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Author(s): Qiao-Ming Li, Tian-Hua He, Zai-Fu Xu
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Generic relationships of *Parashorea chinensis* Wang Hsie (Dipterocarpaceae) based on cpDNA sequences

Qiao-Ming Li[1,3], Tian-Hua He[2] & Zai-Fu Xu[1]

[1] *Kunming Branch of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Xuefu Rd. 88, Kunming, Yunnan 650223, P.R. China. lqm@xtbg.ac.cn (author for correspondence); xzf@xtbg.org.cn*
[2] *Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, P. R. China. thhe@public2.east.net.cn*
[3] *Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla 666303, P. R. China.*

The systematic position of *Parashorea chinensis* (Dipterocarpaceae) was investigated by using maximum parsimony analysis of nucleotide sequences of *matK*, *trnL* intron, and *trnL-trnF* intergenic spacer region of chloroplast DNA. Previous studies have placed the taxon in *Parashorea* or *Shorea*. Analyses indicate that *P. chinensis* belongs within *Parashorea* (80% bootstrap) rather than within *Shorea*. Furthermore, *P. chinensis* Wang Hsie and *P. chinensis* Wang Hsie var. *kwangsiensis* Lin Chi are further confirmed to be the same taxon.

KEYWORDS: *matK*, *Parashorea chinensis*, *Parashorea chinensis* var. *kwangsiensis*, *Shorea*, *trnF*, *trnL-trnF*.

INTRODUCTION

The family Dipterocarpaceae consists of three subfamilies distributed widely throughout tropical regions of the world (Ashton, 1982): Dipterocarpoideae (Asia), Monotoideae (Africa and South America), and Pakaraimoideae (South America). The Asian subfamily occurs mostly in closed forest; they dominate the emergent canopy of most lowland rainforests. Many of these species are major timbers in the hardwood markets in Asia (Ashton, 1982; 1988). China represented the northernmost distribution of this group. According to the *Flora Reipublicae Popularis Sinicae* (Tao & Tong, 1990), there are 13 species in five genera, distributed in southeastern Sitsang (Tibet), southern Yunnan, southwestern Guangxi, and Hainan.

Parashorea chinensis Wang Hsie is a rare and valuable new tree species discovered in Yunnan province of China in 1975 (Fig. 1). It is not only the canopy tree in the tropical rain forests, but also the keystone species, which is listed as specially conserved, Grade One, in China (Fu, 1992). The taxon was first found in Xishuangbanna (Cooperation Group of "*Parashorea chinensis*", 1977) and recognized as a new species in *Parashorea* Kurz. Another taxon found in Guangxi and Hekou of Yunnan was recognized as a variety of *P. chinensis* Wang Hsie, var. *kwangsiensis* ("Chingtienshu" Research Group, 1977). The only differences were the position and size of the sepals in fruit. Tao & Tong (1990) synonymized two taxa based on additional morphological investigations. Zhu (1992) also compared populations of this taxon in these three areas, finding that they have statistical differences in morphological characters, but the variance is continuous—not adequate for recognizing infraspecific taxa.

Despite clarity of the specific limits of *Parashorea chinensis*, the generic placement is still uncertain. Zhu & Wang (1992) listed 10 characters of *Parashorea*, *Shorea*, and *P. chinensis* (after Ashton, 1982, and Smitinand & al. 1980; Table 1). In *Parashorea*, seedling leaves are plicate; the fruit nut is globose, verrucose, and lenticellate; the bark surface has numerous conspicuous large pale corky lenticels; fruit sepals are valvate to narrowly imbricate. By contrast, in *Shorea*, the seedling leaves are not plicate; the fruit nut is ovoid and smooth; the lenticels are small, usually inconspicuous; and the fruit sepals are usually prominently imbricate. In comparison with these characters, *P. chinensis* clearly belongs in *Shorea* rather than in *Parashorea*. The taxon was transferred to *Shorea*, therefore, as *Shorea chinensis* (Wang Hsie) H. Zhu. Yang & Wu (1994) subsequently considered it as an later homonym of *Shorea chinensis* Merr., and renamed it as *Shorea wangtianshuea* Y. K. Yang et J. K. Wu nom. nov.

