Polyphyly of the genus *Actinodaphne* (Lauraceae) inferred from the analyses of nrDNA ITS and ETS sequences

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Abstract   A phylogenetic analysis of the genus *Actinodaphne* (Lauraceae) was conducted using sequences from the nrDNA (nuclear ribosomal DNA) internal transcribed spacer (ITS) and external transcribed spacer (ETS) regions. Maximum parsimony (MP), maximum likelihood (ML) and Bayesian phylogenetic analysis methods were employed to analyse the data sets (ITS, ETS and ITS/ETS). All analyses suggested that the sampled *Actinodaphne* species were not monophyletic, clustering instead as several clades amongst other genera in the tribe Laureae. This result indicates that the generic delimitations between *Actinodaphne* and related genera need to be reevaluated, and that inflorescence features, which are mostly consistent with the current molecular inferences, might be the both important and reliable characters for their redefinition. However, because of the relatively small number of taxa sampled, and conflicts between the separate analyses, more detailed studies are required to clarify the relationships which emerged in our study and to allow for more precise generic delimitation and hypotheses about phylogeny in *Actinodaphne*.

Key words   *Actinodaphne*, ETS, ITS, Lauraceae, phylogenetic analysis, Bayesian analysis.

The genus *Actinodaphne* Nees (Lauraceae) with about 100 species occurs mainly in tropical-subtropical Asia and is an important component of tropical forests (Rohwer, 1993; van der Werff, 2001). In China 19 species are distributed from the southwest to east, with four species in Taiwan (Li et al., 1984). This genus is distinguished from other Lauraceae by its whorled, usually penninerved, rarely sub-triplinerved leaves; (pseudo-) racemose or pseudo-umbellate inflorescences; and imbricate, deciduous involucral bracts at the base of the inflorescence (Nees, 1836; Rohwer, 1993). Many species are used locally for wood or medicine, i.e. the widely used wood of *A. nantoensis* (Hay.) Hay. and *A. mushanensis* (Hay.) Hay. for architecture and furniture, and the important medical properties of the roots of *A. cupularis* (Hemsl.) Gamble and leaves of *A. pilosa* (Lour.) Merr. (Li et al., 1984).

The systematic position of this genus had been in dispute. Nees (1836) and Allen (1938) placed *Actinodaphne* into the tribe Laureae based on its introrse anther cells. Because of its lack of decussate and persistent involucral bracts, however, Kostermans (1957) suggested that *Actinodaphne* was closely related to *Ocotea* Aubl., *Cinnamomum* Trew and *Sassafras* J. Presl, and therefore placed it into the tribe Cinnamomineae. Recently, Li et al. (1984), Rohwer (1993) and van der Werff (1991, 2001) argued that *Actinodaphne* should be returned to the Laureae, mainly based on the reexamination of its inflorescence involucre, and this was

further supported by recent molecular phylogenetic studies (Rohwer, 2000; Chanderbali et al., 2001). Using data from the chloroplast gene *matK* and nuclear ribosomal DNA sequences, Li et al. (2004) similarly observed close relationships between *Actinodaphne, Litsea* Lam., *Lindera* Thunb. and *Neolitsea* Merr., further strengthening its placement in the Laureae.

Despite the agreement on the systematic position of *Actinodaphne* in Laureae, the circumscription of this genus remains unresolved. In the phylogenetic analyses of the "core" Laureae, Li et al. (2004) suggested that *Actinodaphne* might be separated into two groups with different origins, and this was also supported by previous morphological analyses (Li & Christophel, 2000). Van der Werff (2001) also noticed that there were different inflorescence forms in *Actinodaphne* and it was difficult to distinguish some *Actinodaphne* species from *Litsea* ones with similar inflorescences. In light of these studies, it appears that *Actinodaphne* might be polyphyletic, although all the authors agree that many more samples and characters are required before a well-resolved phylogeny and classification might be produced.

The nrDNA internal transcribed spacers (ITS) have become widely used as a source of characters for phylogenetic studies of closely related plant species (e.g. Baldwin et al., 1995; Ge et al., 1997; Hong et al., 2001; Gao et al., 2003; Roalson & Friar, 2004), and this DNA fragment was demonstrated to be powerful in the recent Lauraceae phylogenetic constructions (Chanderbali et al., 2001; Li et al., 2004). In addition, the external transcribed spacer (ETS) has shown potential for phylogenetic studies of angiosperms as it often shows greater variation than ITS and should be considered a good candidate when the ITS lacks sufficient phylogenetic signal (Baldwin & Markos, 1998; Bena et al., 1998), although exceptions exist in some genera (Soltis & Soltis, 1998).

Combined analyses of sequences from these two nuclear DNA fragments could be expected to produce a more robust phylogeny and the goals of our study using maximum parsimony (MP), maximum likelihood (ML), and Bayesian analysis of sequence data from ITS and ETS are to: (1) test whether *Actinodaphne* is monophyletic; (2) examine the relationships of *Actinodaphne* within the tribe Laureae; and (3) evaluate the species relationships within this genus against previous morphological investigations.

1 Material and methods

1.1 Plant material

A total of 13 *Actinodaphne* species were included in this study, 11 from China, plus one each from Malaysia and Singapore. Because *Actinodaphne* might be polyphyletic (Li et al., 1984; Rohwer, 1993, 2000; van der Werff & Richter, 1996; Chanderbali et al., 2001; Li et al., 2004), five representative genera from the tribe Laureae were selected as outgroups in our analysis. A complete list of the species sampled, along with collection and voucher information is provided in Table 1.

