International Symposium on Development of Improved Methods to Identify *Shorea* Species Wood and its Origin

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東南アジア産木材の樹種識別および産地特定技術に関する国際シンポジウム

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Preface

Illegal logging and the trade of such timbers harvested illegally have led to not only a growing number of problems both in producing and consuming countries but also to affect global environment seriously. The timber produced thorough the sustainable forest management is disturbed by the timber supplied with such illegal behaviors, resulting to the rupture of forest environment. Japan advocated this problem in the G8 summit, and contributes to act against these problems. There is a need to have practicable control methods to support the identification of illegally logged timbers and the discrimination from those produce thorough the sustainable forest management. Among actions of countries concerning to the illegal logging presented during "International Seminar in Tokyo for Tacking Illegal Logging, 2007" held in February, 2007, the labeling system of tree species and place of origin of the timber presented by the Forest-Products Identification Promotion Conference in Japan is admitted to have a deterrent effect on trade of timber harvested through illegal logging. To support the labeling system, it is very desirable to establish a system to verify the accuracy of the labeling by scientific methods, in the future.

The main purpose of the symposium is to look into how scientific knowledge can contribute to the traceability of timbers produced in Southeast Asia. It is necessary to enhance our knowledge of identification of tree species and its origin through the exchange of information concerning the wood products, tree species, and situation of the forests in each region of Southeast Asia. We are discussing about the effective method to verify the identity of the timber, to promote of labeling system of the tree species and its provenance. In this symposium, we share the importance of the international corporation for the establishment an international database of wood samples for accurate identification of tree species and its origin of the timber in Southeast Asia and Western Pacific regions.

September 14, 2007

Tomoyuki FUJII
Symposium chairman

CONTENTS

Tree species of timbers imported to Japan from Southeast Asia

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Abstracts

Consumption of timbers in Japan greatly influences forest resources in Southeast Asia and the Pacific regions, because Japan is one of the biggest importers of timbers from these regions. The timbers imported from Malaysia and Indonesia are mainly in the form of plywood recently. We examined 8,543 sheets of plywood imported in 2002, and identified tree species of veneers on one or both surfaces of the plywood. Timbers of red meranti (*Shorea* section *Rubroshorea* spp., Dipterocarpaceae) were used in more than 55% of the plywood examined. The shares of red meranti were 59.2% of plywood from Indonesia and 51.6% of one from Malaysia. It was significantly high marking 71.4% of one from Kalimantan. We compared these results and tree species of logs imported from the regions to Japan in 1991. In spite of the decrease of share of red meranti according to the exhausting natural forest resources, red meranti is still the most important species for timber industries in Malaysia and Indonesia.

Introduction

Japan has imported a large amount of timbers from Southeast Asia after the Second World War, especially since 1949. It accounted for about 14% of total timber consumption in Japan (1). Most of the timbers used to be imported in the form of logs until the early 1980s, but turned decreasing later gradually. About three fourths of the logs have been used for the materials of plywood (2).

Instead of the decrease in the import of logs, timber in the form of products such as plywood and sawn lumber lincreased. Especially, the amount of plywood increased drastically, it became 26 times in 1989 larger than that in 1984 (3). This abrupt increase was mainly caused by the increase in the amount of plywood from Indonesia, because Indonesian government prohibited the export of logs in 1988. The amount of plywood imported from Malaysia began to increase in 1992, as the government of Sabah State in Malaysia also prohibited the log export in 1992. The shares of plywood imported from Indonesia and Malaysia accounts for about 60% and 30% of the plywood imported foreign countries in 2002, respectively (3).

Tree species of logs imported to Japan from Southeast Asia used to be identified by the private companies at each custom until 1991. The identification has been suspended until now, because of the decrease in the number of imported logs. With the increase in the import of timbers as products, especially plywood, the selection of tree species for production of plywood is taking on importance for the reason of effective utilization of natural resources. However, it takes a lot of time and efforts to identify tree species of plywood, because a microscopic investigation is necessary to check the samples from thin slice of each veneer. We investigated tree species of plywood imported from mainly Southeast Asia at each custom in Japan in 2002. We compared the results and tree species of logs imported from the region 1991.

Trees species of plywood imported to Japan from Asia in 2002

We examined 8,638 sheets of plywood imported to Japan in 2002, and identified tree species of veneers on one or both surfaces of the plywood. The plywood that we investigated was imported from Indonesia (53.13%), Malaysia (39.53%; Sarawak 27.69%, Sabah 8.20%), Singapore (1.53%), China (1.46%), Korea (1.40%), and other or unknown places (2.95%). Timbers of red meranti (*Shorea* section *Rubroshorea* spp., Dipterocarpaceae) were used in more than 55% of the plywood examined. The shares of red meranti were 59.2% of plywood from

Indonesia and 51.6% of one from Malaysia. It was significantly high marking 71.4% of one from Kalimantan. In the case of plywood from Sarawak, red meranti shared 53.49%. In addition to red meranti, yellow meranti and kapur shared 8.52%, and 5.87%, respectively. Timbers of Dipterocarpaceae accounted for 78.63%.

Trees species of logs imported to Japan from Southeast Asia in 1991

The logs investigated, were imported from Malaysia in 1991 (92.19%; Sarawak 80.78%, Sabah 11.41%), PNG (5.74%), Solomon (1.91%) and other or unknown places (0.15%). Red meranti logs accounted for 61.79% of total logs investigated. In the case of logs from Sarawak, red meranti shared 71.65%. In addition to red meranti, kapur, yellow meranti and keruing shared 8.84%, 5.27%, 5.05%, respectively. Timbers of Dipterocarpaceae accounted for 94.60%.

Comparison of the tree species of 1991 and of 2002

As most of logs from Southeast Asian were used for the materials of plywood in 1991, it is reasonable to compare tree species of imported logs in 1991 and imported plywood in 2002. The decrease of the share of red meranti was obvious. In the case of Sarawak, the share of red meranti decreased from 71.65% to 52.0%. However, an increase in the share of species that has not been used often before is obvious such as the non-dipterocarp species, nyatoh, bintangor, geronggang, and trees belonging to Burseraceae are increased, which had not been used so often before. The tree species used for timber are possibly reflecting the natural resources of the tree species in the forest, in which the timbers were harvested. Our results suggest that dipterocarp trees have been decreasing in the forests in Southeast Asia in the last couple of decades.

Despite the exhausted natural forest resources, dipterocarp species are the most important tree species for the timber industries in Malaysia and Indonesia even now. It is necessary to control the forest resources for utilizing them effectively. Investigation of the tree species of timber products is the most fundamental scientific behavior to monitor the resources in the forests.

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Second growth forest as the future source of wood from the natural forest – their availability, utilization and identification

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Introduction

Malaysia is a major exporter of tropical timber products with an export value of RM21.5 billion in 2005 (MTC 2006) making the forestry sector as one of the major income earners for the country. The country still has a relatively large area of production forests that are being managed for continuous supply of timber resources and they are likely to consist solely of logged forests in the near future. There are concerns that the yield from second growth forests (SGF) will be low and less of the more valuable commercial species.

Present forest resources

Total forested areas in Malaysia amounted to 19.48 million ha or almost 60% of the total land area in 2005 (MTC 2006). Out of this 14.39 million ha or about 74% has been designated as Permanent Reserved Forest (PRF) to be sustainably managed for the benefit of present and future generations. The bulk of the forest areas in Malaysia comprises of dipterocarp forests, which makes up almost 82% (16 million ha) of the available resources. Other major forests types (Table 1) include peat swamp (7.8%), mangrove (3%) and planted (7%) forests.

Stocking in Second Growth forests

Cannonizado (1978) and Pinnard (1995) reported that SGF is highly variable and may not yield the desired species and volume due to logging impacts. To assess the stocking and composition of SGF, a targeted research project was undertaken by FRIM in Tekam Forest Reserve located in Jerantut, central Pahang, Peninsular Malaysia. The study site was about 11,000 ha and it was logged 17 to 28 years ago. Prior to logging, the area consisted of Meranti (*Shorea* spp.) and Meranti Seraya (*Shorea curtisii*) forests classified under the Malaysian National Inventory (NFI) as superior forests, which is the most highly stocked forest type with volume in excess of 150 m^3/ha.

Table 1 Types of forest in Malaysia

	Land area (million ha)	Inland dipterocarp forest	Swamp forest	Mangrove	Plantation forest	Total forest area	Forest area as % of land area
Peninsular Malaysia	13.16	5.40	0.3	0.10	0.08	5.88	44.7
Sabah	7.37	3.68	0.12	0.36	0.20	4.36	59.2
Sarawak	12.30	6.92	1.10	0.13	1.09	9.24	75.1
Malaysia	32.83	16.00	1.52	0.59	1.37	19.48	59.3

Source: MTC 2006

However, the results from the study site in Tekam indicated that the productivity within the SGF has dwindled (Table 2). The average volume of 145 m^3/ha for Tekam is low compared to the NFI results. Its productivity is even lower then the poor forest strata stocking. However, the

density of trees within the study remains high at 382 trees/ha for Tekam. In Tekam, pioneer *Macaranga* spp. such as Mahang Puteh (*Macaranga hypoleuca*) and Mahang Gajah (*Macaranga gigentea*) are very dominant (Fig. 3) indicating the creation of large gaps during the previous logging operations. The gaps allowed these light demanding and fast growing species an excellent opportunity to flourish and to colonise.

Utilisation of timber – present scenario

At present, the utilization of timber in the country can be classified into two main categories, they are (a) the use of timber is very specific i.e a certain timber group is specified e.g rubberwood (*Hevea brasiliensis*) for furniture, balau (*Shorea* spp.) for structural purposes, treated kempas (*Koompassia malaccensis*) for roof trusses etc and (b) mixed hardwoods where the identity of the timbers are not specified resulting in the use of multiple timber groups in a single usage e.g. roof truss.

The use of multiple timber groups in construction is very common, particularly in the medium to low cost housing projects. Based on a total of 840 specimens sent to the Forest Research Institute Malaysia (FRIM) for identification in 2006, members of the family Leguminosae such as keranji (*Dialium* spp.), kekatong (*Cynometra* spp.), kempas (*Koompassia malaccensis*), tualang (*Koompassia excelsa*) and merbau (*Intsia palembanica*) dominated the specimen received followed by members of the family Dipterocarpaceae such as balau (*Shorea* spp.), chengal (*Neobalanocarpus heimii*), dark red meranti (*Shorea* spp.), kapur (*Dryobalanops* spp.), red balau (*Shorea* spp.), merawan (*Hopea* spp.) and resak (*Cotylelobium/Vatica* spp.). Other more common timber families received include Lauraceae :belian (*Eusideroxylon zwageri*) and medang; Sapotaceae: bitis (*Madhuca utilis/Palaquium* spp.), nyatoh (*Palaquium* spp.); Burseraceae: kedondong and Anacardiaceae :rengas (*Gluta/Melanochyla* spp.) and merpauh (*Swintonia* spp.). Lesser-known timber groups from the families Euphorbiaceae : perah *(Elateriospermum tapos*), mahang (*Macaranga* spp.); Fagaceae: mempening (*Lithocarpus/Quercus* spp.); Bombacaceae: durian (*Durio* and other genera), kekabu (*Bombax* spp.); Meliaceae: bekak (*Aglaia* spp.); Polygalaceae: minyak berok (*Xanthophyllum* spp.) and many other families.

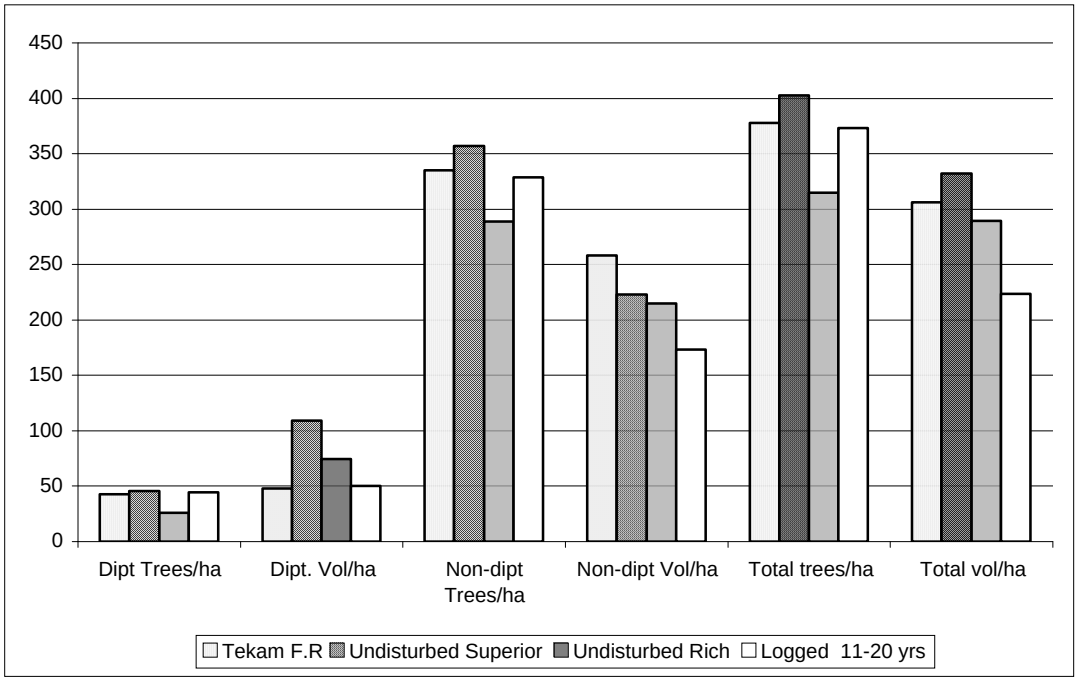

Figure 1 Stocking by species groups of Tekam F.R and other forest types

Table 2 Stocking of trees by species groups and diameter classes for the second rotation forests located in Tekam F.R, Pahang

Species Groups	Volume/ha (m^3/ha) by diameter classes					
	>15 cm	**>30 cm**	**>45cm**	**>60cm**	**>75cm**	**>90cm**
Dipterocarp	29.18	26.30	20.70	14.91	8.21	3.64
Non Dipterocarp	34.11	29.07	20.04	12.75	6.43	2.50
Non Commercial	81.52	58.77	29.02	14.20	5.57	2.14
Grand Total	144.80	114.14	69.75	41.86	20.21	8.29
	Trees/ha by diameter classes					
	>15cm	**>30cm**	**>45cm**	**>60cm**	**>75cm**	**>90cm**
Dipterocarp	43.56	20.25	7.50	2.60	0.97	0.27
Non Dipterocarp	80.90	24.94	7.57	2.25	0.51	0.11
Non Commercial	257.34	62.63	15.09	3.15	0.85	0.14
Grand Total	381.79	107.82	30.15	8.00	2.33	0.52

Woods from the Second Growth Forests

Future supply of log from the natural forest is likely to come from SGF. As such, their composition is likely to be less dominance of the Dipterocarpaceae but a mixed bag of timber from various families like Myristicaceae (penarahan), Sterculiaceae (Kembang semangkok), Moraceae (Keledang), Fagaceae (mempening), Euphorbiaceae (perah, mahang), Burseraceae (kedondong), Lauraceae (medang), Myrtaceae (kelat) and some other left over from the first crop (Figures 3). Log quality may not be as good as much of the prime quality logs had already been harvested earlier. As a result, sawn timbers are likely to be (a) short length (b) easily splits (c) high shrinkage as most trees are likely to contain a large percentage of juvenile wood (d) high variation in properties and density due to the presence of multiple species and (e) easily infected with fungi and insects due the presence of high proportion of sapwood.

Identification of timbers from the Second Growth Forest

The identity of tree in the forest can be normally identified by well-trained foresters and rangers without any difficulties. However, once the trees are felled, the identity of the logs may not be known so easily. Log-yard supervisors need to be trained by looking at the general appearance of the bark. Appendix 1 provides some spot characteristics of 20 types of timbers expected to come from the SGF.

