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Phylogenetic relationships within the 'core' Laureae (*Litsea* complex, Lauraceae) inferred from sequences of the chloroplast gene *mat*K and nuclear ribosomal DNA ITS regions

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Abstract. A phylogenetic analysis of the 'core' Laureae (*Litsea* complex) was conducted using the chloroplast gene *mat*K and nuclear ribosomal DNA ITS sequences to investigate generic relationships and boundaries within the complex. Despite low genetic divergence for *mat*K, rooting of the tree with *Sassafras* resulted in *Iteadaphne* as the basal member of the complex and five resolved clades: a *Neolitsea* clade and then *Laurus*, *Parasassafras*, *Litsea* and *Lindera* clades in a large polytomy with unresolved *Lindera* sections plus *Umbellularia*. A combined analysis of the data (identical to the ITS results) provided a more resolved phylogeny of the Laureae, with four major lineages: the *Laurus*, *Litsea*, *Lindera* and *Actinodaphne* II clades. These clades also appear to reflect the importance of inflorescence structure and ontogeny within the Laureae, as well as data from cuticular micromorphology, but there was no support for traditional generic characters such as 2- versus 4- celled anthers. As a result, genera such as *Actinodaphne*, *Litsea*, *Neolitsea* and *Lindera* were polyphyletic in all analyses. *Parasassafras* was related to *Sinosassafras* by the *mat*K data, but distant from it in the ITS and combined analyses.

Key words: Lauraceae, Laureae, *mat*K, ITS, molecular phylogeny, *Litsea*, *Lindera*, classification.

The 'core Laureae' or *Litsea* complex consists of ten genera with 500–700 species concentrated in tropical-subtropical Asia, but with representatives in Australasia, the Mediterranean, North and Central America. The monophyly of the complex is well supported (Chanderbali et al. 2001; Rohwer et al. 1991; Rohwer 2000; Li 1985, 1995; van der Werff and Richter 1996) and it is distinguished from other Lauraceae by possessing basically thyrsoid open, pseudo-racemose inflorescences bearing involucral bracts and which appear to be umbellate due to short axes, and mainly introrse anthers in the third whorl (Li and Christophel 2000, Li 2001).

Generic delimitation within the complex has traditionally been problematic (Kostermans 1957, Hutchinson 1964, Richter 1981), the major point being the significance of

2-locular versus 4-locular anthers as generic descriptors, although modern Lauraceae classifications, tend to downplay the significance of anther cell number in generic delimitation (e.g. Rohwer et al. 1991, Rohwer 1993, van der Werff and Richter 1996). Li and Christophel (2000) incorporated traditional morphological characters with leaf cuticles in a phylogenetic analysis of the complex, but although they also concluded that anther cell number did not have strong phylogenetic value at the generic level, they also demonstrated that morphological data were insufficient to resolve relationships within the Laureae.

The genus *Litsea* was divided into four sections under the classification of Li et al. (1984), with sect. *Litsea* defined as evergreen with alternate, penninerved leaves, a racemiform inflorescence and non-enlarged perianth tubes with absent or reduced perianth lobes. Sect. *Conodaphne* (Bl.) Benth. et Hook. f. is evergreen with alternate or opposite penninerved leaves and non- to slightly enlarged perianth tubes. Sect. *Cylicodaphne* (Nees) Hook. f. was considered to be evergreen with alternate, penninerved leaves, an enlarged perianth tube and cup-shaped fruiting cupule. Sect. *Tomingodaphne* (Bl.) Hook. f. has deciduous, alternate, penninerved leaves and non-enlarged, 6-lobed perianth tubes.

Lindera was similarly divided into eight sections by Tsui (1987) as follows: sect. *Lindera* with deciduous, penninerved leaves and well-developed terminal buds on shortened brachyblasts; sect. *Sphaerocarpae* Tsui with deciduous; triplinerved or trinerved leaves and well-developed terminal buds on shortened brachyblasts and sect. *Palminervia* Meissn. with deciduous, lobed, trinerved leaves and well-developed terminal buds on shortened brachyblasts. Sect. *Aperula* (Bl.) Benth. has evergreen, penninerved leaves, well-developed terminal buds on shortened brachyblasts and long-pedunculate, racemiform inflorescences; Sect. *Cupuliformis* Tsui possesses evergreen, penninerved leaves, funnel-shaped glands on the third anther whorl and enlarged perianth tubes forming cup-shaped fruiting cupules; sect. *Daphinidium* (Nees) Benth. has evergreen, trinerved leaves and un-developed terminal buds on shortened brachyblasts; sect. *Polyadenia* Nees is evergreen with penninerved leaves and well-developed terminal buds on shortened brachyblasts and sect. *Uniumbellatae* Tsui has evergreen, trinerved leaves, and the long-pedunculate pseudo-umbel is solitary in the axil of a normally developed leaf.

In previous Lauraceae classifications, the number of anther cells was a critical character in distinguishing the different genera and Kostermans (1957) considered it as the third most important character after inflorescence type and fruiting cupule shape. This view was followed by Li (1985, 1995), Tsui (1987) and Hyland (1989), although Li (1985, 1995) considered that the relationship may not be as close as originally thought, and that the similarities between *Litsea* and *Lindera* may be the result of parallel evolution. Li (1985) and Tsui (1987) also suggested that there is parallel evolution between and within *Litsea* and *Lindera* at the generic and sectional levels, making generic delimitation difficult using traditional morphological characters.

Because morphological data did not provide strong phylogenetic signal, our study involving molecular sequences was undertaken to find a more robust phylogeny for the Laureae. The *mat*K gene is one of the most rapidly evolving coding regions found so far in the chloroplast genome (Olmstead and Palmer 1994), and its high rates of synonymous and especially non-synonymous substitutions mean that it is considered appropriate for constructing infrafamilial phylogenies (e.g. Johnson and Soltis 1994, 1995; Steele and Vilgalys 1994; Plunkett et al. 1996, 1997; Liang and Hilu 1996; Kron 1997; Gadek et al. 1996; Manos and Steele 1997; Xiang et al. 1998; Denda et al. 1999), Nevertheless, Rohwer (2000) found that *mat*K sequence divergence in Lauraceae was surprisingly low (only 9.7% informative characters), so a second marker was incorporated to improve recovery of phylogenetic signal (Chase et al. 1997).

