ORIGIN AND RELATIONSHIPS OF *ALPINIA GALANGA* (*ZINGIBERACEAE*) BASED ON MOLECULAR DATA

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Alpinia galanga is an important species cultivated as a culinary spice and is the type species of the genus. It is hence a member of sect. *Alpinia* subsect. *Alpinia* (with non-tubular bracteoles). However, molecular phylogenetic analyses suggest that *A. galanga* is closely related to *A. nigra* in sect. *Allughas* (with tubular bracteoles). This clade, which includes *A. conchigera*, is strongly supported with a bootstrap value (BS) of 100% and a decay index (DI) of $> +6$. These results are based on the internal transcribed spacer (ITS) region of the 18S–25S nuclear ribosomal DNA. The region (405–423bp) was sequenced from 17 accessions representing 16 taxa of *Zingiberaceae*, including 15 species of *Alpinia* and one outgroup. The sequence divergence ranged from 0.5 to 15.6% among the ingroup and from 10.1 to 13.3% between the ingroup and the outgroup. The results also strongly support the sister relationship of *A. rafflesiana* and *A. javanica* in section *Allughas* (BS = 100%, DI = $> +6$), thus the whole section is paraphyletic. Section *Alpinia* subsect. *Catimbium* is monophyletic (BS = 100%, DI = $+5$). On the other hand, sect. *Alpinia* subsect. *Alpinia* is paraphyletic (BS = 100%, DI = $> +6$) with respect to sect. *Alpinia* subsect. *Catimbium*. The results from a phylogenetic analysis of a subset of the taxa using the spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA) of chloroplast DNA confirmed the position of *A. galanga* in sect. *Allughas*. It appears that *A. galanga* has evolved within sect. *Allughas* and the absence of tubular bracteoles is a convergence with sect. *Alpinia*, possibly as a result of evolution under domestication.

Keywords. *Alpinia galanga*, chloroplast DNA, internal transcribed spacer (ITS) region, phylogeny, *Zingiberaceae*.

INTRODUCTION

Taxonomic history of Alpinia *Roxb.*

The genus *Alpinia* Roxb. (*Zingiberaceae*) comprises c.227 species (Smith, 1990). It is distributed from India and Sri Lanka in the west to China, and Japan and south-eastwards to Australia and Fiji. The generic name *Alpinia* was first used by Linnaeus (1753: 2) for the tropical American species *A. racemosa*. Later the younger Linnaeus (1781: 79) described a related species, also from tropical America, as *Renealmia exaltata*. Subsequent authors placed Asiatic species in both genera. However, Roxburgh (1812), Schumann (1904) and some later authors applied the name *Alpinia* mainly to Asiatic species, and used the name *Renealmia* for those in tropical America

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and West Africa. This generic nomenclature was not satisfactory because the type species of *Alpinia* was an American species. Eventually the name *Renealmia* was conserved for the American and African species and *Alpinia* L. was rejected so a new name was required for the Asiatic species. To solve this problem the committee appointed at Amsterdam proposed that the Asiatic species previously in *Alpinia* L. be transferred to the conserved *Alpinia* Roxb. 1812, non L. 1753 with *A. galanga* (L.) Willd. as its type.

The lectotypification of A. galanga *(L.) Willd.*

In 1762 Linnaeus (p. 3) described *Maranta galanga* and in the protologue, he referred solely to Rumphius (1747) citing the excellent plate (t. 63). There is no specimen of *Maranta galanga* at LINN (Burtt & Smith, 1972; Smith, 1990) or at BM, UPS or in S. Hence, it appears that Rumphius's (1747) illustration is the sole basis for the lectotypification of *Maranta galanga* L. (*A. galanga* (L.) Willd.). This accords with the comment of Valeton (1917) that '*Galanga major* Rumph. is the whole basis of *Maranta galanga* Linn., which typifies *Alpinia galanga* Sw.; the plate is very generally cited in botanical literature under *Alpinia galanga* (L.) Sw.' Following this, Rumphius's (1747) illustration is chosen here explicitly as a lectotype of *Maranta galanga* L., and therefore, of *Alpinia galanga* (L.) Willd.

Swartz (1791: 8) treated *Maranta galanga* L., associated it with *Alpinia* and cited Rumphius's (1747) illustration. However, his short account does not constitute an explicit new combination. Willdenow (1797: 12) therefore, was the first person to establish the name *Alpinia galanga* for *Maranta galanga*. Throughout our paper therefore, we use Willdenow's authority for *A. galanga*.

The history of the classification of A. galanga *(L.) Willd.*

The concordance of *Alpinia galanga* with the *Galanga major* of Rumphius (*Maranta galanga* L.) was affirmed by Roxburgh (1812) for his plant grown in Calcutta from rhizomes sent to him by Dr Charles Campbell from Sumatra. In 1832, Roxburgh placed *A. galanga* with *A. allughas* Roscoe (*A. nigra* (Gaertn.) B.L.Burtt), together with all species having terminal inflorescences. Ridley (1899) on the other hand, grouped *A. galanga* in sect. *Hellenia*, along with *A. conchigera* Griff., *A. melanocarpa* (Teijsm. & Binn.) Ridl. (*A. aquatica* (Retz.) Roscoe) and *A. scabra* (Blume) Baker based on the characteristics of their small flowers, narrow lips, crested anthers and small globose fruits which contain few seeds. In 1904, Schumann classified *A. galanga* in subgenus *Autalpinia* sect. *Hellenia*, on the basis of characters of the bracts and bracteoles, along with several species such as *A. scabra*, *A. pubiflora* K.Schum., *A. modesta* (F.Muell.) K.Schum. and *A. vitiensis* Seem. *Alpinia galanga* however, was placed in a separate genus, *Languas*, by Holttum (1950) with two other Malayan species, *A. scabra* and *A. aquatica*, based on similar characters (small flowers, small fruits with few seeds) used by Ridley

(1899) with further details of bracteoles which are never funnel-shaped nor hood-like. Smith (1990) also used forms of bracts and bracteoles to separate the sections of *Alpinia* in subgenus *Alpinia*. She used the non-tubular bracteole character as the main differentiating feature, which resulted in the placement of *A. galanga* with twenty-three other species, including *A. polyantha* D. Fang, *A. maclurei* Merr. and *A. intermedia* Gagnep., in sect. *Alpinia* subsect. *Alpinia*.

The origin of the wild and cultivated A. galanga *(L.) Willd.*

Alpinia galanga has long been cultivated. In the thirteenth century, Marco Polo (Hanbury, 1876) observed that supplies of galangal destined for Europe were produced in southern China as well as in Java. In 1563, Garcia Da Orta (Hanbury, 1876) was the first writer to point out that there were two kinds of galanga – the one with smaller rhizome and a strong scent (*Galanga minor* Rumph. or *Alpinia officinarum* Hance) brought from China, and the other with a thicker and less aromatic rhizome (*Galanga major* Rumph. or *Alpinia galanga* (L.) Willd.) produced in Java (Burkill, 1935).

Alpinia galanga is widely cultivated in East Bengal, South India and South East Asia. Most authors have assumed that the plant is native to Sumatra and Java (Roxburgh, 1812; Watt, 1883; Burkill, 1935; Singh *et al*., 1983), but Dalzell & Gibson (1861) claimed that it grew truly wild on Wag Donger in the Warree Country, India. The plant is common in Peninsular Malaysia, both in cultivation and half-wild near villages where the ground is abandoned (Burkill, 1935). Due to its long history of cultivation and its ability to persist in adverse conditions, Ridley (1899) believed *A. galanga* did not occur in a wild state anywhere, and that apparently wild plants were naturalized from cultivations.

In 1827, Blume (p. 58) described *A. pyramidata*, of Java, Borneo and the Philippines, as closely related to *A. allughas* and being different from *A. galanga* only in having more hair on the undersides of the leaves and the rachises. Thus, it was treated by Schumann (1904) as a variety of *A. galanga* (*A. galanga* (L.) Willd. var. *pyramidata* (Blume) K.Schum.) and later by Smith (1990) merely as *A. galanga*. Valeton (1917) and Burkill (1935) suggested that *A. pyramidata* represents a wild form of *A. galanga*, but Ridley (1909) contended that *A. pyramidata* and *A. galanga* were very different in terms of the characters of the lip and staminodes. In this confusion, therefore, it cannot be assumed that *A. galanga* var. *galanga* is merely a cultigen derived from the variety *pyramidata*.

Uses of A. galanga *and confusion with* A. nigra

In South and South East Asia the aromatic rhizomes of *Alpinia* are used as a spice or as an ingredient in traditional medicine. The species grown, referred to as kha in Thai and langkwas in Malay, is said to be *A. galanga* although, unfortunately, herbarium specimens are rarely cited (e.g. Chopra *et al*., 1956; Mitsui *et al*., 1976;

Perry, 1980; Itokawa *et al.*, 1987; Ahamad & Ahmed, 1991; Jitoe *et al.*, 1992; Haragushi *et al.*, 1996). In Thailand, at least, there appears to be confusion between *A. galanga* and *A.* aff. *nigra* (sect. *Allughas*), the rhizomes of which are used for the same purpose and which are both called kha (e.g. *A.* aff. *nigra*, Newman 5 (E)). This is despite the fact that the former has bracteoles which are open to the base (Fig. 1A) and the latter has them more or less cup- or funnel-shaped (Fig. 1B) which results in the two species being classified in different sections. Since *A. galanga* is economically important and is also the type species of the genus it is desirable that it is correctly classified. The conflation of *A. galanga* with *A. nigra* is at variance with the use of the single bracteole character to classify the two species in different sections.

