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Molecular Phylogeny of Dipterocarpaceae in Thailand Using *trnL-trnF* and *atpB-rbcL* Intergenic Spacer Region in Chloroplast DNA

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Abstract: Dipterocarpoideae is a species-rich subfamily of Dipterocarpaceae. Dipterocarpaceae in Thailand is represented by 65 species in 8 genera. In recent years molecular taxonomist constructed a phylogenetic tree of dipterocarps found in South-East Asia. However, many species of dipterocarps found in Thailand are not included on that tree. In an effort to complete the tree, the phylogenetic relationships among 23 species belonging to 7 genera of dipterocarps found in Thailand were studies using nucleotide sequences of two intergenic spacer region between *trnL-trnF* and *atpB-rbcL* in chloroplast DNA. With *Neobalanocarpus heimii* as an out group, the molecular tree was consistent with the morphological classification and it was clearly resolved two major groups; Vavate-dipterocarpi and Imbricate-Shorea. The analysis showed that each member genera have similar characteristics and base chromosome number of 11 and 7, respectively. The first group consisted of genus *Diptercarpus*, *Anisoptera*, *Cotylelobium* and *Vatica*. *Shorea* and *Hopea* were in the second group.

Key words: Dipterocarpaceae, chloroplast DNA, molecular phylogenetic, *trnL-trmF* region, *atpB-rbcL* region

INTRODUCTION

A large family of massive trees, Dipterocarpaceae is a dominant and important timber species found in tropical forest. The family include over 500 species in 17 genera and is divided into three subfamilies: Monotoideae, Pakarimoideae and Dipterocarpoideae (Astoon, 1982). The subfamily Monotoideae comprises over 30 species in three genera and is confined in Africa, whereas 1 species is in Pakarimoideae and is distributed in South America and Guyana. The Asian dipterocarps, Dipterocarpoideae, includes 470 species in 13 genera. The Dipterocarpoideae subfamily is subdivided into two tribe: Dipterocarpeae and Shoreae.

In Thailand, The first recording of Dipterocarpaceae consisted of 10 genera, 46 species and 7 varieties (Craib, 1925). More recently records however, show Dipterocarpaceae in Thailand represented by 65 species in 8 genera: *Anisoptera* (3), *Nebalanocarpus* (1), *Cotylelobium* (1), *Dipterocarpus* (16), *Hopea* (14), *Parashorea* (1), *Shorea* (22) and *Vatica* (7) (Phemgklia and Niyomdhum, 1999). The majority of Dipterocarpaceae in Thailand belong to the evergreen species which is scattered all over the country: in gallery forests along the hill stream, in the low-lying land and on hill slopes. Only five deciduous species of *D. obtusifolius*, *D. tuberculatus*, *D. intricatus*, *S. obtusa* and *S. siamensis* are distributed at high elevation (Smitinand, 1969).

Dipterocarpaceae is a divers family tree and a species, especially rich in genus of *Shorea*, *Hopea*, Dipterocarpus and *Vatica*. In general, identification is based on morphology, wood anatomy, palinology and fossil record (Aston, 1980). However, identification of this family is not an easy task, because some characteristics vary with age of the tree and it's habitat (Symington, 1974). Rajaseger *et al.* (1997) reported that polymorphism of DNA as molecular marker, are suitable for discriminating closely related genotypes. The advantage of DNA-based markers is that they are not influenced by the environment or by the development stage of the plant. Currently, molecular techniques such as RAPD (Rath *et al.*, 1998), RFLP (Tsumura *et al.*, 1996) and nucleotide sequences (Kajita *et al.*, 1998; Kamiya *et al.*, 1998; Dayanandan *et al.*, 1999) are alternative methods that are used to investigate the relationships between dipterocarps. Beside these, base sequence data base can be used to identify in species level.

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In order to obtain a refined phylogeny among species of Dipterocarpaceae in Thailand, phylogenetic analyses based on the nucleotide sequences of intergenic spacer (IGS) region of *trn*L-*trn*F and *atpB-rbcL* of chloroplast DNA were performed.