The ITS spacer region, has been utilised widely in phylogenetic studies of many taxa of flowering plants (e.g. Baldwin et al. 1995) and recent work on Lauraceae by Chanderbali et al. (2001) found that it provided good signal resolution and therefore should be suitable for the Laureae. However, because ITS is a nuclear gene spacer, phylogenetic conclusions using ITS sequences are advised to be corroborated with data from other sources, including plastid genes such as *mat*K (Donoghue and Sanderson 1992).

The goals of our study using maximum parsimony analysis of sequence data from nuclear ITS (internal transcribed spacer of ribosomal genes) and the chloroplast *mat*K gene are to: (1) examine the circumscription of the Laureae; (2) test the hypothesis that *Litsea*, *Lindera*, *Actinodaphne* and *Neolitsea* are each monophyletic; and (3) investigate major lineages in order to try to explain evolution within the complex.

Materials and methods

Ingroup sampling. Taxon sampling included representatives of all previously recognised generic segregates in the complex, as well as sections of larger genera such as *Litsea* and *Lindera* as defined by Nees von Esenbeck (1836), Meissner (1864), Bentham (1880), Pax (1889), Hooker (1890), Kostermans (1957), Hutchinson (1964), Li (1984, 1985), Long (1984) and Tsui (1987). A complete list of the species sampled, along with collection and voucher information is provided in Table 1.

Outgroup selection. *Sassafras* is generally considered to be closely related to the Laureae due to its dioecious breeding system, inflorescences with involucral bracts and introrsely positioned locelli in all staminal whorls, (Kostermans 1957, Rohwer 1993), as well as being sister to the clade in the ITS study of Chanderbali et al. (2001). However, as Rehder (1920) noted: ''From all the genera of the tribe Litseae the genus *Sassafras* is easily separated by its racemes of slender pedicellate flowers in the axils of the basal scales of the terminal branch-bud, while in other genera the flowers are arranged in lateral umbels or heads, sometimes reduced to 1 flower, subtended by an involucre of 4–6 bracts, or as in *Actinodaphne* in lateral sub-sessile fascicles''. van der Werff and Richter (1996) also considered *Sassafras* to be close to the Laureae, noting that both lack marginal parenchyma and possess similar phloem fibres in the wood, nevertheless, it is separated from the Laureae by its unique accentuated growth ring structure in both secondary xylem and phloem.

Similarly, although *Umbellularia* is usually placed within the Laureae (Kostermans 1957, Rohwer 1993, van der Werff and Richter 1996), Chanderbali et al. (2001) and Rohwer (2000) suggested that it should be placed in the Cinnamomeae variously on the basis of ITS, *trn*L-F, *psb*A-*trn*H and *mat*K sequences and morphology (bisexual flowers and extrorse anthers in the innermost staminal whorl). As a result, we have included it in our study, but treated it in the analyses as an outgroup.

DNA extraction. Total DNA was extracted using the CTAB method of Doyle and Doyle (1987). Approximately 1 cm^2 leaf tissue was ground with a minute amount of sterile sand and 1 ml of warmed (60 °C) CTAB + BME in a warmed (60 °C) mortar and pestle; an additional 1 ml CTAB was added and mixed, then the slurry was poured into two 1.5 ml tubes and incubated at 60 °C in a water bath for 45 minutes. Two volumes of chloroform: isopropanol (24:1) were added, the tubes rocked for at least 10 minutes and then microfuged for 10 minutes at 14,000 rpm. The aqueous phase was transferred to a fresh tube to which 1 ml cold 100% EtOH was added and incubated at −20 °C for between 30 minutes and overnight to precipitate DNA. The tube was then centrifuged for 10 minutes at 14,000 rpm, the supernatant decanted, and the pellet washed for 5 minutes using 500 µl wash buffer (70% EtOH, l0 mM NH_4OAc). After centrifugation for another 10 minutes at 14,000 rpm, the supernatant was again decanted, and the pellet dried and then resuspended in 100 µl ddH_2O (50 µl for herbarium specimens).

Where DNA was difficult to extract by this method, the QIAquick® spin column was used to extract DNA (M. Chase, pers. comm.), 150 µl of the aqueous phase mentioned above was added to the column, after which the QIAquick® protocol for cleaning PCR products was followed. This method avoided precipitating the DNA and generally produced good results within a few minutes.