Conclusion

The stand structure of SGF is highly variable and yielding crops of much lower volume and value. Thus, efforts to enhance the productivity of SGF through silvicultural interventions and more efficient in utilization is necessary. New technologies to minimize wastes and maximize recovery of log are required. Log-yard supervisors and quality control personnel will be required to undergo training so that tree species from the SGF could be readily identified and sorted out according to their groups.

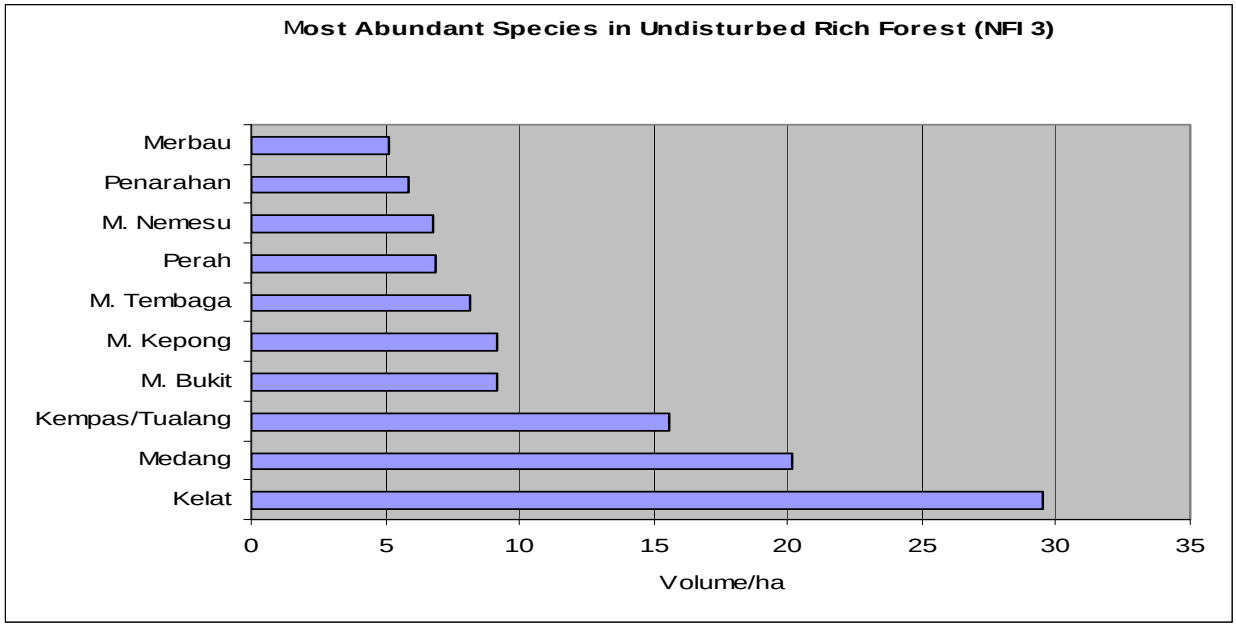

Figure 2 Most abundant species in an undisturbed forest

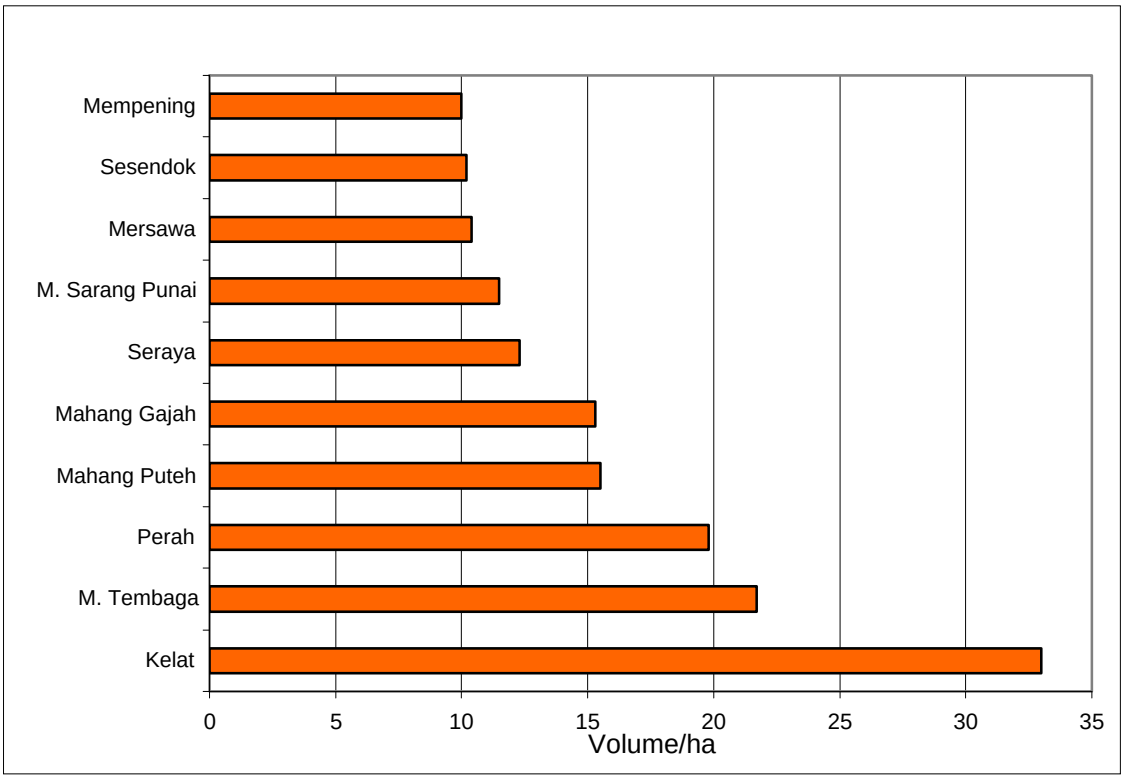

Figure 3 Most dominant species in terms of volume in Tekam F.R

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Appendix 1

Spot characteristics of some timbers from the second growth forests

No	Timber	Spot Characteristics
1	Bekak (*Aglaia* spp.)	Heartwood brick-red to dark red brown; moderately heavy; vessels oval and medium-sized, mainly in radial groups of 2 to 4; rays not visible to naked eye
2	Bintangor (*Calophyllum* spp.)	Heartwood pink to orange brown; light to medium density; growth rings prominent due to terminal bands; vessels exclusively solitary and arrange in bunches and radially oblique; rays very fine, not visible to naked eye.
3	Dark red meranti (*Shorea* spp.)	Heartwood red brown to dark-red and well defined from the yellowish coloured sapwood; wood light to medium weight; vessels moderately large to large without any fixed arrangement; wood parenchyma mainly as short aliform; rays medium-sized and visible to the naked eye; intercellular canals in concentric series containing white resin.
4	Durian (mainly *Durio* spp.)	Heartwood pink brown to deep red-brown or orange brown; light to heavy; vessels large; parenchyma mainly as diffuse strands extending from ray to ray; rays of two distinct sizes; traumatic canals occasionally present
5	Kedondong (spp. of Burseraceae)	Heartwood pink brown or red brown; light to heavy; vessels fine to medium, mostly solitary with the rest in radial arrangement; wood parenchyma indistinct or absent
6	Kelat (*Eugenia/Syzygium* spp.)	Heartwood grey-brown weathering to deep brown; medium to heavy; vessels small to medium-sized and generally angular in shape, mostly in solitary and radial pairs; wood parenchyma as slanting aliform and occasionally confluent; rays very fine and barely visible to the naked eye due to its poor contrasting background.
7	Keledang (*Artocarpus* spp.)	Heartwood orange yellow-brown to gold brown; vessels medium to large-sized, few but fairly evenly scattered; wood parenchyma as vasicentric, aliform or confluent and the colour of wood parenchyma is typically orange
8	Kelempayan (*Neolamarckia cadamba*)	Heartwood white weathering to creamy yellow on exposure; vessels large with most of them in radial pairs or multiples; parenchyma diffuse
9	Kembang semangkok (*Scaphium* spp.)	Heartwood yellow brown to light brown; growth rings distinct due to parenchyma layers; vessels medium-sized to large, few, solitary and in radial groups; parenchyma vasicentric, aliform. confluent and irregularly spaced bands; occasionally with traumatic canals.
10	Mahang (*Macaranga* spp.)	Heartwood light yellow brown with pinkish tinge; Vessels small to medium-sized, few, solitary and in radial groups; wood parenchyma aliform with tendency to confluent and bands; rays

		fine.
11	Medang (spp. of Lauraceae)	Heartwood light straw to olive green; Vessels small to medium-sized, mostly in radial arrangement; Parenchyma scanty
12	Mempisang (spp. of Annonaceae)	Heartwood light yellow with a greenish or pinkish tinge; texture coarse and uneven; vessels medium-sized; wood parenchyma as narrow closely spaced lines from ray to ray; rays of two distinct sizes, conspicuous on all surfaces
13	Mempening (*Lithocarpus/Quercus* spp.)	Heartwood yellow brown or reddish brown; vessels medium to large-sized, exclusively solitary and arranged in groups; wood parenchyma mainly as short tangential lines running from ray to ray; rays of two distinct sizes and prominent silver grain figure on radial surface.
14	Merpauh (*Swintonia* spp.)	Heartwood grey to reddish-brown; growth rings figure on tangential surface; vessels medium to large sized, few; wood parenchyma as irregularly spaced bands; light coloured rays and parenchyma bands are fairly distinct from the dark-coloured background.
15	Minyak berok (*Xanthophyllum* spp.)	Heartwood white to bright yellow weathering to darker yellow; vessels few but large; wood parenchyma as vasicentric, aliform and confluent, apotracheal parenchyma as narrow and regular bands; rays very fine and not visible to the naked eye
16	Penarahan (spp. of Myristicaceae)	Heartwood pale brown or pink brown, some with a dark corewood; growth rings distinct due to irregularly spaced parenchyma bands; vessels predominantly in radial pairs.
17	Perah (*Elateriospermum tapos*)	Heartwood brown with pinkish tinge, corewood dark and hard; vessels few, medium-sized, solitary and in radial groups, tyloses present, white-yellowish deposit common; wood parenchyma as narrow and regularly spaced bands.
18	Pulai (*Alstonia* spp.)	Heartwood cream-white; vessels medium-sized, in radial multiples of 3 or more vessels; wood parenchyma as irregularly spaced bands; latex traces on tangential surface common
19	Rengas (*Gluta/Melanochyla* spp.)	Heartwood blood-red in colour and streaky and distinct from light brown sapwood; growth rings features on tangential surface; vessels medium-sized, solitary and in radial multiples, tyloses common; wood parenchyma as irregularly spaced bands, paratracheal parenchyma as narrow border to the vessels.
20	Sesendok (*Endospermum diadenum*)	Heartwood bright yellow with a greenish tinge; Vessels large, mainly radial multiples; Wood parenchyma as regularly spaced bands; rays visible to the naked eye.

Sources: Menon, revised by Ani S & Lim S.C (1993) & Wong T.M (1976)

Timber species and identification of wood in Indonesia (tentative)

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DNA fingerprinting databases of *Neobalanocarpus heimii* (Dipterocarpaceae) throughout Malaysia for individual identification

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Abstract

Illegal logging poses a significant threat to the sustainability of Malaysian forest ecosystems. Presently, foresters have to depend on wood anatomical evidences to link the suspected timber thefts to the source trees but this is inconclusive. This study was aimed to utilize DNA markers in plant DNA fingerprinting for forensic applications using *Neobalanocarpus heimii* as a model.

To generate a comprehensive DNA database of *N. heimii* for individual identification, 30 natural populations were identified from 27 forest reserves, and a total of 1081 individuals were collected throughout Peninsular Malaysia. An extensive evaluation of 51 short tandem repeat (STR) primers developed for Dipterocarpaceae managed to identify 12 STR loci, which showed specific amplification, absence of null alleles, single-locus mode of inheritance, and absence of mononucleotide repeat motifs in *N. heimii*.

Cluster analyses via assignment test and genetic distance divided the 30 populations into three genetic clusters, corresponding to three geographical regions: Region A (west), Region B (central and south) and Region C (northeast). DNA databases of *N. heimii* were constructed and characterized at the levels of population, region and Peninsular Malaysia. Independence tests showed that the majority of the loci significantly deviated from Hardy-Weinberg equilibrium due to population substructuring and inbreeding. Thus, the match probability of *N. heimii* should be estimated using the 'subpopulation-cum-inbreeding model' that adjusted for coancestry (θ) and inbreeding (f) coefficients. The conservativeness tests showed that both the regional and Peninsular Malaysian databases were conservative and should be adequate to predict allele and genotype frequencies of *N. heimii* throughout Peninsular Malaysia. With a combined power of discrimination of >0.99999999999999999, the Peninsular Malaysian database should be able to provide legal evidences for court proceedings against illegal loggers on *N. heimii*.

The comprehensive DNA fingerprinting databases developed for *N. heimii* are the first reported for a tropical tree species and the methodology developed should be able to serve as a model for the study of other important timber species in Malaysia. The availability of DNA fingerprinting databases for the majority of important timber species in Malaysia would enhance the capacity of Forest Department officials to curb the problem of illegal logging and this would indirectly ensure the conservation and sustainable utilization of forest resources in Malaysia.

Geographical origin identification of four species of *Shorea* (Dipterocarpaceae) in Indonesia using chloroplast DNA

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Abstracts

The variation of chloroplast DNA were analysed in four species of *Shorea* (Dipterocarpaceae), i.e. *S. leprosula*, *S. parvifolia*, *S. ovalis*, and *S. johorensis* originated from populations in Kalimantan and Sumatra island. The haplotype variation is relatively low, *S. leprosula* and *S. ovalis* even showed no chloroplast variation. There is a total of 7 haplotypes and indicated that none of share haplotype variant among species is found. Phylogeographic structure of *Shorea* spp. is not clearly supported. Two haplotypes indicated as a specific cpDNA character for *S. parvifolia* in west Kalimantan and *S johorensis* in east Kalimantan.

Introduction

Shorea Roxb. *ex* Gaertn.f. (Dipterocarpaceae) is one of the most important timber in the Asian humid tropics. As a member of tribe Dipterocarpoideae, the natural distribution of *Shorea* comprises about 194 species in Sri Lanka, India, Myanmar, Thailand Indochina, and about 163 species in Malesia region [1]. Except in Java, the most abundant species of this genus located on the west side of Wallace's line, includes Kalimantan (137 species.) and Sumatra (50 species), while on the east side of Wallace's line the species number of *Shorea* is dramatically decreased with only one species in Sulawesi and three species in Moluccas [1, 2]. In compare to the other genera, *Shorea* is also the most affluent genus of lowland dipterocarp rain forest in Kalimantan with 12,3% of abundance [3].

The previous analysis found that the isozyme variation is exceptionally high (He=0.41) in *S. leprosula* [4]. Other genetic analysis using microsatellite markers revealed the high heterozigosity of *Shorea* spp., i.e. He=0.69-0.71 in *S. leprosula* [5, 6, 7], He=0.62-0.67 in *S. ovalis* [6], He=0.68-0.73 in *S. curtisii* [8], He=0.33-0.85 in *S. parvifolia* and He=0.42-0.76 in *S. acuminata* [9].

Although *Shorea* has widespread natural distribution, the ongoing timber harvesting and intensive forest exploitation in Indonesia could reduce the diversity and eliminate the unique genetic identity in the natural population. The logging activities caused the reduction of outcrossing rate significantly in *S. curtisii* and may result inbreeding depression and/or a decrease in genetic variation in future generations [8].

The chloroplast DNA in most angiosperms is transmitted from female parent [10]. In present study, the haplotypic diversity of four *Shorea* species within and among population in Kalimantan and Sumatra were observed using chloroplast DNA. This study is also an initial method to present the geographical distribution mapping of cpDNA haplotypes of *Shorea* spp and to explore the possibility of using molecular marker as a tool to prove the geographical origin of the individual trees.

Material and Methods

Four species of *Shorea* were investigated in this study, namely *S. leprosula* Miq., *S. parvifolia* Dyer, *S. ovalis* (Korth.) Blume, and *S. johorensis* Foxw. These species were chosen due to the broad distribution in Southeast Asia. In Indonesia, these four species are found in Sumatra and Kalimantan [1].