MATERIALS AND METHODS

Taxon sampling: Leaf samples were obtained from 5 national parks of Thailand. They are Centennial Botanic Gaden, Peninsula Botanic Garden, Pa Klang Ao, Sakaerat Environmental Research Station and Queen Sirikit Botanic Garden. Samples were collected from 23 dipterocarp tree species representing 7 genera during the rainy season of 2002 and 2003. The collected leaf were dried in silica gel while in the field and transport to the laboratory for DNA extraction. The *trnL-trnF* nucleotide sequences from 10 species that also distributed in Thailand (*Cotylelobium lanceolatum*, *Dipterocarpus baudii*, *D. kerrii*, *Hopea pierrei*, *H. odorata*, *Neobalanocarpus heimii*, *Shorea faguetiana*, *S. guiso*, *S. roxburghii* and *Vatica odorata*) were retrived from the Genbank database. The nucleotide sequences of *atpB-rbcL* from 2 species of *D. baudii* and *D. kerrii* were a gift from Dr. Choong and Dr. Wickenswari.*** The information on each species is show in Table 1.

DNA extraction, *trnl-trnf* and *atpbrbcl* spacer region amplification and sequencing : Total genomic DNA was extracted from leaf samples using the methods of Doyle and Doyle (1987). The *trnL-trnF* intergenic spacer was amplified by the polymerase chain reaction (PCR) using oligonucleotide primers e and f (Taberlet *et al*., 1991). Oligonucleotids of *atpB* (Fofana *et al*., 1997) and rbcL (Demesure *et al*., 1995) were developed for amplifying the *atpB-rbcL* spacers. Amplification reactions contained 1 μL of 10 mM each of dATP, dCTP, dTTP, dGTP, 1 μL of 20 σmol/μL of each primer, 2.5 units of Taq DNA polymerase and 4 μL of 25 mmol/L $MgCl_2$ in a total volume of 50 μL. Thermal cycling for *trnL-trnF* IGS region was performed in a thermalcycler at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min. The reactions were completed by a 7 min extension at 72°C and held on 4°C. PCR thermal cycling conditions for *atpB-rbcL* intergenic region were initial denature at 94°C for 3 min, 30 cycles of 40 sec at 94°C, 40 sec at 59°C and 2 min at 72°C, followed by extension at 72°C for 7 min and then a soak at 4°C. The sequencing reactions were performed by using ABI PRISM™ big dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Sequencing primers were the same as those for gene amplification. Reactions were then eletrophoresed on an ABI 377 automate sequencer (Perkin-Elmer Applied Biosystems, Inc.).

Table 1: Dipterocarpaceae species selected for tmL-tmF and atpB-rbcL sequencing and their sources

		Source of tmL-tmF		Source of atpB-rbcL	
No.	Species	This study*	Accession No.**	This study*	Other
1	*A. costata*	PBG		PBG	
2	*C. lanceolatum*		AY02582	PBG	
3	*D. alatus*	PKA		PKA	
4	*D. baudii*		AB006410		***
5	*D. chartaceus*	CBG		CBG	
6	*D. costatus*	QSBG		QSBG	
7	*D. dyeri*	CBG		CBG	
8	*D. kerrii*		AB006409		***
9	*D. intricatus*	CBG		CBG	
10	*D. obtusifolius*	CBG		CBG	
11	*D. tuberculatus*	CBG		CBG	
12	*D. turbinatus*	CBG		CBG	
13	*H. pierrei*		AY026598	CBG	
14	*H. odorata*		AB006419	PBG	
15	*N. heimii*		AB006417	CBG	
16	*S. faguetiana*		AY026607	CBG	
17	*S. guiso*		AY026609	CBG	
18	*S. henryana*	CBG		CBG	
19	*S. roxburghii*		AY026630	SERS	
20	*S. siamensis*	SERS		SERS	
21	*S. thorelii*	CBG		SERS	
22	*V. diospyroides*	PBG		PBG	
23	*V. odorata*		AB006407	PBG	