1.2 DNA extraction, PCR and sequencing

Total DNA was extracted using a modified CTAB protocol (Doyle & Doyle, 1987) and performed as described by Li et al. (2004). DNA was cleaned using QIAquick® PCR purification Kit (Qiagen, Germany), and DNA concentration was determined by visual comparison with a positive control (λ100 ladder, concentration 10, 20 ng) on an ethidium bromide-stained agarose gel.

Templates of the whole nrDNA ITS region were amplified successfully in most cases using primer pair of ITSF/ITS4 (White et al., 1990; Chanderbali et al., 2001), but if these failed, especially for poor-quality DNA, the primer combinations of ITSF/ITS2 and ITS3/ITS4 (White et al., 1990; Chanderbali et al., 2001) were used to amplify ITS1 and ITS2 (including the 5.8S nrDNA region) separately. For most individuals, PCR amplification

Table 1 Species of the genus *Actinodaphne* and outgroup taxa included in the analysis

Species	Locality	Voucher	GenBank number for ITS	GenBank number for ETS
Ingroups				
Actinodaphne kweichowensis Yang & P. H. Huang 黔桂黄肉楠	Dongshan, Guangxi (广西东山), China	H. Q. Li (黎焕奇) 40091 (KUN 0106643)	AY817114	AY817124
A. omeiensis (Liou) Allen 峨眉黄肉楠	Mt. Emeishan, Sichuan (四川峨眉山), China	G. H. Yang (杨光辉) 55824 (KUN 0047252)	AY817117	AY817127
A. pilosa (Lour.) Merr. 毛黄肉楠	Yongning, Guangxi (广西邕宁), China	L. S. Xie (谢立山) 613 (KUN 0047277)	AY817115	AY817125
A. trichocarpa Allen 毛果黄肉楠	Daguan, Yunnan (云南大关), China	B. S. Sun (孙必兴) 0757 (KUN 0047286)	AY817116	AY817126
A. tsaii Hu 马关黄肉楠	Malipo, Yunnan (云南麻栗坡), China	K. M. Feng (冯国楣) 22638 (KUN 0047322)	AY817119	AY817129
A. cupularis (Hemsl.) Gamble 红果黄肉楠	Shidian, Guizhou (贵州施甸), China	Wuyishan Exped. (武夷山考察队) 1693 (KUN 0601976)	AY817113	AY817123
A. henryi Gamble 思茅黄肉楠	Mengla, Yunnan (云南勐腊), China	J. Li (李捷) 2002032 (HITBC)	AY817120	AY817130
A. obovata (Nees) Bl. 倒卵叶黄肉楠	Mengla, Yunnan (云南勐腊), China	H. W. Li (李锡文) 1 (HITBC)	AY265398	AY934880
A. forrestii (Allen) Kosterm. 毛尖树黄肉楠	Mengla, Yunnan (云南勐腊), China	H. W. Li (李锡文) 2 (HITBC)	AY265399	AY934881
A. paotingensis Yang & P. H. Huang 保亭黄肉楠	Baoting, Hannan (海南保亭), China	Hainan East Exped. (海南东路队) 962 (IBK 00003425)	AY817118	AY817128
A. lecomtei Allen 柳叶黄肉楠	Without precise locality, Guangxi (广西, 具体地点不详), China	C. Q. Li (李彩祺) 3979 (IBK 00003410)	AY817112	AY817122
Actinodaphne sp.	Botanical Garden, Singapore	SING	AY817112	AY817122
A. sesquipedalis Hook. f. & Thoms. ex Hook. f.	Kuala Lumpur, Malaysia	Saw Leng Guan s.n. (KEP)	AF272247	–
Outgroups				
Lindera megaphylla Hemsl.	Mengla, Yunnan (云南勐腊), China	H. W. Li (李锡文) 7 (HITBC)	AY265406	AY934882
Litsea glutinosa (Lour.) C. B. Rob.	Mengla, Yunnan (云南勐腊), China	H. W. Li (李锡文) 21 (HITBC)	AY265403	AY934883
Neolitsea levinei Merr.	Mengla, Yunnan (云南勐腊), China	H. W. Li (李锡文) 29 (HITBC)	AY265401	AY934884
Parasassafras confertiflora (Meisner) D. G. Long	Ximeng, Yunnan (云南西盟), China	Y. Y. Qian (钱义咏) 921 (KUN 0104560)	AY265395	AY934885
Sinosassafras flavinervia (Allen) H.W. Li	Mt. Ailaoshan, Yunnan (云南哀牢山), China	Y. H. Liu (刘玉洪) s.n.	AY265394	AY934886

resulted in a single band, but for some specimens, two size classes of PCR products were obtained. To isolate each of the two bands, the PCR products were blunt-end ligated into the *Eco*RV sites of the PMD 18-T Simple Vector after purification, using the Original TA® Cloning Kit (available from TaKaRa Biotechnology) and the fragments obtained following the manufacturer's protocol.

Fragments of the nrDNA ETS region were amplified using the primer pair ETS1/18S-IGS (Li et al., unpublished; Baldwin & Markos, 1998), with the ETS1 primer

designed by Li et al. following the method of Baldwin and Markos (1998). The primer sequences used are shown in Table 2. In order to reduce the likelihood of selective amplification of pseudogenes or paralogous ITS copies, 10% DMSO was included in all amplifications (Buckler & Holtsford, 1996; Buckler et al., 1997) and a negative control was used for every primer combination to detect PCR contamination.

