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THE PHYLOGENY OF TRIBE *ZINGIBEREAE* (*ZINGIBERACEAE*) BASED ON ITS (nrDNA) AND *trn*L–F (cpDNA) SEQUENCES

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A phylogenetic analysis of the tribe *Zingibereae* (*Zingiberaceae*) was performed using nuclear ribosomal DNA (ITS1, 5.8S and ITS2) and chloroplast DNA (*trn*L (UAA) 5′ exon to *trn*F (GAA)). The tribe is monophyletic with two major clades, the *Curcuma* clade and the *Hedychium* clade. *Paracautleya*, sampled for the first time, comes out as predicted while *Caulokaempferia* comes out in a different position from that found in another recent study. The genera *Boesenbergia* and *Curcuma* are apparently not monophyletic.

Keywords. Molecular systematics, phylogeny, *Zingiberaceae*, *Zingibereae*.

INTRODUCTION

The tropical monocotyledonous family *Zingiberaceae* is highly natural, containing some 53 genera mostly occurring from India to New Guinea. Until recently the genera were grouped into three or four tribes, mainly according to the nature of the lateral staminodes. Four subfamilies are currently recognized, two of them monogeneric, the other two with two tribes each (Kress *et al.,* 2002).

The tribe *Zingibereae* contains 25 genera, *Caulokaempferia* K. Larsen being unplaced. All genera so far examined have the plane of leaf distichy parallel to the direction of rhizome growth, and most have large, petaloid lateral staminodes. The ovary is trilocular with axile placentation, or is clearly derived from this condition, and chromosome numbers mostly vary according to the genus.

Kress *et al.* (2002) were able to sample widely across the *Zingiberaceae*, providing sequences of the ITS and *mat*K (chloroplast DNA) genes for 104 species in 41 genera. However, they were unable to obtain sequences of 12 genera.

Previous studies in the *Zingibereae* at the Royal Botanic Garden Edinburgh (RBGE) have focused on the genus *Roscoea* Sm. (Ngamriabsakul *et al.*, 2000). Here we build on that study, examining the genera of the *Zingibereae* in detail. We have obtained material of *Paracautleya* R.M. Sm., which was among the genera not available to Kress *et al.* (2002), and have used the same nuclear gene as Kress *et al.* (2002) but a different chloroplast gene, *trn*L–F.

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Materials and methods

Plant material

Many of the samples were taken from the research collections at RBGE. Another important source was a field trip in Thailand during July and August 1999 (C.N. and M.F.N.). Other *Zingiberaceae* researchers have also provided samples. Table 1 gives details of the plants sequenced, as well as authorities for all species.

Outgroup selection

Early phylogenetic studies within the family (Soltis *et al.*, 2000; Wilf *et al.*, 2000; Wood *et al.*, 2000) appear to show unambiguously that the tribe *Alpinieae* was the basal branch in the family, followed by *Globbeae*, *Hedychieae* and *Zingibereae*. Our study was conducted before Kress *et al.* (2002) discovered that *Siphonochilus* J.M. Wood & Franks was basal so we chose three species in the *Alpinieae*, *Alpinia galanga*, *Renealmia battenbergiana* and *Pleuranthodium schlechteri*, as the outgroup because living plants were available at RBGE and had been used in a previous study (Rangsiruji *et al*., 2000b). This choice does not affect the results adversely since these three species are still clearly outside the clade being studied.

Ingroup selection

For the ingroup taxa, 34 species from 16 genera were sequenced (see Table 1). The species were chosen to represent variation within each genus, in terms of both total number of species and distribution. At least 10% of the species in each genus were included to avoid any excess heterogeneity in rates of molecular evolution that may be found. Table 1 shows the genus names, the numbers of species so far described and the number included in this study.

ITS sequences of some missing genera and additional species of *Zingibereae* were obtained from GenBank:

- *Haniffia cyanescens* (Ridl.) Holttum (AF202407); two species in genus
- *Hitchenia glauca* Wall. (AF202413); three species in genus
- *Pommereschea lackneri* Wittm. (AF202405); two species in genus
- *Rhynchanthus beesianus* W.W. Sm. (AF202415; Wood *et al*., 2000); seven species in genus
- *Camptandra ovata* Ridl. (AJ388302); three species in genus
- *Boesenbergia cordata* R.M. Sm. (AJ388277; Searle & Hedderson, 2000); c.50 species in genus.

The remaining five genera in *Zingibereae*, as circumscribed by Kress *et al.* (2002), which could not be included owing to lack of material, are *Haplochorema* K. Schum. (three or four species), *Laosanthus* K. Larsen & Jenjitt. (monotypic), *Nanochilus* K. Schum. (monotypic), *Parakaempferia* A.S. Rao & D.M. Verma (monotypic), and *Stadiochilus* R.M. Sm (monotypic).

TABLE 1. Taxa sequenced in this study with source, accession number, voucher (if available) and GenBank accession numbers

Taxon	Source, accession number and voucher	GenBank accession number		Total number of species/ Number of species included in this study
		ITS1, 5.8S and ITS2	*trn*L–F	
Outgroup				
Alpinia galanga (L.) Willd.	RBGE 19771077; *A. Rangsiruji* 3 (E)	AY424739	AY424775	—
Pleuranthodium schlechteri (K. Schum.) R.M. Sm.	WAI 75p168; *C. Cory* 5 (E)	AY424740	AY424776	—
Renealmia battenbergiana Cummins ex Baker	RBGE 19740104; *A. Rangsiruji* 27 (E), C8482 (E)	AY424741	AY424777	—
Ingroup				
1. *Boesenbergia aurantiaca* R.M. Sm.	RBGE 19850843; *Ngamriabsakul* 29 (E)	AY424742	AY424778	50/5
2. *B. basispicata* K. Larsen ex Sirirugsa	RBGE 19851662; *Ngamriabsakul* 26 (E)	AY424743	AY424779	
3. *B. gelatinosa* K. Larsen	Thailand; *Newman* 905 (BKF, E)	AY424744	—	
4. *B. longiflora* (Wall.) Kuntze	Thailand; *Newman* 904 (BKF, E)	AY424745	—	
5. *B.* aff. *longiflora*	Thailand; *Newman* 934 (BKF, E)	AY424746	—	
6. *Camptandra parvula* (Baker) Ridl.	Malaysia, Ibrahim; –	AY424747	AY424780	4/2
7. *Caulokaempferia violacea* K. Larsen & Triboun	Thailand; *Ngamriabsakul* 61 (BKF, E)	AY424748	AY424781	10/1
8. *Cautleya spicata* (Sm.) Baker	RBGE 19590760; *Ngamriabsakul* 30 (E)	AY424749	AY424782	2/1
9. *Cornukaempferia longipetiolata* Mood & K. Larsen	Thailand, RBGE 19991165; *Ngamriabsakul* 32 (E)	AY424750	AY424783	2/1

TABLE 1. (*Cont'd*)

Taxon	Source, accession number and voucher	GenBank accession number		Total number of species/ Number of species included in this study
		ITS1, 5.8S and ITS2	*trn*L–F	
10. *Curcuma alismatifolia* Gagnep.	Thailand; *Newman* 944 (BKF, E)	AY424751	AY424784	50/6
11. *C. amada* Roxb.	RBGE 19810001; *M. Ardiyani* 27 (E)	AY424752	AY424785	
12. *C. ecomata* Craib	Thailand; *Ngamriabsakul* 38 (BKF, E)	AY424753	—	
13. *C. harmandii* Gagnep.	Thailand; *Ngamriabsakul* 48 (BKF, E)	AY424754	—	
14. *C. parviflora* Wall.	Thailand; *Ngamriabsakul* 32 (BKF, E)	AY424755	—	
15. *C. rubescens* Roxb.	Thailand, Sirirugsa; –	AY424756	—	
16. *Distichochlamys citrea* M.F. Newman	RBGE 19901463; *Ngamriabsakul* 24 (E)	AY424757	AY424786	2/1
17. *Hedychium coccineum* Sm.	RBGE 19751806; –	AY424758	—	50/5
18. *H. gardnerianum* Roscoe	RBGE 19910120; *Ngamriabsakul* 27 (E)	AY424759	AY424787	
19. *H.* ×*raffillii* A.W. Hill	RBGE 19662631; –	AY424760	—	
20. *H. villosum* Wall.	RBGE 19901454; –	AY424761	—	
21. *H.* sp.	Thailand; *Newman* 916 (BKF)	AY424762	AY424788	
22. *Kaempferia angustifolia* Roscoe	RBGE 19621457; –	AY424763	AY424789	40/4
23. *K. elegans* Wall.	Thailand; *Newman* 879 (BKF, E)	AY424764	AY424790	
24. *K. marginata* Carey	RBGE 19860057; –	—	—	
25. *K. rotunda* L.	RBGE 19590678; *Ngamriabsakul* 28 (E)	AY424765	AY424791	
26. *Paracautleya bhatii* R.M. Sm.	India, Bhat; K.G.B. 11349 (E)	AY424766	AY424792	1/1

