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Molecular phylogeny of Magnoliaceae based on plastid DNA sequences with special emphasis on some species from continental Southeast Asia

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ABSTRACT. A molecular phylogenetic tree of Magnoliaceae was constructed based on four cpDNA regions (*trnK* intron including *matK*, *trnH–psbA*, *atpB–rbcL*, and *ndhF*; total ca 5800 bp) to reveal the phylogenetic positions of some *Magnolia* species from Peninsular Malaysia, Thailand, and Vietnam. A total of 100 accessions, including 75 *Magnolia*, two *Liriodendron* species, three putative hybrids (including *M.* x *alba*), and two outgroups (*Degeneria* and *Galbulimima*), were included, of which 20 species plus three putative hybrids (total 34 accessions) were collected in continental Southeast Asia. Phylogenetic analyses (maximum parsimony and maximum likelihood) indicated that most of these newly examined species were placed in expected taxonomic groups (e.g., *M. villosa* in subsection *Blumiana*; *M. garrettii*, *M. hookeri*, and *M. utilis* in section *Manglietia*; *M. thailandica* in section *Kmeria*; *M. mediocris*, *M. scortechinii*, and *M. sirindhorniae* in subsection *Michelia*; *M. macklottii* var. *beccariana* in subsection *Maingola*; and *M. pahangensis* in subsection *Aromadendron*). Circumscription of the taxonomic groups matched the phylogenetic clades except in subsections *Gwillimia* and *Blumiana*. Phylogenetic relationships among major large clades were still unresolved and interspecific relationships within some large clades (e.g., subsection *Michelia* and section *Manglietia*) were also not suggested.

KEY WORDS: chloroplast DNA, *Magnolia*, Thailand, Vietnam, Peninsular Malaysia.

INTRODUCTION

Magnoliaceae comprise ca. 220 species of trees or shrubs distributed in temperate and tropical regions from the Himalayas to East Asia and Southeast Asia and the Americas, and is divided into two subfamilies, Liriodendroideae and Magnolioideae (Nooteboom, 1993; 2000; Figlar & Nooteboom, 2004). The subfamily Magnolioideae had formerly been divided into six (or more) genera (*Magnolia* L., *Manglietia* Blume, *Michelia* L., *Elmerrillia* Dandy, *Kmeria* Dandy, *Pachylarnax* Dandy: Chen & Nooteboom, 1993; Nooteboom, 1993; Frodin & Govaerts, 1996), but several morphological and molecular studies have shown all genera to be embedded in *Magnolia*, thus the family now consists of just two genera, *Liriodendron* L. and *Magnolia* (Figlar & Nooteboom, 2004).

Molecular phylogenetic analysis based on DNA sequence data has been found to be very useful for understanding phylogenetic relationships among species as well as evolutionary trends of morphological and other taxonomic characters (e.g., chromosome number and chemical compounds) including geographical distribution. Thus, it has become a powerful tool for construction and/or evaluation of taxonomic systems (Judd et al., 2008). Many molecular phylogenetic studies were conducted on Magnoliaceae by using plastid (chloroplast) (Azuma et al., 1999; 2001; 2004; Shi et al., 2000; Kim et al., 2001; Suh & Kim, 2001; Wang et al., 2006) or nuclear DNA sequences (Nie et al., 2008). Several early studies gave many insights into the recent classification of Magnoliaceae (Figlar & Nooteboom, 2004; Nooteboom & Chalermglin, 2009).

Although these molecular studies commonly recognized some well-supported clades, especially at terminal nodes of the trees, we have not yet attained a fully resolved phylogenetic tree of Magnoliaceae because of a limited number of species analysed and low resolution among major

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clades in the tree. For example, Kim et al. (2001) constructed a phylogenetic tree of 99 species by using only *ndhF* sequences (ca 2200 bp). This resulted in a tree with little or no resolution among the major clades. By contrast, Suh & Kim (2001) analyzed a combined plastid DNA sequence data set of *ndhF*, *rbcL*, *trnK* intron including *matK*, *trnH–psbA* spacer region, *atpB–rbcL* spacer regions, *trnL* intron, *trnL–trnF* spacer region and ORF350 regions (total ca. 8700bp). The resulting tree did slightly improve resolution among the major groups, but the tree consisted of only 48 taxa (mostly temperate species) of Magnoliaceae. Therefore, it is important to continue additional molecular work to fully understand the phylogenetic systematics of Magnoliaceae, especially in regard to tropical species most of which have not yet been included in the molecular phylogenetic analyses. Moreover, new species are still being discovered in the tropics, for example *M. sirindhorniae* Noot. & Chalermglin and *M. thailandica* Noot. & Chalermglin, both of which have been discovered in Thailand during the past decade (Nooteboom & Chalermglin, 2000; 2002; see also Figlar, 2011). Previous molecular studies dealt with only a few species widely distributed or cultivated in Southeast Asia [e.g., *M. champaca* (L.) Baill. ex Pierre, *M. insignis* Wall., and *M. liliifera* (L.) Baill.], thus phylogenetic relationships among most *Magnolia* species found in this area are poorly understood.

The purpose of this study is to construct a molecular phylogenetic tree of Magnoliaceae and to know the phylogenetic positions of especially tropical Asian species. In total, 75 species plus three putative hybrids (a total of 98 accessions) including 20 newly analyzed species collected from Peninsular Malaysia, Thailand, and Vietnam were analyzed using plastid DNA sequence data of four regions (*trnK*^{UUU} intron including *matK* gene, *ndhF* gene, *trnH*^{GUG}*–psbA* and *atpB–rbcL* spacer regions; total ca 5800 bp). We also tried to evaluate the taxonomic system of Magnoliaceae by Figlar and Nooteboom (2004) in which the genus *Magnolia* is divided into three subgenera and twelve sections (thirteen subsections in five sections; Table 1). In this study we included representatives of all subgenera, sections, and subsections except for subsection *Dugandiodendron* (Lozano) Figlar & Noot. (tropical American group) in section *Talauma* Baill.

MATERIALS AND METHODS

A list of species (samples) involved in this study is shown in Table 1 giving information or references, and DDBJ/EMBL/GenBank accession numbers of sequences. Most sequences were obtained from DDBJ (DNA Data Bank of Japan), but sequences of 20 species plus three putative hybrids collected in Peninsular Malaysia, Thailand, and Vietnam (total 34 samples) were newly determined in this study. We also determined *ndhF* sequences of 42 samples (taxa) which have been used in previous molecular studies(Azuma et al., 1999; 2001) in which the other three regions (*trnK* intron, *trnH–psbA*, and *atpB–rbcL*) had already been sequenced (Table 1). We also retrieved or newly sequenced corresponding regions of *Degeneria vitiensis* I.W.Bailey & A.C.Sm. or *D. roseiflora* John M.Mill. (Degeneriaceae) and *Galbulimima belgraveana* (F.Muell.) Sprague (Himantandraceae), which were added to the data matrix as outgroup taxa, because they are members in Magnoliales and seem to be more closely related to Magnoliaceae than Annonaceae and Myristicaceae (Soltis et al., 2000; Hilu et al., 2003; Qiu et al., 2010). Finally, DNA sequence data of a total of 100 accessions from 77 species and three putative hybrids including *M*. x *alba* were compiled and analysed (Table 1).

Sequences of the following four regions of plastid DNA were retrieved or sequenced; the *trnK*^{UUU} intron including the *matK* gene (ca 2500 bp), the *ndhF* gene (ca 2090 bp), and *trnH*^{GUG}*–psbA* (ca 430 bp) adn *atpB–rbcL* (ca 800 bp) spacer regions. These regions have often been used in phylogenetic analyses of Magnoliaceae (e.g., Azuma et al., 1999; 2001; 2004; Kim et al., 2001).

