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Molecular database for classifying *Shorea* species (Dipterocarpaceae) and techniques for checking the legitimacy of timber and wood products

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Abstract The extent of tropical forest has been declining, due to over-exploitation and illegal logging activities. Large quantities of unlawfully extracted timber and other wood products have been exported, mainly to developed countries. As part of the export monitoring effort, we have developed methods for extracting and analyzing DNA from wood products, such as veneers and sawn timbers made from dipterocarps, in order to identify the species from which they originated. We have also developed a chloroplast DNA database for classifying *Shorea* species, which are both ecologically and commercially important canopy tree species in the forests of Southeast Asia. We are able to determine the candidate species of wood samples, based on DNA sequences and anatomical data. The methods for analyzing DNA from dipterocarp wood products may have strong deterrent effects on international trade of illegitimate dipterocarp products. However, the method for analyzing DNA from wood is not perfect for all wood products and need for more improvement, especially for plywood sample. Consequently, there may be benefits for the conservation of tropical forests in Southeast Asia.

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Introduction

The importance of conserving biodiversity is increasingly recognized globally, especially since the Convention on Biological Diversity was adopted in 1992 (Balmford et al. 2005). The developing countries in Southeast Asia have concerns about losses of their biological resources and strong motives to ensure that they are sustainably used. However, valuable plant resources are still being illegally extracted and taken to other countries. Certification of sustainable forestry and environmental conservation activities have recently started in these countries (Viana et al. 1996), which can significantly help to reduce illegal logging activities. However, introducing effective implementation of forest certification programs in all of these countries will take considerable time and, until then, tropical forests will continue to decline, due to over-exploitation and illegal logging activities. The unlawfully extracted timber and other wood products are mainly exported to developed countries. Thus, robust inspections of the species and origins of internationally traded timber and other wood products might provide authoritative deterrents for illegal logging activities. Indeed, it has been shown that identification of the species that wood products originate from can dramatically reduce the frequencies of false declarations by their importers in Japan (Itoh et al. 2004). The most reliable and convenient tools for identifying the species and geographic origins of such products are likely to be molecular techniques that can be used in conjunction with a DNA database of appropriate marker sequences.

The botanical family Dipterocarpaceae is a key family, both ecologically and commercially, in the forests of Southeast Asia. Members of the family are predominantly distributed in tropical lowland and hill forests within this region, where they accounted for nearly 55% of the stand volume in lowland forests, according to figures presented by Symington (1943). The family consists of 10 genera with 386 species and shows a high rate of endemicity, which means that more than half of the species are distributed in very restricted regions (Ashton 1982). *Shorea* is the largest genus in the family, consisting of 192 species (163 of which can be found in Malesia, Ashton 1982). Among these, 108 species are distributed in restricted areas of Southeast Asia. In Peninsular Malaysia, over 57% of the dipterocarp species have distribution patterns restricted to specific zones within the Peninsula and 124 out of the 157 dipterocarp species of Peninsular Malaysia were listed in some category of threat (Saw and Sam 2000). For species

with such a restricted distribution, we can obviously identify the area from which material originates, and the threatened species should be conserved not only for genetic resources but also ecosystem of tropical forests. In Sarawak, there are also strictly protected plant species including eight *Shorea* species by law (http://www.forestry.sarawak.gov.my/forweb/ourfor/flora/pp/pp.htm).

The genus *Shorea* has been divided into four sections (Shorea, Rubroshorea, Richetioides and Anthoshorea), which closely follow the four color-based types of timber designated for commercial purposes: Balau, Red Meranti, Yellow Meranti and White Meranti, respectively (Symington 1943). Ashton (1982) divided the genus into ten sections: the sections Shorea, Pentacme, and Neohopea form the timber group of color type Balau, the section Richetioides is the timber group Yellow Meranti, the section Anthoshorea is the timber group White, and the sections Rubella, Brachypterae, Pachycarpae, Mutica and Ovalis are the timber group Red color type. In Japan, the amount of tax paid to import wood derived from *Shorea* trees depends on which of these groups it is assigned to; the highest tax is for Red Meranti timber (Itoh et al. 2004). For this reason, importers sometimes make false declarations to reduce their tax bills. Tax rates are even lower for a few species that taxonomically belong to Red Meranti, such as *Shorea albida* and *S. rugosa* var. *uliginosa*, probably because of miss-classification for tax.

Timber trees have been utilized by local communities for millennia. However, since commercial logging started, the extent of tropical forests has declined dramatically. Illegal logging activities have contributed to this decline in many regions. The extracted wood is processed to make products, such as sawn timber, veneer and plywood, which are then exported to other countries. Once the timber has been processed, it is generally difficult to identify the species used to make the products, using current techniques. There are exceptions for a few species that yield wood with readily identifiable features. Anatomical analysis of *Shorea* wood provides sufficient information to classify it to respective timber groups. However, such analysis cannot identify wood to the species level and requires several working days by well-trained and experienced personnel (Desch 1941; Ogata et al. 2008). In such cases, DNA analysis may provide much more powerful and convenient means for identifying the species. Attempts have been made to isolate DNA from woody products of various species, including several oak species, to identify the origin of the wood (Deguilloux et al. 2004). Chloroplast DNA (cpDNA) is frequently used for these analyses, because (1) it is much easier to amplify cpDNA than nuclear DNA, due to the higher copy numbers of chloroplast genomes per cell (Régis and Lerbs-Mache 2001), and (2) a number of cpDNA regions are known to have

sufficient nucleotide polymorphism to investigate the phylogenetic relationship between species (Soltis and Soltis 1998). Therefore, if robust methods for extracting and analyzing DNA from imported wood products, such as veneers and sawn woods, can be developed, they should be capable of identifying their origins (species and possibly geographic region). This could be very useful for checking the legality of wood products, and for deterring trade in illegal products. The phylogeny of the Dipterocarpaceae has been assessed using several kinds of molecular methods, such as PCR-RFLP analysis of chloroplast genes (Tsumura et al. 1996; Indrioko et al. 2006), and analyses of sequences of cpDNA regions (Kajita et al. 1998; Kamiya et al. 1998; Dayanandan et al. 1999), internal transcribed spacer (ITS) regions (Yulita et al. 2005), and AFLPs (Cao et al. 2006). However, there is still insufficient information for robust molecular classification, and further sequence data are required, covering a greater range of species.