TABLE 1. (*Cont'd*)

Taxon	Source, accession number and voucher	GenBank accession number		Total number of species/ Number of species included in this study
		ITS1, 5.8S and ITS2	*trn*L–F	
27. *Pyrgophyllum yunnanensis* (Gagnep.) T.L. Wu & Z.Y. Chen	RBGE 19901313; *Ngamriabsakul* 33 (E)	AY424767	AY424793	1/1
28. *Roscoea bhutanica* Ngamriab.	RBGE 19841747; *Ngamriabsakul* 23 (E)	AY424768	AY424794	19/2
29. *R. humeana* Balf.f. & W.W. Sm.	RBGE 19871610; *Ngamriabsakul* 8 (E)	AY424769	AY424795	
30. *Scaphochlamys kunstleri* (Baker) Holttum	RBGE 19643232; *Ngamriabsakul* 25 (E)	AY424770	AY424796	20/2
31. *S. lanceolata* (Ridl.) Holttum	RBGE 19782413; –	AY424771	AY424797	
32. *Smithatris supraneanae* W.J. Kress & K. Larsen	Thailand, Paisooksantivatana; *Y. Paisooksantivatana* 00081101 (BK)	AY424772	AY424798	1/1
33. *Stahlianthus involucratus* (Baker) R.M. Sm.	RBGE 19981701; *Ngamriabsakul* 34 (E)	AY424773	AY424799	6/1
34. *Zingiber junceum* Gagnep.	Thailand, RBGE 19991169; *Newman* 954 (BKF, E)	AY424774	AY424800	85/1

Total genomic DNA extraction

The CTAB method (Doyle & Doyle, 1990) was used to obtain total DNA of plant cells. Fresh leaf samples were taken and kept in dry silica gel before DNA extraction. The modified protocol for DNA extraction followed our previous study (Ngamriabsakul *et al.*, 2000). The QIAgen Dneasy kit (QIAGEN, 1997) with liquid nitrogen was also used, with few modifications, to give high quality total DNA. Incubation times were increased to 30 and 10 minutes, instead of 10 and 5 minutes, in steps three and four of the protocol, respectively.

PCR amplification and DNA sequencing

Each PCR reaction was 50µl in volume. The PCR reaction mix was prepared before aliquoting to each tube and adding template DNA as the last component. The components and the conditions of the PCR followed Ngamriabsakul *et al.* (2000), but with primer volume decreased to 2µl instead of 5µl. No significant reduction in products was detected. The ITS1, 5.8S and ITS2 complete region was amplified by using primers '5P' and '8P' (Möller & Cronk, 1997). ITS1 and ITS2 had to be amplified separately for some species. Primer '5P' and primer '2K' (Rangsiruji, 1999) were then used to amplify ITS1, while primer '3P' and primer '8P' were used for ITS2.

PCR amplification of *trn*L–F with primers 'c' and 'f' (Taberlet *et al.*, 1991) was found to show more than one distinct band in some species, e.g. two when using the conditions described for ITS (Ngamriabsakul *et al.*, 2000). Various conditions for the PCR reaction were then tried. It was found that when using primers 'c' and 'f' to amplify some *Zingiberaceae* DNA, optimal conditions were needed, e.g. a smaller volume of primer and a well-calibrated thermocycler. In cases where a second band could still be observed after amplification, the *trn*L–F region was then further amplified using two sets of primers. Primers 'c' and 'd', along with 'e' and 'f', were used to separate amplifications of *trn*L intron and *trn*L–F spacers, respectively. All the products of primers 'c' and 'f' (a complete region of *trn*L intron and *trn*L–F spacer), 'c' and 'd' (*trn*L intron), and 'e' and 'f' (*trn*L–F spacer) were successfully obtained as a single band. PCR products were purified before automated cycle sequencing using a QIAquick™ PCR purification kit. Forward and reverse sequencings, using the same primers as for PCR reactions, were performed for sequence confirmation as described in Ngamriabsakul *et al.* (2000). The primer sequences used in this study are (5′ to 3′), 5P = GGA AGG AGA AGT CGT AAC AAG G, 8P = CAC GCT TCT CCA GAC TAC A, 2K = GGC ACA ACT TGC GTT CAA AG, 3P = GCA TCG ATG AAG AAC GTA GC, c = CGA AAT CGG TAG ACG CTA CG, d = GGG GAT AGA GGG ACT TGA AC, e = GGT TCA AGT CCC TCT ATC CC, f = ATT TGA ACT GGT GAC ACG AG.

Sequence analysis

All sequences were verified by comparison of their forward and reverse sequences in Autoassembler™ (Applied Biosystems Division). Sequence boundaries of the

range of ITS1, 5.8S and ITS2 in all taxa were determined by comparison with published sequence data for *Roscoea* (Ngamriabsakul *et al*., 2000) and *Alpinia* Roxb. (Rangsiruji *et al*., 2000a). All sequences are deposited in GenBank (see Table 1).

The sequences were aligned using CLUSTAL_X (Thompson *et al*., 1997; Hickson *et al*., 2000) with default values (e.g. gap-opening cost = 15) and manual adjustment in only the first alignment. Because of the high similarity in length and nucleotides of the *trn*L–F sequences (see Table 2), a sensitivity test of alignment was performed only for the ITS data set by varying the gap-opening cost to 5, 10, 20 and 25 to yield four other different alignments (Jeanmougin *et al*., 1998). The alignments were directly submitted to parsimony analysis. This was to determine the effects of alignment and gaps in the ITS data set on the resulting phylogenetic estimates. Character congruence is advocated as both an internal criterion (Bogler & Simpson, 1996) and an external criterion (Giribet & Wheeler, 1999) for choosing the best alignment based on parsimony. Thus we chose a rescaled consistency (RC) index for each analysis (Bogler & Simpson, 1996) and *P*-values of the homogeneity test for each of the differently aligned ITS data sets and the *trn*L–F data set as indicators of optimal alignment.

A transition/transversion ratio was determined using MacClade version 3.0 (Maddison & Maddison, 1992) on one of the most parsimonious trees from the unweighted initial analysis. The G + C content and sequence divergence among taxa

TABLE 2. Sequence characteristics of nuclear ribosomal DNA (ITS1, 5.8S, ITS2) and chloroplast DNA (*trn*L–F)

Parameter	ITS1, 5.8S, ITS2	*trn*L–F
Length range (total) (bp)	573–672	894–960
Length mean (total) (bp)	591.24	913.04
Length range (ingroup) (bp)	577–672	894–960
Length mean (ingroup) (bp)	592.00	913.52
Length range (outgroup) (bp)	573–591	906–914
Length mean (outgroup) (bp)	582.00	909.33
Aligned length (bp)	722 (662)*	1008
G + C content range (%)	52.30–59.82	31.35–33.41
G + C content mean (%)	55.71	32.78
Sequence divergence (ingroup) (%)	0.00–23.89	0.11–2.50
Sequence divergence (in/outgroup) (%)	9.98–23.75	1.79–3.88
Number of variable sites (% in parentheses)	342 (51.66)*	123 (12.20)
Number of constant sites (% in parentheses)	320 (48.34)*	885 (87.80)
Number of informative site (% in parentheses)	213 (32.17)*	38 (3.77)
Number of autapomorphic sites (% in parentheses)	129 (19.49)*	85 (8.43)
Transitions (unambiguous)	483	30
Transversions (unambiguous)	258	38
Transitions/transversions (ts/tv)	1.87	0.79
Average number of steps per character	1.414	0.149

*662 bp is the length of the ITS data set used in the analyses.

were calculated using 'Base frequencies' and 'Show pairwise distance' options in PAUP* Version 4.0b4 (Swofford, 1998).

Phylogenetic analysis

Phylogenetic trees were generated using PAUP* Version 4.0b4 (Swofford, 1998), with character states unordered and at first equally weighted. The heuristic search was set to 1000 replicates with random addition sequence and TBR (tree bisection–reconnection) branch swapping. Polymorphic characters were treated as uncertain. Gaps were treated as missing values. 'Mulpars' and 'Steepest descent' were the search options selected. 'Acctran' (accelerated transformation) was chosen for character optimization.