The principal aim of this paper is to determine whether *P. chinensis* belongs in *Parashorea* or *Shorea*. Here, molecular phylogenetic analysis was performed based on nucleotide sequences of *matK*, *trnL* intron, and *trnL-trnF* intergenic spacer region (IGS) in chloroplast DNA. These genes are known to be evolve fast in the chloroplast genome (Neuhaus & Link, 1987; Gielly & Taberlet, 1994) and often are used to study relationships among genera (Johnson & Soltis, 1994; Plunkett & al., 1997), among species (Gielly & Taberlet, 1994), and also within species (Fujii & al., 1995, 1997).

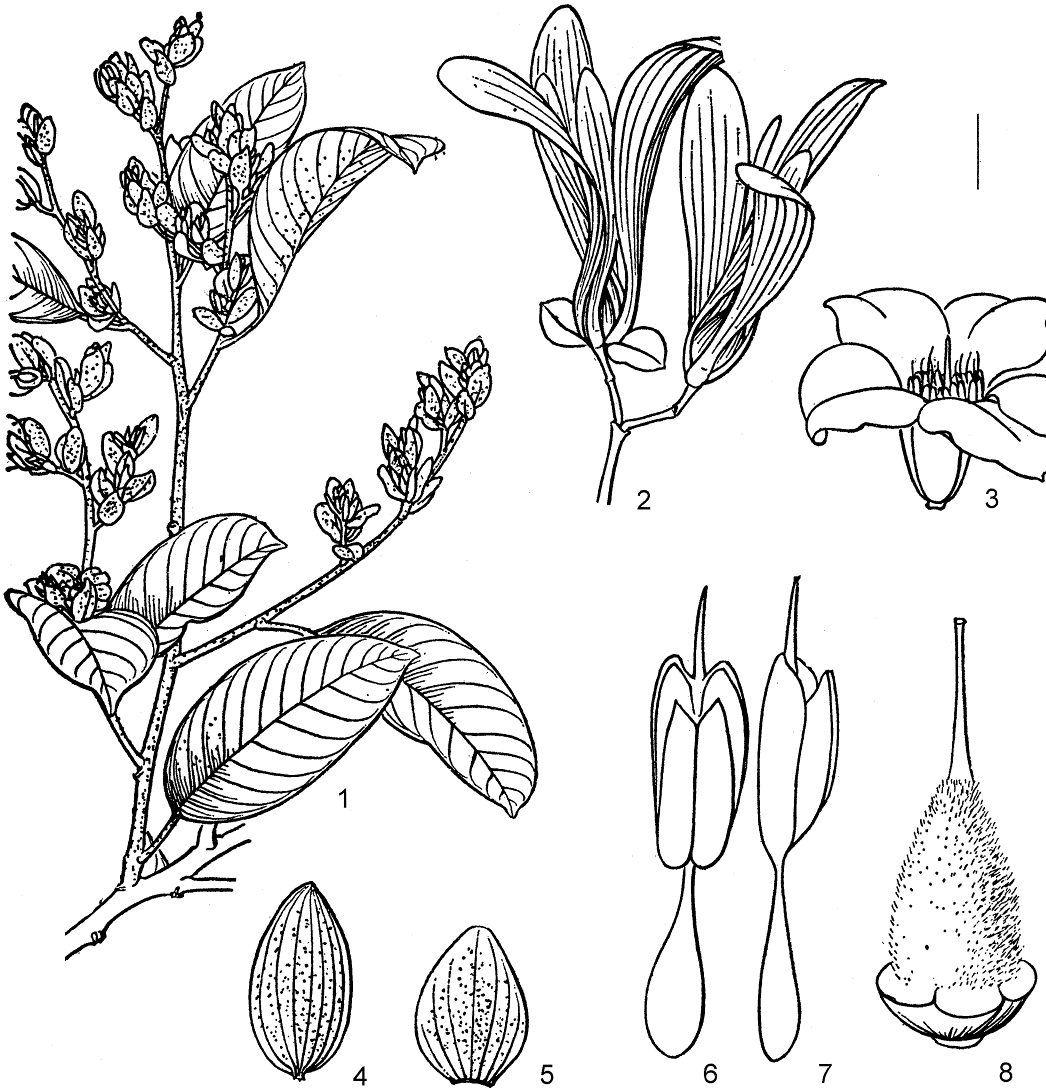

Fig. 1. *Parashorea chinensis* Wang Hsie (Tao & Tong, 1990). 1, flowering branch; 2, fruiting branch (scale = 1.5 cm); 3, flower (scale = 4.0 mm); 4, bract (scale = 4.0 mm); 5, stipule (scale = 4.0 mm); 6, stamen (front; scale = 0.7 mm); 7, stamen (lateral); 8, ovary (scale = 1.5 mm).

