GENETIC VARIATION OF *DRYOBALANOPS AROMATICA* GAERTN. F. (DIPTEROCARPACEAE) IN PENINSULAR MALAYSIA USING MICROSATELLITE DNA MARKERS

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ABSTRACT

Nine microsatellite DNA markers, developed for *Shorea curtisii* (i.e. Shc01, Shc02, Shc03, Shc04, Shc07, Shc08, Shc09, Shc11 and Shc17), and one, developed for *Dryobalanops lanceolata* [DL(GT)202], were tested on *D. aromatica*. The primer developed for *D. lanceolata* showed no amplification in *D. aromatica*. Although all the primers designed for *S. curtisii* produced amplifications, primer Shc01 and Shc08 yielded many non-specific bands. The other seven codominant microsatellite DNA loci were used to analyse the genetic variation of *D. aromatica* from five populations in Peninsular Malaysia, namely Lenggor and Ulu Sedili (Johore), Lesong (Pahang), Kanching (Selangor) and Bukit Sai (Terengganu). The expected mean heterozygosity (H_e) was high (0.709) with values ranging from 0.684 (Lenggor) to 0.735 (Lesong). Most of the populations showed high and positive fixation indices indicating an excess of homozygotes. This implies a high level of inbreeding which may be caused by selfing or/and mating between closely related individuals. A gene diversity analysis showed that 93.3 % of the observed genetic diversity was contained within populations and 6.7 % (G_{ST} = 0.067) was due to differences among populations. The smallest genetic distance (D) was between Bukit Sai and Lesong (0.068) and the largest, between Ulu Sedili and Lesong (0.477). Mean gene flow (N_m) was high (2.94) with the highest value being between Lenggor and Bukit Sai (~6.98). The lowest gene flow occurred between Kanching and Ulu Sedili (1.95). The findings from this study imply that the four natural populations of *D. aromatica* in the east coast of Peninsular Malaysia may have been a single unfragmented population in the recent past. The results of this study also supports an earlier alternative hypothesis that the Kanching population in the west coast of Peninsular Malaysia originated from the east coast populations. This is supported by the dendrogram derived from the UPGMA cluster analysis of genetic distances in that the Kanching population is closely related to the Lesong and Bukit Sai populations from the east coast.

Key words: genetic variation, genetic differentiation, natural population, *Dryobalanops aromatica*, Dipterocarpaceae, microsatellite

INTRODUCTION

Dryobalanops aromatica Gaertn. F., locally known as kapur, occurs naturally in Sumatra, Peninsular Malaysia, the Riau Archipelago and Borneo (SYMINGTON 1943) (Figure 1). In Peninsular Malaysia, this tree is found naturally only in the east coast, south of latitude 5° N, except for small pockets in Rawang, Selangor (WYATT-SMITH 1963). In Terengganu, Pahang and Johore the tree is gregarious, covering nearly 90 % of the total timber volume (FOXWORTHY 1927). The population history of *D. aromatica* in the west coast of Peninsular Malaysia is not well understood. BURKILL (1935) suggested that the tree was introduced from the east coast by traders of crystalline camphor. This substance is obtained from the kapur tree and was traded from the seventh century among Europe, the Malacca port and other ports in the west coast.

Kapur is an important timber because of its economic value. The medium hard wood of kapur is suitable for heavy construction, to make poles, rafts, flooring, furniture, window panels, doors, stairs and railway sleepers (SER 1981). Kapur also produces crystallized camphor and oleo-resin used in traditional medicine.

Kapur is one of the local species identified for planting as quality timber in Peninsular Malaysia (APPANAH & WEINLAND 1993). However, before effective selection and breeding can be carried out, information on genetic parameters like mating system,

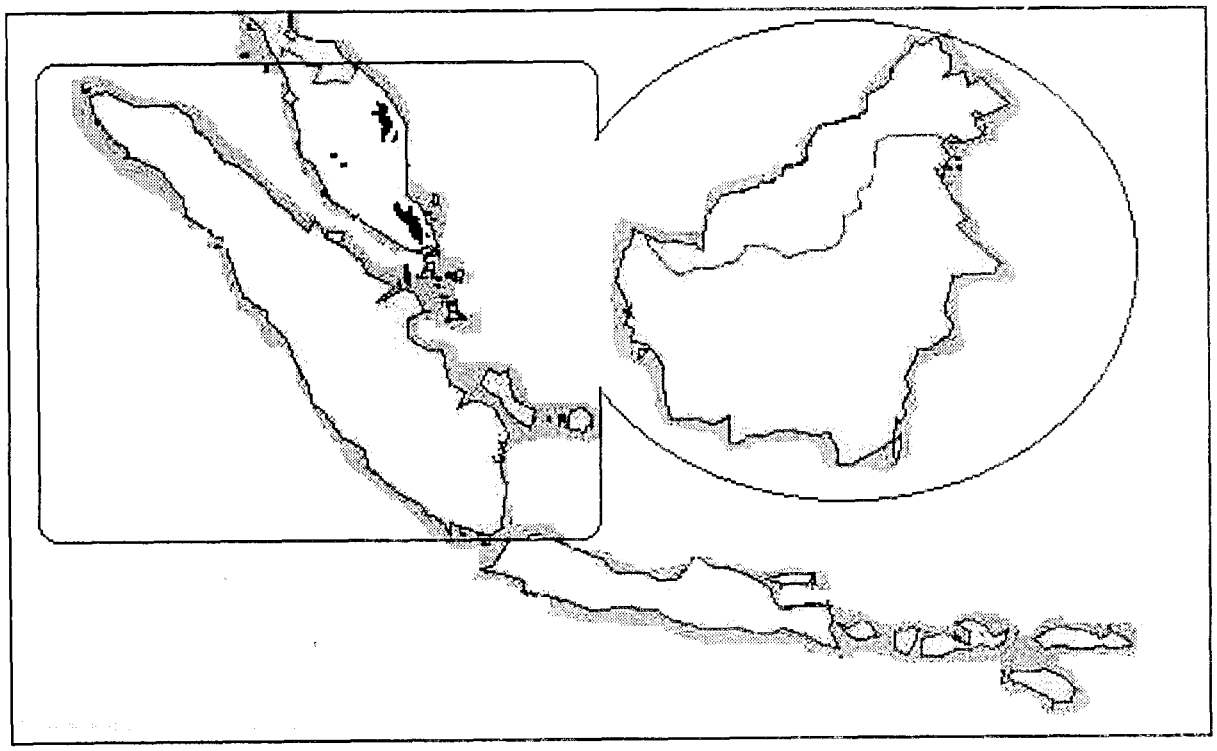

Figure 1. Distribution of *D. aromatica*. General distribution area in rectangle and circle. Distribution in Peninsular Malaysia in black. Source: ASHTON (1982) and SYMINGTON (1943).