Table 2 Primers used for amplification and sequencing ITS and ETS in the *Actinodaphne*

Primers (ITS, ETS)	Sequence 5'-3'	Source
Forward		
ITSF	GCTACGTTCTTCATCGATGC	Chanderbali et al., 2001
ITS3	GCATCGATGAAGAACGTAGC	White et al., 1990
Reverse		
ITS2	GCTACGTTCTTCATCGATGC	White et al., 1990
ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990
Forward		
ETS1	CCAGAACTCGCACTTGCTGAGCTT	Li et al., unpublished
Reverse		
18S-IGS	GAGACAAGCATATGACTACTGGCAGGATCAACCAG	Baldwin & Markos, 1998

The PCR products were purified using the Qiagen QIAquick® PCR Purification Kit following the manufacturer's protocols. To sequence the ITS nrDNA fragments, a series of reactions were run, each with one different internal primer (Table 2), thus creating overlapping fragment sequences that between them covered the entire spacer and 5.8S nrDNA regions along both strands. Sequencing of the nrDNA ETS region using the primer 18S-IGS obtained the fragment that included the 3' ETS region. Cycle sequencing was carried out directly on the purified PCR product using the ABI Prism Big Dye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA), using 1 µL of primer, 10 ng of DNA template, 1.5 µL of Big Dye (version 3.1), and then using ddH$_2$O to make up a final reaction volume of 5 µL. Cycle sequencing reactions were as follows: (1) 30 s denaturation (96 °C), (2) 15 s annealing (50 °C), and 4 min elongation (60 °C) with 25 cycles. Cleaned products were then sequenced directly in an Applied Biosystems 3100 DNA automated sequencer.

1.3 Sequence alignment

Sequences were aligned individually using the software program Lasergene/Megalign (DNASTAR, 1998), allowing uncertainties either to be resolved or recorded as ambiguities. The boundary of ITS and 3' ETS sequences was determined through comparison with other Lauraceae species from GenBank and all the obtained ETS sequences respectively. The sequences were then truncated to include ITS and 3' ETS of the nrDNA gene. The consensus sequences for the analyzed taxa were then realigned using ClustalX (Thompson et al., 1997) and modified manually, if necessary, using BioEdit version 5.0.6. (Hall, 1999).

1.4 Phylogenetic analysis

The aligned submatrices were analyzed both individually and together. Only phylogenetic informative characters were analyzed, and gaps were scored as missing data. Because the two sequenced regions (ITS, ETS) used in this study are part of a tandem repeat within the diploid nuclear genome, possible conflict between the data sets was evaluated with an incongruence length difference (ILD) test (Farris et al., 1994, 1995) prior to combining the data. This test, implemented as the partition homogeneity test in PAUP* version 4.0b10 (Swofford, 1998), determines whether the original data partitions differ significantly from randomly shuffled partitions of the combined data set.

Both MP and ML analyses were performed using PAUP* version 4.0b10 (Swofford,

1998). Heuristic searches were employed (ACCTRAN, 1000 random addition cycles, TBR branch swapping, STEEPEST DESCENT, MULTREES in effect). Clade support was estimated using 1000 heuristic bootstrap replicates with 100 random addition cycles per replicate, 20 trees saved from each addition cycle, TBR branch swapping, and STEEPEST DESCENT options (Felsenstein, 1985; Hills & Bull, 1993; Li et al., 2004; Roalson & Friar, 2004).

The Tamura and Nei (1993) model of evolution with rate heterogeneity and among-site rate variation was used in the ML analysis based on the result of Modeltest 3.06 (Posada & Crandall, 1998). The modeltest analysis tested the fit of various ML models to the data set and estimated base change frequencies, proportion of variable characters, shape of the gamma distribution, and chose the model that best fitted the data using the Hierarchical Likelihood Ratio Tests (Posada & Crandall, 1998; Roalson & Friar, 2004). The parameters assigned to the data set for this analysis are shown in Appendix A.

Bayesian phylogenetic analyses were performed using MrBayes version 3.0b4 (Hall, 2001; Huelsenbeck & Ronquist, 2001). The same model and number of base change frequencies used in the ML search were used, with Bayesian analysis started from a random tree. 10^6 generations were computed for four parallel chains applying six possible substitution types and gamma-distribution of substitution rates and the Markov chains were sampled at intervals of 100 generations, resulting in a final set of 10001 sample points. Plotting likelihood values for the four analyses shows that "stationary" was achieved for each sequence analysis as follows: (1) ITS: 116; (2) ETS: 905; (3) ITS/ETS: 123. These were therefore discarded as "burn-in", with the remaining trees presented as 50% majority rule consensus trees, and the percentage of sample points recovered any particular clade represented its posterior probability (Huelsenbeck & Ronquist, 2001).

2 Results

2.1 Sequence characteristics

The length of the unaligned ITS sequences covering the entire spacer and 5.8S nrDNA regions varied from 605 to 627 bp. The aligned ITS data matrix was 668 bp in length, of which 64 characters (9.6%) were informative. The percentage of G+C in the *Actinodaphne* ITS sequences varied from 61.6% to 67.2%. The whole nrDNA ITS region of three species (*A. lecomtei*, *Actinodaphne* sp. and *A. paotingensis*) could not be amplified successfully using primer pair of ITSF/ITS4 (White et al., 1990; Chanderbali et al., 2001), so their ITS sequences were acquired through separate amplification of the ITS1, 5.8S nrDNA and ITS2 regions.

