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Comprehensive Molecular Phylogeny of the Sub-Family Dipterocarpoideae (Dipterocarpaceae) Based on Chloroplast DNA Sequences

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Dipterocarpoideae, the largest sub-family of well-known plant family Dipterocarpaceae, dominates in South Asian rain forests. Although several previous studies addressed the phylogeny of the Dipterocarpaceae family, relationships among many of its genera from the Dipterocarpoideae sub-family are still not well understood. In particular, little is known about the relationships of the genera *Vateriopsis*, *Stemonoporus*, *Vateria* and inconsistence remains between phylogenetic results and taxonomic classifications of *Shorea* and *Hopea* species. We studied molecular phylogeny of the sub-family Dipterocarpoideae using the *trnL-trnF* spacer, *trnL* intron and the *matK* gene sequences of chloroplast DNA (cpDNA). This study is the first comprehensive phylogeny reconstruction for the sub-family Dipterocarpoideae based on cpDNA, as it includes most genera (14) and a large number of species (79) with most species endemic to Sri Lanka, as well as one species from Seychelles and one species from the genus *Monotes* from Madagascar. Phylogenetic trees were constructed using the Neighbor Joining (NJ) and Maximum Likelihood (ML) methods using combined set of sequences including all three cpDNA regions. The topologies of the NJ and ML trees were to a certain extent, consistent with the current taxonomy of Dipterocarpoideae based on morphology and with previous molecular phylogenies based on cpDNA. Furthermore, our results provided new evidence regarding the relationships of the following genera: *Vateriopsis* and *Stemonoporus* and about the validity of the previous morphology based classifications of *Shorea* species. In addition, the topology of our trees was consistent with the classification of *Shorea* species proposed by Maury (1978), Maury-Lechon (1979) and Symington (1943). Finally, our results provided evidence for the affinity of the genus *Monotes* to Asian Dipterocarpoideae rather than to Tiliaceae and indicated that it is a good candidate for outgroup species for future studies of the former sub-family.

Key words: chloroplast DNA, Dipterocarpoideae, *Doona*, molecular phylogeny, *Monotes*

INTRODUCTION

Dipterocarpaceae is a well-known plant family with approximately 580–680 species (Ashton, 1977; Ashton, 1982; Maury-Lechon and Curtet, 1998). Many members of this family are large forest emergent trees, typically reaching heights of 40–70 m. Their distribution is pan tropical, from northern South America to Africa, Seychelles, Sri Lanka, Philippines, India, China, Thailand, Indonesia and Malaysia with the greatest diversity and abundance in western Malaysia.

The Dipterocarpaceae family is divided into three sub-families: *(i)* Monotoideae, with three genera and about 30 species, distributed across Africa, Madagascar and South America, *(ii)* Pakaraimoideae with a single species

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Pakaraimaea roraimae found in the Guaianan highlands of South America and *(iii)* Dipterocarpoideae, the largest of the sub-families, with 13 genera and about 470 species (Ashton, 1982), which distribute mainly in South Asian countries such as India, Sri Lanka, Philippines, China, New Guinea, Indonesia, Thailand and Malaysia with the exception of *Vateriopsis seychellarum*, which is endemic to Seychelles.

The phylogenetic position of the genus *Monotes*, which is often placed in the sub-family Monotoideae (e.g., Maury, 1978) is still unclear. Initially, it was associated with the family Tiliaceae (Heim, 1892). Later however, it was moved to the sub-family Monotoideae of the family Dipterocarpaceae (Gilg, 1925). On the other hand, based on morphology, Maury (1978) and Kostermans (1989) treated Monotoideae as a separate family.

The sub-family Dipterocarpoideae can be further divided into two tribes: Dipterocarpeae and Shoreae (Brandis, 1895). The genera of the first tribe (*Anisoptera*, *Cotylelobium*, *Dipterocarpus*, *Stemonoporus*, *Upuna*, *Vateria*, *Vateriopsis* and *Vatica*) have valvate sepals in fruits, solitary vessels, scattered resin canals and the basic chromosome number n = 11. The genera of the second tribe (*Dryobalanops*, *Neobalanocarpus, Hopea, Parashorea* and *Shorea*) have imbricate sepals in fruits, grouped vessels, resin canals in tangential bands and basic chromosome number n = 7 (Ashton, 1982; Brandis, 1895; Jong, and Kaur, 1979). However, there is still much controversy regarding the number of genera of the Dipterocarpoideae sub-family, especially in the Shoreae tribe, which varies depending on the author between nine and 19 (Ashton, 1977; Ashton, 1982; Kostermans, 1978; Kostermans, 1982; Kostermans, 1984; Kostermans, 1992; Maury, 1978; Maury-Lechon, 1979; Meijer, and Wood, 1964; Meijer, and Wood, 1976). Perhaps the most controversial is classification of the genus *Shorea*. Based on embryo and leaf epidermal characters Maury (1978) divided this genus *Shorea* into the following separate genera: *Shorea*, *Anthoshorea*, *Rubroshorea*, *Richetia*, *Doona*, and *Pentacme*. On the other hand, Ashton (1977), Ashton (1980) and Ashton (1982) included them in a single genus *Shorea*, which was further divided into 11 sections: *Shorea*, *Pentacme*, *Neohopea*, *Richetioides*, *Anthoshorea*, *Rubella*, *Brachypterae*, *Pachycarpae*, *Mutica*, *Ovalis* and *Doona.* Yet another classification was proposed by Symington (1943) who divided the genus *Shorea* into three separate genera: *Shorea*, *Pentacme* and *Parashorea*. Further, based on wood anatomy he divided it into the four following wood groups: Balau, Red Meranti, White Meranti and Yellow Meranti.