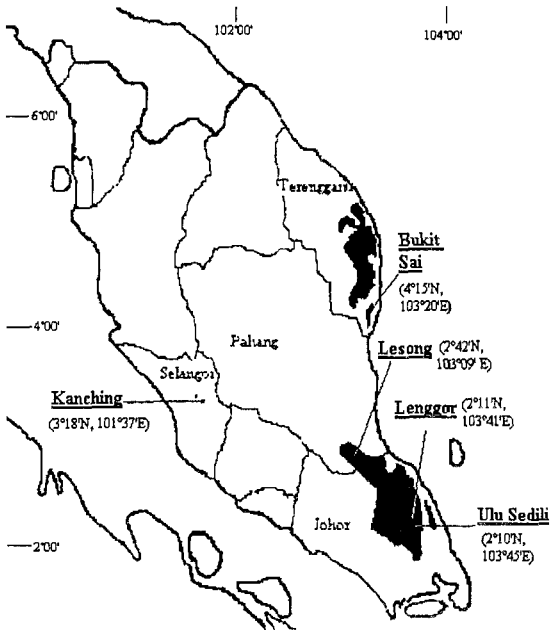

Figure 2. Location of *D. aromatica*. populations sampled in Peninsular Malaysia. Source: SYMINGTON (1943).

genetic variation and genetic structure of the species is needed. Only a few studies have been conducted on the genetic aspects of kapur. SHIRAISHI *et al.* (1994) used isozyme markers to study the population genetics of this species in Brunei. KITAMURA *et al.* (1994) also used isozymes to estimate outcrossing rates of *D. aromatica* in primary and secondary forests in Brunei. LEE (2000) studied the mating system of this species in three different forest types and a seed orchard in Peninsular Malaysia using isozyme markers. The first comprehensive study on genetic diversity of this gregarious species in Peninsular Malaysia was carried out by LEE *et al.* (2000b) using isozyme markers. The objective of this study is to further understand the genetic variation and genetic differentiaton of *D. aromatica* in Peninsular Malaysia using highly polymorphic microsatellite DNA markers.

MATERIALS AND METHODS

Leaf samples from 50 to 70 seedlings were collected from five natural populations of *D. aromatica* in Peninsular Malaysia i.e. Kanching (Selangor), Ulu Sedili and Lenggor (Johore), Bukit Sai (Terengganu) and Lesong (Pahang) (Figure 2). A transect-line sampling method as described by LEE *et al.* (2000a) was utilised as a guide for the sampling activities. One-two seedlings per mother tree were sampled. Adult trees were not sampled due to problems related to collecting

Table 1. Details of *Shorea curtisii* dan *Dryobalanops lanceolata* microsatellite markers.

Locus	Repeats	PCR Primer (5' to 3') *	PCR product length	Annealing temperature (° C)
Shc01	$(CT)_8(CA)_{10}CT(CA)_4C$ TCA	GCT ATT GGC AAG GAT GTT CA CTT ATG AGA TCA ATT TGA CAG	152	56
Shc02	$(CT)_2CA(CT)_5$	CAC GCT TTC CCA ATC TG TCA AGA GCA GAA TCC AG	149	54
Shc03	$(CT)_8$	TTG AAG GGA AGG CTA TG CTT CTC AAC TAC CTT ACC	124	54
Shc04	$(CT)_{16}$	ATG AGT AAC AAG TGA TGA G TAT TGA CGT GGA ATC TG	95	52
Shc07	$(CT)_8CA(CT)_5CACCC$ $(CTCA)_3CT(CA)_{10}$	ATG TCC ATG TTT GAG TG CAT GGA CAT AAG TGG AG	169	54
Shc08	$(CT)_{16}$	GAG TCT GTG GTT GAT ATG TTC TAT GCA AGG GCT TTT TAG	247	52
Shc09	$(CT)_{12}$	TTT CTG TAT CCG TGT GTT G GCG ATT AAG CGG ACC TCA G	197	51.6
Shc11	$(CT)_4TT(CT)_5$	ATC TGT TCT TCT ACA AGC C TTA GAA CTT GAG TCA GAT AC	166	54
Shc17	$(CT)_4TT(CT)_5$	CTA GAA TCC GCC ATT TCC CAC AAA TAC GTC TCC ATA TC	78	56
DL(GT) 202	$(GT)_5GC(GT)_5(GA)_6$	CAG CAC TTT TCT TTG ACA CA CAA TGG TGT AAT AGA AAG TT	201	50

* First row represents forward primer sequence (F)
Second row represents reverse primer sequence (R)

leaves from these tall emergent trees (~60 m high) and poor DNA quality of their inner bark tissues. Use of seedlings also allows good comparison with an earlier study of the same species using isozymes (LEE *et al.* 2000b) DNA was isolated with a modified method of hexadecyltrimethylammonium bromide (CTAB) (MURRAY & THOMPSON 1980). DNA purification followed the modification of MURRAY & THOMPSON (1980). Extracted DNA templates were further purified with a High Pure PCR Template Preparation Kit® (Boehringer Mannheim).

Nine microsatellite DNA markers, developed for *Shorea curtisii,* i.e. Shc01, Shc02, Shc03, Shc04, Shc07, Shc08, Shc09, Shc11 and Shc17 (UJINO *et al.* 1998), and one developed for *Dryobalanops lanceolata,* DL(GT)202 (TERAUCHI 1994), were tested on *D. aromatica*. Details of the markers appear in Table 1.

Polymerase chain reaction (PCR) was performed using GeneAmp® PCR System 9700 (Perkin Elmer) with 25 mL reaction volumes containing 10 ng genomic DNA. Annealing temperature was 50–56 °C, as appropriate for each primer pair (Table 1). PCR reaction mixtures contained 1x PCR buffer, 1.8mM $MgCl_2$, 0.2mM of each dNTP, 5pmol of each primer, 0.8 units of Platinum *Taq* DNA polymerase (Gibco BRL®). A PCR amplification was carried out for 3 min. at 95 °C, followed by 35 cycles of 45 sec. at 94 °C, 30 sec. at 50–56 °C and 45 sec. at 72 °C, with a final 5 min. incubation at 72 °C.

