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An analysis of generic circumscriptions in tribe Alpinieae (Alpiniodeae: Zingiberaceae)

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Abstract

Recent investigations based on molecular phylogenies have resulted in new insights into the evolutionary relationships and classification of the Zingiberaceae and various genera within the family, e.g., *Globba*, *Hedychium*, *Roscoea*, *Etlingera*, *Alpinia*, and *Amomum*. At the same time taxonomic boundaries of many traditionally recognized genera have been challenged, e.g., *Curcuma*, *Boesenbergia*, *Caulokaempferia*, *Alpinia*, and *Amomum*. Within the subfamily Alpinioideae the results of our analyses will require the recircumscription of many of the genera included in the tribe Alpinieae. These phylogenetic results are based on a supermatrix analysis of ITS and *matK* sequence data and are discussed in the context of complementary morphological features and geographic distributions. Seventeen clades are recognized at the generic level although some remain tentative and in need of additional analysis before final taxonomic circumscriptions can be made. A revised classification will require that many species be placed in new or different genera, which will greatly facilitate identification and our understanding of morphological evolution in the family as well as species and genera therein.

Introduction

During the last few years molecular data have been routinely used in determining generic boundaries and evolutionary relationships of the genera in the ginger family, the Zingiberaceae (e.g., Harris *et al.*, 2000; Ngamriabsakul *et al.*, 2000; Rangsiruji *et al.*, 2000a, b; Searle and Hedderson, 2000; Wood *et al.*, 2000; Kress *et al.*, 2002; Pedersen, 2004; Williams *et al.*, 2004; Xia *et al.*, 2004). The study by Kress *et al.* (2002) is the most thorough paper to date addressing relationships among genera in the Zingiberaceae. In that study, sequence data from both the Internal Transcribed Spacer (ITS) and *matK* regions were used to establish well-resolved phylogenetic relationships among the genera, and a new classification of the Zingiberaceae was proposed that recognized four subfamilies and four tribes. Kress *et al.* (2002) also demonstrated that a number of the larger genera in the family (*Amomum*, *Alpinia*, *Boesenbergia*, and *Curcuma*) may be para- or polyphyletic and suggested that more extensive sampling was necessary for these taxa. Subsequent studies have been carried out in some of them (*Alpinia*: Kress *et al.*, 2005; *Amomum*: Xia *et al.*, 2004).

With respect to the tribe Alpinieae, the results of investigations by Rangsiruji *et al.,* (2000a, b), Kress *et al.,* (2002, 2005), and Xia *et al.* (2004) are most pertinent. In the first study, in which 47 species of *Alpinia* and a small number of outgroup taxa were sampled, the authors demonstrated significant statistical support for several monophyletic groups of species of *Alpinia*, but suggested that the genus itself may not be monophyletic. In a broader analysis of genera of Alpinioideae, Kress *et al.* (2002) identified four separate groups of alpinias (Alpinia I–IV) for the 11 *Alpinia* species sampled (Fig. 1). These four groups did not form a monophyletic assemblage, were scattered throughout the tribe, and corresponded to at least some of the clades recognized in the molecular analyses of Rangsiruji *et al.* (2000b). Further sampling in the tribe was also conducted by Xia *et al.* (2004) in their analysis of the generic boundaries of *Amomum*. They identified at least three major non-monophyletic groups of species within the current circumscription of *Amomum*. Within Alpineae, the phylogenetic position of the presumed extinct *Leptosolena* and a second species of the formerly monotypic *Vanoverberghia* were determined by Funakoshi et al. (2005) based on molecular sequence data, providing more information on generic relationships within the tribe.

Kress *et al.* (2005) conducted the most exhaustive phylogenetic analysis of tribe Alpinieae to date sampling 99 species in the Alpinioideae with an emphasis on the genus *Alpinia*. Their results (Fig. 2) demonstrated six polyphyletic clades of *Alpinia* and at least two clades of *Amomum* while resolving the evolutionary relationships among a number of genera in the Alpinieae with a slightly different topology than earlier analyses had indicated (Kress *et al.*, 2002). Although their analyses confirmed the division of both *Alpinia* and *Amomum* into numerous polyphyletic groups, the authors were reluctant to propose a new classification until a number of issues, especially taxon sampling, were resolved.