In this study we aimed: (1) to construct a cpDNA sequence database for the genus *Shorea*, which is the largest genus of the family Dipterocarpaceae and widely distributed within Southeast Asia, and (2) to develop methods for extracting and analyzing DNA from wood samples made from dipterocarp species, in order to check the legality of internationally traded wood and wood products. In addition to describing these efforts, we discuss the feasibility of applying the species identification procedure to wood products in practice, and its limitations.

Materials and methods

Materials for molecular classification of *Shorea*

We collected leaf tissue samples of *Shorea* species from the arboretums of the Forest Research Institute, Malaysia, the Forest Research Centre, Sabah, and permanent ecological research plots in various locations, such as the Lambir and Pasoh forest reserves in Malaysia. We also collected samples directly from natural forests in Indonesia (for recording purposes, we also deposited herbarium specimens for each collected sample at the Georg-August University of Göttingen). In total, 200 individuals were sampled, representing 84 *Shorea* species (Supplement Table 1), which covered ca. 70% of the major commercial timber species in Indonesia and Malaysia (Soerianegara and Lemmens 1994). We also included four numbers of *Hopea* species and one species of *Neobalanocarpus* in this study, because the two genera were found to be taxonomically nested in *Shorea* in a previous study (Tsumura et al. 1996) and it is important to understand the phylogenetic positions of these two genera within *Shorea*.

DNA extraction and sequencing of intergenic spacer regions of cpDNA

Total DNA was extracted using the modified CTAB method (Tsumura et al. 1996), or a DNeasy Plant Mini Kit (Qiagen). Four non-coding regions of the chloroplast genome were selected for polymerase chain reaction (PCR) amplification, based on the high degrees of polymorphism found in preliminary studies: *trn*L (UAA) intron, *trn*L (UAA) 3′ exon and *trn*F (GAA), *trn*H(GUG) and *trn*K(UUU), and *psb*C and *trn*S(UGA) (Table 1).

The PCR reaction mixtures contained PCR buffer (10 mM Tris–HCl, 50 mM KCl, 100 μM of each dNTP, 0.02% Triton X-100 and 0.01% gelatin), 1.5 mM MgCl$_2$, 0.4 U *Taq* DNA polymerase (Promega), 0.2 μM of each primer, and 10 ng template DNA in a total volume of 20 μl. The PCR conditions consisted of an initial 3 min denaturation at 94°C then 30 cycles of 30 s denaturation at 94°C, 45 s annealing at 56°C, and 45 s extension at 72°C, with a final 5 min extension at 72°C.

The PCR products were purified using a QIAquick PCR purification kit (Qiagen). Purified PCR products were used as templates for cycle sequencing reactions using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and DNA sequencing was performed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All four selected regions were sequenced in both directions for all individuals, and the obtained sequences were aligned manually and/or using CLUSTAL_W software (Thompson et al. 1997).

Analysis of chloroplast sequence data

We constructed a molecular phylogenetic tree using the neighbor-joining method (Saitou and Nei 1987) to test for clustering according to wood groups, which are defined according to color—Red Meranti, Yellow Meranti, White Meranti and Balau (Symington 1943)—and are monophyletic. The robustness of the resulting phylogenetic tree was tested by bootstrap analysis (Felsenstein 1985) with 1000 replicates using MEGA4 software (Tamura et al. 2007). The species *Vatica bella*, *V. oblongifolia*, *Anisoptera laevis* and *Cotylelobium lanceolatum* were used as an outgroup for the evaluation. In expectation that each wood color group would prove to be monophyletic, nucleotide substitutions that could be used to discriminate the four groups were sought, using the sequence alignment data. We also searched for nucleotide substitutions capable of discriminating between species within groups, especially for *Shorea albida*, since the tax rate applied to wood of this species is different from the rate applied to other *Shorea* species in Japan.

Table 1 Analyzed regions of chloroplast DNA and their sequence primers

Region	Names of primers	Sequences (5′ → 3′)		Length (bp)[a]
*trn*L(UAA) intron	cp2F	CGAAATCGGTAGACGCTACG	Taberlet et al. (1991)	506
	cp2R	GGGGATAGAGGGACTTGAAC	Taberlet et al. (1991)	
*trn*L(UAA)-*trn*F(GAA)	cp3F	GGTTCAAGTCCCTCTATCCC	Taberlet et al. (1991)	441
	cp3R	ATTTGAACTGGTGACACGAG	Taberlet et al. (1991)	
*trn*H(GUG)-*psb*A-*trn*K(UUU)	cp5F	ACGGGAATTGAACCCGCGCA	Demesure et al. (1995)	1780
	cp5R	CCGACTAGTTCCGGGTTCGA	Demesure et al. (1995)	
	cp5iF1	TTTTCTGTGGTTTCCCTGAT		
	cp5iF2	GCRATGAAGGCRATAATAAA		
	cp5iR1	TGCTCAYAACTTCCCTCTA		
	cp5iR2	TCCCTATTCAGTGCTATGC		
	cp5iR3	CCGCAACTTCTGTATTTATT		
*psb*C-*trn*S(UGA)	cp6F	GGTCGTGACCAAGAAACCAC	Demesure et al.(1995)	1559
	cp6R	GGTTCGAATCCCTCTCTCTC	Demesure et al.(1995)	
	cp6iF1	TGGAGGAGAAGGATGGATTG		
	cp6iF2	GCCACCTCTCATTTTGTTCT		
	cp6iR1	ACACTCACAATCCATCCTTC		
	cp6iR2	CCCAGAACAAAATGAGAGGT		
Primers for amplification of small fragment for plywood				
*trn*L(UAA) intron	2AF	CGAAATCGGTAGACGCTACG		
	2AR	GAATAGGTGTCCCAGTCGTT		306
	2BF	ACTGGGACACCTATTCTT		
	2BR	CTATCTTTATTCTCGTCCAA		190
	2CF	AATGGATTAATTGGACGAGAA		
	2CR	GGGGATAGAGGGACTTGAAC		129

[a] Lengths include alignment gaps

Species identification of dipterocarp wood products using anatomical characteristics

Four veneer samples imported from Sabah, Malaysia, were obtained from the Maruhi Plywood Co., Tokyo, Japan, four other veneer samples from Key Tec Co. Ltd, Tokyo, Japan, and two sawn timber samples were also used in this study. In order to identify the wood species of the veneers by light microscopy, thin sections were cut from transverse, radial and tangential surfaces of each sample using razor blades. Each section was then mounted in a drop of glycerol on a glass slide and covered with a cover slip. The glass slides were heated on a hot plate for several minutes at 120–150°C to remove bubbles in the sections. After the samples had cooled down, the sections were observed under a light microscope. Common anatomical feature of genus *Shorea* is the existence of axial resin canals which arrange concentrically. Anatomical characteristics of wood groups are (1) Balau; thick fiber wall and crystal in chambered axial parenchyma, (2) Red Meranti; red color in wood and crystals in idioblasts, (3) Yellow Meranti; yellow color in wood and the existence of radial resin canals in rays, and

(4) White Meranti; silica grains deposited in rays. The other special characteristics, radial resin canals with extremely large diameter are existed in three species such as *S. leprosula*, *S. ovata*, and *S. teysmaniana* of Red Meranti. To observe the woody characters in greater detail, we also used a scanning electron microscope. For this purpose, the samples were treated with aqueous sodium hypochlorite for 3 min to remove residual cell contents from the surfaces of vessel walls, rinsed copiously with water, then dehydrated with 99% ethanol, and finally dried in air. The samples were observed under a scanning electron microscope with or without metal coating. The structures of the xylem vessel walls were then classified.