Amplified fragments of DNA were separated using 3.5 % high resolution agarose Metaphor® (FMC Corporation) in TBE buffer for about 3.5 hours at 80 V. 50 bp and 100 bp (Gibco BRL®) markers were used. 10^{-5}x Gelstar Gelstain® (FMC Corporation) was used to stain the gel before it was checked under ultraviolet light with Gelstar Gelstain® (FMS) filter. Gels were documented using 667 Polaroid film.

Analysis of allele segregation at microsatellite loci in *D. aromatica*

Inner bark tissues of two isolated mother trees at Forest Research Institute Malaysia (FRIM) were collected for DNA extraction. Leaves from ten seedlings surrounding each of the two isolated mother trees were also sampled. Genotypes were inferred from banding patterns of DNA fragments separated after PCR using seven primer pairs i.e. Shc02, Shc03, Shc04, Shc07, Shc09, Shc11 and Shc17 (Table 2).

Analysis of microsatellite DNA diversity in *D. aromatica*

PCR was performed using seven primer pairs i.e. Shc02, Shc03, Shc04, Shc07, Shc09, Shc11 and Shc17 (Table 2) that showed specific amplification in *D. aromatica*. Allele sizes were estimated for each locus and scored. The program BIOSYS–1 (SWOFFORD & SELANDER 1989) was used to test for deviation from Hardy Weinberg equilibrium, and to calculate allele frequencies, mean sample size per locus, mean number of alleles per locus, observed and expected heterozygosities, fixation index, genetic differentiation (G_{ST}) and genetic distance (NEI 1978). Effective number of alleles per locus was calculated using the formula of CROW & KIMURA (1970). Correlation between genetic and geographic distance for pairs of populations was tested using the Mantel matrix-correspondence test (ROHLF 1990, NTSYS-pc, version 2.0).

A statistic derived from microsatellite DNA loci, R_{ST} (GOODMAN 1997), was used to estimate levels of genetic differentiation, R_{ST} and gene flow, N_m among populations. As R_{ST} is based on stepwise mutation model, those loci which do not follow this model, namely Shc07 and Shc11 were excluded from this estimation. A jackknife procedure (without Kanching) was made to determine the effect the west coast population had on the overall diversity.

RESULTS

Allele segregation at microsatellite loci

Allele segregation of half-sib families and the inferred mother tree genotypes are shown in Table 2. No segregation of genotypes was detected for locus Shc11 and Shc17 in both families. Loci which were polymorphic i.e. Shc02, Shc03, Shc04, Shc07 and Shc09 shared a similar common allele in both families.

Population structure

The genetic diversity parameters for the five *D. aromatica* populations are given in Table 3. Mean number of alleles per locus ranged from 4.9 to 5.4 with a mean of 5.1 ± 0.2. Effective number of alleles per locus A_e was between 3.3 to 3.9 with a mean of 3.6 ± 0.02. The mean observed heterozygosity, H_o was 0.491 ± 0.060 with the lowest value found in the Ulu Sedili population (0.407) and the highest value in the Kanching population (0.555). Expected heterozygosity, H_e, ranged from 0.684 in the Lenggor population and 0.735 in the Lesong population with mean value 0.709 ± 0.020. Mean expected heterozygosity (H_e) was higher than mean observed heterozygosity (H_o) by 0.218 ± 0.154.

Allele frequency

Allele frequencies for the seven loci are presented in Table 4. The most frequent allele at each locus differed from population to population. Some alleles were present only in certain populations.

Hardy-Weinberg equilibrium

Generally, genotypic frequencies at most loci in the five populations did not fit Hardy-Weinberg equilibrium (Table 5). Only Shc17 in the Kanching population and Shc02 in the Ulu Sedili population fit Hardy-Weinberg equilibrium. In the Lenggor population, three loci fit Hardy-Weinberg equilibrium, i.e. Shc02, Shc03 and Shc04. Lesong had three loci which segregated according to the Hardy-Weinberg equilibrium, namely Shc02, Shc03 and Shc17. Genotypes at loci Shc04, Shc09 and Shc17 also conformed to Hardy-Weinberg equilibrium in the Bukit Sai population.

Fixation index

Overall fixation index, F_{is} was positive in each population, for all loci, except for Shc02 (Table 6). Mean F_{is} was high for all populations, ranging between 0.225 and 0.428. Negative values for Shc02 showed an excess of heterozygotes in all populations. Positive values at all other loci in all populations showed an excess of homozygous individuals.

Genetic differentiation

From values of microsatellite data, total gene diversity of *D. aromatica* was very high with the highest value at locus Shc07 (0.850) and the lowest value at locus Shc04 (0.637) (Table 7). Mean total gene diversity, H_T for *D. aromatica* was 0.759. The degree of genetic

Table 2. Allele segregation of half-sib families and inferred mother tee genotype.

Locus	1st half-sib family		2nd half-sib family	
	Inferred parent genotype	Progeny genotype	Inferred parent genotype	Progeny genotype
Shc02	6_	36 (4), 66 (2), 67 (1), 68 (3)	6_	36 (3), 66 (4), 67 (3)
Shc03	1_	11 (6), 12 (4)	1_	11 (6), 12 (4)
Shc04	1_	11 (5), 12 (5)	1_	11 (5), 12 (5)
Shc07	8_	68 (3), 78 (4), 88 (3)	8_	68 (6), 78 (4)
Shc09	3_	23 (1), 33 (3), 35 (3), 37 (3)	3_	33 (1), 35 (3), 37 (6)
Shc11	3_/4_	34 (10)	3_/4_	34 (10)
Shc17	1_/3_	13 (10)	1_/3_	13 (10)

No. of genotypes present given in parentheses

Table 3. Genetic diversity parameters in the five *D. aromatica* populations.