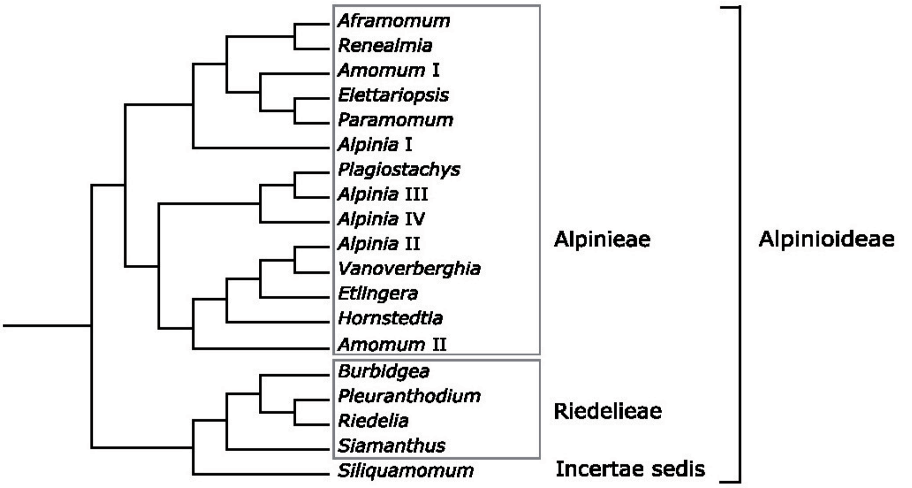

Figure 1. Early results on the phylogenetic relationships among the genera of the subfamily Alpinioideae of the Zingiberaceae based on a parsimony analysis of ITS and matK sequence data for 45 species in the subfamily (from Kress *et al.*, 2002). Note the four polyphyletic clades of *Alpinia* and two clades of *Amomum*.

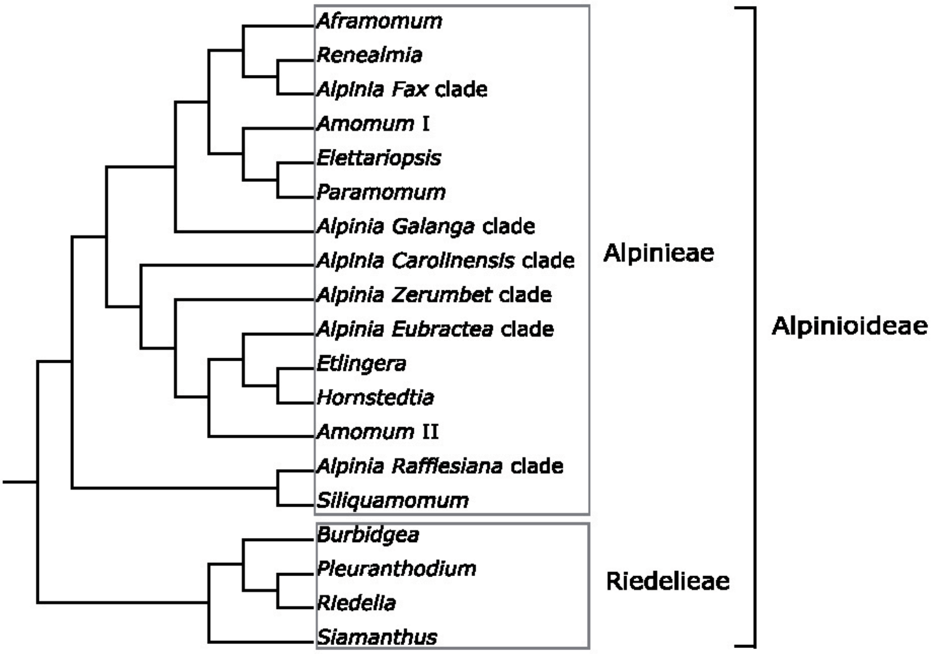

Figure 2. Most recent results on the phylogeny of the Alpinioideae resulting from further analysis of the combined ITS and matK sequence data for 99 species in the subfamily (from Kress et al. 2005). Note the six clades of *Alpinia*. The *Alpinia Zerumbet* clade includes the genus *Plagiostachys*; the *Alpinia Eubractea* clade includes the genera *Vanoverberghia* and *Leptosolena*.