Fragments of the 3' ETS region for all species were amplified successfully using the ETS1/18S-IGS primer pair (Li et al., unpublished; Baldwin & Markos, 1998). The ETS sequencing primer (18S-IGS) produced a fragment that covered the whole 3' ETS region and in the *Actinodaphne* species sampled, this varied from 392–398 bp. The resulting aligned ETS matrix was 393 bp long, of which 32 characters (8.1%) were informative. The percentage of G+C in *Actinodaphne* ETS sequences was more invariable than for ITS, ranging from 50.8% to 52.3%. The 3' ETS sequence for *A. sesquipedalis* was unavailable and so was excluded in the ETS submatrix, but treated as missing in the combined analyses.

2.2 ITS analysis

MP analysis of the ITS submatrix resulted in 545 most parsimonious trees (tree length=161, *CI*=0.4845, *RI*=0.5389), and the 50% majority rule consensus tree is shown in Fig. 1. ML analysis yielded three trees (−ln*Likelihood*=734.32927), with the resulting Bayesian analysis cladogram presented as a 50% majority consensus tree excluding the 116

burn-in trees, and the posterior probabilities from that analysis were mapped along with the MP bootstrap support percentages onto the MP consensus tree (Fig. 1).
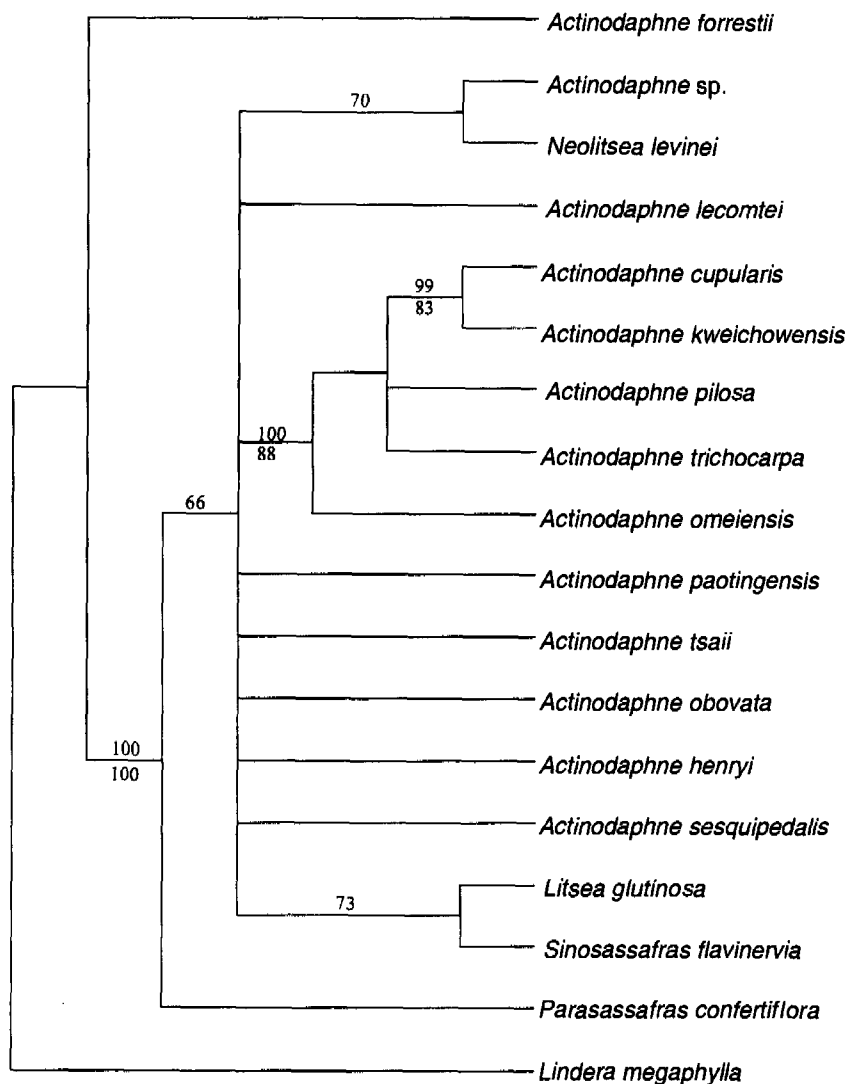
Fig. 1. 50% majority rule consensus cladogram of 545 most parsimonious trees (tree length=161 steps, *CI*=0.4845, *RI*=0.5389) derived from an analysis of ITS sequence data. Bayesian posterior probability values greater than 50% and bootstrap values greater than 50% are indicated above and below branches respectively.

All three analyses (MP, ML and Bayesian) indicate that *Actinodaphne* was polyphyletic and this is congruent with former studies (Chanderbali et al., 2001; Li et al., 2004). The MP analysis cladogram (Fig. 1) indicates that *A. forrestii* was basal above the outgroup of *Lindera megaphylla. Parasassafras confertiflora* was then sister to the remainder with strong bootstrap (100%) and posterior probability support (100%), but above this was a polytomy of three small clades (*Litsea glutinosa* with *Sinosassafras flavinervia*; *A. omeiensis*, *A. trichocarpa*, *A. pilosa* and *A. kweichowensis* with *A. cupularis*; and *Actinodaphne* sp. with

Neolitsea levinei), plus the remainder of *Actinodaphne* as a fourth clade.