Population	N	P	A_a	A_e	Mean heterozygosity	
					H_o	H_e
Kanching	19.1 (1.2)	100	4.86 (0.80)	3.69	0.555 (0.078)	0.721 (0.035)
Lenggor	17.4 (0.5)	100	5.14 (0.67)	3.36	0.454 (0.082)	0.684 (0.057)
Lesong	17.1 (0.7)	100	5.14 (0.59)	3.92	0.506 (0.104)	0.735 (0.039)
Bukit Sai	17.0 (0.6)	100	5.14 (0.70)	3.60	0.531 (0.087)	0.707 (0.048)
Ulu Sedili	18.7 (0.9)	100	5.43 (0.69)	3.60	0.407 (0.108)	0.700 (0.049)
Mean	17.9 (1.0)	100 (0)	5.14 (0.20)	3.63 (0.20)	0.491 (0.060)	0.709 (0.020)

Standard deviations given in parentheses;
N – Mean number of samples per locus; P – Percent polymorphic loci; A_a – Mean number of alleles per locus; A_e – Effective

differentiation among populations was low (0.067), that is 93.3 % of the observed genetic diversity were among individuals within population and 6.7 % were among populations. Analysis without loci Shc07 and Shc11 did not show much difference with the degree of genetic differentiation being 0.062, that is 93.8 % diversity within population and 6.2 % among population. Mean H_T and gene diversity among populations, D_{ST} were not very different with only an increase of 2.7 % and a decrease of 0.5 % each.

Jackknife analysis without Kanching (Table 7) showed that the degree of genetic differentiation in Kanching was not much different from the differentiation in the other populations. Lowest gene diversity was at locus Shc09 (0.014), whereas highest gene diversity was seen at locus Shc04 (0.112).

Comparison of G_{ST} analysis (Table 7) and R_{ST} (Table 8) showed that genetic differentiation from R_{ST} analysis was higher by 2.8 %.

Gene flow

Mean R_{ST} for all populations was 0.09 and ranged from 0.03 to 0.15 (Table 8). The smallest mean R_{ST} value was between populations Bukit Sai and Lenggor (0.03). Whereas the largest values were between populations Kanching and Ulu Sedili and also Bukit Sai and Ulu Sedili (0.15).

Mean gene flow, Nm was high (2.94). Negative values of gene flow between Bukit Sai and Kanching populations (–1.16) and Lenggor and Ulu Sedili populations (–6.98) showed that gene flow was extremely high. Lowest gene flow was between Ulu Sedili and Kanching (1.95) and Ulu Sedili and Bukit Sai (1.96).

Genetic distance

Genetic and geographic distance of the five populations are given in Table 9. Bukit Sai and Lesong populations gave the lowest genetic distance (0.068). Genetic distance between Ulu Sedili and Lesong populations were the highest (0.477). Geographically, Lenggor is nearest to Ulu Sedili (5 km) whereas Kanching is furthest from Ulu Sedili (280 km). Genetic distance between Lenggor and Ulu Sedili was low (0.172) whereas Kanching and Ulu Sedili was high (0.442).

Table 4. Microsatellite loci allele frequency in the five populations of *D. aromatica*.

Loci	Allele	Population				
		Kanching	Lenggor	Lesong	Bukit Sai	Ulu Sedili
Shc02	1	0.023	0.056	0.079	0.132	0.114
	2	0.000	0.028	0.000	0.026	0.023
	3	0.182	0.111	0.211	**0.237**	0.182
	4	0.136	**0.472**	0.132	**0.237**	0.182
	5	**0.227**	0.111	**0.316**	**0.237**	0.068
	6	0.068	0.000	0.079	0.026	0.114
	7	0.182	0.083	0.132	0.053	0.045
	8	0.182	0.139	0.053	0.053	**0.273**
Shc03	1	**0.441**	0.079	0.300	0.316	0.026
	2	0.324	**0.789**	**0.475**	**0.447**	0.368
	3	0.235	0.079	0.050	0.158	**0.526**
	4	0.000	0.053	0.175	0.079	0.079
Shc04	1	0.250	0.206	0.250	0.029	0.250
	2	0.250	0.382	0.250	0.294	**0.688**
	3	**0.500**	**0.412**	**0.500**	**0.676**	0.063
Shc07	1	0.050	**0.289**	0.083	0.059	**0.306**
	2	0.200	0.158	0.083	**0.353**	0.250
	3	0.025	0.237	**0.306**	0.235	0.000
	4	0.225	0.053	0.139	0.147	0.028
	5	0.175	0.184	0.083	0.088	0.194
	6	**0.275**	0.053	0.167	0.059	0.111
	7	0.025	0.000	0.000	0.059	0.083
	8	0.025	0.026	0.139	0.000	0.028
Shc09	1	0.000	0.029	0.000	0.133	0.000
	2	0.065	0.265	0.156	0.133	0.024
	3	**0.348**	0.059	**0.250**	0.067	0.071
	4	0.152	**0.324**	0.156	**0.333**	**0.357**
	5	0.043	0.118	0.125	0.000	0.143
	6	0.130	0.029	0.156	0.133	0.214
	7	0.261	0.176	0.156	0.200	0.190
Shc11	1	0.048	0.294	0.375	0.412	0.250
	2	0.214	**0.382**	0.125	0.059	**0.406**
	3	0.286	0.176	0.094	0.088	0.313
	4	**0.452**	0.147	**0.406**	**0.441**	0.031
Shc17	1	0.333	0.033	0.100	0.300	0.079
	2	0.133	**0.500**	0.033	0.067	**0.500**
	3	0.000	0.267	**0.567**	0.200	0.237
	4	**0.533**	0.200	0.267	**0.433**	0.079
	5	0.000	0.000	0.000	0.000	0.053
	6	0.000	0.000	0.033	0.000	0.053

Most frequent allele at each locus in bold.

Mantel matrix-correspondence test revealed no significant correlation ($r = 0.278$, $p = 0.229$) between genetic and geographic distance for the five populations of *D. aromatica*.

UPGMA dendrogram showed two main clusters with Kanching, Lesong and Bukit Sai as one cluster and Lenggor and Ulu Sedili as another cluster (Figure 3). Kanching was separated from Lesong and Bukit Sai.

Table 5. χ^2 test for deviation from Hardy-Weinberg equilibrium

Locus	Kanching	Lenggor	Lesong	Bukit Sai	Ulu Sedili
Shc02	40.331	10.540*	32.785*	55.206	33.641*
Shc03	10.364	8.325*	12.718*	15.072	20.410
Shc04	20.185	7.012*	16.172	3.386*	8.357
Shc07	58.259	72.907	51.590	54.664	34.281
Shc09	62.830	51.11	45.985	23.183*	62.367
Shc11	58.530	17.946	47.012	51.684	35.629
Shc17	4.162*	29.121	17.138*	8.167*	73.175

* Significantly different at $p \leq 0.05$

Table 6. Fixation index (F) in the five *D. aromatica* populations.