MATERIALS AND METHODS

Plant materials. — Leaves of *Parashorea chinensis* (including var. *kwangsiensis*) were collected from Mengla county (HDH), in the south of Yunnan province, (*Q. M. Li 2000411*, XTBG) and Napo county (NP), southwest Guangxi autonomous region (*Q. M. Li 2000412*, XTBG). The leaves were dried in silica gel in Apr 2000. Voucher specimens were deposited at the Xishuangbanna Tropical Botanical Garden Herbarium (XTBG).

Total DNA was isolated according to the protocol of Doyle & Doyle (1990) with some modifications. DNA fragments of the *matK*, *trnL-trnF* IGS region, and *trnL* intron were amplified by PCR using *Tag* polymerase, universal primers for *matK* ("AF" and "8R" of Ooi & al., 1995), *trnL-trnF* spacer region, and *trnL* intron ("c" and "f" of Taberlet & al., 1991). PCR amplification for *matK* consisted of initial denaturation at 94°C (2 min), 50°C annealing (20 s), and 72°C extension (2 min) for 4 cycles, followed by 36 cycles of 94°C denaturation (20 s), 50°C annealing (20 s), and 72°C extension (2 min), with a final extension for 6 min at 72°C. PCR amplification for *trnL-trnF* IGS region, *trnF* intron consisted of initial denaturation at 94°C (3 min), followed by 32 cycles of 94°C denaturation (1 min), 48°C annealing (1 min), and 72°C extension (2 min), with a final extension for 7 min at 72°C. Amplification products were purified

Table 1. Comparison of diagnostic characters among *Parashorea*, *Shorea* and *P. chinensis* after Ashton (1982), Smitinand & al. (1980), Zhu & Wang (1992), and Yang & Wu (1994).

Characters	*Parashorea* (24 spp.)	*Parashorea chinensis*	*Shorea* (357 spp.)
1. Bark surface	with numerous conspicuous large pale corky lenticels	lenticels small and inconspicuous	lenticels small, usually inconspicuous
2. Leaf lateral nerves	straight, running at a more acute angle to the midrib, curving only near the margin	run at a less acute angle to midrib, curved from their bases	run at a less acute angle to the midrib, curved from their bases
3. Leaf undersurface	more or less glaucous	not glaucous	not glaucous
4. Stipules	usually linear-lanceolate	ovate	linear to oblong, usually larger
5. Seedling leaves	plicate	not plicate	not plicate
6. Sepals	equal, very narrowly imbricate or valvate	3 outer longer than 2 inner, prominently imbricate	3 outer longer, narrower than 2 inner, prominently imbricate
7. Petals	falling separately	usually connate at base when falling	usually connate at base when falling
8. Fruit nut	globose, verrucose, lenticellate	ovoid, smooth	ovoid, smooth
9. Sepals in fruit (relative length)	subequal or 3 outer slightly larger than 2 inner	3 outer larger than 2 inner	3 outer usually much larger than 2 inner
10. Sepals in fruit (arrangement)	valvate to narrowly imbricate	prominently imbricate (immature), imbricate or narrowly imbricate (mature)	usually prominently imbricate

by Wizard PCR Preps DNA Purification System, following manufacturer's instructions. Sequencing reactions consist of 15 s at 95°C, 8 s at 52°C, 4 min 30 s at 60°C for 30 cycles. All the amplification reactions were performed in PT-200. DNA sequencing was performed using an Applied BioSystems 377 automated sequencer with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit following the supplier's instructions. Sequencing primers for the *matK*, *trnL-trnF* spacer region and *trnL* intron were the same ones as those used as PCR primers.

