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Phylogeny of *Neolitsea* (Lauraceae) inferred from Bayesian analysis of nrDNA ITS and ETS sequences

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Abstract. ITS and ETS-based sequence analyses of 29 *Neolitsea*, six *Actinodaphne* and five outgroup 'core' Laureae taxa show that *Neolitsea* is monophyletic with two large subclades, whereas most of the sampled *Actinodaphne* are paraphyletic below it. Inflorescence features appear to be among the more reliable morphological characters for explaining relationships between *Neolitsea* and other genera within the 'core' Laureae, with the *Neolitsea*/*Actinodaphne* clade defined by inflorescences lacking vegetative terminal buds in the main axis. Although the relationships within *Neolitsea* are still poorly resolved, there is enough structure to suggest that the genus seems to divide into two groups based on fruit shape: elliptic or ovoid, versus globose, although more evidence (both molecular and morphological) and wider taxon sampling are required to confirm this.

Keywords: Lauraceae; Laureae; ITS; ETS; *Neolitsea*; *Actinodaphne*; phylogeny; character evolution

Introduction

Lauraceae are one of the most important tropical woody families (Gentry 1988), but relationships within the family are still poorly understood. The 'core' Laureae, in addition to *Laurus*, consist primarily of four large, tropical, Old World genera: *Actinodaphne*, *Lindera*, *Litsea* and *Neolitsea* (Chanderbali et al. 2001, Li et al. 2004). Recent studies (Rohwer 1993, van der Werff and Richter 1996, Li and Christophel 2000, Rohwer 2000, Chanderbali et al. 2001, Li et al. 2004) have revealed a close relationship between these genera; all are dioecious and most have umbellate inflorescences subtended by involucral bracts. Amongst these, *Neolitsea* is an important component with about 100 species in tropical Asia and three in Australia (Hyland 1989, Rohwer 1993, van der Werff 2001). China is a centre of diversity for the genus, with 45

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species distributed across the south of the country (Li et al. 1984).

Traditionally, *Neolitsea* was considered to be close to *Litsea* (Kostermans 1957, Hyland 1989, Rohwer 1993, van der Werff 2001) and *Actinodaphne* (Allen 1938, Li et al. 2006), with *Neolitsea* treated as a section within *Litsea* by Bentham (1880) and Merrill (1906). *Neolitsea* is often characterised by its dimerous flowers, although other characters can help separate it from *Litsea* and *Actinodaphne*. *Neolitsea* usually has six anthers, clustered, triplinerved leaves and sessile sub-umbels, the latter frequently borne on the bare, inter-leaf cluster stem regions. In contrast, *Litsea* and *Actinodaphne* generally have 12 anthers and penninerved leaves, and the stalked sub-umbels are either axillary or borne on short shoots; *Litsea* having alternate (rarely opposite) leaves, persistent bracts (until anthesis) and largely unmodified calyx tubes in fruit, whereas *Actinodaphne* has generally verticillate leaves, caducous bracts and cyathiform, lobed calyx tubes subtending the fruit (Allen 1938). Van der Werff (2001) considered that *Actinodaphne* is ''best recognized by its usually rather large, whorled leaves, and perulate terminal buds'', and ''differs from *Litsea* species in the presence of bract scars at the base of the inflorescence.''

Neolitsea differs from most other large Old World Lauraceae genera in lacking formal subgenera or sections. The only (but nomenclaturally invalid) attempt to subdivide the genus was that of Liao (1988), where the Taiwanese *Neolitsea* species were divided into two sections, each with two subsections. Although his classification is problematic (e.g. two varieties of *N. aciculata* (Bl.) Koidz. listed in his key are placed into different subsections, despite still being regarded by him as conspecific), taxa were separated largely on venation characteristics such as adaxially elevated or plane veinlets (sections), and the distinctness and direction of secondary veins (subsections). The importance of these features has not been explored widely within the genus, but merits further study.

Li et al. (2004) using *mat*K sequences showed that *Neolitsea* seems to be monophyletic with very high bootstrap support. However, because of the limited sampling within *Neolitsea* in that study, the phylogeny within the genus, and its position relative to other genera, especially *Litsea* was unclear. In a molecular study of *Actinodaphne*, Li et al. (2006) found that although that genus was polyphyletic, the single included 'outgroup' *Neolitsea* species was embedded deep within a clade containing most of their sampled *Actinodaphne*. This unexpected result means that the relationship between these two genera in particular needs to be resolved, and if they are closely related, morphological character evolution to help explain this needs to be explored. Therefore, in order to develop a more robust phylogeny and to establish species relationships and character evolution patterns within *Neolitsea*, our current study uses data from more extensive taxon sampling, in particular from Chinese *Neolitsea* spp.

Improved understanding of relationships within Lauraceae has come in recent years from the addition of new of character sets including wood and bark anatomy, inflorescence structure (van der Werff and Richter 1996), leaf venation and cuticular patterning (Klücking 1987, Li and Christophel 2000). This has been further expanded through sequence-based analyses of various chloroplast and nuclear markers, mainly *mat*K, ITS and ETS (Rohwer 2000, Chanderbali et al. 2001, Li et al. 2004, Li et al. 2006). In particular, the ITS region of 18S-26S nuclear rDNA is used widely in phylogenetic analysis of closely related plant species (Baldwin 1992, Baldwin et al. 1995). Chanderbali et al. (2001) and Li et al. (2004, 2006) found that ITS gave good phylogenetic signal in both Lauraceae and Laureae, making it ideal for a phylogenetic analysis of *Neolitsea*. Similarly, the ETS region of 18S-26S nuclear rDNA seems to be evolving under similar functional constraints and at comparable rates to ITS (Musters et al. 1990, Good et al. 1997, Hitchen et al. 1997) and ETS sequence data have augmented ITS, increasing resolution and/or support in

rDNA-based phylogenies (e.g. Baldwin and Markos 1998). ETS is also useful in Lauraceae, providing additional characters to help resolve relationships and demonstrate polyphyly in *Actinodaphne* s. l. (Li et al. 2006).

Accordingly, our study made use of both ITS and ETS to investigate inter-species relationships in *Neolitsea* to:

(1) Produce a more resolved molecular phylogeny with stronger support that can show the relationships between *Neolitsea* and other genera within the 'core' Laureae, especially *Actinodaphne*.
(2) Test support for the hypothesis that genus *Neolitsea* is monophyletic.
(3) Investigate relationships within *Neolitsea* based on molecular markers and relate these to morphology.

Materials and methods

Sampling. A total of 41 ingroup taxa were sampled, representing 29 *Neolitsea*, six *Actinodaphne*, four *Litsea*, and one each from *Lindera* and *Laurus*. Although two of the *Neolitsea* species were Australian and one from Japan, the remainder were Chinese taxa. *Cinnamomum pittosporoides* was chosen as the outgroup on the basis of previous studies (Rohwer 2000, Chanderbali et al. 2001). A list of the taxa sampled, voucher information and GenBank accession numbers are provided in Table 1.

DNA extraction. Total DNA was extracted from either field-collected, silica gel-dried leaves or herbarium specimens, using a modified CTAB procedure (Doyle and Doyle 1987). For older herbarium specimens, 3 × CTAB was added and mixed with the ground leaf tissue, following by 24-hour incubation at 60°C before the CI (24:1 chloroform: isopropanol) was added into the mixture. For silica gel-dried leaves, 2 × CTAB was added and incubation was reduced to 6 hours. In both cases, DNA was precipitated in ethanol overnight at −20°C, followed by purification using the QIAquick® Purification Kit (Qiagen) prior to PCR amplification.