Extraction and analysis of DNA from dipterocarp wood products

The ten veneer and timber samples were cut into smaller pieces with a pair of pruning scissors, and ca. 1 g samples were powdered with a disruptor (Multi-Beads shocker; Yasui Kikai Co., Japan). DNA was extracted using the DNeasy Plant Mini Kit and a QIAshredder Maxi Spin

Column from the DNeasy Plant Maxi Kit (Qiagen). DNA concentration was determined using a fluorescence spectrophotometer (F-3010; Hitachi) with λDNA as a standard. Four cpDNA regions were amplified by PCR (Table 1). The PCR reaction mixtures consisted of 12.5 μl of 2× Ampdirect Plus (Shimadzu), 0.25 μl (0.625 unit) of Blend *Taq*-Plus (Toyobo), 0.5 μM primers and 5 μl of template DNA in a total volume of 25 μl. Amplification was performed with an initial denaturation step at 94°C for 90 s followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 50 s. PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Sequences were determined in both directions by direct sequencing with the same primers that were used in the PCR.

An attempt was also made to extract and analyze DNA, to species level, from a plywood sample originating from an unknown dipterocarp species. Plywood is made by a process involving heating wood veneers and joining them with adhesives, most likely resulting in strong degradation of the DNA. We peeled away the surface of the plywood and powdered ca. 200 mg of the wood in liquid nitrogen using a mortar and pestle. The DNeasy Plant Mini Kit was used for DNA extraction, applying a modification in which fourfold more buffer was used for all steps and the incubation time at 65°C was increased to 120 min. PCR amplification was done using a Qiagen Multiplex PCR kit according to the manufacturer's protocol. We amplified the four cpDNA regions used for the molecular classification of *Shorea* and three small fragments of the *trn*L using newly designed primers (Table 2), since we expected the DNA to be highly degraded. The sequencing and sequence alignment procedures were the same as those described above for the molecular classification of *Shorea*.

Results

Molecular classification of *Shorea*

We aligned sequences obtained from each of the samples for the following regions: *trn*L intron (506 bp), *trn*L-*trn*F (441 bp), *trn*H-*trn*K (1780 bp), and *psb*C-*trn*S (1559 bp). Sequences obtained were deposited in the DNA Data Bank of Japan database, DDBJ (accession numbers AB451979–AB452810, AB458531–AB458538). The total sequence length was 4286 bp, including some gaps. The numbers of polymorphic sites that could be used to classify the species into wood groups ranged from 17 for Yellow Meranti to 128 for Red Meranti, and the number of informative sites of the four cpDNA regions increased with sequence length (Table 2). Within-group nucleotide diversity was highest for the White Meranti and lowest for the Yellow Meranti group (Table 3). Among the four cpDNA regions, the *trn*L-*trn*F region had the highest nucleotide diversity, two- to threefold higher than each of the other three examined regions.

The phylogenetic tree suggested that each group based on wood color is monophyletic (Fig. 1a), except for *S. roxburghii* (Fig. 1c), implying that we should be able to find nucleotide substitutions capable of distinguishing each of the four groups. Numbers of unique difference nucleotide sites detected in the White, Yellow, Balau and Red Meranti groups amounted to 9, 11 6 and 5, respectively (Table 4). These nucleotide substitutions provide diagnostic DNA

Table 2 Polymorphic sites (singleton and parsimony-informative sites) used to classify the species within wood groups

Group	*trn*L 506 bp	*trn*L-*trn*F 441 bp	*trn*H-*psb*A-*trn*K 1780 bp	*psb*C-*trn*S 1559 bp	Total
White Meranti	11 (4, 7)	14 (0, 14)	42 (7, 35)	39 (4, 35)	106 (15, 91)
Yellow Meranti	3 (2, 1)	4 (1, 3)	5 (3, 2)	5 (4, 1)	17 (10, 7)
Balau	7 (2, 5)	18 (6. 12)	35 (24, 11)	41 (12, 29)	101 (44, 57)
Red Meranti	11 (7, 4)	26 (9, 17)	48 (27, 21)	43 (30, 13)	128 (73, 55)

Table 3 Nucleotide diversity of four chloroplast DNA regions in the four wood groups