Table 1. Species of the Laureae and outgroup taxa included in the analysis

Taxon	Voucher	Source	GB number for *mat*K	GIB number for ITS
Litsea section Tomingodaphne				
Litsea cubeba (Lour.) Pers.	Li H.-W. 28 (HITBC)	Yunnan, China	AF 244398	AY265402
Section *Litsea*				
Litsea glutinosa (Lour.) C.B. Rob.	Li H.-W. 21 (HITBC)	Yunnan, China	AF 244396	AY265403
Section *Conodaphne*				
Litsea umbellata (Lour.) Merr.	Li H.-W. 24 (HITBC)	Yunnan, China	AF 244395	AY265404
Section *Cylicodaphne*				
Litsea dilleniifolia P.Y. Pai et P.H. Huang	Li H.-W. 19 (HITBC)	Yunnan, China	AF 244397	AY265405
Lindera				
Section *Cupuliformes*				
Lindera megaphylla Hemsl.	Li H.-W. 7 (HITBC)	Yunnan, China	AF 244404	AY265406
Section *Lindera*				
Lindera reflexa Hemsl.	Nei M.X. & Lai S,.K. 3768 (KUN 0100201)	Jiangxi, China	AF 244401	AY'265407
Section *Aperula*				
Lindera metcalifiana Allen	Li H.-W. 8 (HITBC) Li H.-W. 4 (HITBC)	Yunnan, China	AF 244403	AY265408
Section *Polyadenia*				
Lindera communis Hemsl.	Li G.F. 4 (HITBC)	Yunnan, China	AF 244406	AY265409
Section *Sphaerocarpae*				
Lindera fruticosa Hemsl.	Li G.F. 63966 (KUN 0104915)	Sichuan, China	AF 244405	AY265410
Section *Palminerviae*				
Lindera obtusiloba Bl.	Sino-Amer. Exped. 1308 (KUN 0151469)	Hubei, China	AF 244402	AY265411
Section *Uniumbellatae*				
Lindera tienchuanensis W.P. Fang et H.S. Kung	Sichuan Exped. 2258 (KUN0101388)	Sichuan, China	AF 244399	AY265412
Section *Daphnidium*				
Lindera thomsonii Allen	Li H.-W. 9 (HITBC)	Yunnan, China	AF 244400	AY265413
Actinodaphne				
I: *Actinodaphne obovata* (Nees) Bl.	Li H.-W. 1 (HITBC)	Yunnan, China	AF 244410	AY265398
II: *Actinodaphne forrestii* (Allen) Kosterm.	Li H.-W. 2 (HITBC)	Yunnan, China	AF 244411	AY265399
Neolitsea				
I: *Neolitsea confertifolia* (Hemsl.) Merr.	GaoX.P. 53971 (KUN 0106507)	Guangdong, China	AF 244394	AY265400
II: *Neolitsea levinei* Merr.	Li H.-W. 29 (HITBC)	Yunnan, China	AF 244393	AY265401
Iteadaphne				
Iteadaphne caudata (Nees) H.-W. Li	Li H.-W. 27 (HITBC)	Yunnan, China	AF 244408	AY26.5396
Dodecadenia				
Dodecadenia grandiflora Nees	Wu C.Y. et al 75-1048 (KUN 0049206)	Tibet, China	AF 244409	AY265397

Table 1 (continued)

Taxon	Voucher	Source	GB number for *mat*K	GIB number for ITS
Parasassafras				
Parasassqfras confertiflora (Meissn.) Long	Qian Y.Y. 682 (KUN 0104558)	Yunnan, China	AF 244392	AY265395
Sinosassafras				
Sinosassafras flavinervia (Allen) H.-W. Li	Yang Z.H. 101437 (KUN 0150376)	Yunnan, China	AF 244390	AY265394
Laurus				
Laurus nobilis L.	Li H.-W. 16 (HITBC)	Yunnan, China	AF 244407	AY265392
Outgroup taxa:				
Sassafras tzumu (Hemsl.) Hemsl.	Li H.-W. 15 (HITBC)	Yunnan, China	AF 244391	AY265391
Umbellularia californica (Hooker et Arnott) Nuttall	van der Werff s.n. (MO)	North America	AF 244389	AY265393

PCR and sequencing. The main primers used to amplify the whole *mat*K gene were 909 and 2288 (Johnson and Soltis 1995). However, if these were unsuccessful, various internal primer combinations were utilised using the primers 909, 5500, 5400, 5300, 5200, 5200F, 1245, 4100, 5800 and 2288 (Table 2). The entire ITS region was amplified successfully in most cases using LAUR 1 and ITSB (Chanderbali et al. 2001), but if these failed, primer combinations using the universal primers of White

Table 2. Primers used for amplification and sequencing *mat*K and ITS in the Laureae

*mat*K	Primer sequence 5′-3′	Source
Forward		
909(*trn*K3914F)	GGGGTTGCTAACTCAACGG	Johnson et al. (1996)
1244(*mat*K7)	GTATTAGGGCATCCCATT	Steele and Vilgalys (1994)
1245(KPS5)	GGATCCTTTCATGCATTATG	Steele and Vilgalys (1994)
5400	CTCAAATGATATCGAAGGG	L. Jones unpubl. Primer
5500	GATGGATTTCGGCAACAATA	L. Jones unpubl. primer
5600	GTGTACGACTAAACTCTTCG	L, Jones unpubl. primer
5200F	CCTTTCTTGAGCGAACAC	L. Jones unpubl. primer
Reverse		
2288 (*trn*K-2R)	AACTAGTCGGATGGAGTAG	Johnson et al. (1996)
4100	TAGAACTAGATAGATCTCAGC	K. Edwards unpubl. primer
2520	GATCCTTCCTGGTTGAAACCAC	L. Jones unpubl. primer
5200	GTGTTCGCTCAAGAAAGG	L. Jones unpubl. primer
5300	GCATCTTGTATCCAAGAGTG	L. Jones unpubl. primer
5800	GGTTCTCTATGTGACCTATG	L. Jones unpubl. primer
ITS	Primer sequence 5′-3′	Source
Forward		
LAUR 1	ACCACCACCGGCGAACCA	Chanderbali et al. (2001)
ITS3	GCATCGATGAAGAACGTAGC	White et al. (1990)
USA	GGAAGGAGAAGTCGTAACAAGG	Blattner (1999)
Reverse		
1TS2	GCTACGTTCTTCATCGATGC	White et al. (1990)
ITSB	CTTTTCCTCCGCTTATTGATATG	Blattner (1999)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)

et al. (1990) with LAUR 1 and ITS B were also used to amplify regions of poor-quality template. The primer sequences used are shown in Table 2.

To prevent PCR contamination, a negative control was used for every primer combination in every reaction. Once the required fragment was successfully amplified, the DNA was purified using the Qiagen QIAquick® PCR Purification Kit, following protocols provided by the manufacturers. To sequence the DNA fragment, a series of reactions were set up, each with one different internal primer (Table 2), thus producing single stranded DNA in both directions to produce overlapping sequences. Sequencing reactions were performed in a 0.2 ml PCR tube, using 2 µl of primer, ~90 ng of DNA template, 4 µl of Big Dye and 4 µl of Big Dye Buffer, using ddH_2O to make up a final reaction volume of 20 µl. This mixture was then temperature cycled at 1) 96 °C for 30 seconds, 2) 50 °C for 15 seconds, 3) 60 °C for 4 minutes with 25 cycles. Cleaned products were then directly sequenced in an Applied Biosystems 3100 DNA automated sequencer.