The samples were obtained from four sites in Kalimantan and one site in Sumatra (Table 1). The sites in Kalimantan are geographically widely separated. Each species was represented by 6

single adult trees from every site. Leaf tissue from adult trees was collected from Tebo natural forest, Jambi, Sumatra. Seeds were collected from natural forest in Kalimantan populations and identified according to the mother trees.

Total DNA was isolated with DNeasy® 96 Plant Kit (Qiagen GmbH, Hilden) or using CTAB method adapted from [11]. The quality of DNA was verified elecrophoretically in comparison to a standard of 50 ng λ DNA (Roche, Mannheim) on 0.8% agarose gel. Extracted DNA was diluted to 1–10% prior to PCR.

According to previous analyses of the phylogenetic relationships of Dipterocarpaceae [12], chloroplast DNA was analysed using three primer - restriction enzyme combinations for further population genetic studies on *S. leprosula*, *S. parvifolia*, *S. ovalis*, and *S. johorensis*., i.e. *rbcL* with *Alu* I and *Msp* I, and *petB* with *Rsa* I. Two cpSSR primers, i.e. *ccmp6* and *ccmp10*, were also selected to analyse these species. PCR conditions followed previous inverstigation [13, 14] with slight modifications.

Table 1. The population sites of samples obtained in Kalimantan and Sumatra

Species	**Approximate geographic location**	
	Latitude	**Longitude**
S. leprosula and *S. parvifolia*		
• West Kutai, East Kalimantan	0°00'–0°45' S	115°30'–115°45' E
• Muara Teweh, Central Kalimantan	0°00'–0°20' S	114°30'–115°10' E
• Ketapang, West Kalimantan	1°00'–1°15' S	110°45'–111°00' E
• Tebo, Jambi, Sumatra	1°00'–1°45' S	102°00'–102°45' E
S. ovalis (Korth.) Blume		
• Malinau, East Kalimantan	2°45'-3°21' N	115°48'-116°34' E
• Muara Teweh, Central Kalimantan	0°00'–0°20' S	114°30'–115°10' E
• Ketapang, West Kalimantan	1°00'–1°15' S	110°45'–111°0' E
• Tebo, Jambi, Sumatra	1°00'–1°45' S	102°00'–102°45' E
S. johorensis Foxw.		
• Malinau, East Kalimantan	2°45'-3°21' N	115°48'-116°34' E
• Muara Teweh, Central Kalimantan	0°00'–0°20' S	114°30'–115°10' E
• Ketapang, West Kalimantan	1°00'–1°15' S	110°45'–111°00' E

DNA fragments were separated after restriction on 2.5% agarose gels. Fragment size was estimated using DNA Molecular Weight Marker XIV (Roche, Mannheim) as a standard. The cpSSRs were amplified using fluorescence with one primer (the forward primer) that was labeled with the fluorescent dyes 6-FAM (blue), HEX (green) and NED (yellow). Amplification products were separated by capillary electrophoresis on the ABI Prism 3100® Genetic Analyzer (Applied Biosystems). GS 500 ROX (fluorescent dye ROX) was used as an internal size standard. Length variants of cpSSRs were recognized with the software packages GeneScan 3.7 and Genotyper 3.7.

The restriction site data of polymorphic cpDNA regions were transformed into a binary matrix (0,1) for further analysis. Length variants of restriction fragments and chloroplast microsatellites were coded as multistate characters. In order to analyse the genetic diversity, the hierarchical analysis of allelic variation among populations is calculated as follows [15]:

$$G_{ST} = (H_T - H_S) / H_T$$

Where H_T equals the gene diversity $1-\Sigma p_i^2$ in the total population of pooled demes and H_S is the average gene diversity within each of the population, p is the frequency of haplotype in each population.

Results

Haplotype variation

S. leprosula and *S. ovalis* showed no chloroplast DNA variation (haplotype A and B, respectively). Haplotype variation can be found in *S. parvifolia* (three haplotypes: C1 as a common haplotype, C2 and C3) and *S. johorensis* (two haplotypes: D1 as a common haplotype and D2). The variation among haplotypes in *S. parvifolia* has been detected in the *rbcL* gene digested with *Alu* I, and an amplification product with only one base pair difference in *ccmp6* (96-97 bp) and *ccmp10* loci (98-99 bp), with a total of four diagnostic characters. Likewise, haplotype variation in *S. johorensis* has been found in *rbcL* digested with *Msp* I and *petB* digested with *Rsa* I, and an amplification product with only one base pair difference in *ccmp6* (96-97 bp), with a total of three diagnostic characters. Haplotypes revealed in this research have low variation with only 0.0073 - 0.0292 mean character distances among haplotypes as shown in Tables 2 and 3.

Genetic variation within and among populations

The species observed, i.e. *S. leprosula*, *S. ovalis*, *S. parvifolia* and *S. johorensis* do not share haplotypes among species. The distribution of each haplotype per species in each population is shown in Table 4 and Figures 1-4. *S. leprosula* and *S. ovalis* did not exhibit chloroplast DNA variation in any population. Differentiation between populations of *S. parvifolia* is characterized by a G_{ST} value of 0.15. In West Kalimantan there were three haplotypes, whereas in East Kalimantan there was only one. The separate population in Jambi has two haplotypes. *S. johorensis* populations observed in Kalimantan show a G_{ST} value of 0.25. More than one haplotype has been found only in the East Kalimantan population.

Table 2. Pairwise distance matrix between haplotypes in *S. parvifolia*: total character differences (below diagonal) and mean character differences (above diagonal).

Haplotypes	C1	C2	C3
C1	-	0.0219	0.0073
C2	3	-	0.0292
C3	1	4	-

Table 3. Pairwise distance matrix between haplotypes in *S. johorensis*: total character differences (below diagonal) and mean character differences (above diagonal).

Haplotypes	D1	D2
D1	-	0.0219
D2	3	-

Table 4. Number of samples per geographical origin that contain each haplotype in each species

Geographical	***S. leprosula***	***S. ovalis***	***S. parvifolia***			***S. johorensis***	
Origin	**A**	**B**	**C1**	**C2**	**C3**	**D1**	**D2**
East Kalimantan	6	6	6	0	0	4	2
Central Kalimantan	6	6	5	1	0	6	0
West Kalimantan	6	6	3	2	1	6	0

Jambi	6	6	5	1	0	-	-

Discussion

In order to reconstruct the geographical origin of species with genetic markers, the analysis of the geographical structure of haplotypes is useful. Based on previous phylogenetic analyses on Dipterocarpaceae, four cpDNA genes (*rbcL*, *petB*, *psaA* and *psbA*) and two cpSSR loci (*ccmp6* and *ccmp10*) are polymorphic in genus *Shorea* [12]. In this study, further analysis in four populations of four *Shorea* species (*S. leprosula, S. ovalis, S. parvifolia* and *S. johorensis*) also revealed no shared haplotype among the species.

S. leprosula and *S. ovalis* populations showed no variation and have identical haplotypes as compared to the same species found in the phylogenetic analysis, namely haplotypes A and B, respectively. The four populations of *S. parvifolia* showed variation with three different haplotypes, i.e. C1 (as the common haplotype), C2, and C3. The geographical structure of haplotypes is not clear. Three haplotypes occurred in Kalimantan, whereas only two haplotypes occurred in Jambi, Sumatra. Observation on *S. parvifolia* revealed the low variation (4%) in *rbcL* gene digested with *Alu* I, and in loci *ccmp6* and *ccmp10*. Likewise, low variation (3%) in 3 populations of *S. johorensis* have been found in the *rbcL* gene digested with *Msp* I, the *petB* gene digested with *Rsa* I and at the locus *ccmp6*. Only 2 haplotypes, i.e. D1 (as the common haplotype) and D2, have been found in the east Kalimantan population, whereas only one haplotype has been found in west and central Kalimantan populations.

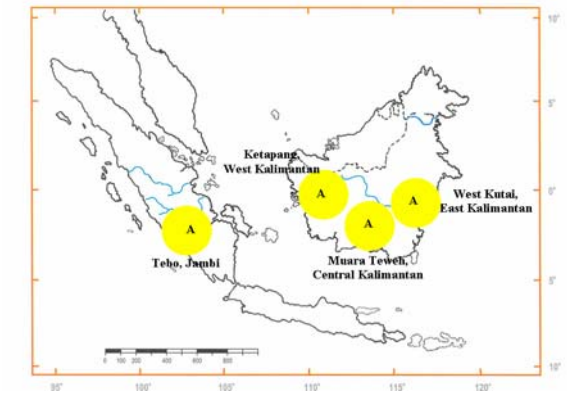

Figure 1. The haplotype distribution in four populations of *S. leprosula*

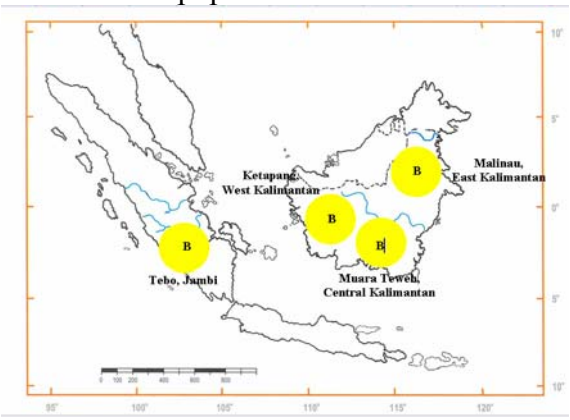

Figure 2.The haplotype distribution in four populations of *S. ovalis*

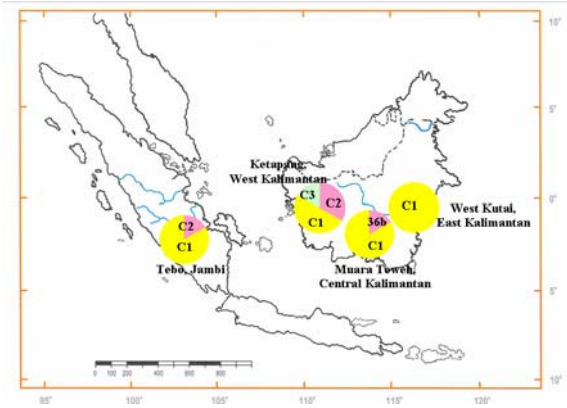

Figure 3. The haplotype distribution in four populations of *S. parvifolia*

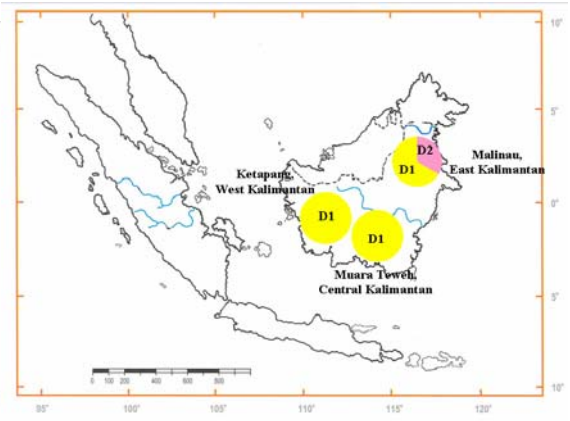

Figure 4.The haplotype distribution in four populations of *S. johorensis*

These species have a wide distribution; *S. johorensis* and *S. ovalis* occur in Kalimantan, Sumatra, and Peninsular Malaysia, whereas *S. leprosula* and *S. parvifolia* occurred in the same region but also in Thailand. It has been hypothesized that a number of widespread genera and species of Dipterocarpaceae may have originated during the late Cretaceous - early Tertiary period in these regions [16]. In Kalimantan, dipterocarps have evolved in all parts of the island between sea level and about 2000 m altitude in all kinds of habitats and it seems unlikely that speciation can be explained by adaptation to different niches [17]. Theoretically, it might be assumed that the origin of a series of closely allied species was the result of only a few mutations causing changes directly in a relatively small number of characters, but leading indirectly, through the consequent changes in morphogenesis involving different plant organs, to changes in many other characters [18]. Observation on all natural distributions is needed in order to confirm the evolutionary steps of these species, including the chloroplast DNA evolution.

Geographical structure of haplotypes is not always reflected by the natural distribution of certain species. The phylogeographic structure of species observed in white oaks / *Quercus* spp. [19, 20] and *Fagus sylvatica* [21] at the regional scale in Europe, showed the interrelationships between haplotype distribution and the origin of the samples. The molecular method for testing the geographic origin of such species has also been developed [22]. On the contrary, observation on *Prunus spinosa* populations revealed incongruency between the phylogeny of haplotypes and their geographic locations on the European continent [23]. Colonization history of some tropical species is also indicated by the distribution of haplotypes. *Cedrela odorata*, a tropical tree occurred in Mesoamerica, has five haplotypes and phylogenetically grouped into three distinct lineages [24], whereas observation on *Cyclobalanopsis glauca* populations in Taiwan and East Asia revealed 13 haplotypes and it can be concluded that the derived cpDNA variations are confined only to Taiwan [25].

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Outline of the research project "Development of Improved Methods to Identify *Shorea* Species Wood and its Origin"

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Timber identification, i.e. of tree species and their origin, is technically essential for monitoring, control and thus enforcement of specific trade regulations. Forestry and Forest Products Research Institute (FFPRI, Tsukuba) has been conducting this research project under the collaboration with Forest Research Centre, Sabah (FRC) and Forest Research Institute Malaysia (FRIM).

The genus *Shorea* (Dipertocarpaceae), the most important timber producing group in Southeast Asia, can be divided wood-anatomically into 4 sections which correspond to the characteristics of wood utilization as follows: Sect. *Rubroshorea* (Red Meranti Group), Sect. *Richetioides* (Yellow Meranti Group), Sect. *Anthoshorea* (White Meranti Group) and Sect. *Shorea* (Balau or Selangan Batu Group).

The TLC (Thin-layer chromatography)was developed to identify timbers of Sect. *Rubroshorea*, especially suitable for plywood, from other sections owing to the distinctive gallic acid content. In addition to common wood anatomical features of the genus *Shorea* such as axial resin canals in continuous concentric bands, vestured pits, heterogeneous size of vessel-ray pits and so on, novel helical thickenings with warts were found in some species. Also it was ascertained that horizontal resin canals are only in restricted species and crystals are contained in idioblasts in some species and in chambered in some others.

Specific DNA-sequences, varying among populations or regions or species, are considered as marker. Molecular classification of *Shorea* species based on chloroplast DNA 4 regions (ca.3500bp) have ever been undertaken using 104 samples, 34 species from Sect. *Rubroshorea*, 18 samples, 10 species from Sect. *Richetioides*, 16 samples, 8 species from Sect. *Anthoshorea*, and 24 samples, 13 species from Sect. *Shorea*. For DNA analysis, amplification of DNA from wood samples was required using polymerase chain reaction (PCR). Currently, chloroplast and mitochondrial genes can be successfully detected from the sapwood of larch (*Larix gmelinii)* and oak (*Quercus crispula*) heated at up to 180 ˚C.

Chemical markers such as extractives and inorganic elements, which have been already in use for the identification of the geographical origin of agricultural products, may have a potential regional or local variation in composition or contents due to genetic or ecological influences. Stable isotope ratios (delta^{18}O and delta ^{13}C) of wood specimens showed significant correlations to the latitudes and longitudes of their geographical origins and therefore indicated some possibility to identify the geographical origin.

Identification of red meranti based on the heartwood extractives

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Introduction

Shorea is the largest and economically most important genus in Dipterocarpaceae. It is classified into the white meranti, yellow meranti, red meranti, and balau (selanagan batu) groups, corresponding to the 4 sections *Anthoshorea*, *Richetioides*, *Rubroshorea*, and *Shorea*, respectively. Because the tariff on plywood made from red meranti is higher than that on the plywood obtained from the other sections of *Shorea* in Japan, a simple and rapid method is required for the identification of the section *Rubroshorea*.

