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PHYLOGENY OF *PGIC* GENE IN *SHOREA* AND ITS CLOSELY RELATED GENERA (DIPTEROCARPACEAE), THE DOMINANT TREES IN SOUTHEAST ASIAN TROPICAL RAIN FORESTS[1]

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Dipterocarpaceae, trees that dominate tropical rain forests in Southeast Asia consist of many economically and ecologically important species. We determined partial sequences of the *PgiC* gene from species of *Shorea*, *Hopea*, *Neobalanocarpus*, and *Parashorea* to elucidate phylogenetic relationships among the species of these genera, which have been regarded as interrelated. The sequences generated a gene tree with better resolution than previous cpDNA trees. The *PgiC* tree is essentially consistent with cpDNA trees, except for the placement of *Neobalanocarpus*. The *PgiC* tree shows that *Neobalanocarpus* is nested within White Meranti of *Shorea*, whereas this genus forms a clade with *Hopea* in cpDNA trees. This conflict suggests that *Neobalanocarpus* is derived via hybridization between White Meranti of *Shorea* and *Hopea*. Species belonging to each of three timber groups (Yellow Meranti, Balau, and Red Meranti) within *Shorea* are monophyletic. Together they form a monophyletic clade distinct from White Meranti. Botanical sections within Red Meranti appear not to be monophyletic. An extensive number of shared polymorphisms among species and consequential lack of monophyly of intraspecific haplotypes are found in Red Meranti. Potential causes of this phenomenon, including persistence of ancestral polymorphisms and gene flow via interspecific hybridization, are discussed.

Key words: Dipterocarpaceae; *Hopea*; interspecific hybridization; molecular phylogeny; *Neobalanocarpus*; *PgiC*; *Shorea*.

The tropical rainforests in Southeast Asia are characterized by a high species diversity of trees (Whitmore, 1984). The extreme floristic richness is largely due to co-occurrence of a great number of species within the same community (Whitmore, 1998). In particular, Borneo has one of the highest species diversity of trees among the world's tropical rainforests. In lowland Southeast Asian tropical forests, dipterocarp species dominate the forest canopy (Ashton, 1988). Because of their economic and ecological significance, the dipterocarp trees have served as representative species in a number of tropical biological studies. However, human impact on tropical rainforests has been increasing in the past half-century, and many primary forests have been degraded by logging and shifting cultivation (Richards, 1996; Whitmore, 1998).

Dipterocarpaceae consist of more than 500 species and are divided into three subfamilies, Dipterocarpoideae, Monotoideae, and Pakaraimoideae (Ashton, 1982). Although the phylogenetic placement of Dipterocarpaceae within angiosperms has been problematic, a recent molecular phylogenetic analysis suggests that this family should be assigned to the order Malvales and that Sarcolaenaceae is the closest relative of the Dipterocarpaceae (Dayanandan et al., 1999).

The Asian subfamily Dipterocarpoideae includes 13 genera and 470 species (Ashton, 1982). Detailed taxonomic study by Ashton, initially focused on Borneo, substantially relied on androecium and bark characters for classification (Ashton, 1962, 1963, 1964, 1967). These subsequently led to a regional monograph (Ashton, 1982). Ashton has retained most of the previous classification of Symington (1943), but some of the groups were reclassified at lower taxonomic rank. For *Shorea*, which we study here, Symington (1943) divided this genus into four groups that are equivalent to the timber groups classified by timber characters (i.e., Balau, White Meranti, Yellow Meranti, and Red Meranti) and treated *Pentacme* as an independent genus being closely related to *Shorea*. Ashton (1982), primarily on the basis of shared fruit calyx characters and differences in androecium and bark morphology, reduced *Doona* Thw. and *Pentacme* A. DC. as sections within *Shorea* and divided the genus into 11 sections (see Fig. 1). Although Ashton treated all 11 sections as having equivalent status (section), Maury (1978, 1979; summarized in Maury-Lechon and Curtet, 1998) argued, primarily on the basis of embryo and leaf epidermal characters, that some sections of *Shorea* have unequal hierarchic ranks. She concluded that Ashton's sections *Doona*, *Pentacme*, and *Anthoshorea*, *Shorea* and *Richetioides*, which correspond to Symington's informal groups White Meranti, Balau and Yellow Meranti, respectively, should have higher ranks than sections such as *Ovalis* and *Rubella*, which are members of the Red Meranti. Thus, a molecular phylogenetic approach would be helpful to clarify controversial relationships in the family.

Recently, several cpDNA sequences have been analyzed to

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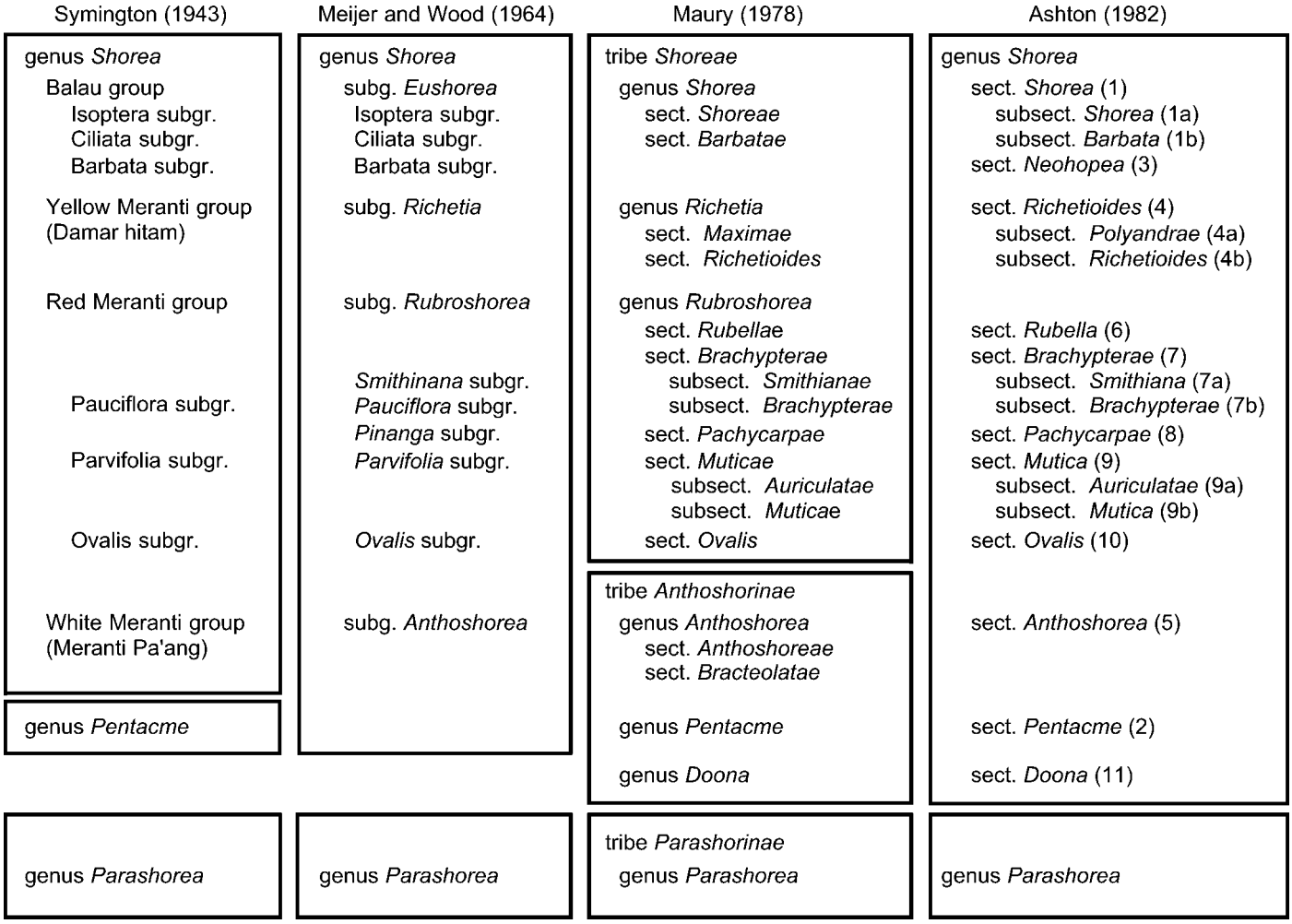


. Fig. 1. Comparison of classifications of *Shorea* and closely related genera, modified from table 8 in Maury-Lechon and Curtet (1998)

examine the relationships within Dipterocarpaceae (Tsumura et al., 1996; Kajita et al., 1998; Kamiya et al., 1998; Dayanandan et al., 1999). The phylogenetic analyses based on the cpDNA data revealed two distinct clades within Dipterocarpoideae, corresponding to the two tribes, *Dipterocarpeae* and *Shoreae*. Those studies demonstrated that cpDNA phylogenies are largely consistent with morphological classification at higher taxonomic levels, but several generic circumscriptions conflict with each other. Kajita et al. (1998) and Dayanandan et al. (1999) showed that genus *Shorea* is not monophyletic; the White Meranti of *Shorea* together with *Doona* form a clade that is sister to the clade of *Hopea* and *Neobalanocarpus*, while other members of *Shorea* form a cluster with *Parashorea*. In other phylogenetic studies with large numbers of species of *Shorea*, Tsumura et al. (1996) and Kamiya et al. (1998) also supported the paraphyletic relationships of *Shorea*, but these studies could not resolve most of the intrageneric relationships due to an insufficient number of informative characters in the cpDNA.