A partition homogeneity test (Farris *et al*., 1994), also known as the incongruence length difference (ILD) test, was carried out in PAUP* with the heuristic search set to 1000 replicates and 10 replicates of random addition sequence, TBR and 'Mulpars', to test the hypothesis that the two data sets, ITS and *trn*L–F, contain the same phylogenetic information. The ITS data set was reduced to 26 taxa to match the 26 taxa *trn*L–F data set for the test. The results suggest that both data sets are congruent (P-value > 0.05) and can be combined. A combined analysis of both data sets was performed using the same phylogenetic methods and parameters as above.

Successive weighting searches were performed using a rescaled consistency (RC) index (mean value; Swofford, 1993) until the resulting tree length remained unchanged in two consecutive rounds. Due to the high value of transitions found in the ITS data matrix (65%), the transition/transversion ratio (ts/tv = 1/1.87) was applied to a parsimony analysis of the data set to weight transversions over transitions.

Support for individual clades was given a bootstrap value (Felsenstein, 1985) and a decay index (Bremer, 1988; Donoghue *et al*., 1992). Bootstrap analysis was performed using PAUP* set to heuristic search with 1000 replicates, TBR and 10 random addition sequence replicates per heuristic search. In the results and discussion presented here, clades with bootstrap (BS) values of 50–74% represent weak support, 75–84% moderate support and 85–100% strong support (Richardson *et al*., 2000). The decay index (DI) was calculated using Autodecay version 4.0 (Eriksson, 1998) with 10 random addition sequence replicates per heuristic search.

Maximum likelihood analysis was performed for the ITS data set in PAUP* by applying the best fit likelihood model, TrN + G, resulting from the likelihood ratio test (Huelsenbeck & Rannala, 1997) using MODELTEST version 3.0 (Posada & Crandall, 1998). The substitution model used allows unequal base frequencies, unequal transition and transversion rates, and among-site rate heterogeneity.

RESULTS

The ITS sequences of 36 species were obtained in this study and sequences of six other ingroup taxa were taken from GenBank. The sequences of *Kaempferia*

marginata were unreadable due to sequence polymorphism. In total, there are 42 taxa in the ITS data matrix and 26 taxa in the *trn*L–F data matrix. The *trn*L–F data matrix is smaller than the ITS data matrix for two reasons. Firstly, the taxa in GenBank have not been sequenced for *trn*L–F (or the sequences are not yet available) and secondly, the *trn*L–F region in some of our own DNA samples proved difficult to amplify and sequence.

Best alignment of the ITS data set

The alignment of the ITS data set with default values (i.e. gap-opening cost = 15) in CLUSTAL_X gave the highest RC value when the data set was analysed to find the most parsimonious trees. Four other values of gap-opening in CLUSTAL_X, namely 5, 10, 20 and 25, gave alignments different from the default value. RC values of these different alignments by parsimony analysis were lower than those of the first alignment without manual adjustment (data not shown). The default value alignment that gave the highest RC value was further improved by manual adjustment and when analysed, the resulting RC was slightly higher than the alignment without manual adjustment (data not shown).

The *P*-value of the initial homogeneity test of both data sets suggests that the phylogenetic signals contained in the data sets are homogeneous and can be combined (P-value > 0.05). It is assumed that the alignment of the ITS data set that yields the highest *P*-value when used in the homogeneity test represents the best alignment. The assumption is that the data sets are parts of one big data set of all taxa and that any partition of it will lead to the same phylogenetic estimate. The *P*-value for the homogeneity test of the *trn*L–F data set and the first alignment of the ITS data set, with default value and manual adjustment, was higher than values derived from other alignments of the ITS data set (data not shown). This is thus the best alignment of the ITS data set found and is that used in the rest of this study.

Sequence analysis of the ITS region

Alignment of the ITS sequences of the 42 taxa analysed resulted in a 722-bp long data matrix. As 60 bp were excluded because of alignment ambiguities, a data matrix 662 bp long was subject to analyses. Its characteristics are given in Table 2. Two sequences taken from GenBank, *Boesenbergia cordata* and *Camptandra ovata*, lacked the first 23 and 25 bp of ITS1, respectively. *Scaphochlamys kunstleri* and *S. lanceolata* lacked most of their 5.8S sequences.

The lengths of the complete ITS sequences were 573–672 bp. Of these aligned sites, 320 (48.34%) were constant, 213 (32.17%) had at least two nucleotide states in two or more sequences and were potentially phylogenetically informative, and 129 (19.49%) were autapomorphies (Table 2).

The sequence divergence of ITS1, 5.8S and ITS2 among ingroup species ranged from 0.0 to 23.9% whereas sequence divergence between ingroup and outgroup

ranged from 10.0 to 23.8%. The maximum sequence variation among ingroup species was 23.9%, between *Kaempferia angustifolia* and *Scaphochlamys lanceolata*. The maximum sequence variation between ingroup and outgroup was 23.8%, between *Alpinia galanga* and *Scaphochlamys lanceolata*. Apart from the identical ITS sequences of *Hedychium coccineum*, *H. gardnerianum* and *H.* × *raffillii*, the lowest sequence variation among ingroup species was 0.09%, between *Curcuma alismatifolia* and *C. parviflora.*

The sequence of *Kaempferia elegans* is the longest in this study (672 bp), and the highest ITS variation within a genus is 17.93%, between *K. angustifolia* and *K. elegans*. The maximum levels of variation of ITS within other genera are 15.37% (*Scaphochlamys kunstleri* and *S. lanceolata*), 11.83% (*Boesenbergia cordata* and *B. gelatinosa*), 7.06% (*Camptandra ovata* and *C. parvula*), 6.96% (*Curcuma alismatifolia* and *C. ecomata*), 2.75% (*Roscoea bhutanica* and *R. humeana*), and 1.88% (*Hedychium coccineum* and *H.* sp.).

Sequence analysis of the trn*L–F region*

Alignment of *trn*L–F sequences of the 26 taxa analysed resulted in a data matrix 1008 bp long (Table 2). Ranges of the sequence at primer sites 'd' and 'e' of three taxa, *Cornukaempferia longipetiolata*, *Hedychium* sp. and *Kaempferia rotunda*, lacked 25, 66 and 32 bp, respectively. The sequence of *Distichochlamys citrea* lacked the last 126 bp.

The length of the complete *trn*L–F was 894–960 bp. Of these aligned sites, 885 (87.80%) were constant, 38 (3.77%) had at least two nucleotide states in two or more sequences and were potentially phylogenetically informative, and 85 (8.43%) were autapomorphies (Table 2).

The sequence divergence of the *trn*L–F intron and the *trn*L–F spacer among ingroup species ranged from 0.1 to 2.5%, whereas the sequence divergence between ingroup and outgroup ranged from 1.8 to 3.9%. The maximum sequence variation among ingroup species was 2.5% between *Kaempferia angustifolia* and *Pyrgophyllum yunnanensis*. The maximum sequence variation between ingroup and outgroup was 3.9%, between *Renealmia battenbergiana* and *Curcuma alismatifolia*. The lowest sequence variation among ingroup species was 0.1%, between *Boesenbergia aurantiaca* and *Caulokaempferia violacea*. However, when comparing the sequences of these two taxa, two indels are present, 1 and 7 bp long.

Phylogenetic analysis of the ITS region

Twenty-three most parsimonious trees from two islands, size 2 and 21, were obtained from parsimony analysis of the ITS1, 5.8S and ITS2 data sets from 42 taxa, with length of 936, consistency index (CI) = 0.5417, retention index (RI) = 0.6374 and RC = 0.3452. The strict consensus tree of the 23 most parsimonious trees is given in Fig. 1 with bootstrap values and decay indices. The average