Loci	Population				
	Kanching	Lenggor	Lesong	Bukit Sai	Ulu Sedili
Shc02	–0.101	–0.154	–0.175	–0.043	–0.100
Shc03	0.362	0.126	0.002	0.292	0.547
Shc04	0.400	0.175	0.200	0.224	0.458
Shc07	0.249	0.537	0.254	0.397	0.151
Shc09	0.033	0.393	0.469	0.073	0.501
Shc11	0.428	0.259	0.907	0.717	0.907
Shc17	0.205	0.791	0.440	0.016	0.533
Mean	0.225	0.304	0.300	0.239	0.428

Table 7. Genetic differentiation parameters of the five *D. aromatica* populations

Locus	H_T		H_S		D_{ST}		G_{ST}	
	+KC	–KC	+KC	–KC	+KC	–KC	+KC	–KC
Shc02	0.836	0.832	0.815	0.807	0.021	0.025	0.025	0.030
Shc03	0.665	0.646	0.597	0.580	0.068	0.066	0.102	0.102
Shc04	0.637	0.633	0.579	0.562	0.058	0.071	0.092	0.112
Shc07	0.850	0.843	0.816	0.816	0.034	0.027	0.039	0.032
Shc09	0.823	0.820	0.802	0.808	0.021	0.012	0.025	0.014
Shc11	0.744	0.736	0.689	0.688	0.055	0.048	0.073	0.065
Shc17	0.754	0.747	0.655	0.667	0.099	0.080	0.132	0.107
Mean	0.759	0.751	0.708	0.704	0.051	0.047	0.067	0.062
Mean (without Shc07, Shc11)	0.786		0.737		0.049		0.062	

+ KC – analysis including Kanching population,
– KC – analysis excluding Kanching population.

DISCUSSION

DOWNEY & IEZZONI (2000) succeeded in amplifying black cherry (*Prunus serotina*) using primers developed for other species developed for the same genus i.e. sweet cherry (*P. avium*), peach (*P. persica*) and sour cherry (*P. cerasus*). In this study application of interspecific microsatellite primers Primer DL(GT)202 developed for *D. lanceolata* did not show any amplifi-

Table 8. R_{ST} and *Nm* values of five *D. aromatica* populations (without Shc07 and Shc11).

	Kanching	Lenggor	Lesong	Bukit Sai	Ulu Sedili	Mean *Nm*
Kanching		9.66	5.71	-1.16	1.95	2.94
Lenggor	0.06		21.00	-6.98	2.74	
Lesong	0.06	0.04		13.48	2.19	Mean R_{ST}
Bukit Sai	0.09	0.03	0.04		1.96	0.09
Ulu Sedili	0.15	0.11	0.10	0.15		

Nm – values given above diagonal line
R_{ST} – values given below diagonal line

Table 9. Genetic distance and geographic distance among five *D. aromatica* populations.

	Kanching	Lenggor	Lesong	Bukit Sai	Ulu Sedili
Kanching		270	190	210	280
Lenggor	0.352		80	240	5
Lesong	0.183	0.197		180	90
Bukit Sai	0.113	0.208	0.068		250
Ulu Sedili	0.442	0.172	0.477	0.474	

Geographic distance above diagonal (in km)
Genetic distance below diagonal

cation which maybe because of that there was no annealing site in *D. aromatica*. Primer Shc01 and Shc08 developed for *S. curtisii* were non-specific for *D. aromatica*. Non-specific amplifications may have been due to the presence of more than one annealing site.

Other primers, i.e., Shc02, Shc03, Shc04, Shc07, Shc09, Shc11 and Shc17 also developed for *S. curtisii* revealed specific amplifications on agarose gel, showing that these primers can be used on *D. aromatica*.

Number of alleles detected at microsatellite loci, which had mixed repeats, i.e., Shc02 and Shc07, were high. Whereas loci Shc03 and Shc04 which had simple CT repeats, had low number of alleles, i.e., only four and three respectively. However, locus Shc09, which also contained simple CT repeats, revealed a high number of alleles (seven). BRUFORD & WAYNE (1993) reported that number of alleles were higher at loci that were developed specific for a particular primer compared to loci which are not specific. This maybe because during sequence selection of the original primer, longer sequences (typically 12 repeats) are selected as primers. When used for different species, alelle variation at these loci may be shorter or contain other repeats in between, hence reducing the mutation rate and

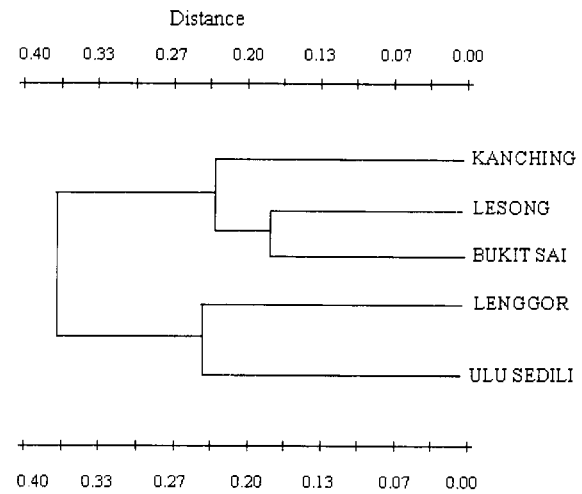

Figure 3. Dendrogram from UPGMA cluster analysis based on NEI's genetic distance (1978) among five *D. aromatica* populations.

diversity in spite of population size. This reduced detection of number of alelles still needs to be studied. This was observed in amplifications among sea turtles (FITZSIMMONS *et al.* 1995) and acacias (BUTCHER *et al.* 2000), but not among wombats (TAYLOR *et al.* 1994).