Accordingly, to clarify the relationships at lower taxonomic levels, nuclear gene sequences are required to obtain additional sources of characters (Kamiya et al., 1998). In addition, a comparison between cpDNA and nuclear DNA phylogenetic analyses sometimes provides a strongly conflicting signal due to hybridogenous genomic constitution (Shi et al., 2001; Oh and Potter, 2003; Mummenhoff et al., 2004). Therefore, we use the nuclear gene *PgiC*, which encodes cytosolic phosphoglucose isomerase, an essential enzyme of glycolysis and gluconeogenesis, to clarify the phylogenetic relationships between *Shorea* and its allied genera, and among taxonomic groups within *Shorea*. In this study, we examined partial sequences of the *PgiC* gene from species of *Shorea* and its allied genera *Hopea*, *Neobalanocarpus*, and *Parashorea*. The aims of our study are to (1) elucidate phylogenetic relationships among the species of *Shorea* using nuclear *PgiC* sequences, (2) compare the nuclear *PgiC* phylogeny with cpDNA based phylogenies, and (3) investigate the utility of the *PgiC* sequences for phylogenetic reconstruction at lower taxonomic levels.

MATERIALS AND METHODS

Sample collection—Some of the samples were collected from the two permanent plots, the Canopy Biology Plot (8 ha) and the Long Term Ecological Research Plot (52 ha) in Lambir Hills National Park, Sarawak, Malaysia (Lee et al., 2002). All individuals in the plots at Lambir have been tagged, mapped, and identified to species by Sarawak Forest Department staff in collaboration with the Smithsonian's Center for Tropical Forest Science, the Center for Ecological Research in Kyoto University and Osaka City University. DNA samples that we used in this study include those used in the cpDNA analysis

of Kamiya et al. (1998). Additional leaf samples were collected from the Dipterocarp Arboretum in FRIM (Forest Research Institute Malaysia), and some DNA samples were kindly provided by S. L. Lee. A total of 78 accessions throughout 48 species of *Shorea*, representing all recognized sections of Ashton (1982) except for the Sri Lanka endemic section *Doona* and also *Pentacme*, six species representing all the sections and subsections of *Hopea*, the monotypic genus *Neobalanocarpus*, and one species from *Parashorea* were collected. *Dipterocarpus palembanicus* subsp. *bornensis* (tribe *Dipterocarpeae*) was used as an outgroup taxon. *Dipterocarpus* is known as the closest sister genus of tribe *Shoreae* based on previous molecular phylogenies (Tsumura et al., 1996; Kajita et al., 1998; Kamiya et al., 1998; Dayanandan et al., 1999). Total DNA was extracted using the CTAB procedure of Doyle and Doyle (1990) or DNeasy Plant Mini kit (Qiagen, Valencia, CA). Information on the specimens is shown in Table 1.

Molecular methods—A partial region of the *PgiC* (ca. 1250 bp) was amplified by PCR using the following primers, PgiCF3 (5′ CATTTCTATTCAGCACCTTT 3′) and PgiCR4 (5′ ATTAGATGCTGTGGAACATTCTC 3′) designed by T. Kado (Kyushu University, personal communication). The PCR was performed with 50 μL of reaction mixture containing 10 ng genomic DNA, 1× PCR buffer for KOD -plus-, 2.5 mM $MgSO_4$, 0.2 mM dNTPs, 0.1 μM of each primer, and 1 U of KOD -Plus- DNA polymerase (TOYOBO, Osaka, Japan). The cycling profile consisted of a primary denaturing of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2.5 min of 72°C, and a final extension of 10 min at 72°C. Initially, PCR products were directly sequenced after purification using a MiniElute PCR Purification kit (Qiagen). When two or more positions that each had two overlapping heterozygous peaks in the electoropherogram were found, the PCR products were cloned with a pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA) following the manufacturer's protocol. At least two independent clones were sequenced for every haplotype to avoid PCR artifacts. DNA sequencing was performed using an ABI BigDye Terminator Cycle v3 (or v3.1) Sequencing kit and an ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer, Foster City, CA) following the manufacturer's instructions. We designed and used the following internal sequencing primers: PgiC2F (5′ TTCTTGGATAGCCACCAAGG 3′) at position 417–436; PgiC2R (5′ AAATCACATGGAATTACACG 3′) at position 933–914; PgiC5F (5′ TCAGTTGCAAACTTGACTACCA 3′) at position 1016–1037; and PgiC5R (5′ CCTGAAGAGCCAAAAGATTCTT 3′) at position 380–359.

Data analyses—The nucleotide sequences were aligned with ClustalX (Thompson et al., 1997) and then manually edited using SeqPup 0.6 (available at website http://iubio.bio.indiana.edu/soft/molbio/seqpup). The aligned sequence data were analyzed by maximum-parsimony and neighbor-joining (Saitou and Nei, 1987) methods implemented in PAUP* 4b10 (Swofford, 2002). A heuristic search was conducted with random sequence addition with 100 replicates, tree-bisection-reconnection (TBR) branch swapping, and the number of rearrangement was limited to 100 000 for each replication. All characters were equally weighted, and gaps were treated as missing data. Relative robustness for clades was examined using a bootstrap analysis (Felsenstein, 1985) with 5000 replications of fast bootstrapping. Mort (2000) indicated that fast bootstrapping (without branch swapping) generates a smaller value than the standard bootstrap analysis (with branch swapping), and this method is suitable for analyzing large data sets because of easier and faster computation. The neighbor-joining tree was constructed based on Kimura's two-parameter distance (1980), and 1000 replicates of bootstrap were performed to obtain cluster supports.

RESULTS

Characteristics of the PgiC sequences—PCR amplifications using the primer set produced mostly single clear bands in the agarose gel electrophoresis. Furthermore, sequences from multiple clones from one individual were homologous to the *PgiC* sequence of *Arabidopsis* in all cases, indicating nonspecific products were not generally amplified. Therefore, each PCR product must be a result of amplification from a single specific region coding for PGIC. The cloned sequences from some of the accessions contained polymorphic sites where the direct sequencing has double peaks, and thus such individuals are heterozygous at the locus. Length variations were rare within individuals, allowing us to determine heterozygous sites easily. As expected for diploid outcrossers, 63 of 84 (except for four accessions of polyploid species) had two different haplotypes per individual. Each individual of *Shorea ovalis* subsp. *sericea*, *Hopea odorata*, and *H. subalata*, which are known to be polyploid species (Ashton, 1982), had three or four haplotypes, as expected with polyploidy. In total, we identified 161 *PgiC* haplotypes throughout 88 accessions. Most variable sites were found in introns or at synonymous sites. Newly determined DNA sequences were deposited in the DNA Data Bank of Japan database under accession numbers AB189478 to AB189638.

The *PgiC* sequences that we determined corresponded to exons 13 to 19 of the *PgiC* of *Arabidopsis thaliana* (e.g., Kawabe and Miyashita, 2000). The coding sequences of the dipterocarp *PgiC* were about 85% identical to that of *Arabidopsis*, although the introns of dipterocarps and *Arabidopsis* could not be aligned. Numbers of exons and their lengths in *Arabidopsis* and dipterocarp were the same. Lengths of the sequences vary from 1233 to 1449 bp due to insertions and deletions (indels) found in the introns. Alignment of the sequences was unambiguous in both exons and introns, and the alignment matrix used for phylogenetic analyses contained 1597 characters.

Level of variation in PgiC gene sequences—As expected, sequences of the nuclear *PgiC* had a higher proportion of informative characters (21.5% with 141 sequences) than noncoding regions of cpDNA (3.8% with 30 sequences based on data obtained from Kamiya et al., 1998) for the *Shorea* species. Sequence variation at the *PgiC* locus (2.4%) is about 2.5 times as high as that in the chloroplast noncoding region (0.9%), due to a lower substitution rate in chloroplast than nuclear genomes (Wolfe et al., 1987).

Mean pairwise sequence divergences among species within each clade and among clades are shown in Table 2. Mean divergences between species range from 0.0089 to 0.0422 within clades. The divergences range from 0.0247 to 0.0585 between clades within the ingroup, and 0.0623 to 0.0711 between the ingroup and outgroup.

Although the primary focus of this study is the phylogenetic relationships among species of *Shorea*, in most cases, two haplotypes were detected from a single accession, and in some cases, several different individuals were investigated to look for additional intraspecific diversity. At the intraspecific level, the *PgiC* gene sequences had considerable amounts of DNA variation in several species (>0.01), with values that were sometimes larger than the interspecific divergences (Tables 1 and 2).

Phylogenetic relationships inferred from the PgiC sequences—One of the 6376 most parsimonious trees (length [L] = 827; CI = 0.748; RI = 0.921) is shown in Fig. 2 and the neighbor-joining (NJ) tree is shown in Fig. 3. Most parsimonious (MP) and NJ trees are basically congruent with each other, with some differences between poorly supported nodes. Figure 2 shows several polytomies where supports for the corresponding nodes in the NJ tree are low. The analyses using *Dipterocarpus* as an outgroup identify six clades: I, White

TABLE 1. Taxa used in this study, with timber group, taxonomic attributions (genus/section/subsection) according to classifications of Ashton (1982), and source. Number of observed haplotypes and mean pairwise distance among haplotypes (nucleotide diversity) for each species are shown. Monophyly (M) and lack of monophyly (P) of intraspecific haplotypes in each species are determined based on the NJ tree (Fig. 3).