PCR and sequencing. PCR amplification of the whole ITS region succeeded in most cases using the primers ITS F (Chanderbali et al. 2001) and ITS 4 (White et al. 1990). For some older herbarium specimens, from which poor-quality DNA templates were obtained, amplification using that primer pair failed and the primer combinations of ITS F/ITS 2 and ITS 3/ITS 4 (White et al. 1990, Chanderbali et al. 2001) were used to amplify the ITS1 and ITS2 regions (including the 5.8S nrDNA region) separately. The primer pair of ETS1/18S-IGS (Baldwin and Markos 1998 and Li et al. unpublished) was used in the amplification of the 3′ end fragment of the ETS region. Sequences of these primers are listed in Table 2. To minimize the selective amplification of pseudogenes or paralogous ITS copies, all reactions included DMSO to a final concentration of 10% (Buckler and Holtsford 1996, Buckler et al. 1997). All PCR amplifications were conducted in an ABI Gene Amp 9700™ PCR system, including negative controls to detect contamination.

PCR products were visualized with ethidium bromide staining after electrophoresis in a 1% agarose gel. If a single band was detected (most cases), the PCR product was purified with the Qiagen QIAquick PCR Purification Kit®. Where multiple bands were identified, the ITS fragment was chosen based on its size (approximately 700 bp, the size of ITS fragment within *Neolitsea*), then purified through electrophoresis in a 1.5% agarose gel and extracted by using QIAquick Gel Extraction Kit® (Qiagen). In a very few cases, two bands of similar size were obtained, but which were so close in length that purification through electrophoresis in agarose gel was inefficient. We cloned the multiple PCR products into the pMD18-T Vector (TaKaRa), sequencing at least five clones for each sample and compared the obtained sequences with ITS sequences from other samples to find the correct product.

The same amplification primers were also used to sequence the purified fragments in both directions. An ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit® with Big Dye Terminator® ver. 3.1 chemistry was used for cycle sequencing and the products were then sequenced on an Applied Biosystems 3100 DNA automated sequencer after purification.

Building the molecular data matrix. Forward and reverse sequences of each fragment were edited and combined into a single consensus sequence. In very few cases, uncertainties in ETS sequences were recorded as ambiguities. The ITS1, 5.8S and ITS2 sequences were combined creating a single sequence. Complete GenBank ITS sequences for 13

Table 1. Taxa of the genus *Neolitsea* and related taxa included in the analysis

Taxon	Voucher	Source	ETS	ITS
Ingroups				
***Neolitsea* (29)**				
Neolitsea aurata (Hay.) Koidz. var. *aurata*	Li J. 2002181 (HITBC)	Guangxi, China	DQ120557	DQ124270
Neolitsea aurata var. *chekiangensis* (Nakai) Yang et P.H. Huang	Zhang S.-Y. 5482 (KUN 0162041)	Zhejiang, China	DQ120558	DQ124271
Neolitsea aurata var. *paraciculata* (Nakai) Yang et P.H. Huang	Zhang S.-Y. 4794 (KUN 0162049)	Zhejiang, China	EF192150	_
Neolitsea brassii Allen	Gray, B. 03911 (KUN 0793628)	Queensland, Australia	DQ120559	DQ124272
Neolitsea cambodiana Lec. var. *glabra* Allen	Li X.-G. 202474 (IBK 00009945)	Guangdong, China	DQ120560	DQ124273
Neolitsea chrysotricha H.W. Li	Wu S.-G. 7095 (KUN 0106438)	Yunnan, China	DQ120561	DQ124274
Neolitsea chuii Merr.	Li J. 2002063 (HITBC)	Yunnan, China	DQ120562	DQ124275
Neolitsea confertifolia (Hemsl.) Merr.	Xi X.-Y. 414 (PE 1272040)	Hunan, China	DQ120563	DQ124276
Neolitsea dealbata (R.Br.) Merr.	Gray. B. 03993 (KUN 0793630)	Queensland, Australia	DQ120564	DQ124277
Neolitsea homilantha Allen	Li J. 2002071 (HITBC)	Yunnan, China	DQ120565	DQ124278
Neolitsea kwangsiensis Liou	Wu S.-J. 3419 (IBK 00010186)	Hongkong, China	DQ120566	DQ124279
Neolitsea levinei Merr.	Li H.-W. 29 (HITBC)	Yunnan, China	AY934884	AY265401
Neolitsea lunglingensis H.W. Li	Li J. 2002058 (HITBC)	Yunnan, China	DQ120567	DQ124280
Neolitsea oblongifolia Merr. et Chun	Deng L. 3070 (KUN 0108298)	Hainan, China	EF192151	_
Neolitsea ovatifolia Yang et P.H. Huang var. *ovatifolia*	Wu S.-J. 3246 (IBK 00010360)	Hongkong, China	DQ120568	DQ124281
Neolitsea ovatifolia var. *puberula* Yang et P.H. Huang	Mao P.-Y. 03875 (KUN 0108307)	Yunnan, China	DQ120569	DQ124282
Neolitsea pallens (D. Don) Momiyama	Qinhai-Xizang Exped. 5972 (KUN 0108358)	Xizang China	DQ120570	DQ124283
Neolitsea phanerophlebia Merr.	Deng L. 7511 (KUN 0108338)	Guangdong, China	DQ120571	DQ124284

Neolitsea pingbienensis Yang et P.H. Huang	Mao P.-Y. 04139 (KUN 0108220)	Yunnan, China	DQ120572	DQ124285
Neolitsea pinninervis Yang et P.H. Huang	Li J. 2002187 (HITBC)	Guangxi, China	DQ120573	DQ124286
Neolitsea polycarpa Liou	Zhou Z.-K. et al. EXLS-0252 (KUN 0695675)	Yunnan, China	DQ120574	DQ124287
Neolitsea pulchella (Meissn.) Merr.	Li J. 2002166 (HITBC)	Guangxi, China	DQ120575	DQ124288
Neolitsea sericea (Bl.) Koidz.	K. Midorikawa 2180 (KUN 0108215)	Honshu, Japan	DQ120576	DQ124289
Neolitsea shingningensis Yang et P.H. Huang	Chen S.-Q. 9594 (KUN 0108166)	Guangxi, China	EF192152	_
Neolitsea sutchuanensis Yang var. *sutchuanensis*	Zhao Z.-X. 64 (KUN 0108178)	Sichuan, China	DQ120577	DQ124290
Neolitsea sutchuanensis var. *gongshanensis* H.W. Li	Feng G.-M. 6987 (KUN 0108134)	Yunnan, China	DQ120578	DQ124291
Neolitsea undulatifolia (Level.) Allen	Li J. 2002203 (HITBC)	Guangxi, China	DQ120579	DQ124292
Neolitsea wushanica (Chun) Merr. var. *wushanica*	Deng L. 1515 (KUN 0108200)	Guangdong, China	EF192153	_
Neolitsea wushanica var. *pubens* Yang et P.H. Huang	Liu L.-H. 15149 (KUN 0162057)	Hunan, China	DQ120580	DQ124293
***Actinodaphne* (6)**				
Actinodaphne forrestii (Allen) Kosterm.	Li H.-W. 2 (HITBC)	Yunnan, China	AY934881	AY265399
Actinodaphne kweichowensis Yang et P.H. Huang	Li H.-Q. 40091 (KUN 0106643)	Guangxi, China	AY817124	AY817114
Actinodaphne obovata (Nees) Bl.	Li H.-W. 1 (HITBC)	Yunnan, China	AY934880	AY265398
Actinodaphne paotingensis Yang et P.H. Huang	Hainan Exped. 962 (IBK 00003425)	Hannan, China	AY817128	AY817118
Actinodaphne pilosa (Lour.) Merr.	Xie L.-S. 613 (KUN 0047277)	Guangxi, China	AY817125	AY817115
Actinodaphne tsaii Hu	Feng G.-M. 22638 (KUN 0047322)	Yunnan, China	AY817129	AY817119
***Laurus* (1)**				
Laurus nobilis L.	Li H.-W. 16 (HITBC)	Yunnan, China	DQ120553	AY265392

Table 1. (Continued)