Total DNA was extracted from silica-gel dried leaves or herbarium specimens using the modified CTAB method by Doyle and Doyle (1987). The PCR mixture (20 µL) contained 1 µL of template DNA, 2 µL of dNTPs (2.5 mM each), 1 µL of each primer (10 µM), 2 µL of 10x *Taq* buffer (containing 20 mM MgCl2), 0.5 U of *Taq* polymerase (TaKaRa Ex*Taq*, Takara Bio Inc., Japan). The PCR was performed with a GeneAmp PCR System 2700 (Applied Biosystems Japan Ltd., Japan) starting at 94ºC (5 min), followed by 35 cycles of denaturation at 94ºC (30 sec), annealing at 50ºC (30 sec), and extension at 72ºC (30 sec or 1 min), and a final extension at 72ºC (7 min). After checking for a single band by electrophoresis on an 1% agarose TAE gel stained with ethidium bromide, the PCR products were purified by enzymatic treatment. That is, 2 μL of mixture containing 0.3 Us of Exonuclease I (Takara Bio Inc., Japan) and Calf Intestine Alkaline Phosphatase (TOYOBO Co., Ltd., Japan) was added to each PCR tube to degrade remaining primers and to dephosphorylate remaining dNTPs. The tubes were heated at 37ºC for 30 min then at 80ºC for 15 min. Direct sequencing of both DNA strands was conducted on an ABI 3100 Genetic Analyzer (Applied Biosystems Japan Ltd., Japan) using a BigDye Terminator version 3.1 Cyclic Sequencing Ready Reaction Kit (Applied Biosystems Japan Ltd., Japan) following the manufacturer's protocol. Primers used for amplification and/or sequencing are listed in Table 2 and the relative positions are illustrated in Fig. 1.

Alignment of sequence data was manually carried out in MEGA 4 (Tamura et al., 2007). Four regions were combined as a single data matrix. Three and one short inversions were found in the *matK* region (all 33 species in subgenera *Yulania* and *Gynopodium*, and section *Kmeria* showed –TCTATT– instead of –AATAGA–; 20 species, i.e., *M. dawsoniana*, all 10 species in section *Magnolia*, all 8 species in section *Gwillimia*, and *L. chinense* showed –GAA– instead of –TTC–; and 2 species, *M. nitida* and *M. sinica*, showed –GA– instead of –TC–) and in the *trnH–psbA* spacer region [7 species, i.e., *M. figo*, *M. vrieseana* (syn. *M. ovalis*), *M. acuminata*, *M. nitida*, *M. fraseri* ssp. *fraseri* and ssp. *pyramidata*, *M. delavayi*, and *L. tulipifera*, showed –TTCTAT– instead of –ATAGAA–], respectively (Azuma et al., 1999). These inversions were excluded from the data matrix. Samples which showed completely identical sequence were represented by one OTU (operational taxonomic unit) in phylogenetic analysis. Indels were treated as missing data.

Maximum parsimony analysis (MP) was conducted in PAUP* 4.0b10 (Swofford, 2003). For the heuristic search we used a random stepwise addition sequence with TBR (tree bisection and reconnection) branch swapping and 10 replication of the random addition sequence. One thousand bootstrap replications were performed using the same setting as in the heuristic search. Maximum likelihood analysis (ML) was conducted in MEGA 5.05 (Tamura et al., 2011). The best substitution model was selected based on BIC (Bayesian Information Criterion) values of 24 possible combinations of substitution models and rate description analyzed in MEGA. The GTR+G model, the lowest BIC value, was choosen for our data set. For ML analysis, gaps were partialy deleted by 95% "site coverage cutoff" option. NNI (Nearest-Neighbor-Interchange) was used as ML heuristic method and the initial tree was made automatically. One hundred bootstrap replications were performed using the same setting.

RESULTS AND DISCUSSION

A combined data matrix of four plastid regions was composed of 5960 characters after alignment (total 85 ingroup and 2 outgroup OTUs). There are 785 (13.2%) variable sites among all OTUs, of which 374 (6.3%) were parsimony-informative, and 420 (7.0%) variable sites among *Magnolia* species, of which 223 (3.7%) were parsimony-informative. The MP analysis generated 334 equally most parsimonious trees with a tree length 999; the consistency index (CI) and the CI excluding uninformative characters were 0.8649 and 0.7648, respectively, and the retention index (RI) was 0.8985. The strict consensus tree of 334 MP trees is shown in Fig. 2 with subgenera, sections and subsections of the Magnoliaceae according to Figlar and Nooteboom (2004). Maximum likelihood tree (lnL = -14976.16) is shown in Fig. 3. There is no topological contradiction between the MP and ML trees.

The Magnoliaceae were first divided into two clades which correspond to the two subfamilies, i.e., Liriodendroideae (only *Liriodendron*) and Magnolioideae (only *Magnolia*) (Fig. 2). In the clade of the Magnolioideae, three well supported clades [clade B-D, >98% bootstrap percentage] and one moderately supported clade (clade A, 79% in MP tree and 80% in ML tree) were recognized. The relationships among these clades were not suggested in the MP tree(Fig. 2), but two tropical American clades (C and D) formed a weakly supported clade (60%) in the ML tree (Fig. 3). Clade A is a relatively large clade and contains taxa from sub-genera *Magnolia* [except for sects. *Gwillimia* DC.

(tropical Asia; clade B) and *Talauma* (tropical America; clades C and D)], *Yulania* (Spach) Rchb., and *Gynopodium* Figlar & Noot., indicating that subgenus *Magnolia* is at least paraphyletic. All temperate and some subtropical to tropical species were found in this clade.

Monophyletic relationship of members in sect. *Gwillimia* (clade B), sect. *Talauma* subsect. *Splendentes* (Dandy ex A.Vasquez) Figlar & Noot. (clade C), and sect. *Talauma* subsect. *Talauma* (clade D) were suggested, respectively. Section *Gwillimia* is taxonomically divided into two subsections, *Gwillimia* and *Blumiana* (Blume) Figlar & Noot. (Figlar & Nooteboom, 2004). However, neither representatives of subsect. *Gwillimia* nor representatives of subsect. *Blumiana* form well supported clades. That is, *M. pterocarpa* Roxb. and *M. henryi* Dunn belong to subsect. *Gwillimia* with *M. coco* (Lour.) DC. and *M. delavayi* Franchet., but the former two species seem to be more closely related to *M. hodgsonii* (Hook.f. & Thom.) H.Keng in subsect. *Blumiana* (clade B). The mode of fruit dehiscence is a key character to distinguish the two subsections; carpels of subsect. *Gwillimia* show longitudinally dehiscence which is widely observed in *Magnolia*, while those of subsect. *Blumiana* show circumscissile dehiscence (Figlar & Nooteboom, 2004), which means that identification of individuals or specimens having no fruits to either subsect. *Gwillimia* or *Blumiana* is rather difficult. In addition, it is hard to obtain good specimens (with fruits) of these tree species in tropical forest. Therefore, results of our molecular analysis suggest that subsections *Gwillimia* and *Blumiana* should be abolished and only section *Gwillimia* should be recognized. Fruits occurring in *Blumiana* as well as in South American subsect. *Talauma* probably are a parallel adaptation to the tropical rain forest. Apart from the fruits the morphology of *M. henryi* (subsect. *Gwillimia*) and *M. hodgsonii* (subsect. *Blumiana*) is so similar that, in the herbarium, they are very difficult to recognize as separate species (observation by Nooteboom). More detailed morphological studies linked with identification by means of molecular techniques may be helpful to solve this problem. This may also be true for the other paraphyletic relationships found in *M. liliifera* with *M. villosa* (Miq.) K.Keng (clade B). *Magnolia liliifera* is a complex species which contains at least four varieties (Frodin & Govaerts, 1996), therefore, our finding is supporting the complex status of *M. liliifera*, and we need to re-evaluate the taxonomic statuses of the varieties and a related species, *M. villosa*.