Sequence alignment. Sequences of the 23 taxa were aligned using DAPSA ver. 3.8 (Harley 1995), allowing uncertainties either to be resolved or recorded as ambiguities. When all the overlapping sequences had been checked, a consensus sequence for each species was generated and aligned to the database sequences and any differences reverified. Consensus sequences for all taxa were then realigned and a final data matrix produced.

Phylogenetic analysis. The data matrix for the 23 taxa consisted of a *mat*K submatrix comprising 1406 base pairs (bp) without alignment gaps in the coding region, and an ITS submatrix comprising 673 bp with 16 alignment gaps. Only phylogenetically informative characters were analysed, gaps were scored as missing data, and all characters were unweighted initially and treated as unordered multistate. Resulting trees were rooted by outgroup comparison with *Sassafras* and *Umbellularia*.

Parsimony analysis was performed using PAUP* version 4.0b10 (Swofford 1998), analysing the aligned submatrices alone and then combined. An initial heuristic search with 100 replicates with random addition sequence, tree-bisection-reconnection (TBR) branch-swapping, MULPARS on, and steepest descent off, was followed by successive weighting (Farris 1989), using heuristic searches with 10 replicates and random sequence addition, TBR branch-swapping, and characters re-weighted using their rescaled consistency indices and the maximum value (best fit) criterion. All trees from these 10 replicates were then swapped to completion, after which re-weighting was repeated until tree length stabilised. Homoplasy was estimated by consistency (excluding uninformative sites) and retention indices. Internal support was evaluated using Bootstrap analysis (Felsenstein 1985), with 1000 replicates performed on the weighted data matrix, using the following PAUP* settings: retain groups compatible with 50% majority-rule consensus, sample characters with equal probability but apply weights, 10 replicate random sequence addition, TBR branch-swapping and MULPARS off.

Results

Sequence characteristics. DNA was extracted successfully from all 23 samples, including eight from herbarium samples more than 20 years old. The amplified *mat*K regions were 1406 base pairs (bp) long, except for those of *Lindera tienchuanenis* W. P. Fang et H. P. Kung from *Lindera* sect. *Uniumbellatae* and *Lindera thomsonii* Allen from *Lindera* sect. *Daphnidium* which were missing 108 bp and 31 bp, respectively (possibly due to primer mismatch or poor-quality templates). With no insertions or deletions alignment posed no problem, however, as *mat*K is highly conservative in Lauraceae (Rohwer 2000) the number of nucleotide substitutions was limited. Informative sites comprised only 1.14% of 1,406 sites (16 bp), and the Guanine and Cytosine (GC) content ranged from. 36.1–36.6%, with an average of 36.3%.

In contrast, the ITS region was quite variable between taxa, and alignment required 16 indels varying from. 1–44 bp. As a result, only regions which could be aligned unequivocally were analysed, making our ITS phylogenetic estimates conservative. These aligned sequences included the 5.8S gene and resulted in 299 constant, 426 uninformative, and 247 parsimony informative characters. The total aligned ITS region contained 673 bases with 36.7% parsimony informative characters, although due to the poor quality of DNA

from some herbarium specimens, data for ITS1 were missing in *Lindera tienchuanensis*. The GC content of ITS was much higher than *mat*K, ranging from 51.0% in *Neolitsea confertifolia* (Helms.) Merr. to 67.5% in *Litsea glutinosa* (Lour.) C.B. Rob.

The combined data matrix therefore contained 23 OTUs and 2,079 characters, of which 263 were parsimony informative.

***mat*K analysis.** Analysis of the *mat*K submatrix resulted in 45 equally parsimonious trees (length = 19; CI = 0.8947; RI = 0.9048). The results from the successive weight analyses were identical to those of the preweighted analyses, and the topology of the 50% majority rule consensus tree is shown in Fig. 1.