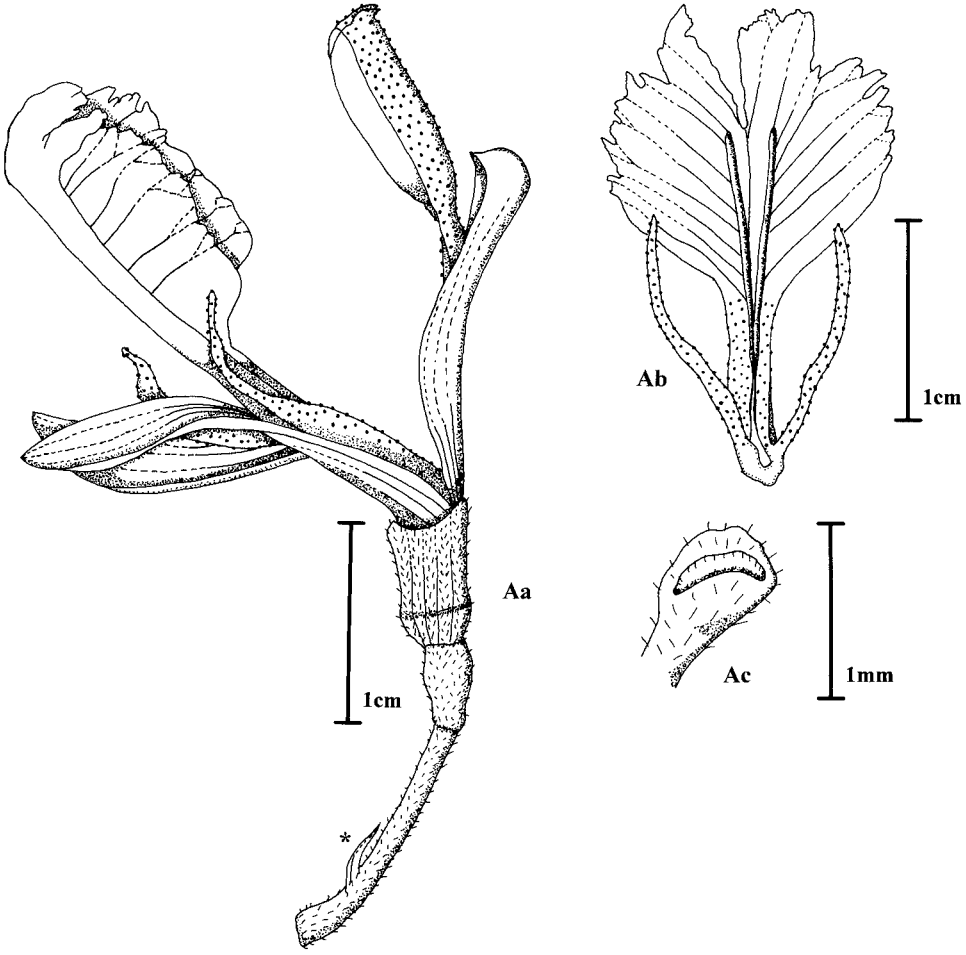

FIG. 1A. *Alpinia galanga* (L.) Willd.: Aa, cincinnus with mature flower, * indicates a non-tubular bracteole; Ab, labellum; Ac, stigma, from above (from spirit material).

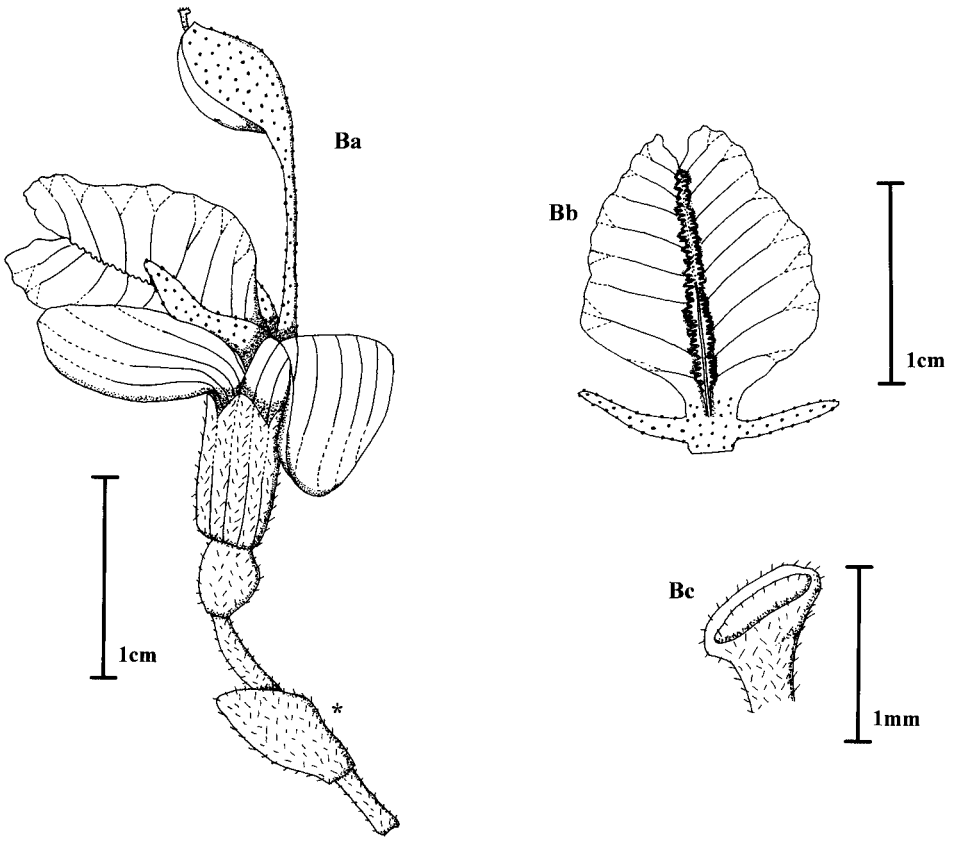

FIG. 1B. *Alpinia nigra* (Gaertn.) B.L. Burtt: Ba, cincinnus with mature flower, * indicates a tubular bracteole; Bb, labellum; Bc, stigma, from above (from spirit material).

The use of molecular approaches to determine the correct infrageneric placement of A. galanga

In order to confirm the relationship of *A. galanga* to other species in sections *Alpinia* and *Allughas*, a molecular study was undertaken which used the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA). In the plant nuclear genome these genes are arranged in repeating units in nucleolar organiser regions (reviewed in Hamby & Zimmer, 1992). Each unit consists of two spacers, ITS1 and ITS2. ITS1 is located between conserved regions of the small subunit (18S) and 5.8S and ITS2 is located between the 5.8S and large subunit (25S). In yeast (*Saccharomyces cerevisiae*), it was found that both ITS1 and ITS2 regions play a role in primary RNA processing (Musters *et al*., 1990; van der Sande *et al*., 1992). Due to its small size, highly conserved flanks and high copy number (Rogers & Bendich, 1987) the ITS region appears to be ideal for use in a PCR-based sequencing strategy. Rapid concerted evolution (Arnheim *et al*., 1980; Zimmer *et al*., 1980;

Arnheim, 1983) of the nrDNA also enables us to study plant phylogenetic relationships at low taxonomic levels such as within subtribes, among genera or species (e.g. Baldwin, 1992; Baldwin, 1993; Suh *et al.*, 1993; Sun *et al.*, 1994; Campbell *et al.*, 1995; Oxelman & Lidén, 1995; Soltis & Kuzoff, 1995; Downie & Katz-Downie, 1996; Möller & Cronk, 1997).

As an additional check on the infrageneric placement of *A. galanga* a study of two non-coding chloroplast regions: i, between *trn*L (UAA) 5′ exon and *trn*L (UAA) 3′ exon; and ii, between *trn*L (UAA) 3′ exon and *trn*F (GAA) was carried out. Although these regions are less variable than the ITS region their sequences can also be used for phylogenetic studies of closely related species, for instance, at the interspecific level in *Acer* (Taberlet *et al.*, 1991).

MATERIALS AND METHODS

Origin of plant material

For this study 17 accessions representing 16 taxa of *Zingiberaceae* were selected. All plant material was taken from living plants at the Royal Botanic Garden Edinburgh (E, UK), the National Museum of Natural History (Smithsonian Institution, USA), Waimea Arboretum and Botanical Garden (Hawaii, USA), Harold L. Lyon Arboretum (the University of Hawaii at Manoa, Hawaii, USA) and the Department of Botany, University of Malaya (Malaysia) (Table 1) – see acknowledgements. The majority of the voucher herbarium specimens were freshly prepared at E. The rest were either prepared elsewhere and loaned to E, or sent dried and unmounted to E with further flowers sent moist in cotton wool soaked with alcohol. Herbarium specimens were then prepared at E. Flowers were preserved in Copenhagen mixture (10 : 1 : 8 mixture of methylated spirit, glycerol and water) in a spirit collection (E). Of 17 taxa, four have no voucher specimens. This is mainly because some of these species flower infrequently. Nonetheless, they were used in this study following identification by an authority on *Zingiberaceae*, Prof W.J. Kress at the National Museum of Natural History (Smithsonian Institution, USA). Other plant specimens with accession numbers could be checked from their sources.