Taxon	Voucher	Source	ETS	ITS
***Lindera* (1)**				
Lindera obtusiloba Bl.	Sino-Amer. Exped. 1308(KUN 0151469)	Hubei, China	DQ120546	AY265411
***Litsea* (4)**				
Litsea cubeba (Lour.) Pers.	Li H.-W. 28 (HITBC)	Yunnan, China	DQ120523	AY265402
Litsea glutinosa (Lour.) C.B. Rob.	Li H.-W. 21 (HITBC)	Yunnan, China	AY934883	AY265403
Litsea umbellata (Lour.) Merr.	Li H.-W. 24 (HITBC)	Yunnan, China	DQ120528	AY265404
Litsea dilleniifolia P.Y. Pai et P.H. Huang	Li H.-W. 19 (HITBC)	Yunnan, China	DQ120532	AY265405
Outgroup				
***Cinnamomum* (1)**				
Cinnamomum pittosporoides Hand.-Mazz.	Li H. 5252 (KUN 0108156)	Yunnan, China	DQ120554	DQ124269

taxa from Li et al. (2004, 2006): *Actinodaphne forrestii*, *A. kweichowensis*, *A. obovata*, *A. paotingensis*, *A. pilosa*, *A. tsaii*, *Laurus nobilis*, *Lindera obtusiloba*, *Litsea cubeba*, *L. dilleniifolia*, *L. glutinosa*, *L. umbellata*, and *Neolitsea levinei* were also used. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) searches of the new data against GenBank sequences confirmed that they were Lauraceae. Both the ETS and ITS datasets were aligned separately, each automatic alignment was generated using ClustalX 1.81 (Thompson et al. 1997) and then edited manually using BioEdit 7.0.1 (Hall 1999).

Congruence between the ETS and ITS datasets was checked using the Incongruence Length Difference (ILD) or Partition Homogeneity Test (Farris et al. 1995), as implemented in PAUP* version 4.0b10 (Swofford 2002). Sequences were analysed with heuristic searching, 1000 replicates each with 10 random sequence addition replicates, MULTREES and TBR branch swapping. Based on these results, the ETS and ITS data sets were then also combined into a single data set.

Bayesian analysis. Bayesian Inference (BI) was applied in order to overcome some of the conditions that may lead to Long-Branch Attraction (LBA) in Maximum Parsimony (MP) methods. BI differs from MP in being a model-based protocol with *a priori* assumptions, but possesses advantages over other model-based methods such as Maximum Likelihood (ML) in terms of its ability to use complex models of evolution with greater computational efficiency (Huelsenbeck et al. 2001, Nylander et al. 2004).

An evolutionary model for each molecular data set (ETS: TrNef + I + G; ITS: TrN + I + G; ETS & ITS: TrN + I + G) was selected based on simultaneous evaluation of 56 models of sequence evolution using Modeltest (Posada and Crandall 1998). Bayesian phylogenetic analysis were performed with the MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001), using 1,000,000 generations with four chains (three chains ''heated'' using the default parameters) and sampling from the Markov Chain every 100th cycle. For each data set, Markov Chain Monte Carlo (MCMC) chains runs were repeated twice as a safeguard against spurious results. To ensure sampling of topologies after chain convergence, we discarded the first 1000 trees as burn-in. Examination of the log-likelihood values and the observed consistency between runs suggested that

Table 2. Primers used for ITS and ETS amplification and sequencing in the study

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	CCACAACTCCTTGCTGAGCTT	Li et al. unpublished
Reverse		
18S-IGS	GAGACAAGCATATGACTAC TGGCAGGATCAACCAG	Baldwin and Markos 1998

this burn-in period was sufficiently long. A Bayesian majority-rule consensus tree was constructed from the remaining trees using PAUP*.

Morphology. Thirty morphological characters (Tables 3 and 4) were chosen for character evolution and mapping because they have been regarded as important in delimiting *Neolitsea* from other 'core' Laureae, and/or between groups within *Neolitsea* (e.g. Bentham 1880, Merrill 1906, Kostermans 1957, Li et al. 1984, Liao 1988, Hyland 1989, van der Werff and Richter 1996, Li and Christophel 2000, Li et al. 2004).

An initial Maximum Parsimony (MP) analysis was run on the morphological character matrix using WinClada ver. 1.00.08 (Nixon 2002) employing an heuristic search (maxtree = 10,000; mult*n = 10,000; hold/ = 100; and multiple TBR + TBR mult*max* branch-swapping in effect). All characters were unordered, equally weighted, and *Cinnamomum pittosporoides* was the outgroup taxon. Bootstrap support values were calculated from 10,000 re-samplings holding 10 trees per run and TBR off. Morphological character state distribution patterns were also mapped on the Bayesian tree obtained from the ETS & ITS combined analysis using MacClade 4.0 (Maddison and Maddison 1996) to determine which characters were phylogenetically informative and might help to clarify the morphological relationships between *Neolitsea* and other Laureae, as well as relationships within the genus.

Results

Sequence characteristics. Complete ITS sequences were obtained from 38 of the 42 samples. The ITS lengths from the 25 *Neolitsea* that sequenced completely ranged from 578 to 610 bp, and from 579 to 627 bp in the other ingroup taxa. The percentage G + C content of *Neolitsea* ITS sequences ranged from 69.87% to 72.17%, with 69.13% to 73.11% for the other ingroup taxa. Aligned ITS sequences were 675 bp long, with 147 variable characters, of which 80 were informative (11.85%). ETS 3'-end sequences were obtained for all 42 taxa and were all 395 bp long (except for *Actinodaphne tsaii* at 394 bp). Aligned ETS sequences were 395 bp long, and with only one indel. Of these, 88 positions were variable and 66 informative (16.71%). The percentage G + C content of the ETS sequences ranged from 50.13% to 53.67% in *Neolitsea*, and 51.39% to 53.42% in the other ingroup taxa.

The incongruence length difference (ILD) test indicated that the ITS and ETS data sets were significantly incongruent ($P = 0.001$) in their phylogeny estimates. Nevertheless, the two regions were analysed simultaneously in order to get a more resolved and better-supported topology. The combined matrix contained 1,070 characters, of which 145 were informative (13.55%). The percentage G + C content of the ITS / ETS matrix in ranged in *Neolitsea* from 62.76% to 64.32% and between 62.39% to 65.16% for other ingroup taxa.

ITS Analysis. The monophyly of *Neolitsea* was strongly supported by the ITS data (Fig. 1) with 25 *Neolitsea* forming a clade with

Table **3.** Character coding for morphological analysis

1. Young branchlets: glabrous (0), pubescent (1)
2. Leaf arrangement: always alternate (0), alternate or crowded on top of branchlets (1), always verticillate or subverticillate (2)
3. Leaf texture: papery (0), leathery (1)
4*. Leaf venation: always penninerved (0), penninerved or triplinerved (1), always triplinerved (2)
5. Transverse veins: not conspicuous adaxially (0), conspicuous adaxially (1)
6. Transverse veins: not conspicuous abaxially (0), conspicuous abaxially (1)
7*. Lateral veins: without veinlets at one side near the margin (0), with veinlets at one side near the margin (1)
8. Leaves: glabrous adaxially (0), pubescent adaxially (1)
9. Leaves: glabrous abaxially (0), pubescent abaxially (1)
10. Leaves: not glaucous abaxially (0), glaucous abaxially (1)
11. Petiole: glabrous (0), pubescent (1)
12. Petiole length: minimum length $<$ 10 mm and maximum length $<$ 20 mm (0), minimum length $\geq$10 mm and maximum length $\geq$ 20 mm (1)
14*. Inflorescence type: without vegetative terminal bud in the main axis (0), with vegetative terminal bud in the main axis (1)
15. Inflorescence: sessile (0), stipitate (1)
16. Pedicel length: $\leq$ 2 mm (0), $>$ 2 mm (1)
17. Peduncle: glabrous (0), pubescent (1)
18*. Involucres: imbricate (0), decussate (1)
19*. Involucres: deciduous (0), persistent (1)
20*. The basic floral number: dimerous (0), trimerous (1)
21*. Anthers: 2-locular (0), 4-locular (1)
22. Perianth segments: elliptic (0), ovate (1)
23. Filaments: glabrous (0), pubescent (1)
24. Male flowers: rudimentary pistil absent (0), rudimentary pistil present (1)
25. Male flowers: glands of the 3rd whorl filaments sessile (0), glands of the 3rd whorl filaments stipitate (1)
26*. Fruit shape: globular (0), ovoid or ellipsoid (1)
27. Fruits: not seated on the cup-shaped or flat-disc-like perianth tube (0), seated on the cup-shaped or flat-disc-like perianth tube (1)
28. Fruiting pedicel: without persistent tepals (0), without persistent tepals (1)
29. Fruiting pedicel: glabrous (0), pubescent (1)
30. Fruiting pedicel length: $\leq$ 7 mm (0), $>$ 7 mm (1)

* Selected morphological characters mapped on the Bayesian tree from the ITS & ETS combined data set.