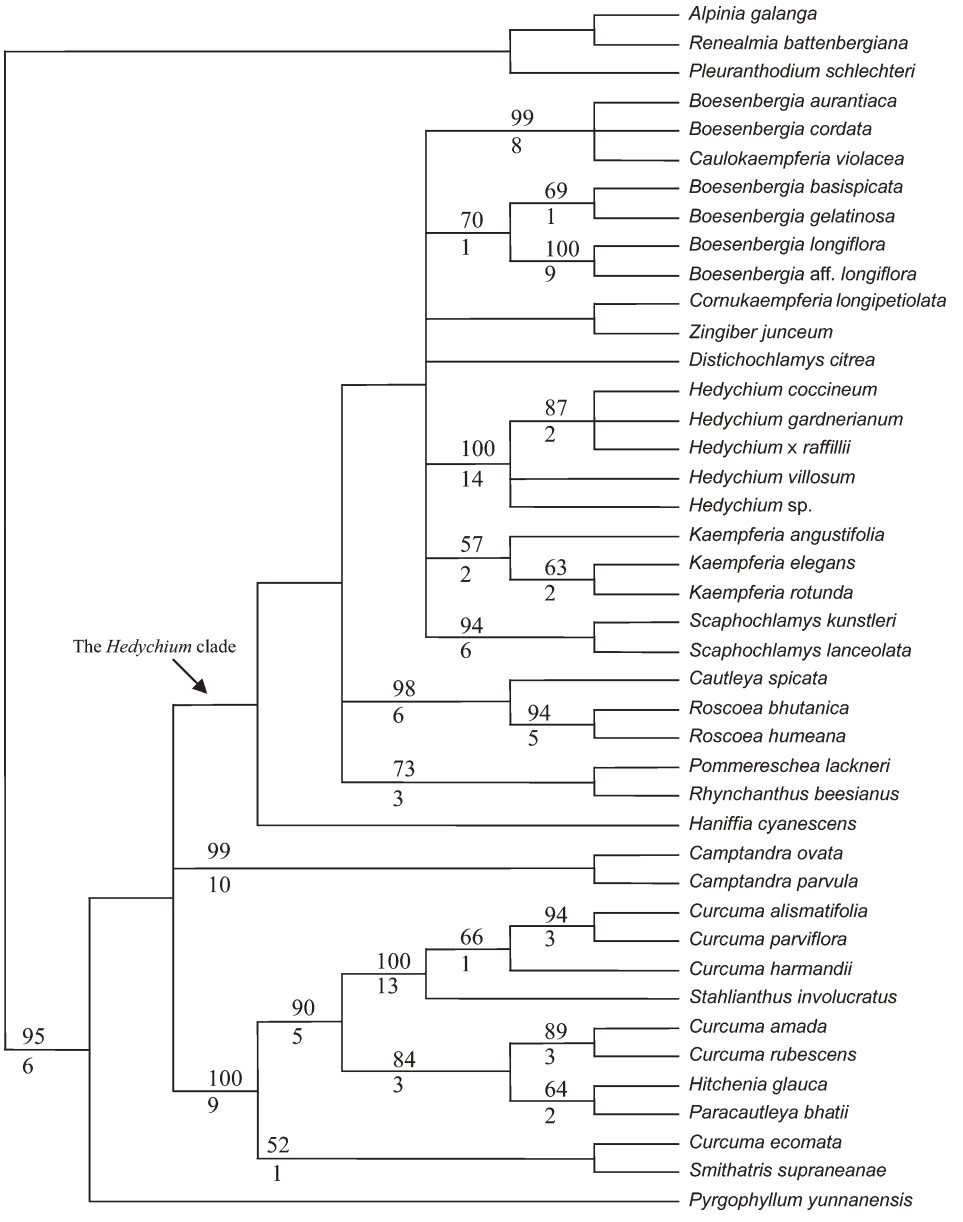

FIG. 1. Strict consensus tree of the 23 most parsimonious trees resulting from analysis of ITS data for 42 taxa. Upper numbers are bootstrap values of 1000 replicates. Lower numbers are decay indices (CI = 0.542, RI = 0.637, RC = 0.345).

number of nucleotide substitutions per character was high: 1.414 compared with 0.149 for the *trn*L–F data set.

The strict consensus tree accords with that of Kress *et al.* (2002). Three major clades are recognized, namely the *Curcuma* clade, the *Camptandra* clade and the

Hedychium clade. *Pyrgophyllum* resolves as sister to these clades, the relationships of which are unresolved. Only the *Curcuma* clade and the *Camptandra* clade are strongly supported. Resolution within the *Curcuma* clade is rather high (most subclades having BS $\geq$ 84 and DI = 3–13) showing that *Curcuma* L. is paraphyletic. The *Curcuma* clade also contains four morphologically very similar genera, *Hitchenia* Wall., *Smithatris* W.J. Kress & K. Larsen, *Stahlianthus* Kuntze and *Paracautleya*, the last of which was not sampled by Kress *et al.* (2002). *Curcuma ecomata* and *Smithatris supraneanae* form a subclade separate from the rest, but with weak support. *Stahlianthus* is found to be sister to *Curcuma* subgen. *Hitcheniopsis* and the *Hitchenia*/*Paracautleya* clade is sister to *Curcuma* subgen. *Curcuma*.

Although relationships within the *Hedychium* clade are not resolved, there are some well-supported clades. *Scaphochlamys* Baker species are grouped as a clade with strong support and *Cautleya spicata* is sister to *Roscoea*. *Hedychium* J. König species are grouped as a strongly supported clade while *Kaempferia* species are grouped as a clade with weak support. Our strict consensus tree shows one conflict with that of Kress *et al.* (2002). Where they found *Caulokaempferia* K. Larsen formed a clade, sister to all other members of the *Zingibereae*, we find it to be sister to *Boesenbergia aurantiaca* and *B. cordata*. As in Kress *et al.* (2002), the other *Boesenbergia* taxa form two clades, though as there are no species in common between the two studies it is not possible to say whether our data support their *Boesenbergia* I and *Boesenbergia* II clades. The other four species, *B. basispicata*, *B. gelatinosa*, *B. longiflora* and *B.* aff. *longiflora*, form a weakly supported clade, as do *Pommereschea* and *Rhynchanthus*.

Successive weighting analyses produced a single most parsimonious tree (Fig. 3). However, this is not one of the 23 shortest trees resulting from an unweighted analysis. Figure 2 shows one of the 23 most parsimonious trees. Besides the clearer pattern of relationships, the positions of *Pyrgophyllum* (Gagnep.) T.L. Wu & Z.Y. Chen and *Camptandra* Ridl. in the successive weighting tree and in the strict consensus tree from an unweighted analysis are the most significant differences. Because the successive weighting analysis fails to replicate any of the most parsimonious trees from an unweighted analysis, we consider the result unreliable and do not discuss it further.

The weighting of transversion over transition by an observed ratio (2/1) of the data set produced 14 most parsimonious trees (CI = 0.5620, RI = 0.6342, RC = 0.3564). The strict consensus tree of these 14 is nearly identical to the strict consensus tree of an unweighted analysis, but with higher resolution, particularly within the *Hedychium* clade (Fig. 2).

Maximum likelihood analysis recovered two optimal trees (ln-likelihood = 5551.712). One of the two optimal trees is presented in Fig. 4. Two main subclades, as found in the strict consensus tree of ts/tv-applied search, can be recognized, namely the *Hedychium* clade and the *Curcuma* clade. *Pyrgophyllum* is basal in the *Curcuma* clade. The *Pyrgophyllum*/*Camptandra* clade is sister to the *Curcuma* complex.