Outgroup taxa

To investigate the origin of *Alpinia galanga* and its relationships to other members of *Alpinia*, appropriate outgroup selection was essential. *Renealmia battenbergiana*, a species native to tropical Africa, was used as the outgroup because it is morphologically close to *Alpinia*, yet not so close as to be nested in *Alpinia*. *Renealmia* differs from *Alpinia* in the presence of stellate hairs on the leaves and the erect, petaloid labellum. In a much larger study based on molecular and morphological evidence, we have used species in *Burbidgea*, *Pleuranthodium* and *Elettariopsis* as the outgroup (Rangsiruji *et al.*, 2000). That study indicates that *Renealmia* is the closest relative of *Alpinia*, among those studied. Material of the Asiatic genera *Geostachys* and

TABLE 1. Sources, and voucher specimens for the 16 species (17 accessions) of Zingiberaceae from which ITS sequences were obtained for this study. Ten species marked by * were included in the analyses of the spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA). Abbreviations used in the table are E: The Royal Botanic Garden Edinburgh; herb.: DNA extraction from a herbarium specimen; HLA: Harold L. Lyon Arboretum; NMNH (SI): The National Museum of Natural History (Smithsonian Institution); UM: University of Malaya; USNH: United States National Herbarium; WAI: Waimea Arboretum & Botanical Garden; – denotes no voucher. Accession numbers follow the abbreviations (when available)

Species number	Sect./subsect.	Source & accession number	Voucher	GenBank accession	
				ITS1	ITS2
Renealmia battenbergiana Cummins ex Baker*		E, 19740104	A.Rangsiruji 27 (E), C 8482 (E)	AF192707	AF192708
Alpinia blepharocalyx K.Schum. var. *blepharocalyx*	*Alpinia*/*Catimbium*	NMNH (SI), AC 95–5521	3308901 (USNH)	AF192709	AF192710
Alpinia blepharocalyx K.Schum. var. *glabrior* (Hand.-Mazz.) T.L.Wu*	*Alpinia*/*Catimbium*	E, 19901453	A.Rangsiruji 21 (E)	AF192711	AF192712
A. conchigera Griff.*	*Allughas*/*Strobidia*	NMNH (SI), AC 95–5512	3308910 (USNH)	AF192713	AF192714
A. coriacea T.L.Wu & S.J.Chen	*Alpinia*/*Alpinia*	NMNH (SI), AC 95–5539	3308886 (USNH)	AF192715	AF192716
A. galanga (L.) Willd.*	*Alpinia*/*Alpinia*	E, 19771077	A.Rangsiruji 3 (E)	AF192717	AF192718
A. galanga (L.) Willd.	*Alpinia*/*Alpinia*	KMN 3543 (E) (herb.)	KMN 3543 (E)	AF192717	AF192718
A. intermedia Gagnep.*	*Alpinia*/*Alpinia*	NMNH (SI), AC 94–5330	—	AF192719	AF192720
A. japonica Miq. var. *kiushiana* Kitam.*	*Alpinia*/*Alpinia*	HLA, L-84.0177	—	AF192721	AF192722
A. javanica Blume*	*Allughas*/*Allughas*	UM	A.Rangsiruji 53 (E)	AF192723	AF192724
A. maclurei Merr.	*Alpinia*/*Alpinia*	NMNH (SI), AC 95–5540	—	AF192725	AF192726

TABLE 1. Continued

A. malaccensis (Burm.f.) Roscoe*	*Alpinia*/*Catimbium*	E, 19751793	A.Rangsiruji 14 (E)	AF192727	AF192728
A. nigra (Gaertn.) B.L.Burtt*	*Allughas*/*Allughas*	WAI, 80p172	A.Rangsiruji 55 (E)	AF192729	AF192730
A. polyantha D.Fang	*Alpinia*/*Alpinia*	NMNH (SI), AC 94–3744 JK	—	AF192731	AF192732
A. rafflesiana Wall. ex Baker	*Allughas*/*Allughas*	UM	A.Rangsiruji 52 (E)	AF192733	AF192734
A. suishaensis Hayata	*Alpinia*/*Alpinia*	E, 19791028	A.Rangsiruji 37 (E), C 8468 (E)	AF192735	AF192736
A. zerumbet (Pers.) B.L.Burtt & R.M.Sm.	*Alpinia*/*Catimbium*	E, 19751777	A.Rangsiruji 18 (E)	AF192737	AF192738

Riedelia, which are also morphologically close to *Alpinia*, might have been investigated but this awaits a generic-level study of the Alpineae and is not expected to alter the relationships of the infrageneric taxa in *Alpinia*.

Ingroup taxa

Following Smith's (1990) infrageneric classification of *Alpinia*, this study included four species of sect. *Allughas* (subsect. *Allughas*: *A. javanica*, *A. nigra*, *A. rafflesiana* and subsect. *Strobidia*: *A. conchigera*) and eleven species of sect. *Alpinia* (subsect. *Alpinia*: *A. coriacea*, *A. galanga*, *A. intermedia*, *A. japonica*, *A. maclurei*, *A. polyantha*, *A. suishaensis* and subsect. *Catimbium*: *A. blepharocalyx* var. *blepharocalyx*, *A. blepharocalyx* var. *glabrior*, *A. malaccensis*, *A. zerumbet*).

In this study we look closely at the placement of the type species, *A. galanga* so the sampling is restricted to the relevant groups. Much wider sampling can be found in Rangsiruji *et al.* (2000) where we discuss the infrageneric classification of *Alpinia* as a whole.

Total genomic DNA extraction

For each taxon, fresh leaf material of no larger than $2cm^2$ was collected. For studies involving PCR it is crucial that any material used for DNA extraction is healthy (i.e. free from insect, fungal and viral damage). The material was placed in a snap-top plastic bag, dried and preserved with approximately one teaspoonful of self-indicating silica gel. Prior to extraction of the DNA, the leaves were usually stored at 4°C overnight to be destarched. Total genomic DNA was isolated from the leaf material using a modification of the CTAB method of Doyle & Doyle (1987), with no further purification.

A small amount of silica gel dried leaf material (a single paper-punch size) was placed into a 1.5ml microcentrifuge tube where sand and 200µl of 2x CTAB extraction buffer were added. The material was then ground with a plastic pestle. A further 800µl of the extraction buffer was added, the contents were mixed gently, and incubated at 65°C for 30min with occasional inversion. The tube was allowed to cool to room temperature before 200µl 'wet' chloroform (24 : 1 mixture of chloroform and isoamyl alcohol) was added. Invert the tube gently 3 or 4 times (each time to obtain a momentary single phase), followed by 2min of centrifugation at 13,000rpm to separate the layers. The aqueous (upper) phase was removed to a clean tube where it was re-extracted with 200µl 'wet' chloroform. Again the aqueous (upper) phase obtained was removed to a clean tube and 600µl of cold (−20°C) isopropanol was added. The contents were mixed gently and allowed to stand for 15min. Then the tube was centrifuged for 2min at 13,000rpm. The supernatant was removed and 1ml of wash buffer (76% ethanol and 10mM ammonium acetate) was added. The tube was vigorously agitated to release the pellet from the bottom, and then left standing for at least 30min. The tube was centrifuged for 2min at 13,000rpm and the super-

natant was removed. To dry the pellet, the tube was inverted and placed in an oven at 45°C for 5–10min. The pellet was then resolved in 50μl sterile distilled water and stored at −20°C until required.

In the case of the herbarium specimen, DNA was isolated using the 'hot' CTAB method. This method followed the above procedure except: i, 2x CTAB buffer was heated to 65°C prior to use; ii, liquid nitrogen was used instead of sand to ease the grinding of leaf material; iii, 50μl RNase A (1mg/ml) was added to a mixture of the CTAB and ground leaf material, and the contents were kept at 65°C for 1h (instead of 30min) before proceeding to the next step of 'wet' chloroform extraction; iv, in the presence of the wash buffer DNA was left at 4°C overnight.