The cladograms for the Bayesian (not shown) and MP analyses were very similar; however, a major incongruence appeared between these and the ML analysis. In the ML cladogram, one clade placed above *Actinodaphne forrestii* was sister to the remainder of the sampled taxa. This clade contained two sister-taxon pairs: *Parasassafras confertiflora* and *A. lecomtei*; *Litsea glutinosa* and *Sinosassafras flavinervia*. Above this clade, *A. tsaii* was basal to the remainder, within which *A. obovata* and *A. henryi* formed a sister pair in an unresolved polytomy with two other clades (*A. omeiensis*, *A. trichocarpa*, *A. pilosa*, *A. kweichowensis* and *A. cupularis* versus *A. paotingensis*, *A. sesquipedalis*, *Actinodaphne* sp. and *Neolitsea levinei*).

2.3 ETS analysis

MP analysis of the ETS data resulted in 132 most parsimonious trees (length=70 steps, CI=0.5143, RI=0.6634) and the 50% majority rule consensus tree is shown in Fig. 2. ML analysis yielded only one tree and the "burn-in" for Bayesian analysis was the first 905 trees.

The resulting MP cladogram indicates that *Lindera megaphylla*, *Actinodaphne forrestii*, *Actinodaphne* sp. were basal and successive sisters to the remainder. Above them were a terminal pair (*Parasassafras confertiflora* and *Sinosassafras flavinervia*) and then *Litsea glutinosa* was sister to the remainder, which divided into two major clades. The first clade consisted of *A. trichocarpa, A. omeiensis, A. tsaii, A. cupularis, Neolitsea levinei, A. obovata, A. henryi* and *A. pilosa*, of which the first five formed a subclade (94% posterior probability), sister to the other three species (77% posterior probability). The second major clade comprised *A. kweichowensis, A. paotingensis* and *A. lecomtei*, in which the latter two formed a terminal pair (50% bootstrap, 54% posterior probability) sister to the former (63% bootstrap support, 55% posterior probability).

Although the relationships described above were not as well resolved in the Bayesian cladogram, they were relatively congruent with the MP analysis. However, the ML cladogram was once again highly incongruent with the MP and Bayesian results as follows: In the ML analysis, *A. paotingensis*, *A. lecomtei* and *A. kweichowensis* did not cluster into one clade, instead forming successive sister relationships above *Lindera megaphylla* and *A. forrestii*. *Litsea glutinosa*, *Parasassafras confertiflora* and *Sinosassafras flavinervia* were clustered into a clade in which the latter two formed a terminal pair and this clade was then sister to the remainder of the tree.

2.4 ITS/ETS analysis

The Incongruence Length Difference Test (ILD) indicates that ITS and ETS data sets were relatively incongruent in the estimates of phylogeny (P=0.01). However, these two spacer regions were analyzed simultaneously in order to address consistency problems. The combined data matrix contained 18 operational taxonomy units (OTUs) and 1061 characters, of which 96 were informative (9.05%). The percentage G+C content for the combined ITS/ETS *Actinodaphne* data ranged from 58.1% to 61.4%.