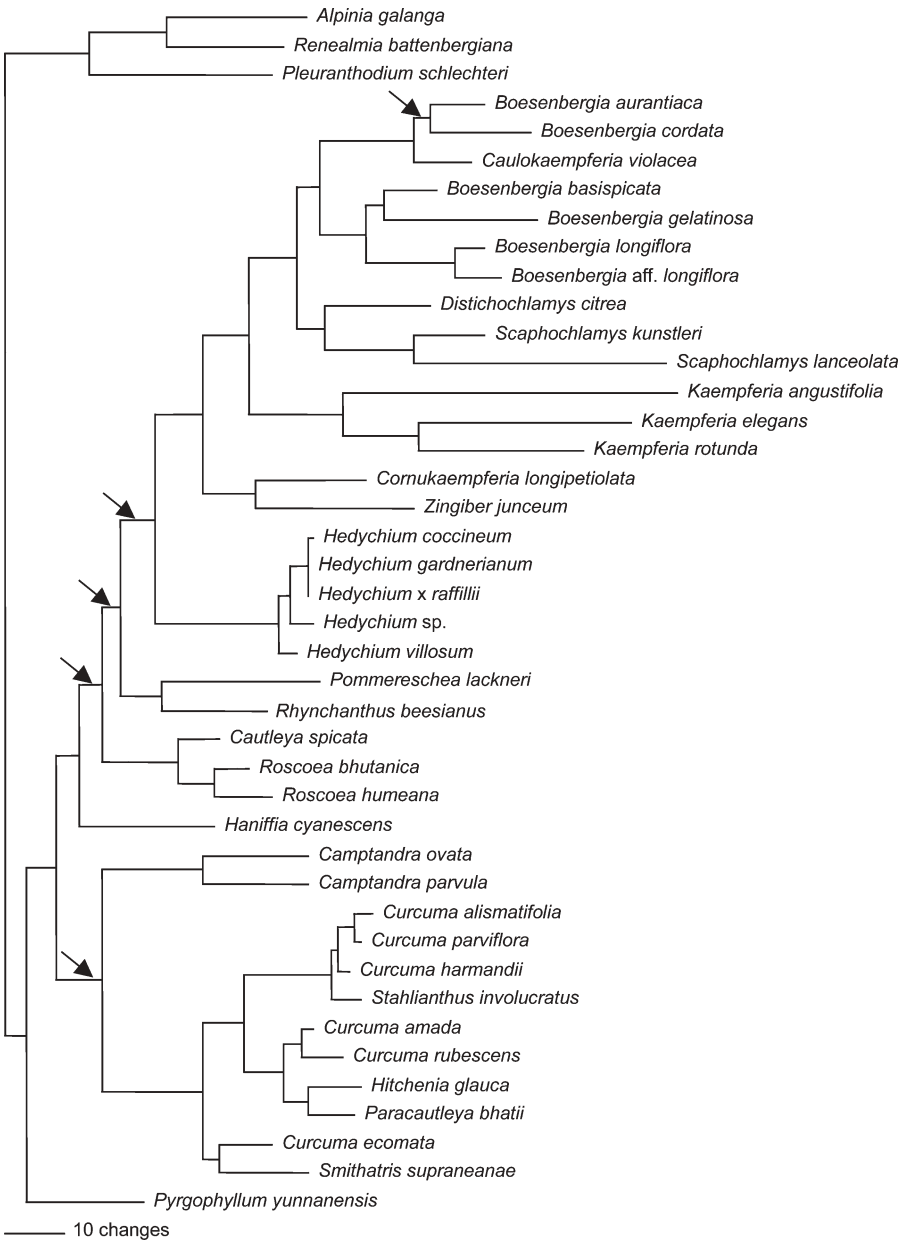

FIG. 2. One of 23 most parsimonious trees resulting from unweighted analysis of ITS data for 42 taxa. Arrows denote collapsed branch in the strict consensus tree of the 14 most parsimonious trees resulting from the transition/transversion ratio applied analysis.

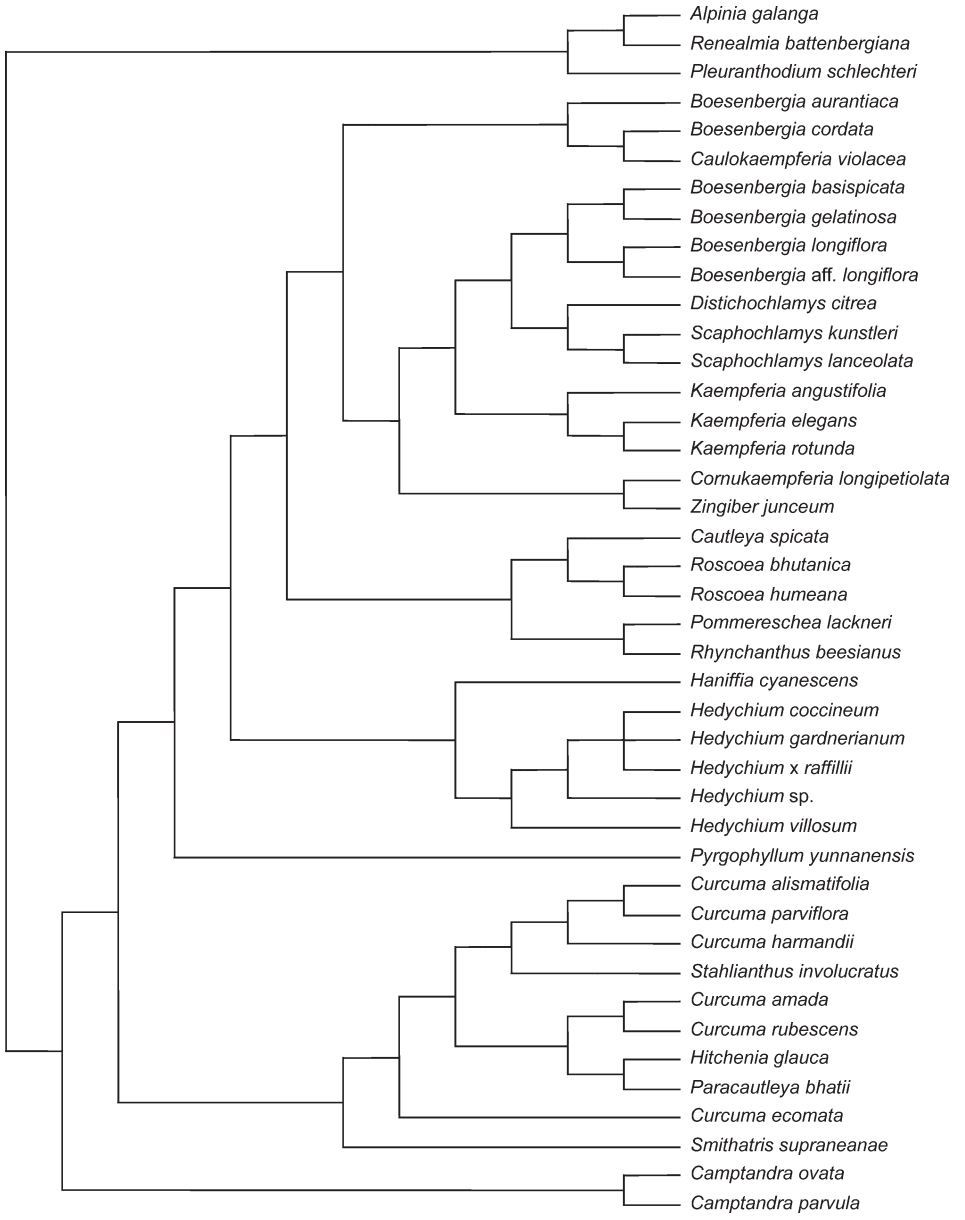

FIG. 3. The single most parsimonious tree resulting from successive weighting searches of ITS data for 42 taxa using a rescaled consistency index. Note that this tree is not one of the 23 most parsimonious trees from an unweighted search.

Topologies of this complex are identical to those found in the strict consensus tree of the ts/tv-applied search. Within the *Hedychium* clade, the subclade *Cautleya*/*Roscoea*/*Pommereschea*/*Rhynchanthus* is sister to *Hedychium*. In turn, this *Cautleya*/*Roscoea*/*Pommereschea*/*Rhynchanthus*/*Hedychium* clade is sister to the *Boesenbergia* group.

