森林総研研報 Bull. For. & For. Prod. Res. Inst. No.366, 1994 129～137

Genecological Study on *Agathis borneensis* WARB. in Brunei Darussalam Using Isozyme Electrophoresis

By

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Summary : The genus *Agathis* is a conifer which is distributed in tropical lowland forest from South-east Asia to New Zealand. A genecological study on 5 populations of *Agathis borneensis* in Brunei Darussalam was carried out using isozymes (17 loci; 15 enzyme systems) as gene markers. The heterozygosity of the total population, which is an indicator of genetic diversity, was H_T= 0.122. The proportion of between-population heterozygosity was G_{ST}=0.140. The low level of the genetic diversity and the large proportion of differentiation among populations were opposite results to those of conifers in the temperate zone. Genotype frequencies of each population did not deviate significantly from Hardy-Weinberg expectations. In the Badas Forest Reserve, the inbreeding coefficient was estimated as a parameter of a mating system by comparing genotype frequencies between adult trees and seedlings. There was no differentiation of allele frequencies between adult trees and seedlings. In Badas, the Hardy-Weinberg Equilibrium was maintained among both adult trees and seedlings, indicating that *A. borneensis* is predominantly an outcrossing species.

1 Introduction

Agathis is a genus of conifers, which is one of the most valuable tree species in tropical rainforest. There are 13 species in the genus of *Agathis* whose range extends from Sumatra and Malaya, through the Malay archipelago to Queensland, Fiji, New Caledonia, and the north-west peninsula of the North Island, New Zealand (WHITMORE, 1977, 1984). *Agathis* is, like other conifers, a climax tree species mainly found in the lowlands of the tropics. It is a valuable timber species because of its rapid growth, self pruning, and good quality softwood. It is sought for and felled wherever the genus grows, and today many stands have been exhausted (WHITMORE and PAGE, 1980).

In Brunei, there are two *Agathis* species: *Agathis borneensis* WARBURG and *Agathis dammara* (LAMBERT) L. C. RICHARD (WHITMORE, 1980; WONG, 1990). *A. dammara* occurs as isolated populations on steep mountain terrain. *A. borneensis* occurs on the low-land podzolized sand called 'Kerangas'. All of the remaining *A. borneensis* populations in Brunei are small but well-conserved natural stands. In other parts of the world, however, natural populations of *Agathis* have been considerably reduced by logging. For successful establishment of plantations and the enrichment of logged forests using this genus, investigation of these natural forests is necessary in order to obtain basic information.

The genecological survey is an indispensable means of obtaining primary knowledge for gene conservation and forest regeneration. There is a rich variety of isozymes within a species, making

Received December 2, 1992 生物機能－6 Bio-resources Technology－6

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isozyme electrophoresis a suitable method to estimate genetic variability. Many studies on conifers in temperate forests have been conducted using isozyme analysis (MITTON, 1983), however few studies of conifers in tropical rainforests have been reported.

In this study, the genetic variation and differentiation among 5 populations of *A. borneensis* in Brunei Darussalam was investigated using isozymes as a gene marker. The Hardy-Weinberg Equilibrium (HWE) of seedlings was estimated to evaluate the existence of inbreeding in Badas Forest Reserve (F. R.). The seedlings from this reserve are used for reforestations.

2 Materials and Methods

Five known natural stands of *A. borneensis* were investigated (Fig. 1). In Badas, most of the trees which reach to the top of canopy are *A. borneensis* and have large numbers of seedlings. In Andulau, the vegetation is similar to that of Badas and there are a large number of saplings and seedlings. In the 1960's, trees in Andulau which were >6ft. in circumference at chest height were harvested. Ulu-badas is a mixed forest of *A. borneensis* and *Shorea albida*. In Bukit (Bt.) Patoi, *A. borneensis* occurs at the top of the hill, and also forms a mixed forest with other tree species. In Labi,