100% posterior probability support (PPS) above a paraphyletic *Actinodaphne* grade. Within *Neolitsea*, *N. kwangsiensis* was isolated, but the remainder of the sampled species fell into two clades (A and B). Clade A, with 13 *Neolitsea* taxa, received 97% PPS. Within it, *N. pallens* and *N. chrysotricha* formed a basal trichotomy with the remainder, in which *N. lunglingensis* subtended two subclades, each with five taxa and all branches showing moderate PPS.

In contrast, Clade B (68% PPS) was internally poorly resolved, with its 11 *Neolitsea* taxa largely forming a polytomy, although there were three small subclades each with at least 97% posterior probability: *N. phanerophlebia* plus *N. pulchella*; both varieties of *N. aurata* with *N. cambodiana* var. *glabra*; and *N. dealbata* with *N. ovatifolia* var. *puberula*, but not var. *ovatifolia*.

Sister to the *Actinodaphne* - *Neolitsea* clade were (in order) *Litsea umbellata*, *Lindera*

Table 4. Data matrix of morphological characters in the study

Taxa	12	34	56	78	1 90	11 12	11 34	11 56	11 78	12 90	22 12	22 34	22 56	22 78	23 90
Actinodaphne forrestii	12	10	01	00	10	11	21	00	10	01	10	01	11	10	11
Actinodaphne kweichowensis	12	10	01	00	10	11	20	00	10	01	1?	11	10	10	10
Actinodaphne obovata	12	12	01	01	10	11	10	11	10	01	11	11	01	10	10
Actinodaphne paotingensis	12	10	00	00	11	11	10	01	10	01	10	01	1?	10	1?
Actinodaphne pilosa	12	10	01	01	10	11	10	11	10	01	1?	11	10	10	10
Actinodaphne tsaii	12	00	01	00	10	10	10	01	10	01	10	11	11	10	10
Cinnamomum pittosporoides	10	10	01	10	10	10	0?	11	1?	?1	11	11	10	10	11
Laurus nobilis	10	10	11	00	00	10	21	10	11	10	01	?1	11	??	??
Lindera obtusiloba	01	12	01	10	10	11	01	1?	?1	11	00	01	11	00	1?
Litsea cubeba	12	00	00	00	00	00	11	1?	11	11	11	11	10	00	00
Litsea glutinosa	10	10	00	01	10	11	11	1?	11	11	1?	11	10	00	00
Litsea umbellata	10	10	00	00	10	10	21	10	11	11	11	10	00	11	10
Litsea dilleniifolia	00	10	11	00	00	01	21	11	11	11	11	11	10	10	10
Neolitsea aurata var. *aurata*	11	12	00	00	10	10	00	10	11	10	10	11	11	10	10
Neolitsea aurata var. *chekiangensis*	11	12	00	00	11	10	00	10	11	10	10	11	11	10	10
Neolitsea brassii	10	11	01	10	1?	01	00	0?	11	10	1?	11	10	??	??
Neolitsea cambodiana var. *glabra*	12	11	01	00	01	10	00	00	11	10	11	11	10	11	10
Neolitsea chrysotricha	10	12	11	10	10	11	00	??	?1	10	1?	??	?1	00	10
Neolitsea chuii	01	12	11	00	00	01	00	01	11	10	11	11	01	00	00
Neolitsea confertifolia	12	10	00	00	10	10	00	00	11	10	11	11	11	11	10
Neolitsea dealbata	1?	11	01	10	1?	10	00	0?	11	10	1?	11	10	11	1?
Neolitsea homilantha	01	02	00	00	01	00	00	01	11	10	11	11	11	00	11
Neolitsea kwangsiensis	01	12	11	10	00	0?	00	0?	11	10	11	01	10	00	10
Neolitsea levinei	12	12	01	10	11	11	00	01	11	10	11	01	10	00	11
Neolitsea lunglingensis	10	12	00	00	10	11	00	01	11	10	11	11	?1	00	11
Neolitsea ovatifolia var. *ovatifolia*	01	12	00	00	00	00	00	0?	11	10	10	01	00	00	00
Neolitsea ovatifolia var. *puberula*	11	12	00	00	00	10	00	0?	11	10	10	01	00	00	00
Neolitsea pallens	10	12	00	00	00	10	00	??	?1	10	1?	??	?0	10	11
Neolitsea phanerophlebia	12	02	01	10	10	11	00	01	11	10	11	10	?0	00	10
Neolitsea pingbienensis	01	12	00	00	01	10	00	01	11	10	10	1?	11	01	1?
Neolitsea pinninervis	01	10	00	00	00	01	10	1?	11	10	10	11	10	00	11
Neolitsea polycarpa	11	12	00	00	10	10	00	11	11	10	10	11	11	10	01
Neolitsea pulchella	11	12	00	00	10	10	00	00	11	10	10	10	10	10	10
Neolitsea sericea	10	12	11	11	10	11	00	01	11	10	10	11	10	10	10
Neolitsea sutchuanensis var. *sutchuanensis*	00	12	11	00	00	01	00	??	?1	10	1?	??	?1	10	11
Neolitsea sutchuanensis var. *gongshanensis*	00	12	11	00	10	01	00	??	?1	10	1?	??	?1	10	10
Neolitsea undulatifolia	12	10	00	00	00	10	10	00	11	10	11	01	11	10	11
Neolitsea wushanica var. *pubens*	10	11	00	00	01	10	10	0?	11	10	11	01	?0	10	00

obtusiloba with moderate PPS, and then a small, well-supported clade of *Litsea glutinosa*, *L. dilleniifolia*, and *Laurus nobilis*. *Actinodaphne forrestii* and *Litsea cubeba* were placed at the base of the tree with *Cinnamomum pittosporoides*.

ETS Analysis. The ETS analysis (Fig. 2) was very much less resolved. In this tree,

Lindera obtusiloba was basal with the outgroup, as sister to a large but well-supported (94% PPS) polytomy. Within the polytomy, four clades of varying support were detected. Clade I (100% PPS) consists of five taxa, with *Actinodaphne paotingensis* sister to *Litsea dilleniifolia* and a small subclade of both *Neolitsea sutchuanensis* varieties plus *N. wushanica* var. *pubens*. Clade II with nine *Neolitsea* taxa (55% PPS), had *N. phanerophlebia* basal to a trichotomy of *N. chrysotricha*; a subclade with *N. oblongifolia*, *N. shingningensis* and *N. pingbienensis*; and a subclade of *N. aurata* var. *paraciculata* (separate from the other varieties) + *N. cambodiana* var. *glabra,* and *N. ovatifolia* var. *puberula* (but again, not var. *ovatifolia*) + *N. wushanica*.

In Clade III, *Laurus nobilis* and *Litsea glutinosa* formed a moderately supported pair, but the main other group (Clade IV) was a strongly supported *Neolitsea* clade (99% PPS). *N. kwangsiensis* and *N. chuii* formed a basal polytomy with an unresolved but well-supported (93% PPS) polytomy of five *Neolitsea* spp. including both sampled varieties of *N. aurata*, *N. confertifolia*, *N. undulatifolia* and *N. dealbata*.