Despite the apparent sequence incongruity, the combined ITS/ETS analyses removed the polytomies present in the separate analyses of ITS and ETS, and generally resulted in more robust cladograms and support the hypothesis that *Actinodaphne* was polyphyletic. MP analysis yielded three most parsimonious trees (tree length=445 steps, CI=0.4635, RI=0.4931) and ML analysis yielded only one tree (−ln*Likelihood*=1192.8063). The Bayesian analysis excluded the first 123 burn-in trees and the posterior probabilities from that analysis along with the MP bootstrap support percentages presented on a MP 50% majority consensus tree (Fig. 3).
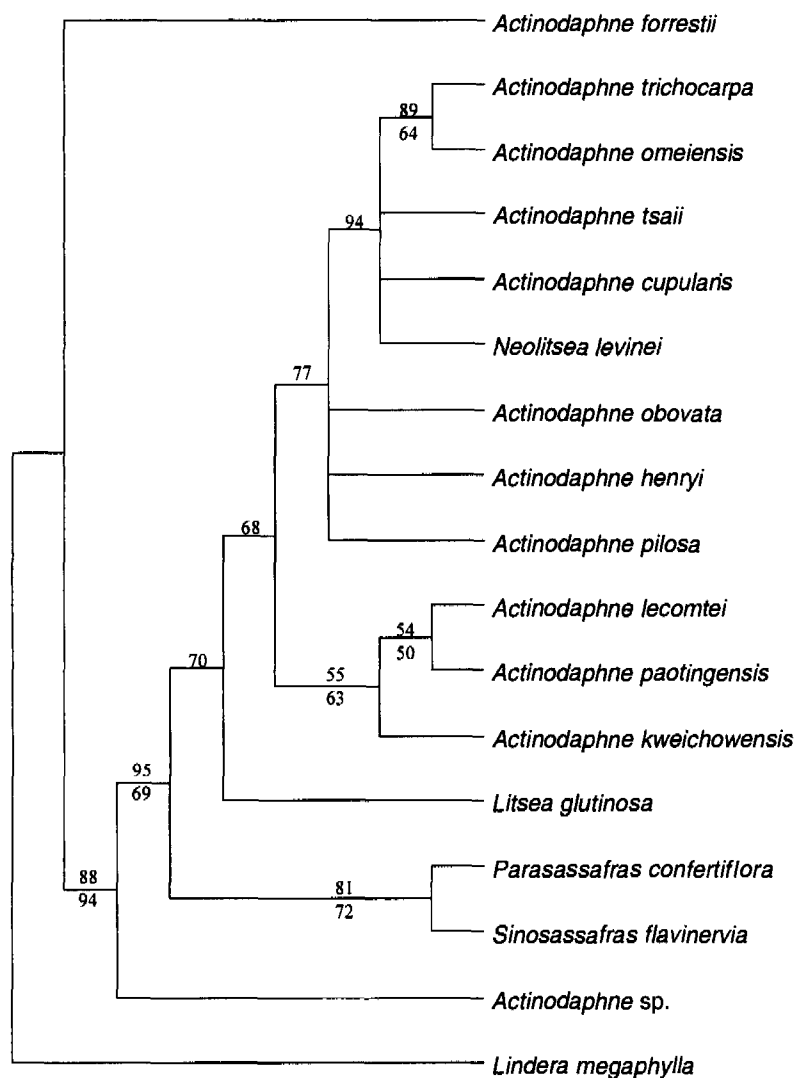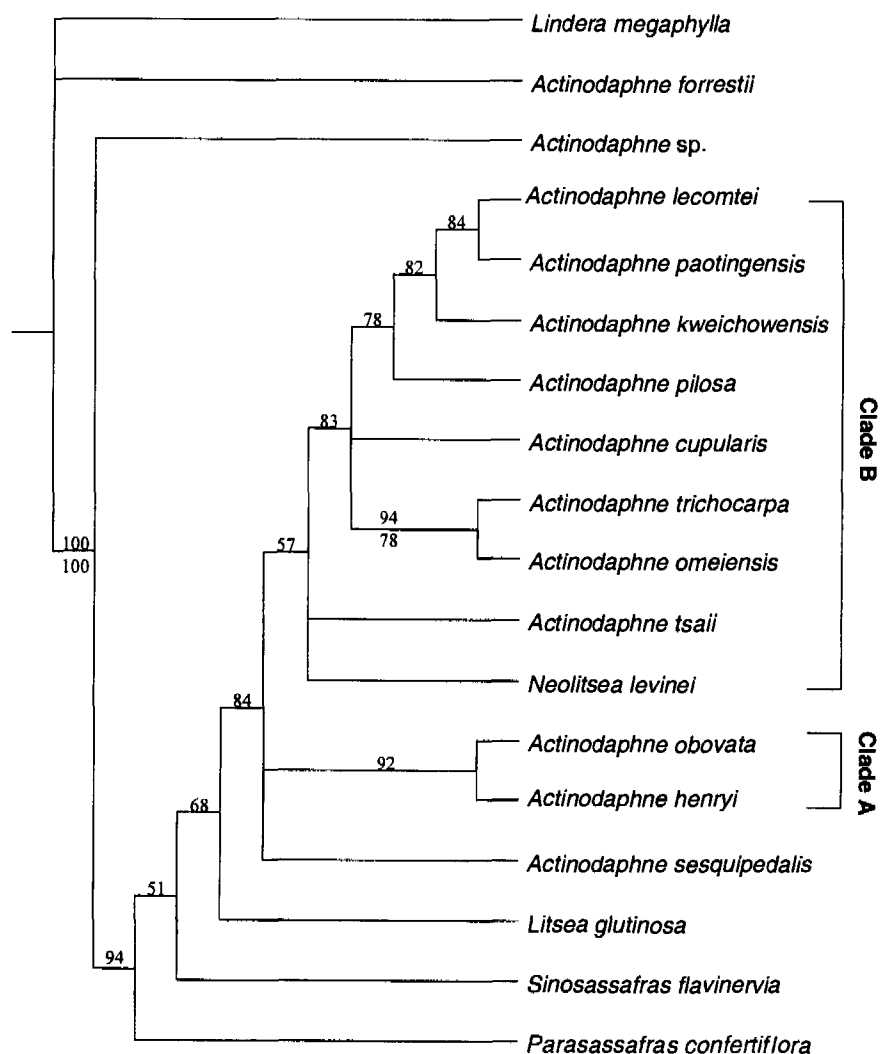
Fig. 2. 50% majority rule consensus cladogram of 132 most parsimonious trees (tree length=70 steps, *CI*=0.5143, *RI*=0.6634) derived from an analysis of ETS sequence data. Bayesian posterior probability values greater than 50% and bootstrap values greater than 50% are indicated above and below branches respectively.

All analyses (MP, ML and Bayesian) indicate that *Actinodaphne* was polyphyletic. The resulting cladograms of Bayesian and MP analyses were relatively congruent, but the former was slightly more resolved and gave relatively high posterior probability values (Fig. 3). The Bayesian cladogram successively placed *Lindera megaphylla, A. forrestii, Actinodaphne* sp., *Parasassafras confertiflora, Sinosassafras flavinervia* and *Litsea glutinosa* in a basal grade, above which was a polytomy (84% posterior probability) of *A. sesquipedalis* and the remainder of the sampled species, divided into two clades. Clade A consisted of *A. obovata* and *A. henryi* (92% posterior probability), whereas clade B contained *Neolitsea levinei, A. tsaii, A. trichocarpa, A. omeiensis, A. cupularis, A. pilosa, A. kweichowensis, A. paotingensis*

and *A. lecomtei*, in which the first two species together with a subclade formed an unresolved polytomy (57% posterior probability).