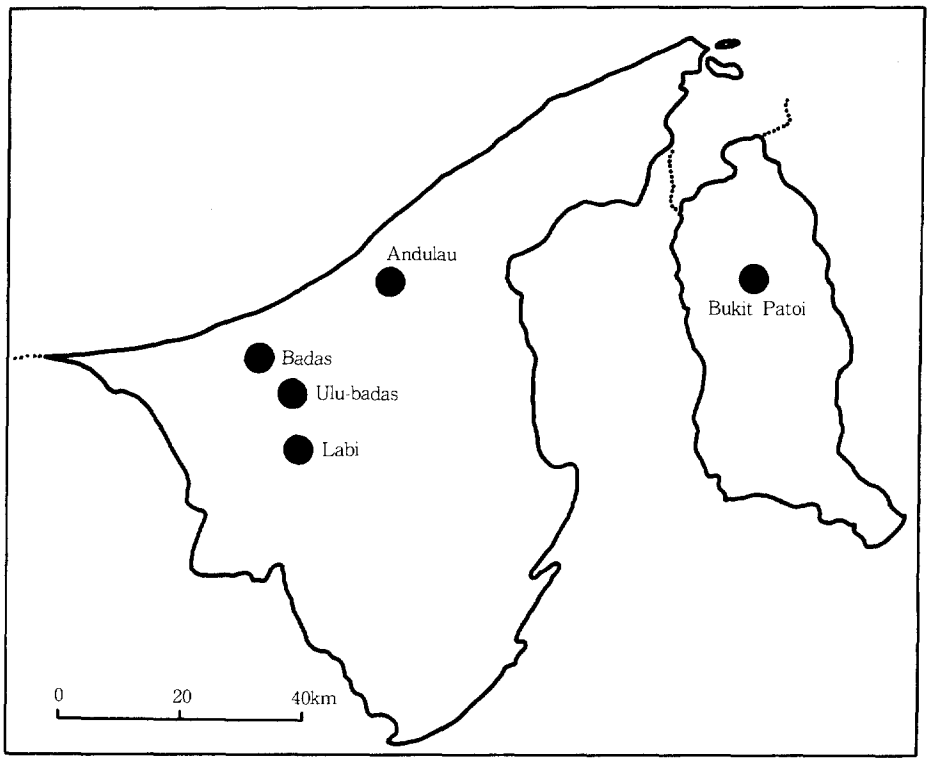

Fig.1. Sampling site of *Agathis borneensis*

A. borneensis occurs rarely and was damaged by forest fires in the past. All of the populations of *A. borneensis* in Brunei are small and isolated from each other.

In 1991, forty trees of >40cm diameter at breast height (DBH) (YAMADA, 1987) were chosen at random from Badas and Ulu-badas populations, individually. Forty trees of >10cm DBH in Bt. Patoi, twenty of >10cm DBH in Labi and fifteen of >40cm DBH in Andulau were also chosen at random. Their inner bark tissues (SHIRAISHI *et al.*, 1990) were collected from the lower trunk. In Badas, the leaf tissues from 80 seedlings were also collected in order to compare the genotype frequencies between adult trees and seedlings. All samples were brought to the laboratory of the Brunei Forest Centre at Sungai Liang under low temperature conditions, and stored at 2℃ for later use. The procedures used in gel electrophoresis and detection of enzyme activities followed those of SHIRAISHI *et al.* (1989).

Allele frequencies for each population were calculated from the phenotypes of the sample trees. Observed (H_o) and expected (H_e) heterozygosities, total genetic diversity (H_T), and within-population diversity (H_S), were calculated from allele frequencies (NEI, 1973, 1978). The genetic differentiation between populations (G_{ST}) is measured as the proportion of genetic diversity between them (D_{ST}) to H_T.

3 Results and Discussions

3.1 Survey of Enzyme Systems as Gene Markers

Initially the extraction buffer for *Agathis* (SHIRAISHI *et al.*, 1989) was modified with three different pH ranges; 7.0, 7.5, 8.0. When extracted with the pH7.0 buffer, leaf tissues showed no activity for some enzyme systems. Extracted at pH8.0, enzyme activities were observed to be higher than at pH7.5. Thus, we decided to use the extraction buffer with pH8.0 for *A. borneensis*. The components of the extraction buffer are as follows: 0.1M Tris-HCl (pH8.0), 25% (w/v) Glycerol, 1% (w/v) Tween 80, 10mM DTT, 0.4%(v/v) β-ME, and 70mg/ml PVPP.