Clade A was further divided into five clades (clades E–I), but the relationships among them was not suggested in the MP tree (Fig. 2). The ML tree suggested that sister relationship between clades F (section *Kmeria*) and H (section *Macrophylla*) with less than 50% of bootstrap value (Fig. 3). Clade E, which was weakly supported in the MP and ML trees (60% and 57%, respectively), consists of three well supported subclades (clades J, K, and L), which correspond to section *Michelia* (L.) Baill. (clade J; 99% in MP and 100% in ML), and sect. *Yulania* (clade K; 83% in MP and 89% in ML), both in subg. *Yulania*, and subg. *Gynopodium* (clade L; 100% in MP and ML), respectively. Section *Michelia* (clade A1a) taxonomically consists of four subsects., *Michelia* (L.) Figlar & Noot., *Elmerrillia* (Dandy) Figlar & Noot., *Maingola* Figlar & Noot., and *Aromadendron* Figlar & Noot. The molecular phylogenetic trees clearly indicated close relationships between subsects. *Michelia* and *Elmerrillia* (with 91% in MP and 95% in ML), and between *Maingola* and *Aromadendron* (with 73% in MP and 76% in ML), which are also supported by morphological features, i.e., the former two subsections have flowers on proleptic brachyblasts and two to six ovules per carpel, the latter has terminal flowers (rarely proleptic) and two ovules per carpel (Figlar, 2000; Figlar & Nooteboom, 2004).

We included three morphologically putative hybrids in the molecular analysis. Because plastid (chloroplast) DNA of most flowering plants is essentially maternally inherited (Mogensen, 1996), the phylogenetic tree presented here may represent a maternal lineage of *Magnolia*. *Magnolia* x *alba* (DC) Figlar, is a well known taxon and ornamentally cultivated in tropical and subtropical Asian countries. The plant is often sterile and is believed to be a hybrid between *M. champaca* and *M. montana* (Blume) Figlar & Noot. (Frodin & Govaerts, 1996; Nooteboom & Chalermglin, 2009), although we do not have any molecular or direct evidence to support this hypothesis. The phylogenetic tree indicated that *M.* x *alba* was sister to *M. montana* (98%). The other undescribed putative hybrids, *M. champaca* x *baillonii* (“Champa khao”; Nooteboom &

Chalermglin, 2009) and *M. champaca* x *M.* x *alba*, showed identical sequences with *M. champaca*, indicating mothers of these hybrids should be *M. champaca*, although further evidence is needed to further confirm whether they are truly hybrids by detailed molecular and morphological studies.

Phylogenetic relationships among species collected from Peninsular Malaysia and Vietnam, e.g, *M. foveolata* (Merr. ex Dandy) Figlar, *M. mediocris* (Dandy) Figlar, *M. sirindhorniae*, and *M. scortechinii* (King) Figlar & Noot., in subsect. *Michelia* were not suggested. *Magnolia foveolata* and *M. mediocris* showed the same sequences of all regions examined, suggesting they are genetically closely related and morphologically recently diversified, but we need an additional examination to confirm the sister relationship because we used sterile individuals for the samples. Because sect. *Michelia* is the largest group in *Magnolia* (ca 65 spp.; ca 50 spp. in subsect. *Michelia*; Figlar & Nooteboom, 2004), we need more comprehensive sampling and sequencing to reveal relationships among species in this group and additional taxonomic examination to better understand the species boundaries.

Clade F (100% in MP and ML) corresponds to sect. *Kmeria* (Pierre) Figlar & Noot., including *M. thailandica* from Thailand (Nooteboom & Chalermglin, 2009), which is characterized by unisexual flowers (only this group). However, a sister group of this peculiar group in Magnoliaceae was not suggested.

Clade G (59% in MP and 60% in ML) contains sects. *Magnolia* (American group; clade M, 100% in MP and ML), *Manglietia* (Blume) Baill. (100% within clade N) and *Rhytidospermum* Spach (86% and 78% within clade N in MP and NL). In the *Manglietia* clade there are two relatively well-supported clades; one clade (86% in MP and 88% in ML) consists of *M. aromatica* (Dandy) V.S.Kumar, *M. grandis* (Hu & W.C.Cheng) V.S.Kumar, *M. hookeri* (Cubitt & W.W.Sm.) D.C.S.Raju & M.P.Nayar, and *M. insignis*, the other (98% in MP and ML) consists of *M. conifera* (Dandy) V.S.Kumar, *M. garrettii* (Craib) V.S.Kumar, *M. blumei* Prantl. However, relationships among the species in each clade are not resolved and morphological characters supporting each clade are uncertain. In addition, three individuals (samples) of *M. utilis* (Dandy) V.S.Kumar gave different sequences and did not form a single clade, suggesting that we need additional taxonomic and molecular works on these species as well as on sect. *Gwillimia*.

Clades H (sect. *Macrophylla* Figlar & Noot.) and I (sect. *Auriculata* Figlar & Noot.) consist of temperate American species and had previously been treated as section *Rhytidospermum sensu* Dandy (1978), which is characterized by having leaves arranged in false whorls. Morphologically they are very similar, but molecular data does not indicate their close relationship, thus supporting their exclusion from subsect. *Rhytidospermum sensu* Figlar & Nooteboom (2004).

The molecular phylogenetic tree constructed in this study provides information on the phylogenetic positions of *Magnolia* species from continental Southeast Asia, some of which have not been suggested in previous molecular phylogenetic studies (Azuma et al., 1999; 2001; 2004; Shi et al., 2000; Kim et al., 2001; Suh & Kim, 2001; Wang et al., 2006). In addition, circumscription of taxonomic groups (sections and subsections) proposed by Figlar & Nooteboom (2004) matches the phylogeny (monophyletic clades) except for subsects. *Gwillimia* and *Blumiana*. However, phylogenetic relationships among major clades are still unresolved and interspecific relationships within some large clades are also not suggested. Additional sequence data may improve resolution of the phylogeny, and taxonomic re-examination of species boundaries noted in this study should be conducted along with molecular studies.