The phylogenetic relationships of Dipterocarpaceae have been studied using distribution, fossil and morphological data by Ashton (1982) and the first phylogeny based on molecular data was reported by Tsumura et al. (1996). Since then, several other phylogenetic studies on Dipterocarpaceae were reported based on chloroplast (cp) DNA sequences (Dayanandan et al., 1999; Gamage et al., 2003; Kajita et al., 1998; Kamiya et al., 1998; Morton et al., 1999) and the nuclear gene *PgiC* (Kamiya et al., 2005). However, previous studies on molecular phylogeny of the Dipterocarpaceae included either limited number of species (Kajita et al., 1998; Morton et al., 1999; Tsumura et al., 1996) or informative sites (Gamage et al., 2003; Kamiya et al., 1998) or both (Dayanandan et al., 1999). The most recent work by Kamiya et al. (2005) has mainly focused on the relationships of *Shorea*, *Hopea, Neobalanocarpus* and *Parashorea* genera and did not include species from the Dipterocarpeae tribe and species of the *Doona* genus (Kostermans, 1984; Kostermans, 1992; Maury, 1978; Maury-Lechon, 1979). As a result, phylogenetic placement of many species and genera, which belong to sub-family Dipterocarpoideae is still unclear. In particular, little is known about the relationships of the following genera: *Vateriopsis*, *Stemonoporus* and *Vateria* and species from the *Doona* genus created by Kostermans (1984), Kostermans (1992) and Maury (1978).

It is therefore necessary to examine a larger number of species representing all genera and distribution areas. The main objective of the present work was to provide comprehensive assessment of phylogenetic relationships among Dipterocarpoideae species from Southeastern Asia. Another objective of our study was to ascertain the placement of the genus *Vateriopsis* (endemic to Seychelles) and classification of many endemic Sri Lankan species from the tribes Dipterocarpeae and Shoreae. In addition, our aim was to investigate the familial affinity of the genus *Monotes*. Finally, we wanted to determine, which of the two species from outside the Dipterocarpoideae sub-family included in our study (*Tilia kuisiana* or *Monotes madagascariensis*) is a better candidate for outgroup species for future studies of Dipterocarpoideae phylogeny.

Among the 79 Dipterocarpaceae species included in our present study, 42 species were from Malaysia, 34 species were from Sri Lanka, one species was from Thailand, one species was from Seychelles and one species was from Madagascar. The species used here represent 14 genera of the family Dipterocarpaceae and thus provide the first comprehensive material for phylogeny reconstruction. To address the issues of outgroup choice and the family placement of the genus *Monotes*, we have also included one species from the Tiliaceae family: *Tilia kiusiana*. The chloroplast DNA (cpDNA) used in the present study included the following three regions: *trnL-trnF* spacer, *trnL* intron and the partial region of the *matK* gene, which encodes a splicing-associated maturase (Neuhaus, and Link, 1987).

MATERIALS AND METHODS

Species sampling The total number of Dipterocarpaceae species included in this study was 79. This includes 42 species from Malaysia, 34 species from Sri Lanka and a single species from each of the following regions: Seychelles, Madagascar and Thailand (Table 1). In addition to sequences obtained in the present study (24 sequences for *trnL-trnF* spacer and *trnL* intron regions and 65 sequences for *matK*), we used data reported in the previous studies (Table 1). For the *trnL-trnF* spacer and *trnL* intron regions we used seven sequences obtained by Kamiya et al. (1998), 34 sequences obtained by Gamage et al. (2003) and 14 sequences reported by Kajita et al. (1998). For the *matK* region, we used 14 sequences from Malaysian species reported by Kajita et al. (1998). The

Table 1. List of species used in this study and the database accession numbers of the DNA sequences

Species	Source	Database Accession No.			Reference
		trnL-trnF	*trnL*	*matK*	
Family: Dipterocarpaceae					
Sub family: Dipterocarpoideae					
Tribe: Shoreae					
Dryobalanops aromatica C. F. Gaertn	Frim, Kepong, Malayasia	AB006411	AB006394	AB006377	Kajita et al. (1998)
Dryobalanaops oblongofolia Dyer	Frim, Kepong, Malayasia	AB006412	AB006395	AB006378	Kajita et al. (1998)
Hopea nervosa King	Frim, Kepong, Malayasia	AB006418	AB006401	AB006384	Kajita et al. (1998)
Hopea jucunda Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246524	AB246589	AB246459	Present study, Gamage et al. (2003)
Hopea discolor Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246523	AB246588	AB246458	Present study, Gamage et al. (2003)
Hopea jucunda ssp. modesta DC.	Kanneliya Forest Reserve, Sri Lanka	AB246525	AB246590	AB246460	Present study, Gamage et al. (2003)
Hopea subalata Sym.	Frim Arboretum, Malayasia	AB246520	AB246585	AB246455	Present study, Gamage et al. (2003)
Hopea wightiana Wall.	Frim Arboretum, Malayasia	AB246526	AB246591	AB246461	Present study, Gamage et al. (2003)
Hopea odorata Roxb.	Frim, Kepong, Malayasia	AB006419	AB006402	AB006385	Kajita et al. (1998)
Hopea latifolia Sym.	Frim Arboretum, Malayasia	AB246521	AB246586	AB246456	Present study
Hopea helferi (Dyer) Brandis	Frim Arboretum, Malayasia	AB246522	AB246587	AB246457	Present study
Neobalanocarpus heimii (King) Ashton	Frim, Kepong, Malayasia	AB006417	AB006400	AB006383	Kajita et al. (1998)
Parashorea lucida (Miq.) Kurz	Frim, Kepong, Malayasia	AB006416	AB006399	AB006382	Kajita et al. (1998)
Shorea seminis (de Vriese) Slooten	Frim Arboretum, Malayasia	AB246515	AB246580	AB246450	Present study
Shorea elliptica Burck	Engkabang, Semengoh, Malayasia	AB246509	AB246574	AB246444	Present study
Shorea splendens Ashton	Engkabang, Semengoh, Malayasia	AB246508	AB246573	AB246443	Present study
Shorea pinanga Scheff.	Engkabang, Semengoh, Malayasia	AB246510	AB246575	AB246445	Present study
Shorea acuminata Dyer	Mersing Johor, Malayasia	AB246505	AB246570	AB246440	Present study
Shorea leprosula Miq.	Seremban, Negeri Sembilan, Malayasia	AB246504	AB246569	AB246439	Present study
Shorea xanthophylla Sym.	Frim Arboretum, Malayasia	AB246517	AB246582	AB246452	Present Study, Kamiya et al. (1998)
Shorea bullata Ashton	Engkabang, Semengoh, Malayasia	AB246500	AB246565	AB246435	Present Study, Kamiya et al. (1998)
Shorea curtisii Dyer ex King	Mersing Johor, Malayasia	AB246498	AB246563	AB246433	Present Study, Kamiya et al. (1998)
Shorea macroptera Dyer	Frim, Kepong, Malayasia	AB246503	AB246568	AB246438	Present study
Shorea parvifolia Dyer	Seremban, Negeri Sembilan, Malayasia	AB246502	AB246567	AB246437	Present study, Gamage et al. (2003)
Shorea quadrinervis Sloot.	Kubah National Park, Malayasia	AB246501	AB246566	AB246436	Present study, Kamiya et al. (1998)
Sorea bracteolata Dyer	Frim, Kepong, Malayasia	AB006415	AB006398	AB006381	Kajita et al. (1998)
Shorea ovalis (Korth.)	Frim, Kepong, Malayasia	AB006414	AB006397	AB006380	Kajita et al. (1998)
Shorea macrophylla (de Vriese) Ashton	Frim Arboretum, Malayasia	AB246506	AB246571	AB246441	Present study, Kamiya et al. (1998)
Shorea fallax Meijer	Kubah National Park, Malayasia	AB246499	AB246564	AB246434	Present study, Kamiya et al. (1998)
Shorea richetia Sym.	Kubah National Park, Malayasia	AB246507	AB246572	AB246442	Present study, Gamage et al. (2003)
Shorea laevis Ridl.	Frim Arboretum, Malayasia	AB246514	AB246579	AB246449	Present study
Shorea multiflora (Burck) Sym.	Semengoh Arboretum, Malayasia	AB246516	AB246581	AB246451	Present study
Shorea assamica Dyer	Frim Arboretum, Malayasia	AB246518	AB246583	AB246453	Present study, Gamage et al. (2003)
Shorea congestiflora Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246528	AB246593	AB246463	Present study, Gamage et al. (2003)
Shorea worthingtonii Ashton	Kanneliya Forest Reserve, Sri Lanka	AB246534	AB246599	AB246469	Present study
Shorea pallescens Ashton	Kanneliya Forest Reserve, Sri Lanka	AB246513	AB246578	AB246448	Present study, Gamage et al. (2003)