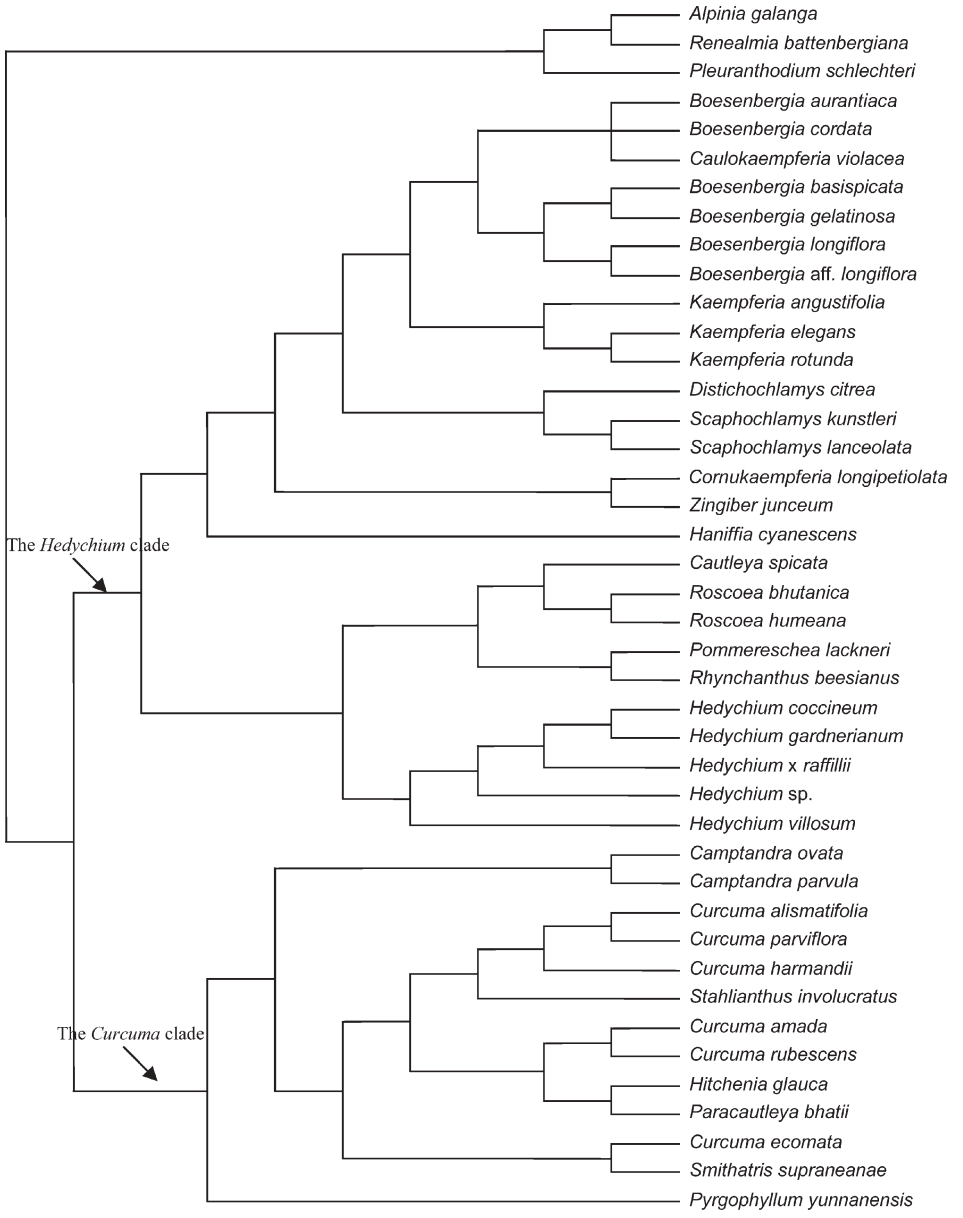

FIG. 4. The strict consensus tree of two equally optimal trees resulting from maximum likelihood analysis of ITS data for 42 taxa (ln-likelihood = 5551.712).

Haniffia may be sister to the remaining taxa. One difference in the topologies found in the maximum likelihood tree and the ts/tv-applied tree is the swapping of the *Distichochlamys*/*Scaphochlamys* clade and the *Kaempferia* clade, while the *Boesenbergia* clade, with *Caulokaempferia* nested in it, is the last branch and is identical in both trees.

Phylogenetic analysis of the trn*L–F region*

Five most parsimonious trees on one island were obtained from parsimony analysis of the 26 taxa *trn*L–F complete region data set, with a length of 150, CI = 0.9067, RI = 0.7879 and RC = 0.7143. Successive weighting analyses produced the same set of trees as found in the unweighted analysis. The majority consensus tree of the five most parsimonious trees is given in Fig. 5 with bootstrap values and decay indices.

Although there is less resolution in the consensus tree compared to that of the ITS data set, the *trn*L–F tree gives some phylogenetic information. The clades with moderate to strong support are those supported in the *mat*K analysis of Kress *et al.* (2002). This is what would be expected from two genes in the chloroplast genome.

The analysis of *trn*L–F gives moderate support to the hypothesis that *Zingiber* belongs in the *Hedychieae* (sensu Schumann). It also confirms that *Caulokaempferia* is derived within *Boesenbergia*. An obscure relationship, not found in the strict ITS consensus tree, was revealed when *Camptandra parvula* and *Pyrgophyllum yunnanensis* were grouped together, though with weak support. This relationship does not appear in the *mat*K analysis of Kress *et al.* (2002). The same *Curcuma* complex as found in the strict ITS consensus tree (*Curcuma*, *Paracautleya*, *Smithatris* and *Stahlianthus*) was again revealed by the *trn*L–F data set, though only with moderate support. *Curcuma* (subgen. *Hitcheniopsis*), *Smithatris* and *Stahlianthus* were further, though weakly, supported as a clade. *Hedychium* appears as sister group to the *Curcuma* complex according to the *trn*L–F data set, yet with weak support. The species within *Kaempferia*, *Roscoea* and *Scaphochlamys* were each grouped together with weak to moderate support (BS = 69, DI = 2 in *Kaempferia*, BS = 80, DI = 3 in *Roscoea* and BS = 51, DI = 1 in *Scaphochlamys*).

Phylogenetic analysis of the combined data sets

The *P*-value, 0.734, resulting from the partition homogeneity test of both data sets indicates that there is considerable congruence in the phylogenetic information from the ITS and *trn*L–F data sets. The data sets were thus combined for a simultaneous parsimony analysis. Two most parsimonious trees from one island were obtained, with length of 882, CI = 0.6406, RI = 0.5681 and RC = 0.3639. The strict consensus tree is shown in Fig. 6. The tree recognizes the monophyly of the *Zingibereae* with strong support. Three major clades were identified in the *Zingibereae*, *Cautleya*/*Roscoea*, the *Curcuma* complex, and the *Hedychium* clade. However, there is no strong support for the relationships among these clades. *Cautleya* (Benth.) Hook.f. is identified as sister group to *Roscoea*. *Camptandra*/*Pyrgophyllum* appears as sister to the *Curcuma* complex, though the bootstrap value is less than 50%. Within the *Curcuma* complex, *Smithatris* is moderately supported as sister to the rest of the complex. *Stahlianthus* is grouped with *Curcuma* subgen. *Hitcheniopsis*, and *Paracautleya* is grouped with *Curcuma* subgen. *Curcuma*.

The *Hedychium* clade is weakly supported and shows *Hedychium* as sister to the rest of the clade. The monophyly of *Hedychium* is strongly supported (BS = 100,

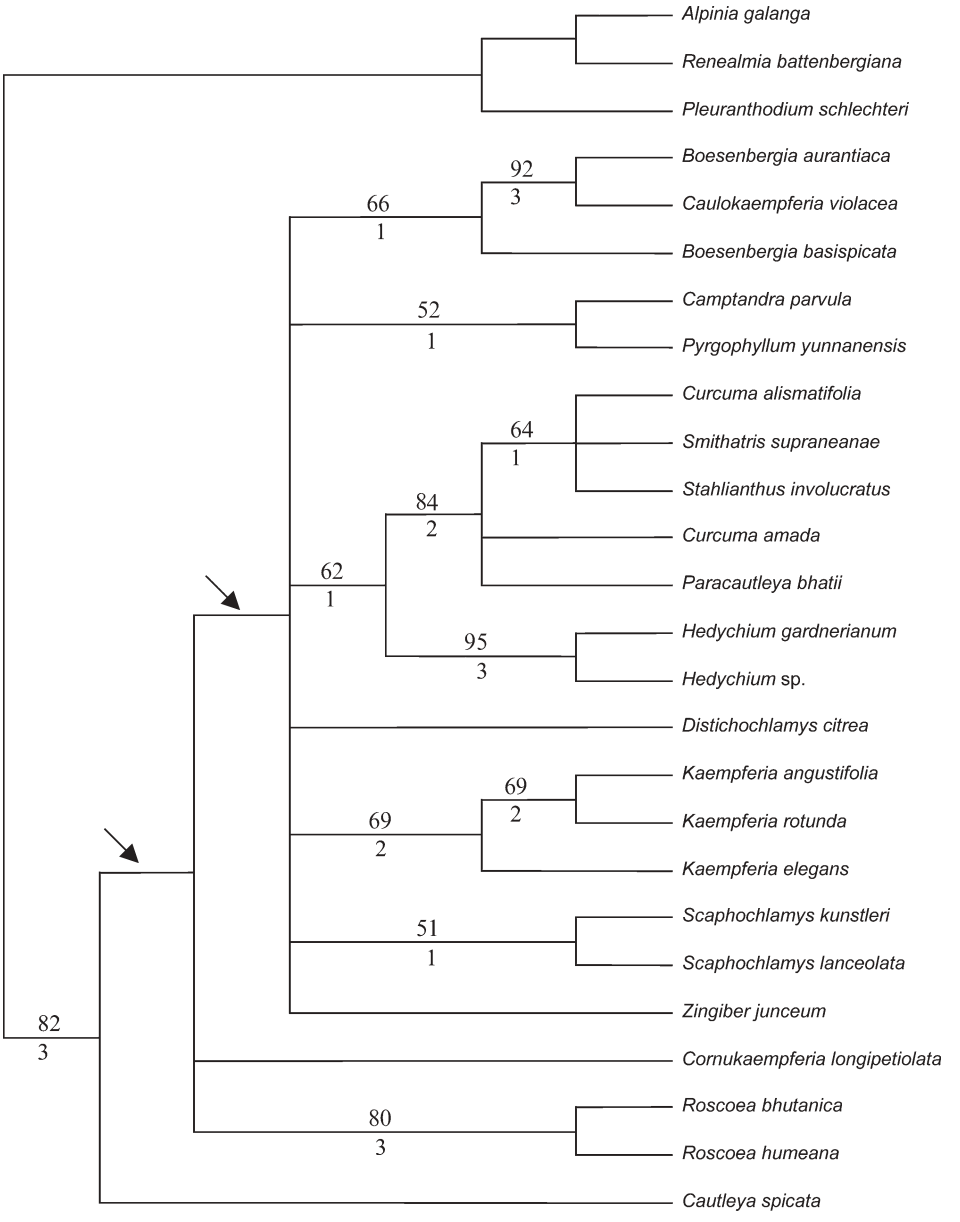

FIG. 5. The majority consensus tree of five most parsimonious trees resulting from analysis of *trn*L–F data for 26 taxa. Upper numbers are bootstrap values of 1000 replicates. Lower numbers are decay indices (CI = 0.907, RI = 0.788, RC = 0.714). Arrows denote collapsed branch in the strict consensus tree.