The ML combined analysis cladogram was, however, still incongruent with those of the Bayesian and MP analyses, with *Actinodaphne* sp. no longer in the basal clade, instead forming a subclade with *Actinodaphne lecomtei* and *A. paotingensis*, as sister to a subclade which was similar to the Bayesian clade B, only without *A. lecomtei* and *A. paotingensis*.

Fig. 3. Bayesian consensus of 9977 trees, and 50% majority rule consensus cladogram of the most parsimonious trees (tree length=445 steps, *CI*=0.4635, *RI*=0.4931) derived from a combined analysis of ITS/ETS sequence data. Bayesian posterior probability values greater than 50% and bootstrap values greater than 50% are indicated above and below branches respectively.

3 Discussion

3.1 Data compatibility

One of the outstanding issues in systematics is how to test phylogenetic conflict between different data sets (Kluge, 1989; Bull et al., 1993; de Queiroz et al., 1995; Miyamoto & Fitch, 1995; Cunningham, 1997), and two alternative approaches have generally been suggested, i.e., separate analysis and combined analysis (Kluge, 1989; Barrett et al., 1991; Lanyon, 1993; Miyamoto & Fitch, 1995). In our study the incompatibility of ITS and ETS was high for the ILD analysis ($P=0.01$). Nevertheless, it is deemed more reasonable to combine the data sets in a simultaneous analysis for the following reasons. Firstly, nrDNA ITS and ETS regions occur within the same transcriptional unit and there is evidence indicating a similar and interdependent role in the maturation of rRNAs (e.g., Good et al., 1997). Secondly, even with significant incompatibility between different DNA data sets ($P<0.01$ or even <0.001), a combined analysis can still produce a more satisfactory estimate of phylogenetic relationships than separate analysis of each data set alone (Cunningham, 1997). Thirdly, the incongruent clades in our analyses received little or no bootstrap support or posterior probabilities in the consensus trees for the separate ITS and ETS analyses. In the phylogenetic analysis of the "core" Laureae based on *mat*K and ITS sequences, a similar issue emerged but the combined analysis provided a more resolved phylogeny (Li et al., 2004).

Because of this improved resolution effect, conflicting ITS and ETS data are generally still deemed to be combinable (e.g. Mason-Game & Kellogg, 1996; Elodenäs & Linder, 2000; Barker et al., 2003; Li et al., 2004), and combined data sets generally produce more robust phylogenies (Chase et al., 1997). Nevertheless, caution is recommended, and conflict between data sets should be taken into consideration (Li et al., 2004).

3.2 Selecting Bayesian inference

Bayesian inference is a relatively recent addition to the analytical toolbox for phylogenetics (Hall, 2001), but Steane et al. (2003) considered that Bayesian analysis has the following advantages over other phylogenetic analysis methods. Firstly, Bayesian estimation is based on the likelihood function and thus related to maximum likelihood analysis; however, it resolves problems such as long branch attraction. Secondly, Bayesian analysis requires fewer computational resources, so that large data sets could be analyzed more readily. Thirdly, because the estimation of branch support accompanies tree estimation, additional bootstrap analyses are not required. Finally, it provides a practical alternative, with the resulting phylogeny providing additional support for the major clades identified by maximum parsimony analysis. Therefore, by comparing the resulting trees, we regarded the Bayesian ITS/ETS tree (Fig. 3) as being the most resolved and well-supported reconstruction of the underlying phylogeny.

3.3 Polyphyly of *Actinodaphne*

Our results agree with those or earlier studies, both morphological and molecular, that *Actinodaphne* is a member of the Laureae (Nees, 1836; Allen, 1938; Li et al., 1984; Rohwer, 1993, 2000; van der Werff, 1991, 2001; Chanderbali et al., 2001; Li et al., 2004). Nevertheless, although the genus was traditionally delimitated by whorled, penninerved leaves; pseudo-racemose or pseudo-umbellate inflorescences and imbricate, deciduous involucral bracts at the base of the inflorescence (Nees, 1836; Rohwer, 1993), these characters, especially the inflorescence types, also occur in other Laureae. For example the inflorescences of *Parasassafras* D. G. Long are similar to those of *Actinodaphne* species with umbellate inflorescences, both of which originate from scales underneath a vegetative bud that would later sprout (Rohwer, 1993). Similarly, van der Werff (2001) suggested that there is no clear difference between the inflorescence architecture of *Actinodaphne* and that of some

Litsea species, noting that the generic delimitation between them is unsatisfactory. Based on the analyses of cpDNA *mat*K and nrDNA ITS sequences, Li et al. (2004) suggested that *Actinodaphne* might be polyphyletic, and this is further supported by ongoing leaf micromorphology studies (Li & Christophel, unpublished).

The present ITS and ETS analyses also show polyphyly in *Actinodaphne*, with the sampled species variously clustering into clades with other Laureae genera. Furthermore, these admittedly preliminary clades recovered within *Actinodaphne* and related Laureae are partly consistent with inflorescence structure (van der Werff & Richter, 1996; van der Werff, 2001; Li et al., 2004). The basal clade in the combinative analysis (Fig. 3) consisting of *A. forrestii*, *Actinodaphne* sp., *Lindera megaphylla*, *Parasassafras confertiflora* and *Sinosassafras flavinervia*, is characterized by clustered or fasciculate pseudo-umbels with the usually vegetative terminal bud in the main axis, and the vegetative bud sprouting later forms a leafy short-shoot. Such pseudo-umbels may result from the shortening of the brachy-blast, and the peduncles of these pseudo-umbels shorten sequentially, ultimately resulting in the clustered or fasciculate pseudo-umbels (Tsui, 1987; Li et al., 2004).