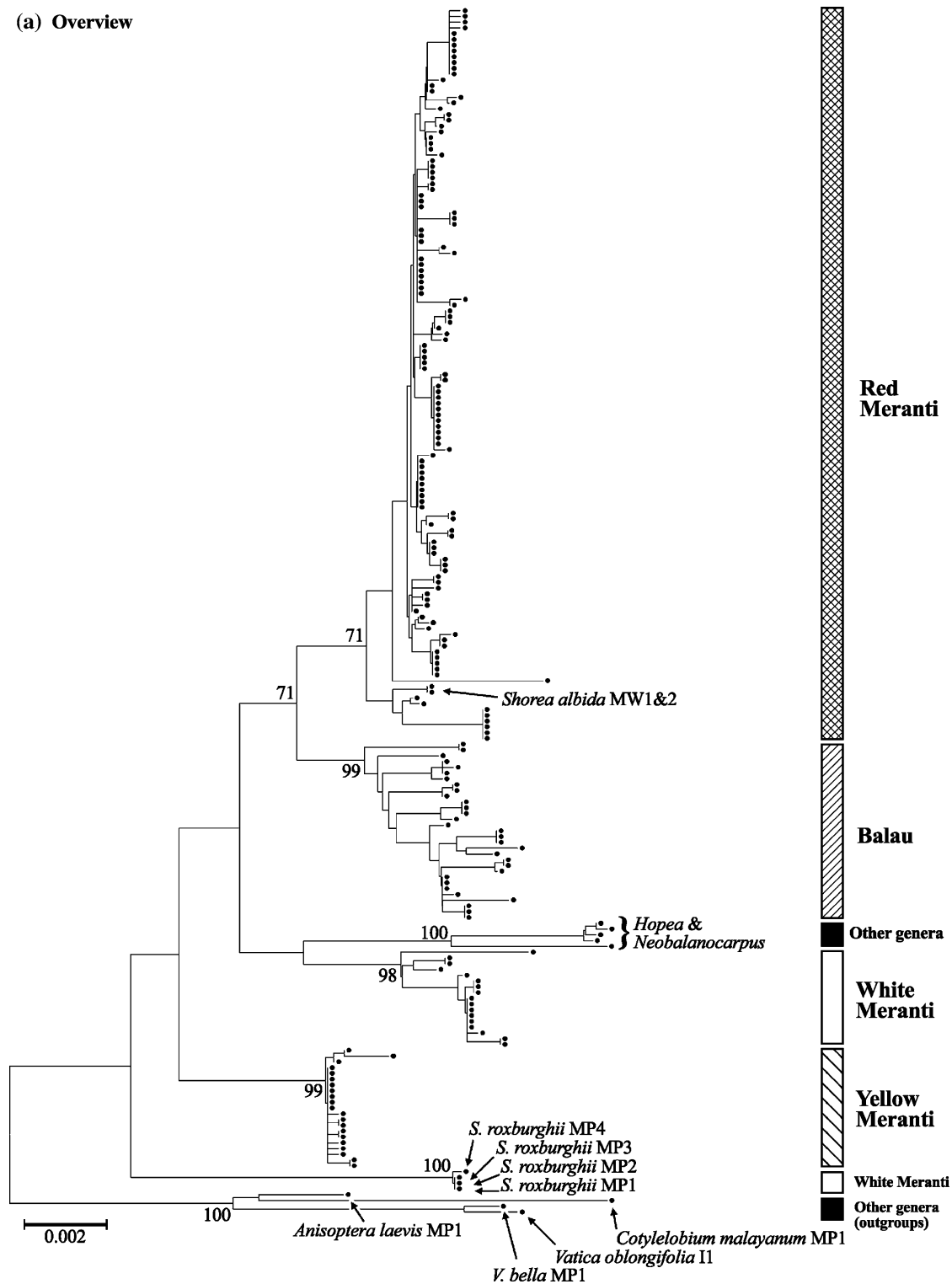| Group	*trn*L (SD)	*trn*L-*trn*F (SD)	*trn*H-*psb*A-*trn*K (SD)	*psb*C-*trn*S (SD)
White Meranti	0.00468 (0.00098)	0.01043 (0.00194)	0.00679 (0.00148)	0.00555 (0.00161)
Yellow Meranti	0.00075 (0.00030)	0.00164 (0.00039)	0.00060 (0.00013)	0.00037 (0.00022)
Balau	0.00176 (0.00050)	0.00674 (0.00097)	0.00282 (0.00050)	0.00418 (0.00048)
Red Meranti	0.00149 (0.00019)	0.00515 (0.00052)	0.00142 (0.00024)	0.00129 (0.00027)

(a) Overview

Fig. 1 Phylogenetic tree of *Shorea* species based on sequence data obtained for four chloroplast DNA regions: **a** overview of the phylogeny, **b** Red Meranti group, **c** Balau, **d** White Meranti, **e** Yellow Meranti groups. The *letters in parentheses* indicate origins of the species (*MP* Peninsular Malaysia, *MW* Sarawak Malaysia, *MS* Sabah Malaysia, *I* Indonesia) and the following numbers indicate numbers assigned to individuals when we analyzed multiple individuals of each species from the same geographic region. *Numbers* above and below branches indicate percentages of 1,000 bootstrap replications exceeding 50%
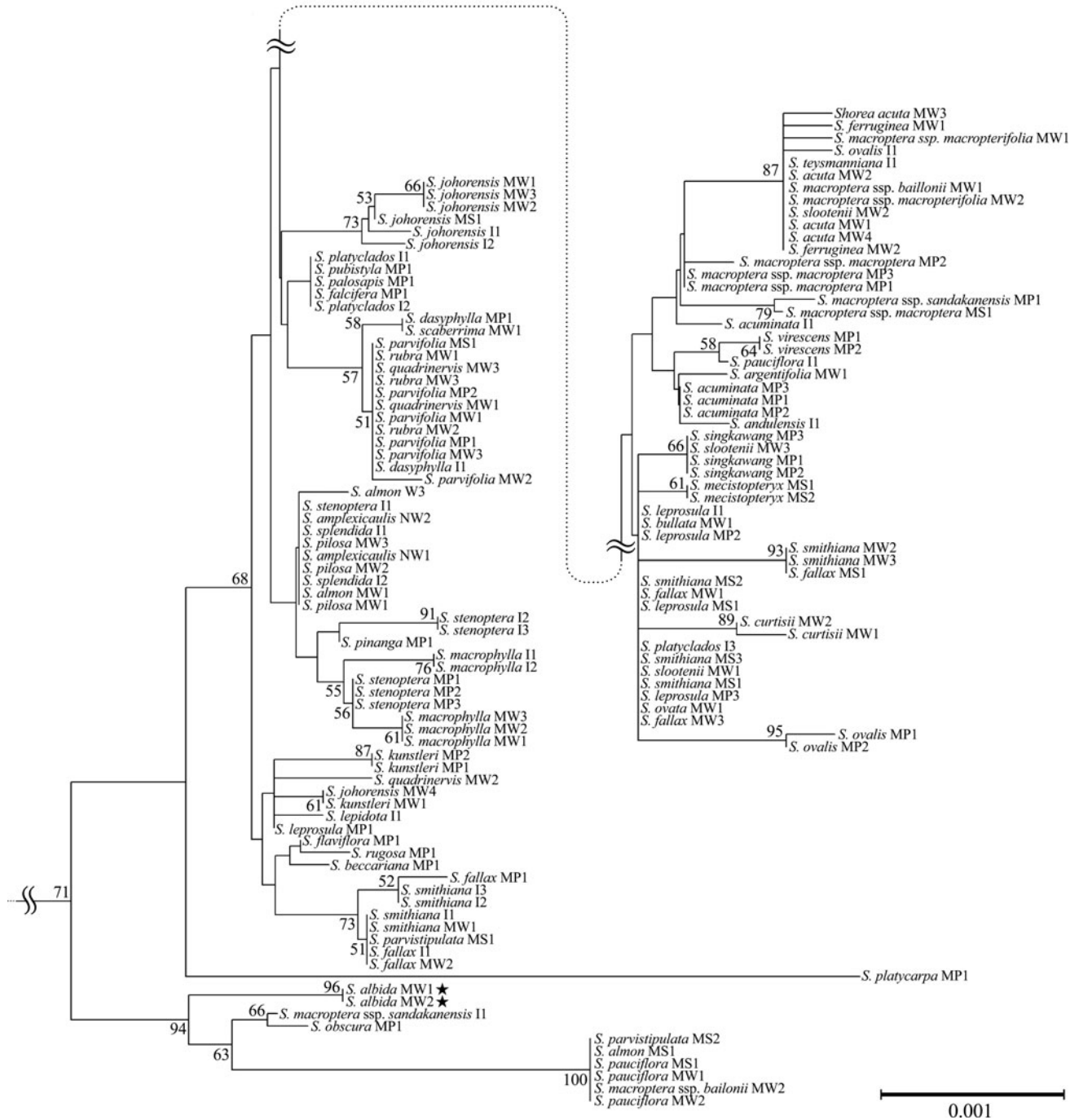
(b) Red Meranti