Study site*, CBG = Centennial Botanic Garden, Chachaengsao / Sakaew province, Thailand, PBG = Peninsular Botanic Garden (Thung Khai), Trang province, Thailand, PKA = Pa Klang Ao, Prachuab Kiri Khan province, Thailand, SERS = Sakaerat Environmental Research Station, Nakonrachasrima province, Thailand, QSBG = Queen Sirikit Botanic Garden, Chiang Mai province, Thailand, **Accession Number of DNA Data Bank of GenBank, ***Form Dr. Choong, C.Y. and Dr. Wickenswari, R. School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, University Kebangsaan, Malaysia, 43600 UKM Bangi, Selangor

Alignment and phylogenetic analysis: Multiple alignment of the sequences were performed by Clustal W (Thompson, *et al*., 1994). Alignments were visually adjusted. Sequence data from two nucleotide region were combined in a cladistic analysis using PAUP v. 3.1.1 (Swofford, 1993). Phylogenetic analyses were carried out by maximum parsimony and Neighbor-joining (NJ) method. Parsimony trees were constructed using heuristic search TBR branch swapping, stepwise addition of 10 random replicates, an unconstrained number of maximum trees and retention of multiple most parsimonious trees and all characters trees were unweighted. Both strict and 50% majority-rule consensus trees were roots at *Neobalanocarpus heimii*. NJ analyses were conducted using HKY85 distance.

RESULTS

The combined sequences of the *trnL-trnF* and *atpB-rbcL* intergenic spacer region of 23 species were analyzed. The final alignments included 1339 sites. There was a total of 273 informative characters in equally weight of maximum parsimony. The analysis generated 124 mostly equal parsimonious trees, each with a length of 752 steps with Consistency Index (CI) of 0.871 and Rescaled Consistency (RC) index of 0.736 (after excluding uninformative characters). The strict consensus tree of

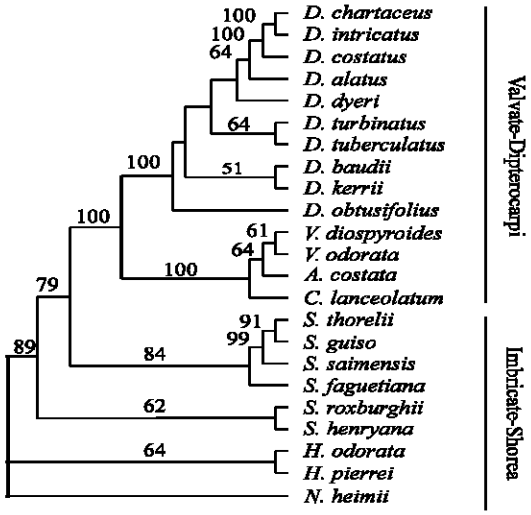

Fig. 1: The strict consensus tree of 124 most parsimonious trees of combined non-coding region: *trnL-trnF* and *atpB-rbcL* of Dipterocarpaceae. The number above the branches indicate the percentage of bootstrap values (higher than 50) from 1000 replicates. The total length is 752. Consistency index is and retention index are 0.871 and 0.736, respectively. This tree is rooted with *N. heimii*

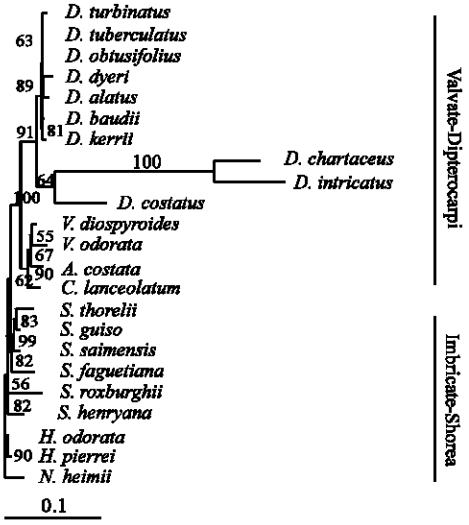

Fig.2: Neighbor-joining tree obtained from combined sequence of two non-coding region: *trnL-trnF* and *atpB-rbcL* of Dipterocarpaceae. Numbers above the branches indicate bootstrap values from 1000 replicates. *N. heimii* is using as an outgroup