Table 1. Continued

Species	Source	Database Accession No.			Reference
		trnL-trnF	*trnL*	*matK*	
Shorea disticha (Thw.) Ashton	Gilimale Forest, Sri Lanka	AB246530	AB246595	AB246465	Present study
Shorea megistophylla Ashton	Royal Botanical Garden, Sri Lanka	AB246529	AB246594	AB246464	Present study, Gamage et al. (2003)
Shorea zeylanica (Thw.) Ashton	Royal Botanical Garden, Sri Lanka	AB246535	AB246600	AB246470	Present study, Gamage et al. (2003)
Shorea trapezifolia (Thw.) Ashton	Kanneliya Forest Reserve, Sri Lanka	AB246531	AB246596	AB246466	Present study, Gamage et al. (2003)
Shorea affinis (Thw.) Ashton	Kottawa Arboretum, Sri Lanka	AB246536	AB246601	AB246471	Present study, Gamage et al. (2003)
Shorea cordifolia (Thw.) Ashton	Kanneliya Forest Reserve, Sri Lanka	AB246527	AB246592	AB246462	Present study, Gamage et al. (2003)
Shorea stipularis Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246519	AB246584	AB246454	Present study, Gamage et al. (2003)
Shorea lissophylla Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246512	AB246577	AB246447	Present study
Shorea ovalifolia (Thw.) Ashton	Gilimale Forest, Sri Lanka	AB246532	AB246597	AB246467	Present study
Shorea gardneri (Thw.) Ashton	Bambarabotuwa Forest Reserve, Sri Lanka	AB246533	AB246598	AB246468	Present study
Shorea dyeri Thw.	Bambarabotuwa Forest Reserve, Sri Lanka	AB246511	AB246576	AB246446	Present study
Tribe: Dipterocarpeae					
Anisoptera oblonga Dyer	Frim, Kepong, Malayasia	AB006405	AB006388	AB006371	Kajita et al. (1998)
Anisoptera laevis Dyer	Frim, Kepong, Malayasia	AB006404	AB006387	AB006370	Kajita et al. (1998)
Cotylelobium malayanum V. Sl.	Frim Arboretum, Malayasia	AB246479	AB246544	AB246414	Present study
Cotylelobium scabriusculum (Thw.) Brandis	Kottawa Forest, Sri Lanka	AB246480	AB246545	AB246415	Present study, Gamage et al. (2003)
Dipterocarpus alatus A. DC.	Samui Island, Thailand	AB246538	AB246603	AB246473	Present study
Dipterocarpus cornutus Dyer	Frim Arboretum, Malayasia	AB246537	AB246602	AB246472	Present study
Dipterocarpus baudii Korth	Frim, Kepong, Malayasia	AB006410	AB006393	AB006376	Kajita et al. (1998)
Dipterocarpus kerrii King	Frim, Kepong, Malayasia	AB006409	AB006392	AB006375	Kajita et al. (1998)
Dipterocarpus glandulosus Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246542	AB246607	AB246477	Present study, Gamage et al. (2003)
Dipterocarpus hispidus Thw.	Wilpita Forest, Sri Lanka	AB246541	AB246606	AB246476	Present study, Gamage et al. (2003)
Dipterocarpus zeylanicus Thw.	Diyadawa Forest, Sri Lanka	AB246539	AB246604	AB246474	Present study, Gamage et al. (2003)
Dipterocarpus insignis Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246540	AB246605	AB246475	Present study, Gamage et al. (2003)
Stemonoporus acuminatus (Thw.) Beddome	Kanneliya Forest Reserve, Sri Lanka	AB246487	AB246552	AB246422	Present study, Gamage et al. (2003)
Stemonoporus lancifolius (Thw.) Ashton	Kanneliya Forest Reserve, Sri Lanka	AB246495	AB246560	AB246430	Present study
Stemonoporus kanneliyensis Kosterm.	Kanneliya Forest Reserve, Sri Lanka	AB246494	AB246559	AB246429	Present study, Gamage et al. (2003)
Stemonoporus canaliculatus Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246490	AB246555	AB246425	Present study, Gamage et al. (2003)
Stemonoporus bullatus Kosterm.	Kanneliya Forest Reserve, Sri Lanka	AB246491	AB246556	AB246426	Present study, Gamage et al. (2003)
Stemonoporus reticulatus Thw.	Kanneliya Forest Reserve, Sri Lanka	AB246492	AB246557	AB246427	Present study, Gamage et al. (2003)
Stemonoporus scalarinervis Kosterm.	Gilimale Forest, Sri Lanka	AB246489	AB246554	AB246424	Present study, Gamage et al. (2003)
Stemonoporus wightii Thw.	Gilimale Forest, Sri Lanka	AB246493	AB246558	AB246428	Present study
Stemonoporus gilimalensis Kosterm.	Gilimale Forest, Sri Lanka	AB246488	AB246553	AB246423	Present study
Upuna borneensis Sym.	Frim, Kepong, Malayasia	AB006408	AB006391	AB006374	Kajita et al. (1998)
Vatica coriacea Ashton	Kubah National Park, Malayasia	AB246483	AB246548	AB246418	Present study
Vatica micrantha V. Sl.	Kubah National Park, Malayasia	AB246484	AB246549	AB246419	Present study, Gamage et al. (2003)
Vatica affinis Thw.	Kottawa, Sri Lanka	AB246486	AB246551	AB246421	Present study, Gamage et al. (2003)
Vatica pauciflora (Korth.) BI.	Frim Arboretum, Malayasia	AB246482	AB246547	AB246417	Present study, Gamage et al. (2003)
Vatica odorata Roxb.	Frim, Kepong, Malayasia	AB006419	AB006402	AB006385	Kajita et al. (1998)
Vatica bella V. Sl.	Frim Arboretum, Malayasia	AB246481	AB246546	AB246416	Present study, Gamage et al. (2003)
Vatica chinensis L.	Gilimale, Sri Lanka	AB246485	AB246550	AB246420	Present study, Gamage et al. (2003)
Vateria copallifera (Retzius) Alston	Kanneliya Forest Reserve, Sri Lanka	AB246496	AB246561	AB246431	Present study, Gamage et al. (2003)
Vateriopsis seychellarum Dyer	Seychelles	AB246497	AB246562	AB246432	Present study, Gamage et al. (2003)
Sub-family: Monotoideae					
Monotes madagascariensis Humb.	Fenetrede I'Isalo, Madagascar	AB246543	AB246608	AB246478	Present study, Gamage et al. (2003)
Family: Tiliaceae					
Tilia kiusiana Makino et Shirasawa	Kyushu University, Japan	AB006420	AB006403	AB006386	Kajita et al. (1998)