Fig. 1 continued

markers for the four groups. The bootstrap values of the nodes for the White, Yellow, Balau and Red groups were 97, 99, 95 and 65, respectively (Fig. 1a).

Sixteen combinations of species have identical sequences in the four examined cpDNA regions (Table 5) and the total number of combinations having identical sequence was 124, of which 67 combinations was identical within species of different individuals and 57 combinations was identical between species because we analyzed multiple individuals for each species. For example, the sequences of the four regions were found to be identical in *S. pilosa*, *S. splendida*, and *S. amplexicaulis*, all of which belong to the same section of *Shorea* (Pachycarpae). The probability of two species having an identical sequence between
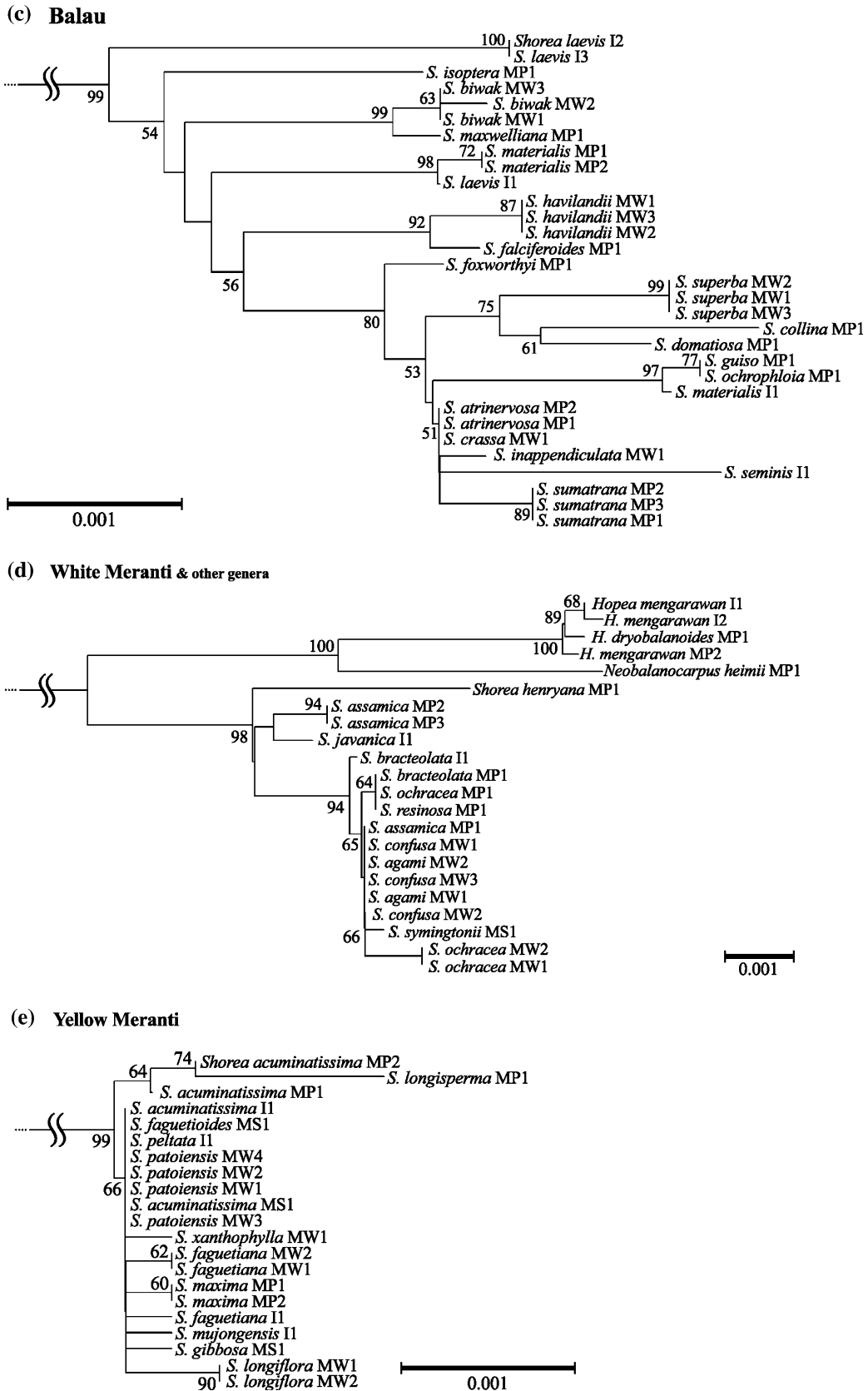
(c) Balau

(d) White Meranti & other genera

(e) Yellow Meranti

Fig. 1 continued

Table 4 Discriminated nucleotide substitutions between the four wood groups (indicated by bold type)

Group	trnL (bp)					trnL-trnF (bp)							trnH-psbA-trnK (bp)					
	190	214	285	296	358	17	52	203	220	245	269	300	92	637	1,416	1,509	1,532	1,681
White Meranti	C	T	**T**	T	T	G	C	**A**	C	T	C	**C**	**C**	**A**	C	G	C	T
Yellow Meranti	**T**	**G**	C	**G**	T	G	C	G	**T**	**G**	**G**	T	T	G	**A**	G	**A**	T
Balau	C	T	C	T	**A**	G	**T**	G	C	T	C	T	T	G	C	**T**	C	T
Red Meranti	C	T	C	T	T	**A**	C	G	C	T	C	T	T	G	C	G	C	**C**