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Table 1. A list of species used in molecular phylogenetic analysis with accession numbers and voucher information. Accession numbers with underline are newly determined sequences.

	trnK intron	*psbA-trnH*	*atpB-rbcL*	*ndhF*	Voucher & source or Reference
Magnoliaceae					
Subfamily Magnolioideae					
Genus *Magnolia* L.					
Subg. *Magnolia*					
Sect. *Magnolia*					
M. grandiflora L.	AB020990	AB021020	AB021050	AB623373	HA & SK 95051701 **KYO** USA
M. guatemalensis Donn.Sm.	AB020991	AB021021	AB021051	AB623374	*Thien 20007* **NO** cult.
M. iltisiana Vazquez	AB055520	AB055551	AB055569	AB623375	*Thien 12002* **NO** Mexico
M. pacifica ssp. *pugana* Iltis & Vazquez	AB055521	AB055552	AB055570	AB623376	*Rico-Gray & Thien 12003* **NO** Mexico
M. panamensis Vazquez & Iltis	AY008996	AY009026	AY008965	AF216255	Kim et al. (2001) & unpub.
M. schiedeana Schltdl.	AB055550	AB055568	AB055586	AB623377	*Thien & Azuma 12004* **NO** Mexico
M. sharpii Meranda	AB020993	AB021023	AB021053	AB623378	*Thien 20009* **NO** cult.
M. tamaulipana Vazquez	AB020994	AB021024	AB021054	AB623379	*Dieringer 878* **NO** Mexico
M. yoroconte Dandy	AB055522	AB055553	AB055571	AB623380	*Thien 12006* **NO** Mexico
M. virginiana L. (1)	AB020988	AB021018	AB021048	AB623381	HA & SK 95052601 **KYO** USA
M. virginiana (2)	AB020989	AB021019	AB021049	AB623382	HA & SK 95051713 **KYO** USA
Sect. *Gwillimia* DC.					
Subsect. *Gwillimia*					
M. coco (Lour.) DC.	AB021004	AB021034	AB021064	AB623401	*Thien 10540* **NO** cult.
M. delavayi Franchet.	AB021005	AB021035	AB021065	AB623402	*Thien 590645* **NO** cult.
M. henryi Dunn (1)	AY008997	AY009027	AY008966	AF107918	Kim et al. (2001) & unpub.
M. henryi (2)	AB623292	AB623318	AB623344	AB623403	*Nooteboom 6004* **L** Thailand

	trnK intron	*psbA-trnH*	*atpB-rbcL*	*ndhF*	Voucher & source or Reference
M. pterocarpa Roxb.	AY008999	AY009029	AY008968	AF107920	Kim et al. (2001) & unpub.
Subsect. *Blumiana* (Blume) Figlar & Noot.					
M. gigantifolia (Miq.) Noot.	AY009000	AY009030	AY008969	AF107944	Kim et al. (2001) & unpub.
M. hodgsonii (Hook.f. & Thom.) H.Keng (1)	AB021013	AB021043	AB021073	<u>AB623404</u>	*Thien 20006* **NO** cult.
M. hodgsonii (2)	AB623293	AB623319	AB623345	AB623404	*Nooteboom 6002* **L** Thailand
M. liliifera (L.) Baill.	AY008998	AY009028	AY008967	AF107919	Kim et al. (2001) & unpub.
M. liliifera var. *liliifera*	AB623294	AB623320	AB623346	AB623405	Yao FRI 55902 **KEP** W Malaysia
M. villosa (Miq.) H.Keng (1)	AB623295	AB623321	AB623347	AB623406	*Kamarudin FRI 51353* **KEP** W Malaysia
M. villosa (2)	AB623296	AB623322	AB623348	AB623407	*Chew FRI 53649* **KEP** W Malaysia
Sect. *Talauma* Baill.					
Subsect. *Talauma*					
M. dodecapetala (Lam.) Govaerts	AB055534	AB055560	AB055578	<u>AB623410</u>	*Rainer 1625* **XAL** Cuba
M. mexicana DC.	AB055536	AB055562	AB055580	<u>AB623412</u>	*Rico-Gray & Thien 12001* **NO** Mexico
M. minor (Lozano) Govaerts	AB055535	AB055561	AB055579	<u>AB623411</u>	*Thien s.n.* **NO** Cuba
M. ovata (A.St.Hil.) Spreng.	AB055537	AB055563	AB055581	<u>AB623413</u>	*Thien 12000* **NO** Brazil
Subsect. *Splendentes* (Dandy ex A.Vazquez) Figlar & Noot.					
M. portoricensis Bello	AB055523	AB055554	AB055572	<u>AB623408</u>	*Thien 11800* **NO** Puerto Rico
M. splendens Urban	AB055524	AB055555	AB055573	<u>AB623409</u>	*Thien 11801* **NO** Puerto Rico
Sect. *Manglietia* (Blume) Baill.					
M. aromatica (Dandy) V.S.Kumar	AY008993	AY009023	AY008962	AF107983	Kim et al. (2001) & unpub.
M. blumei Prantl	AY008995	AY009025	AY008964	AF107988	Kim et al. (2001) & unpub.
[=*Manglietia glauca* Blume]					

	trnK intron	*psbA-trnH*	*atpB-rbcL*	*ndhF*	Voucher & source or Reference
M. conifera (Dandy) V.S.Kumar (1)	AB055541	AB055564	AB055582	<u>AB623391</u>	*Azuma 384* **KYO** cult.
M. conifera (2)	AB623285	AB623311	AB623337	AB623392	*Nooteboom* Vietnam1 **L** Vietnam
M. conifera (3)	AB623285	AB623311	AB623337	AB623392	*Nooteboom* Vietnam2 **L** Vietnam
M. decidua (Q.Y.Zheng) V.S.Kumar	AB055542	AB055565	AB055583	<u>AB623393</u>	*Azuma 386* **KYO** cult.
M. utilis (Dandy) V.S.Kumar (1)	AB623286	AB623312	AB623338	AB623394	*Chew FRI 53676* **KEP** W Malaysia
M. utilis (2)	AB623288	AB623314	AB623340	AB623396	*Chew FRI 53678* **KEP** W Malaysia
M. utilis (3)	AB623287	AB623313	AB623339	AB623395	*Yao FRI 55912* **KEP** W Malaysia
M. garrettii (Craib) V.S.Kumar	AB623289	AB623315	AB623341	AB623397	*Nooteboom 6001* **L** Thailand
M. grandis (Hu & W.C.Cheng) V.S.Kumar	AY008992	AY009022	AY008961	AF107989	Kim et al. (2001) & unpub.
M. hookeri (Cubitt & W.W.Sm.) D.C.S.Raju & M.P.Nayar	AB623290	AB623316	AB623342	AB623398	*Nooteboom 6003* **L** Thailand
M. insignis Wall. (1)	AB055543	AB055566	AB055584	<u>AB623399</u>	*Azuma 385* **KYO** cult.
M. insignis (2)	AB623291	AB623317	AB623343	AB623400	*Nooteboom 6005* **L** Thailand
Sect. *Kmeria* (Pierre) Figlar & Noot.					
M. kwangsiensis Figlar & Noot. [=*Kmeria septentrionalis* Dandy]	AY009007	AY009037	AY008976	AF107930	Kim et al. (2001) & unpub.
M. thailandica Noot. & Chalermglin	AB623284	AB623310	AB623336	AB623372	*Nooteboom 6006* **L** Thailand
Sect. *Rhytidospermum* Spach					
Subsect. *Rytidospermum*					
M. obovata Thunb.	AB020999	AB021029	AB021059	<u>AB623384</u>	HA 96070301 **KYO** Japan
M. officinalis Rehd. & Wilson	AY009001	AY009031	AY008970	AF107926	Kim et al. (2001) & unpub.