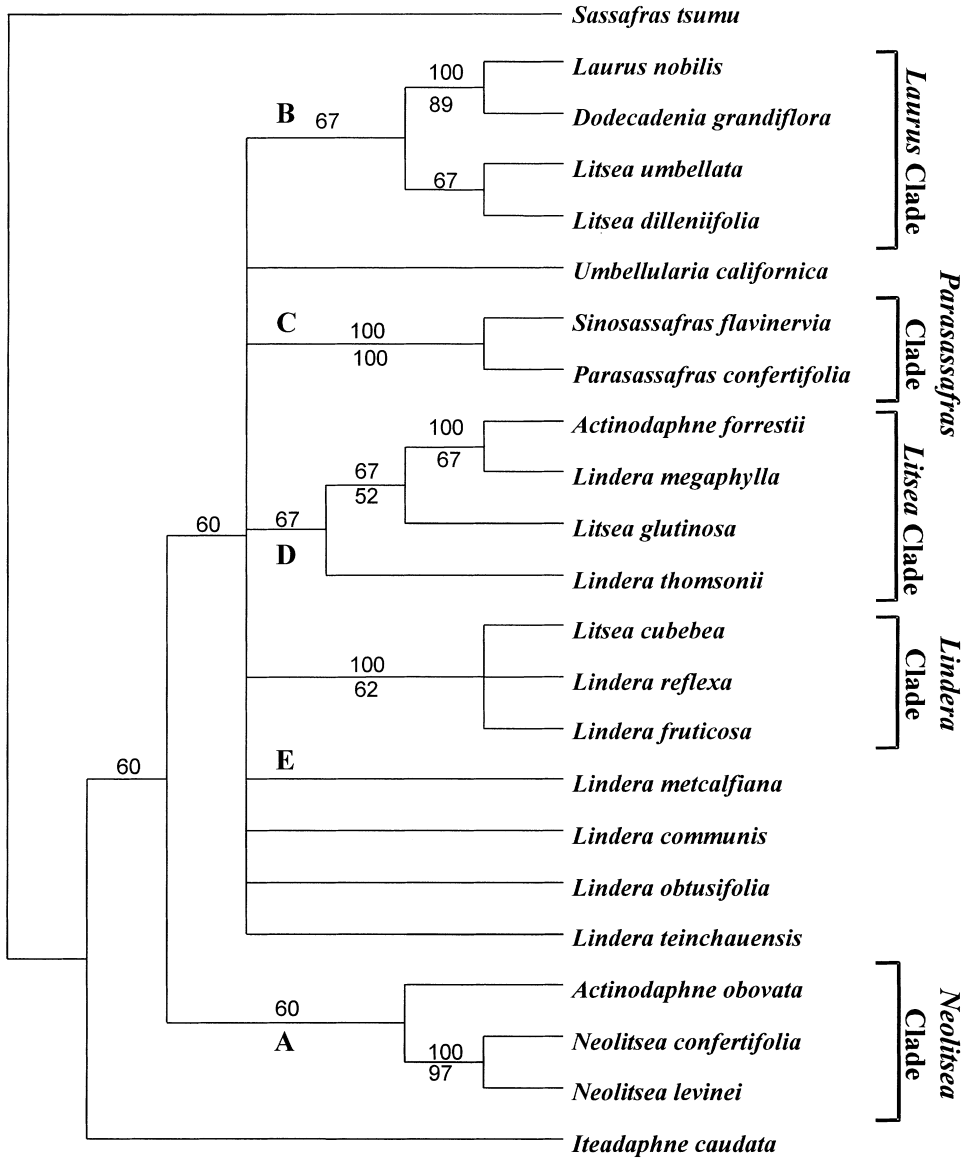

Fig. 1. The majority consensus tree of 45 equally parsimonious trees obtained from the successive weighting analysis of the *mat*K submatrix. Majority rule percentages are indicated above branches and bootstrap support below branches

The resulting cladogram was poorly resolved with *Actinodaphne*, *Lindera* and *Litsea* all polyphyletic, and only five small terminal clades common to all 45 trees. The first of these (A) represented the two sampled *Neolitsea* accessions (97% bootstrap support) weakly supported as sister to *Actinodaphne* I, the second (B) a *Laurus* – *Dodecania* pair with 89% bootstrap as unsupported sisters to a *Litsea* sects. *Conodaphne* and *Cylicodaphne* pair. Clade C contained *Sinoassafras* and *Parasassafras* with 100% bootstrap support whereas the fourth clade (D) consisted of *Actinodaphne* II and *Lindera* sect. *Cupiliformes* (67% support) along with *Litsea* sects. *Litsea* and *Daphnidium*. The final clade (E) represented a polytomy of three *Lindera* sections (*Lindera*, *Sphaerocarpae* and *Tomingodaphne*) but only poor support (62%). Rooting the tree to *Sassafras* meant that *Umbellularia* (although a nominated outgroup) fell within the Laureae as part of the large unresolved polytomy, but without bootstrap support.

ITS analysis. Analysis of the ITS sequences resulted in 15 most parsimonious trees (length = 575; CI = 0.6330; RI = 0.6719). Successive weighting produced 3 shortest trees (length = 364; CI = 0.7482, RI = 0.7914), all of which were congruent with the trees from the unweighted analysis, and the topology of the weighted strict consensus tree is shown in Fig. 2.

Four major clades occur in the strict consensus tree, although none of these had bootstrap support. The first and most basal of these (Clade A) contained *Lindera* section *Palminerviae*, *Litsea* section *Tomingodaphne*, *Lindera* section *Cupuliformes*, and *Actinodaphne* II. Within the clade, *Actinodaphne* II and *Lindera* section *Cupuliformes* formed a terminal pair with 100% bootstrap support, and sister to *Litsea* section *Tomingodaphne* with 74% bootstrap support. *Lindera* section *Palminerviae* was basal within Clade A, but without bootstrap support.

Clade B comprised *Parasassafras*, *Lindera* section *Sphaerocarpae*, *Lindera* section *Lindera* and *Litsea* section *Conodaphne*, the latter two forming a terminal pair clade with relatively low bootstrap support (64%), which was sister to *Lindera* section *Sphaerocarpae* (51% support).

Actinodaphne I, *Iteadaphne*, *Lindera* section *Aperula*, *Dodecadenia*, *Litsea* sections *Litsea* and *Cylicodaphne* constituted Clade C, with *Actinodaphne* I sister to the remainder. *Litsea* sections *Litsea* and *Cylicodaphne* constituted a monophyletic pair with 73% bootstrap support and allied with *Dodecadenia* (78%). *Lindera* section *Aperula* was sister to that trio, but with only weak support (57%).

In Clade D, *Sinosassafras*, *Neolitsea* I and *Lindera* section *Uniumbellatae* formed an unresolved but well-supported polytomy (100% bootstrap support), which formed a strongly supported (100%) sister group to *Lindera* section *Daphnidium*. *Neolitsea* II was weakly allied to them (61% support), and *Laurus* was basal to the clade, but without bootstrap support.

Combined *mat*K and ITS. Heuristic analysis of all phylogenetically informative nucleotide characters from the combined chloroplast and nuclear DNA data produced 15 trees each 611 steps long (CI = 0.6236; RI = 0.6536). Successive weighting resulted in three most parsimonious trees, each 381 steps long (CI = 0.7373; RI = 0.7773) (Fig. 2).

The strict consensus tree from the combined analysis is topologically identical to the ITS submatrix analysis, the same clades reappearing in the combined analysis, and with similar bootstrap support values. However, only one of the bootstrap-supported clades from the *mat*K analysis clades (*Actinodaphne* II + *Lindera* section *Cupuliformes*) occurred in the combined matrix.