Group	psbC-trnS (bp)													
	74	109	484	526	637	655	709	766	1,139	1,378	1,394	1,414	1,445	1553
White Meranti	C	C	C	**G**	T	**G**	**C**	T	A	**A**	T	A	C	G
Yellow Meranti	**T**	C	C	A	T	T	T	T	A	T	T	A	**T**	**A**
Balau	C	C	T	A	**C**	T	T	**C**	A	T	T	**G**	C	G
Red Meranti	C	**T**	T	A	T	T	T	T	**T**	T	**C**	A	C	G

Table 5 *Shorea* species having identical sequence data based on four region of cpDNA

Species	Species with identical sequence
S. agami (MW2), 5	*S. assamica* (MP1), 5, *S. confusa* (MW2), 5, *S. confusa* (MW1), 5, *S. agami* (MW1), 5
S. acuta (MW4), 9a	*S. macroptera* ssp. *macropterifolia* (MW2), 9b, *S. acuta* (MW2), 9a, *S. acuta* (MW1), 9a
S. pilosa (MW2), 8	*S. almon* (MW1), 7b, *S. amplexicaulis* (MW1), 8, *S. amplexicaulis* (MW2),8, *S. splendida* (I1), 8, *S. splendida* (I2), 8, *S. pilosa* (MW3), 8, *S. pilosa* (MW1), 8
S. dasyphylla (I1), 9b	*S. parvifolia* (MP2), 7b, *S. parvifolia* (MP1), 7b, *S. parvifolia* (MW3), 7b, *S. parvifolia* (MW1), 7b
S. falcifera (MP1), 1a	*S. palosapis* (MP1), 7b
S. fallax (MS1), 7b	*S. smithiana* (MW3), 7a
S. fallax (MW2), 7b	*S. smithiana* (MW1), 7a, *S. parvistipulata* (MS1), 7b
S. ferruginea (MW2), 9a	*S. slootenii* (MW2), 9a, *S. macroptera* ssp. *baillonii* (MW1), 9a, *S. teysmanniana* (I1), 9b
S. guiso (MP1), 1a	*S. ochrophloia* (MP1), 1a
S. quadrinervis (MW1), 9b	*S. rubra* (MW3), 9b, *S. rubra* (MW1), 9b, *S. rubra* (MW2), 9b, *S. quadrinervis* (MW3), 9b

Letters in parenthesis indicate the sample number and the following number indicates the section of the species based on Ashton (1982)

1, Section Shorea, 1a, Subsection Shores, 1b, Subsection Barbata, 2, Section Pentacme, 3, Section Neohopea, 4, Section Richetioides, 4a, Subsection Polyandrae, 4b, Subsection Richetioides, 5, Section Anthoshorea, 6, Section Rubella, 7, Section Brachypterae, 7a, Subsection Smithiana, 7b, Subsection Brachypterae, 8, Section Pachycarpae, 9, Section Mutica, 9a, Subsection Auriculatae, 9b, Subsection Mutica, 10, Section Ovalis

different species was only 1.64% [57/((84 × 83)/2)], thus most of species can be identified by our cpDNA data.

We found multiple haplotypes in 28 out of 47 species when analyzing multiple samples (2–10 samples with an average of 3.45) from the same species, but all of the haplotypes within a species were very similar to each other (Fig. 1b, c). Some species that shared haplotypes with other species (Table 5), as described above, appeared to have more complex relationships with other species, especially in the Red Meranti group (Fig. 1b). However, species from different wood varieties did not share chloroplast haplotypes. The *Hopea* and *Neobalanocarpus* group was found to be a sister of the White Meranti group (Fig. 1c). Based on our data, we have developed an online

molecular database to identify *Shorea* and closely related species in Dipterocarpaceae using FASTA software (http://f5002.ffpri-108.affrc.go.jp/shorea/).

Discrimination of *S. albida*

We analyzed two individuals that were collected in different forests, but their sequences of the four examined regions of cpDNA were identical. We found two unique nucleotide sites in *S. albida* that were not present in any of the other *Shorea* species examined. These were the 11th and 1225th sites of the *trnH-psbA-trnK* sequence, at which G and C, respectively, were present in *S. albida*, while A and G were present in all of the other *Shorea* species.

Species identification of woody products by anatomical observations

Eight veneer samples and one sawn timber sample were identified as wood of species from the sections Brachypterae, Pachycarpae, Mutica, and Ovalis (Red Meranti), based on the wood's anatomical features (Desch 1941; Ogata et al. 2008). The other sawn timber sample (no. 2) was identified as originating from the Yellow Meranti group, because of the presence of small radial resin canals. Veneer 1 had crystals in idioblasts in its axial and ray parenchyma tissue, diffuse-porous wood with warts on its vessel wall surfaces and thin-walled fibers (Table 6). Therefore, candidate species include: *S. fallax*, *S. parvistipulata*, *S. macrophylla*, *S. almon*, *S. johorensis*, *S. palembanica* and *S. ovalis* (see details in Desch 1941; Ogata et al. 2008). Veneer samples 2, 3, 4, 6, 7 and 8 shared a number of anatomical features, notably crystals in idioblasts in axial parenchyma (but not in rays), warts present on vessel walls, and thin fiber walls. Therefore, candidate species for these samples were *S. parvifolia*, *S. smithiana*, *S. mecistopteryx*, *S. ovalis*, and/or *S. rubra* (Table 6). Sawn timber 1 had anatomical features of the Red Meranti group including large radial resin canals, which are a typical feature of *S. leprosula*.

Species identification of woody products using DNA sequence data

DNA was extracted from eight veneer samples collected before the drying step in the manufacturing process. The amount of extracted DNA ranged from 0.4 to 4.2 μg per g fresh weight sample. The four non-coding regions of the chloroplast genome (*trn*L intron, *trn*L-*trn*F, *trn*H-*psb*A and *psb*C-*trn*S) were successfully amplified from the extracted DNA. The sequences of the PCR products were searched against a database constructed in this study using FASTA software. *Shorea* species whose sequences completely matched the query sequences across all four regions were regarded as candidate source species of the veneers. For veneer 1, *S. fallax* and *S. johorensis* were identified as candidates; only one nucleotide out of ca. 4.2 kbp did not match the query sequence in this case and for the other samples we could also find the candidate species (Table 6).