The species of clade A (*Actinodaphne sesquipedalis* and *Litsea glutinosa*) share pseudo-racemose inflorescences, but lack development at the terminal tip. This inflorescence type might also originate from shortening of brachy-blast internodes and distal internodes; however, the peduncles of the pseudo-umbels do not shorten, making the pseudo-umbels appear pseudo-racemose (Rohwer, 1993; Li et al., 2004).

The species of clade B (except for *Actinodaphne pilosa*) also have similar pseudo-umbels to those of the basal clade, but the inflorescences of these Clade B species usually fail to develop at the terminal bud, a feature also seen in *Neolitsea levinei* which nested within this clade. In contrast, *A. pilosa* has a thyrsoid inflorescence more like the members of clade A. This might indicate that the inflorescence type in clade B might have originated from a thyrsoid cymose inflorescence, which is congruent with former studies (Rohwer, 1993; van der Werff, 2001; Li et al., 2004). The evolution of this inflorescence type would then be as follows: every secondary peduncle of the thyrsoid cyme could form a pseudo-umbel, and then by shortening of the peduncle form this clustered or fasciculate pseudo-umbellate inflorescence type. Thus, although the clade B inflorescences appear identical to those of the basal grade taxa, they may have different origins. However, because this is largely speculative, further study is required to determine why *A. pilosa* occurred in the mainly pseudo-umbellate clade B, and whether this represents a sample-size artifact or evidence of convergence in inflorescence types.

Accordingly, although the clades seen here might explain the phylogeny of the genus *Actinodaphne*, whether the Bayesian tree using two combined data sets accurately reflects the phylogeny of *Actinodaphne* and related genera remains to be tested in the future. All these conclusions should be tentative because of the following reasons: Firstly, topological conflicts were obvious between separate analyses of ITS and ETS data, and most of the major clades received relatively poor bootstrap support, even in the combined analyses of the two data sets. Secondly, because of relatively limited taxon sampling, more detailed studies are needed to clarify relationships within the genus and to allow for more precise taxon boundary definitions and hypotheses about phylogeny in *Actinodaphne*. Nevertheless, the available data in our study have further confirmed earlier suggestions that *Actinodaphne* is polyphyletic, and suggest that inflorescence type and ontogeny might be among the more reliable morphology-based characters for examining phylogenetic signal in *Actinodaphne* and possibly the Laureae in general.

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Appendix A. Likelihood settings from best-fit model (TrN+I+Γ) selected by Akaike Information Criterion (AIC) in Modeltest Version 3.06

Model selected:

TrN+I+Γ: (1) ITS: –ln*Likelihood*=2106.4932; AIC=4226.9863 (2) ETS: –ln*Likelihood*=1092.1334; AIC=2190.2668 (3) ITS & ETS: –ln*Likelihood*= 3316.5947; AIC=7396.2100

Base frequencies: (1) ITS: freq$A$=0.1667; freq$C$=0.3586; freq$G$=0.3587; freq$T$=0.1161 (2) ETS: Equal frequencies (3) ITS & ETS: –ln*Likelihood*=3691.1050; AIC=7396.210

Substitution model:

Rate matrix: (1) ITS: R(a) [A-C] =1.0000 R(*b*) [A-G] =3.5278; R(*c*) [A-T] =1.0000; R(*d*) [C-G] =1.0000; R(*e*) [C-T] =5.1798; R(*f*) [G-T] =1.0000 (2) ETS: R(*a*) [A-C] =1.0000; R(*b*) [A-G] =3.5840; R(*c*) [A-T] =1.0000; R(*d*) [C-G] =1.0000; R(*e*) [C-T] =6.9717; R(*f*) [G-T] =1.0000 (3) ITS/ETS: R(*a*) [A-C] =1.0000; R(*b*) [A-G] =3.7325; R(*c*) [A-T] =1.0000; R(*d*) [C-G] =1.0000; R(*e*) [C-T] = 5.7495; R(*f*) [G-T] =1.000

Proportion of invariable sites: (1) ITS: (I) =0.3171 (2) ETS: (I) =0.7954 (3) ITS/ETS: (I) =0.459

Gamma distribution shape parameter: (1) ITS: 0.5460 (2) ETS: Equal rates (3) ITS/ETS: 0.5017.

樟科黄肉楠属是一个复系类群——基于 nrDNA ITS 和 ETS 序列分析

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摘要   应用nrDNA ITS和ETS序列探讨了樟科Lauraceae黄肉楠属*Actinodaphne*的系统演化关系。对得到的3个序列矩阵(ITS、ETS和ITS/ETS), 采用MP (maximum parsimony), ML (maximum likelihood)和Bayesian 3种分析方法进行了系统发育分析。结果显示, 本文选的黄肉楠属*Actinodaphne*物种与所选的月桂族中的外类群靠近并混和在一起, 进一步证实了本属为一个复系类群。结合对传统的形态学性状的重新认识, 认为花序类型特征可能是重新界定黄肉楠属的最重要的性状, 具有相同花序类型的物种可能具有相同的起源。然而, 由于取样数量相对较少以及对矩阵的单独分析存在一定的差异, 还需更详细的研究来验证本文对黄肉楠属系统演化关系的假设, 并进一步更精确地重建本属的系统发育关系。

关键词   黄肉楠属; ETS; ITS; 樟科; 系统学研究; 贝叶斯分析