Discussion

Data combinability. The combinability of different data sets is a contentious issue and there are numerous suggested methods for approaching the problem (e.g. De Queiroz et al. 1995, Huelsenbeck et al. 1996, Nixon and Carpenter 1996), although if incongruence

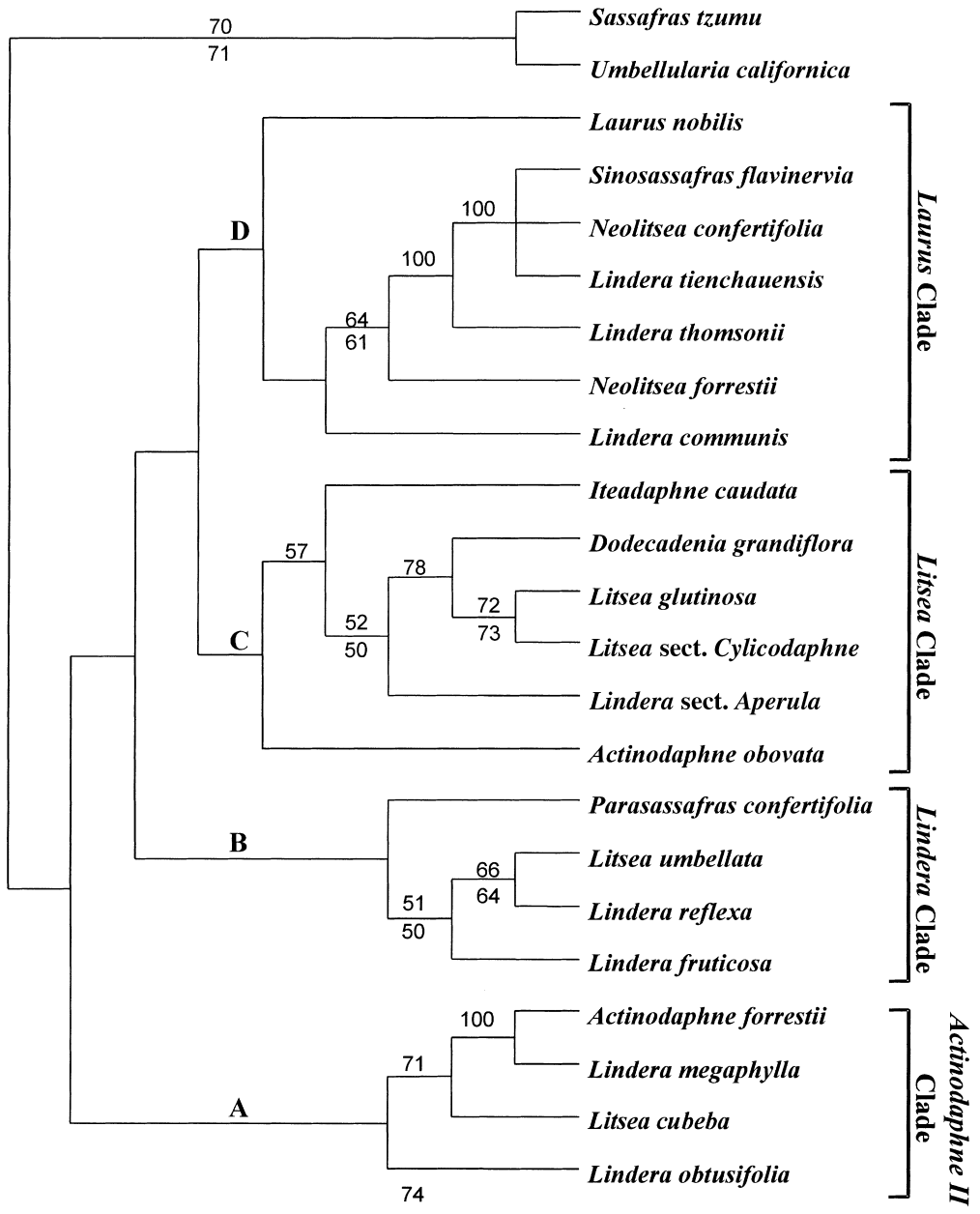

Fig. 2. The strict consensus tree of the three equally most parsimonious trees resulting from the successive weighting analysis of a combined *mat*K and ITS matrix, and identical to the topology of the ITS only analysis. Bootstrap support percentages for the combined analysis are indicated above the branches, those for ITS only, below the branches

is low, or there is relatively low (<70%) bootstrap support for the incongruent nodes then data can be deemed to be combinable (e.g. Mason-Gamer and Kellogg 1996, Eldenäs and Linder 2000, Barker et al. 2003). As there was mostly lower bootstrap support in at least one of the two trees for the incongruent clades, our Laureae data were considered to be sufficiently compatible to be combined. Despite the small number of

parsimony informative sites found in *mat*K for the Laureae (1.14% of the total) compared to ITS (36.7%), limiting its usefulness for classification in our study, it showed low levels of homoplasy. ITS had a greater number of parsimony informative sites, but the region was also more homoplasious, resulting in lower indices of consistency and retention.

When the *mat*K and ITS sequences were combined, the results were identical to those obtained with ITS alone, probably because ITS had more than thirty times the number of informative sites, and combining the two did not substantially alter the levels of bootstrap support for the nodes. Combined data sets generally produce more robust phylogenies (Chase et al. 1997, Davis et al. 1998), especially where nuclear and chloroplast genes may have different histories due to mutation, introgression, transfer into different lineages or loss of polymorphisms in descendant species (Judd et al. 1999) Nevertheless, caution is still recommended, and conflict between data sets should at least be taken into consideration.

Circumscription of the Laureae. The inclusion of *Sassafras* and/or *Umbellularia* within the Laureae had been advocated by several authors (Kostermans 1957, Rohwer 1993, van der Werff and Richter 1996) based on introrsely positioned anther locelli and/or the umbellulate, involucrate inflorescences. However, in the Laureae introrse locelli occur only in the outer two staminal whorls (mainly in the third), not in all whorls as in *Sassafras*, and the inflorescences in *Sassafras* are botryose not umbellulate.

Similarly, despite possessing umbellulate, involucrate inflorescences, *Umbellularia* has bisexual flowers and extrorse locelli in the innermost staminal whorl. Although *Umbellularia* fell inside Laureae in our *mat*K analysis (if the tree is rooted to *Sassafras*) the more extensive family-level *mat*K study by Rohwer (2000) found that the former was instead associated with *Licaria* and *Endlicheria* in a terminal clade with members of Cinnamomeae. Chanderbali et al. (2001) using ITS and cpDNA sequences also found that *Umbellularia* was part of what they called the *Ocotea* complex, with similar relationships to Rohwer's *mat*K results, and that it was not part of the Laureae. It is possible that in the absence of its near relatives, *Umbellularia* fell inside Laureae in our *mat*K analysis due to the very small number of informative sites and/or long-branch attraction.