PCR amplification of the *trn*L intron, *trn*L-*trn*F, *trn*H-*psb*A and *psb*C-*trn*S regions of cpDNA from plywood failed, but three small fragments of the *trn*L intron were successfully amplified. After alignment of the sequence data, we searched against a database constructed in this study using FASTA software, and identified *Hopea* spp. as a candidate species (Table 6).

Discussion

Molecular classification and the limitations of using *Shorea* cpDNA sequences

We have sequenced 4.2 kbp of cpDNA for identifying *Shorea* species. However, some species belonging to the same sections have identical sequences, as described above, indicating that it may be difficult to discriminate between closely related species within the genus solely using cpDNA sequence data. ITS regions may be much better for classifying species of the genus (Yulita et al. 2005), but their complicated and uninferable evolutionary history may reduce their utility for phylogenetic analysis, for which single-copy nuclear genes are generally more robust (Álvarez and Wendel 2003).

In our dataset we found many nucleotide substitutions between the four woody groups, which provide convenient candidate sites for developing markers to use with techniques such as multiplex allele-specific PCR (Vallone et al. 2004). The woody group with the highest nucleotide diversity was White Meranti (Table 3), partly because one of its members, *S. roxburghii*, has a unique sequence that is close to sequences of Yellow Meranti species (Fig. 1). However, even when we estimated the nucleotide diversity of the White Meranti group excluding the *S. roxburghii* data, we still found it to be more diverse than the other groups (data not shown). We investigated 21, 43, 11 and 15 species, representing 2, 5, 1 and 1 sections of Balau, Red, White and Yellow Meranti (Ashton 1982), respectively. The classification based on morphological data is not entirely consistent with the variations in their chloroplast sequences, probably because cpDNA evolves more slowly than nuclear DNA, which contains genes that are strongly associated with most morphological and physiological characters. This inconsistency is prominent in the species of Red Meranti, the largest group in *Shorea*. For more detailed molecular classification of Red Meranti species, it will be necessary to obtain nuclear sequence information in further studies. However, *S. albida,* which belongs to Red Meranti taxonomically (section Rubella, Ashton 1982) and its wood is subjected to different tax rates from other members of Red Meranti in Japan, was clearly discriminated by cpDNA sequence data. Therefore, these nucleotide variations should be useful for discriminating *S. albida* from other Red Meranti species to detect illegal trading. Chloroplast sequence data can also be used to discriminate the compact sections of *Shorea*, such as the sections Rubella, Neohopea, Ovalis and also section Shorea subsection Shorea. The rate of sharing identical sequences was only 57 (1.64%) between species among 3486 combinations of species, which is quite low. Therefore, our cpDNA

Table 6 Investigated woody samples of dipterocarps and their candidate species using anatomical and cpDNA sequence analyses

Investigated samples	Anatomical data			Candidate species	Chloroplast DNA sequence Candidate species	Final candidate species	Distribution of candidate species
	Crystals in axial parenchyma	Crystals in rays	Warts on vessel walls				
Veneer no. 1	Present	Present	Present	*S. fallax, S. parvistipulata, S. macrophylla, S. almon, S. johorensis, S. palembanica, S. ovalis*	*S. fallax, S. johorensis,*	*S. fallax S. johorensis*	N.E. Sarawak, Sabah, Berau Malaysia, Sumatra, Borneo
Veneer no. 2	Present	Absent	Present	*S. parvifolia, S. smithiana, S. mecistopteryx, S.ovalis, S. rubra*	*S. parvifolia, S. dasyphylla*	*S. parvifolia*	Thailand, Malaysia, Indonesia
Veneer no. 3	Present	Absent	Present	*S. parvifolia, S. smithiana, S. mecistopteryx, S.ovalis, S. rubra*	*S. parvifolia, S. dasyphylla*	*S. parvifolia*	Thailand, Malaysia, Indonesia
Veneer no. 4	Present	Absent	Present	*S. parvifolia, S. smithiana, S.ovalis, S. rubra*	*S. parvifolia, S. dasyphylla*	*S. parvifolia*	Thailand, Malaysia, Indonesia
Veneer no. 5	Absent	Absent	Present	*S. smithiana, S.curtisii, S. scaberrima, S. waltonii, S. ovalis, S. rubra*	*S. quadrinervis, S. rubra*	*S. rubra*	Northern Borneo
Veneer no. 6	Present	Absent	Present	*S. parvifolia, S. smithiana, S. ovalis, S. rubra*	*S. parvifolia, S. dasyphylla*	*S. parvifolia*	Thailand, Malaysia, Indonesia
Veneer no. 7	Present	Absent	Present	*S. parvifolia, S. smithiana, S. mecistopteryx, S. ovalis, S. rubra*	*S. argentifolia*	*S. argentifolia*	N. E. Borneo
Veneer no. 8	Present	Absent	Present	*S. parvifolia, S. smithiana, S. mecistopteryx, S. ovalis, S. rubra*	*S. argentifolia*	*S. argentifolia*	N. E. Borneo
Sawn timber no. 1	Present	Present	Present	*S. leprosula*	*S. leprosula*	*S. leprosula*	Thailand, Malaysia, Indonesia
Sawn timber no. 2	–	–	–	Sect. Rechetioides	*S. patoiensis*	*S. patoiensis*	Northern Borneo
Plywood	–	–	–	–	*Hopea* spp.		

database can be used robustly to identify *Shorea* species in most cases.

Kamiya et al. (1998) reported that the Yellow Meranti group, White Meranti group and Selengan Batu (Balau) were each monophyletic, but they concluded that the Red Meranti group was paraphyletic. However, all four woody groups seem to be monophyletic except for *S. roxburghii* according to our results, with bootstrap value for the divergence of Yellow, White, Balau and Red of 99, 98, 99 and 71%, respectively (Fig. 1a). Kamiya et al. (1998) used two regions of cpDNA of less than 900 bp to construct their phylogenetic tree of dipterocarps, but our length was approximately 4200 bp. The limited 900 bp dataset may explain why the cited authors concluded that the Red Meranti group was paraphyletic. Our larger dataset also confirms that the *Hopea* and *Neobalanocarpus* group is closest to the White Meranti group, which was also previously showed by Kamiya et al. (1998, 2005).