Table 2. Primers used in the present study

Primer	Sequence (5'-3')	Usage	Reference
trnL-e	GGTTCAAGTCCCTCTATCCC	PCR and sequencing	Taberlet et al. (1991)
trnL-f	ATTTGAACTGGTGACACGAG	PCR and sequencing	Taberlet et al. (1991)
trnL-c	CGAAATCGGTAGACGCTACG	PCR and sequencing	Taberlet et al. (1991)
trnL-d	GGGGATAGAGGGACTTGAAC	PCR and sequencing	Taberlet et al. (1991)
matK-AF	CTATATCCACTTATCTTTCAGGAGT	PCR and sequencing	Ooi et al. (1995)
matK-R	CTGCATATACGCCCAAATCGGTCAA	PCR and sequencing	Ooi et al. (1995)
matK-5F	GAAATGCGGGTTCGACA	PCR and sequencing	Present study
matK-990R	GGACAATGATCCAATCAAGGC	PCR and sequencing	Present study
matK-37F	TCAGTTTACTGATTGTAAAACG	PCR and sequencing	Present study
matK-983R	TTTGGACAATGATCCAATCAAG	PCR and sequencing	Present study
matK-1125R	TCCAGATCGGCTTACTAATG	Sequencing	Kajita et al. (1998)
matK-392R	GATGGATGGGATGAGGTATTAGT	Sequencing	Kajita et al. (1998)
matK-IR	AATGGATTCGTATTCACA	Sequencing	Present study
matK-IF	GTATGTGAATACGAATCCAT	Sequencing	Present study

remaining *matK* sequences (65) were obtained in the present study. In addition, we included sequences of the *trnL-trnF* spacer, *trnL* intron and the *matK* gene of *Tilia kiusiana*, which belongs to the family Tiliaceae.

DNA isolation, PCR and sequencing Total DNA was extracted as described by Gamage et al. (2003). The intergenic spacer region between *trnL* and *trnF* genes, *trnL* intron region and a partial region of the *matK* gene were amplified by polymerase chain reaction (PCR). The primers designed by Taberlet et al. (1991) were used to amplify the *trnL-trnF* spacer and *trnL* intron regions. The primers designed by Ooi et al. (1995) were used to amplify the partial *matK* gene region. Since for most samples the *matK* gene region could not be amplified, several new primers for both PCR and sequencing were designed. The primers used in this study are listed in Table 2. Amplification was carried out after denaturing the DNA at 94°C for 3 minutes followed by 30 cycles of 1 minute at 94°C, 1 minute at 52–55°C for annealing, 1.3 minutes at 72°C, and ending with 7 minutes at 72°C for extension.

PCR products were purified using MiniElute PCR Purification QIAGEN Kit according to the manufacturer's instructions. Sequencing reactions were carried out using the BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. Purified PCR products were directly sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequences were determined in both directions. For the *trnL-trnF* spacer and *trnL* intron regions sequencing primers were the same as those used for PCR, while sequencing primers designed by Kajita et al. (1998) and newly designed PCR and internal primers were used for sequencing of the *matK* region (Table 2). Nucleotide sequence data obtained in this study and the sequences used by Kamiya et al. (1998) are diposited in the DDBJ/EMBL/GenBank databases under accession numbers AB246414 through AB246478, AB246479 through AB246543 and AB246544 through AB246608 for the *matK*, *trnL-trnF*, *and trnL* intron regions respectively.

Data analyses Sequences for the *trnL-trnF* spacer, *trnL* intron and *matK* regions were aligned individually and as combined data set using the ClustalX program (Thompson et al., 1997). The aligned sequences were corrected manually using the BioEdit program (Hall, 1999).