	trnK intron	*psbA-trnH*	*atpB-rbcL*	*ndhF*	Voucher & source or Reference
M. tripetala (L.) L.	AB021001	AB021031	AB021061	AB623385	HA & SK 95051311 **KYO** USA
Subsect. *Oyama* (Nakai) Figlar & Noot.					
M. sieboldii ssp. *japonica* K.Ueda	AB021003	AB021033	AB021063	AB623383	HA 960717 **KYO** Japan
M. wilsonii (Finet. & Gagnep.) Rehder	AY009002	AY009032	AY008971	AF107934	Kim et al. (2001) & unpub.
Sect. *Auriculata* Figlar & Noot.					
M. fraseri var. *fraseri* Walt.	AB020995	AB021025	AB021055	AB623386	HA & SK 950524 **KYO** cult.
M. fraseri var. *pyramidata* (Bartram) Pampanini	AB020996	AB021026	AB021056	AB623387	HA & SK 95051711 **KYO** USA
Sect. *Macrophylla* Figlar & Noot.					
M. dealbata Zucc.	AB055525	AB055556	AB055574	AB623388	*Thien & Rico-Gray 12005* **NO** cult.
M. macrophylla ssp. *macrophylla* Michx.	AB020997	AB021027	AB021057	AB623389	HA & SK 95051705 **KYO** USA
M. macrophylla ssp. *ashei* (Weatherby) D.Johnson	AB020998	AB021028	AB021058	AB623390	*Thien 10502* **NO** cult.
Subg. *Yulania* (Spach) Rchb.					
Sect. *Yulania*					
Subsect. *Yulania*					
M. biondii Pampan	AY008986	AY009017	AY008956	AF107953	Kim et al. (2001) & unpub.
M. campbellii Hook.f. & Thomson	AY008988	AY009019	AY008958	AF107947	Kim et al. (2001) & unpub.
M. cylindrica Wilson	AY008989	AY009020	AY008959	AF107957	Kim et al. (2001) & unpub.
M. dawsoniana Rehder & Wilson	AY008987	AY009018	AY008957	AF107948	Kim et al. (2001) & unpub.
M. denudata Desr.	AB021007	AB021037	AB021067	AB623365	HA 97052701 **KYO** cult.
M. kobus DC.	AB021008	AB021038	AB021068	AB623366	HA 96062401 **KYO** Japan

	trnK intron	*psbA-trnH*	*atpB-rbcL*	*ndhF*	Voucher & source or Reference
M. liliiflora Desr.	AB021012	AB021042	AB021072	AB623367	HA 97052601 **KYO** cult.
M. salicifolia (Sieb. & Zucc.) Maxim.	AB021009	AB021039	AB021069	AB623368	HA 96062404 **KYO** Japan
M. stellata (Sieb. & Zucc.) Maxim.	AB021010	AB021040	AB021070	AB623369	HA 96061501 **KYO** Japan
Subsect. *Tulipastrum* (Spach) Figlar & Noot.					
M. acuminata (L.) L.	AB021011	AB021041	AB021071	AB623370	HA & SK 95051708 **KYO** USA
Sect. *Michelia* (L.) Baill.					
Subsect. *Michelia* (L.) Figlar & Noot.					
M. baillonii Pierre	AY008981	AY009012	AY008951	AF107979	Kim et al. (2001) & unpub.
M. cavaleriei (Finet & Gagnep.) Figlar	AY008978	AY009009	AY008948	AF107961	Kim et al. (2001) & unpub.
M. champaca (L.) Baill. ex Pierre (1)	AY008980	AY009011	AY008950	AF107962	Kim et al. (2001) & unpub.
M. champaca (2)	AB623273	AB623299	AB623325	AB623351	*Yao FRI 55901* **KEP** W Malaysia
M. compressa Maxim.	AB021014	AB021044	AB021074	AB623354	HA 97052702 **KYO** cult.
M. figo (Lour.) DC.	AB021015	AB021045	AB021075	AB623355	HA 97052602 **KYO** cult.
M. foveolata (Merr. ex Dandy) Figlar	AB623272	AB623298	AB623324	AB623350	*Nooteboom* Vietnam3 **L** cult.
M. macclurei (Dandy) Figlar	AB055546	AB055567	AB055585	AB623356	*Azuma 134* **KYO** cult.
M. mediocris (Dandy) Figlar	AB623276	AB623302	AB623328	AB623357	*Nooteboom* Vietnam4 **L** Vietnam
M. montana (Blume) Figlar & Noot. (1)	AB623277	AB623303	AB623329	AB623358	*Yao FRI 55911* **KEP** W Malaysia
M. montana (2)	AB623277	AB623303	AB623329	AB623358	*Yao FRI 55903* **KEP** W Malaysia
M. montana (3)	AB623277	AB623303	AB623329	AB623358	*Yao FRI 55921* **KEP** W Malaysia

	trnK intron	*psbA-trnH*	*atpB-rbcL*	*ndhF*	Voucher & source or Reference
M. odora (Chun) Figlar & Noot.	AY008982	AY009013	AY008952	AF107978	Kim et al. (2001) & unpub.
M. scortechinii (King) Figlar & Noot.	AB623278	AB623304	AB623330	AB623359	*Yao FRI 55908* **KEP** W Malaysia
M. sirindhorniae Noot. & Chalermglin	AB623279	AB623305	AB623331	AB623360	*Nooteboom 6007* **L** Thailand
described putative hybrid					
M. x *alba* (DC.) Figlar & Noot. (1)	AB623271	AB623297	AB623323	AB623349	*Yao FRI 55890* **KEP** W Malaysia
M. x *alba* (2)	AB623271	AB623297	AB623323	AB623349	*Nooteboom FRI 55927* **KEP** W Malaysia
undescribed putative hybrids					
M. champaca x *M. baillonii* (1)	AB623274	AB623300	AB623326	AB623352	*Chalermglin 490416* **BKF** Thailand
M. champaca x *M.* x *alba* (2)	AB623275	AB623301	AB623327	AB623353	*Chalermglin 490420* **BKF** Thailand
Subsect. *Elmerrillia* (Dandy) Figlar & Noot.					
M. vrieseana (Miq.) Baill. ex Pierre [=*Elmerrillia ovalis* (Miq.) Dandy]	AY008983	AY009014	AY008953	AF107982	Kim et al. (2001) & unpub.
Subsect. *Maingola* Figlar & Noot.					
M. cathcartii (Hook.f. & Thoms.) Noot.	AY008984	AY009015	AY008954	AF107945	Kim et al. (2001) & unpub.
M. gustavii King	AB623280	AB623306	AB623332	AB623361	*Chalermglin 450416* **L** Thailand
M. macklottii Dandy var. *beccariana* (Agostini) Noot. (1)	AB623281	AB623307	AB623333	AB623362	*Yao FRI 55922* **KEP** W Malaysia
M. macklottii var. *beccariana* (2)	AB623281	AB623307	AB623333	AB623362	*Yao FRI 55907* **KEP** W Malaysia
M. macklottii var. *beccariana* (3)	AB623281	AB623307	AB623333	AB623362	*Yao FRI 55906* **KEP** W Malaysia