PCR amplication and conditions

The ITS region was amplified via the polymerase chain reaction (PCR) using primer 'ITS5P' (5′-GGA AGG AGA AGT CGT AAC AAG G-3′) and primer 'ITS8P' (5′-CAC GCT TCT CCA GAC TAC A-3′) (Möller & Cronk, 1997) each at 10μM in a PCR reaction which yielded double-stranded DNA of approximately 800bp. The PCR amplication of the ITS region was performed in a Perkin-Elmer DNA Thermal Cycler. The reaction mixture contained: 31.5μl sterile distilled water (33.5μl for the negative control), 5.0μl of 10× Dynazyme reaction buffer (1×: 10mM Tris HCl, pH 8.8 at 25°C, 1.5mM $MgCl_2$, 50mM KCl, 0.1% Triton X-100; Finnzymes Oy, Espoo, Finland), 1.0μl of 10mM deoxyribonucleoside triphosphate (dNTP) mix (Sigma Chemicals, Poole, Dorset, UK), 5.0μl of each primer (final concentration 1μM) (Oswel DNA Service, Southampton, UK), 2.0μl of total genomic DNA (none for the negative control) and 0.5μl (1U) of Dynazyme II thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland). After an initial denaturation step at 94°C for 3min the double-stranded PCR products were produced via 30 cycles of denaturation (94°C for 1min), primer annealing (55°C for 2min), and extension (72°C for 1.5min). A 5min final extension cycle at 72°C followed the 30th cycle. In the case of the herbarium specimen, the cycle started at 94°C for 10min as an initial incubation, with 40 cycles. Each PCR product (3μl) was then checked and quantified by electrophoresis in a 1.5% agarose (Promega, Madison, WI, USA) gel using 1x TBE as the gel buffer.

For the two non-coding regions between *trn*L (UAA) 5′ exon and *trn*L (UAA) 3′ exon, and *trn*L (UAA) 3′ exon and *trn*F (GAA) of chloroplast DNA (cpDNA), PCR amplications of the ten taxa (marked with * in Table 1) required four universal primers (primers c, d, e and f) (reviewed in Taberlet *et al.*, 1991). These primers were synthesized by and purchased from Oswel DNA Service, Southampton, UK. To obtain the whole region between *trn*L (UAA) 5′ exon and *trn*F (GAA), primer c (5′-CGA AAT CGG TAG ACG CTA CG-3′) and primer f (5′-ATT TGA ACT GGT GAC ACG AG-3′) were used, with some modifications of the PCR protocol for the ITS (i.e. primer annealing: 50°C for 1min, extension: 72°C for 2min, number of PCR cycles: 35). However, resulting gels showed that the amplification contained

more than one product. Multiple bands persisted even after several attempts were made to adjust the PCR conditions. As a result, each region had to be amplified individually. For the region between *trn*L (UAA) 5′ exon and *trn*L (UAA) 3′ exon, primer c and primer d (5′-GGG GAT AGA GGG ACT TGA AC-3′) were employed. Primer e (5′-GGT TCA AGT CCC TCT ATC CC-3′) and primer f were used to amplify the region between *trn*L (UAA) 3′ exon and *trn*F (GAA). The results obtained were as follows: for each taxon, the combination of primer c and primer d produced multiple bands while that of the primer e and primer f produced only one clear and distinctive band. Methods used to increase the stringency of the PCR conditions were carried out, but failed to improve the amplification with primer c and primer d. Therefore, only the PCR products between *trn*L (UAA) 3′ exon and *trn*F (GAA) generated from the pair of primer e and primer f were used as potential templates for cpDNA sequencing.

Prior to sequencing, PCR-amplified templates of both the ITS region and the region between *trn*L (UAA) 3′ exon and *trn*F (GAA) were purified using the QIAquick PCR Purification Kit (Qiagen Ltd, Dorking, Surrey, UK), and subsequently DNA concentrations between 10–30ng/μl were obtained.

Sequencing protocol

Cycle sequencing with dye-termination was used (a Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) with AmpliTaq DNA Polymerase, FS following the manufacturer's directions. Samples were analysed on an ABI model 377 Prism Automatic DNA Sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA), according to the manual supplied. For each taxon, two external primers ('ITS5P' and 'ITS8P' – identical to those used for PCR) and two internal primers ('ITS2K': 5′-GGC ACA ACT TGC GTT CAA AG-3′ and 'ITS3P': 5′-GCA TCG ATG AAG AAC GTA GC-3′), each at 3.2pMol, were employed. Primer 'ITS2K' was designed during this course of study based on the 5.8S sequences of several *Alpinia* species when aligned with their outgroup (Rangsiruji, 1999). Primer 'ITS3P' on the other hand, was employed following Möller & Cronk (1997). The use of all four primers allowed confirmation of the accuracy of the ITS sequences.

For the region between *trn*L (UAA) 3′ exon and *trn*F (GAA), a similar protocol used for the ITS sequencing was employed, using primer e and primer f.

Sequence analysis

Following the published sequences of the 5.8S rDNA gene and the ITS region in carrot and broad bean ribosomal DNA (Yokota *et al*., 1989), both ITS1 and ITS2 regions of the 16 taxa under study were determined. Sequence Navigator version 1.0.1 software (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) was used with the CLUSTAL option for the initial alignments of both ITS regions.

These alignments were subsequently adjusted by eye. The number and size of insertion/deletion events (indels) were examined and the G+C content was analysed. In PAUP version 3.1.1 (Swofford, 1993), sequence characteristics such as sequence divergence, number of constant sites, variable sites, informative sites and autapomorphic sites were calculated. In addition, the number of transitions, transversions and their respective ratios were obtained using MacClade version 3.01 (Maddison & Maddison, 1992).

To determine the sequence boundaries of the intergenic spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA), sequences of all ten taxa of *Zingiberaceae* under study (data not shown) were compared with published data of the complete sequence of the tobacco chloroplast genome (Shinozaki *et al.*, 1986). The remaining procedures for sequence analysis of this cpDNA followed those for the ITS, with the addition of a summary of the insertion/deletion characteristics in Table 4.

Phylogenetic analysis

Sequence data were analysed using PAUP version 3.1.1 (Swofford, 1993). All characters were unordered and equally weighted. Gaps were treated as missing values and multistate taxa were interpreted as uncertain. A branch-and-bound search was then conducted with TBR (tree bisection-reconnection) swapping algorithm. In the analysis, options COLLAPSE, MULPARS and ACCTRAN optimization were in effect. Additional analyses were carried out with the exclusion of uninformative characters, with coded gaps (0 was coded for an insertion and 1 was coded for a deletion), and with weighting by transition/transversion ratio.

To demonstrate the robustness of the phylogenetic trees produced, bootstrap values (BS: Felsenstein, 1985) and decay indices (DI: Bremer, 1988; Donoghue *et al.*, 1992) were calculated. Using PAUP, bootstrapping was performed under the heuristic search option with SIMPLE addition sequence of 1000 replicates. Only branch support of $>50\%$ was retained. To obtain the decay indices, SIMPLE addition sequence with TBR swapping was employed in PAUP, comparing strict consensus trees based on saving trees of progressively longer lengths. In addition, fit measures of the phylogenetic trees, the consistency index (CI: Kluge & Farris, 1969), retention index (RI: Farris, 1989), and rescaled consistency index (RC: Farris, 1989) were also calculated.

In the case of the *trn*L (UAA) 3′ exon and *trn*F (GAA) spacer sequence, methods used to obtain the most parsimonious tree(s), the essential statistic values and indices followed those applied for the ITS.

RESULTS

Sequence analysis

Alignment of ITS sequences of the 16 taxa of *Zingiberaceae* under study resulted in a 431bp long data matrix (without coded gaps) as shown in Fig. 2. The alignment

```
                                  10        20        30        40        50        60        70        80        90
                                   .         .         .         .         .         .         .         .         .

Taxon                             ITS1

Renealmia battenbergiana          TTGTTGAGGGAGAGCATTGAATGATGGATGGTTGTGAATGTGTTAACGTGCCCCTT-CCTT-GCCCCATGTTGGTGTGCAATTGACCGTA
Alpinia conchigera                TTGTTGA--GAGTGCATTGAATGATGGATGGTTGCGAATGTGTCAACGTGCCCCTTTCCTT-GCCCCATGTTGTTGGGCAATTGATCGTA
Alpinia galanga                   TTGTTGA--GAGTGCATTGAATGATGGATGGTTGCGAATGTGTCAACGTGCCCCTTTCCTT-GCCCCATGTTGCTGGGCAATTGATCGTA
Alpinia nigra                     TTGTTGA--GAGTGCATTGAATGATGGATGGTTGCGAATGTGTCAACGTGCCCCTTTCCTT-GCCCCATGTTGTTGGGCAATTGATCGTA
Alpinia rafflesiana               TTGTTGA--GAGAGCATTGAATGACGGATGATTGTGAATGTGTCAACGTGCCCCATTCCTT-GCCCCATGTTGGTGGGCGACTGACCGGA
Alpinia javanica                  TTGTTGA--GAGAGCATTGAATGACGGATGATTGTGAATGTGTCAACGTGCCCCATTCGTT-GCCCCATGTTGGTGGGCGACTGACCGGA
Alpinia coriacea                  TTGTTGA--GAGAGCATTGAACGACGGATGGTTGTGAATGTGTCAACGTGCCCCTTTTGTTTGCCCCATGTTGGCAGCTGATTGATCGTA
Alpinia polyantha                 TTGTTGA--GAGAGCATTGAACGACGGATGGTTGTGAATGTGTCAACGTGCCCCTTTTGTT-GCCCCATGTTGGCAGCTGATTGACCGTA
Alpinia suishaensis               TTGTTGA--GAGAGCATTGAACGACGAATGACTGTGAATGTGTCAACGTGCCCCTTTCGCT-GCCCCATGTTGGCAGCTGATTGACCGTA
Alpinia intermedia                TTGTTGA--GAGAGCATTGAACGACGAATGATTGTGAATGTGTCAACGTGTCCCTTTCGTT-GCCCCATGTTGGCAGCyGATTGACCGTA
Alpinia japonica                  TTGTTGA--GAGAGCATTGAACGACGGATGATTGTGAATGTGTCAACGTGCCCCTTTTGTT-GCCCCATATTGGCAGCCGATTGACCGTA
Alpinia maclurei                  TTGTTGA--GAGAGCATTGAACGACGAATGACTGTGAATGTGTCAACGTGCCCCTTTCGCT-GCCCCATGTTGGCAGCTGATTGACCGTA
Alpinia zerumbet                  TTGTTGA--GAGAGCATTGAACGACGGATGGCTGTGAATGTGTCAACGyGCCCCTTTCGCT-GCCCCATGTyGGCAGTTGATTGATCGTA
Alpinia blepharocalyx 1           TTGTTGA--GAGAGCATTGAACGACGGATGGATGTGAATGTGTCAACGCGCCCCTTTCGCT-GCCCCATGTTGGCAGTTGATCGATCGTA
Alpinia blepharocalyx 2           TTGTTGA--GAGAGCATTGAACGACGGATGGCTGTGAATGTGTCAACGCGTCCCTTTCGCT-GCCCCATGTTGGCAGTTGATCGATCGTA
Alpinia malaccensis               TTGTTGA--GAGAGCATTGAACGACGGATGGCTGTGAATGTGTCAACGCGCTCCTTTCGCT-GCCCCATGTTGGCAGTTGATTGATCGTA
```