Kimura's two-parameter distance (Kimura, 1980) was used to calculate the genetic distances for all pairs of sequences. Neighbor-joining (NJ) trees (Saitou, and Nei, 1987) for both individual and combined data sets were constructed excluding and including alignment gaps using the ClustalX program (Thompson et al., 1997). Pair-wise deletion option was used when gaps were included in the distance calculations. Phylogenetic tree using Maximum Likelihood (ML) method (Felsenstein, 1981) was obtained based on the combined data using the SEQBOOT, DNAML and CONSENSE programs from the PHYLIP v. 3.6 package (Felsenstein, 2004). In this analysis, empirical nucleotide frequencies were used and the transition/transversion ratio was set to 0.5 as estimated from the combined data set. The statistical support for the nodes of the trees was determined using bootstrap (BT) method (Felsenstein, 1985) based on 1000 replicates.

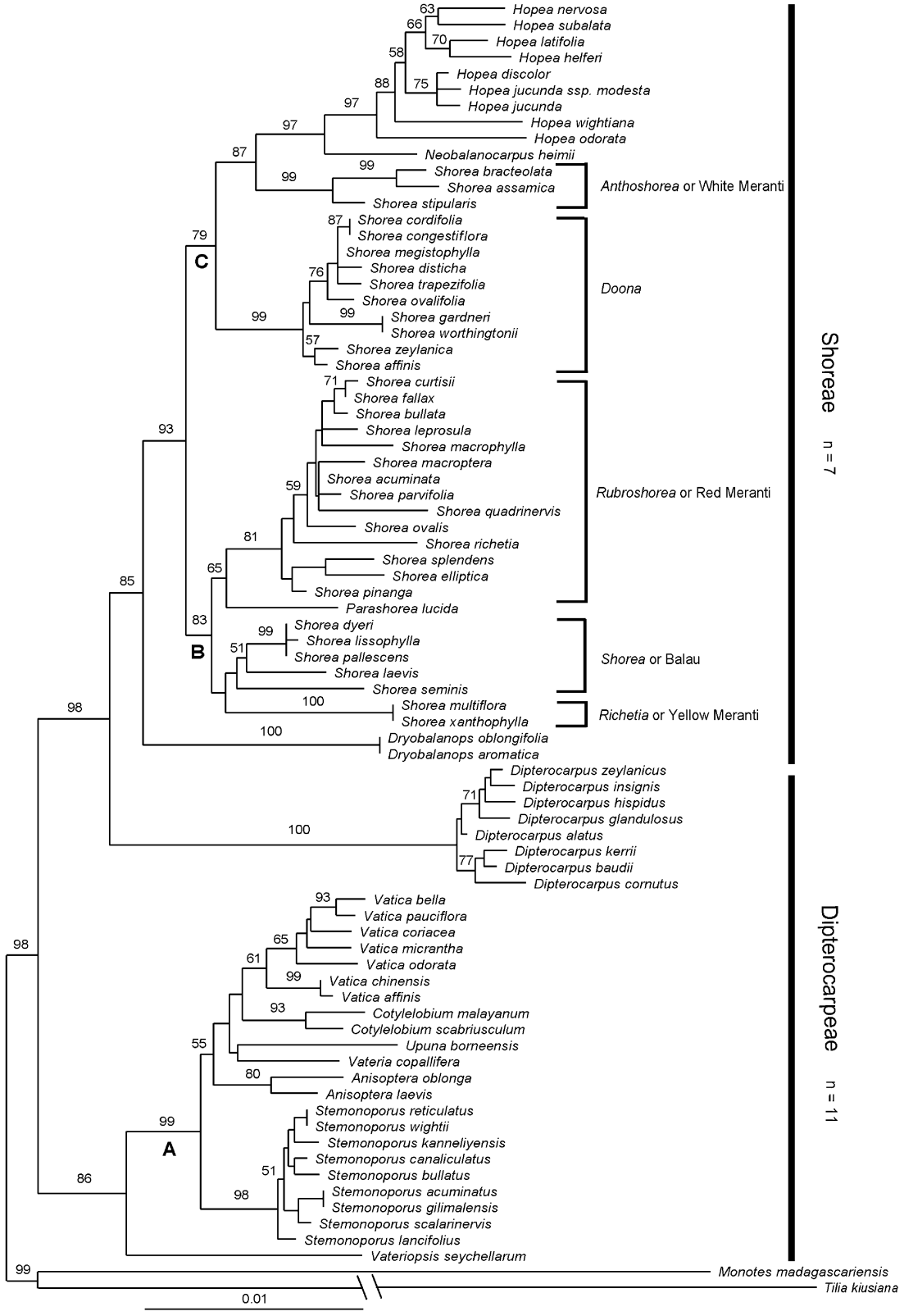

Fig. 1. Neighbor joining tree constructed using the combined data set for the *trnL-trnF* spacer, *trnL* intron and *matK* regions, based on Kimura's two parameter distance (Kimura, 1980). Bootstrap values (BT) in percent from 1000 replicates are indicated above the nodes. The BT values, which were < 50% are not shown. The tree is unrooted and branch lengths are proportional to the scale given in nucleotide substitution per site.

RESULTS

Characteristics of *trnL-trnF* spacer, *trnL* intron and *matK* sequences In the present study, we determined sequences of the *trnL-trnF* spacer and *trnL* intron regions for additional 24 species, which were not included in our previous study (Gamage et al. 2003). For the *trnL-trnF* spacer region the total number of sites, including gaps was 408 after alignment of which 101 sites were variable and 49 were parsimony informative. For the *trnL* intron region the total number of sites after alignment, including gaps was 560 of which 165 sites were variable and 39 were parsimony informative. There were 14 indels ranging from 1 bp to 89 bp in the *trnL-trnF* spacer while 30 indels ranging from 1 bp to 101 bp were found in the *trnL* intron. A long indel of 89 bp was found in the *trnL-trnF* spacer region of *Hopea latifolia* and *H. helferi.* The longest indel, 101 bp was found in the *trnL* intron of all *Stemonoporus* species. Sri Lankan endemic species belonging to the *Doona* genus established by Kostermans (1984) and Maury (1978) had a common 33 bp indel in the *trnL* intron. All species from the genus *Vatica* had one 6 bp indel in both the *trnL-trnF* spacer and the *trnL* intron regions. *Monotes madagascariensis* had two indels of 1 bp and 4 bp in the *trnL-trnF* spacer and one indel of 7 bp in the *trnL* intron. *Tilia kiusiana* had eight indels (<4bp) in the *trnL-trnF* spacer and three indels (two of 1 bp and one of 38 bp) in the *trnL* intron.