Species	Timber group	Section	Subsection	Source (locality/voucher)	No. of observed haplotypes	Nucleotide diversity	Monophyly of intraspecific haplotypes
Shorea isoptera Ashton	Balau	*Neohopea*		FRIM/540	2	0.0056	M
S. biawak Ashton	Balau	*Shorea*	*Barbata*	Lambir, Sarawak, Malaysia/T36	2	0.0121	M
S. laevis Ridl.	Balau	*Shorea*	*Barbata*	FRIM/SM275	2	0.0129	M
S. maxwelliana King	Balau	*Shorea*	*Barbata*	FRIM/SM267	2	0.0016	M
S. atrinervosa Sym.	Balau	*Shorea*	*Shorea*	FRIM/SM268	2	0.0032	P
S. falciferoides Foxw.	Balau	*Shorea*	*Shorea*	Lambir, Sarawak, Malaysia/T288	2	0.0000	M
S. geniculata Sym. ex Ashton	Balau	*Shorea*	*Shorea*	Lambir, Sarawak, Malaysia/0901-089	2	0.0064	M
S. havilandii Brandis	Balau	*Shorea*	*Shorea*	Lambir, Sarawak, Malaysia/T132	2	0.0016	M
S. materialis Ridl.	Balau	*Shorea*	*Shorea*	FRIM/SM318	2	0.0032	M
S. lumutensis Sym.	Balau	*Shorea*	*Shorea*	1) Sungai Pinang FR, Malaysia/ LeeLU006	1	0.0000	M
				2) Sungai Pinang FR, Malaysia/ LeeLU010	1		
				3) Sungai Pinang FR, Malaysia/ LeeLU023	1		
S. obtusa Wall.	Balau	*Shorea*	*Shorea*	Chang Mai, Thailand/no voucher	2	0.0048	M
S. seminis (de Vriese) Sloot.	Balau	*Shorea*	*Shorea*	FRIM/SM288	2	0.0016	M
S. sumatrana (Sloot. Ex Thorenaar) Sym. ex Desch.	Balau	*Shorea*	*Shorea*	FRIM/SM266	2	0.0088	P
S. almon Foxw.	Red Meranti	*Brachypterae*	*Brachypterae*	Lambir, Sarawak, Malaysia/T464	2	0.0203	P
S. bullata Ashton	Red Meranti	*Brachypterae*	*Brachypterae*	1) Lambir, Sarawak, Malaysia/T261	2	0.0016	M
				2) Lambir, Sarawak, Malaysia/T501	2		
S. fallax Meijer	Red Meranti	*Brachypterae*	*Brachypterae*	1) Lambir, Sarawak, Malaysia/T495	2	0.0120	P
				2) Lambir, Sarawak, Malaysia/ Tanaka01	2		
				3) Lambir, Sarawak, Malaysia/ Tanaka02	2		
				4) Lambir, Sarawak, Malaysia/ Tanaka03	2		
S. kunstleri King	Red Meranti	*Brachypterae*	*Brachypterae*	FRIM/SM300	2	0.0138	P
S. pauciflora King	Red Meranti	*Brachypterae*	*Brachypterae*	1) Lambir, Sarawak, Malaysia/ Kamiya014	2	0.0130	P
				2) FRIM/SM313	2		
S. scaberrima Burck	Red Meranti	*Brachypterae*	*Brachypterae*	1) Lambir, Sarawak, Malaysia/T568	1	0.0107	P
				2) FRIM/no voucher	2		
S. smithiana Sym.	Red Meranti	*Brachypterae*	*Smithiana*	FRIM/SM308	2	0.0153	P
S. ferruginea Dyer ex Brandis	Red Meranti	*Mutica*	*Auriculatae*	1) Lambir, Sarawak, Malaysia/T18	2	0.0045	P
				2) Lambir, Sarawak, Malaysia/T296	2		
S. macroptera Dyer subsp. *bailonii* (Heim) Ashton	Red Meranti	*Mutica*	*Auriculatae*	1) Lambir, Sarawak, Malaysia/T103	1	0.0032	M
				2) Lambir, Sarawak, Malaysia/ Kamiya011	2		
S. macroptera Dyer subsp. *macroptera* King	Red Meranti	*Mutica*	*Auriculatae*	1) Sungai Lalang FR, Malaysia/ LeeSM039	2	0.0052	P
				2) Sungai Lalang FR, Malaysia/ LeeSM047	2		
S. macroptera Dyer subsp. *macropterifolia* Ashton	Red Meranti	*Mutica*	*Auriculatae*	Lambir, Sarawak, Malaysia/T508	2	0.0080	P
S. myrionerva Sym. ex Ashton	Red Meranti	*Mutica*	*Auriculatae*	Niah, Sarawak, Malaysia/Kamiya031	2	0.0064	M
S. slootenii Wood ex Ashton	Red Meranti	*Mutica*	*Auriculatae*	Niah, Sarawak, Malaysia/Kamiya012	2	0.0024	M
S. acuminata Dyer	Red Meranti	*Mutica*	*Mutica*	1) Sungai Lalang FR/LeeSA101	2	0.0073	P
				2) Sungai Lalang FR/LeeSA102	1		
				3) Sungai Lalang FR/LeeSA109	2		

TABLE 1. Continued.

Species	Timber group	Section	Subsection	Source (locality/voucher)	No. of observed haplotypes	Nucleotide diversity	Monophyly of intraspecific haplotypes
S. argentifolia Sym.	Red Meranti	*Mutica*	*Mutica*	Lambir, Sarawak, Malaysia/T316	1	—	—
S. curtisii Dyer ex King	Red Meranti	*Mutica*	*Mutica*	1) Lambir, Sarawak, Malaysia/ Kamiya016	1	0.0023	M
				2) Sungai Lalang FR/LeeSC002	2		
				3) Sungai Lalang FR/LeeSC003	1		
S. leprosula Miq.	Red Meranti	*Mutica*	*Mutica*	1) Lambir, Sarawak, Malaysia/T481	2	0.0029	M
				2) Pasoh FR/LeeSL001	2		
				3) Pasoh FR/LeeSL002	1		
				4) Pasoh FR/LeeSL003	1		
				5) Pasoh FR/LeeSL004	2		
S. ovata Dyer ex Brandis	Red Meranti	*Mutica*	*Mutica*	Lambir, Sarawak, Malaysia/ Kamiya009	2	0.0024	M
S. parvifolia Dyer	Red Meranti	*Mutica*	*Mutica*	1) Lambir, Sarawak, Malaysia/T406	2	0.0080	P
				2) Sungai Lalang FR, Malaysia/ LeeSP090	2		
				3) Sungai Lalang FR, Malaysia/ LeeSP112	1		
				4) Sungai Lalang FR, Malaysia/ LeeSP116	1		
				5) Sungai Lalang FR, Malaysia/ LeeSP120	2		
S. quadrinervis Sloot.	Red Meranti	*Mutica*	*Mutica*	Lambir, Sarawak, Malaysia/1622-112	2	0.0008	M
S. singkawang (Miq.) Miq.	Red Meranti	*Mutica*	*Mutica*	FRIM/SM315	2	0.0032	M
S. ovalis (Korth) Bl. subsp. *sarawakensis*	Red Meranti	*Ovalis*		Lambir, Sarawak, Malaysia/1814-184	1	—	—
S. ovalis (Korth) Bl. subsp. *sericea* (Dyer) Ashton	Red Meranti	*Ovalis*		1) Pasoh FR/LeeSO002	3	0.0191	P
				2) Pasoh FR/LeeSO003	4		
S. amplexicaulis Ashton	Red Meranti	*Pachycarpae*		Lambir, Sarawak, Malaysia/1814-140	2	0.0008	M
S. beccariana Burck	Red Meranti	*Pachycarpae*		1) Lambir, Sarawak, Malaysia/T226	2	0.0081	P
				2) Lambir, Sarawak, Malaysia/T258	2		
S. macrophylla (de Vriese) Ashton	Red Meranti	*Pachycarpae*		Kuching, Sarawak, Malaysia/ no voucher	2	0.0040	M
S. pilosa Ashton	Red Meranti	*Pachycarpae*		Lambir, Sarawak, Malaysia/T289	2	0.0024	M
S. rubella Ashton	Red Meranti	*Rubella*		Lambir, Sarawak, Malaysia/ Kamiya007	2	0.0072	M
S. agami Ashton	White Mreranti	*Anthoshorea*		Lambir, Sarawak, Malaysia/ Kamiya003	2	0.0056	P
S. assamica Dyer	White Mreranti	*Anthoshorea*		FRIM/SM284	2	0.0024	M
S. bracteolata Dyer	White Mreranti	*Anthoshorea*		FRIM/Kajita et al. (1998)	1	—	—
S. henryana Pierre	White Mreranti	*Anthoshorea*		FRIM/no voucher	2	0.0008	M
S. ochracea Sym.	White Mreranti	*Anthoshorea*		1) Lambir, Sarawak, Malaysia/T165	2	0.0005	M
				2) FRIM/SM309	2		
S. roxburghii G. Don	White Mreranti	*Anthoshorea*		FRIM/SM282	2	0.0030	M
S. faguetiana Heim	Yellow Meranti	*Richetioides*	*Richetioides*	Lambir, Sarawak, Malaysia/T282	2	0.0048	P
S. maxima (King) Sym.	Yellow Meranti	*Richetioides*	*Richetioides*	Fraser's Hill, Pahang, Malaysia/ no voucher	1	—	—
S. patoiensis Ashton	Yellow Meranti	*Richetioides*	*Richetioides*	Lambir, Sarawak, Malaysia/T743	2	0.0056	M
S. xanthophylla Sym.	Yellow Meranti	*Richetioides*	*Richetioides*	1) Lambir, Sarawak, Malaysia/T394	1	0.0144	P
				2) Lambir, Sarawak, Malaysia/T408	1		
Parashorea lucida (Miq.) Kurz				FRIM/SM317	2	0.0016	M
Hopea dryobalanoides Miq.		*Dryobalano-ides*	*Dryobalano-ides*	Lambir, Sarawak, Malaysia/0510-013	2	0.0024	M
H. nervosa King		*Dryobalano-ides*	*Sphaerocarpae*	FRIM/Kajita et al. (1998)	1	—	—

TABLE 1. Continued.