No amplification was obtained from the mother trees which maybe due to the fact that there was a higher level of secondary metabolites that caused more oxidation and accumulation of polysaccharide compounds. NAGAMITSU *et al.* (2001) also could not amplify microsatellites from inner bark tissues of mature trees of *Shorea leprosula*. Segregation of genotypes according to Mendelian ratios could not be determined due to limited sample size of the half-sibs and failure to genotype the mother trees sampled.

xation index

Genotype frequencies of the microsatellite loci that deviated from Hardy-Weinberg equilibrium may be caused by inbreeding and small population sizes. Seedlings have been reported to have high homozygosity (MUONA 1989). Samples that could not be amplified due to oxidation by phenolic compounds caused the sample size to drop. Therefore, estimation of homozygosity might not be accurate. However, sampling was done at random for this study. Thus there was no bias in selection of monomorphic or polymorphic loci. EDWARDS *et al.*'s (1992) study on populations of different human races in North America also gave a genotype ratio that deviated from Hardy-Weinberg equilibrium due to a lack of heterozygotes.

Outcrossing rates (t_m) in *D. aromatica* can vary from 0.86–0.92 in primary forests to 0.77–0.79 in logged forests, 0.67 in artificial forest and 0.55 in seed orchard (KITAMURA *et al.* 1994, LEE 2000). High correlated mating (r_p=0.08) and biparental mating ($t_m - t_s$=0.39) have been detected in *D. aromatica* (LEE 2000). Thus, the high homozygosity observed from positive fixation indices in seedling samples may have been produced through selfing or half-sib mating. A similar observation had also been shown through the study on *Pinus* sp. (MUONA 1989). According to MUONA (1989), positive fixation indices at seedling level might be due to self-pollination. Seedlings produced through inbreeding usually die off before maturity since they are weaker than outbred progenies. This follows the theory of natural selection which states that selection is the dominant force in evolution (HARTL 1980). Excess of heterozygosity in locus Shc02 shown by a negative value might mean that this locus maybe co-segregating with a trait responding to selection.

Wahlund effect states that natural populations that are divided into sub-populations might have genotype frequencies that deviate from Hardy-Weinberg equilibrium due to natural selection or random genetic drift in the case of small populations (WAHLUND 1928). The effect is homozygous genotype frequency of the whole population becomes more than Hardy-Weinberg equilibrium, whereas heterozygotes of the whole population decreases from the Hardy-Weinberg equilibrium. In the case of *D. aromatica*, it could be said that all the populations in Peninsular Malaysia originated from one large population which had been sub-divided into a few sub-populations. This would explain the high fixation indices observed in all *D. aromatica* populations. Excess of homozygotes was also detected in *D. aromatica* populations in Peninsular Malaysia using isozyme markers (LEE *et al.* 2000b)

Genetic diversity in *D. aromatica* populations

Out of the seven microsatellite loci studied, 40 alleles were observed, which is a mean of 5.14 ± 0.20 alelles per locus. This value is higher than those obtained from isozyme markers for conifers (2.29) and tropical species (2.02) (HAMRICK & LOVELESS 1989). LEE *et al.* (2000b) obtained 4.1 alleles per locus in *D. aromatica*, using isozyme markers. Microsatellite markers give a high number of alleles due to length mutation that causes differences in repeat units. AMOS *et al.* (1993) obtained 54 alelles from one whale microsatellite locus and FORNAGE *et al.* (1992) obtained 15 alelles in one locus located on the first intron of the C-II apoliprotein gene in a French human population. In tropical trees slightly lower number of alleles were detected i.e. 25 alleles at one locus (Shc07) in *S. leprosula* (NAGAMITSU *et al.* 2001) and 17 alleles at one locus (Nhe015) in *Neobalanocarpus heimii* (IWATA *et al.* 2000).

Expected heterozygosity was more than the observed value by 0.218, showing an excess in homozygotes. This may be because leaf samples of seedlings were used in this study. Expected heterozygosity (H_e = 0.709) was higher than other tropical timber species (H_e = 0.125, HAMRICK *et al.* 1992) and also higher compared to temperate tree species, especially conifers (H_e = 0.145, HAMRICK *et al.* 1992), based on isozyme markers. LEE *et al.*'s (2000b) study on *D. aromatica* using isozyme yielded H_e = 0.459, which is lower than the value obtained from this study. Estimates of expected heterozygosity from microsatellite data for tree species are generally based on one or two populations where the focus was mainly on development of microsatellite primers and their applicability in related species. Thus, they do not provide good comparisons to this study.

According to HAMRICK *et al.* (1979), long-lived trees have a higher level of hetorozygosity. This is

important for the survival of species in a changing environment especially when natural selection occurs. This was proven by NIKOLIĆ & TUCIĆ (1983) on *Pinus nigra*, a long-lived plant. HIEBERT & HAMRICK (1983) supported this theory with a study on *P. longaeva* that showed a high level of H_e.

High levels of genetic diversity can be associated with population history, strategy and life history of the species like outcrossing, long life, distribution and high fecundity.

The highest effective number of alleles per locus (A_e) was found in Lesong population whereas the lowest was found in Lenggor population. Studies on these five populations with isozyme markers showed that Ulu Sedili population had the highest effective number of alleles per locus (2.7), whereas Kanching population had the lowest (2.4) (LEE *et al.* 2000b). Lesong population showed the highest expected heterozygosity (0.735), whereas Lenggor population revealed the lowest (0.684) from microsatellite markers. This differed from the isozyme study carried out by LEE *et al.* (2000b) where out of these five *D. aromatica* populations, Lenggor population showed the highest expected heterozygosity (0.475) and Kanching population revealed the lowest (0.459). Isozymes are markers that are limited to coding regions of genes. Meanwhile microsatellite markers can be found dispersed in the whole eukaryote genome. Therefore, microsatellite markers may give a clearer picture of the genetic diversity of species.

Allele frequency at each locus differed from population to population. This was also portrayed in the study of human populations of Negro, Mexican whites and Asians in America (EDWARDS *et al.* 1992). FORNAGE *et al* (1992) also reported a complex allele frequency distribution at the first intron of the apoliprotein gene C–II. High mutation rate at microsatellite loci, that is 10^{-4} to 10^{-6} (DALLAS 1992, EDWARDS *et al.* 1992) may cause the presence of high number of alleles. Therefore, a large sample size may be needed to represent all the alleles present in the population and for the all the alleles to be in Hardy-Weinberg equilibrium.