In the present study, we determined 65 sequences of the partial *matK* gene region. The aligned matrix of the *matK* region comprised 972 bp. There were 235 polymorphic sites, of which 106 sites were parsimony informative. One 6 bp long indel was found in the *T. kiusiana* sequence.

Phylogenetic analysis Topologies of the NJ trees obtained separately for the *trnL-trnF* spacer, *trnL* intron, and the partial *matK* gene region were generally congruent, with small differences in the resolution of some genera such as *Cotylelobium*, *Dipterocarpus, Hopea* and *Vateriopsis* (data not shown). The topology of the NJ tree based on the *matK* sequence was identical with that of the tree based on the combined data set including all three regions used in the present study (data not shown). There were no considerable topological differences between the trees constructed including and excluding alignment gaps. The topology and BT support of the ML tree based on the combined data set were very similar to those of the corresponding NJ tree (data not shown). Therefore, only the NJ tree constructed using the combined data set including alignment gaps is presented (Fig. 1).

Monotes madagascariensis was grouped together with *Tilia kiusiana*. Next to this group was *Vateriopsis seychellarum*, which occupied a single branch sister to the clade containing species from the Dipterocarpeae tribe.

The genera: *Stemonoporus*, *Anisoptera*, *Vateria*, *Upuna*, *Cotylelobium* and *Vatica* formed distinct clade (designated as A on Fig. 1) supported by high bootstrap (BT) probability (99%). It contained two clades: the *Stemonoporus* clade, and the clade with the following genera: *Anisoptera*, *Vateria, Upuna, Cotylelobium* and *Vatica*. Within the latter clade *Anisoptera*, *Cotylelobium* and *Vatica* were monophyletic, while *Upuna* and *Vateria* formed sister clades. Except for the *Upuna, Vateria* and *Vatica* clades, other generic clades had high BT support (80%–99%).

Most of the remaining genera included in our study formed separate clades on our tree. Here, the genera *Dipterocarpus* and *Dryobalanops* formed two separate monophyletic clades each with 100% BT support. *Dipterocarpus* clade was divided into two groups, one (BT = 77%) with Malaysian species (*D. kerrii*, *D. baudii* and *D. cornutus*) and the other (BT = 71%) with three Sri Lankan species (*D. zeylanicus*, *D. insignis*, *and D. glandulosus*) and *D. alatus* from Thailand.

The remaining genera formed two main clades (designated as B and C on Fig. 1). Clade B contained species belonging to the genus *Richetia* (BT = 100%) (Maury, 1978) or Yellow Meranti (Symington, 1943) wood group, *Shorea* (Maury, 1978) or Balau (Symington, 1943) wood group, *Parashorea* (BT = 65%), *Rubroshorea* (BT = 81%) (Maury, 1978) or Red Meranti wood group (Symington, 1943). The clade C contained the following genera: *Doona* (BT = 99%) (Kostermans, 1984; Kostermans, 1992; Maury, 1978), *Anthoshorea* (BT = 99%) (Maury, 1978) or White Meranti wood group (Symington, 1943), *Neobalanocarpus heimii* (BT = 97%) and *Hopea* (BT = 97%). The clades B and C had 93% BT support. Species belonging to the genera *Richetia* and *Shorea* formed separate clades. *Parashorea* and *Rubroshorea* were grouped separately with the former genus as a sister clade. *Richetia* clade was sister to the *Shorea* clade.

DISCUSSION

Phylogeny reconstruction We obtained two NJ trees (with and without alignment gaps) for the combined data set including *trnL-trnF* spacer, *trnL* intron, and the partial region of the *matK g*ene. Except for the differences in the bootstrap (BT) support for some nodes, the topologies of these trees were similar. Thus, we think that alignment gaps had little effect on the topology of our phylogenetic trees. Furthermore, topologies of our NJ and ML trees were nearly identical. Hence, only the NJ tree based on combined data set including gaps is discussed.

Generic relationships The generic relationships revealed by our NJ tree are mostly in agreement with previous molecular phylogenies based on cpDNA (Dayanandan et

al., 1999; Gamage et al., 2003; Kajita et al., 1998; Kamiya et al., 1998). However, our current results provide new evidence regarding the relationships of additional genera such as *Monotes*, *Vateriopsis* and *Stemonoporus*, which were not included or discussed well (except *Monotes*) in most previous studies (Dayanandan et al., 1999; Kajita et al., 1998; Kamiya et al., 1998). Our study also gives new information about the validity of the previous classifications of the genus *Shorea*.

To date, the genus *Monotes* was included only in three previous studies of Dipterocarpaceae phylogeny (Dayanandan et al., 1999; Gamage et al., 2003; Morton et al., 1999). Moreover, no effort employing molecular data was made to directly test its placement in the family Tiliaceae suggested by Heim (1892) based on flowers and fruit features. Morphological similarity of *Monotes* to Tiliaceae was also suggested by Kostermans (1985). Other classifications however, placed it in a separate family Monotaceae, which includes two sub-families Monotoideae and Pakaraimoideae (Kostermans, 1989). Based on the *rbcL* sequences, Dayanandan et al. (1999) suggested that *Monotes* is more related to the Asian Dipterocarpaceae than to Tiliaceae. Our present results showed that *Monotes madagascariensis* was placed together with *Tilia kiusiana*. However, the *M. madagascariensis* branch was much shorter (0.0243) than the branch with *T. kiusiana* (0.1159). Furthermore, the internal branch of the clade containing these two species was relatively short (0.0080). This result supports suggestion by Dayanandan et al. (1999) to place the genus *Monotes* within the Dipterocarpaceae family. Furthermore, it also indicates that *M. madagascariensis* appears to be better candidate for outgroup species than *T. kiusiana*, which was used for this purpose by Kajita et al. (1998).

The topology of the present phylogenetic tree, was to a certain extent, consistent with the current division of the sub-family Dipterocarpoideae into two tribes: Dipterocarpeae (n = 11) and Shoreae (n = 7) (Ashton, 1982; Brandis, 1895; Jong, and Kaur, 1979; Maury-Lechon, 1979). The two tribes formed two monophyletic clades (BT = 98%) on our tree except for the genus *Dipterocarpus* from the Dipterocarpeae tribe, which was placed as a sister clade to species from the Shoreae tribe.