Chemotaxonomy is the classification of plants based on the results of chemical analyses. The chemical constituents of woods are classified into major components (cellulose, hemicellulose, and lignin), and minor components extractable with solvents (wood extractives). The extractives consist of carbohydrates, waxes, terpenoids, phenols, tannins, flavonoids, etc. Generally, wood extractives have a stable chemical composition, since their biosyntheses are governed by the regulation of a few genes, with some compounds are limited to certain species only.

The chemotaxonomy of the genus *Shorea* based on the analyses of resins[1)] and leaves[2)] has been carried out; the occurrence of sesquiterpene alcohols and triterpenes in the resins of this genus has enabled the section *Anthoshorea* to be distinguished from other sections. Few systematic studies have been conducted on chemotaxonomy based on heartwood extractives; however, some resveratrol oligomers[3)] (Fig.1) and hydrolysable tannins[4)] (Fig.2) have been isolated,

In this study, the distribution of extractives in the genus *Shorea* was investigated, and a simple chromatographic method for the identification of red meranti was developed.

Determination of chromatographic method

Our preliminary research on heartwood extractives suggested that many resveratrol oligomers are widely distributed in the genus *Shorea*; however, the occurrence of gallic acid appears to be limited to red meranti. The chemical structures of resveratrol oligomers are very complex and many isomers occur; on the other hand, gallic acid (3,4,5-trihydroxybenzoic acid) is a simple phenolic acid and is readily available. Gallic acid is found in tea leaves, oak bark, and other plants, both free and as a constituent of tannins, and appears to be a good taxonomic marker.

Thin layer chromatography (TLC) is a common separation technique; it offers many advantages such as low cost, simplified sample preparation, high flexibility, and the parallel separation of many samples with minimal time requirement. Therefore, TLC conditions[5)] suitable for the detection of gallic acid have been considered. In the silica gel TLC, the spot of gallic acid often exhibits tailing, probably due to the interaction of the carboxyl and pyrogallol groups with the stationary phase. A considerable amount of acid in the developing solvent is required to avoid this phenomenon. The best result has been obtained using chloroform - ethyl acetate - formic acid, (v/v, 5:4:1), as the developing solvent. With regard to the spraying reagents, ferric chloride is considered suitable for the detection of gallic acid because it is highly sensitive to pyrogallol groups that turn dark blue in the presence of this compound; however, it is insensitive to resveratrol oligomers (Fig.3). The detection limit was approximately 0.2μg for gallic acid.

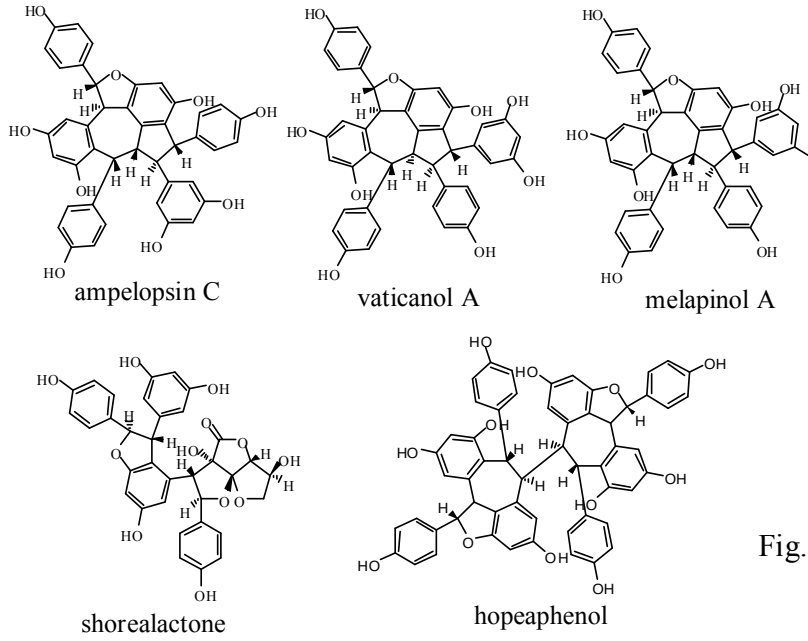

Fig.1 Resveratrol oligomers isolated from the heartwood of genus Shoprea

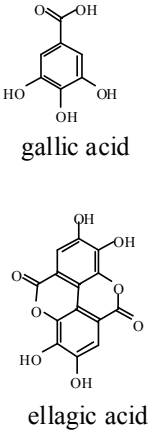

Fig.2 Tannin-related compounds isolated from the heartwood of genus Shoprea

Distribution of gallic acid in the genus *Shorea*

The saw dust of heartwood specimens, stored in the xylarium of the Forestry and Forest Products Research Institute (TWTw), were extracted with aqueous acetone, and the extracts were subjected to TLC analysis. The white meranti, yellow meranti, and balau groups sample gave no spots corresponding to gallic acid. In contrast, all the red meranti samples gave dark blue spots suggesting the existence of gallic acid (Table 1). The spots for dark red meranti were clearer and darker than those for light red meranti, suggesting a correlation between color and the presence of gallic acid. Gallic acid, which is colorless, turns red on oxidation. Its presence in red meranti was also confirmed using different developing solvents in TLC and by ^{1}H-NMR spectra.

From a practical point of view, gallic acid has been proven to be a good taxonomic marker, and a TLC method has been developed for the identification of the red meranti group. The distribution of gallic acid in the other genera in Dipterocarpaceae should be clarified for the further chemotaxonomic classification of this family.

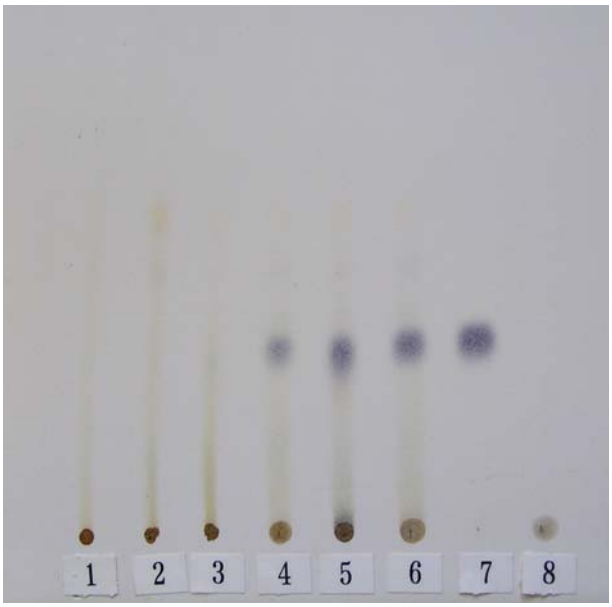

Fig.3 TLC of the heartwood extractives and the standard

1: *S.hypochra* (White Meranti) 2: *S.gibbosa* (Yellow Meranti)
3: *S.laevis* (Balau) 4: *S.polysperma* (Red Meranti)
5: *S.negrosensis* (Red Meranti 6: *S.pauciflora* (Red Meranti)
7: gallic acid 8: shorealactone

Table 1 Distribution of gallic acid in the genus *Shorea*

White Meranti (*Anthoshorea*)		Yellow Meranti (*Richetioides*)		Red Meranti (*Rubroshorea*)				Balau (*Shorea*)	
S.ochracea	-	*S.xanthophylla*	-	*S.dasyphylla*	+	*S.quadrinervis*	+	*S.inaequilateralis*	-
S.polita	-	*S.faguetiana*	-	*S.parvistipulata*	+	*S.negrosensis*	+	*S.kunstleri*	-
S.symingtonii	-	*S.gibbosa*	-	*S.curtisii*	+	*S.macroptera*	+	*S.venulosa*	-
S.floribunda	-	*S.hopeifolia*	-	*S.leprosula*	+	*S.amplexicaulis*	+	*S.havilandii*	-
S.gratissima	-	*S.patoensis*	-	*S.pauciflora*	+	*S.monticola*	+	*S.gisok*	-
S.hypochra	-	*S.illiasii*	-	*S.amplexicaulis*	+	*S.parvifolia*	+	*S.teng*	-
S.philippinensis	-	*S.kudatensis*	-	*S.almon*	+			*S.robusta*	-
S.bracteolata	-	*S.multiflora*	-	*S.gysbertsiana*	+			*S.laevis*	-
S.agami	-	*S.mujongensis*	-	*S.leptoclados*	+			*S.malibato*	-
S.talura	-	*S.acuminatissima*	-	*S.polysperma*	+			*S.domatiosa*	-
				S.scabrida	+			*S.guiso*	-
				S.rubella	+			*S.seminis*	-

Procedure for identification of red meranti using TLC method

1) Approximately 20mg of the sawdust of the heartwood is suspended in 4ml of 70% aqueous acetone (v/v), allowed to stand for 10 h at room temperature with stirring, and is then filtered.
2) The filtrate is evaporated under reduced pressure to dryness, and the resulting material is dissolved in 50 μl of acetone.
3) Five microliters of acetone solutions of the sample and standard gallic acid is spotted onto the TLC plate (Merck precoated Silica Gel 60F-254; layer thickness, 0.2mm) using a glass capillary tube.
4) The plate is dipped in the developing solvent chloform-ethyl acetate-formic acid (v/v, 5:4:1), in a glass container.
5) After development, the plate is heated to remove organic solvents.
6) The plate is placed under a UV lamp to detect UV-absorbing phenolic compounds as dark spots on a fluorescent background.
7) Ferric chloride solution (1%, in methanol) is sprayed on the plate.
8) The spot of gallic acid turns dark blue.

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Anatomical Identification of Wood of section Rubros*horea* species.

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Introduction

Trees of the section *Rubroshrea*, which is generally called red meranti (Malay, Indonesia), red seraya (Sabah), red lauan (Philipinnes) are the most dominant group in forests and their wood is utilized heavily in Malaysia, Indonesia and the Philippines. In this study, we would focus on the anatomical identification of the section *Rubroshrea* in genus *Shoreas*, and establish a database of anatomical characteristics based on the observation with light microscopy and electron microscopy.

Materials and Methods

For the database establishment, we collected data of anatomical characteristics of each specimen of section *Rubroshrea* in genus *Shorea* of the xylariums of Forest Research Institute Malaysia (FRIM), Forest Research Centre of Sabah (FRC) and FFPRI. With the observation with light microscopy, we mainly investigated the crystals and distribution of crystals in 343 samples of 45 species.

With the observation with scanning electron microscopy (SEM), we mainly investigated the modification of vessel walls such as warts and helical thickenings in 117 samples of 45 species.

Results and Discussion

Light Microscopy

With the observation with light microscopy, the existence of crystals was recognized in the axial parenchyma of 235 samples, and in the ray parenchyma of 133 samples of the section *Rubroshrea*. Crystals were always existed in idioblasts, which are enlarged cell, in the axial and the ray parenchyma (Figs. 1). It is often observed that a few axial parenchyma having crystals arrayed in a line. The distribution pattern of crystals is divided into 4 types: (1) crystals absent, (2) crystals present in axial parenchyma and absent in rays, (3) crystals present in axial and ray parenchyma, (4) no uniform pattern in crystal distribution.

Electron microscopy

The existence of warts on the surface of vessel walls was recognized 19 species, and was not recognized in 21 species with the observation with SEM. Existence of warts depended on the specimen in 5 species. As distribution pattern of warts are various, warts distributed like helical thickenings in some species such as *Shorea albida, S. monticola, S. waltonii, S. platyclados, S. parvifolia and S. macrophylla*. Helical thickenings were observed in some samples of these species (Fig. 2)

The patterns of distribution of crystals in the idioblasts and warts on the vessel walls are useful characteristics to identify wood of red meranti into the species level.

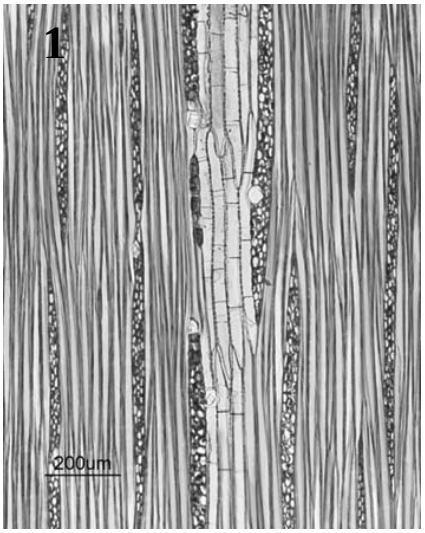

Figure 1. Crystals in axial and ray parenchyma. *Shorea fallax.*
Figure 2. Warts distributing like helical thickenings on the inner surface of a vessel wall. *Shorea macrophylla*.

Manual of identification of wood species based on anatomical characteristics

Light microscopy

1) Thin sections are cut from the transverse, radial and tangential surfaces of the samples with a razor blade or a hand knife.
2) These sections are put on a glass slide, and are mounted in glycerol. The glass slide is covered by a cover glass, and is heated with a temperature of 120-150˚C on a hot plate.
3) Bubbles are coming out from the sections, because of water evaporation. This causes the removal of air bubbles in the sections.
4) As bubbles stop to come out, the samples are observed under a microscope.
5) The characteristics of red meranti
   Continuous concentric bands of axial resin canals present
   Vestured pits
   Vessel-ray pits not very common, simple, and round to oval, irregular in size.
   Silica grains absent.
   Crystals in long chambered axial parenchyma absent.
   Wood color red.
   Horizontal resin canals with small diameter absent (Horizontal resin canals with

Electron microscopy

1) Specimens are treated with aquatic of sodium hypochlorite for 3minutes to remove the residua of cell contents form the surface of vessel walls.
2) The specimen are rinsed with sufficient water, and dehydrated with an graded alcohol. The specimen are dried in air condition.
3) The specimen are observed by a SEM with or without metal coating.
4) The modification of vessel walls is investigated.

Characteristics of the specimen obtained with the observation with light microscopy and SEM are compared to the list of table 1.

Table 1 Subdivisions of *Shorea* section *Rubroshorea* by characteristics of crystals, warts and fiber wall thickness

○: present, ×: absent, △: various amonog specimens, S: warts distributing like helical thickening.

	Species	Crystals	Crystals in rays	Warts	Fiber wall thickness
Species with radial resin canals					
A	ovata	○	×	△	thin–thick
	teysmaniana	○	×	×	thin
	leprosula	○	○	○S	thin
Species without radial resin canals					
B	kunstleri	○	○	○	thick
	minor	○	○	○	thick
	fallax	○	○	△	thin–thick
	parvistipulata	○	○	△	thin–thick
	andulensis	△	△	○	thick
C	ovalis	△	△	△	thin
	fallax	○	○	△	thin–thick
	parvistipulata	○	○	△	thin–thick
	macrophylla	○	○	○S	thin
	almon	○	○	○	thin
	joholensis	○	○	○	thin
	palembanica	○	○	○	thin
D	uliginosa	○	○	×	thick
	pauciflora	○	○	×	thin–thick
E	pauciflora	○	○	×	thin–thick
	pinanga	○	○	×	thin
	scabrida	○	○	×	thin
	platycarpa	○	△	×	thin
	amplexicaulis	△	△	×	thin
F	platyclados	△	×	○S	thick
	inaequilateralis	○	×	○	thick
	andulensis	△	△	○	thick
G	parvifolia	○	×	○S	thin
	smithiana	△	×	○	thin
	mecistopteryx	○	×	△	thin
	ovalis	△	△	△	thin
H	dasyphylla	○	×	×	thick
	venulosa	○	×	×	thick
	ferruginea	○	×	×	thin–thick
I	ferruginea	○	×	×	thin–thick
	ovalis	△	△	△	thin
	argentifolia	○	×	×	thin
	macroptera	○	×	×	thin
	pilosa	○	×	×	thin
	polysperma	△	×	×	thin
J	andulensis	△	△	○	thick
	bullata	×	×	○	thick
	platyclados	△	×	○S	thick
	albida	×	×	○S	thick
	monticola	×	×	○S	thick
K	smithiana	△	×	○	thin
	curtisii	×	×	○	thin
	scaberrima	×	×	○	thin
	waltonii	×	×	○S	thin
	ovalis	△	△	△	thin
L	ovalis	△	△	△	thin
	polysperma	△	×	×	thin
	negrosensis	×	×	×	thin
	beccariana	×	×	×	thin
	palosapis	×	×	×	thin
	quadrinervis	×	×	×	thin
	rubella	×	×	×	thin

Extraction and Detection of DNA from Wood for Species Identification

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Abstract

DNA analysis is an effective method for identifying tree species, but it requires a sufficient quantity and quality of DNA. We investigated how the position in xylem and heat treatment affect the efficiency of DNA extraction and the quality of DNA from wood. The efficiency of DNA extraction was higher for sapwood and for the outer parts of the xylem of species with an indistinguishable sapwood/heartwood boundary (ripewood) than for heartwood and for the inner parts of the xylem of ripewood. A large portion of extracted DNA ranged in size from 250 to 2,000 base pairs. A chloroplast gene (*rbcL*), a mitochondrial gene (*cox1*) and a nuclear gene (rDNA) were detected in the DNA extracted from the sapwood of 3 species and from the outer parts of the xylem of 3 ripewood trees by polymerase chain reaction. In heat-treated sapwood of *Larix gmelinii* var. *japonica* and *Quercus crispula*, DNA became more degraded at treatment temperatures of 140ºC and over. The detection of the genes was reproducible for the DNA from *Larix gmelinii* var. *japonica* specimens heated up to 160ºC, but it was inconsistent for specimens treated at 180ºC. PCR performed with Ampdirect Plus, a reagent recalcitrant to PCR inhibitors, enabled the detection of genes in DNA from some sapwood and heat-treated wood at 180 ºC, which could not be done by PCR with an ordinary reagent. These results suggest that DNA for the detection of genes can be extracted from the outer parts of the xylem of several tree species, and from sapwood heated to temperatures up to 180 ºC.