Genetic differentiation and gene flow

Tropical species have been reported to have low genetic differentiation (HAMRICK & LOVELESS 1989). G_{ST} values of locally distributed tropical species, seeds which are pollinated by animals and seeds dispersed by gravity have values of 0.119, 0.092 and 0.131 each (HAMRICK *et al.* 1992). Mean G_{ST} of conifer is 0.068 (HAMRICK 1989). Tropical pine species showed low genetic differentiation among populations (HAMRICK 1989). LEE *et al.* (2000a) reported a G_{ST} of 0.117 for *Shorea leprosula*, a dipterocarp in Peninsular Malaysia. G_{ST} for *D. aromatica* based on isozyme analysis also revealed low values (0.042, LEE *et al.* 2000b). From this study, genetic differentiation was higher (G_{ST} = 0.062, R_{ST} = 0.09) than that obtained using isozyme markers for *D. aromatica* in Peninsular Malaysia. This is because microsatellite loci are more polymorphic than isozyme loci. GAGGIOTTI *et al.* (1999) showed that in small sample sizes ($n < 10$) and a few loci ($n < 20$), R_{ST} gave higher estimates. In this study R_{ST} estimates exceeded G_{ST} by 0.028.

High gene flow level caused low values of genetic differentiation among populations (MITTON 1992). LEE *et al.* (2000b) obtained high gene flow among ten *D. aromatica* populations in Peninsular Malaysia with gene flow Nm = 5.70 and level of genetic differentiation G_{ST}= 0.042.

Negative gene flow, Nm values showed that allele size variation within population was higher than variation among populations. Therefore, most genetic variation was within population and variation among population was less. This meant that these populations have little genetic variation and are panmictic by nature (GOODMAN 1997). Negative Nm are also considered to be too high to be estimated using this approach (GOODMAN 1997), which can clearly be seen from microsatellite data of *D. aromatica* obtained from this study which has a low mean genetic differentiation (0.09) and a high mean gene flow (2.94).

Genetic differentiation analysis, R_{ST} that was designed especially for microsatellite data showed correlation between level of genetic differentiation, R_{ST} and gene flow, Nm. Bukit Sai and Lenggor populations which had the lowest genetic differentiation, showed the highest level of gene flow (–6.98). Whereas Ulu Sedili and Kanching populations and Ulu Sedili and Bukit Sai populations, that had the highest genetic differentiation, had the lowest level of gene flow.

Geographically, Kanching is the furthest from Ulu Sedili. Gene flow decreases with distance. This was clear from gene flow and genetic differentiation values which showed low gene flow and high genetic differentiation between these two populations.

Analysis without Kanching population showed that Kanching was not much different from the other populations in the east coast of Peninsular Malaysia. This supports BURKILL's (1935) hypothesis that the Kanching population was introduced from other populations in the east coast. The low genetic differentiation value supports the hypothesis that all the populations of *D. aromatica* in Peninsular Malaysia might have been one large population that had been divided into subpopulations due to fragmentation.

Genetic distance

Mantel matrix-correspondence test revealed no significant correlation between genetic distance (*D*) and geographic distance. Even though Ulu Sedili was nearest to Lenggor in terms of geographic distance, genetic distance between these two places was not the lowest. The same applied for the furthest genetic distance, between Lesong and Ulu Sedili, which was not reflected geographically, with the furthest distance being between Ulu Sedili and Kanching.

This may be because *D. aromatica* populations in Peninsular Malaysia are sub-populations formed from fragmentation and limited migration of one original large population. Because of high gene flow among sub-populations in the east coast, genetic distance correlated positively with geographic distance. However, the correlation was not significant.

In situ conservation of *D. aromatica* only requires a few populations. This is due to the higher diversity within populations compared to among populations. For *ex-situ* conservation, it is suggested that focus is given to variation among individuals within populations so that all the variations are sampled. Population selection should be done based on the highest diversity in terms of alelle or genotype.

High diversity within populations also gives a wider base for improvement programs. The basis for selection for this process is similar to *ex-situ* conservation but is more focused upon the highest level of heterozygosity. To carry out a selection program effectively, selection of more individuals within population needs to be done so that high genetic diversity can be maintained.

The samples for this study were from leaves of seedlings. It is suggested that further studies are carried out with samples from different age classes to give a better picture of the genetic differentiation among populations. As there is no study that relates microsatellite genotypes with morphological and physiological characteristics thus far, results from this study cannot be used as the sole criterion for sampling with the aim of conservation and breeding. Empirical studies on the level and distribution of genetic diversity for morphological and physiological characteristics are suggested. For breeding programs, selection criteria like wood quality, growth rate and adaptability to certain environments have to be taken into consideration.

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REFERENCES

APPANAH, S. & WEINLAND G. 1993: Planting quality timber trees in Peninsular Malaysia – a review. *Malayan Forest Record No. 38.* Forest Research Institute Malaysia, Kepong, Malaysia.

AMOS, B., SCHLOTTERER, C. & TAUTZ, D: 1993: Social structure of pilot whales revealed by analytical DNA profiling. *Science* **260**:670–672.

BRUFORD, M. W. & WAYNE, R. K. 1993: Microsatellites and their application to population genetic studies. *Current Opinion in Genetics & Development* **3**:939–943.

BUTCHER, P.A., DECROOCQ, S., GRAY, Y. & MORAN G. F. 2000: Development, inheritance and cross-species amplification of microsatellite markers from *Acacia mangium. Theoretical and Applied Genetics* 101: 1282 –1290.

BURKILL, I. H. 1935: A Dictionary of the Economic Products of the Malay Peninsula. Crown Agents for the Colonies, London.

DALLAS, J. F. 1992: Estimation of microsatellite mutation rates in recombinant inbred strains of mouse. *Mammalian Genome* 5:32–38.

DOWNEY, S. L. & IEZZONI, A. F. 2000: Polymorphic DNA markers in black cherry (*Prunus serotina*) are identified using sequences from sweet cherry, peach, and sour cherry. Journal of the American Society for Horticultural Science 125(1) : 76–80.

EDWARDS A., HAMMOND, H. A., JIN, L., CASKEY, C. T. & CHAKRABORTY, R.. 1992: Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. *Genomics* 12:241–253.

FITZSIMMONS, N. N., MORITZ, C. & MOORE, S. S. 1995: Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. *Molecular Biology & Evolution* 12:432–440.

FORNAGE, M., CHAN, L., SIEST, G. & BOERWINKLE, E. 1992: Allele frequency distribution of the $(TG)_n(AG)_m$ microsatellite in the apoliprotein C–II gene. *Genomics* **12**:63–68.