Twenty-six enzyme systems were surveyed to determine isozyme loci useful as gene markers. Finally, 17 putative loci (15 enzyme systems): 6-phosphogluconate dehydrogenase (6PGD), Diaphorase (DIA), Glucose-6-phosphate dehydrogenase (G6PD) , Glutamate dehydrogenase (GDH), Glucokinase (GK), Glutamate oxalomate transaminase (GOT), Glutatione reductase (GR), Leucineaminopeptidase (LAP), Malic enzyme (ME), Maleic acid dehydrogenase (MDH, 3 loci), Phosphoglucose isomerase (PGI), Phosphoglucomutase (PGM), Shikimate dehydrogenase (SkDH), Superoxide dismutase (SOD), and Sorbitol dehydrogenase (SORDH) were detected. Five loci of the above 17 showed polymorphisms, of which the most common allele frequency was lower than 0.99. Other polymorphisms were also detected in acid phosphatase and isocitric acid dehydrogenase, but genetic interpretation of the variation was uncertain.

The nomenclature of each allele followed that of SHIRAISHI *et al.* (1989). The phenotypes and the corresponding genotypes are shown in Fig.2 (monomorphic loci) and Fig.3 (polymorphic loci).

3.2 Genetic Diversity of Natural *A. borneensis* Forest

The allele frequencies of each population at the 5 polymorphic loci (Table 1) were estimated from observed genotype frequencies. Some of the allele frequencies were different among populations. The common allele of *Dia* locus was Dia^a in the Ulu-badas, Andulau and Labi population, but Dia^b was common in the Badas and Bt. Patoi populations. Also the Badas and Andulau populations hold a different common allele from the other populations in the case of *Pgi*,

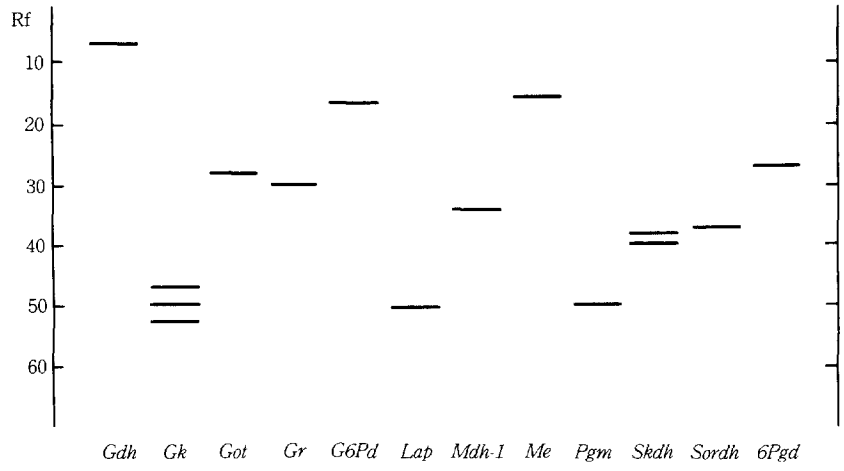

Fig.2. Zymograms of 12 monomorphic loci
Rf: Relative value to the front

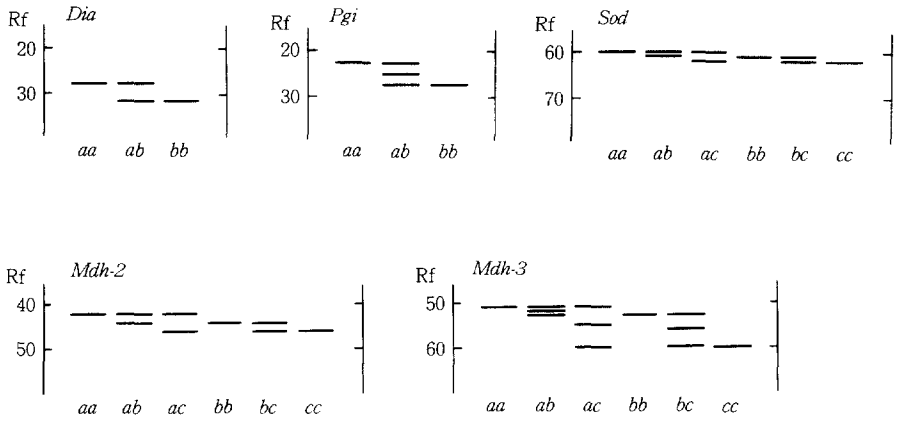

Fig.3. Zymograms of 5 polymorphic loci
Rf: Relative value to the front
aa,ab,ac. . . . . . ; Corresponding genotypes