Introduction

Wood identification has been carried out based on morphological and/or chemotaxonomical methods. However, it is often difficult to identify species solely by using those methods. As molecular DNA technology advances, it is being used in forensics, parentage tests, identifying the origin of food materials and so on. This technology would also be applicable to wood identification. For that purpose, it is necessary to isolate a sufficient quality and quantity of DNA from wood, and to amplify suitable DNA region(s) by polymerase chain reaction (PCR).

There are some reports about extraction and amplification of DNA from various types of wood including fresh wood[1)], dry wood[2-6)], herbarium specimens[5,7)], buried wood[8)] and submerged wood[9)]. Although the efficiency of DNA extraction and successful DNA amplification by PCR are known to depend on the conditions and duration of storage, it is not clear how the efficiency of DNA extraction is affected by the position in xylem of trees belonging to different families. In addition, the influence of heat treatment of wood on the extraction and amplification of DNA has not been systematically examined. Thus, we investigated how the position in xylem and heat treatment affect the efficiency of DNA extraction and the amplification of DNA from wood. We also tested the efficacy of a PCR reagent, which is recalcitrant to PCR inhibitors, in DNA amplification from wood DNA that was not amenable to PCR amplification with an ordinary PCR reagent.

Materials and Methods

Wood samples

Ten types of broadleaved and coniferous wood were used for this experiment (Table 1). The xylem of the wood specimens was divided into 4 parts (one part from sapwood and 3 parts from

heartwood, along the radial direction). When the wood had an indistinguishable sapwood/ heartwood boundary (ripewood), the xylem was equally separated into 4 parts. For heat treatment, sapwood of *Larix gmelinii* var. *japonica* and *Quercus crispula* was used.

The sapwood was cut into slices of 2 mm in thickness, and the pieces were heated in an air-circulated heater for 5 min at either 60, 100, 140, 160 or 180ºC.

Table 1. Origin of wood samples

Species	Collection year	Collection site	Specimen No. (TWTw)
Morus australis Poir.	2004	Sapporo	21871
Prunus sargentii Rehder	2004	Sapporo	21795
Styrax obassia Sieb. et Zucc.	2004	Sapporo	21840
Quercus crispula Blume	2004	Sapporo	21861
Salix udensis Trautv. et C.A.Mey.	2004	Sapporo	21803
Juglans mandshurica Maxim.	2004	Sapporo	21802
var. *sieboldiana* (Maxim.) Makino			
Larix gmelinii Rupr. ex Kuzen.	2004	Sapporo	21814
var. *japonica* (Maxim. ex Regel) Pilg.			
Pinus koraiensis Sieb. et Zucc.	2004	Sapporo	21832
Abies firma Sieb. et Zucc.	2004	Sapporo	21864
Ginkgo biloba L.	2004	Sapporo	21789

Disruption of wood

Small, chopstick-like pieces were cut from wood with a chisel. The pieces were further cut into smaller ones with a pair of pruning scissors, and powdered with a disruptor (Multi-Beads shocker; Yasui Kikai, Japan). About 1 g of the pieces was placed into a 50 ml centrifuge tube (NALGENE; 3117-0500), together with a metal cone (Yasui Kikai; MC-5035PC). A cap (Yasui Kikai; CP-5010) and an aluminum spacer (Yasui Kikai; ALSP50(S)) were then put on the tube. After placing the tube in a suitable sample holder in the Multi-Beads shocker, the shocker was run at maximum frequency with a 10-sec disruption/10-sec rest cycle for 5 to 15 repeats. Another disruptor, Wig-L-Bug Model 30 (International Crystal Laboratories, USA) may be also utilized for pulverization. Wood powder can be prepared with a fret saw without the use of a mill or disruptor.

Extraction of DNA

DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN) and a QIAshredder Maxi Spin Column in a DNeasy Plant Maxi Kit (QIAGEN). During the extraction process, the authors wore disposable gloves, and used sterile filtered pipette tips. The procedure was as follows.

1) Transfer ca. 1 g of wood powder to a mortar (inner diameter: 9 cm) and add 4 ml of Buffer AP1 (when precipitates form in Buffer AP1, dissolve them by warming to 65 ºC). Leave it for 10 min for swelling.
2) Spread the wet powder with a pestle. Freeze the sample by pouring liquid nitrogen on it and grind it with the pestle. Continue grinding for a few minutes after the sample thawed.
3) Transfer the sample to a new 50 ml screw-cap centrifuge tube with a small spoon. Wash off

the spoon with 1 ml of Buffer AP1.
4) (Option: add 8 μl of RNaseA.)
5) Incubate the mixture for 10 min at 65 ºC. Mix 2-3 times during incubation.
6) Add 1.625 ml of Buffer AP2 to the mixture, and mix. Place the tube on ice for 5 min.
7) Centrifuge the tube for 10 min at maximum speed.
8) Decant the aqueous phase to a QIAshredder Maxi Spin Column, and centrifuge at 3,000-5,000 x g for 5 min at room temperature in a swing-out rotor.
9) Transfer flow-through to a new 15 ml screw-cap tube (e.g., Falcon; 2097).
10) Transfer the remaining debris in the 50 ml tube to the QIAshredder Maxi Spin Column (reused) with a spoon, and centrifuge at 3,000-5,000 x g for 5 min at room temperature in a swing-out rotor.
11) Decant the flow-trough to the same 15 ml tube. Add 1.5 volumes of Buffer AP3/E, and mix by inverting the tube.
12) Place a DNeasy Mini Spin Column on a vacuum manifold (QIAvac 24 Plus). Apply the mixture prepared in Step 11 to the Spin Column, and pass it through. (This step can be performed by centrifugation: Apply 650 μl of the mixture from Step 11 to the DNeasy Mini Spin Column sitting in a 2 ml collection tube. Centrifuge for 1 min at 6,000 x g, and discard flow-through. Repeat this process.)
13) Place the DNeasy Mini Spin Column in a new 2 ml collection tube. Add 0.6 ml of Buffer AW to the DNeasy Mini Spin Column, and centrifuge for 1 min at 6,000 x g. Discard flow-through and reuse the collection tube.
14) Add 0.5 ml of Buffer AW to the DNeasy Mini Spin Column, and centrifuge for 5 min at 20,000 x g.
15) Transfer the DNeasy Mini Spin Column to a new 1.5 ml tube and pipette 100 μl of Buffer AE. Incubate for 5 min at room temperature, and then centrifuge for 1 min at 6,000 x g.
16) Repeat Step 15 once.

Determination of DNA concentration

If RNase A is included in the above procedure (at Step 4), concentration and purity of DNA can be determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer. The sample DNA solution should be adequately diluted for measurement. An absorbance of 1.0 at 260 nm corresponds to 50 μg of DNA per ml. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9.

When RNase was not included in the above procedure, we measured DNA concentration with a fluorescence spectrophotometer (Hitachi; F-3010). Then we mixed 2 μl of DNA solution and 1 ml of measuring buffer (10 mM Tris-HCl (pH7.5), 1 mM EDTA, 100 mM NaCl and 0.1 μg ml^{-1} Hoechst33258 (Invitrogen; H1398)). Fluorescence at 455 nm was measured with excitation at 352 nm. DNA concentration was determined by comparing fluorescence strength with that of standard DNA (λDNA).

Determination of DNA length

DNA was separated on a 0.7% agarose gel containing 0.25 μg ml^{-1} of ethidium bromide. After electrophoresis, DNA was made visible under UV light. DNA length was determined by referring to DNA size markers.

Polymerase chain reaction (PCR)

A chloroplast gene *rbcL* (ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit), a mitochondrial gene *cox1* (cytochrome oxidase subunit 1) and a nuclear DNA ITS1 (internal transcribed spacer region 1) of ribosomal DNA were selected for amplification by PCR. The primers used for the PCR are listed in Table 2.

For the amplification of ITS1, the following combinations of primers were used: *Prunus sargentii*, *Quercus crispula*, *Salix udensis* and *Ginkgo biloba*, rDNA-F1 and rDNA-R1; *Morus australis* and *Styrax obassia*, rDNA-F2 and rDNA-R1; *Larix gmelinii* var. *japonica*, LAITS1B and rDNA-R2; *Pinus koraiensis*, ITS1N and rDNA-R3.

PCR was performed with two independent reagents. One was GoTaq Green Master Mix (Promega). The reaction mixture

for PCR was composed of 12.5 μl of GoTaq green master mix, 0.5 μM primers and an adequate amount of DNA (e.g., 50 ng or 5 μl) in a total volume of 25 μl. The other was Ampdirect Plus (Shimadzu). The reaction mixture for PCR was composed of 12.5 μl of 2 x Ampdirect Plus, 0.25 μl (0.625 unit) Blend Taq -Plus- (Toyobo), 0.5 μM primers and an adequate amount of DNA (e.g., 50 ng or 5 μl) in a total volume of 25 μl. Amplification was performed with an initial denaturation step at 95ºC for 90 sec followed by 35 or 40 cycles of denaturation at 95ºC for 30 sec, annealing at 56ºC for 30 sec, and extension at 72ºC for 50 sec. The PCR products were analyzed on 0.7% agarose gels containing ethidium bromide.

Table 2. Primer sequences used for the PCR

Primer name	Sequence (5' - 3')	Reference
rbcL-F	GGACTTACCAGTCTTGATCG	(10)
rbcL-R	TCACATGTACCTGCAGTAGC	(10)
cox1-F	CGGTCTTCGGGTATCTAGGC	(10)
cox1-R	TCCATCCAGCGTAAGCATCT	(10)
rDNA-F1	GAACCTGCGGAAGGATCATTG	(10)
rDNA-F2	CGTGATGGGGATAGATCATTGC	(10)
LAITS1B	CCAAGGGCCTTGCATCAT	(11)
ITS1N	CGTAACAAGGTTTCCGTAGG	(12)
rDNA-R1	AGTCCCGCCTGACCTG	(10)
rDNA-R2	CAGCGACAACAAGCAATGC	(10)
rDNA-R3	TCCCTTGACCCAACCACC	(10)

Sequencing analysis

PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Sequences were determined by direct sequencing with the same primers used in the PCR. Sequences were searched against a public DNA database (e.g., DNA Data Bank of Japan (DDBJ), http://blast.ddbj.nig.ac.jp/top-j.html) by BLASTN. The origin of the sequence which showed the highest similarity was compared with the species of the sample.

Results and Discussion

DNA extraction and DNA amplification from different positions of xylem

When manufacturing wood products such as plywood, the xylem part in the lumber is not considered in most cases. Therefore, wood products contain various parts of xylem. To reveal the relationship between the efficiency of DNA extraction from wood and its position in the xylem, DNA was extracted from 4 different parts in the radial direction of xylem from 6 broadleaved trees and 4 conifers (Table 3).

For the wood from species which have a distinguishable sapwood/heartwood boundary (*Morus australis*, *Prunus sargentii*, *Quercus crispula*, *Larix gmelinii* var. *japonica* and *Pinus*

koraiensis), the efficiency of DNA extraction was higher for sapwood than for heartwood. It was also higher for the outer parts of the xylem of species with an indistinguishable sapwood/heartwood boundary (ripewood; *Styrax obassia*, *Salix udensis*, *Ginkgo biloba* and *Abies firma*) than for the inner parts of the xylem. This tendency was common in both the broadleaved and coniferous species in this experiment.

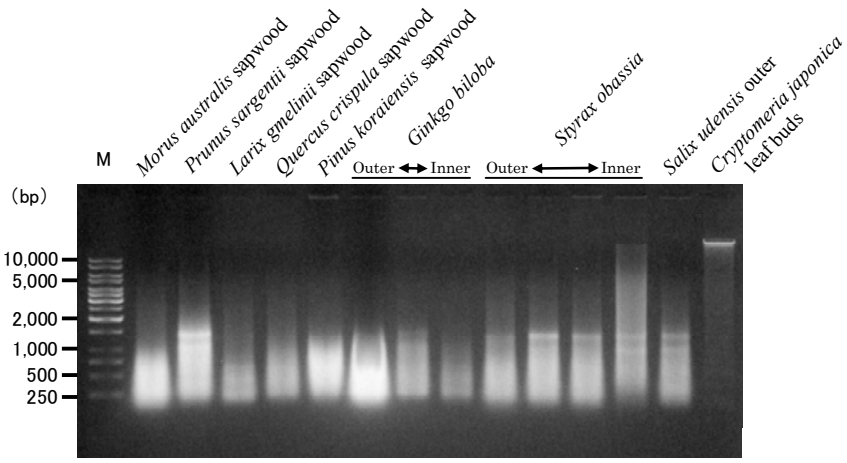

Fig. 1. DNA extracted from various wood specimens
M, DNA size marker
Outer, Outer part of xylem
Inner, Inner part of xylem

The length of extracted DNA was examined by agarose gel electrophoresis (Fig. 1). DNA isolated from *Cryptomeria japonica* leaf buds was nearly 20 kbp (base pairs) in size. In contrast, a large portion of the DNA extracted from wood ranged in size from 250 to 2,000 bp. DNA prepared from submerged wood[9)] and herbarium specimens[5)] was reported to range from 125 to 23,000 bp and from 50 to 10,000 bp, respectively. It appears that DNA obtained from wood is generally degraded.

Table 3. DNA extraction efficiency from different positions of xylem along the radial direction

Species	DNA extraction efficiency (mg g^{-1} wood) Position along the radial direction			
	Outer side	⟵		Inner side
Morus australis	13.9	2.3	1.6	1.0
Prunus sargentii	8.3	0.8	0.3	0.2
Styrax obassia	7.5	6.1	5.2	1.5
Quercus crispula	7.1	0.8	0.4	0.3
Salix udensis	1.5	0.5	0.2	0.3
Juglans mandshurica var. *sieboldiana*	0.3	0.3	0.3	0.1
Larix gmelinii var. *japonica*	17.2	0.0	0.0	0.0
Ginkgo biloba	4.2	2.4	1.4	0.1
Pinus koraiensis	1.0	0.1	0.1	0.0
Abies firma	0.5	1.2	0.1	0.0

Underlined values denote heartwood position (and discolored part for *Styrax obassia* and *Ginkgo biloba*).

DNA extracted from plants contains DNA of the nucleus, chloroplast and mitochondria. Chloroplast and mitochondrial genes are easy to detect because of their high copy number in a cell.