	trnK intron	*psbA-trnH*	*atpB-rbcL*	*ndhF*	Voucher & source or Reference
Subsect. *Aromadendron* Figlar & Noot.					
M. elegans (Blume) H.Keng (1)	AB623282	AB623308	AB623334	AB623363	*Imin FRI 50778* **KEP** W Malaysia
M. elegans (2)	AB623282	AB623308	AB623334	AB623363	*Yao FRI 55889* **KEP** W Malaysia
M. elegans (3)	AB623282	AB623308	AB623334	AB623363	*Imin FRI 50777* **KEP** W Malaysia
M. pahangensis Noot.	AB623283	AB623309	AB623335	AB623364	*FRI 68502* **KEP** W Malaysia
Subg. *Gynopodium* Figlar & Noot.					
Sect. *Gynopodium*					
M. nitida W.W.Smith	AB021006	AB021036	AB021066	<u>AB623371</u>	*Thien 20005* **NO** cult.
Sect. *Manglietiastrum* (Y.W.Law) Noot.					
M. sinica (Law) Noot.	AY008990	AY009021	AY008960	AF107937	Kim et al. (2001) & unpub.
Subfamily Liriodendroideae					
Genus *Liriodendron* L.					
L. chinense (Hemsley) Sarg.	AB021016	AB021046	AB021076	<u>AB623414</u>	HA 970518 **KYO** cult.
L. tulipifera L.	AB021017	AB021047	AB021077	<u>AB623415</u>	HA & SK 95051703 **KYO** USA
Himantandraceae					
Genus *Galbulimima* F.M.Bailey					
G. belgraveana (F.Muell.) Sprague	<u>AB332090</u>+ AB055547+ <u>AB332091</u>	<u>AB332085</u>	<u>AB332087</u>	# AY218176+ <u>AB332083</u>	*Thien 11200* **NO** Australia #Sauquet et al. (2003)
Degeneriaceae					
Degeneria I.W.Bailey & A.C.Sm.					
D. roseiflora John M.Mill.	AB055548			#AY218174 +	SV 1/12022000 **SUVA** Fiji
D. vitiensis I.W.Bailey & A.C.Sm.		<u>AB332084</u>	<u>AB332086</u>	<u>AB332082</u>	SV 1/17022000 **SUVA** Fiji #Sauquet et al. (2003)

Table 2. Primer sequences used in this study.

Foward (5'-3')		Reverse (5'-3')	
***trnK* intron**			
C7FM	TGCTAACTCAACGGTAGAGT	C7RM	CTAGTCGGATGGAGTAGATAA
TMF	TTTCTCTATTATGGAGTG	C10RM	TCTAGCACACGAAAGTCGAA
C10FM	TCCGCTTCTATTTCAGGAGT	MR2	CAAGGTGAGATTTCCATTTC
MF1	CTGCTGGATACAAGATGCCC	MR1	GGGCATCTTGTATCCAGCAG
MF2	GAAATGGAAATCTCACCTTG	MR	GTTCGTAAAAAATCGATCCA
MF3	CGATTTGGGCGGATATACAG	TMR	CACTCCATAATAGAGAAA
trnK-3914F[1)]	GGGGTTGCTAACTCAACGG	C7RG1[1)]	TAGGAAGTGTTGTTGCCGAG
C7FG1[2)]	GGTCACATAGAGAACGGATT	*trnK*-2R[2)]	AACTAGTCGGATGGAGTAG
ndhF			
C11FM	AGACATATCAATATGCGTGG	C11RM	ACATATTTGATACCTTCTCC
C11FM2	TTCCTTGGTGCAGTTGCTAA	C11RM2	TTCCCCATATAGATATTGAA
C11FM3	CAAAATTACAGTGGCACTAAAA	C11RM3	TCCATGGCATCAGGTAACCA
C11FG1[3)]	GGGTATAAGAGGATTAGCCG	350-2R[3)]	GGAAGAAAAGGAGGATCCGG
trnH-psbA			
C8F	CGCATGGTGGATTCACAATC	C8R	AGACCTAGCTGCTATCGAAG
atpB-rbcL			
C9F	AGAACCAGAAGTAGTAGGAT	C9R	ACACCAGCTTTGAATCCAAC
ATM	CATGGATGAATTCTGCCTA	RBM	TAGGCAGAATTCATCCATG
C9F2-138[4)]	GGCTGTTGTTCCTTATTTCAG	C9R2-138[4)]	AATTGACTCTTGACAGTGAT
		RBM2[5)]	GAAATTGACTCTTGACAGTGA

[1)] for a sequence between *trnK*5’ and *matK* of Galbulimima

[2)] for a sequence between *matK* and *trnK*3’ of Galbulimima

[3)] for parts of *ndhF* sequences of Degeneria and Galbulimima

[4)] for M. thailandica instead of ATM or RBM

[5)] for M. sirindhorniae and M. scortechinii instead of RBM

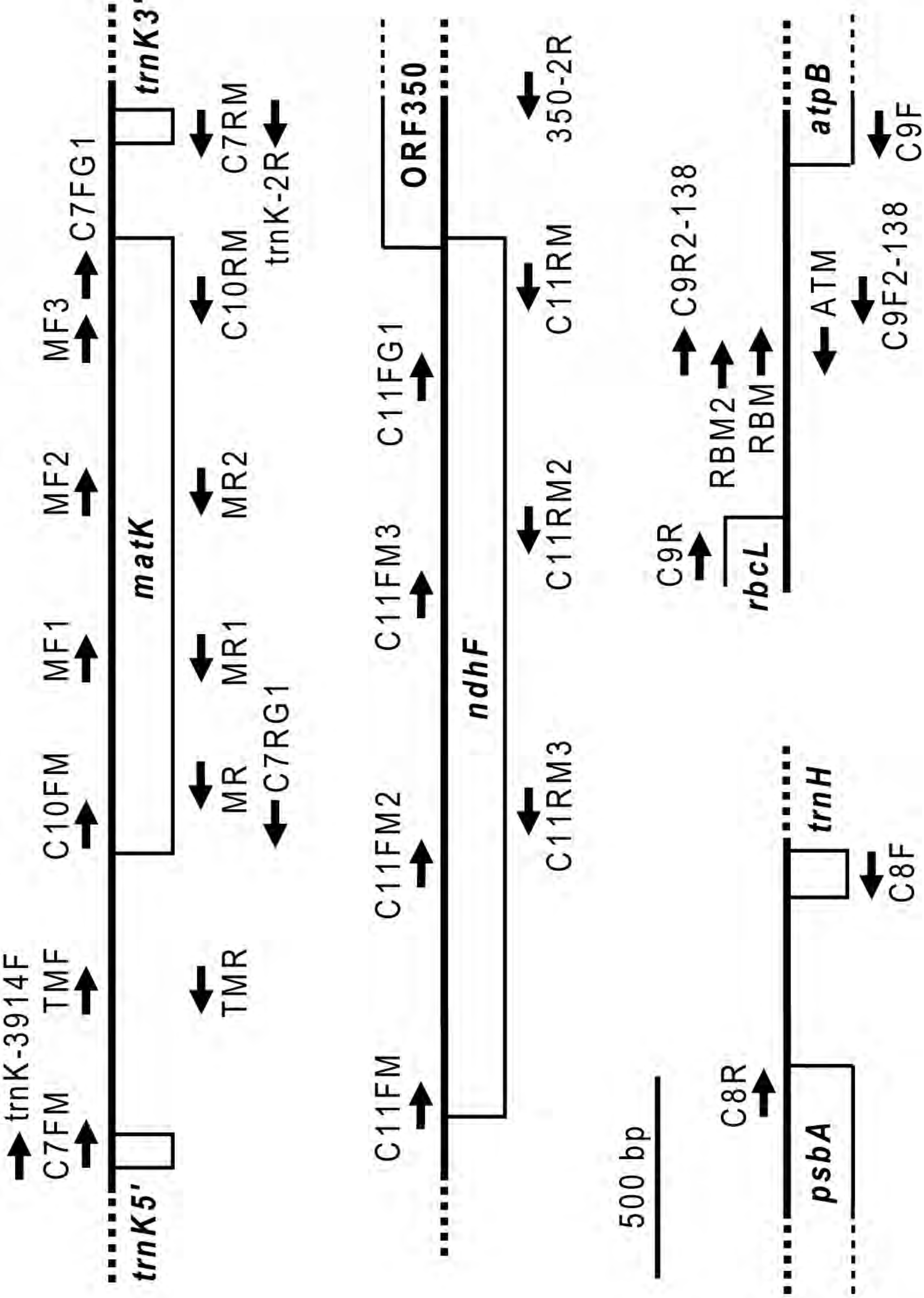

Figure 1. Relative positions of amplification and sequencing primers on schemed maps of four cpDNA regions.