DI = 20) as is that of *Kaempferia* (BS = 91, DI = 7) and *Scaphochlamys* (BS = 98, DI = 9), and a clade containing *Boesenbergia aurantiaca* and *Caulokaempferia violacea* is strongly supported (BS = 100, DI = 11). Nevertheless, relationships

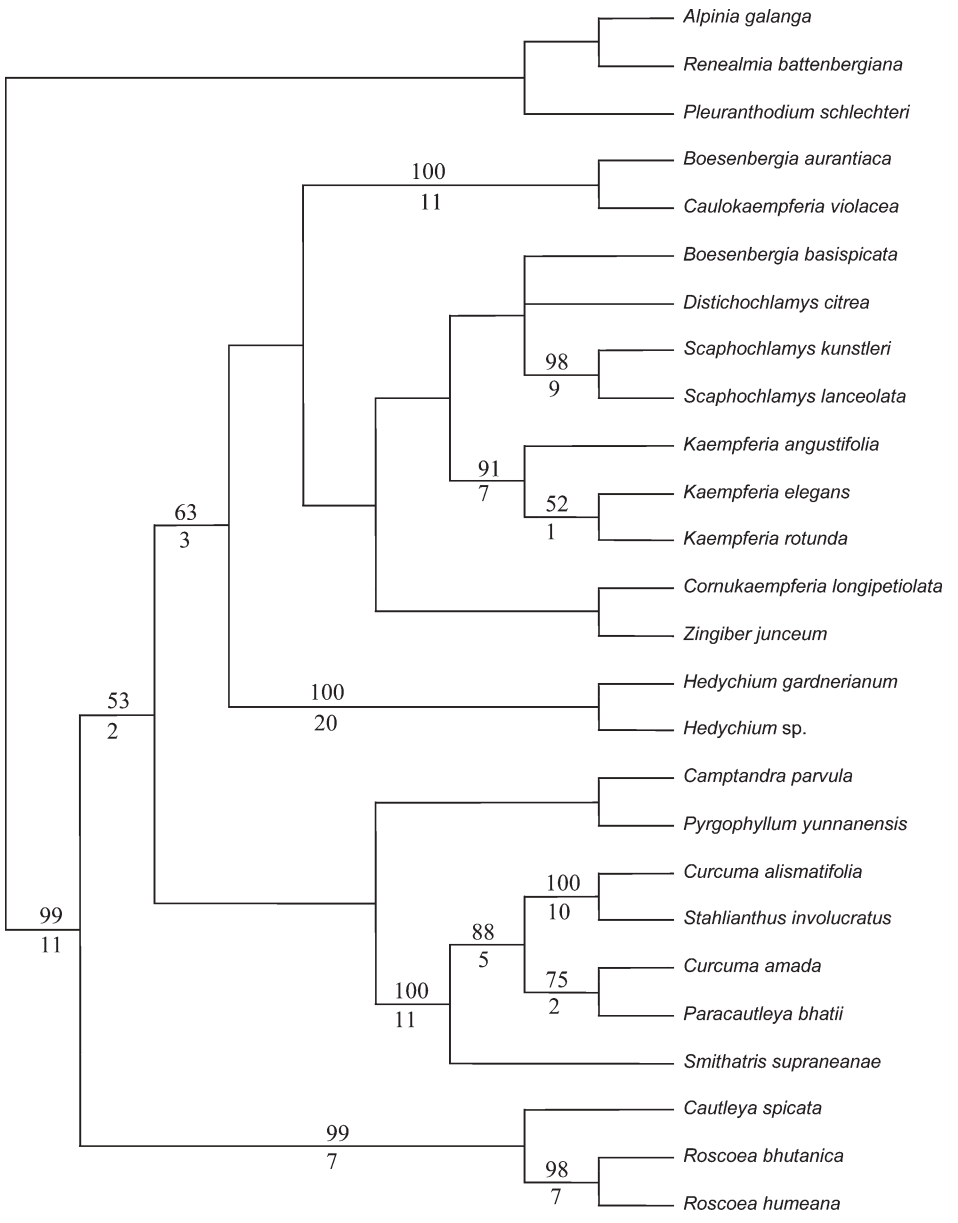

FIG. 6. The strict consensus tree of the two most parsimonious trees resulting from analysis of the combined data set (ITS and *trn*L–F) for 26 taxa. Upper numbers are bootstrap values of 1000 replicates. Lower numbers are decay indices (CI = 0.641, RI = 0.568, RC = 0.364).

among these genera are not resolved with any significant support in this combined analysis.

Successive weighting searches of the combined data set using the RC index produced a single most parsimonious tree (Fig. 7). The same two major clades

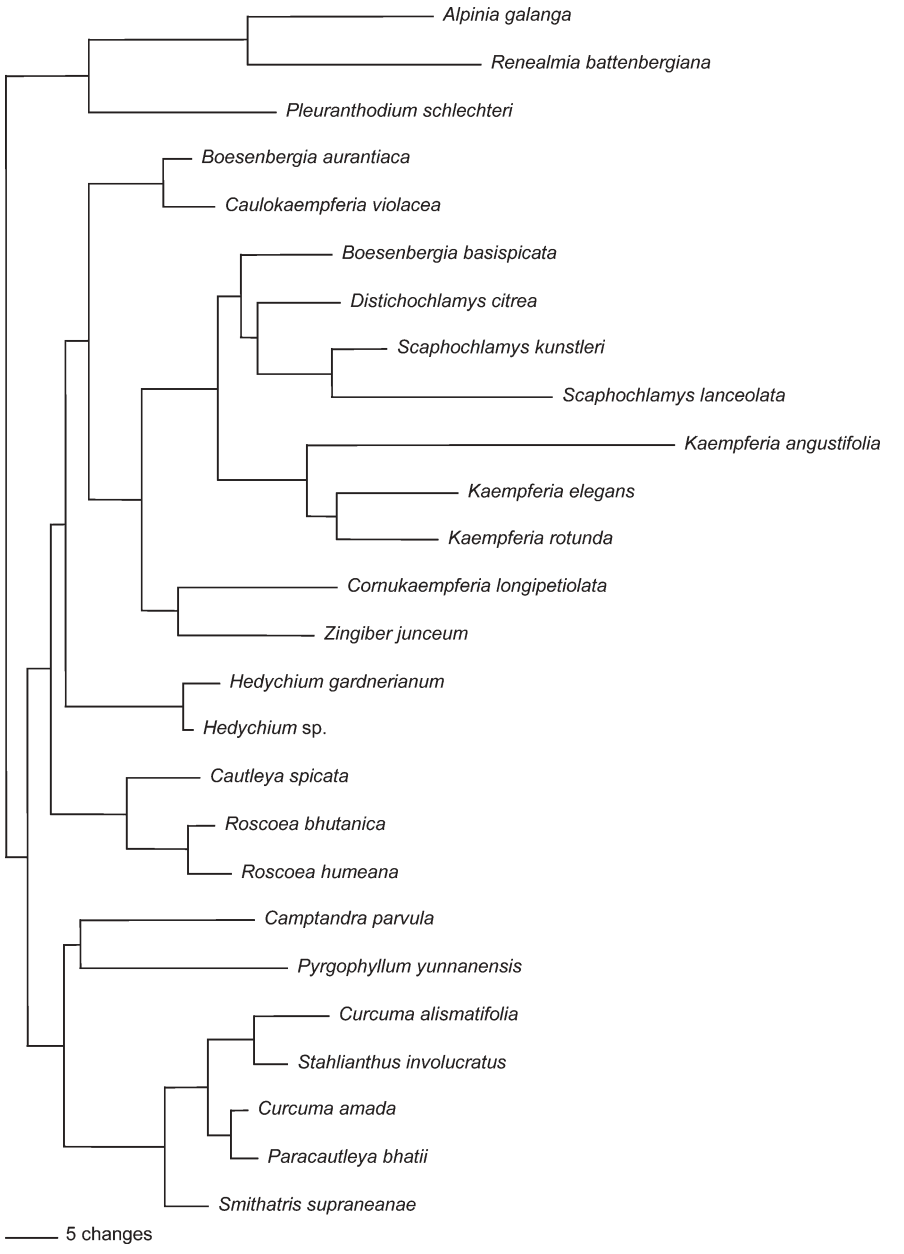

FIG. 7. The single most parsimonious tree resulting from successive weighting searches of the combined data set (ITS and *trn*L–F) for 26 taxa using a rescaled consistency index.

can be recognized, the *Curcuma* clade and the *Hedychium* clade. In the *Curcuma* clade, *Camptandra* and *Pyrgophyllum* are sister to a set of four morphologically very similar genera, *Curcuma*, *Paracautleya*, *Smithatris* and *Stahlianthus*. *Smithatris* is sister to *Paracautleya*/*Curcuma* (subgen. *Curcuma*) and *Stahlianthus*/*Curcuma* (subgen.