Species	Timber group	Section	Subsection	Source (locality/voucher)	No. of observed haplotypes	Nucleotide diversity	Monophyly of intraspecific haplotypes
H. subalata Sym.		*Dryobalanoides*	*Sphaerocarpae*	Kanchin FR/LeeHS001	4	0.0071	M
H. bilitonensis Ashton		*Hopea*	*Pierrea*	Kinta FR/LeeHB006	1	—	—
H. helferi (Dyer) Brandis		*Hopea*	*Hopea*	FRIM/SM272	2	0.0032	M
H. odorata Roxb.		*Hopea*	*Hopea*	FRIM/LeeHB037	3	0.0022	M
Neobalanocarpus heimii (King) Ashton				1) Pasoh FR/LeeNH43	2	0.0103	M
				2) Pasoh FR/LeeNH44	1		
Dipterocarpus palembanicus Sloot. subsp. *bornensis* Ashton				Lambir, Sarawak, Malaysia/T7	2	0.0016	M

TABLE 2. Average numbers of nucleotide changes within species, among species in each clade, and among clades.

Clade name	Corresponding taxa	No. of species used	No. of sequences determined	Within species (range)	Among species in clade (range)	Among clades					
						Out	I	II	III	IV	V
Outgroup	*Dipterocarpus*	1	2	0.0016 (N.A.)	N.A.						
Clade I	White Meranti of *Shorea* and *Neobalanocarpus*	7	16	0.0033 (0.0000–0.0056)	0.0315 (0.0044–0.0538)	0.0655					
Clade II	*Hopea*	6	13	0.0025 (0.0000–0.0071)	0.0422 (0.0078–0.0636)	0.0711	0.0585				
Clade III	*Parashorea*	1	2	0.0016 (N.A.)	N.A.	0.0631	0.0515	0.0549			
Clade IV	Yellow Meranti of *Shorea*	4	7	0.0063 (0.0000–0.0144)	0.0089 (0.0060–0.0121)	0.0652	0.0547	0.0549	0.0340		
Clade V	Balau of *Shorea*	13	27	0.0047 (0.0000–0.0129)	0.0175 (0.0036–0.0361)	0.0666	0.0545	0.0560	0.0350	0.0309	
Clade VI	Red Meranti of *Shorea*	25	94	0.0066 (0.0000–0.0203)	0.0133 (0.0037–0.0216)	0.0623	0.0511	0.0528	0.0317	0.0281	0.0247

Meranti of *Shorea* + *Neobalanocarpus*; II, *Hopea*; III, *Parashorea*; IV, Yellow Meranti of *Shorea*; V, Balau of *Shorea*; and VI, Red Meranti of *Shorea*, with diversification of the groups estimated to have occurred in this order. All of these clades are supported by >70% of bootstrap values (BS), except for clade I (BS = 62% in the MP tree and 84% in the NJ tree), and clade II (BS = 50% in the MP and 63% in the NJ trees). In White Meranti/*Neobalanocarpus* clade (I), three subclades, consisting of *Shorea roxburghii*, *Neobalanocarpus heimii*, and White Meranti of *Shorea*, are recognized. *Shorea roxburghii*, a widely distributed species throughout Indian subcontinent to Malaysia, is a typical member of White Meranti (Ashton, 1982), but our result shows that this species is more distantly related to the other Malaysian species of White Meranti than *Neobalanocarpus*. Clade II consists of two subclades, corresponding to sections *Dryobalanoides* and *Hopea* of Ashton (1982). In Balau (clade V), four distinct subclades are identified: (1) two species of section *Shorea* subsection *Barbata*, (2) *S. isoptera* (section *Neohopea*), (3) *S. laevis* (subsection *Barbata*), and (4) nine species of subsection *Shorea*. Red Meranti (clade VI) involves five distinct sections according to the classifications of Ashton (1982), but these taxonomical circumscriptions are only partially resolved in the gene tree. Although several well-supported subclades are identified, and species within Ashton's sections are largely concentrated within them, the species in some sections are placed in more than one separate subclade while the species of section *Brachypterae* and the subspecies of *Shorea ovalis* (sole member of section *Ovalis*) in particular, and some others to less extent, are spread over several subclades.

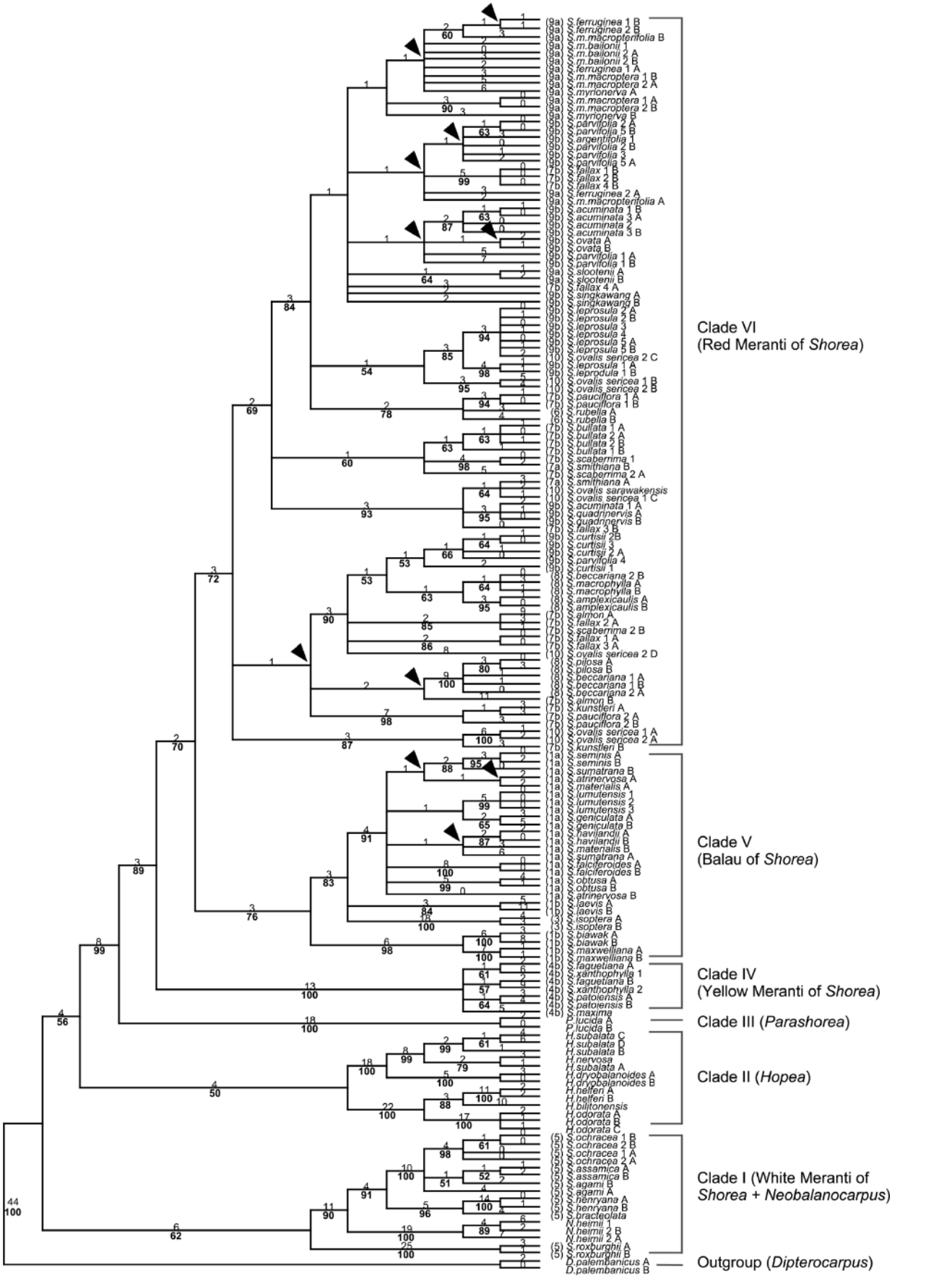
Fifty-one species and three subspecies of *S. macroptera* are found to have more than one haplotypes within each species (subspecies). Haplotypes of each species (or subspecies) appear in sister positions in 36 of the 54 species, but not in the remaining 18 species (Table 1). In the former cases, haplotypes not in sister positions may appear if we increase sample sizes. In the latter cases, most of the species show that their haplotypes are closely related to each other. In several species, only one haplotype is distantly related with the other haplotypes, which are clustered with each other (e.g., *S. acuminata* 1A and *S. beccariana* 2B). Although only one individual is used for most of the species of section *Brachypterae*, haplotypes of the same individual are notably divergent from each other in this section (Figs. 2 and 3), and thus they have larger intraspecific sequence variation (>0.01 in Table 1) except for *S. bullata*. Surprisingly, haplotypes of *S. fallax* and *S. ovalis* are positioned throughout the clade of Red Meranti (VI); the divergent haplotypes of each of these species fall into five different subclades (Figs. 2 and 3). Note that this is not due to misidentification because for the samples that have a haplotype in an unexpected position, the alternative haplotype clusters with that from samples of same species; in the example of two haplotypes determined from *S. acuminata* 1, one haplotype (B) clusters with those from samples of the same species (*S. acuminata* 2, 3A and 3B), while another haplotype (A) is diverse from them.