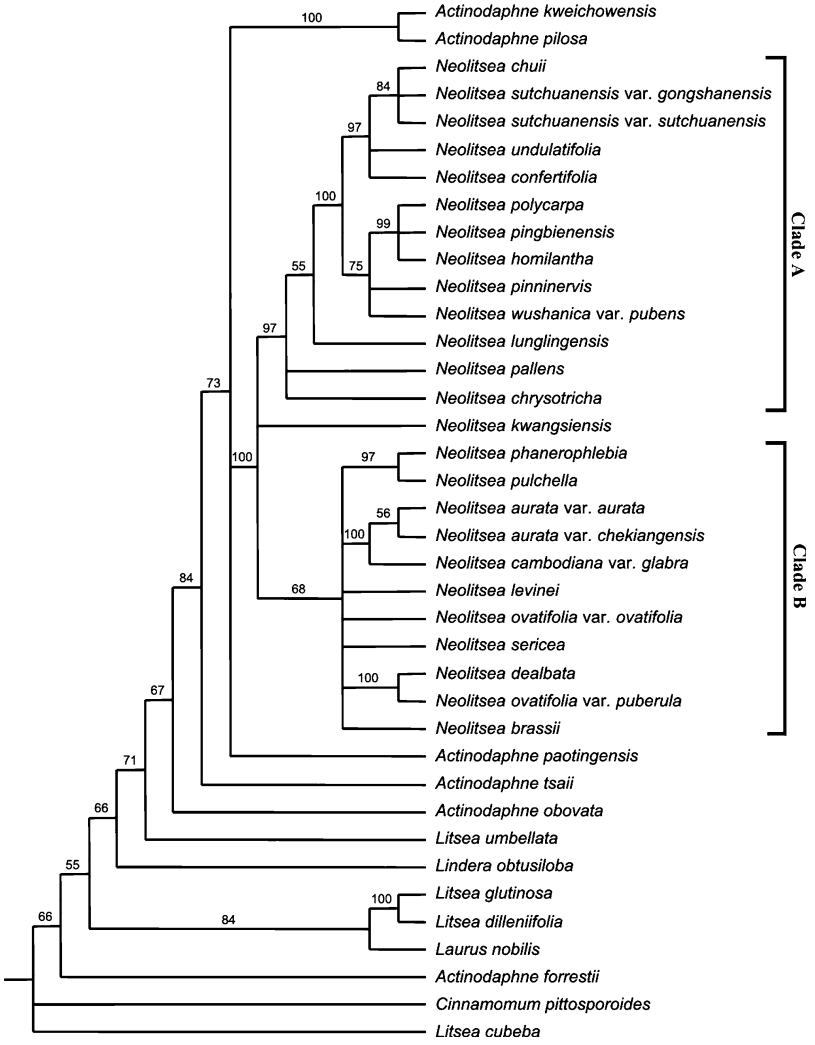

Fig. 1. Bayesian consensus of 9001 trees derived from the analyses of ITS sequence data. Bayesian posterior probability values greater than 50% are shown above branches

Combined analysis. The phylogenetic tree generated from the combined ETS & ITS data (Fig. 3) is very similar to the ITS tree, and the monophyly of *Neolitsea* is also strongly supported with 100% posterior probability value in the analysis of the combined data. Within the *Neolitsea* clade, *N. kwangsiensis* and a small subclade including *N. aurata* (both varieties) were placed between two large subclades (referred to here as Clade A′ and B′), which between them contain the rest 22 *Neolitsea* taxa. The components of the Clade A' are same as those of Clade A in the ITS tree but with different structure. Within Clade A′, *N. pallens* and *N. lunglingensis* are placed at the base, next to a polytomy formed by 11 *Neolitsea* taxa. Clade B' differs from Clade B of the ITS tree by excluding *N. aurata* var. *aurata* and var. *chekiangensis*. Within Clade B', nine *Neolitsea* taxa form a large polytomy, and eight others form three small subclades. Sister to the large *Neolitsea* monophyletic clade is *Actinodaphne tsaii*, and the remaning three *Actinodaphne* taxa are also very closely related to *Neolitsea*. All *Neolitsea* and *Actinodaphne* taxa (except *A. forrestii*) formed a

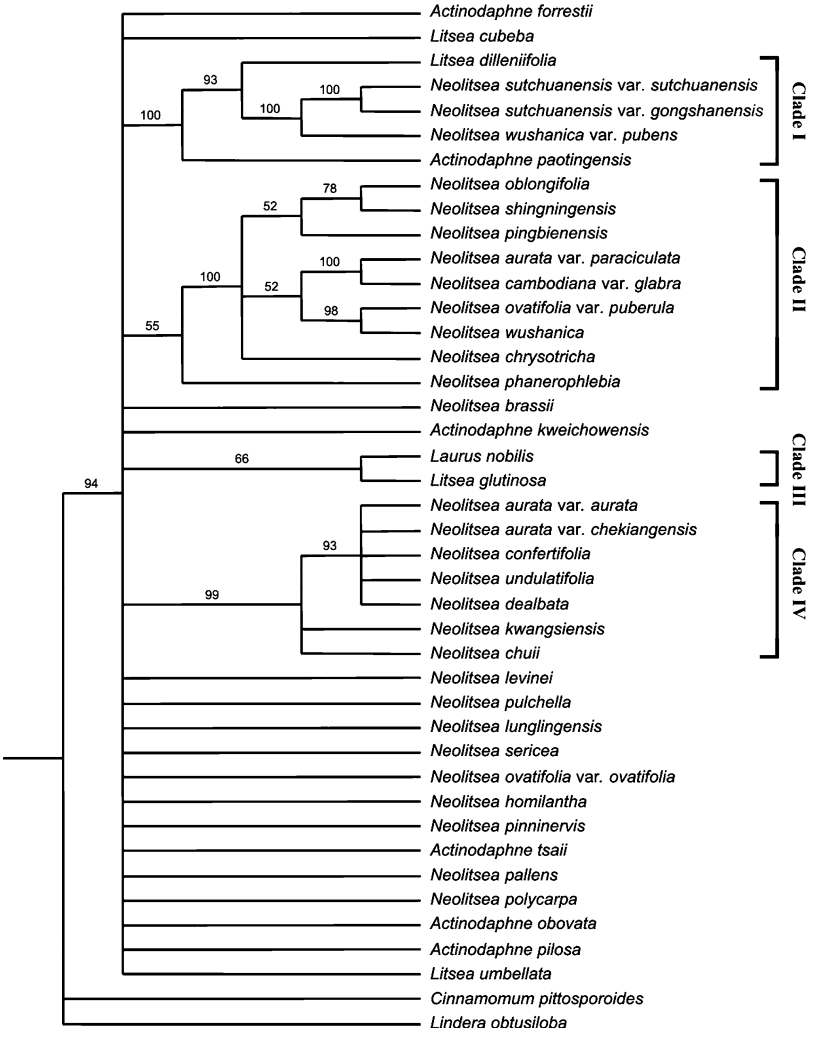

Fig. 2. Bayesian consensus of 9001 trees derived from the analyses of ETS sequence data. Bayesian posterior probability values greater than 50% are shown above branches

monophyletic clade (95% PPS). Below this was *Litsea umbellata*, then a small *Laurus* clade consisting of *Litsea glutinosa*, *L. dilleniifolia*, and *Laurus nobilis* (86% PPS). *A. forrestii* and *Litsea cubeba* formed a polytomy with most of the Laureae (95% PPS), except for *Lindera obtusiloba* which sat in a basal polytomy with *Cinnamomum pittosporoides*.