FIG. 2. Sequence data matrix of aligned ITS1 and ITS2 regions of nuclear ribsomal DNA for 16 taxa of *Zingiberaceae.* Nucleotide sequence displayed from 5′ to 3′. ITS1 ranges from site 1 to 192 and ITS2 ranges from site 193 to 431. Uncertian nucleotide states are coded as follows: N = A/C/G/T, K = G/T, R = A/G, S = CG, W = A/T, Y = C/T. Hyphens denote alignment gaps. Nucleotides in bold show phylogenetically informative insertions. Numbers in square brackets at the end of sequence indicates the actual spacer length of the combined ITS1 and ITS2 regions. *Alpinia blepharocalyx* var. *glabrior*, and *Alpinia blepharocalyx* 2 is A. *blepharocalyx.*

```
                                100       110       120       130       140       150       160       170       180
                                .         .         .         .         .         .         .         .         .
Taxon

Renealmia battenbergiana        GCTCGGTGCGATCGGCACCAAGGAACAATGAAcTTAGAAGCAGAGGGCCC-TCGATGTGCGCGGGGAGCCCAATGCGGAGGAGATGCCAC
Alpinia conchigera              GCTCGGTGCGATCGGCACCAAGGAATAATAAACTGAGAAGCAGAGGGCCC-TCGGTGTGTGCGGGGAGCCCAATGCGTCGGAGAAGCCTC
Alpinia galanga                 GCTCGGTGCGATCGGCACCAAGGAATAATAAACTGAGAAGCAAAGGGCCC-TCGGTGTGTGCGGGGAGCCCAATGCGTCGGAGAAGCCTC
Alpinia nigra                   GCTCGGTGCGATCGGCACCAAGGAATAATAAACTGAGAAGCAGAGGGCCC-TCGGTGTGTGCGGGGAGCCCAATGCGTCGGAGAAGCCTC
Alpinia rafflesiana             GCTCGGTGCGATCGGCACCAAGGAACAATGAACTCAGAAGCAAAGGGCTC-TCGGTGTGCGTGCGAGGCCCAATGCTTCGGAGATGCCTC
Alpinia javanica                GCTCGGTGCGATCGGCACCAAGGAACAATGAACTCAGAAGCAAAGGGCTC-TCGGTGTACGTCCAAGGCCCAATGCTTCGGAGATGCCTC
Alpinia coriacea                GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCAGATGGCCC-TCAGCGTGCGCGGGGAGGCCAATGCATCGGAGATGCCTC
Alpinia polyantha               GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCAGATGGCCC-TCGGCGTGCGTGAGGAGGCCAATGCATCAGAGATGCCTC
Alpinia suishaensis             GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCAGATGGCCC-TCAGCGTGCGCGAGGAGGCCAATGCATCGGAGATGCCTC
Alpinia intermedia              GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCAGATGGCCC-TCAGCGTGCGCGAGGAGGCCAATGCATCGGAGATGCCTC
Alpinia japonica                GCTCGGTGCGATCTGCACCAAGGAATAATGAACTCAGAAGCAGATGGCCC-TCAGCGTGCGCGAGGAGGCCAATGCATCGGAGATGCCTC
Alpinia maclurei                GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCAGATGGCCC-TCAGCGTGCGTGAGGAGGCCAATGCATCGGAGATGCCTC
Alpinia zerumbet                GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCATATGGCCCCTCAGCGTGCGCGGGGAGGCCATTGCATCGGAGATGCCTC
Alpinia blepharocalyx 1         GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCATATGGCCCCTCAGCGTGCGCGGGGAGGCCAATGCATCGGAGATGCCTC
Alpinia blepharocalyx 2         GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCATATGGCCCCTCAGCGTGCGCGGGGAGGCCAATGCATCGGAGATGCCTC
Alpinia malaccensis             GCTCGGTGCGATCTGCACCAAGGAACAATGAACTCAGAAGCATATGGCCCCTCAGCGTGCGCGGGGAGGCCAATGCATCAGAGATGCCTC
```