124 mostly equal parsimonious trees is given in Fig. 1. All branches were well resolved and showed 2 major groups of Valvate-Dipterocarpi and Imbricate-Shorea. The first major clade contained 4 genera of *Anisoptera*, *Cotylelobium*, *Vatica* and *Dipterocarpus* with a bootstrap value of 100%. This major clade split into 2 subclades *Dipterocarpus* and Valvate both of with bootstrap value of 100%. Four taxa within subclade Valvate were well resolved with bootstrap supported over 60%. The second subclade of *Dipterocarpus* formed monophyletic clade sister to valvate subclade. However, the relationships among this subclade remain unresolved. The second major clade, a paraphyletic clade of tribe Shorea (*Hopea* and *Shorea*), was well supported with bootstrap value over 60%. Species of *Shorea* (*S. thorelii*, *S. guiso*, *S. siamensis* and *S. faguetiana*) formed a clade sister to the clade of *S. roxburghii* and *S. henryana*. *Hopea* clade (*H. odorata* and *H. pierrei*) formed a monophyletic clade sister to the clade of *Shorea*.

Neighbor-Joining analysis were conducted on HKY85 distance matrix. A tree was shown in Fig. 2. All branches showed a bootstrap consensus value of over 50%. Although topology of the NJ tree was not fully consistent with the parsimony trees, all branches were mostly agree with the parsimony trees except Dipterocarpus clade.

DISCUSSION

In this study, phylogenetic relationships within 23 dipterocarp species based on *trnL-trnF* and *atpB-rbcL*

sequences are mostly in agreement with present taxonomic treatment (Aston, 1980; 1982). Using morphology, pollen analysis and fossil records, (Aston, 1982) classified two tribes in Dipterocarpoideae.The first tribe Dipterocarpeae or Valvate-Dipterocarpi group consist of *Anisoptera*, *Cotylelobium*, *Dipterocarpus*, *Stemonoporus*, *Upuna*, *Vatica*, *Vateriopsis* and *Vatica*. The genera of this tribe have valvate sepals in fruit, solitary vessels, scattered resin canals and basic chromosome number x = 11. Present results showed that the genera Dipterocarpus is monophyletic with of the *Anisoptera-Cotylelobium-Vatica* clade with a bootstrap value of 100% (Fig. 1and 2). The genera *Anisoptera*, *Cotylelobium* and *Vatica* clustered together (bootstrap probability was 100% in Fig. 1) consistent with the morphological classification of Aston (1982). Our tree showed *D. kerrii* was closely related with *D. baudii* as the same result of (Kamiya *et al*., 1998) and Kajita *et al*., (1998). The second tribe or Imbricate-*Shorea* group consist of *Balanocarpus*, *Hopea*, *Parashorea* and *Shorea*. The genera of this group have imbricate sepals in fruit, grouped vessels, resin canals in tangential bands and basic chromosome number x = 7. Therefore the species rich genera of *Hopea* and *Shorea*, can be distinguished by a single characteristic of a number of long fruit calyxes. RFLP analysis (Tsumura *et al*., 1996) and combined sequences analysis of *matK*, *trnL-trnF* IGS region and *trnL* intron (Kajita *et al*., 1998) the genus *Hopea* is sister to the genus *Shorea*. This is also confirmed by Kamiya *et al*. (1998). In this study *Hopea* is also closely related genera forming a sister grouping to *Shorea*.

Shorea is the largest genus of Dipterocarpaceae, out of 194 species, 163 species which occur in Malesia. Phylogenetic tree of Kamiya *et al*. (1998) showed *S. roxburghii* was closely related with *S. saimensis* and *S. obtusa* fromed a clade with the Malayan species. Beside these they also suggested that the deciduous species of the genus *Shorea* in Thailand may evolve in at least independent lineages. In this study, the Imbricate-*Shorea* group consisted of 3 subclade: they are 2 clade of *Shorea* and 1 clade of *Hopea*. Our tree, therefore, indicated that the genus *Shorea* is paraphyletic. However, our trees were different from the tree of Kamiya *et al*., (1998) in the case of *S. siamensis* was not closely affinity with *S. roxburghii*. Our tree showed *S. roxburghii* was closely related with *S. henryana* and *S. siamensis* fromed a clade with *S. faguetiana*, *S. guiso* and *S. thorelii*.

In the future, the study on Dipterocarpacea is an easy task. Because molecular techniques are an universal tools to access all the problems and get the new knowledge.

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