FOXWORTHY, F. W. 1927: Commercial timber trees of the Malay Peninsula. *Malayan Forest Record No. 3*, Forest Research Institute, Kepong, Malaya.

GAGGIOTTI, O. E., LANGE, O., RASSMANN, K. & GLIDDON, C. 1999: A comparison of two indirect methods for estimating average levels of gene flow using microsatellite data. *Molecular Ecology* 8(9) :1513–1520.

GOODMAN, S. J. 1997: RST CALC: a collection of computer programs for calculating unbiased estimates of genetic differentiation and determining their significance for microsatellite data. *Molecular Ecology* 6 :881–885.

HAMRICK, J. L. 1989: Isozyme and analysis of genetic structure in plant populations. *In:* Isozymes in plant biology. (ed. D.E. Solstis & P.S. Soltis). pp. 87–105. Dioscorides Press, Portland.

HAMRICK, J. L. & LOVELESS, M. D. 1989: The genetic structure of tropical tree populations: Association with reproductive biology. *In*: The evolutionary ecology of plants. (ed. J. Bock & Y.B. Linhart). pp. 129–146. Westview Press, Colorado.

HAMRICK, J. L., GODT, M. J. W. & SHERMAN-BROYLES, S. L. 1992: Factor influencing levels of genetic diversity in

woody plant species. *New Forests* 6:95–124.

HAMRICK, J. L., LINHART, Y. B. & MITTON, J. B. 1979: Relationship between life history characteristics and elecrophoretically detectable genetic variation in plants. *Annual Review of Ecology and Systematics* **10**:173–200.

HARTL, D. L. 1980: Principles of Population Genetics. Sinauer, Sunderland, MA.

HIEBERT, R. D. & HAMRICK, J. L. 1983: Patterns and levels of genetic variation in Great Basin bristlecone pine, *Pinus longaeva*. *Evolution* 37(2):302–310.

IWATA, H., KONUMA , A. & TSUMURA, Y. 2000: Development of microsatellite markers in the tropical tree *Neobalanocarpus heimii* (Dipterocarapaceae). *Primer Notes* 1684 –1685.

KITAMURA, K., ABDUL RAHMAN, M. Y., OCHIAI, Y. & YOSHIMURA, H. 1994: Estimation of outcrossing rate on *Dryobalanops aromatica* Gaertn. F. in primary and secondary forest in Brunei, Borneo, Southeast Asia. *Plant Species Biology* 9 : 37–41.

LEE, S. L. 2000: Mating system parameters of *Dryobalanops aromatica* Gaertn. f. (Dipterocarpaceae) in three different forest types and a seed orchard. *Heredity* 85:338–345.

LEE, S. L., WICKNESWARI, R. MAHANI, M. C. & ZAKRI, A. H. 2000a: Genetic diversity of a tropical tree species, *Shorea leprosula* Miq. (Dipterocarpaceae), in Malaysia: Implications for conservation of genetic resources and tree improvement. *Biotropica* 32(2) :213–224.

LEE, S. L., ANG, K. C. & NORWATI, M. 2000b: Genetic diversity of *Dryobalanops aromatica* Gaertn. f. (Dipterocarpaceae) in Peninsular Malaysia and its pertinence to genetic conservation and tree improvement. *Forest Genetics* 7(3) :209–217.

MITTON, J. B. 1992: The dynamic mating system of conifers. *New Forests* 6:197–216.

MUONA, O. 1989: Population genetics in forest tree improvement. *In*: Plant population genetics, breeding and genetic resources. (ed. A.H.D. Brown, M.T. Clegg, A.L. Kahler & B.S. Weir). pp. 287–298. Sunderland: Sinauer Associates Press.

MURRAY, M.G. & THOMPSON, W.F. 1980: Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* 8:4321–4325.

NAGAMITSU,T., ICHIKAWA, S., OZAWA, M., SHIMA MURA, R., KACHI, N., TSUMURA, Y. & NORWATI, M. 2001: Microsatellite analysis of the breeding system and seed dispersal in *Shorea leprosula* (Dipterocarpaceae). *International Journal of Plant Science* 162(1) : 155–159.

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

NIKOLIĆ, D. J., & TUCIĆ, N. 1983: Clinal variation of null alleles in European black pine (*Pinus nigra* Am.). *Genetika* 15:105–113.

ROHLF, F. J. 1990: NTSYS-pc, Numerical taxonomy and multivariate analysis system, version 2.0. Applied Biostatics Inc., New York.

SER, C. S. 1981: Malaysian Timbers – Kapur. *Malaysian Forest Service Trade Leaflet No. 46.*

SHIRAISHI, S., LATIFF, N. H., MIYATAKE, S., OCHIAI, Y. & FURUKOSHI, T. 1994: Population-genetical studies on *Dryobalanops* (kapur) species. *Bulletin of Forest and Forestry Research Institute* 366:107–114.

SWOFFORD, D. L. & SELANDER, R. B. 1989: BIOSYS–1: A computer program for the analysis of allelic variation in population genetics and biochemical systematics. Illinois Natural History Survey, IL.

SYMINGTON, C. F. 1943: Foresters' Manual of Dipterocarps. Malayan Forest Records No. 16. Universiti Malaya, Malaysia.

TAYLOR, A. C., SHERWIN, W. B. & WAYNE, R. K. 1994: Genetic variation of microsatellite loci in a bottlenecked species: The northern hairy-nosed wombat *Lasiorhinus krefftii*. *Molecular Ecolology* 3:277–290.

TERAUCHI, R. 1994: A polymorphic microsatellite marker from the tropical tree *Dryobalanops lanceolata* (Dipterocarpaceae). *Japanese Journal of Genetics* 69:567–576.

UJINO, T., KAWAHARA, T., TSUMURA, Y., NAGAMITSU, T., YOSHIMURA, H. & WICKNESWARI, R. 1998: Development and polymorphism of simple sequence repeat DNA markers for *Shorea curtisii* and other dipterocarpaceae species. *Heredity* 81:422–428.

WAHLUND, S. 1928: Zusammensetzung von Populationen und Korrelationserscheinungen von Standpunkt der Vererbungslehre aus betrachtet. *Hereditas* 11:65–106.

WYATT- SMITH, J. 1963: Manual of Malayan Silviculture for Inland Forests. *Malayan Forest Record No. 23.* Forest Research Institute, Kepong., Malaysia.