Sequences of *Parashorea lucida*, *Shorea bracteolata, S. macroptera*, *S. ovalis*, and other 12 taxa of Dipterocarpaceae (*Anisoptera laevis*, *A. oblonga*, *Cotylelobium lanceolatum*, *Dipterocarpus baudii*, *D. kerrii*, *Dryobalanops aromatica*, *D. oblongifolia*, *Neobalanocarpus heimii*, *Hopea nervosa*, *H. odorata*, *Vatica odorata*, *Upuna borneensis*), with *Tilia kiusiana* as outgroup, were downloaded from GenBank. Their accession numbers are shown in Table 2. The final dataset comprised 19 taxa.

Phylogenetic analysis. — Alignments of obtained sequences were performed by Clustal V (Higgins, 1994). The aligned matrix is available on request from the corresponding author. Phylogenetic analysis was performed by the maximum parsimony method, using a branch-and-bound search with PAUP 4.0b4a (Swofford, 1998). Bootstrap analysis was conducted using 1000 replications. Genetic distances (mean distances and absolute distances) were calculated using pairwise distance for all pairs of sequences.

RESULTS

Sequences of *matK*, *trnL-trnF* IGS region, and *trnL* intron of chloroplast DNA. — The characteristics of *matK*, *trnL-trnF* IGS region, and *trnL* intron were described in detail by Kajita & al. (1998). The *matK* fragments studied here were 1011 bp except for the outgroup *Tilia kiusiana* with 1005 bp. There were no indels among sequences of Dipterocarpaceae, but a 6-base indel was found between dipterocarp species and *Tilia kiusiana*.

For the *trnL-trnF* IGS region, the length of the intergenic spacer region varies from 379 to 401 bp. Only considering the genera *Shorea* and *Parashorea*, *Shorea bracteolata* had a 6 bp deletion between positions 19 and 24; *Parashorea chinensis* and *P. chinensis* var. *kwangsiensis* had a 9 bp deletion between positions 125 and 133; *Shorea ovalis* had a 4 bp deletion between positions 182 and 185; at position 222, *S. bracteolata* had one insertion; *Parashorea lucida* had one deletion at position 342.

In the intron of *trnL*, the length varies from 463 to 537 bp. Indels are seen in *Shorea bracteolata*: one at positions 20 and 21 (2 bp), one at positions 33 and 34 (2 bp), one between positions 50 and 57 (8 bp), one at positions 69, 70, 77, 123, 124 and 320 (all 2 bp). Furthermore, one insertion also occurred at position 271 in *S. bracteolata*. In other species of these two genera, *Parashorea lucida* and *P. chinensis* had one deletion at position 38, and *Shorea ovalis* had one insertion at position 37.

The aligned length of *matK*, *trnL-trnF* IGS region and *trnL* intron is 1982 bp, which provides 134 phyloge-

Table 2. List of taxa included in the analysis. Accession numbers (*matK*, *trnL* intron, *trnL/F*) except those for *P. chinensis* are from GenBank. All other sequences are from Kajita & al. (1998).

Anisoptera laevis Dyer, AB006370, AB006387, AB006404; *A. oblonga* Dyer, AB006371, AB006388, AB006405; *Cotylelobium malayanum* Sloot., AB006372, AB006389, AB006406; *Dipterocarpus baudii* Korth., AB006376, AB006393, AB006410; *D. kerrii* King, AB006375, AB006392, AB006409; *Dryobalanops aromatica* Gaertn., AB006377, AB006394, AB006411; *D. oblongifolia* Dyer, AB006378, AB006395, AB006412; *Hopea nervosa* King, AB006384, AB006401, AB006418; *H. odorata* Roxb., AB006385, AB006402, AB006419; *Neobalanocarpus heimii* (King) Ashton, AB006383, AB006400, AB006417; *Parashorea chinensis*, AY305717, AY305719, AY305721; *P. chinensis* var. *kwangsiensis*, AY305718, AY305720, AY305722; *Parashorea lucida* (Miq.) Kurz, AB006382, AB006399, AB006416; *Shorea bracteolata* Dyer, AB006381, AB006398, AB006415; *S. macroptera* Dyer, AB006379, AB006396, AB006413; *S. ovalis* (Korth.) Bl., AB006380, AB006397, AB006414; *Tilia kiusiana* Makino et Shirasawa, AB006386, AB006403, AB006420; *Upuna borneensis* Sym., AB006374, AB006391, AB006408; *Vatica odorata* (Griff.) Sym., AB006373, AB006390, AB006407.