Chloroplast genes are commonly used for phylogenetic studies of plants[13]. Mitochondrial genes are utilized to identify animal species. In nuclear DNA, ribosomal DNA is amenable to analysis because it forms clusters of repeat units, and is used for forensics and parentage tests.

We examined whether *rbcL*, *cox1* and rDNA could be detected by PCR in DNA from sapwood and ripewood from which at least 1.4 μg g^{-1} of DNA was obtained (Fig. 2). Among the species with a distinguishable sapwood/heartwood boundary, the genes were detected in sapwood DNA of *Morus australis*, *Prunus sargentii* and *Larix gmelinii* var. *japonica*, but not in sapwood DNA of *Quercus crispula* and *Pinus koraiensis*. In ripewood species (*Ginkgo biloba*, *Styrax obassia* and *Salix udensis*), the genes were detected in DNA from the outer part of the xylem, but not from the inner part. Sequencing of PCR products and searching them against a public DNA database confirmed the origin of DNA from each sample wood.

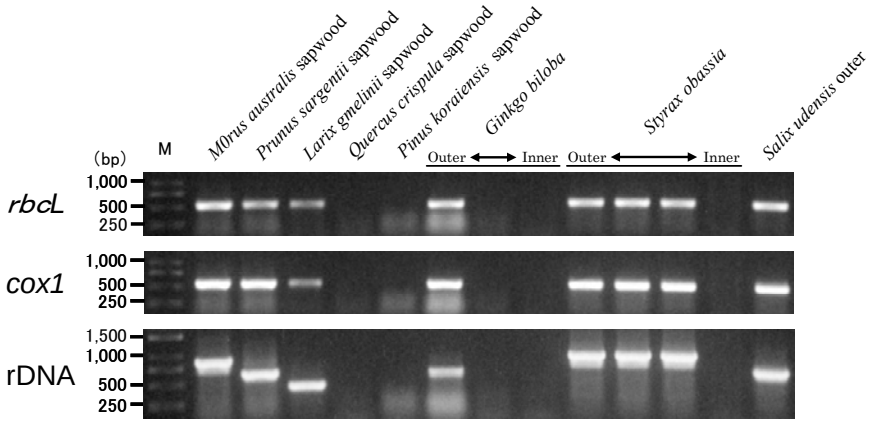

Fig. 2. Detection of genes in DNA extracted from different positions of xylem
M, DNA size marker
Outer, Outer part of xylem; Inner, Inner part of xylem

These results suggest that DNA suitable for DNA analysis can be extracted from wood, but extraction efficiency largely depends on the species and/or the position in xylem. Furthermore, genes are not always detected by PCR, if DNA is obtained from wood.

Influence of heat treatment

Lumber is frequently artificially dried before use. In the manufacturing process of plywood and laminated wood, raw wood is treated at high temperature for drying and adhesion. We examined the influence of heating on the efficiency of DNA extraction

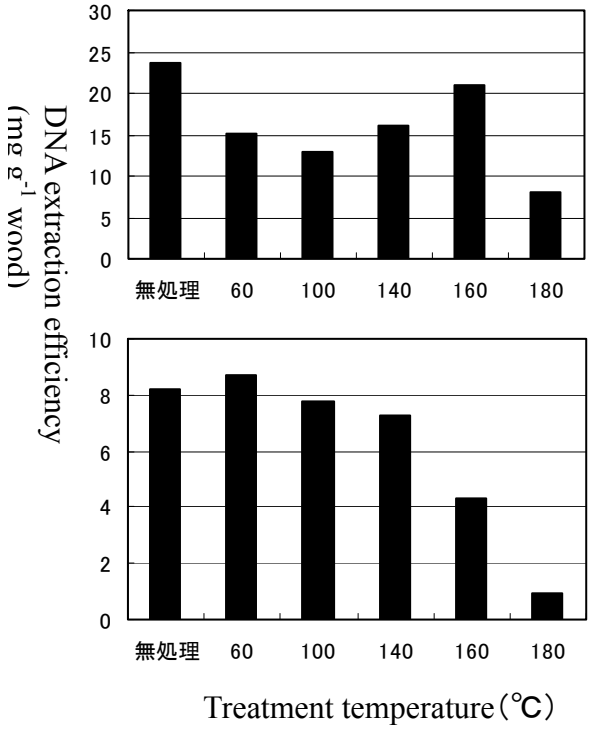

Fig. 3. DNA extraction efficiency from heat-treated wood specimens
A, *Larix gmelinii* var. *japonica* (n=2), B, *Quercus crispula*, Room temp., 140ºC (n=2); 60, 100, 160, 180ºC (n=1)

from wood.

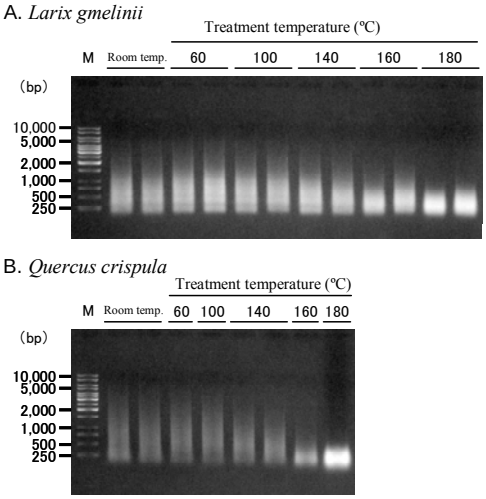

Fig. 4. DNA extracted from heat-treated wood specimens
M, DNA size marker

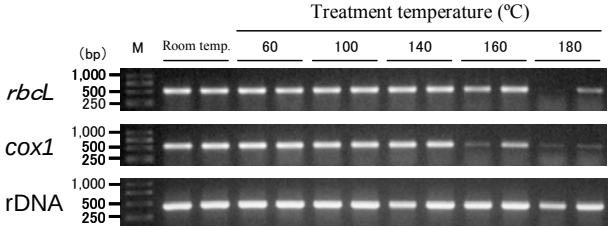

Fig. 5. Detection of genes in the DNA extracted from heat-treated *Larix gmelinii* var. *japonica* wood
M, DNA size marker

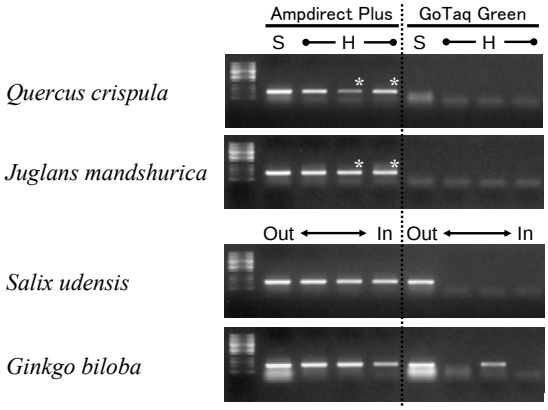

Fig. 6. Comparison of PCR reagents for amplification of DNA from different position of xylem
S, Sapwood; H, Heartwood; Out, Outer part of xylem; In, Inner part of xylem; *, Amplification based on contaminated DNA

Quercus crispulaLarix and *gmelinii* var. *japonica* were selected because the former and the related species (*L. kaempferi*) of the latter are used for plywood. Efficiency of DNA extraction from the sapwood of *L. gmelinii* var. *japonica* did not vary up to 160 ºC, but it declined at 180 ºC (Fig. 3A). Although the efficiency from the sapwood of *Q. crispula* was consistent below 100 ºC, it gradually decreased over the temperature (Fig. 3B). In heat-treated sapwood of *L. gmelinii* var. *japonica* and *Q. crispula*, DNA became more degraded at treatment temperatures of 140ºC and over (Fig. 4).

The detection of the genes by PCR was reproducible for the DNA from *L. gmelinii* var. *japonica* sapwood heated up to 160ºC, but it was inconsistent for specimens treated at 180ºC (Fig. 5). As the temperature of heat treatment rose, DNA became increasingly degraded (Fig. 4) and this may have hindered the amplification of DNA.

Efficacy of a PCR reagent that is recalcitrant to PCR inhibitors

It was difficult to detect genes by PCR in DNA extracted from heartwood, sapwood of several species, and sapwood treated at high temperature. It was assumed that PCR inhibitors were present or generated in such samples. Thus, we tested a commercial PCR reagent (Ampdirect Plus) that can neutralize PCR inhibitors. In our study, genes were not detected in the DNA extracted from sapwood and heartwood of *Quercus crispula* and *Juglans mandshurica* var. *sieboldiana*, and from the inner part

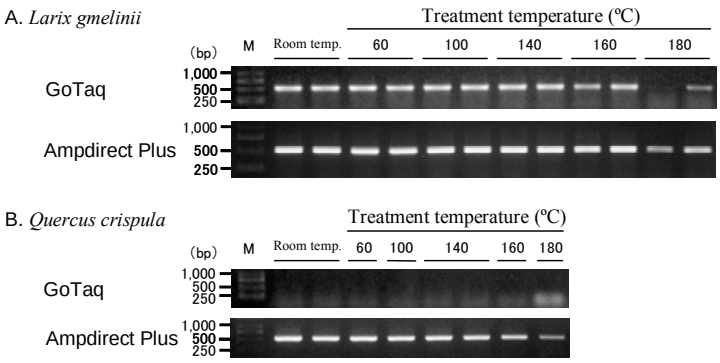

Fig. 7. Comparison of PCR reagents for amplification of DNA from heat-treated wood

of *Salix udensis* and *Ginkgo biloba* xylem. By using Ampdirect Plus, *rbcL* were amplified from those DNA (Fig. 6).

Although amplification of DNA was inconsistent with an ordinary PCR reagent for *Larix gmelinii* var. *japonica* sapwood treated at 180 ºC (Fig. 5), the efficiency of amplification was improved by Ampdirect Plus (Fig. 7A). For *Quercus crispula*, genes could not be detected even in the DNA from untreated sapwood. However, *rbcL* was detected in DNA from sapwood treated at all temperatures (Fig. 7B).

These results indicate that Ampdirect Plus is effective for amplifying DNA from wood, and can be utilized for identifying species used in wood products.

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Molecular database for species classification of *Shorea* species (Dipterocarpaceae)

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Introduction

Dipterocarpacea is keystone species in ecosystem and forestry of Southeast Asia. This family is predominantly distributed in tropical lowland and hill forests and occupied nearly 55 % of the stand volume of the forest in lowland forest (Symington 1943). This family consists of 10 genera with 386 species and shows a high rate of endemicity (Ashton 1982), which means that some species distribute in the very restricted region.

The trees have been utilized in local communities for long time. But once commercial logging has been started, the tropical forests have been dramatically reduced and declined, and illegal logging sometimes occurred in many places of countries. Illegal logging might not disappear without the solution of the poverty and economic problems in these regions. However, if we develop the species identification system using DNA information from woods that appear in market, this system might act as deterrent power to prevent the illegal logging.

Therefore, we have been developing the DNA database to identify the species of dipterocarp using sequence data of chloroplast DNA.

Materials and methods

Materials for molecular classification of *Shorea*

We collected leave tissue samples of *Shorea* species in arboretums in Forest Research Institute Malaysia and Sabah Forest Research Center, and permanent ecological research plots such as Lumbir forest reserve and Pasoh forest reserve in Malaysia. We also collected samples from natural forest directly in Indonesia, in this case, we made herbarium specimen for each sample (Table 1).

Sequence of intregenic spacer regions of chloroplast DNA

Total DNA was extracted by a modified CTAB method (Tsumura et al. 1995), or Dneasy Plant Mini Kit (Qiagen). Four non-coding regions of chloroplast genome were selected for polymerase chain reaction (PCR) amplification because these regions were expected to show high polymorphisms from preliminary studies: (1) *trn*L (UAA) intron; (2) *trn*L (UAA) 3 exon and *trn*F (GAA); (3) *trn*H(GUG) and *trn*K(UUU); and (4) *psb*C and *trn*S(UGA) (Table 2).

The PCR reaction mix contained PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 100 lM each dNTP, 0.02% Triton X-100, 0.01% gelatin), 1.5 mM MgCl2, 0.02 U Taq polymerase, 0.2 lM of each primer, and 10 ng template DNA in a total volume of 20 μ L. The PCR amplification was an initial 3 min denaturation at 94 °C; 30 cycles of 30 s denaturation at 94 °C, 45 s annealing at 50 °C, and 45 s extension at 72 °C; with a final 5 min extension at 72 °C.

The PCR products were purified by using the QIAquick PCR purification Kit (Qiagen). The obtained DNA was sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). All four selected regions were sequenced in both directions for all individuals. Sequencing data were aligned manually with CLUSTALW (Thompson et al., 1994).

Table 1 Number of species and individuals in Shorea species

Groups	Number of species	Number of individuals
White meranti	8	16
Yellow meranti	10	18
Balau	13	24
Red meranti	34	104
Total	65	162

Table 2 Number of SNPs to discriminate the four groups of Shorea

Group	Fixed difference between group	Putative mulitiple mutations	Variation only within group
White meranti	9	5	29
Yellow meranti	11	2	7
Balau	6	6	34
Red meranti	5	8	48

Data analysis

We constructed molecular phylogenetic tree using neighbor-joining method (Saito and Nei 1987) to confirm whether each woody color group (Symington 1943) such as red meranti, yellow meranti, white meranti and balau are monophyletic group or not. We used *Vatica micrantha* as an outgroup of *Shorea* species to know the evolutionary relationship between the four groups. In case that each woody color group was monophyletic group, single nucleotide polymorphisms (SNP) to discriminate the four groups were searched using alignment sequence data. Then, we classified these SNPs into three categories, which were fixed difference between groups, putative multiple mutations, and variation only within group. We have also searched SNPs to discriminate species within group, especially *Shorea albida*, which is the species categorized the different tax rate to the other *Shorea* species in Japan.

Results and discussion

We have done alignment of obtained sequence regions, which were 506 bp of *trn*L intron, 441bp of *trn*L-*trn*F, 1780 bp of *trn*H-*trn*K, and 1559 bp of *psb*C-*trn*S. The total sequence length was 4286 bp including some gap sequences.

Phylogenetic tree suggested that each group based on woody color is monophyletic origin (Fig. 1), which means that we can find SNPs to discriminate four groups. Number of fixed difference nucleotide sites were 9, 11, 6 and 5 in white, yellow, balau and red metanti group,

respectively (Table 2). These SNPs are good source to make discriminated DNA markers of the four groups.

Within group, some species have completely same sequence for four regions of chloroplast DNA (Fig. 1). For example, sequences of four regions in *S. amplexicaulis*, *S. pinosa*, *S. splendida*, *S. stenoptera* and *S. macrophylla* were identical, which species belong to same section Pachycarpae. However, species belong to different section sometimes have identical sequences. For those species, it is necessary to confirm the result to take more sequence data not only chloroplast DNA but also nuclear DNA. Because if the species was a hybrid origin, the species has the organelle DNA derived from mother species. In this case, it is impossible to distinguish between a hybrid and the mother species only in chloroplast DNA.

In this study, we used sometimes multiple samples in each species that were collected at different regions such as Peninsula Malaysia, Sabah and Sarawak states, and Indonesia. The sequence data of some individuals in same species was not identical, which might indicate the possibility of region discrimination in some species. To confirm that, we have to investigate more samples originated from multiple regions and need to accumulate more data.

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Fig. 1 Plylogenetic tree of *Shorea* species based on sequence data of four chloroplast DNA regions.

Stable isotopes and inorganic elements as potential indicators of geographic origin of Southeast Asian timber.

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Introduction

Analysis of stable isotopes and inorganic elements has been used as chemical fingerprinting tools to identify geographic origin of plant-based agricultural products (Fujita 2003). For example, geographic origin of the following products can be identified through the chemical fingerprinting technique; beverages (wine, juices, coffee and tea; Martin et al. 1988, Robards et al. 1995, Kim and Smith 2002, Weinert et al 1999), grains (soybeans and rice, Homura et al. 2005, Orita et al. 2002) and cocaine (Ehleringer 2000).