The goals of our current analyses are 1) to combine molecular sequence data from previously published analyses with new original sequences to build a "supermatrix" for Alpinieae; 2) to identify major clades defined by molecular data and assess their correspondence to existing generic boundaries; and 3) to provide new circumscriptions of genera and apply new names if necessary. Goals one and two are addressed in this paper. The results of these analyses have established the major lineages within the tribe, have confirmed the validity of previously recognized genera, and have identified clades which will require new generic circunscriptions. Although our sampling is extensive, we will be adding several key taxa to a final analysis to be published in the near future. At that time we will address goal three and provide a new revised classification of the Alpinieae that will recognize more finely circumscribed genera.

Materials and Methods

Taxon and Character Sampling

Taxon sampling was designed to include the full diversity (taxonomic, morphologic, and biogeographic) of the Alpinioideae with a focus on phyletic and biogeographic diversity in *Alpinia* and *Amomum*. A total of 230 taxa are used in the analysis, including 23 outgroup taxa representing the Zingiberoideae, Tamijiodeae and Siphonochiloideae. Previously published sequence data from our own work as well as additional data downloaded from GenBank were combined with new original sequences in the current analysis. Two independent gene regions were sampled for this analysis: the ITS (internal transcribed spacer) region of nuclear ribosomal DNA (White *et al*., 1990) and the intron of the chloroplast transfer RNA gene for lysine *trn*K, including the maturase (*mat*K) coding region and 5´and 3´ flanking introns (Johnson and Soltis 1994; Mohr *et al*. 1993). ITS was obtained for all 230 taxa included in this analysis, while the entire *trn*K region is missing from 84 taxa.

DNA Isolation and Manipulation

Whole genomic DNA was extracted from plants with the Plant DNAeasy kit protocol (Qiagen). DNA fragments were amplified and sequenced for each of the gene regions using the primers and protocols previous published for Zingiberales (Kress *et al*., 2002, 2005; Specht, 2006; Specht *et al*., 2001). Sequencing was carried out on an ABI 3700 automated sequencer equipped with ABI PRISM$^{\text{TM}}$ sequencing analysis software. Sequences were analyzed and edited using Sequence Navigator (Applied Biosystems) and GeneJockey (Taylor, 1994) or Se-Al (Rambaut, 1996). Alignments across taxa were performed using CLUSTAL X (Thompson *et al.* 1994) as a Multiple Alignment option in GeneJockey with both fixed and floating gap penalties set to 10. All manual alignment adjustments follow the criteria of Zurawksi and Clegg (1987) in which gaps are considered as characters and the number of evolutionary events (insertions or deletions) is minimized. All characters are treated as unordered and gapped regions are treated as missing, however insertion-deletion events (indels) are coded as additional individual binary characters using the simple indel coding method (Simmons and Ochoterena 2000). A total of 25 and 17 gaps were coded from within the *trn*K and ITS regions, respectively.

Phylogenetic Analysis

The combined ITS and *trn*K/*mat*K phylogenetic analyses were performed using the parsimony optimality criterion, in each case considering all positions of equal weight for evaluating phylogenetic relationships. Analyses were conducted with PAUP*4.0b4a (Swofford, 2001) for a total of 3820 aligned characters with 28% of the matrix scored as missing as the result of either inability to acquire sequence data for certain taxa or the presence of gap characters. For all parsimony analyses, heuristic searches were performed with TBR as the branch-swapping algorithm; starting trees were obtained using stepwise random addition with 100 replicates and one tree held at each step. Jackknife support values were calculated with 37% deletion and the "emulate Jac resampling" option selected (Farris *et al.*, 1996).

Results and Discussion

The first goal of our analysis was addressed by the parsimony consensus tree of the combined ITS and *trn*K/*matK* sequence data. The consensus tree represents 9,624 equally parsimonious trees with a length of 4,277 steps, including 32 coded gaps (Figs. 3, 4). A total of 920 characters were parsimony informative (2433 were constant, 467 were uninformative). The majority of primary clades defining recognized or tentative genera, including *Etlingera*, *Renealmia, Aframomum*, the *Alpinia rafflesiana* clade, the *Alpinia zerumbet* clade, the *Alpinia carolinenesis* clade, the *Alpinia eubractea* clade, the *Alpinia galanga* clade, the *Alpinia fax* clade, and the *Amomum tsao-ko* clade, are strongly supported with jackknife values ranging between 99-100%. Monotypic genera or genera with only a single species sampled in our analysis, including *Siliquamomum*, *Geostachys*, and *Geocharis*, are clearly differentiated from their sister taxa. The *Amomum villosum* clade, the *Amomum maximum* clade and the *Elettariopsis* clade have slightly lower jackknife values (76-89%) while the seven species of *Hornstedtia* sampled in our analysis form a grade with *Etlingera*. The relationships of only a few of the major lineages are not fully resolved, such as the placement of the *Siliquamomum-Alpinia rafflesiana* clade, and the polytomy formed by the *Geostachys - Amomum tsao-ko* clade, the *Alpinia zerumbet* clade, and the *Alpinia carolinenesis* clade.