and from the Ulu-badas and Andulau in the case of *Sod*. In the Bt. Patoi population, there were two unique alleles, *Mdh-2*^{a} and *Mdh-3*^{a}, with the frequency of *Mdh-3*^{b} indicating a lower value, while the other 4 populations had frequencies higher than 0.90. The Andulau population was monomorphic for *Mdh*-3. The *Sod*^{b} allele was lacking in the Andulau and Bt. Patoi populations. *Pgi*^{a} was common in Ulu-badas, Labi and Bt. Patoi, while *Pgi*^{b} was common in Badas and Andulau. *Sod*^{a} was common in Badas, Labi and Bt. Patoi and most frequent in Bt. Patoi. *Sod*^{c} was common in Ulu-badas and Andulau.

Table 1. Allele frequencies at polymorphic loci for five populations of *A. borneensis*

Locus	Allele	Population				
		Badas N=40	Ulu-badas N=40	Andulau N=15	Labi N=20	Bt. Patoi N=40
Dia	*a*	0.525	0.325	0.233	0.450	0.782*
	b	0.475	0.675	0.767	0.550	0.218*
Mdh-2	*a*	0	0	0	0	0.087
	b	0.663	0.763	0.833	0.850	0.738
	c	0.337	0.237	0.167	0.150	0.175
Mdh-3	*a*	0	0	0	0	0.150
	b	0.938	0.950	1.000	0.975	0.713
	c	0.062	0.050	0	0.025	0.137
Pgi	*a*	0.388	0.612	0.367	0.675	0.888
	b	0.612	0.388	0.633	0.325	0.112
Sod	*a*	0.575	0.400	0.200	0.575	0.825
	b	0.250	0.137	0	0.125	0
	c	0.175	0.463	0.800	0.300	0.175

N:Number of individuals examined.

* Number of individuals examined in *Dia* of the Bt. Patoi population was N=39.

Based on the results, it can be said that there is a difference in genetic structures between the Bt. Patoi and the other 4 populations. The differentiation of genetic structure between the Temburong district and the main-land of Brunei was also observed in *Dryobalanops aromatica* GAERTN. f. populations (SHIRAISHI *et al.*, 1989).

The measures of genetic variation are given in Table 2. The mean allele number per locus was lowest (1.24) in the Andulau population and highest (1.41) in the Bt. Patoi population. The proportion of polymorphic loci was 0.29 in all except the Andulau population (0.23). The observed heterozygosity did not deviate from the expected heterozygosity; i. e. no significant deviation from HWE was observed in any of the 5 populations ($p>0.05$). The expected panmictic heterozygosity as an indicator of genetic diversity was different among populations. The genetic diversity (H_e) of the Andulau population was smallest (0.084) while Badas was largest (0.124). The heterozygosity of the total population (H_T) was 0.122, whereas the average heterozygosity within populations (H_S) was 0.106. The proportion of between-population heterozygosity to total heterozygosity (G_{ST}) was 0.140. This indicates that 14.0% of the total gene diversity was attributed to gene differences among populations, and the remainder was maintained within them. The genetic diversity in the *A. borneensis* populations in Brunei ($H_T=0.122$) was similar to preliminary estimates available for

Table 2. Measures of genetic variation based on seventeen loci in five populations of *A. borneensis*

Population	A	P	H_e	H_o
Badas	1.35	0.29	0.124	0.125
Ulu-badas	1.35	0.29	0.116	0.110
Andulau	1.24	0.23	0.084	0.082
Labi	1.35	0.29	0.106	0.106
Bt. Patoi	1.41	0.29	0.100	0.103

A : Mean allele number per locus
P : Proportion of polymorphic loci
H_e : Expected heterozygosity
H_o : Observed heterozygosity

neotropical rain forest trees (H_T=0.111, HAMRICK and LOVELESS, 1986) but less than those for tropical angiosperms (H_T=0.211, HAMRICK and LOVELESS, 1988) or temperate wind-pollinated conifers (H_T=0.207, HAMRICK *et al.*, 1981). The genetic diversity between populations (G_{ST}=0.140) of *A. borneensis* in this study comprised a greater value than those of other conifers. In general, conifers have small G_{ST} values, but rare species tend to have great G_{ST} values. Genetic drift in small populations may be a factor contributing to the large G_{ST} value. When the species was made up of subdivided small populations, genetic drift is a main factor causing differences in allele frequencies.