Very few studies have applied these chemical fingerprinting methods to identify geographic origin of wood. For example, isotope ratios of strontium ($^{90}Sr/^{88}Sr$) differ among wood from different areas of Japan, indicating potential use of isotopes as a tool to identify geographic origin of wood (Kagawa et al 2002). In this study, we applied the chemical fingerprinting technique such as analysis of stable isotopes and inorganic elements, which are already in use for the identification of geographic origin of agricultural products, to major wood species from Southeast Asia (Rubroshorea spp.) to examine efficacy of the method to identify geographic origin of wood.

Materials and methods

Following specimens of Rubroshorea wood originating from Southeast Asia were selected from the wood library of Forestry and Forest Products Research Institute: *Shorea argentifolia* (n=17), *S. leprosula* (n=15), *S. minor* (n=4), *S. monticola* (n=5), *S. negrosensis* (n=13), *S. pauciflora* (n=19), *S. pinanga* (n=5), *S. platycarpa* (n=5), *S. quadrinervis* (n=5), *S. waltonii* (n=4), etc. Among the 109 specimens, we plotted approximate sampling locations of the 102 specimens on a map, which have data on geographic origin. Then we examined if the longitudes and latitudes of wood origins are correlated to isotope ratios or concentrations of inorganic elements of the wood. Furthermore, we conducted principal components analysis on stable isotope data to see if the statistical treatment improves the separation of the wood groups.

Stable isotope analysis

Stable isotope ratios of wood reflect environmental conditions in which the tree grew. Since the environmental conditions vary year to year, it is desirable to sample wood lath from the wood specimen as wide in radial direction as possible, so that the obtained isotope ratio reflects averaged environmental conditions over a long term. We sliced out a cross section of 2.0 mm thickness from each wood specimen, as long in radial direction as possible, using a circular saw. Each lath was grinded with a ball mill at the particle size of less than 200 microns. Wood powder of 0.1mg, 2.0mg, and 10.0mg was weighed into tin or silver capsules to analyze stable oxygen ($\delta^{18}O$), carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios, respectively. These capsules were later analyzed by a combined system of an elemental analyzer (CE Instruments NC2500) and a mass spectrometer (Thermo Electron MAT252).

Inorganic element analysis

In order to remove potential metal contamination coming from dusts on the surface of wood laths, we scraped off the surfaces in contact with outside atmosphere for a long time from each

specimen using a ceramic knife. About 200mg each of sapwood and heartwood was weighed into a Teflon container. Then we wet-ashed the wood by adding high-purity fuming nitric acid used for toxic metal analysis and later heating the sealed container at 140 ^{o}C for 4 hours. After filtering and then diluting the solution 1100 times with 5% HNO_3, inorganic elements were analyzed using ICP-AES (PerkinElmer Optima 4300DV). Plant leaf standards (Apple leaves, Pine needles etc. by National Institute of Standards and Technology) were used. Nine elements (Al, Ba, Ca, Fe, Mg, Mn, Sr, V, Zn) were analyzed.

Results and Discussion

Correlation between the chemical parameters and the longitude/latitude

Significant correlation was observed between the longitude/latitude of the wood habitat and stable oxygen, carbon and nitrogen isotope ratios of the wood (Fig. 1). However, no significant correlation was observed between the longitude/latitude and concentrations of inorganic elements (Table 1). On a two dimensional map, we plotted the data of oxygen and carbon isotope ratios, with which we found the first and the second highest correlations, respectively, to the longitude/latitude. On the map, we could differentiate wood groups from Philippines and Borneo, indicating possibility of oxygen isotope ratio to identify geographic origin of wood (Fig. 2). However, we could not classify wood from Borneo into smaller areas (Sabah, Sarawak, Brunei) using the isotope ratios. Principal components analysis did not improve the separation. The main reason for the significant difference of oxygen isotopic ratios between Philippines and Borneo wood is probably attributable to oxygen isotopic difference of rain water (IAEA/WMO 2001) and large climatic factors between the two regions. However, we can not rule out the possibility of genetic factor, because all wood specimens from Philippines are *S. negrosensis*.

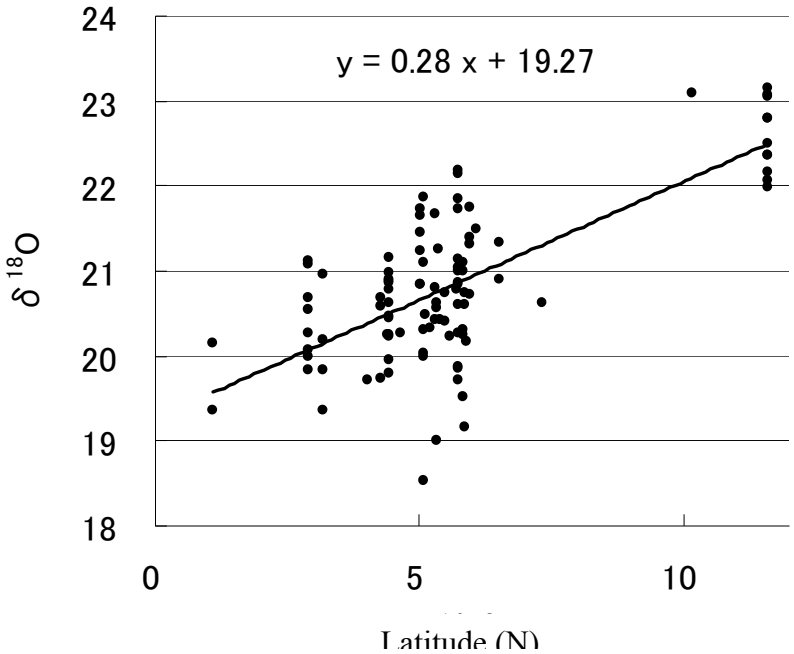

Figure 1. Relationship between oxygen isotope ratios of wood and the latitudes of geographic origin.

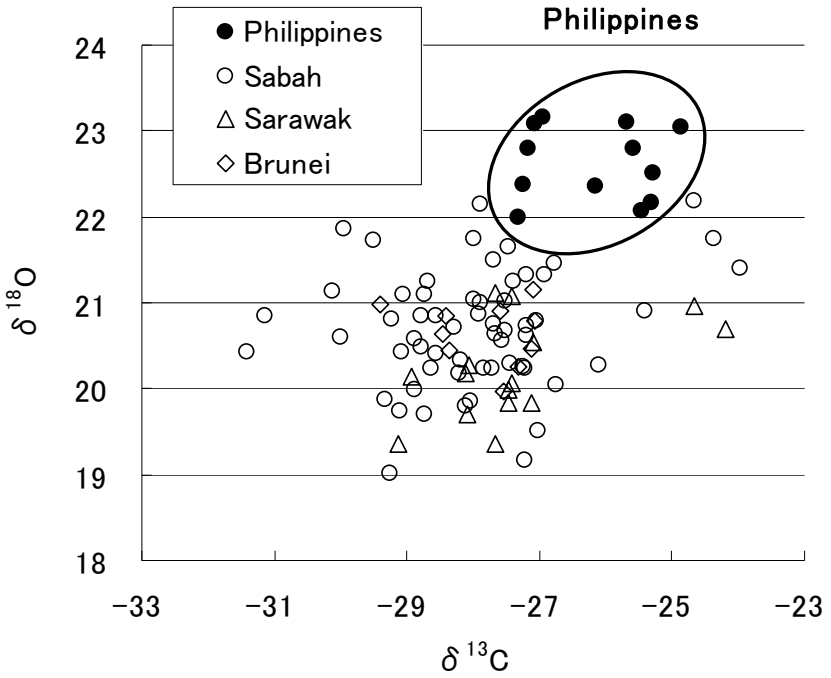

Figure 2. Classification of Philippines and Borneo wood using two-dimensional $\delta^{18}O$-$\delta^{13}C$ map.

Table 1. Correlation between wood isotope ratios/inorganic element concentrations and their geographic origins.

		$\delta^{18}O$	$\delta^{13}C$	$\delta^{15}N$	Ca	Mg	Fe	Al	Sr	V	Ba	Mn
R^2	Lat.	0.51^{**}	0.12^{**}	0.10^{**}	0.03	0.03	0.02	0.02	0.01	0.01	0.00	0.00
	Long.	0.42^{**}	0.04^{*}	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.01

** $P < 0.005$ * $P < 0.05$

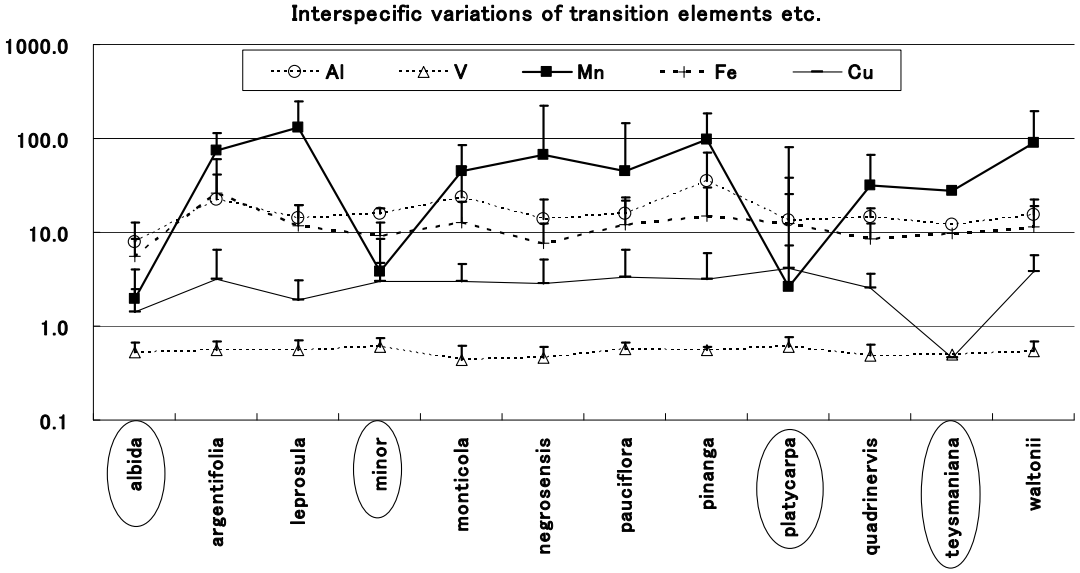

Figure 3 Inorganic element concentrations of wood and water conditions of its habitat (Circled are the species distributed in swampy areas)

Inorganic element concentrations and environmental conditions of habitat

There was no significant correlation between inorganic element concentrations and longitude/latitude of habitats. However, there was significant difference in inorganic element concentrations between the Rubroshorea species. *S. albida, S. minor,* and *S. platycarpa* showed relatively low concentrations of manganese (Mn). All of these species commonly grow in swampy areas. Alkali earth metals also showed a similar trend. Inorganic elements of wood seemed to have been mainly affected by habitat conditions rather than geographical locations (longitudes/latitudes) of their habitats.

Conclusions

Stable isotope ratios of wood were significantly correlated to the latitudes/longitudes of wood geographic origins. Stable isotope analysis seems most promising as a tool to identify geographic origins of wood. Although inorganic element concentrations showed no correlations to the longitudes/latitudes, they seemed to have been affected more strongly by soil water conditions of the tree habitats. We could differentiate wood groups from Philippines and Borneo by using stable isotope ratios. However, it was impossible to differentiate wood groups into smaller areas in Borneo, i.e. (Sabah, Sarawak, Brunei). The main problem associated with working on tropical timber is that they lack clear annual ring boundaries. This prevented us from comparing stable isotope ratios of wood formed in the same year or period.

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Experimental protocol for stable isotope analysis of wood

Oxygen of wood originates from oxygen of soil water absorbed by roots and therefore $\delta^{18}O$ of wood reflects $\delta^{18}O$ of rain as well as environmental conditions at the time of photosynthesis, such as temperature and humidity (Barbour 2007). Similarly, carbon isotope ratio ($\delta^{13}C$) of wood reflects $\delta^{13}C$ of atmospheric CO_2 and also environmental conditions at the time of photosynthesis (water use efficiency), such as temperature and humidity (Farquhar and Richard 1984). Since rain water $\delta^{18}O$ and climatic factors fluctuate spatiotemporally (longitude/latitude and year), wood from different areas show different isotope ratios (Epstein 1990). A review by McCaroll and Loader (2004) on stable isotopes of wood is available.

Separation of wood.

Due to annual fluctuations of climatic factors, stable isotope ratios of wood fluctuate every year. In order to cancel out the fluctuations and obtain averaged isotope value over a long period, it is important to cut out a cross section as wide in radial direction as possible. The cross section was 2mm thick in longitudinal direction and 2cm wide in tangential direction.

Grinding and weighing of wood

A ball mill (Wig-L-Bug Model 30, International Crystal Laboratories, Garfield, NJ, USA) was used to grind wood down to the particle size of less than 200 microns. After homogenizing the wood powder, 2mg, and 10mg were weighed into a tin capsule for carbon and nitrogen isotope analysis, respectively. 0.2g was weighed into a silver capsule for oxygen isotope analysis. High-precision balance (accuracy better than 2 μ g, Mettler Toledo AX26) was used for weighing. No α－cellulose extraction was conducted in this study.

Stable isotope analysis

In order to analyze oxygen and carbon isotope ratios of wood samples, carbon monoxide and carbon dioxide gases are generated, respectively, before introducing them to a mass spectrometer for isotope ratio measurement. A set of 50 samples can be loaded onto the auto sampler and up to 100 samples can be analyzed per day, if the system works fine. As internal standards for oxygen and carbon isotope ratios of organic samples, cellulose, alanine, or tyrosine are used. In order to constantly monitor analytical accuracy of the instrument, standard samples are analyzed every 10 wood samples.

Experimental protocol for inorganic element analysis of wood

A review by Cutter and Guyette (1993) is available on inorganic elements of wood.

Separation of wood

In order to remove surface contamination from aerosols etc., wood surfaces which were in long-term contact with outside atmosphere were scraped off with a ceramic knife. As in the case of samples for stable isotope analysis, we cut out cross sections as wide in radial direction as possible to obtain values averaged over a long-term.

Wet-ashing of wood by fuming nitric acid

- Grinding is not necessary for the analysis of inorganic elements. About 200 mg wood was weighed and put into a Teflon container.
- The Teflon container was put into a high pressure Teflon bomb (Sanai Kagaku HU-25) and 2ml of high purity fuming nitric acid for toxic metal analysis was added before sealing. A blank sample without wood sample was created to monitor the background level.
- These containers were heated for 4 hours at 140^{o}C. After heating, the containers were cooled overnight (opening the container before cooling is dangerous because larger amount of acid vapor is ejected).

- The lid was opened in the following day and 5ml of 1% HNO_3 was added. In order to remove solid remains such as silica, the solution was filtered with a cellulose-acetate filter connected to a plastic syringe.
- After dilution, inorganic element concentrations were measured by ICP-AES or ICP-MS.

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Trial identification of tree species and its origin of commercial veneer

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Samples

Samples were collected at the Plywood Factory of Maruhi Corporation, Tokyo, Japan on March 9, 2007. Logs for plywood in this factory were mainly meranti timbers, i.e. red meranti, white seraya and yellow meranti, imported from Sabah and Sarawak. They partly also use plantation grown timbers from Papua New Guinea.

Fresh veneer samples were collected and some part of them were dried through a commercial drying line heated at 190 to 200 ˚C (Table 1 and Fig.1).