Similar to result reported by Gamage et al. (2003) monotypic species *Vateriopsis seychellarum* endemic to Seychelles Island was placed on a separate branch sister to the Dipterocarpeae clade (Fig. 1). On our tree, the relationship of this species with other species of the tribe Dipterocarpeae, which have the same chromosome number (n = 11) is relatively well supported (BT = 86%). The origin of this species is still unclear and the possibilities of both plate tectonic movements and the human transportation should be considered (Kostermans, 1992). Embryological evidence suggested that it is related to *Dipterocarpus*, *Hopea*, *Shorea* and *Vateria* (Oginuma et al., 1999). However, it more resembles *Dipterocarpus* than the other three genera in having the micropyle formed by both the inner and outer integument and a conspicuously enlarged chalaza (basal part of the ovule opposite the micropyle, where integument and nucellus are joined) with ample vascular tissues (Oginuma et al., 1999). The placement of *Vateriopsis seychellarum* on our tree did not support its relationship with *Dipterocarpus*, which occupied a separate clade, sister to the clade containing species from the Shoreae tribe. Based on our results it appears that it rather represents a relatively diverged member of the Dipterocarpeae or Shoreae tribe.

The genus *Stemonoporus,* which is endemic to Sri Lanka formed a distinct and well supported monophyletic clade (BT = 98%). This is in agreement with the phylogenetic analyses based on *rbcL* data and noncoding cpDNA (Dayanandan et al., 1999; Gamage et al., 2003). Based on comparative morphology *Stemonoporus* was considered as one of the most archaic genera of the Asian sub-family Dipterocarpoideae (Ashton, and Gunatilleke, 1987). We found a long 101 bp indel in the *trnL* intron in all *Stemonoporus* species included in the present study. Divergent status of *Stemonoporus* revealed in the present and other studies is also consistent with its unique morphological features such as peculiar anthers with apical dehiscence and apical leaf traces, which separate from the central vascular cylinder well before the node (Ashton, 1982; Kostermans, 1992).

Our present results showed that (except for *Upuna* and *Vateria*) *Anisoptera*, *Vatica* and *Cotylelobium* clades are monophyletic, although only the *Cotylelobium* clade had high BT support (93%). *Vatica* also showed monophyly in the study by Dayanandan et al. (1999). However, its relationships with *Anisoptera* and *Cotylelobium* were not elucidated. On our tree, *Upuna* and *Vateria* were grouped together but with low BT support. On the other hand, the *rbcL* analysis placed *Upuna* and *Vateria* on two separate branches sister to *Stemonoporus* (Dayanandan et al., 1999). Kostermans (1992) suggested that *Vateria* is closely related with *Vatica* and that there is no consensus whether these two genera should be fused or kept separate. Further studies are necessary to elucidate the relationship of these two genera.

On our NJ tree, the genus *Dipterocarpus* formed a distinct, highly supported monophyletic clade (BT = 100%, Fig. 1). Similar results were reported in the previous molecular phylogenies (Gamage et al., 2003; Kajita et al., 1998; Kamiya et al., 1998). Morphological evidence also supports highly divergent character of this genus. *Dipterocarpus* has many unique characters, including the winged free calyx tube and large flowers (Dayanandan et al., 1999). Some studies suggested that *Dipterocarpus* might represent the basal clade of Dipterocarpoideae subfamily (Meijer, 1979). On the other hand, others placed *Dipterocarpus* (together with other members of the

Dipterocarpeae tribe) as a sister to the group with species of the tribe Shoreae (Maury, 1978). Our present results also indicate that the genus *Dipterocarpus* was among the most diverged genera of the sub-family Dipterocarpoideae. Finally, Sri Lankan *Dipterocarpus* species (*D. glandulosus*, *D. hispidus*, *D. insignis* and *D. zeylanicus*) formed a separate clade but they were not much diverged from the other species of the *Dipterocarpus* clade. Thus, our results suggest early divergence of this genus from other species of the Dipterocarpoideae and independent evolution of Sri Lankan species.

Similar to *Dipterocarpus*, the genus *Dryobalanops* also formed a distinct, highly supported monophyletic clade on our tree (Fig. 1, BT = 100%). Ashton (1979) placed *Dryobalanops* in the tribe Shoreae due to the presence of connate petals. Such placement was also suggested by the presence of solitary vessels (Gotwald, and Parameswaran, 1966) and the chromosome number (n = 7) (Jong, and Kaur, 1979). However, Maury-Lechon (1979) placed it in the tribe Dipterocarpeae based on the presence of valvate fruit sepals. Our results showed that *Dryobalanops* was placed as a sister clade to the cluster containing species from the Shoreae tribe, which supports classification proposed by Ashton (1979).

Relationships of *Shorea*, *Hopea*, *Neobalanocarpus* and *Parashorea* The topology of our tree lends some support to the classification of *Shorea* species proposed by Maury (1978) and Maury-Lechon (1979). On our tree, the genera created by this author (*Richetia*, *Shorea*, *Rubroshorea, Doona* and *Anthoshorea*) are resolved as separate groups, although the clade containing *Shorea* members had weak BT support (<50%). Some of these genera (*Richetia, Shorea* and *Rubroshorea*) also formed separate groups on the tree reported by Kamiya et al. (2005) although their study did not include *Doona* and *Pentacme* species. Our present study also did not include species from the genus *Pentacme* recognized by Maury (1978) and Maury-Lechon (1979). Therefore, it is important to include them in future phylogenies for obtaining further support for the classification of *Shorea* species proposed by this author.

The placement of *Shorea* species on our tree is also in agreement with classification proposed by Symington (1943). White Meranti, Red Meranti, Balau and Yellow Meranti were all monophyletic. Similar result, except monophyly of the Red Meranti, was reported by Gamage et al. (2003) and Kamiya et al. (1998). On our tree, White Meranti, Red Meranti, and Yellow Meranti had high BT support (>81%). However, the Yellow Meranti-Balau clade was sister to the clade with *Parashorea* and Red Meranti. Thus, the topology of our tree is not consistent with that of the tree obtained by Kamiya et al. (2005), where *Parashorea* was placed on a long separate branch sister to Yellow Meranti, Balau and Red Meranti. On the other hand, the placement of *Parashorea* (within the clade containing Balau, Red Meranti and White Meranti) revealed in our present study is similar to that reported in the previous cpDNA based phylogenies (Gamage et al., 2003; Kamiya et al., 1998). Actually, the wood groups recognized by Symington (1943), well correspond with the generic classification proposed by Maury (1978) and Maury-Lechon (1979). That is, Yellow Meranti with *Richetia*, Balau with *Shorea*, Red Meranti with *Rubroshorea* and White Meranti with *Anthoshorea*.