DISCUSSION

Nuclear- vs. chloroplast-based topologies—The topology of the *PgiC* gene tree is essentially consistent with the consensus of previous cpDNA trees (Tsumura et al., 1996; Kajita et al., 1998; Kamiya et al., 1998; Dayanandan et al., 1999), except for the placement of *Neobalanocarpus* (Fig. 4). The *PgiC* tree indicates that *Neobalanocarpus* is nested in White Meranti of *Shorea*, although the previous cpDNA analyses show that this genus forms a clade with *Hopea*. The phylogenetic placements of the *Neobalanocarpus* are supported well by bootstrap values in both phylogenetic trees of the cpDNA (BS > 99% in Kajita et al., 1998) and the *PgiC* (BS > 84% in this study). Overall, the *PgiC* gene tree shows better resolution than the cpDNA trees; our analysis clarifies the relationship of three timber groups as (Yellow Meranti—(Balau + Red Meranti)) where the cpDNA trees could not resolve the relationship. Another incongruence is identified among the *PgiC* and cpDNA-based topologies; the *PgiC* tree shows that White Meranti of *Shorea* is at the basal position of the remaining groups, but the cpDNA topology exhibits a monophyletic clade containing White Meranti of *Shorea*, *Neobalanocarpus* and *Hopea*; this clade is sister to the clade of *Parashorea* and the remaining groups of *Shorea*. Because the basal position of White Meranti of *Shorea* receives lower bootstrap values (<56%) in the *PgiC* tree, we suppose that this incongruence is not the ''hard incongruence'' of Johnson and Soltis (1998).

Incongruence of the placement of Neobalanocarpus between nuclear and chloroplast phylogenies—The phylogeny based on the *PgiC* sequences is incongruent with previous cpDNA phylogenies in terms of the placement of the *Neobalanocarpus* (Fig. 4). *Neobalanocarpus heimii* was originally described as a species of *Balanocarpus* and is confined to the Malay Peninsula (Symington, 1943; Ashton, 1982). This species produces timber of good quality and is locally known as ''chengal.'' On the basis of leaf morphology and wood anatomy, *Neobalanocarpus* seems to be rather close to *Hopea* (Symington, 1943). Yet, *Neobalanocarpus* has unique characters such as a linear anther in the flower, and sub-equal short woody fruit sepals as many species of *Hopea* and many groups of *Shorea*. These obscure both its generic affinity, which is based on the number of wing-like fruit sepals in those species that have them, and its sectional affinity, which is based on anther characters (Ashton, 1982). Therefore, it has been difficult to determine whether the genus is more closely related to *Hopea* or *Shorea*. An interesting feature of this species is irregular segregation of chromosomes during meiosis (Jong and Lethbridge, 1967). These authors hypothesized that the irregular meiotic behavior may imply a hybrid origin of this taxon, and the equivocal placement of *Neobalanocarpus* based on morphology supports this hypothesis. The hybridization hypothesis could explain the incongruence between cpDNA and *PgiC* trees. Our data indicates that this genus could be derived via hybridization between the ancestral lineage leading to White Meranti of *Shorea* and that leading to *Hopea*. Our data further implies the *Hopea* lineage as the maternal progenitor and *Shorea* White Meranti lineage as the paternal progenitor of *Neobalanocarpus*. In this scenario, the maternal haplotype of the nuclear *PgiC* seems to have been eliminated through random genetic drift or excesses of gene flow from the paternal lineage.

Gene duplication is also a potential cause of phylogenetic incongruence (Wendel and Doyle, 1998). Gene duplication at the *PgiC* locus is found in *Clarkia* (Gottlieb and Ford, 1996) and in *Arabidopsis halleri* subsp. *gemmifera* (Kawabe and Miyashita, 2002). Assuming that the cpDNA tree is identical to the true phylogeny, we must hypothesize that gene duplication


(9a) S.ferruginea 1 B
(9a) S.ferruginea 2 B
(9a) S.m.macropteriifolia B
(9a) S.m.bailonii 1
(9a) S.m.bailonii 2 A
(9a) S.m.bailonii 2 B
(9a) S.ferruginea 1 A
(9a) S.m.macroptera 1 B
(9a) S.m.macroptera 2 A
(9a) S.myrioneрva A
(9a) S.m.macroptera 1 A
(9a) S.m.macroptera 2 B
(9a) S.myrioneрva B
(9b) S.parvifolia 2 A
(9b) S.parvifolia 5 B
(9b) S.argentifolia 1
(9b) S.parvifolia 2 B
(9b) S.parvifolia 3
(9b) S.parvifolia 5 A
(7b) S.fallax 1 B
(7b) S.fallax 2 B
(7b) S.fallax 4 B
(9a) S.ferruginea 2 A
(9a) S.m.macropteriifolia A
(9b) S.acuminata 1 B
(9b) S.acuminata 3 A
(9b) S.acuminata 4
(9b) S.acuminata 3 B
(9b) S.ovata A
(9b) S.ovata B
(9b) S.parvifolia 1 A
(9b) S.parvifolia 1 B
(9a) S.slootenii A
(9a) S.slootenii B
(7b) S.fallax 4 A
(9b) S.singkawang A
(9b) S.singkawang B
(9b) S.leprosula 2 A
(9b) S.leprosula 2 B
(9b) S.leprosula 3
(9b) S.leprosula 4
(9b) S.leprosula 5 A
(9b) S.leprosula 5 B
(10) S.ovalis sericea 2 C
(9b) S.leprosula 1 A
(9b) S.leprodula 1 B
(10) S.ovalis sericea 1 B
(10) S.ovalis sericea 2 B
(7b) S.pauciflora 1 A
(7b) S.pauciflora 1 B
(6) S.rubella A
(6) S.rubella B
(7b) S.bullata 1 A
(7b) S.bullata 2 A
(7b) S.bullata 2 B
(7b) S.bullata 1 B
(7b) S.scaberrima 1
(7a) S.smithiana B
(7b) S.scaberrima 2 A
(7a) S.smithiana A
(10) S.ovalis sarawakensis
(10) S.ovalis sericea 1 C
(9b) S.acuminata 1 A
(9b) S.quadrinervis A
(9b) S.quadrinervis B
(7b) S.fallax 3 B
(9b) S.curtisii 2B
(9b) S.curtisii 3
(9b) S.curtisii 2 A
(9b) S.parvifolia 4
(9b) S.curtisii 1
(8) S.beccariana 2 B
(8) S.macrophylla A
(8) S.macrophylla B
(8) S.amplexicaulis A
(8) S.amplexicaulis B
(7b) S.almon A
(7b) S.fallax 2 A
(7b) S.scaberrima 2 B
(7b) S.fallax 1 A
(7b) S.fallax 3 A
(10) S.ovalis sericea 2 D
(8) S.pilosa A
(8) S.pilosa B
(8) S.beccariana 1 A
(8) S.beccariana 1 B
(8) S.beccariana 2 A
(7b) S.almon B
(7b) S.kunstleri A
(7b) S.pauciflora 2 A
(7b) S.pauciflora 2 B
(10) S.ovalis sericea 1 A
(10) S.ovalis sericea 2 A
(7b) S.kunstleri B
(1a) S.seminis A
(1a) S.seminis B
(1a) S.sumatrana B
(1a) S.atrinervosa A
(1a) S.materialis A
(1a) S.lumutensis 1
(1a) S.lumutensis 2
(1a) S.lumutensis 3
(1a) S.geniculata A
(1a) S.geniculata B
(1a) S.havilandii A
(1a) S.havilandii B
(1a) S.materialis B
(1a) S.sumatrana A
(1a) S.falciferoides A
(1a) S.falciferoides B
(1a) S.obtusa A
(1a) S.obtusa B
(1a) S.atrinervosa B
(1b) S.laevis A
(1b) S.laevis B
(3) S.isoptera A
(3) S.isoptera B
(1b) S.biawak A
(1b) S.biawak B
(1b) S.maxwelliana A
(1b) S.maxwelliana B
(4b) S.faguetiana A
(4b) S.xanthophylla 1
(4b) S.faguetiana B
(4b) S.xanthophylla 2
(4b) S.patoleensis A
(4b) S.patoleensis B
(4b) S.maxima
P.lucida A
P.lucida B
H.subalata C
H.subalata D
H.subalata B
H.nervosa
H.subalata A
H.dryobalanoides A
H.dryobalanoides B
H.helferi A
H.helferi B
H.bilitonensis
H.odorata A
H.odorata B
H.odorata C
(5) S.ochracea 1 B
(5) S.ochracea 2 B
(5) S.ochracea 1 A
(5) S.ochracea 2 A
(5) S.assamica A
(5) S.assamica B
(5) S.agami B
(5) S.agami A
(5) S.henryana A
(5) S.henryana B
(5) S.bracteolata
N.heimii 1
N.heimii 2 B
N.heimii 2 A
(5) S.roxburghii A
(5) S.roxburghii B
D.palembanicus A
D.palembanicus B
Clade VI
(Red Meranti of Shorea)
Clade V
(Balau of Shorea)
Clade IV
(Yellow Meranti of Shorea)
Clade III (Parashorea)
Clade II (Hopea)
Clade I (White Meranti of
Shorea + Neobalanocarpus)
Outgroup (Dipterocarpus)

occurred in the stem lineage of clades I to VI. If this is true, at least four independent gene losses at specific positions are necessary to explain the *PgiC* tree topology. The explanation does not seem parsimonious and thus occurrence of gene duplication appears unrealistic although further analyses using other nuclear loci are necessary to confirm it. We favor the hypothesis of the hybrid origin of *Neobalanocarpus*, because of cytological evidence for it (Jong and Lethbridge, 1967) and the confusion about its affinity to *Hopea* and *Shorea* (Symington, 1943; Ashton, 1982).