Morphology and character mapping. Thirty morphological characters (Table 3) were used for a morphological analysis of the relationships between the *Neolitsea* spp. as well as for character mapping onto taxon groups created in the molecular analyses. The morphological analysis produced six equally parsimonious trees (L = 140, CI = 22, RI = 59) and a randomly chosen tree for character evolution exploration is shown in Fig. 4, with those branches that collapsed under strict consensus, as well as bootstrap support values ≥50% indicated. There was almost no supported structure in the resulting consensus tree (only three branches had bootstrap values > 50%), although *Actinodaphne* was monophyletic (57% bootstrap support), united by abaxially conspicuous transverse veins (char 6/1), long petioles (12/1), and imbricate (18/0), deciduous involucres (19/0). The genus was sister to a clade defined by dimerous flowers (20/0), representing all the

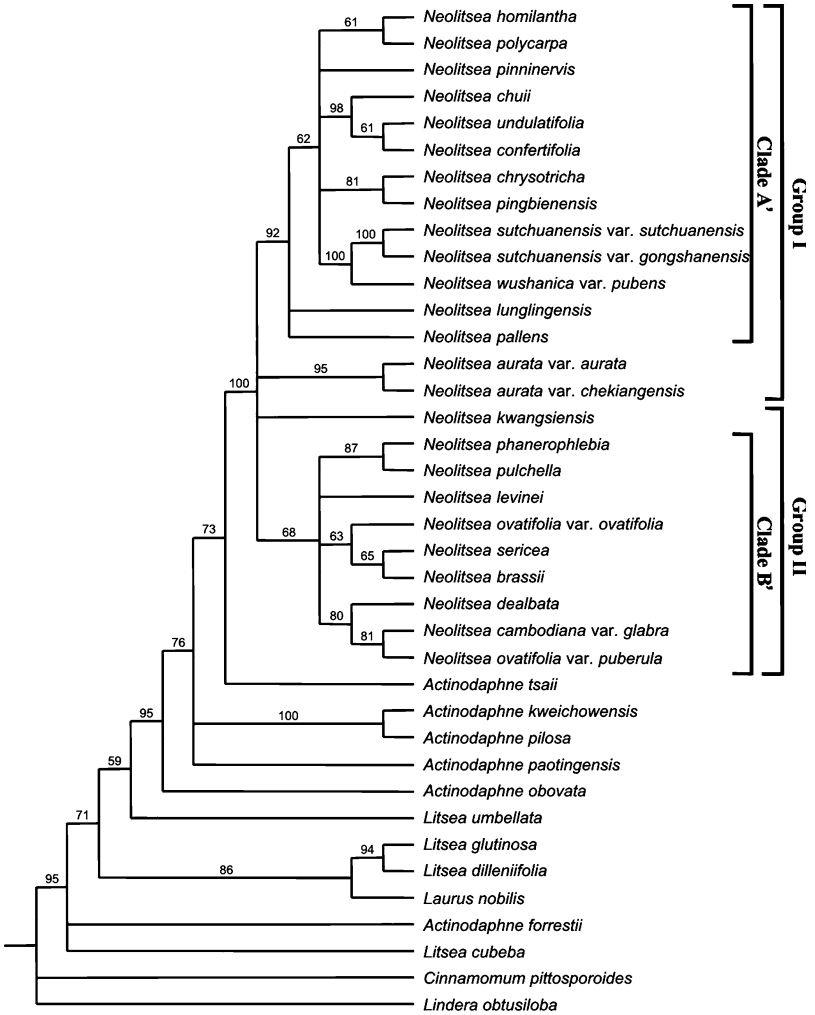

Fig. 3. Bayesian consensus of 9001 trees derived from the analyses of ITS & ETS combined sequence data. Bayesian posterior probability values greater than 50% are shown above branches

Neolitsea taxa, but with *Lindera obtusiloba* deeply embedded within it. The *Actinodaphne*/*Neolitsea* clade was defined by the presence of sessile inflorescences (15/0) that lack a terminal vegetative bud on the main axis (14/0), albeit without bootstrap support. The only other supported branches (both 51%) were a terminal *A. obovata*/*A. pilosa* pair, and the two varieties of *N. ovatifolia* as a terminal pair. None of the morphologically supported branches occurred in the molecular trees. A *Litsea*/*Laurus* grade subtended the *Actinodaphne*/*Neolitsea*/*Lindera* clade.

Although all of the morphological characters were mapped onto the combined ITS/ETS tree, most of them failed to show any clear synapomorphic pattern. However, seven in particular will be discussed here in more detail, either because they have been used previously to discuss intergeneric relationships between *Neolitsea* and related 'core' Laureae, infrageneric patterns within *Neolitsea*, or because when mapped onto the combined molecular consensus tree they showed possible character state evolutionary changes in relation to major, well-supported clades (Fig. 5). Character 14 (inflorescence type) was regarded as a key character for explaining the relationships among *Actinodaphne*, *Lindera*, *Litsea* and *Neolitsea* on the basis of former studies (Rohwer 1993, van der Werff 2001, Li et al. 2004). Characters 18 and 19 (involucral features) have been used to separate *Actinodaphne* from *Neolitsea* and other 'core' Laureae. Character 20 (the basic floral number) is considered to delimit *Neolitsea* from *Actinodaphne*, *Lindera* and *Litsea*, whereas character 21 (the number of anther cells) traditionally separates *Neolitsea* from *Laurus* and *Lindera*. Because Li et al. (2004) found that leaf venation might also be helpful taxonomically within *Neolitsea*, we included character 4 (leaf venation pattern). Characters 7 (lateral veins) and 26 (fruit shape) were also mapped, because their state distributions showed patterns agreeing with clades on the molecular trees and thus they might be useful for explaining relationships within *Neolitsea*.

Discussion

Data combinability. One of the unresolved issues in systematics is how to proceed when different data partitions collected from the same taxa support conflicting phylogenies (Bull et al. 1993, De Queiroz et al. 1995, Miyamoto and Fitch 1995, Cunningham 1997). At the heart of the controversy is whether conflicting data partitions should be analysed separately (Lanyon 1993, Miyamoto and Fitch 1995) or combined in a simultaneous analysis (Kluge 1989, Barrett et al. 1991). Although the ILD test detected significant incongruence between ITS and ETS data sets ($P = 0.001$), we would argue that the two data sets should still be combined in a simultaneous analysis for the following reasons. Firstly, the ILD test does not distinguish whether incongruence between data sets results with different phylogenetic histories and/or rates of evolution. It has also been shown to be less reliable as a measure of incongruence when the data sets differ markedly in size (Dowton and Austin 2002) as in the case of our study (395 bp in ETS; 695 bp in ITS). Secondly, although parts of the same transcriptional unit, ETS seems to evolve more rapidly than ITS (Baldwin and Markos 1998, Bena et al. 1998), a feature which by itself could be sufficient to explain the incongruence detected. Thirdly, comparison of the combined ETS & ITS results with the separate analyses of the ITS and ETS data sets was more resolved and had higher PPS values. Because of this, the ITS and ETS data were deemed to be combinable, although caution is recommended (Li et al. 2004).

Monophyly of *Neolitsea*. Bayesian analyses of the ITS and ETS & ITS combined data strongly support the monophyly of *Neolitsea* with 100% posterior probabilities in both trees. The ETS results do not clearly support the monophyly of *Neolitsea*, but there was such poor resolution of most relationships within the 'core' Laureae (see Fig. 2) that conclusive statements are not possible either way.