Table 1 Veneer samples

Sample No.	Species expected	Origin	Color		Drying condition
Veneer-1	red meranti	Sabah	reddish	fresh	
			gray	dry	190--200˚C
Veneer-2	yeloow meranti	Sabah	reddish	dry	190--200˚C
Veneer-3	white seraya	Sabah		dry	190--200˚C
Veneer-4	campnosperma	PNG		dry	190--200˚C
Veneer-5	red meranti	Sabah	reddish	fresh	
			gray	fresh	
			gray	dry	190--200˚C
Veneer-6	red meranti	Sabah	reddish	fresh	
			gray	fresh	
			reddish	dry	190--200˚C
			gray	dry	190--200˚C
Veneer-7	red meranti	Sabah	gray	fresh	
			gray	dry	190--200˚C

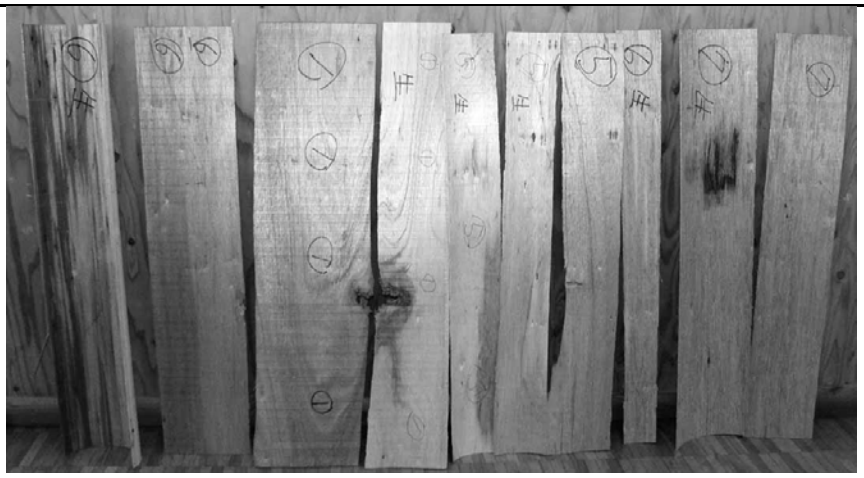


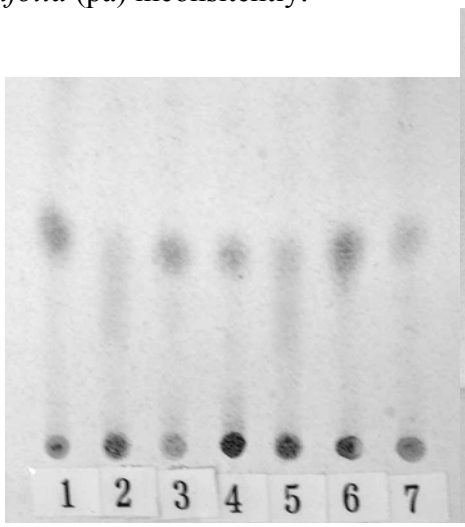
Fig. 1 Veneer samples

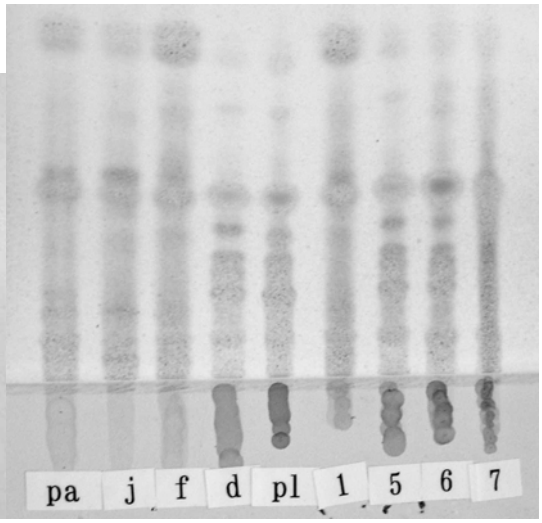
Chemotaxonomy

Gallic acid was detected in all sample veneers with TLC (thin layer chromatography: Fig. 2), although the density of the spots were various from light (veneer-2) to dark (veneer-1 and 6).

The results of veneer samples 1, 5, 6 and 7, which were sampled as red meranti samples, are reasonable. Veneer-2 expected as yellow meranti showed the content of gallic acid even though it was rather faint. However, this result agreed with wood anatomical as mentioned later. Veneer-3 was not a subject of the analyses, because white seraya (*Parashorea* spp.) was not examined well for the gallic acid yet. Veneer-4 expected *Campnosperma* spp. (Anacardiaceae) showed a conspicuous spot of gallic acid. It is easily expectable that wood of *Campnosperma* spp. contains comonly gallic acid as well as others from this family.

According to the result of DNA analysis, wood extractives from veneer samples were compared with those from candidates species (Fig. 3). TLC pattern from veneer-1 resembled to those of *S. johorensis* (j) and *S. fallax* (f) agreeing with the result of wood anatomical identification, but that of veneer samples-5, 6, 7 resembled to *S. dasyphylla* (d) rather than *S. parvifolia* (pa) inconsitently.





(Left) Fig. 2 TLC for gallic acid. 1—7: sample veneer number.
(Right) Fig. 3 TLC for wood extractives. pa: *S. parvifolia*, j: *S. johorensis*, f: *S. fallax*, d: S. *dasyphylla*, pl: *S. platyclados*

Wood anatomical identification

Veneer-1, 2, 5, 6, 7 were identified as wood of the section *Ruburoshorea* spp. based on the wood anatomical features (Fig. 4). Although veneer-2 was expected to be yellow meranti, section *Rhicheitoides* spp., it did not have horizontal resin canal which is a distinct characteristic to this section.

Veneer-3 was identified as white seraya, *Parashorea* sp., as expected having long chain crystals differing from section *Ruburoshorea*. Veneer-4 was *Campnosperma* sp. as expected.

Veneer-1 had crystals in axial and ray idioblasts, also warts in diffuse on vessel wall and thin-walled fibers. According to the results of DNA analysis, only one species, *S. johorensis*, was assorted from several candidates.

Veneer-2 was without crystals neither in axial nor ray parenchyma, had warts and thin walled fibers, so the candidates were *S. scaberrima*, *S. waltonii*, *S. curtisii*, *S. smithiana* and *S. ovalis*.

Veneer-5, 6, and 7 had common anatomical features, i.e. they had crystals in axial parenchyma but not in ray, warts present (Fig. 5), fiber wall thin. Then the candidates were follows; *S. parvifolia*, *S. smithiana*, *S. mecistopteryx*, and *S.ovalis*. Among them, veneer-6 was furter identified into *S. parvifolia* by DNA analysis.

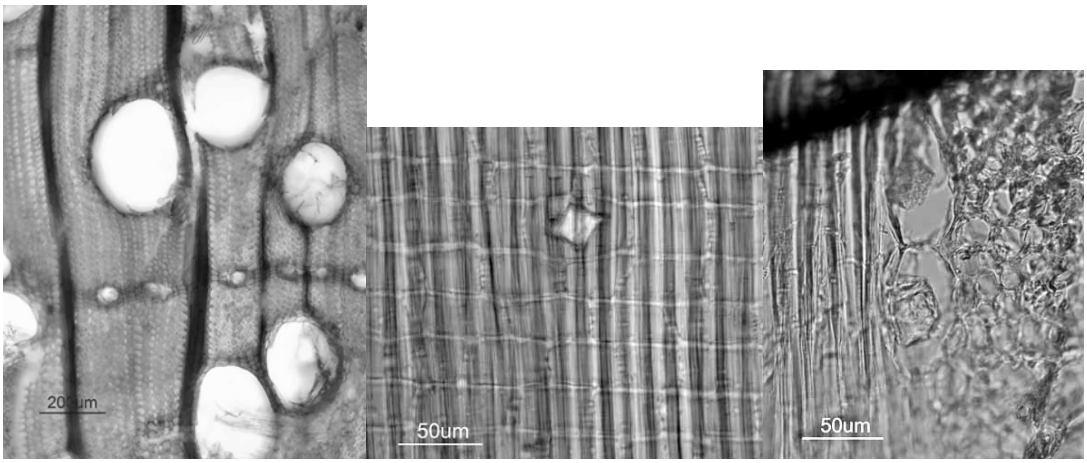


Fig. 4 Microphotographs of veneer-1. Left: axial resin canals in a concentric bans in cross section. Middle: prismatic crystal in a idioblast. Right: prismatic crystal in idioblast in axial parenchuma.

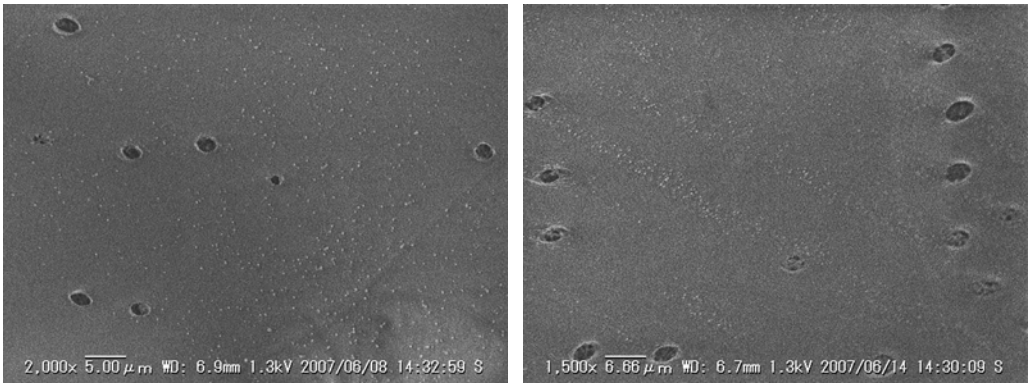


Fig. 5 SEM photographs of vessel walls. Left: veneer-5 with warts in diffuse. Right: veneer-6 with warts in helical thickenings pattern.

DNA analysis

DNA analysis were focused on *trnL* introns, *trnL*(UAA)-*trnF*(GAA), *trnH*(GUG)-*psbA* and *psbC*-*trnS*(UGA) of chloroplast DNA. Primers used in this analysis were listed in the Table 2. Ampdirect Plus (Shimazu Co. Ltd.) was utilized as a PCR reagent.

DNA were extracted from veneer samples-1, 5, 6 and 7 when they were fresh, and the regions targeted were amplified by PCR. From the result of the analysis, the species listed in Table 3 were estimated for each sample.

Table 2 Primers used in the analysis

DNA region	Primer	DNA arrangement (5'—3')
trnL(UAA) intron	B49317	CGAAATCGGTAGACGCTACG
	A49855	GGGGATAGAGGGACTTGAAC
trnL(UAA)-*trnF*(GAA)	B49873	GGTTCAAGTCCCTCTATCCC
	A50272	ATTTGAACTGGTGACACGAG
trnH(GUG)-*psbA*	trnH(GUG)	ACTGCCTTGATCCACTTGGC
	psbA	CGAAGCTCCATCTACAAATGG
psbC-*trnS*(UGA)	psbC-F	GCCACCTCTCATTTTGTTCTGG
	trnS-R	GGTTCGAATCCCTCTCTCTC

Table 3 Results of DNA analysis

No.	Sample distinction	Moisture content (%)	Amount used for extraction (g)	DNA conc. (ng/micro L)	Amplification of trnL-trnF	Estimation by DNA analysis	DNA and anatomy
1	Wet	45.8	1.04	2	○	*Shorea platyclados, S. fallax, S. johorensis*	*S. fallax or S. johorensis*
	Dry	3.3	0.92	0	×		
2	Dry	6.1	1.00	?	×		
5	fresh, reddish	56.3	1.05	8	×	*Shorea parvifolia, S. dasyphylla*	*Shorea parvifolia*
	fresh, gray	36.5	1.10	2.5	○		
	dry	3.1	0.96	0	×		
6	fresh, reddish	53.2	1.38	18	○	*Shorea parvifolia, S. dasyphylla*	*Shorea parvifolia*
	fresh, gray	43.6	1.25	6	○		
	dry, reddish	3.6	0.94	0	×		
	dry, reddish	3.6	0.95	0	×?		
	dry, gray	4.1	0.95	0	○		
	dry, gray	4.1	0.98	0	×?		
7	fresh	24.3	1.10	23	○	*Shorea parvifolia, S. dasyphylla*	*Shorea parvifolia*
	dry	3.2	0.96	0	×		

Estimation of the origin

Isotope analysis results from the five veneer samples were plotted on the two-dimensional map of carbon and oxygen isotope ratios, along with the data obtained from the *Rubroshorea* spp. samples from Borneo and Philippines with known geographic origin (Fig. 6). The five samples examined showed similar oxygen and carbon isotopic values to the *Rubroshorea* spp. from Borneo, while they showed different isotopic values from those from Philippines ($\delta^{18}O$ = 19.0 ∽22.0‰, $\delta^{13}C$ = -29.6∽28.0). These results indicate the possibility that the samples are from Borneo not from Philippines.

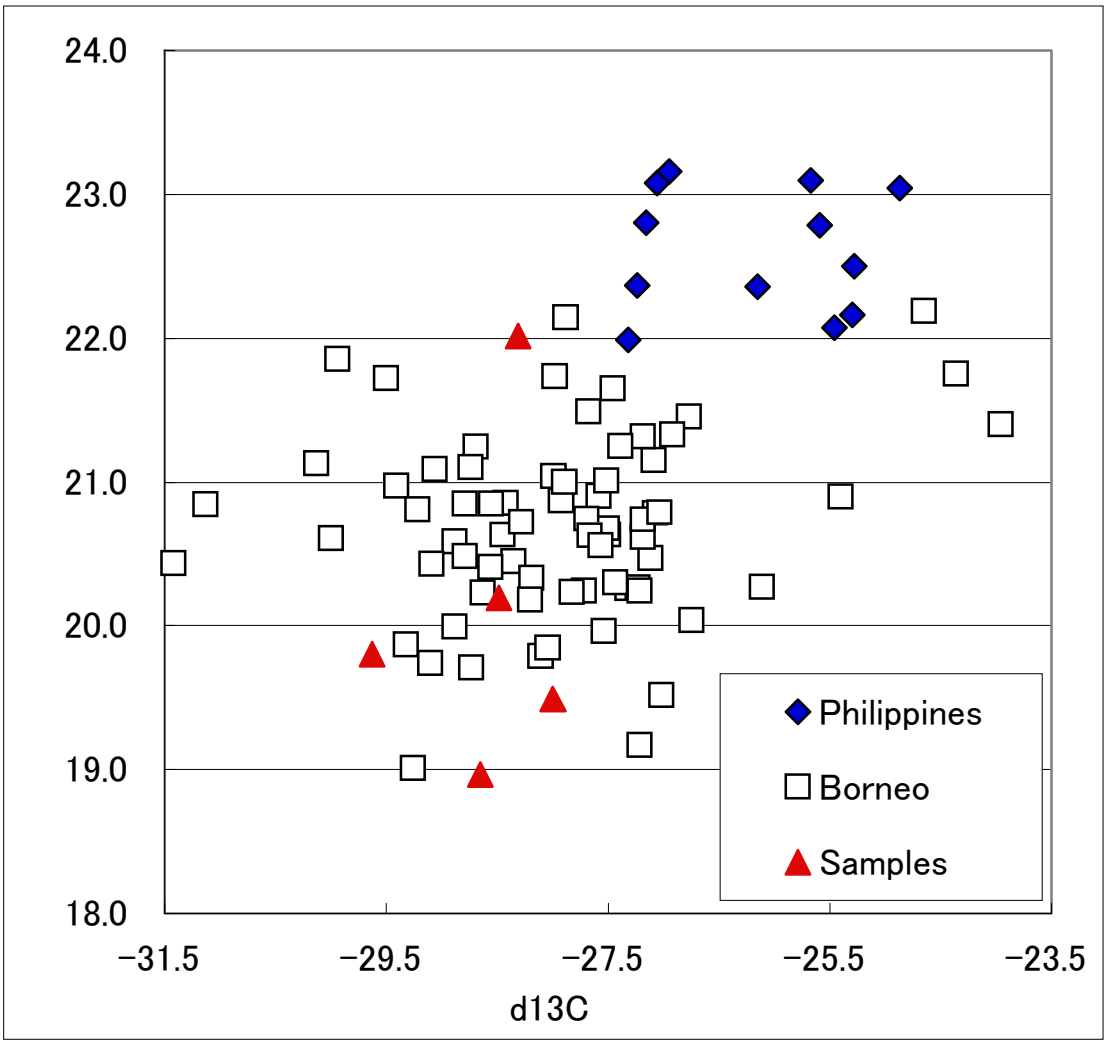


Fig. 6 Two-dimensional map of carbon and oxygen isotope ratios