Intrageneric relationships within genus Shorea—The *PgiC* tree from representative *Shorea* species shows two distinct clades, White Meranti and a clade consisting of Yellow Meranti, Balau and Red Meranti. The paraphyly of the genus *Shorea* and the sister relationship of *Parashorea* to this second group of *Shorea* in the *PgiC* tree correspond to the previous phylogeny of Kajita et al. (1998). One striking difference between the phylogenetic analyses based on *PgiC* sequences and the previously studied cpDNA is the levels of resolution below the genus level. Whereas the cpDNA phylogenies based on PCR-RFLP (Tsumura et al., 1996) and sequences of the noncoding regions (Kamiya et al., 1998) could not resolve many of the relationships within *Shorea*, the *PgiC* tree could identify three distinct lineages that are concordant with the wood anatomical characters.

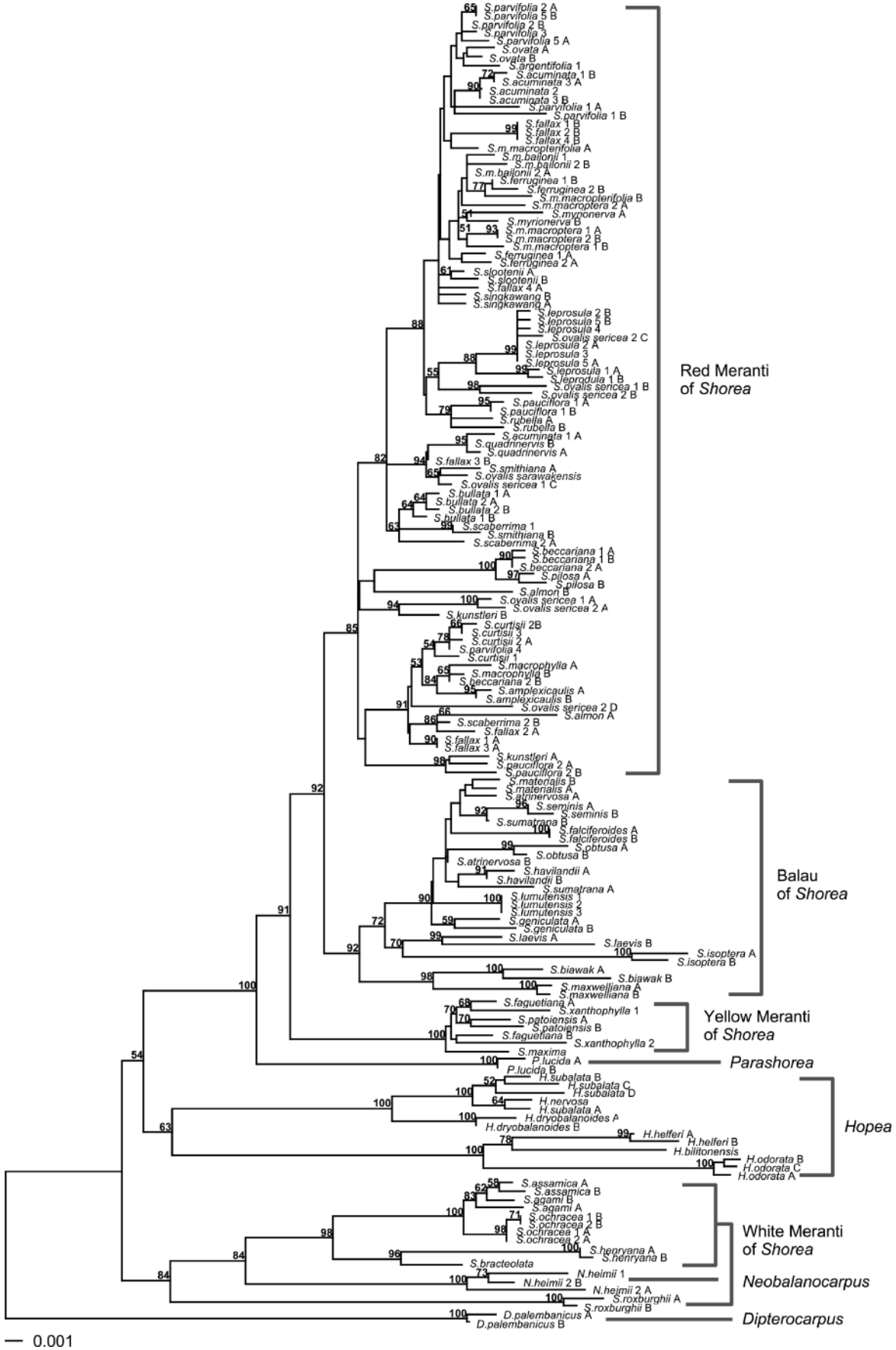
All sampled species of the Yellow Meranti (the botanical section *Richetioides*) form a strongly supported group (BS = 100%), and this group is sister to a clade of Balau (the botanical section *Shorea*) and Red Meranti (section *Rubroshorea* of Meijer) (BS > 70%). The Yellow Meranti group is distributed in the Malay Peninsula, Borneo, Sumatra, and the Philippines (Ashton, 1982). The flowers and bark of members of this group are uniform. The species with short subequal fruit sepals were formerly referred to *Balanocarpus*, but Symington (1938, 1943) first recognized the group as a natural botanical entity, the 'Richetia' group. Later, Ashton (1963) formalized Symington's group as section *Richetioides* Heim. The *PgiC* gene tree confirms that species such as *S. maxima*, *S. patoiensis*, and *S. xanthophylla*, all with wingless fruits, are not separated from the species having wings (*S. faguetiana*). The monophyly of this group was supported from the cpDNA analysis, and the interspecific divergences were fewer than those in other groups (Kamiya et al., 1998). Mean divergence among species in this group is the least compared with the other groups at the nuclear *PgiC* locus (0.0089 in Table 2). These results suggest that the species of Yellow Meranti have experienced recent adaptive radiation.

The Balau group, known to foresters in Borneo as Selangan Batu, produces rather hard and glistening textured timbers; it is widely distributed from India to Malesia, except for East of Wallace's Line (Symington, 1943; Ashton, 1982). This group was formerly divided into three subgroups, named Ciliata, Isoptera, and Barbata, based on the morphology of flower and fruiting calyx, although there are no reliable diagnostic characters for wood anatomy. Symington (1943) suggested that the two subgroups Ciliata and Isoptera, based on differences in the equality of the fruit calyx lobes, are not synapomorphic, and Ashton (1982) further confirmed this when newly described Bornean materials were included. Consequently, Ashton reduced these two groups within section *Shorea*. Furthermore, if new materials from Borneo are added, the Balau contains two sections, *Shorea* and *Neohopea* (Ashton, 1963, 1982). The section *Shorea* is further divided into two subsections *Shorea* and *Barbata*, and the latter corresponds to the Barbata subgroup of Symington (Ashton, 1982). In the *PgiC* tree as well as the previous cpDNA tree (Kamiya et al., 1998), the Balau timber group as a whole is monophyletic (BS > 76%). Within this group, the *PgiC* tree recognizes a distinct group, consisting of the species of section *Shorea* subsection *Shorea* (BS > 90%). The two species of Symington's Isoptera subgroup (*S. seminis* and *S. sumatrana*) are not of distinct lineage from the members of Ciliata group within subsection *Shorea*. The members of subsection *Barbata* form a well-supported group (BS = 98%), except for *S. laevis*, and this species clusters with section *Neohopea* (*S. isoptera*) in the NJ tree (BS = 70%). This suggests that section *Shorea* as well as subsection *Barbata* is not a monophyletic group.

The Red Meranti group, like the Yellow Meranti, is confined to the biogeographic region of western Malesia but with a single species also in the Moluccas; most species are found in Borneo (Symington, 1943). Although the members of Red Meranti are, with some exceptions, easily defined by reddish inner bark and wood in the field, they are botanically heterogeneous (Symington, 1943). Symington suggested, in his monograph on the basis of specimens from the Malay Peninsula, that this group could be divided into three distinct subgroups, Pauciflora, Ovalis, and Parvifolia. Ashton (1963, 1982) defined two new sections, *Pachycarpae* and *Rubella*, neither of which occur on the Malay Peninsula. Finally, Ashton (1982) proposed a total of five sections in Red Meranti based primarily on flower and bark characters: *Brachypterae* Heim referring to Symington's Pauciflora subgroup, *Ovalis* Ashton to his Ovalis subgroup and *Mutica* Brandis to his Parvifolia subgroup, and two new sections, *Pachycarpae* and *Rubella*, which include species confined to Borneo or the Philippines. The previous cpDNA study suggested that Red Meranti is not monophyletic, probably due to an insufficient number of informative molecular characters (Kamiya et al., 1998). In this study, the Red Meranti is a monophyletic group (BS > 72%), while neither Symington's (1943) three subgroups nor Ashton's (1982) five sections within this group are consistently recognized in the gene tree. Although most species in each of these sections cluster together in the subclades and the two subsections of the large section *Mutica* are mostly well defined, *S. ovalis* and several species in section *Brachypterae*,

←

Fig. 2. One of the 6376 most parsimonious trees based on the *PgiC* gene sequences for the species of *Shorea* and its closely related genera. Number above each branch is branch length; number below each branch is a support value (≥50%) resulting from 5000 replicates of ''fast'' bootstrap analysis. The branch labeled with an arrow collapses in the strict consensus tree. Numbers following species names represent accession numbers. Letters following the accession numbers refer to different haplotypes of the same accession. Six main clades corresponding to taxonomic circumscriptions are shown by gray brackets. Sections and subsections of *Shorea* abbreviated according to Ashton (1982): (1a) sect. *Shorea* subsect. *Shorea*, (1b) sect. *Shorea* subsect. *Barbata*, (3) sect. *Neohopea*, (4b) sect. *Richetioides* subsect. *Richetioides*, (5) sect. *Anthoshorea*, (6) sect. *Rubella*, (7a) sect. *Brachypterae* subsect. *Smithiana*, (7b) sect. *Brachypterae* subsect. *Brachypterae*, (8) sect. *Pachycarpae*, (9a) sect. *Mutica* subsect. *Auriculatae*, (9b) sect. *Mutica* subsect. *Mutica*, (10) sect. *Ovalis*.


