.C. Bell, 1989b. *Acacia mangium*: A tropical forest tree of the coastal lowlands with low genetic diversity Evolution, 43: 231-235.

Nei, M., 1972. Genetic distance between populations. Am. Naturalist, 106: 283-292.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proceeding Naturalist of Academic Science, 70: 3321-3323.

Nei, M., 1975. Molecular Population Genetics and Evolution. 1st Edn. American Elsevier, New York, pp: 288.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89: 583-590.

Norwati, M., R. Wickneswari and S.S. Lee, 1997. Genetic Assessment of five Seed Sources of *Azadirachta excelsa*: A Preliminary Insight. In: *Azadirachta excelsa*: A Monograph, Ahmad, N. (Ed.). Research Pamphlet No. 120, Forest Research Institute of Malaysia, Kuala Lumpur, pp: 17-18.

Nor Aini, A.S, K.C. Tee and C. John Keen, 2006. Isozymes variation and relationships of selected *Acacia* species. Pak. J. Biol. Sci., 9: 1047-1051.

Paiva, J.R., P.Y. Kageyama, R. Vencovsky and P.B. Contel, 1994a. Genetics of rubber tree (*Hevea brasiliensis* Willd. ex Adr. de Juss.): Genetic variation in natural populations. Silvae Genetica, 43: 307-312.

Paiva, J.R., P.Y. Kageyama and R. Vencovsky, 1994b. Genetics of rubber tree (*Hevea brasiliensis* Willd. ex Adr. de Juss.): Mating system. Silvae Genetica, 43: 373-376.

Playford, J., C. Bell and G.F. Moran, 1993. Genetic variation of *Acacia melanoxylon*. ACIAR Proc., 32: 92-93.

Rogers, D.J. and T.T. Tanimoto, 1960. A computer analysis program for classifying plants. Science, 132: 1115-1118.

Schnabel, A. and J.L. Hamrick, 1990. Comparative analysis of population genetic structure in *Quercus macrocarpa* and *Q. gambelii* (Fagaceae). Syst. Bot., 15: 240-241.

Schwarzmann, J.F. and H.D. Gerhold, 1991. Genetic structure and mating system of northern red oak (*Quercus rubra* L.) in Pennsylvania. For. Sci., 37: 1376-1380.

Shannon, C.E. and W. Weaver, 1949. The Mathematical Theory of Communication. 1st Edn. University of Illinois Press, Urbana. .

Slatkin, M. and N.H. Barton, 1989. A comparison of three indirect methods for estimating average levels of gene flow. Evolution, 43: 1349-1368.

Weeden, N.F., 1989. Genetics of Plant Isozymes. In: Isozymes in Plant Biology, Soltis, D.E. and P.S. Soltis (Eds.). Dioscorides Press, Portland, Oregon, pp: 46-72.

Wickneswari, R. and M. Norwati, 1993. Genetic diversity of natural populations of *Acacia auriculiformis*. Aust. J. Bot., 41: 65-77.

Wickneswari, R., 1999. Understanding genetic variation of tropical tree species-the way forward 5th Conference on Forestry and Forest Product Research in Genetic and Tree Improvement: Towards Improved Planting Materials and Mass Production for Future Forestry, October 4-5, 1999, Forest Research Institute of Malaysia, Kuala Lumpur,. pp: 6-15.

Wright, S., 1969. Evolution and the Genetics of Populations. Vol. 2, The Theory of Gene Frequencies. 1st Edn. University of Chicago Press, Chicago. IL. .

Wright, S., 1978. Variability Within and Among Natural Population. Vol. 4, 1st Edn. University of Chicago Press, Chicago. IL. .