FIG. 2. Continued

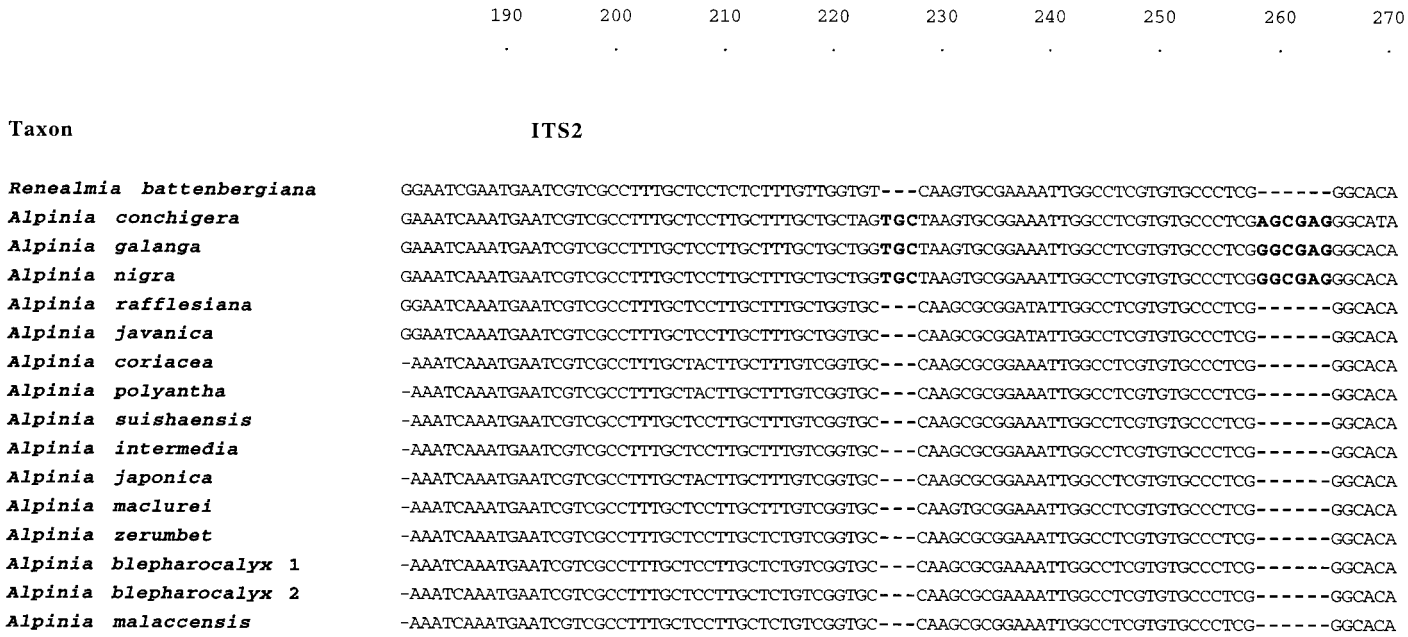

FIG. 2. Continued

```
                                  280        290        300        310        320        330        340        350        360
                                   .          .          .          .          .          .          .          .          .

Taxon

Renealmia battenbergiana          GTCGGCTGAAGAgTGGGTAgTCGGCAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAATTGAACGTTGTCCCCGTCGTGTTG
Alpinia conchigera                GTCGGTTGAAGAGTGGGTAGTCGGTAGACGTCGGGCGCGATGGGTGTTGGTCGCTCTATGCGTGAATCGAACATCGTCCCCGTCGTACTG
Alpinia galanga                   GTCGGTTGAAGAGTGGGTAGTCGGTAGACGTCGGGCGCGATGGGTGTTGGTCACTCTATGCGTGAATCGAACATCGTCCCCGTCGTACTG
Alpinia nigra                     GTCGGTTGAAGAGTGGGTAGTCGGTAGACGTCGGGCGCGATGGGTGTTGGTCACTCTATGCGTGAATCGAACATCGTCCCCGTCGTACTG
Alpinia rafflesiana               GTCGGTTGAAGAGTGGGTAGTCGACAGTCGTCGAGGGCGATGGGTGTTGGTCGCCCTGTGCGTGAATTGAACGTCGTCCTCGTCGTGTTG
Alpinia javanica                  GTCGGTTGAAGAGTGGGTAGTCGGCAGTCGTCGGGGGCGATGGGTGTTGGTCGCCCTGTGCGTGAATTAAACGTCGTCCTCGTCGTGTTG
Alpinia coriacea                  GTCGGCTGAAGAGTGGGTAATCCGCAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGCCGTGTTG
Alpinia polyantha                 GTCGGCTGAAGAGTGGGTAATCCACAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGCCGTGTTG
Alpinia suishaensis               GTCGGCTGAAGAGTGGGTAATCCACAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGCCGTGTTG
Alpinia intermedia                GTCGGCTGAAGAGTGGGTAATCCACAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGTCGTGTTG
Alpinia japonica                  GTCGGCTGAAGAGTGGGTAATCCGCAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGCCGTGTTG
Alpinia maclurei                  GTCGGCTGAAGAGTGGGTAATCCACAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGCCGTGTTG
Alpinia zerumbet                  GTCGGCTGAAGAGTGGGTAATCCGCAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGTCGTGTTG
Alpinia blepharocalyx 1           GTCGGCTGAAGAGTGGGTAATCCGCAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGTCGTGTTG
Alpinia blepharocalyx 2           GTCGGCTGAAGAGTGGGTAATCCGCAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGTCGTGTTG
Alpinia malaccensis               GTCGGCCGAAGAGTGGGTAATCCGCAGTCGTCGGGTGCGATGGGTGTTGGTCGCCCTGTGCGTGAACTGAACGTCGTCCCCGTCGTGTTG
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FIG. 2. Continued

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                                370        380        390        400        410        420        430
                                 .          .          .          .          .          .          .
Taxon

Renealmia battenbergiana        GGATGAGTCCTCAAGTGA-----------TTGCGGCGTCGCGTGAAA--GTGCCGTGTTCGTCATATTGT  [405]
Alpinia conchigera              GGATGAGTCCTCAAGAGACCT--TGTGTGATTGCAGCATCGCATGAAA--GTGCCGTGTTCATTAGATTGT  [423]
Alpinia galanga                 GGATGAGTCCTCAAGAGACCT--TGTGTGATTGCAGCATCGCATGAAA--GTGCCGTGTTCATCATATTGT  [423]
Alpinia nigra                   GGATGAGTCCTCAAGAGACCT--TGTGTGATTGCAGCATCGCATGAAA--GTGCCGTGTTCATCATATTGT  [423]
Alpinia rafflesiana             GGATGAGCCCTCAAGAGACCC--TGTGCGATTGCGGCGTCGCGTGGAA--GCGCCGTGTCCATCAGATTGT  [414]
Alpinia javanica                GGATGAGCCCTCAAGAGACCC--TGTGTGATTGCGGCGTCGCATGGAA--GTGTCGTGTCCATCAGATTGT  [414]
Alpinia coriacea                GGATGAGTCCTCAAGAGACCC--TGTGTGATTGCGGCGTCGCATGAAA--GTGCCGTGTCCATCAAATTGT  [414]
Alpinia polyantha               GGATGAGTCCTCAAGAGACCCCCTGTGTGATTGCGGCGTCGTATGAAA--GTGCCGTGTCCATTAAATTGT  [415]
Alpinia suishaensis             GGATGAGTCCTCAAGAGACCC--TGTGTGATAGCGGCGCCGCATGAAA--GTGCCGTGTCCATCAAATTGT  [413]
Alpinia intermedia              GGATGAGTCCTCAAGAGACCC--TGTGTGATWGCGGCGTCGCATGAAA--GTGCCGTGTCCATCAAATTGT  [413]
Alpinia japonica                GGATGAGTCCTCAAGAGACCC--TGTGTGATTGCGGCGTCGCATGAAA--GTGCCGTGTCCATCAAATTGT  [413]
Alpinia maclurei                GGATGAGTCCTCAAGAGACCC--TGTGTGATAGCGGCGCCGCATGAAA--GTGCCGTGTCCATCAAATTGT  [413]
Alpinia zerumbet                AGATGAGTCCTCAAGAGACCC--TGTGTGATAGCGGCGTCGCATGAAA--GTGTCGTGTCCATCAAATTGT  [414]
Alpinia blepharocalyx 1         AGATGAGTCCTCAAGAGACCC--TGTGTGATAGCGGCGTCGCATATAAGTGTGCCGTGTCCATCAAATTGT  [416]
Alpinia blepharocalyx 2         AGATGAGTCCTCAAGAGACCC--TGTGTGATAGCGGCGTCGCATATAA--GTGCCGTGTCCATCAAATTGT  [414]
Alpinia malaccensis             AGATGAGTCCTCAAGAGACCC--TGTGTGATAGCGGCGTCGCATAAAA--GCGCCGTGTCCATCAAATTGT  [414]
```

FIG. 2. Continued

did not include 5.8S rDNA (162bp) because this coding region is highly conserved and therefore almost completely uninformative for the phylogenetic analysis of *Alpinia*. Sequence characteristics of ITS1, ITS2 and the combined ITS region are summarised in Table 2. The length of ITS1 ranged from 187 to 189bp while that of ITS2 was greater and ranged from 216 to 235bp. In ITS2 there were two indels of three bp (at positions 225–227) and six bp (at positions 259–264) which are critical and apparent synapomorphies uniting *Alpinia galanga*, *A. nigra* and *A. conchigera*. It is unlikely that the identical insertions in these three taxa could have occurred twice independently, and thus both these indels provide additional support for the most parsimonious tree, which unites *A. galanga, A. nigra* and *A. conchigera.*

For the spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA) of cpDNA, PCR products of only ten taxa of *Zingiberaceae* were readily obtainable and their sequences were examined. Sequence characteristics are summarised in Table 3. The total length of the spacer ranged from 295 to 310bp. There were five indels (see Table 4), one of which (a six bp-insertion at positions 256–261) corresponded to the grouping of *A. galanga* with *A. nigra* and *A. conchigera* by the ITS data. The spacer contained 26 (8.4%) variable sites, 285 (91.6%) constant sites, 7 (2.3%) informative sites and 19 (6.1%) autapomorphic sites.

TABLE 2. Sequence characteristics of ITS1 and ITS2 regions for 16 taxa of *Zingiberaceae*

Sequence characteristics	ITS1	ITS2	ITS1 and ITS2
Length range (total) (bp)	187–189	216–235	405–423
Length mean (total) (bp)	187.7	227.3	415.0
Length range (ingroup) (bp)	187–188	226–235	413–423
Length mean (ingroup) (bp)	187.7	228.0	415.7
Length (outgroup) (bp)	189	216	405
Aligned length (bp)	192	239	431
G+C content range (%)	51.3–54.3	54.4–60.2	53.3–57.5
G+C content mean (%)	52.7	58.1	55.7
Sequence divergence (ingroup) (%)	0–16.1	0–14.6	0.5–15.6
Sequence divergence (total) (%)	8.9–15.6	7.4–12.0	10.1–13.3
Number of indels (ingroup)	3	4	7
Number of indels (total)	5	4	9
Size of indels (ingroup) (bp)	1	2–6	1–6
Size of indels (total) (bp)	1–2	2–12	1–12
Number of variable sites (%)	56 (29.2)	55 (23.0)	111 (25.8)
Number of constant sites (%)	136 (70.8)	184 (77.0)	320 (74.2)
Number of informative sites (%)	42 (21.9)	42 (17.6)	84 (19.5)
Number of autapomorphic sites (%)	14 (7.3)	13 (5.4)	27 (6.3)
Transitions (minimum)	55	56	111
Transversions (minimum)	22	13	35
Transition/transversion (ts/tv) ratio	2.5	4.3	3.2
Skewness of tree length distribution (g_1 value for 10,000 random trees)	−0.8	−1.4	−1.0

TABLE 3. Sequence characteristics of the chloroplast spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA) for ten taxa of *Zingiberaceae*

Sequence characteristics	The spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA)
Length range (total) (bp)	295–310
Length mean (total) (bp)	302.4
Length range (ingroup) (bp)	295–310
Length mean (ingroup) (bp)	302.2
Length (outgroup) (bp)	304
Aligned length (bp)	311
G+C content range (%)	34.2–35.8
G+C content mean (%)	35.1
Sequence divergence (ingroup) (%)	0–2.3
Sequence divergence (total) (%)	5.8–6.8
Number of indels (ingroup)	3
Number of indels (total)	5
Size of indels (ingroup) (bp)	1–8
Size of indels (total) (bp)	1–8
Number of variable sites (%)	26 (8.4)
Number of constant sites (%)	285 (91.6)
Number of informative sites (%)	7 (2.3)
Number of autapomorphic sites (%)	19 (6.1)
Transitions (minimum)	13
Transversions (minimum)	14
Transition/transversion (ts/tv) ratio	0.9
Skewness of tree length distribution (g_1 value for 10,000 random trees)	−0.7

Phylogenetic analysis

Phylogenetic analysis of the ITS region yielded a single most parsimonious tree. With the complete data matrix of the region, including uninformative characters, the resulting tree had a length (L) of 150 steps (Fig. 3). The tree had the following fit measures: CI = 0.807, RI = 0.879 and RC = 0.709. When all uninformative characters were ignored, the tree had a length of 124 steps. When coded gaps were added to the data matrix in the presence of the uninformative characters, the analysis yielded one most parsimonious tree identical to the tree shown in Fig. 3 (L = 154, CI = 0.812, RI = 0.885, RC = 0.718).