S.parvifolia 2 A
S.parvifolia 5 B
S.parvifolia 2 B
S.parvifolia 3
S.parvifolia 5 A
S.ovata A
S.ovata B
S.argentifolia 1
S.acuminata 1 B
S.acuminata 3 A
S.acuminata 2
S.acuminata 3 B
S.parvifolia 1 A
S.parvifolia 1 B
S.fallax 1 B
S.fallax 2 B
S.fallax 4 B
S.m.macropterifolia A
S.m.ballonii 1
S.m.ballonii 2 B
S.m.ballonii 2 A
S.ferruginea 1 B
S.ferruginea 2 B
S.m.macropterifolia B
S.m.macroptera 2 A
S.myrionerva A
S.myrionerva B
S.m.macroptera 1 A
S.m.macroptera 2 B
S.m.macroptera 1 B
S.ferruginea 1 A
S.ferruginea 2 A
S.slootenii A
S.slootenii B
S.fallax 4 A
S.singkawang B
S.singkawang A
S.leprosula 2 B
S.leprosula 5 B
S.leprosula 4
S.ovalis sericea 2 C
S.leprosula 2 A
S.leprosula 3
S.leprosula 5 A
S.leprosula 1 A
S.leprodula 1 B
S.ovalis sericea 1 B
S.ovalis sericea 2 B
S.pauciflora 1 A
S.pauciflora 1 B
S.rubella A
S.rubella B
S.acuminata 1 A
S.quadrinervis B
S.quadrinervis A
S.fallax 3 B
S.smithiana A
S.ovalis sarawakensis
S.ovalis sericea 1 C
S.bullata 1 A
S.bullata 2 A
S.bullata 2 B
S.bullata 1 B
S.scaberrima 1
S.smithiana B
S.scaberrima 2 A
S.beccariana 1 A
S.beccariana 1 B
S.beccariana 2 A
S.pilosa A
S.pilosa B
S.almon B
S.ovalis sericea 1 A
S.ovalis sericea 2 A
S.kunstleri B
S.curtisii 2B
S.curtisii 3
S.curtisii 2 A
S.parvifolia 4
S.curtisii 1
S.macrophylla A
S.macrophylla B
S.beccariana 2 B
S.amplexicaulis A
S.amplexicaulis B
S.ovalis sericea 2 D
S.almon A
S.scaberrima 2 B
S.fallax 2 A
S.fallax 1 A
S.fallax 3 A
S.kunstleri A
S.pauciflora 2 A
S.pauciflora 2 B
S.materialis B
S.materialis A
S.atrinervosa A
S.seminis A
S.seminis B
S.sumatrana B
S.falciferoides A
S.falciferoides B
S.obtusa A
S.obtusa B
S.atrinervosa B
S.havilandii A
S.havilandii B
S.sumatrana A
S.lumutensis 1
S.lumutensis 2
S.lumutensis 3
S.geniculata A
S.geniculata B
S.laevis A
S.laevis B
S.isoptera A
S.isoptera B
S.biawak A
S.biawak B
S.maxwelliana A
S.maxwelliana B
S.faguetiana A
S.xanthophylla 1
S.patoiensis A
S.patoiensis B
S.faguetiana B
S.xanthophylla 2
S.maxima
P.lucida A
P.lucida B
H.subalata B
H.subalata C
H.subalata D
H.nervosa
H.subalata A
H.dryobalanoides A
H.dryobalanoides B
H.helferi A
H.helferi B
H.bilitonensis
H.odorata B
H.odorata C
H.odorata A
S.assamica A
S.assamica B
S.agami B
S.agami A
S.ochracea 1 B
S.ochracea 2 B
S.ochracea 1 A
S.ochracea 2 A
S.henryana A
S.henryana B
S.bracteolata
N.heimii 1
N.heimii 2 B
N.heimii 2 A
S.roxburghii A
S.roxburghii B
D.palembanicus A
D.palembanicus B
Red Meranti of Shorea
Balau of Shorea
Yellow Meranti of Shorea
Parashorea
Hopea
White Meranti of Shorea
Neobalanocarpus
Dipterocarpus
0.001

notably, are widely scattered within the Red Meranti clade. This must imply either that Ashton's sections are not monophyletic, or that they have arisen too recently to be resolved by *PgiC* as is suggested by the low level of interspecific variation that we found among some species (discussed later).

Maury (1978, 1979; summarized in Maury-Lechon and Curtet, 1998) was the first to reassess phylogenetic relationships within Ashton's *Shoreae*. She divided Ashton's tribe *Shoreae* into tribes *Hopeae* and *Shoreae*, with *Shoreae* further divided into three subtribes: *Anthoshorinae* (to include *Anthoshorea*, *Doona*, and *Pentacme*), *Shorinae* (*Shorea*, *Richetia*, and *Rubroshorea*), and *Parashorinae* (the genus *Parashorea*, Fig. 1). She therefore concluded that Ashton's 11 sections of *Shorea* have unequal hierarchic levels and that sections such as *Anthoshorea*, *Shorea*, and *Richetioides* (also *Doona* and *Pentacme*), should be raised to generic rank. When we compare our phylogenetic study with current arguments of classification, the results of the topology of *PgiC* gene tree and the level of sequence divergences among the clades support the argument of Maury. Our results support Maury's three genera in *Shoreae*: *Richetia* (clade IV), *Shorea* (clade V), and *Rubroshorea* (clade VI). Moreover, *Parashorea* has an equal level of divergence as those groups of *Shorea* when we consider levels of sequence divergence among the clades (Table 2).

These results urge us to reconsider the taxonomic ranks of *Shorea* in relation to closely related genera such as *Parashorea*. Until now, however, the morphological and anatomical characters by which Maury recognized her supraspecific taxa within *Shorea* and *Parashorea* have been examined in only 20–30% (according to the character) of the c. 200 species in the genus *Shorea*, though these taxa were representative of all of Ashton's taxa. Furthermore, the species examined by Maury were consistently assignable to her proposed genera solely on the position of the hypocotyl and, in the case of some, their stomatal anatomy. Most challenging, no group of characters have yet been identified that consistently identifies species of *Rubroshorea* from other taxa of proposed equivalent rank. It would be imprudent to assign new generic names to a majority of species in such an economically and ecologically important genus as *Shorea*, whose species are so easily recognized in the field, until most species have been examined for the characters proposed for its division and more readily observable characters found.

Inter- and intraspecific variation within the Red Meranti of Shorea—We can also discuss polymorphism and divergence across closely related species in Red Meranti because we collected multiple samples from some of the species. It is noteworthy that some species have large intraspecific variation relative to interspecific divergence (Tables 1 and 2). Accordingly, for such species, no monophyletic clustering of intraspecific haplotypes is revealed (Figs. 2 and 3). This is due to an extensive number of variable sites that are shared among species (shared polymorphisms). Within Red Meranti, the observed 241 variable sites can be divided into three categories: (1) one or more species have a derived nucleotide, which is fixed in the species, but others have the ancestral nucleotide (fixed difference), (2) one species is polymorphic at the site, but others have the ancestral nucleotide (polymorphism exclusive to one species), and (3) more than one species is polymorphic at the site (shared polymorphism). When we classify observed variable sites into these categories, the number of sites in categories 1, 2, and 3 are 6, 160, and 75, respectively. Note that the number of shared polymorphisms may increase as the sample size increases, because the sites currently categorized as fixed may become polymorphic if the sample size becomes larger. Shared polymorphism can be explained by the persistence of polymorphism from the ancestral population, gene flow via interspecific hybridization, or reverse/parallel mutations (Machado et al., 2002). Because the species of Red Meranti have low sequence divergences among species (Table 2), reverse or parallel mutations may explain only a small fraction of shared polymorphisms between species. Consequently, our concern is whether the shared polymorphisms are better explained by persistence of polymorphisms from the common ancestor or by gene flow via interspecific hybridization.

Polymorphisms from a common ancestor persist even at neutral loci if the number of generations after the speciation is insufficient for the ancestral polymorphic alleles to be fixed (Clark, 1997). Because fixations of neutral alleles depend on the number of generations since speciation and effective populations size, the longer generation time of tree species ($>$50 yr) certainly will extend their persistence in terms of absolute time. Although the time when the most recent common ancestor of Red Meranti existed is not known, the low levels of divergences in the nuclear *PgiC* and cpDNA (Kamiya et al., 1998) suggest relatively recent diversification of this group. Morley (2000) has shown that the extensive lowlands under an aseasonal wet climate to which the mixed dipterocarp forests of western Malesia, and the Yellow and Red Meranti groups of *Shorea* are confined, has its origin in the early Miocene c. 20 mya. This can therefore be regarded as the earliest date for the origin of these groups. A reasonable average age to flowering of *Shorea* in primary forests would be 50 years, predicting 400 000 generations in 20 million years. Therefore, retention of ancestral polymorphisms currently seems to be a likely explanation for the shared polymorphisms among several recent diverged species. Alternatively, balancing selection may have been promoting maintenance of polymorphism and thus has increased coalescence time, resulting in slower lineage sorting (Broughton and Harrison, 2003). It is difficult to discriminate these two possibilities—recent speciation or balancing selection—solely with the present data. However, because demographic factors affect all loci similarly while natural selection acts on specific loci, a comparative DNA approach surveying more loci will enable us to discriminate these two possibilities.