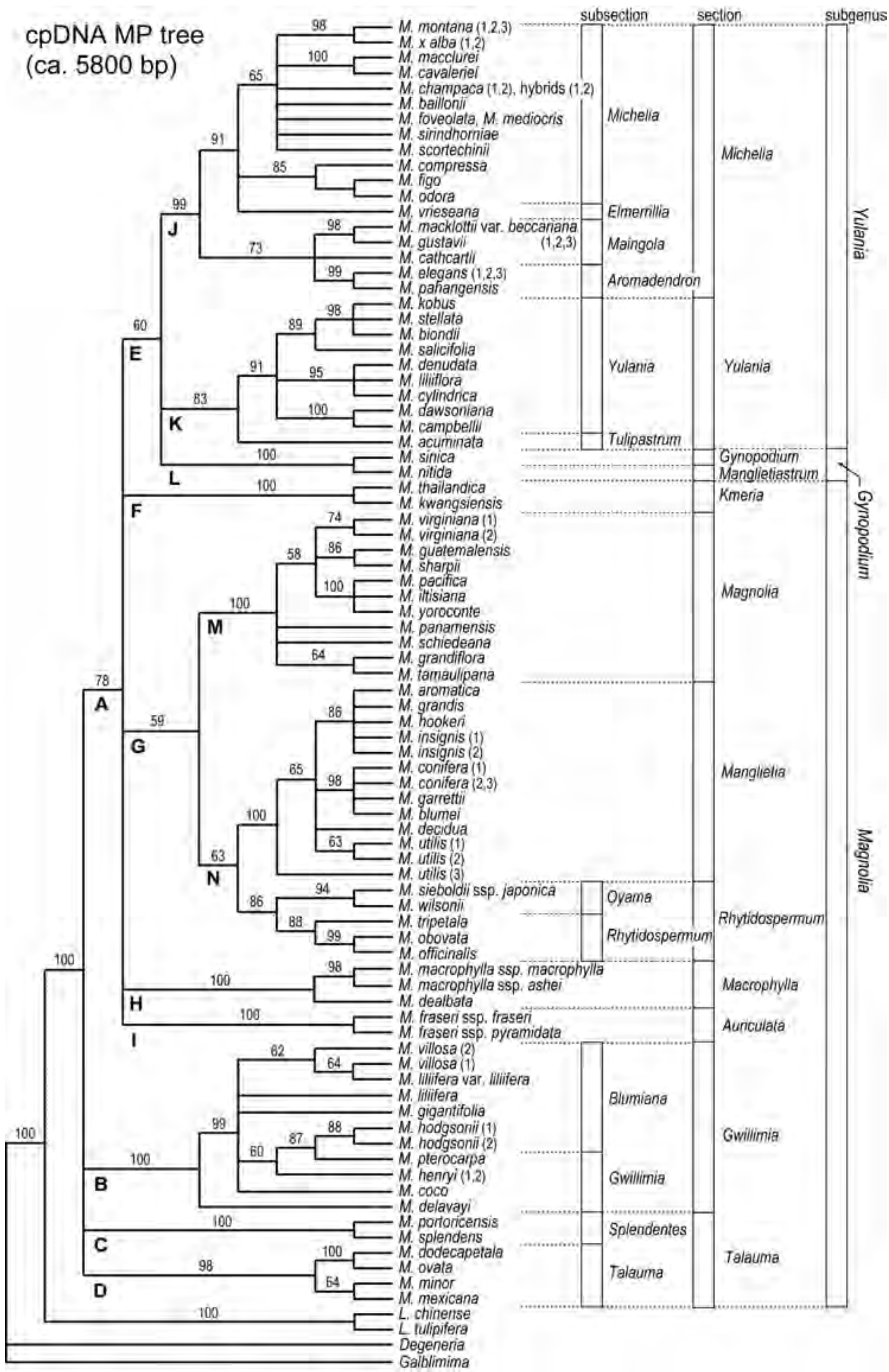

Figure 2. The strict consensus tree of 334 most parsimonious trees of Magnoliaceae based on *trnK* intron including *matK*, *trnH–psbA*, *atpB–rbcL*, and *ndhF* sequence data [total of 5960 characters after alignment; tree length = 999; consistency index (CI) = 0.8649; CI excluding uninformative characters = 0.7648; retention index (RI) = 0.8985]. Numbers on branches indicate bootstrap percentage (>50%). The taxonomic system proposed by Figlar & Nooteboom (2004) is also presented.