Some species belonging to different sections have identical sequences in the four examined cpDNA regions (Table 5), possibly because of hybridization or recent divergence of species from shared origins. In some cases at least, since cpDNA is generally maternally inherited, hybridization may result in such patterns. In accordance with this hypothesis, hybrid dipterocarps are sometimes found in natural forests. For instance, interspecific hybrids between *S. curtisii* and *S. leprosula* have been reported from Peninsular Malaysia and Singapore (Ashton 1982). Kamiya et al. (2005) also suggested that *Neobalanocarpus* originated from hybridization between members of the White Meranti in *Shorea* and *Hopea*. In addition, Ishiyama et al. (2003) found that part of nuclear *Gap*C sequence in one species resembling that in another species of four *Shorea* species, probably resulting from recombination between two divergent haplotypes. To distinguish between species (and test the hybridization hypotheses), it will be necessary to acquire more data on nuclear DNA sequences, as well as their cpDNA sequences, because if a species has hybrid origins its cpDNA will be derived from the maternal species but its nuclear DNA will be derived from both parental species and thus need to use single-copy nuclear genes as mentioned above. However, for assessing the origins of woody samples and/or small samples, the chloroplast genome is still the best choice for analysis, because of its high copy number per cell (Régis and Lerbs-Mache 2001), robustness, and polymorphism.

In this study, we examined multiple samples of each species collected from individuals in various regions, including Peninsular Malaysia, Sabah, Sarawak and Indonesia. The sequences found in some individuals were not identical to those found in some of their conspecifics, indicating that it may be possible to identify the geographic regions from which individuals of some species originated.

However, in some cases, closely related species shared the same haplotypes, especially some Red Meranti members, possibly reflecting hybridization events or recent divergence of species from shared origins. To confirm these possibilities and to distinguish between samples of different species and geographic origins with confidence, we must investigate more samples originating from many regions and accumulate more data.

Our molecular database covers ca. 70% of major timber species in the genus *Shorea* in Indonesia and Malaysia (Soerianegara and Lemmens 1994). It might not be easy to collect samples of all species of *Shorea*, because some species are very rare. However, it is necessary to increase the proportion of species covered in these areas for more accurate identification of the species.

Species identification of woody products

Morphological features, such as wood color, the presence of axial resin canals, vestured pits, silica grains, and crystals in tissue of *Shorea* species, provide information that can be used for identification to the wood group level (Desch 1941; Ogata et al. 2008). In this study, we were able to narrow down the investigated woods to several candidate species using anatomical methods. However, candidate species of veneer samples 7 and 8 by anatomical analysis were incongruent with those by cpDNA sequence (Table 6). This is probably because the anatomical database was constructed by the limited number of analyzed samples for each species and thus the false identification may sometimes be occurred. Although such analysis requires several working days by well-trained and experienced personnel, the information generated can help to deter false declarations regarding imported timber and, hence, reduce the trade of illegal timber. However, in order to protect the genetic resources of *Shorea* species in Southeast Asia, identification at the species level is more important, because nearly half of the species have very restricted distributions and many of them are threatened (Ashton 1982; Saw and Sam 2000). In such cases, we may identify the region the wood sample has originated from. The final candidate species of veneer samples 1, 5, 7, 8 and sawn timber sample 2 have relatively restricted distribution areas, so we know the area where the timber came from (Table 6). If the identified species distributed only in conservation areas or was protected species such as endangered species (Saw and Sam 2000, http://www.forestry.sarawak.gov.my/forweb/ourfor/flora/pp/pp.htm), we can know that the timber was collected illegally. However, the other five samples are widespread species and thus it is necessary to study the phylogeography within species to identify their origins.

Extracting DNA from a plywood sample proved to be difficult, and we did not obtain sufficient DNA to visualize molecules on agarose gels. PCR amplification of the four regions (506, 441, 1780 and 1559 bp) of cpDNA also failed, probably due to physical degradation of the DNA during the plywood manufacturing process. These results are in accordance with previous reports that it is sometimes difficult to extract DNA from woody products or old wood samples (Deguilloux et al. 2002; Tani et al. 2003; Takahashi et al. 2008), although short DNA fragments probably still remain within the wood. Such small DNA fragments of multiple-copy genomes such as cpDNA can be amplified by PCR (Deguilloux et al. 2002; Parducci et al. 2005; Liepelt et al. 2006; Rachmayanti et al. 2009), and in this study we successfully amplified three small fragments of the *trn*L intron region, ranging in size from 129 to 306 bp. These results indicate that even if the DNA in a sample has been seriously degraded, as in the plywood samples, it may still be possible to amplify small fragments (<300 bp) by PCR. In the *Shorea* molecular classification database, we accumulated cpDNA sequence data for most of the major *Shorea* species in Southeast Asia. Using such information, it should be possible to design species-specific PCR primers to amplify short fragments of target species, including discriminating nucleotide substitutions from other species when necessary, and use them to identify source species of recalcitrant woody products such as plywood. In this time we have tried to analyze only one sample from plywood and could obtain the result. However, the developed method for analyzing DNA from especially plywood is not still perfect and probably need to improve it more because DNA of plywood would be more degraded by its making process such as heating than those of veneer and sawn timber.

Conservation perspective

Imports of plywood from Malaysia and Indonesia into Japan have increased dramatically since the 1980s, and nearly 600 million m^3 of plywood were imported in 2002, according to Itoh et al. (2004). However, the proportion of Red Meranti in the surface layers of the plywood imported into Japan reportedly fell from 94.9% in 1994 to 0.4% in 2000. A large fraction of this apparent decline seems to have been due to false customs declarations, prompted by the higher tax rate imposed on Red Meranti wood. When the possibility of discriminating between the four woody groups of *Shorea* species using anatomical analyses was announced recently, there was a dramatic decline in such false declarations (Itoh et al. 2004). Therefore, if we can develop methods and a database that can be used to identify *Shorea* species at the species level, they should help to further reduce frequencies of false declarations and hence reduce illegal logging. Thus, the molecular classification data and methods for identifying the source species of woody products presented here may have potential for deterring illegal logging and illegal international trade in wood products not only for developed countries such as Japan but also the other countries such as exporters of Malaysia and Indonesia. Especially, the developed database may work for conservation of protected *Shorea* species and endangered *Shorea* species (Saw and Sam 2000). The DNA extraction methods from plywood samples should be developed more convenient and simple one because ca. 48% of exported wood products was exported as plywood in Asian countries (ITTO 2008). Consequently, these methods may help to promote the conservation of tropical forests in Southeast Asia. In future studies, it would be helpful to develop DNA markers that can be used to identify the geographical origins of individuals of each species, especially widely distributed species. For this purpose, cpDNA may be particularly suitable, because diversity is generally low within populations but high between populations. Ideally, dense cpDNA haplotype maps covering the entire distributions of each species should be compiled (Petit et al. 2002).

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