Gene flow via interspecific hybridization is another potential source of generating shared polymorphisms. Many tropical forest trees are thought to be highly outcrossed, but low hybrid fitness and rarity of fertile interspecific hybrid populations has been suggested in Dipterocarpaceae of the aseasonal mixed dipterocarp forest zone (Ashton, 1969; Murawski et al., 1994).

←

Fig. 3. Neighbor-joining tree based on the *PgiC* gene sequences for the species of *Shorea* and its closely related genera. Numbers above branches are support values ($\geq$50%) resulting from 1000 replicates of bootstrap analysis. Gray brackets indicate taxonomical circumscriptions. See Fig. 2 for the explanation of taxon labeling.

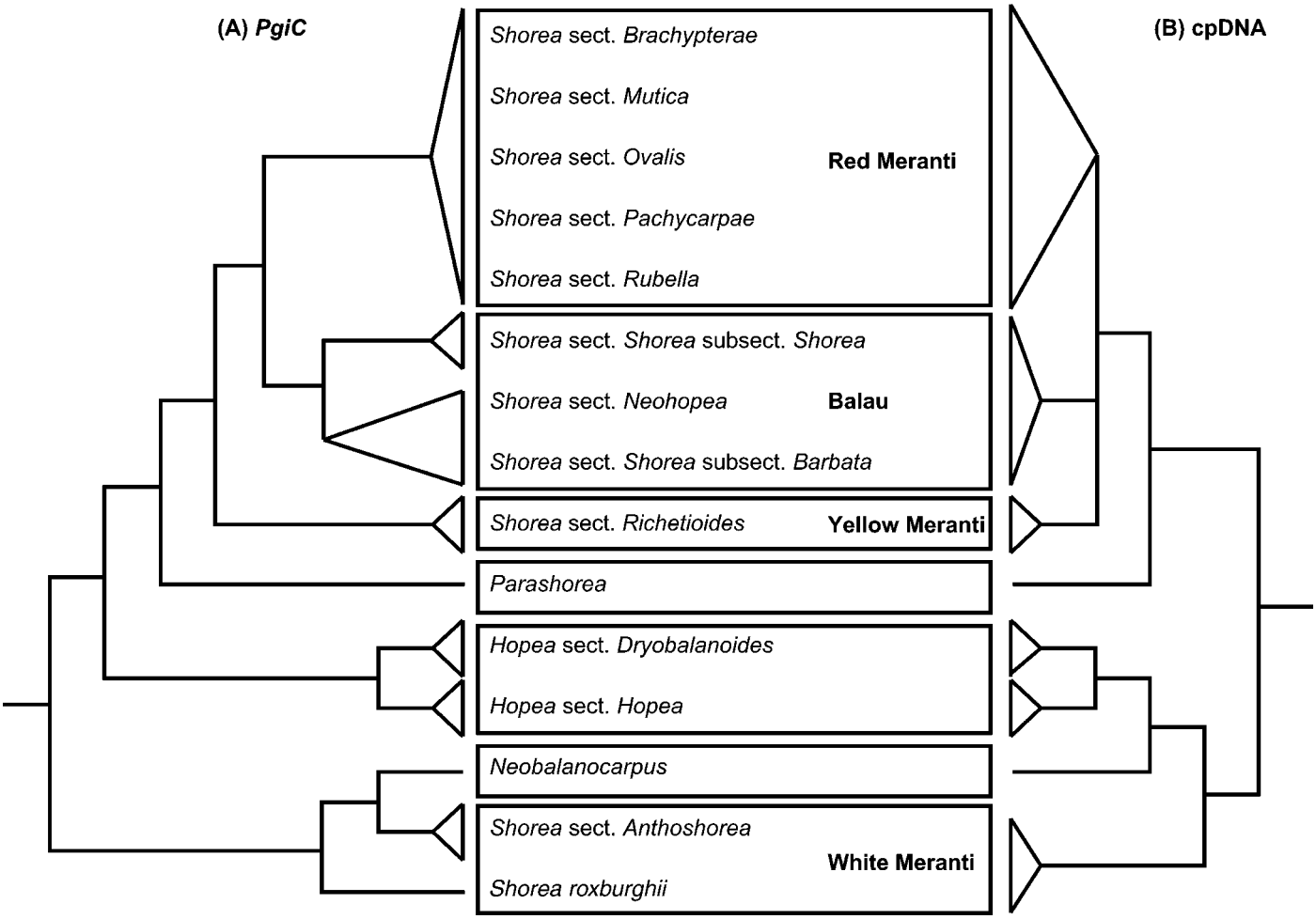


Fig. 4. Simplified summary topology representing the relationships among major clades of *Shorea* and closely related genera based on the nuclear *PgiC* (A) and cpDNA (B). The consensus topology of cpDNA is constructed from previous studies (Tsumura et al., 1996; Kajita et al., 1998; Kamiya et al., 1998; Dayanandan et al., 1999).

However, a few examples of hybridization have been reported among dipterocarps of seasonal Indo-Burma (Ashton, 1982; Murawski et al., 1994; Bawa, 1998). The interspecific hybrids between *S. curtisii* and *S. leprosula* have been reported from several localities in the Malay Peninsula and from Singapore (Ashton, 1982). A recent study of sequence variation at the nuclear *GapC* locus of four *Shorea* species (*S. acuminata*, *S. curtisii*, *S. leprosula*, and *S. parvifolia*) found a part of a sequence in one species resembling that in another species (chimeric haplotypes), probably resulting from recombination between two divergent haplotypes (Ishiyama et al., 2003). From this result, coupled with successful intercrossing between closely related species of *Shorea* (Chan, 1981), Ishiyama and colleagues have suggested that introgressive hybridization is likely to have occurred in natural populations of these *Shorea* species. Such a chimeric sequence is not found at the *PgiC* locus, and no identical haplotypes are found between different species. Although we did not collect enough population samples, the fact that some haplotypes from one species are unexpectedly clustered with haplotypes found in other species suggests the possibility of introgressive hybridization occurring currently and/or in the past. With continuing interspecific gene flows, we would expect identical haplotypes from two different co-occurring species. However, the lack of identical haplotypes from co-occurring species suggests at least that interspecific hybrids are not abundant at the present time, even though it could have occurred to some extent in the past.

In Quaternary glacial periods, most of the regions in Southeast Asia have been covered with savanna and deciduous forests. Consequently, tropical rain forests were confined to a few refugia in northern Borneo, northern Sumatra, and the Mentawai islands (Gathorne-Hardy et al., 2002). This indicates that the tropical rain forests expanded their ranges after the last glacial period. Paleogeographical evidence also suggested that during the glacial periods coinciding with the low sea level, Borneo was connected to Southeast Asian mainland, Java, and Sumatra (Morley, 2000). The forest fragmentation and recolonization caused by such historical climate fluctuations may have influenced the patterns and levels of intraspecific variation, species differentiation, and interspecific hybridization that we observed in *Shorea*.

At this time, we cannot say whether ancestral polymorphism, balancing selection, or introgressive hybridization is the more important to explain the shared polymorphisms among the *Shorea* species. Currently, we do not have enough data to accept either one of those hypotheses. Although shared

polymorphisms lead to ambiguous results in phylogenetic analyses, studies of polymorphisms and divergence between closely related species are important to understand mechanisms of how a great variety of species evolved and now coexist in the tropical ecosystem.

Conclusion—The study presented here assessed phylogenetic relationships among species of *Shorea*, *Hopea*, *Neobalanocarpus*, and *Parashorea* within Dipterocarpaceae, a dominant tree family in Asian tropical rainforests, based on the partial sequences of the nuclear *PgiC* gene. The *PgiC* gene tree is essentially compatible with the previous cpDNA trees, with the exception of the placement of *Neobalanocarpus*. This conflict suggests that *Neobalanocarpus* is derived via hybridization between *Shorea* and *Hopea* (*Hopea* is presumed to be the female parent considering the maternal inheritance of cpDNA usual in angiosperms.).

The nuclear *PgiC* gene sequences have more potentially informative sites than cpDNA as expected and provide a better-resolved phylogenetic tree. Our results with respect to *Shorea* support the phylogeny and proposed classification of Maury (1978). Three timber groups within *Shorea* are shown to constitute monophyletic groups, and the phylogenetic relationship among those groups is clearly elucidated from the *PgiC* gene tree. However, the *PgiC* tree does not yield a consistent grouping of the five sections of Ashton within Red Meranti. Our result suggests that the Ashton's botanical sections such as *Anthoshorea*, *Richetioides*, and *Shorea*, each of which is recognized as a monophyletic group in our analysis, have higher ranks than the other sections of Red Meranti, which are less distinct from each other.

Extensive numbers of shared polymorphisms are found among species within Red Meranti, and this results in lack of monophyly of intraspecific haplotypes. Ancestral polymorphisms, natural selection, and introgressive hybridization could be considered as potential causes of shared polymorphism among these species. Multilocus approaches of polymorphisms and divergences across the closely related taxa are needed to infer accurately phylogenetic relationships and to reveal historical, demographic, and selective factors that have contributed to a high rate of speciation and a great variety of dipterocarp species. In addition, more extensive sampling of individuals and populations are required to assess these aspects.

For the present, though, most species must be examined, and more readily observable key characters found, before it would be wise to divide the well known and easily recognized groups of genus *Shorea* into several separately named entities.

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