These features, and the results of our combined study tend to support the exclusion of both genera from the Laureae, rather than the inclusion of at one of the two, depending on which is chosen as the outgroup, as our *mat*K data might suggest.

Polyphyly in *Litsea* and *Lindera*. Hooker (1890), although following Bentham (1880), expressed similar doubts as to the utility of 2- and 4-celled anthers as generic characters, and Rohwer (1993) considered that *Litsea* and *Lindera* were anatomically and morphologically polyphyletic, and that they should be split into smaller entities. The representatives of the sections of these genera sampled were polyphyletic in our study, agreeing with the morphological and leaf cuticle analysis of Li and Christophel (2000), suggesting that these genera are not monophyletic. This further supports the assertions by Hyland (1989), Rohwer et al. (1991), van der Werff and Richter (1996) and van der Werff (2001) that the use of 2-thecate and 4-thecate anthers in generic delimitation was confusing and likely to result in the recognition of poly- or paraphyletic genera.

Nevertheless, until a much wider-sampled and robust phylogeny can be developed for the tribe, the poly- or paraphyly of both these genera, and the relationships and possibly even monophyly of the sections within them will remain unresolved.

Systematic position of *Sinosassafras*, *Parasassafras*, *Iteadaphne* and *Dodecadenia*. As with the *Litsea*/*Lindera* complex, two other generic pairs traditionally distinguished by anther cell number (*Sinosassafras* vs. *Parasassafras* and *Iteadaphne* vs. *Dodecadenia*) were also unrelated in our ITS and combined

analyses. Nevertheless, *Sinosassafras* and *Parasassafras* were monophyletic with 100% bootstrap support in our *mat*K analysis, agreeing with Rohwer's (1993) assertion that anther locule number was an insufficient ground for generic separation. Although there were very few informative *mat*K sites, the fact that this pair was strongly supported in our data suggests that given the more conservative nature of *mat*K their pairing may well reflect phylogenetic signal.

In contrast, although *Iteadaphne* and *Dodecadenia* have umbels which are reduced to a single flower, the different number and arrangement of involucral bracts caused Rohwer (1993) to treat them as separated genera, and this is supported by our study. Nevertheless, they, along with the core *Litsea* group and part of *Actinodaphne* formed the unsupported *Litsea* clade, partly reflecting Kosterman's (1957) placement of *Dodecadenia* within *Litsea*, but also Rohwer's (1993) suggestion that *Dodecadenia* was possibly close to *Actinodaphne*. Our *mat*K analysis, on the other hand, placed *Dodecadenia* in a well supported clade with *Laurus* again suggesting that much wider sampling is needed in order to determine fully the monophyly and relationships between Laureae genera and sections.

***Actinodaphne* and *Neolitsea*.** Imbricate, deciduous involucral bracts at the base of the inflorescence traditionally delineate *Actinodaphne* (Nees von Esenbeck 1836). However, the genus was polyphyletic in both the *mat*K or ITS analyses, agreeing with the morphological study of Li and Christophel (2000).

Our study suggests that *Actinodaphne* might be separated into two groups, one characterised by thyrsoid inflorescences (*Actinodaphne* I); the other with clustered or fasciculate pseudo-umbels (*Actinodaphne* II), and this separation is also supported by leaf micromorphology, where *Actinodaphne I* is mainly characterised by granulate periclinal walls on the abaxial epidermis, but *Actinodaphne* II has finely papillate periclinal walls with narrow or linear stomatal scales on the abaxial epidermis (Li et al. unpublished data). The apparent polyphyly of *Actinodaphne* is not surprising, given the absence of any clear morphological synapomorphies for the genus, but wider sampling is needed to confirm this.

In *Neolitsea*, at least based on the very limited sample used in our study, the genus divided into pinnately-veined (*Neolitsea* I) and tri-nerved (*Neolitsea* II) entities, which is also consistent with both gross morphology and leaf cuticular features (Li and Christophel 2000). The traditional recognition of *Neolitsea* based on its dimerous flowers was neither supported by ITS nor the combined analysis. Nevertheless, the genus was a monophyletic clade in the *mat*K analysis with very high bootstrap support (97%), and as with the *Sinosassafras*/*Parasassafras* *mat*K pair, the more conservative nature of that gene may be indicating phylogenetic signal which requires further investigation with more robust sampling.

Phylogenetic relationships within the *Laureae*. The results of our study and the molecular phylogenies of Chanderbali et al. (2001) and Rohwer (2000), show that traditional diagnostic generic and sectional characters such as deciduousness, number of leaf nerves, the size and arrangement of involucral bracts, the numbers of floral parts and flowers per pseudo-umbel, number of anther cells, shape of the cupule etc… have arisen repeatedly within the family. Accordingly, the homology of such characters needs to be reconsidered in light of the molecular data, as many appear to be homoplasious. The convergent nature of these characters is evident not only from their distributions within polyphyletic genera, but also because they occur several times in unrelated genera. This means that although some characters appear to support the molecular clades, the higher-level taxonomic utility of morphological data in Laureae must be treated with caution, until it can be determined through more extensive sampling and delineation of robust clades, which, if any, morphological characters provide enough resolution to reflect relationships at the level of genus and/or section.

Based on the molecular results, it was also clear that generic boundaries within the complex seem to require extensive redefinition, but using traditionally morphology it is difficult to find synapomorphies which define these molecular clades. Nevertheless, mapping of some of the more frequently used generic and suprageneric characters in Lauraceae by van der Werff and Richter (1996) suggested that inflorescence features were among the most reliable.