netically informative sites when gaps are treated as missing. The nucleotide sequences of *matK*, *trnL-trnF* IGS region and *trnL* intron of *P. chinensis* and *P. chinensis* var. *kwangsiensis* were identical.

Phylogenetic trees using the three regions. — Maximum parsimony analysis resulted in six equally parsimonious trees. The 50% majority-rule consensus tree of 1000 bootstrap replications is shown in Fig. 2, with a length of 595, a consistency index (CI) of 0.8840, a homoplasy index (HI) of 0.1160, and retention index (RI) of 0.7940. In the consensus tree, we could recognize two groups of genera: one consisting of *Anisoptera*, *Upuna*, *Cotylelobium* and *Vatica* and the other of *Dipterocarpus*, *Dryobalanops*, *Neobalanocarpus*, *Hopea*, *Shorea* and *Parashorea*. Bootstrap probabilities for the two groups are 69% and 68%. *Parashorea chinensis* and *P. chinensis* var. *kwangsiensis* occur in the clade *Dipterocarpus-Dryobalanops-Shorea-Parashorea-Neobalanocarpus-Hopea* and *P. lucida* joins at 80%. *Parashorea chinensis* and *P. chinensis* var. *kwangsiensis* ally together at 99% bootstrap probability. *Parashorea* (*P. lucida*, *P. chinensis* and *P. chinensis* var. *kwangsiensis*) is sister to the clade consisting of two *Shorea* species with 96% bootstrap probability. The topology of the parsimony tree is consistent with that from Kajita & al. (1998).

Pairwise genetic divergence. — The sequences of the three regions were analyzed among the 19 species using PAUP. Pairwise genetic distances are shown in Table 3. *Parashorea chinensis* has the smallest divergence from *P. lucida*; the mean distance is 0.00696 and the absolute distance is 13. The former has smaller divergence from *Shorea ovalis* (mean distance 0.00750 and absolute distance 14), followed by *S. macroptera* (0.01016, 19). *Parashorea chinensis* is very divergent from *S. bracteolata* (mean distance 0.02167 and absolute distance 40). Important is that *P. chinensis* has a closer relationship with *Parashorea* than with the three species of *Shorea*.

DISCUSSION

Evaluation of results from the consensus tree (Fig. 2) allow us to fix the phylogenetic position of *Parashorea chinensis*. The two samples (*P. chinensis* and *P. chinensis* var. *kwangsiensis*) show no sequence variation and together are sisters to *P. lucida* (80% bootstrap probability). *Parashorea* is sister to the clade consisting of two *Shorea* species (*S. macroptera* and *S. ovalis*). Based on the molecular data, therefore, *P. chinensis* has closer rela-