Taking into account many distinctive morphological differences between *Shorea* and *Doona* species, several studies suggested that *Doona* should be regarded as a separate genus (Kostermans, 1984; Kostermans, 1992; Maury, 1978; Maury-Lechon, 1979). In our present study, species placed by these authors in the genus *Doona* (*S. megistophyla*, *S. ovalifolia*, *S. worthingtonii*, *S. gardneri*, *S. trapezifolia*, *S. zeylanica, S. disticha*, *S. cordifolia, S. congestiflora* and *S. affinis*) formed particularly distinct, monophyletic clade with 99% BT support. Based on morphology these species were placed by Ashton (1972), Ashton (1977) and Ashton (1982) in a separate section *Doona*. The common 33 bp indel in the *trnL* intron present in all *Doona* species provides further evidence for distinct character of this group. Our present study also resolved the position of additional two *Doona* species (*S. disticha* and *S. ovalifolia*), which were not included in our previous study (Gamage et al., 2003). Therefore, we could determine phylogenetic position of almost all the *Doona* species present in Sri Lanka.

Parameswaran and Gotwald (1979) reported that the genus *Neobalanocarpus* has close affinity with *Doona* based on wood anatomy. Floral characters such as diurnal anthesis and stamen structure of *Neobalanocarpus* also show similarity to *Doona* (Dayanandan et al., 1999). However, on our tree *Doona* had the sister relationship to the *Neobalanocarpus heimii* branch, which in turn was sister to the *Hopea* clade. This agrees with results of other cpDNA phylogenies (Gamage et al., 2003) but is incongruent with phylogeny based on nuclear (n) DNA, which placed *Neobalanocarpus* together with *Anthoshorea* species in the most basal and first diverged clade sister to clades containing *Shorea*, *Parashorea* and *Hopea* (Kamiya et al., 2005). The different placement of *Neobalanocarpus* in cpDNA and nDNA based phylogenies together with the morphological characters shared by *Neobalanocarpus*, *Anthoshorea* and *Hopea*, and the irregular behavior of *Neobalanocarpus* during meiosis (Jong, and Lethbridge, 1967) lead Kamiya et al. (2005) to suggest that it may be a hybrid between *Anthoshorea* and *Hopea*. Our results also showed that *Neobalanocarpus* has an intermediate position between *Hopea* and *Anthoshorea* (BT = 97%). If this placement is associated with the hybrid nature of *Neobalanocarpus* our present result would also imply the occurrence of recombination in the

cpDNA. This is surprising because it is believed that due to its uniparental inheritance in most plants cpDNA does not undergo recombination (Chiu and Sears, 1985). Therefore, our result suggests that cpDNA in some Dipterocarpaceae species is inherited biparentally and undergoes recombination. Further investigation regarding this mater is necessary.

An unresolved feature in the previous classifications of the Shoreae tribe was that the well recognized genus *Hopea* was placed within the clade containing other species of that tribe. Our results showed that *Hopea* group was monophyletic within the clade containing *Anthoshorea* and *Neobalanocarpus* and had high BT support (97%). The topologies of the *PgiC* (Kamiya et al., 2005) and *rbcL* trees (Dayanandan et al., 1999) also showed the monophyly of *Hopea* and placed it within the clade containing other species of the Shoreae tribe. The floral morphology of the genera *Hopea* and *Anthoshorea* are similar, both having an urceolate corolla and stamens with an acicular connective appendage (Dayanandan et al., 1999). There are also some unique morphological characters shared by *Hopea*, some *Shorea* species and *Neobalanocarpus* (Kamiya et al., 2005). Therefore, it is possible that these genera have yet not reached the generic level of divergence at molecular level, even though they have already evolved some different morphological characters. Further evaluation using morphological and molecular data is important for detailed classification of these genera.

Relationships of Sri Lankan species There are about 58 species of Dipterocarpaceae in Sri Lanka (Kostermans, 1992). They belong to the genera: *Dipterocarpus*, *Shorea*, *Doona*, *Hopea*, *Stemonoporus*, *Cotylelobium*, *Vatica*, and *Vateria*. Ninety eight percent of the species are endemic. Our present phylogeny revealed the monophyly of Sri Lankan endemic genus *Stemonoporus* and *Doona* species while other species formed separate clades. However, the present phylogeny did not reveal much divergence between Sri Lankan and other Dipterocarpideae species. The isolated position of Sri Lankan species on our tree may be due to their independent evolution caused by the geographic isolation. The placement of Sri Lankan *Shorea stipularis,* which belongs to the *Anthoshorea* section (Ashton, 1980; Ashton, 1982) or genus (Maury, 1978) with other *Anthoshorea* species (*S. bracteolata* and *S. assamica*) from Malaysia is in agreement with such taxonomical grouping. Geographical distribution of *S. stipularis* in Sri Lanka and its morphological similarity to Malaysian *Shorea* species suggest that Dipterocarpaceae must have already diverged to generic or infrageneric sections before they entered the Laurasian plate from the Deccan plate according to the Gondwanan origin of Asian Dipterocarpaceae (Dayanandan et al., 1999). The other Sri Lankan *Shorea* species (*S. lissophylla*, *S. dyeri* and *S. pallescens*), which belong to section (Ashton, 1982) or genus (Maury, 1978) *Shorea* were monophyletic and had close relationship with other species from this group. To obtain more refined phylogeny and further insights into the evolutionary history of Sri Lankan species, additional sequence data are necessary. They should also include species from India. Sri Lanka was intermittently connected to mainland India and this could have enabled biotic interchange with southern India during the Pleistocene ice ages (Bossuyt et al., 2004). Thus, Sri Lankan and Indian Dipterocarpaceae species may be closely related. The most likely possibility is that Dipterocarpaceae spread to Sri Lanka through India.

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