The ITS regions from the two accessions of *A. galanga* were identical so only one was used in the analysis. Based on ITS analyses, *A galanga* is closely related to *A. nigra* and *A. conchigera*. This clade is strongly supported with a bootstrap value of 100% and a decay index of $> +6$. *A. rafflesiana* and *A. javanica* also form a strongly supported monophyletic group (BS = 100%, DI = $> +6$). The clade of these two closely related species is located between sect. *Allughas* and sect. *Alpinia* subsect. *Alpinia*, with moderate support (BS = 75%, DI = +2). The species of sect. *Alpinia*

TABLE 4. Characteristics of the five insertion/deletion events inferred from the alignment of the chloroplast spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA) of ten taxa of *Zingiberaceae*

No.	Position	Size (bp)	Potentially informative phylogenetically	Type	Taxa
1	86	1	No	Deletion	*Alpinia blepharocalyx* var. *glabrior*, *A. intermedia*, *A. japonica*, *A. malaccensis*
2	121–128	8	Yes	Deletion	*Alpinia blepharocalyx* var. *glabrior*, *A. malaccensis*
3	195–202	8	Yes	Deletion	*Alpinia javanica*, *A. rafflesiana*
4	256–261	6	Yes	Insertion	*Alpinia conchigera*, *A. galanga*, *A. nigra*
5	298	1	Yes	Insertion	*Alpinia intermedia*, *A. japonica*

subsect. *Catimbium* form a natural group and its monophyly is highly supported with a bootstrap value of 100% and a decay index of +5. However, *Catimbium* is nested within a paraphyletic sect. *Alpinia* subsect. *Alpinia* (BS = 100%, DI = > +6) (although a clade, consisting of *A. coriacea*, *A. polyantha* and *A. japonica*, is only weakly supported with BS < 50%).

For the spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA), analysis of the sequence data with the uninformative characters and without the coded gaps resulted in three equally parsimonious trees (length 27 steps, CI = 0.963, RI = 0.929, RC = 0.894). The strict consensus tree (Fig. 4) shows that *A. galanga* is in the same clade as *A. conchigera* and *A. nigra*. Under the same settings, with the addition of coded gaps, the analysis yielded two parsimonious trees with 32 steps (CI = 0.969, RI = 0.955, RC = 0.925). The strict consensus tree (Fig. 4) obtained confirmed a close relationship of *A. galanga*, *A. nigra* and *A. conchigera* (BS = 79%, DI = +1) although the tree did not provide clear phylogenetic resolution of the taxa examined due to the presence of many invariable sites and the lack of potentially informative sites. The relationship of *A. galanga*, *A. nigra* and *A. conchigera* is however supported by an apparently synapomorphic indel event (indel No. 4 in Table 4).

For all 16 taxa under study, the transition/transversion (ts/tv) ratio was 2.5 for ITS1, 4.3 for ITS2 and 3.2 for the combined ITS region. This suggests a surprisingly high rate of transitions over transversions in these taxa. When the characters were weighted (3x and 5x) to accommodate the ts/tv ratio, the strict consensus trees obtained from the analyses (without coded gaps) still retained the feature of *A. galanga* being placed within sect. *Allughas.*

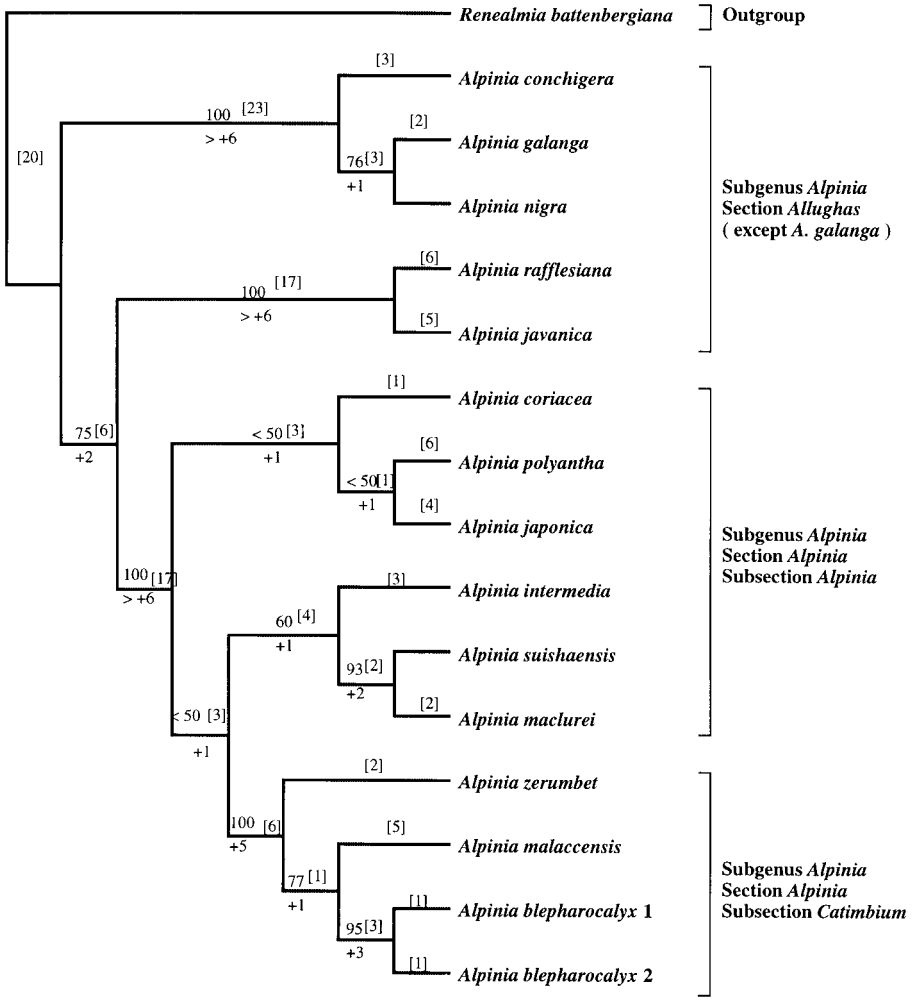

FIG. 3. A single most parsimonious tree of length 150 steps obtained based on equally weighted parsimony analysis of the combined ITS1 and ITS2 sequence data excluding coded gaps. Upper numbers are bootstrap values of 1000 replicates. Lower numbers are decay indices. Numbers in square brackets indicate branch lengths. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior*, and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

DISCUSSION

Molecular evolution of the ITS region

In the *Zingiberaceae* under study ITS1 is shorter than ITS2. The length variation of both spacers is due to the presence of the indels of 1–12bp. It is clear from the ITS sequences that some of these indels support the grouping of several species in the cladogram. In comparison with other substitutions the indels might be less likely to be homoplasious (Baldwin *et al.*, 1995) because they are likely to be involved in a