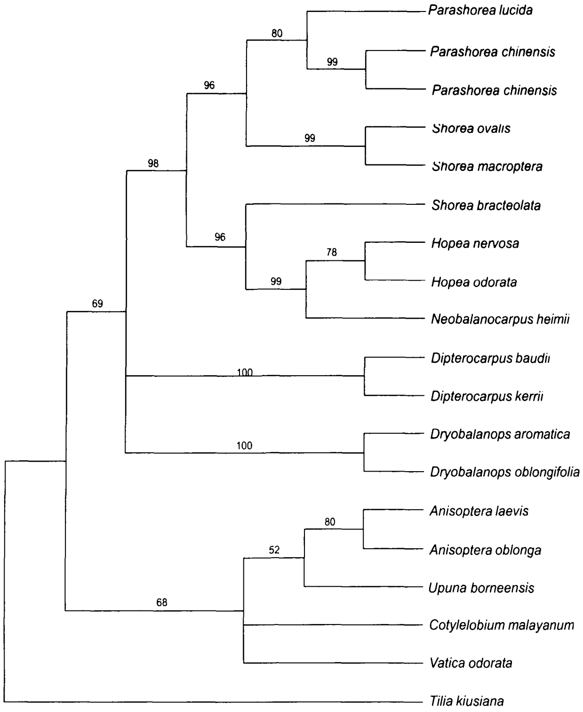

Fig. 2. 50% majority-rule consensus tree of six equally most parsimonious trees for Southeast Asian genera of Dipterocarpaceae constructed by maximum parsimony method on nucleotide sequences of the *matK*, *trnL-trnF* IGS region, and *trnL* intron with gaps being treated as missing data. Length = 595 steps, CI = 0.8840, RI = 0.7940. Bootstrap values are indicated above branches. *Tilia kiusiana* is the outgroup.

Table 3. Pairwise genetic distances among trees baes on *matK*, *trnL-trnF* IGS region, and *trnL* intron gene sequences. Absolute distances are below diagonal, mean distances above.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	-	0.0053	0.0295	0.0316	0.0347	0.0277	0.0277	0.0346	0.0255	0.0314	0.0252	0.0252	0.0277	0.0361	0.0271	0.0261	0.1650	0.0138	0.0129
2	10	-	0.0290	0.0342	0.0347	0.0283	0.0283	0.0351	0.0298	0.0330	0.0268	0.0268	0.0293	0.0366	0.0287	0.0277	0.1651	0.0117	0.0123
3	55	54	-	0.0501	0.0528	0.0446	0.0446	0.0499	0.0467	0.0472	0.0421	0.0421	0.0451	0.0543	0.0445	0.0446	0.1698	0.0295	0.0270
4	59	64	93	-	0.0042	0.0305	0.0305	0.0353	0.0342	0.0316	0.0263	0.0263	0.0289	0.0374	0.0294	0.0284	0.1719	0.0364	0.0339
5	65	70	98	8	-	0.0332	0.0332	0.0380	0.0374	0.0342	0.0285	0.0285	0.0321	0.0379	0.0326	0.0316	0.1778	0.0396	0.0371
6	52	53	83	57	62	-	0.0000	0.0288	0.0293	0.0256	0.0182	0.0182	0.0214	0.0308	0.0213	0.0203	0.1646	0.0304	0.0263
7	52	53	83	57	62	0	-	0.0288	0.0293	0.0256	0.0182	0.0182	0.0213	0.0307	0.0213	0.0203	0.1645	0.0304	0.0263
8	65	66	93	66	71	54	54	-	0.0144	0.0117	0.0198	0.0198	0.0224	0.0243	0.0229	0.0219	0.1708	0.0373	0.0359
9	48	56	87	64	70	55	55	27	-	0.0144	0.0214	0.0214	0.0234	0.0259	0.0240	0.0229	0.1717	0.0330	0.0305
10	59	62	88	59	64	48	48	22	27	-	0.0155	0.0155	0.0181	0.0210	0.0176	0.0165	0.1690	0.0351	0.0332
11	47	50	78	49	53	34	34	37	40	29	-	0.0000	0.0070	0.0217	0.0102	0.0075	0.1648	0.0278	0.0269
12	47	50	78	49	53	34	34	37	40	29	0	-	0.0070	0.0217	0.0102	0.0075	0.1648	0.0278	0.0269
13	52	55	84	54	60	40	40	42	44	34	13	13	-	0.0243	0.0123	0.0101	0.1666	0.0299	0.0295
14	67	68	100	69	70	57	57	45	48	39	40	40	45	-	0.0237	0.0238	0.1740	0.0394	0.0384
15	51	54	83	55	61	40	40	43	45	33	19	19	23	44	-	0.0043	0.1664	0.0298	0.0289
16	49	52	83	53	59	38	38	41	43	31	14	14	19	44	8	-	0.1651	0.0299	0.0290
17	306	306	312	319	332	304	304	316	318	313	304	304	308	318	308	305	-	0.1686	0.1664
18	26	22	55	68	74	57	57	70	62	66	52	52	56	73	56	56	312	-	0.0134
19	24	23	50	63	69	49	49	67	57	62	50	50	55	71	54	54	306	25	-