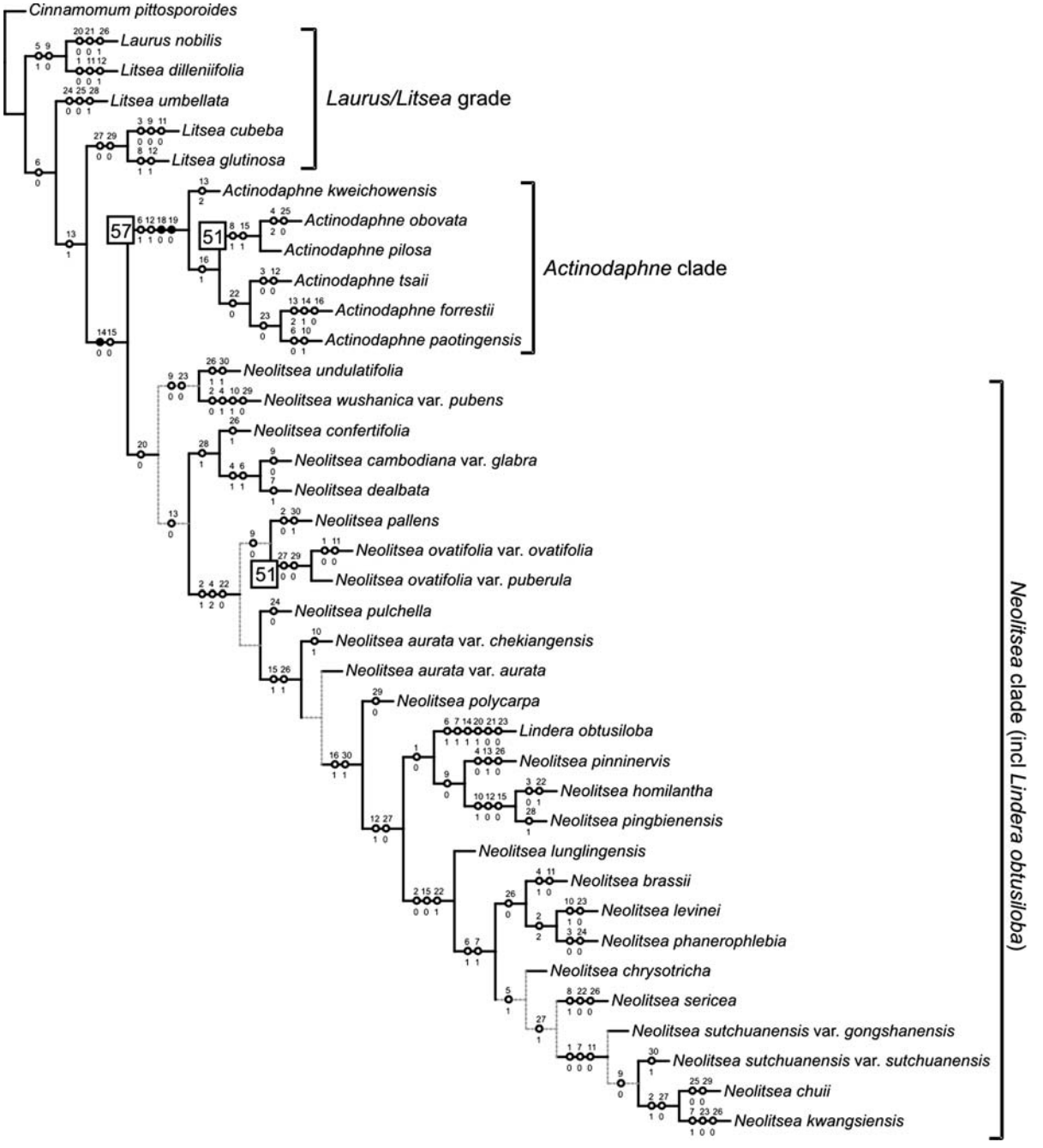

Fig. 4. Randomly selected equally most parsimonious tree from six trees derived by heuristic analysis L = 140; CI = 22; RI = 59). Characters for each branch are shown above, with their states below; open circles represent homoplasious characters. Bootstrap values greater than 50% at nodes are shown in boxes. Dashed lines represent branches which collapse under strict consensus

One of the ways in which *Neolitsea* can be delimitated from other 'core' Laureae is that all *Neolitsea* so far examined lack a 7–27 bp insertion in the ITS region between positions 20 and 49 in ITS1. In contrast, of the other sampled taxa only *Actinodaphne paotingensis* shares this insertion, although this is an anomalous species in *Actinodaphne* as it

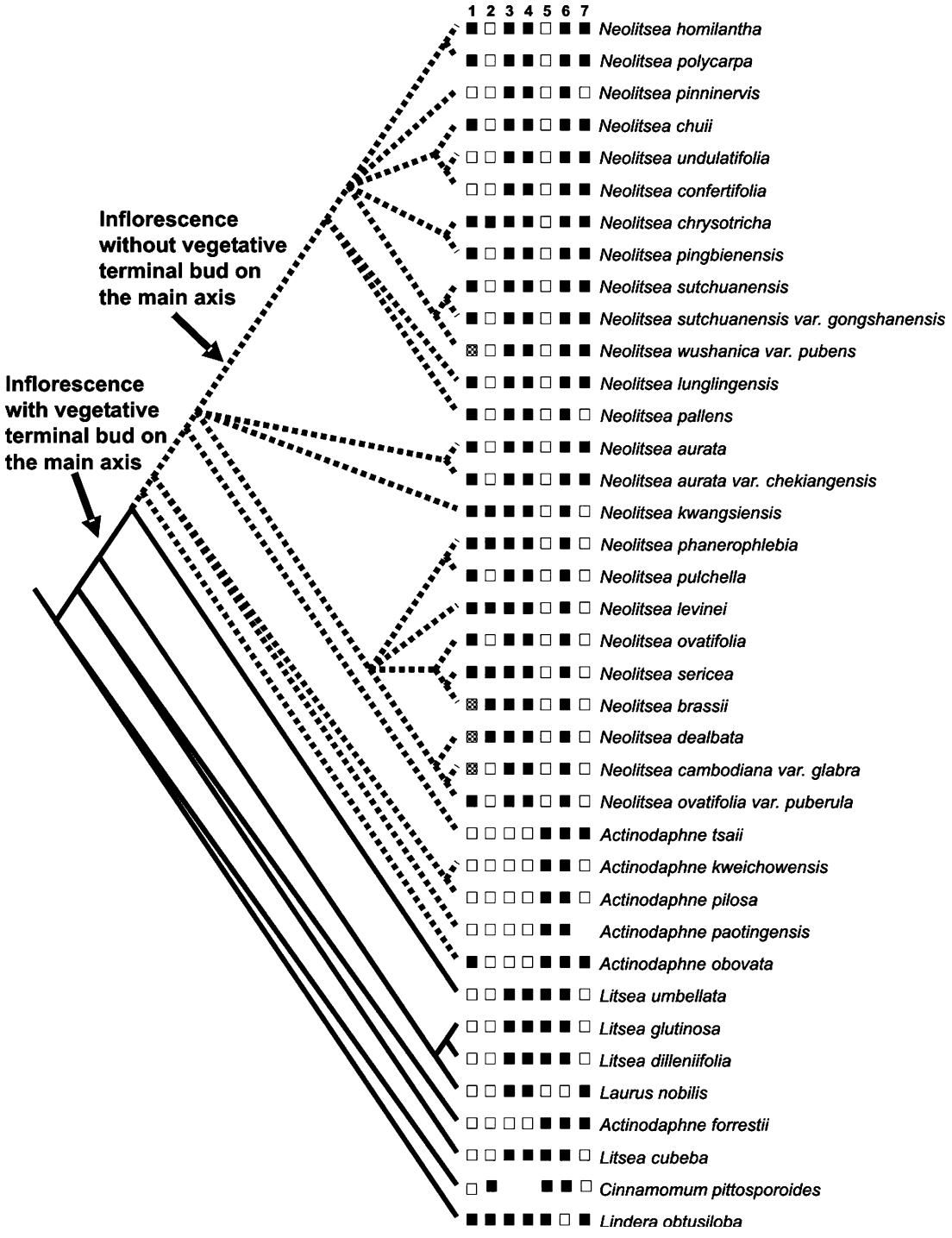

Fig. 5. Selected morphological characters mapped on the Bayesian consensus tree derived from analyses of ITS & ETS combined sequence dataset. Inflorescence types are mapped on the tree. Distribution of the other characters is shown with numbered columns of boxes: 1) Leaf venation: always penninerved (white); penninerved or triplinerved (dots); always triplinerved (black); 2) Leaf lateral veins: without veinlets at one side near the margin (white); with veinlets at one side near the margin (black); 3) Involucres: imbricate (white); decussate (black); 4) Involucres: deciduous (white); persistent (black); 5) The basic floral number: dimerous (white); trimerous (black); 6) Anthers: 2-locular (white); 4-locular (black); 7) Fruit shape: globular (white); ovoid or ellipsoid (black)

possesses 6–8 tepals, 9–15 fertile stamens and the lower pair of anther cells are lateral introrse (Li et al. 1984).