Hitcheniopsis). Within the *Hedychium* clade, *Cautleya*/*Roscoea* is sister to all of the rest. *Hedychium* is next separated as sister to the genera of the '*Boesenbergia* group': *Boesenbergia*, *Caulokaempferia*, *Cornukaempferia*, *Distichochlamys* M.F. Newman, *Kaempferia*, *Scaphochlamys* and *Zingiber*. *Boesenbergia* is found to be paraphyletic; *Caulokaempferia* forms a clade with *Boesenbergia aurantiaca*; *Distichochlamys* is sister to *Scaphochlamys*, and *Cornukaempferia* and *Zingiber* are sister to each other.

Discussion

Comparison with previous sequence

The ITS1 and ITS2 sequences of *Renealmia battenbergiana* and *Pleuranthodium schlechteri* in this study are identical to those of the same taxa obtained by Rangsiruji *et al*. (2000a), and only one nucleotide of ITS2 was found to be different in *Alpinia galanga*. For the *trn*L–F spacer, the sequences of *Alpinia galanga* and *Pleuranthodium schlechteri* are identical to the sequences of Rangsiruji *et al*. (2000a). However, the first 37 nucleotides in the spacer of *Renealmia battenbergiana* of Rangsiruji *et al*. (2000a) were different from this study. Nine unmatched nucleotides and one gap of eight nucleotides were observed in this region. Apart from this, the sequences differed by only two nucleotides. Each sequence obtained in this study was a complete region of *trn*L intron and *trn*L–F spacer from the sequencing of all four primers' products (c, d, e and f). The reason why the sequence of *Renealmia battenbergiana* differs from that observed by Rangsiruji *et al*. (2000a) is unclear. Different PCR conditions amplify different sites of the region. In addition, the problematic site is near the beginning of the primer which makes it more difficult to obtain the correct sequence using only one primer.

Evolution of ITS and trn*L–F*

The rate of mutation in ITS of the *Zingibereae* is about nine times faster than in the *trn*L–F region. As a result, phylogenetic relationships revealed by ITS are more fully resolved than those revealed by the *trn*L–F region. This has also been observed in *Gentiana* L., a genus of perennial dicotyledonous herbs (Gielly *et al*., 1996).

The ITS1, 5.8S and ITS2 sequences of *Hedychium* were found to be markedly less variable than those of other genera in the *Zingibereae*. Their usefulness as phylogenetic markers is thus minimal, as also observed by Wood *et al*. (2000). There are two possible explanations for this. Firstly it may be attributed to an exceptionally low mutation rate of sequences in *Hedychium* compared to other genera in the family. The other explanation is that the diversity of morphology found in the genus is large and outstrips the mutation rate of the ITS genes (rapid radiation). The latter may relate to the theory that morphology is normally held in equilibrium by stabilizing selection for much of evolutionary time but is punctuated by relatively rapid speciation events (Bateman, 1999). This phenomenon may also have occurred in

Curcuma subgen. *Hitcheniopsis* where, though ITS sequence variation is low, the species are distinct morphologically. Another example can be found in *Aframomum* K. Schum. of the *Alpinieae* with 70 species (D.J. Harris, pers. comm.) where ITS variation is exceptionally low: 0–2.74% (Harris *et al*., 2000). The mechanism can be further explained by ecological factors. Most *Aframomum* species are found on the boundary between forest and savanna; the ecological constraints of these habitats are normally large and have a profound effect on the morphology of the species (Harris *et al*., 2000). The different edges have rather specific conditions and these differences could exert a driving force for speciation. It may also be assumed that the distribution of an ancestor species was restricted, thus giving rise to a few species by peripheral isolation or fragmentation.

In contrast, the sequence is very variable in *Kaempferia* which has the highest mutation rate of the genera in this study. It is also noticeable that its ITS sequences are polymorphic, suggesting that there may be more than one copy of the ribosomal gene, or low molecular drive to homogenize the gene. This would allow the presence of different copies of the gene and relaxation of the homogenization process, giving rise to the very variable ITS sequences found among *Kaempferia* species. The big deviation of the ITS mutation rate in *Kaempferia* and *Scaphochlamys* from the mean rate in other genera of the *Zingibereae* poses the potential problem of long branch attraction when analysed under a parsimony criterion (Felsenstein, 1978). Nonetheless, no morphologically implausible groupings in the trees are observed. This may be due to the fact that the sampling in this study is quite representative.

The ITS analyses give more accurate trees than *trn*L–F analyses because they contain more taxa and more informative sites in the data matrix. In addition, there is no strongly contradictory clade revealed by the analyses of the two genomes. Thus, the following discussion is based mainly on trees resulting from the ITS analyses, while the results of the *trn*L–F analyses are used as supporting evidence.

The tribal position of Pommereschea *and* Rhynchanthus

For many years *Pommereschea* and *Rhynchanthus* have been placed in the *Alpinieae*, primarily because they lack lateral staminodes (Smith, 1981; Larsen *et al*., 1998). The present analysis confirms the finding of other recent analyses that they belong in the *Zingibereae* (Wood *et al*., 2000; Kress *et al*., 2002).

Caulokaempferia

Caulokaempferia violacea from northern Thailand is grouped with two *Boesenbergia* species from Borneo in this analysis. Nonetheless, we cannot disregard the possibility that *Boesenbergia* may be polyphyletic and that *Caulokaempferia* may be unplaced, as found by Kress *et al*. (2002). Further sampling of *Boesenbergia* and *Caulokaempferia* species and studies of other lines of evidence are needed before suggesting any reclassification of *Caulokaempferia*.

The Curcuma *complex*

All genera currently recognized in the *Curcuma* complex may be regarded as a single genus, *Curcuma*, though there are some morphological characters supporting the separation of each taxon, as seen in Table 3. These characters are however autapomorphic, as suggested by the present data. The acceptance of *Hitchenia*, *Paracautleya*, *Smithatris* and *Stahlianthus* as distinct genera leaves *Curcuma* as a paraphyletic genus within which infrageneric relationships are more complicated. *Smithatris* may be regarded as a distinct genus, though further sampling of *Curcuma* species may prove otherwise.

Summary

The *Zingibereae* are found to be monophyletic and most of the clades within the tribe agree with those found by Kress *et al.* (2002), lending strong support to their new classification of the *Zingiberaceae*. This is clearly shown by the placement of *Paracautleya bhatii* which we find to be in the *Zingibereae* as predicted by Kress *et al.*

Our results show some divergence from those of Kress *et al.* with regard to the relationships among *Boesenbergia* species and the placement of *Caulokaempferia*. What is clearly shown here is that *Boesenbergia* is paraphyletic with respect to *Caulokaempferia*, but sampling is insufficient for firm conclusions to be drawn.

Two main subclades can be recognized in the tribe *Zingibereae*, namely the *Hedychium* clade and the *Curcuma* clade.

Although few morphological synapomorphies in the tribes are readily observable in the field, exceptions occur, and we feel that the best course for the moment is to consider combinations of characters when placing genera. The classification of the *Zingiberaceae* seems never to be adequate when based just on a few morphological characters. Besides, as more and more molecular data become available for phylogenetic investigation, convergence and reversals of morphological characters turn out to have occurred more often than previously thought.

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TABLE 3. Distinguishing characters of genera in the *Curcuma* complex

Genus	Bracts	Flowers per bract	Bracteoles	Corolla tube	Anthers	Anther crests	Labellum
Curcuma L.	Adnate	Several	Open to base	Not protruding	Versatile	Absent	Emarginate, rarely split
Hitchenia Wall.	Free	Several	Tubular	Protruding	Not versatile	Absent	Emarginate
Laosanthus K. Larsen & Jenjitt.	Free	Several	Open to base	Protruding	Versatile	Present	Split
Paracautleya R.M. Sm.	Free	1	Absent	Not protruding	Versatile	Absent	Split
Smithatris W.J. Kress & K. Larsen	Adnate	1	Open to base	Protruding	Not versatile	Present	Deeply split
Stahlianthus Kuntze	Adnate	Several	Open to base	Not protruding	Not versatile	Present	Split

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