3.3 Mating system of *A. borneensis* in Badas F. R.

In general, wind-pollinated conifers are under random mating and the gene flow is extensive. If seedlings are regenerated by random mating among parents, the relationship between allele frequencies and genotype frequencies follows the HWE. Among the 5 populations mentioned above, the Badas and Andulau have naturally regenerated seedlings. The genetic structure of seedlings in Badas F. R. was investigated in this study.

Comparison of the allele frequencies of 3 polymorphic loci (*Dia*, *Mdh-2* and *Pgi*) between adult trees and seedlings indicated almost the same values (Table 3). Based on these findings, it can be said that the gene flow is not restricted within populations.

Genotype frequencies for seedlings and adult trees are listed in Table 4. Observed genotype frequencies of both mother trees and seedlings were found to agree with HWE ($p>0.05$).

In general, the inbreeding coefficient under panmixia is zero, and will be increased by inbreeding but decreased by selection favouring heterozygotes. The result of the seedling stage of *A. borneensis* indicated a high outcrossing, revealing that *A. borneensis* is a predominantly outcrossing species like other conifers.

Table 3. Allele frequencies for seedlings and adult trees of *A. borneensis* in Badas F. R.

Locus	Allele	Seedlings (N=80)	Adult trees (N=40)
Dia	*a*	0.49	0.53
	b	0.51	0.47
Mdh-2	*b*	0.61	0.66
	c	0.39	0.34
Pgi	*a*	0.42	0.39
	b	0.58	0.61

N:Number of individuals examined

Table 4. Observed and expected numbers of genotype for seedlings and adult trees of *A. borneensis* in Badas F. R.

		Seedlings		Adult trees	
Locus	Genotype	Observed	Expected	Observed	Expected
Dia	*aa*	20	19.0	11	11.0
	ab	38	40.0	20	20.0
	bb	22	21.0	9	9.0
	χ^2		0.200(n.s.)		0.000(n.s.)
Mdh-2	*bb*	34	30.0	16	17.6
	bc	30	38.0	21	17.9
	cc	16	12.0	3	4.6
	χ^2		3.550(n.s.)		1.239(n.s.)
Pgi	*aa*	18	14.0	5	6.0
	ab	31	38.9	21	19.0
	bb	31	27.0	14	15.9
	χ^2		3.330(n.s.)		0.604(n.s.)

n.s. : Not significant ($p>0.05$)

Acknowledgements

We would like to thank Dr. MORNI, the Director of the Forestry Department, and Mr. Hj. HAFNEH, the Deputy Director of the Forestry Department, for their support. We would also like to thank Mr. A. MARUYAMA, the JICA Project Team Leader, for his support.

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ブルネイ国における *Agathis borneensis* WARB.のアイソザイム手法による生態遺伝学的研究

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摘　要

*Agathis*属は東南アジアからニュージーランドにかけての熱帯低地に生育する針葉樹である。ブルネイ国における*Agathis borneensis*天然林5集団についてアイソザイムを標識遺伝子として生態遺伝学的研究を行った。分析に際しては，15酵素種17遺伝子座のアイソザイム遺伝子座を使用した。遺伝的変異性の指標である平均ヘテロ接合体率は $H_T=0.122$であった。分集団間の遺伝子多様度は$G_{ST}=0.140$であり，集団全体の遺伝子多様度は低く分集団間の分化の程度は高いことが明らかになった。これは温帯地域に生育する針葉樹とは逆の結果である。各集団はハーディ・ワインベルグの平衡を保っていた。バダス保護林で，成木と天然更新している実生の遺伝子型頻度を比較して，交配様式のパラメータである近交係数の生育段階による違いを推定した。その結果，成木と実生の間に遺伝子頻度の違いは認められなかった。また，ハーディ・ワインベルグ平衡も両生育段階で保たれていた。これらのことから*Agathis borneensis*は他殖性の樹種であることが示唆された。

1992年12月2日受理

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