1, *Anisoptera laevis*; 2, *A, oblonga*; 3, *Cotylelobium lanceolatum*; 4, *Dipterocarpus baudii*; 5, *D, kerrii*; 6, *Dryobalanops aromatica*; 7, *D. oblongifolia*; 8, *Hopea nervosa*; 9, *H.odorata*; 10, *Neobalanocarpus heimii*; 11, *Parashorea chinensis*; 12, *P. chinensis* var, *kwangsiensis*; 13, *P. lucida*; 14, *Shorea bracteolata*; 15, *S. macroptera*; 16, *S. ovalis*; 17, *Tilia kiusiana;* 18, *Upuna borneensis*; 19, *Vatica odorata*.

tionships with *Parashorea* than with *Shorea*. However, considering only the morphological characters of *P. chinensis* mentioned earlier (Zhu & Wang, 1992; Yang & Wu, 1994; Table 1), it has more similarity with *Shorea* than with *Parashorea*. Zhu & Wang (1992), in fact, placed *P. chinensis* in *Shorea*.

Symington (1974) considered that the most diagnostic traits of *Parashorea* are the glaucescence of immature leaves, the acute angle between main nerves and midrib, and the large corky lenticels at the base of the bole and on the shoulders of the buttresses. Furthermore, the stamens have long pollen sacs. Persistence of stipules in juveniles is also a useful feature. *Parashorea chinensis*, however, lacks most of these characters of *Parashorea*.

According to P. Ashton (pers. comm.), species of *Parashorea* share a range of characters of flower and fruit morphology, wood, bark anatomy and morphology, buttress morphology, and leaf and seedling morphology. *Parashorea chinensis*, however, lacks some of these characters; notably, there are some differences in the stamens, the nut is ovoid (not cylindrical), and the usually prominent pale lenticels are obscured by tomentum. Also, the leaves are not plicately folded, and seedling leaves are not peltate, nor silvery beneath. But in fact, the flowers of *P. chinensis* are not significantly different from some other species in the genus, nor are the sepals of the fully ripe fruit more imbricate than in some other species. *Parashorea chinensis* is not the only *Parashorea* to lack some generic characters, and *P. parvifolia* of Borneo is in some respects intermediate. It differs notably in its relative glabrousness and shares small leaves with absence of clear plication.

According to Li & al. (2002) and Ashton (pers. comm.), *Parashorea chinensis* exhibits bark with prominent lines of lenticels on buttress ridges and in fresh fissures; pubescence on the nut, which obscures the normally distinct pale lenticels, and subequal, narrowly imbricate mature fruit sepals. Ashton (1982) considered the following characters to be distinguishing features between *Parashorea* and *Shorea*: fruit sepals subequal, narrowly imbricate, nut globose, verrucose, lenticellate. Li & al. (2002) considered the following characters to differentiate *Parashorea* and *Shorea*: fruit sepals subequal, imbricate; bark prominently pale lenticellate. On the whole, *Parashorea chinensis* has some characters of *Shorea*, but it has also some characters of *Parashorea*. Although *Parashorea* was divided from *Shorea*, the former is still the closest generic relative, and many of its distinguishing characteristics would justify its status as a section within the latter genus. The subequal fruit sepals (which *P. chinensis* shares) and nut, however, do suggest generic status as much as do characters that define *Hopea* and *Shorea*.

The genus *Shorea* was divided into four groups (encompassing 11 sections) of phylogenetically diverse species by Kamiya & al. (1998). In the present study, molecular evidence indicates that *P. chinensis* has a closer relationship with *Parashorea* than with species of

Shorea. Symington (1943) pointed out that *Parashorea* is a well-defined taxonomic group with close affinity to *Shorea*, in particular to sect. *Anthoshorea* (including *Shorea bracteolata*). In our consensus tree, however, *Parashorea* joins with *Shorea ovalis* (sect. *Ovalis*) and *Shorea macroptera* (sect. *Mutica*). The genus *Shorea* is apparently polyphyletic, but as other sections are not represented in the present study, the full relationships among *Shorea* species cannot be fully assessed. Thus, a larger sample of *Shorea* and *Parashorea* species, plus analysis of nuclear sequences, will be needed for a more accurate determination of the position of the species.

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