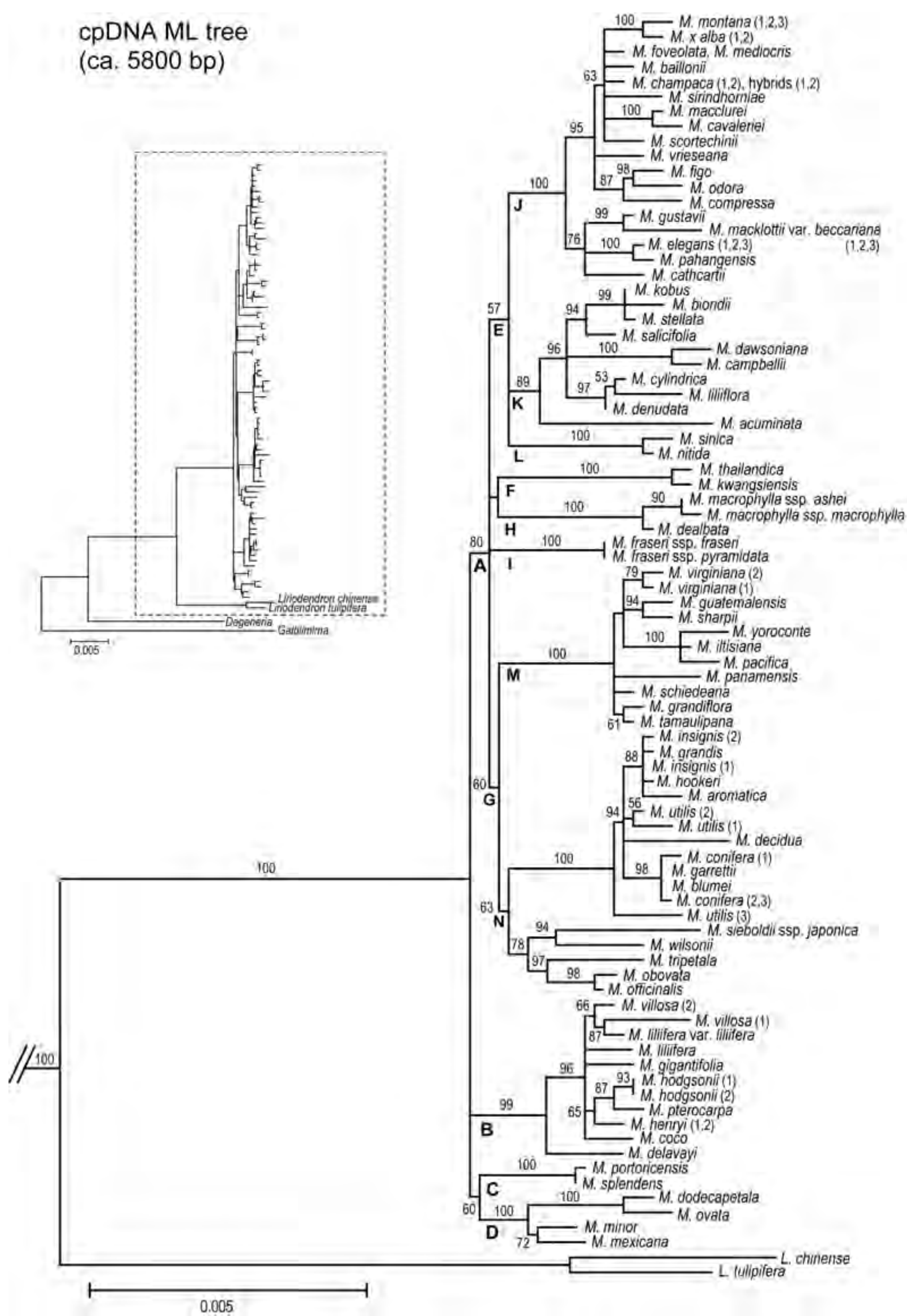

Figure 3. The maxmum likelihood tree of Magnoliaceae based on *trnK* intron including *matK*, *trnH–psbA*, *atpB–rbcL*, and *ndhF* sequence data (total of 5960 characters after alignment). Numbers on branches indicate bootstrap percentage (>50%). Alphabetic marks indicating branches are corresponding to those in Fig. 2.

REFERENCES

Azuma, H., García-Franco, J.G., Rico-Gray, V. & Thien, L.B. (2001). Molecular phylogeny of the Magnoliaceae: The biogeography of tropical and temperate disjunctions. American Journal of Botany 88: 2275–2285.

Azuma, H., Rico-Gray, V., García-Franco, J.G., Toyota, M., Asakawa, Y. & Thien, L.B. (2004). Close relationship between Mexican and Chinese *Magnolia* (subtropical disjunct of Magnoliaceae) inferred from molecular and floral scent analyses. Acta Phytotaxonomica et Geobotanica 55: 167–180.

Azuma, H., Thien, L.B. & Kawano, S. (1999). Molecular phylogeny of *Magnolia* (Magnoliaceae) inferred from cpDNA sequences and evolutionary divergence of floral scents. Journal of Plant Research 112: 291–306.

Chen, B.L & Nooteboom, H.P. (1993). Notes on Magnoliaceae III: the Magnoliaceae of China. Annals of the Missouri Botanical Garden 80: 999-1104.

Dandy, J.E. (1978). A revised survey of the genus *Magnolia* together with *Manglietia* and *Michelia*. In: N.G. Treseder, Magnolias: pp. 29–37. Faber and Faber, London, UK.

Doyle, J.J. & Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19:11–15.

Figlar, R.B. (2000). Proleptic branch initiation in *Michelia* and *Magnolia* subgenus *Yulania* provides basis for combinations in subfamily Magnolioideae. In: Y. Liu, H. Fan, Z. Chen, Q. Wu & Q. Zeng (eds), Proceedings of the International Symposium on the Family Magnoliaceae. pp. 14–25. Science Press, Beijing, China.

______. (2011). Chasing magnolias in Thailand. In: Rhododendrons, Camellias and Magnolias 2011, pp. 73–87. Royal Horticultural Society, London, UK.

Figlar, R.B. & Nooteboom, H.P. (2004). Notes on Magnoliaceae IV. Blumea 49: 87–100.

Frodin, D.G. & Govaerts, R. (1996). World checklist and bibliography of Magnoliaceae. Royal Botanic Gardens, Kew, UK. 72 pp.

Hill, K.W., Borsch, T., Müller, K., Soltis, D.E., Soltis, P.S., Savolainen, V., Chase, M.W., Powell, M.P., Alice, L.A., Evans, R., Sauquet, H., Neinhuis, C., Slotta, T.A.B., Rohwer, J.G., Campbell, C.S. & Chatrou, L.W. (2003). Angiosperm phylogeny based on *matK* sequence information. American Journal of Botany 90: 1758–1776.

Judd, W.S., Campbell, C.S., Kellogg, E.A., Stevens, P.F. & Donoghue, M.J. (2008). Plant Systematics: A Phylogenetic Approach, Third Edition. Sinauer Associates, Sunderland, Massachusetts, USA. 611pp.

Kim, S., Park, C.-W., Kim, Y.-D. & Suh, Y. (2001). Phylogenetic relationships in family Magnoliaceae inferred from *ndhF* sequences. American Journal of Botany 88: 717–728.

Nie, Z.-L., Wen, J., Azuma, H., Qiu, Y.-L., Sun, H., Meng, Y., Sun, W.-B. & Zimmer, E. A. (2008). Phylogeny and biogeographic complexity of Magnoliaceae in the Northern Hemisphere inferred from three nuclear data sets. Molecular Phylogenetics and Evolution 48: 1027–1040.

Mogensen, H.L. (1996). The hows and whys of cytoplasmic inheritance in seed plants. American Journal of Botany 83: 383–404.

Nooteboom, H.P. (1993). Magnoliaceae. In: K. Kubitzki (ed), The families and genera of vascular plants, Flowering plants, vol. II, Dicotyledons, pp. 391–401. Springer-Verlag, Berlin, Germany.

______. (2000). Different looks at the classification of the Magnoliaceae. In: Y. Liu, H. Fan, Z. Chen, Q. Wu & Q. Zeng (eds), Proceedings of the International Symposium on the Family Magnoliaceae, pp. 26–37. Science Press, Beijing, China.

Nooteboom, H.P. & Chalermglin, P. (2000). A new species of *Magnolia* (Magnoliaceae) from Thailand. Blumea 45: 245–247.

______. (2002). A new species of *Magnolia* (Magnoliaceae) from Thailand. Blumea 47: 541–543.

______. (2009). The Magnoliaceae of Thailand. Thai Forest Bulletin 37: 111–138.

Qiu, Y.-L., Li, L., Wang, B., Xue, J.-Y., Hendry, T.A., Li, R.-Q., Brown, J.W., Liu, Y., Hubson, G.T. & Chen, Z.-D. (2010). Angiosperm phylogeny inferred from sequences of four mitochondrial genes. Journal of Systematics and Evolution 48: 391–425.

Shi, S., Jin, H., Zhong, Y., He, X., Huang, Y., Tan, F. & Boufford, D.E. (2000). Phylogenetic relationships of the Magnoliaceae inferred from cpDNA *matK* sequences. Theoretical and Applied Genetics 101: 925–930.

Soltis, D.E., Soltis, P.S., Chase, M.W., Mort, M.E., Albach, D.C., Zanis, M., Savolainen, V., Hahn, W.H., Hoot, S.B., Fay, M.F., Axtell, M., Swensen, S.M., Prince, L.M., Kress, W.,J., Nixon, K.C. & Farris, J.S. (2000). Angiosperm phylogeny inferred from 18S rDNA, *rbcL*, and *atpB* sequences. Botanical Journal of the Linnean Society 133: 381–461.

Suh, Y. & Kim, S. (2001). Phylogeny of the family Magnoliaceae inferred from sequences of chloroplast DNA genes. In: D. S. Cho (ed), Proceedings of the 19th international symposium on plant biology: biodiversity-status, conservation and restoration, pp 62–87. The Catholic University of Korea, Puchon, Korea.

Swofford, D.L. (2003). PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), version 4. Sinauer Associates, Sunderland, Massachusetts, USA.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28: 2731-2739.

Wang, Y.-L., Li, Y., Zhang, S.-Z. & Yu, X.-S. (2006). The utility of *matK* gene in the phylogenetic analysis of genus *Magnolia*. Acta Phytotaxonomica Sinica 44: 135–147 (in Chinese).