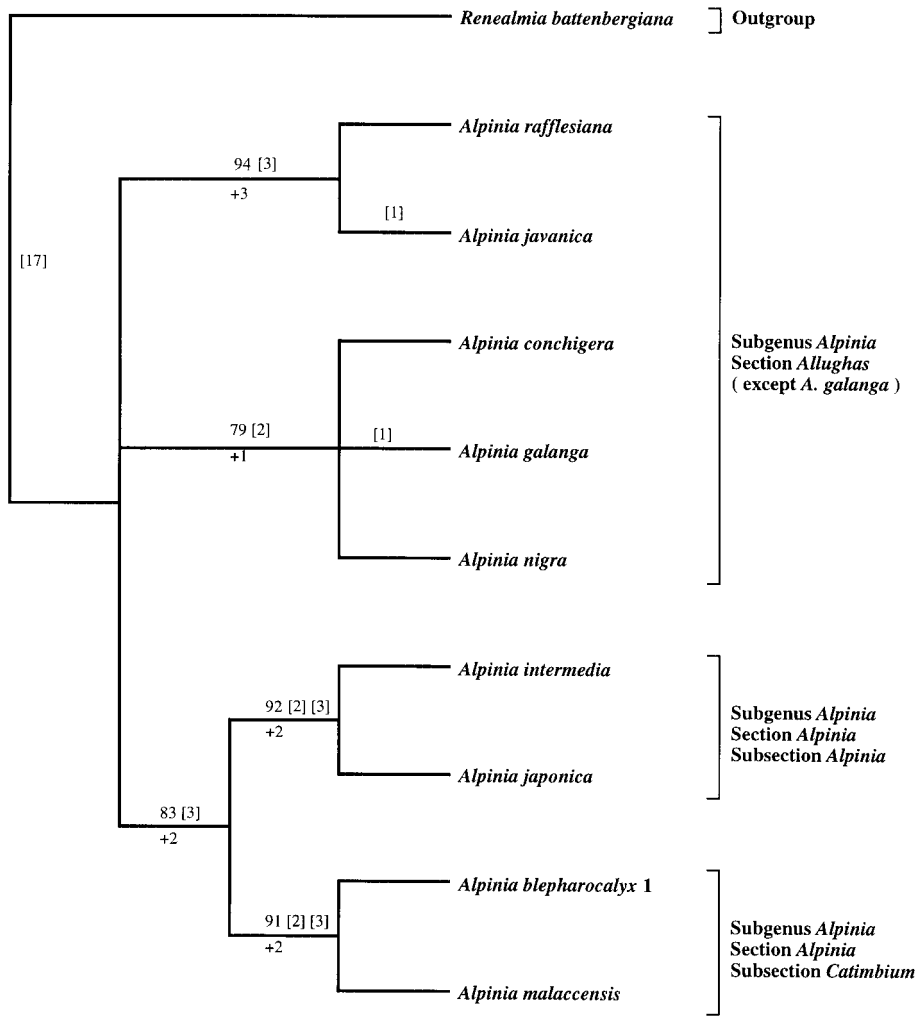

FIG. 4. The strict consensus tree obtained from two equally parsimonious trees of length 32 steps based on equally weighted parsimony analysis of the chloroplast DNA between *trn*L (UAA) 3′ exon and *trn*F (GAA) plus coded gaps. Upper numbers are bootstrap values of 1000 replicates. Lower numbers are decay indices. Numbers in square brackets indicate branch lengths. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior*.

series of ITS structural changes and hence are probably more constrained (Buckler & Holtsford, 1996a,b).

For the 15 species of *Alpinia* analysed, the ranges of sequence divergence values are similar for both ITS1 (0–16.1%) and ITS2 (0–14.6%), indicating a similar substitution rate in both spacers. This similarity of the pairwise sequence divergence is also a feature of other groups of angiosperms such as *Astragalus* (ITS1: 0–10.2%, ITS2: 0–8.8%; Wojciechowski *et al.*, 1993) and *Viburnum* (ITS1: 0–13.6%, ITS2:

0–11.9%; Donoghue & Baldwin, 1993). In addition, the *Zingiberaceae* studied have a more or less uniform G+C content in ITS1 and ITS2, which conforms with other studies in angiosperms (reviewed in Baldwin *et al.*, 1995).

The respective ratio of ts/tv in the combined ITS1 and ITS2 regions of *Alpinia* and its outgroup (3.2) is higher than the expected value (approximately 2.0) for relatively recently diverged sequences (Holmquist, 1983) of other angiosperms such as Poaceae (Hsiao *et al.*, 1994) because of an excess of transitions among nucleotide substitutions (reviewed in Wakeley, 1996). Transitions may be caused by pyrimidine dimerisation, ionisation, and 5-methylcytosine deamination – phenomena which are related to kinetic processes and not to cell replications (Vairapandi & Duker, 1994; von Borstel, 1994). In contrast, transversions are more tightly linked to cell replication cycles and generation time (Buckler & Holtsford, 1996a), therefore the occurrence of transversions is less prevalent in nature.

The chloroplast spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA) evolves more slowly than the ITS region. However, despite the fact that it provides very limited phylogenetic information for the present study, this chloroplast spacer serves as a useful confirmation for the results based on the ITS region.

The origin of A. galanga *and its phylogenetic relationships within* Alpinia

Molecular phylogenetic analyses of the ITS region suggest that *A. galanga* is a close relative of *A. nigra* and *A. conchigera*. This is confirmed by results from a phylogenetic analysis of the spacer between *trn*L (UAA) 3′ exon and *trn*F (GAA). Thus, it appears that *A. galanga* has evolved within sect. *Allughas*. It is interesting to note that some authors (Roxburgh, 1832; Ridley, 1899) also placed *A. galanga* near to *A. nigra* or *A. conchigera*. Other lines of evidence which seem to support the close relationship of these three species have come from studies of fruit and seed anatomy of Chinese *Alpinia* by Liao & Wu (1996a,b). All three species possess the 'Conchigera' type of fruit and seed. However, results based on Liao and Wu's studies should be viewed with caution because no voucher specimens were cited, so the species identification may be uncertain.

Following Smith's (1990) infrageneric classification of *Alpinia*, *A. galanga* was grouped with other species in sect. *Alpinia* subsect. *Alpinia*, while *A. conchigera* and *A. nigra* were placed in sect. *Allughas*. The classification of these two sections is mainly based on one character, the form of bracteoles. In sect. *Alpinia*, if bracteoles are present they are open to the base (non-tubular) whereas in sect. *Allughas*, bracteoles are always present and are cup to funnel-shaped (tubular). It is significant that both these forms of bracteoles have been found within a single inflorescence in *A. abundiflora* of sect. *Fax* (Smith, 1981). Moreover, in Smith's (1990) treatment of sect. *Kolowratia* species with non-tubular and with tubular bracteoles were placed together. Smith (1975) remarked that, although this character is obvious in the fully grown bracteole, it depends on a very minute shift of cell division in the course of development. Hence, it appears that the feature of bracteoles by itself, may not be

adequate for grouping of species at sectional level. Based on this molecular study, it is suggested that the absence of tubular bracteoles in *A. galanga* is a convergence with sect. *Alpinia*.

It was assumed by Holttum (1950) that tubular bracteoles were primitive, which accords with the basal position of *Allughas* in the ITS cladogram (Fig. 3). In addition, the ITS data suggest that large flowers with showy labella, characteristic of sect. *Alpinia* subsect. *Catimbium*, have evolved from smaller flowered ancestors similar to those of sect. *Alpinia* subsect. *Alpinia*.

New systematic position of A. galanga *and its significance*

The reclassification of *A. galanga* as a member of sect. *Allughas* is important in the classification of the genus *Alpinia*. *Alpinia galanga* is the type species of the genus and therefore any infrageneric groups it belongs in must be called *Alpinia* (Greuter *et al*., 1994). Therefore the clade which includes *A. nigra* and *A. conchigera* (Fig. 3) should become sect. *Alpinia* rather than sect. *Allughas*. However, the present ITS analysis suggests that a clade consisting of *A. rafflesiana* and *A. javanica* is located between the clade with *A. conchigera*, *A. nigra* and *A. galanga*, and the clade with *A. coriacea*, *A. polyantha*, and *A. japonica* (sect. *Alpinia* subsect. *Alpinia*) so sect. *Allughas* of Smith (1990) is paraphyletic. Clearly, before suggesting a new classification of these species both at sectional and subsectional levels, more characters including morphological ones, are required, and more taxa should be examined.

Following Smith's (1990) maps of the distribution of *Alpinia* (modified from Schumann, 1904), sect. *Allughas* extends from India and Sri Lanka to China, South East Asia and the east coast of Australia, and over most of its range it co-occurs with members of sect. *Alpinia* (subsections *Alpinia* and *Catimbium*). *Alpinia galanga* and perhaps also *A. nigra* are often cultivated and occur in abandoned cultivations throughout Thailand and Malaysia. Rhizomes of both species have been used for cooking without local people drawing a distinction between them. This implies that *A. galanga* and *A. nigra* possess similar secondary compounds, a fact that also suggests a close relationship between them. Molecular phylogenetic analyses of the ITS sequences strongly support this relationship. In terms of economic botany, if we wish to find other sources of compounds similar to those of *A. galanga* (for instance, for pharmacology or domestic use) we should begin our search with members of sect. *Allughas*, especially, those which are in the same clade with *A. galanga*, and not with sect. *Alpinia* subsect. *Alpinia*.

Following plant domestication, a series of morphologically divergent variants generally develops within each cultivated type as a result of human selection. Examples are given by many authors (Zohary, 1984; Hanelt, 1986; Pickersgill, 1986; Heiser, 1988). This raises the question of whether the morphological changes that have resulted in misclassification of *A. galanga*, such as the change from tubular to non-tubular bracteoles, could merely be the result of accelerated evolution under domestication. At present it is not clear where, if anywhere, *A. galanga* occurs as a native

wild plant. It could be entirely cultivated and derived from an unknown ancestor. Hairiness of the leaves of the possibly wild var. *pyramidata* and the bracteole character of *A. galanga* could be merely a result of single gene mutations in developmental genes. In addition, the hairiness of var. *pyramidata* could be partly environmental in origin. There is some confusion as to the variation and identity of true *A. galanga*, and biosystematic research is required.

This work on *A. galanga* is part of a larger investigation of the infrageneric classification of *Alpinia* (Rangsiruji, 1999). *Alpinia* is a large and complex genus and clearly more work is required which it is hoped will shed further light on general patterns of evolution within the genus.

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