Neolitsea also separates from the other genera in the 'core' Laureae on morphology. Even though the morphological analysis gave poorly supported clades, none of which were recovered in the molecular analyses, character mapping of morphology onto the molecular trees suggests that there are evolutionary patterns that can be used in studies of Laureae evolution. Traditionally *Neolitsea* is considered to be close to *Litsea* (Kostermans 1957, Hyland 1989, Rohwer 1993), since they share inflorescences consisting of pseudo-umbels (*Neolitsea*: clustered or fasciculate pseudo-umbels or single pseudo-umbel, *Litsea*: clustered or fasciculate pseudo-umbels, single pseudo-umbel or pseudo-umbels arranged on a short shoot) with a decussate and persistent involucre (see Fig. 5 columns 3, 4); 4-locular anthers (see Fig. 5 column 6); and pollen sacs arranged in two pairs, one above the other. However, after careful checking of the inflorescences from all available materials, we found that the inflorescences between *Neolitsea* and *Litsea* are structurally different.

Re-examination of the inflorescences in 'core' Laureae showed that they divide into two types. Type I (e.g. *Laurus*, *Lindera*, *Litsea*) is characterized by the presence of a vegetative terminal bud on the main axis. Type II (e.g. *Neolitsea* and *Actinodaphne* except *A. forrestii*) lacks these terminal buds. The inflorescence description for *Neolitsea* by van der Werff (2001): "inflorescence umbellate, umbel with several flowers, single or several close together along a short shoot" is incorrect, because *Neolitsea* actually shares the same inflorescence type as *Actinodaphne* (except for *A. forrestii*), forming a synapomorphy for the clade (Fig. 5). Although the presence or lack of the vegetative terminal bud is the main feature for each inflorescence type, the key difference between them is their different origin, viz. clustered or fasciculate pseudo-umbels to both types produced via different evolutionary pathways. For clustered or fasciculate pseudo-umbels of Type I, they may result from shortening of the brachyblast (or short shoot), with the peduncles of these pseudo-umbels shortening sequentially, ultimately resulting in clustered or fasciculate pseudo-umbels (Li 1985, Tsui 1987, Li et al. 2004, Fig. 6A). For clustered or fasciculate pseudo-umbels of Type II, earlier studies suggested that they may arise from a thyrsoid inflorescence by shortening the main axis and secondary peduncle of the cyme to form the clustered or fasciculate pseudo-umbels (Rohwer 1993, van der Werff 2001, Li et al. 2004, Fig. 6B). Our molecular results similarly place the thyrsoid *A. pilosa* and *A. obovata* close to the clustered or fasciculate pseudo-umbellate *Neolitsea* and *Actinodaphne* (except *A. forrestii*), supporting the importance of this character for phylogenetic affinity.

Dimerous (*Neolitsea*) versus trimerous (*Litsea*) flowers is a feature separating these two genera (Fig. 5 column 5), and *Neolitsea* also usually has verticillate, triplinerved and only rarely penninerved leaves, whereas *Litsea* has usually alternate (rarely opposite or subverticillate), penninerved leaves (Allen 1938).

Neolitsea shares relatively few morphological characters with *Actinodaphne*, despite having same kind of inflorescence; *Neolitsea* has decussate, usually persistent involucral bracts while those of *Actinodaphne* are imbricate and deciduous (Fig. 5 column 3, 4). Similarly, the flowers are dimerous in *Neolitsea* vs. trimerous in *Actinodaphne* (Fig. 5 column 5), and they differ in leaf arrangement (verticillate in *Neolitsea,* subverticillate in *Actinodaphne*) and venation patterns (triplinerved or rarely penninerved in *Neolitsea* vs. penninerved or rarely subtriplinerved in *Actinodaphne*).

Nevertheless, the ITS and ETS & ITS combined trees show that *Actinodaphne* is closely related to *Neolitsea,* both genera together forming a well-supported clade, agreeing with their placement in Li et al. (2006) and supporting the phylogenetic utility of inflorescence characters in the group. The pseudo-umbels seen in *Lindera* and *Litsea* are derived from shortening of the brachyblast; the peduncles shorten sequentially, ultimately resulting in

clustered or fasciculate pseudo-umbels (Li 1985, Tsui 1987, Li et al. 2004). The pseudo-racemose inflorescences arranged in pseudo-umbels seen in *Laurus nobilis*, *Litsea dilleniifolia* and *L. glutinosa* might also have formed by shortening of brachyblast internodes and distal internodes, even though the peduncles of these pseudo-umbels do not shorten, creating the effect of a pseudo-raceme.

The basal position of the *Laurus* clade along with *Actinodaphne forrestii*, *Litsea cubeba* and *L. umbellata*, suggests that Type II inflorescences are derived, whereas those of Type I are ancestral in the Laureae.

Relationships within *Neolitsea*. Although relationships within *Neolitsea* are relatively poor resolved in our study, some patterns were generated by the ETS & ITS data and the sampled *Neolitsea* taxa can be divided into two groups (I and II) largely on fruit shape (Fig. 5 column 7). In Group I (Clade A and *N. aurata*) fruit shape is elliptic, ovoid or in *N. pallens* and *N. pinninervis*, globose. In contrast, all members of Group II (Clade B plus *N. kwangsiensis*) possess globose fruit (Fig. 5 column 7). Fruit shape might be therefore be more phylogenetically informative in *Neolitsea* than the leaf venation characters suggested by Liao (1988) and Li et al. (2004) (Fig. 5 column 1). Nevertheless, some venation features show promise; Group II seems to possess lateral veins with veinlets at one side near the margin as a synapomorphy (albeit with loss in some taxa, see Fig. 5 column 2), whereas in Group I only *N. chrysotricha* shows that feature.

An unexpected result in our analyses was the consistent separation of *N. ovatifolia* var. *ovatifolia* from var. *puberula*, and further study is needed to clarify taxonomic and varietal limits in this species.

Despite the reasonable support for many of the branches in the combined molecular phylogeny, the lack of more robust and/or supporting evidence from morphology or other gene regions means that we cannot yet resolve the relationships between *Neolitsea* species. The relatively small sample size means that more evidence (both molecular and morphological) and as wide a range of sampled taxa as possible need to be employed in further studies in order to resolve the question of relationships and character evolution within *Neolitsea*.

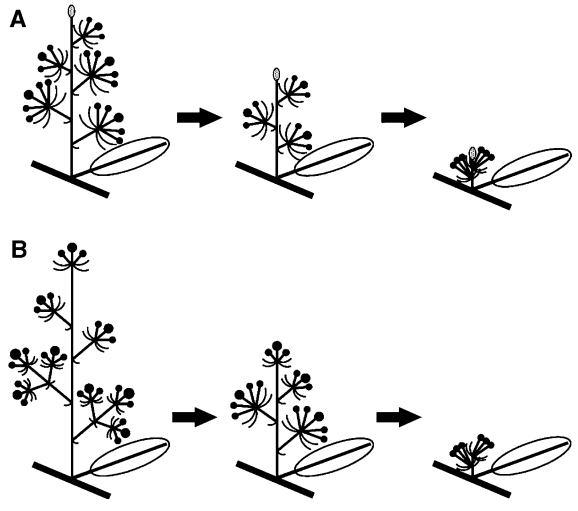

Fig. 6. Evolutionary patterns of inflorescence Types I and II. **A** Type I: clustered or fasciculate pseudo-umbels with terminal vegetative bud; **B** Type II: clustered or fasciculate pseudo-umbels lacking a terminal vegetative bud

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