According to Li (1985, 1995) and Tsui (1987), the evolutionary trends in Laureae, and *Litsea* and *Lindera* in particular, are as follows: evergreen to deciduous, pinnate to triple-nerved or trinerved leaf venation, 3-merous to 2-merous flowers, and the evolutionary trend of progressive reduction in the inflorescence, each section representing a different stage of inflorescence development, and this is largely reflected in the ITS sequence-derived topology (Chanderbali et al. 2001). In our study, each of the clades in the combined analysis is homogenous, but each clade represents different characters: some have 2-merous flowers, some have trinerved leaves, and some others show different stages of inflorescence evolution.

Kostermans' (1957) regarded *Sassafras* of tribe Cinnomomeae to be part of the ancestral lineage leading to the Laureae, but recent molecular data (Rohwer 2000, Chanderbali et al. 2001) showed that Cinnamomeae (including both *Sassafras* and *Umbellularia*) are sister to the Laureae, hence our use of them as outgroup taxa.

The Laureae are characterised by basically thyrsoid inflorescences that are often protected by decussate or alternate bracts. Although there is general agreement that pseudo-umbels result from shortening of the inflorescence axis (Li 1985, Tsui 1987, Rohwer 1993, van der Werff and Richter 1996), there are relatively few studies of inflorescence evolution within the Lauraceae and Laureae in particular (Weberling 1985). Li (1985) and Tsui (1987) suggested an evolutionary series for the inflorescence based on studies of *Litsea* and *Lindera*, which we here term the Brachyblast Type, and which is exemplified by the *Laurus* Clade (D). The ancestral thrysoid inflorescence is reduced to a pseudo-umbellate one, which then condenses to multiple shortened brachyblasts and, by reduction, to solitary apparently racemose pseudo-umbels. Elongation of the axis could then lead to the condition of a solitary, axillary, long-pedunculate pseudo-umbel, a condition which may have arisen several times.

A second inflorescence development series noted by Rohwer (1993) can be seen in the *Litsea* Clade (C) where a thyrsoid cymose inflorescence is reduced by shortening of brachyblast internodes to the pseudo-racemose arrangement seen in *Lindera* section *Aperula*, *Litsea* sections *Litsea* and *Cylicodaphne*. In the final stage the number of flowers per involucre is reduced to one; the apparently convergent condition seen in both *Iteadaphne* and *Dodecadenia*.

The third type of inflorescence development seen in the *Lindera* Clade (B), is where a pair of pseudo-umbels occurs on each side of a terminal bud which then develops into a leafy shoot. In contrast, the alternative clustered inflorescence form seen in the *Actinodaphm* II Clade (A) consists of several pseudo-umbels clustered around a terminal bud from which either a new leafy shoot or several pseudo-umbels and a terminal bud develops, resulting in a mixed bud enclosed by imbricate scales. Tsui (1987) considered that these clustered inflorescences also resulted from shortening of the brachyblast, resulting in the two or more pseudo-umbels making the terminal bud appear to be axillary.

However, the lack of bootstrap support for the clades in our combined analyses as well as the small sample size relative to the numbers of species in the tribe as a whole make statements about character evolution highly speculative. Nevertheless, the fact that there are apparent developmental patterns suggests that inflorescence development may well be one of the more important sources of

morphology-based phylogenetic signal in Laureae. Certainly inflorescence features were regarded by Rohwer (1993) as being important in discussing generic affinities, and they were also seen in broad terms (paniculate versus thyrsoid versus involucrate) to map well onto the major lineages within the family (Rohwer 2000). Whether the finer scale developmental series within the Laureae hold up under broader, more robust sampling remains to be seen, but these patterns merit further investigation.

Biogeography. Li (1995) inferred on the basis of the large number of endemic genera and species found in tropical and subtropical Asia that the Laureae possibly originated in the southern part of Laurasia or northern part of Gondwana along the tropical coast area of the Tethys Sea not earlier than the mid-Cretaceous. Particularly for *Litsea* and *Lindera*, which are the core genera in the complex, the area around South China to Indo-Malaysia was proposed as the centre of origin and speciation, with later migration into tropical America and Australasia. The fossil record for Australasia shows that unlike earlier theories of recent invasion (Barlow 1981), the families predate the breakup of Gondwana and are part of the autochthonous Australian rainforest flora (Christophel 1994), supporting an early widespread Gondwanan distribution. Whereas Rohwer (2000) found that the basal Lauraceae (Cryptocaryeae) were Gondwanan, the Laureae were part of a terminal Laurasian/South American group (although including taxa from Australia and New Zealand). Similarly, Chanderbali et al. (2001) placed the Laureae within a boreotropical group, and in both studies the Laurasian Perseae were basal to the Laureae/Cinamomeae clades. This, combined with the absence of any clear Gondwanan versus Laurasian clades emerging in our study tends to support the idea of a Laurasian origin but with early and perhaps repeated migration and diversification into Gondwana, although more extensive sampling may help to improve the picture.

Conclusions

Phylogenetic analysis of the 'core' Laureae (*Litsea* complex) using *mat*K and ITS sequences provided a relatively resolved but very poorly bootstrap-supported phylogeny of the Laureae, with genera such as *Actinodaphne*, *Litsea*, *Neolitsea* and *Lindera* polyphyletic in the analyses. Four major clades resulted which are referred as the *Laurus*, *Litsea*, *Lindera* and *Actinodaphne* II clades, based on the placement of the various type sections of the genera within each group. These clades appear to reflect the importance of inflorescence structure and ontogeny within the Laureae, as well as data from cuticular micromorphology, but there was no support for traditional generic characters such as 2- versus 4-celled anthers.

However, because of the apparent fragmentation of these large genera based on relatively small taxon samples, as well as conflict between some well-supported clades in the *mat*K tree versus the combined analysis, much more detailed studies are required to clarify the relationships which emerged in our study and to allow for more precise generic boundary definitions and hypotheses about the historical biogeography of the tribe. Similarly, the phylogenetic significance of inflorescence and other morphological characters needs to be determined in the light of these new alignments.

Our study also highlighted several areas in need of further study. More data, including both more characters and more taxa, are required before a well-resolved robust phylogeny can be produced. In particular, extensive sampling is needed within the large polyphyletic such as *Litsea*, *Lindera* and *Neolitsea*.

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