The second goal of our analyses, to assess the correspondence of the major clades defined by the molecular data to existing generic concepts, has also been accomplished. The 17 clades recognized here (Fig. 4) can be classified into three categories: 1) well-supported lineages that correspond to formerly recognized genera whose taxonomic names should be maintained, 2) well-supported lineages in need of new (or previously used) taxonomic names, and 3) problematic lineages in need of additional data prior to making final taxonomic decisions.

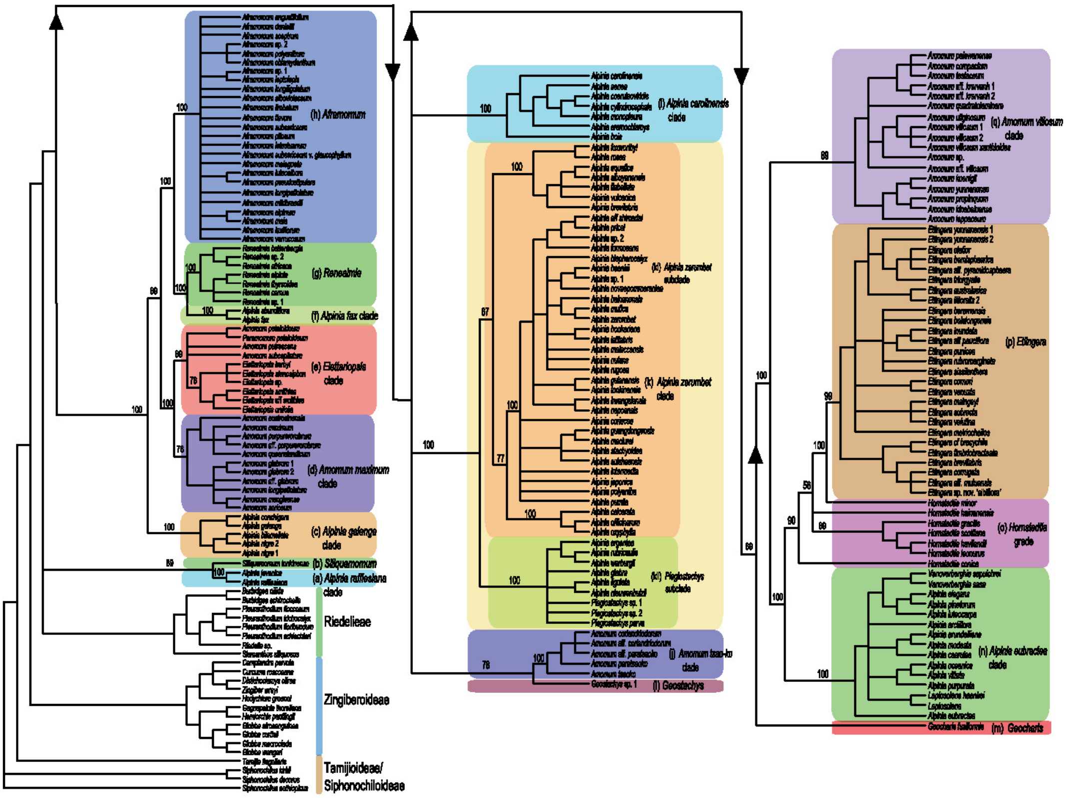

Figure 3. The strict consensus supertree of 9,624 equally parsimonious trees of the Alpinioideae (with an emphasis on tribe Alpinieae) in the analysis of combined ITS and *trn*K/*mat*K sequence data for 207 species in the subfamily (length = 4,277 steps) showing bootstrap values from the parsimony analysis. The seventeen major clades in Alpinieae are indicated with small letters (a through q) and variously color shading. For discussion of each clade see text.

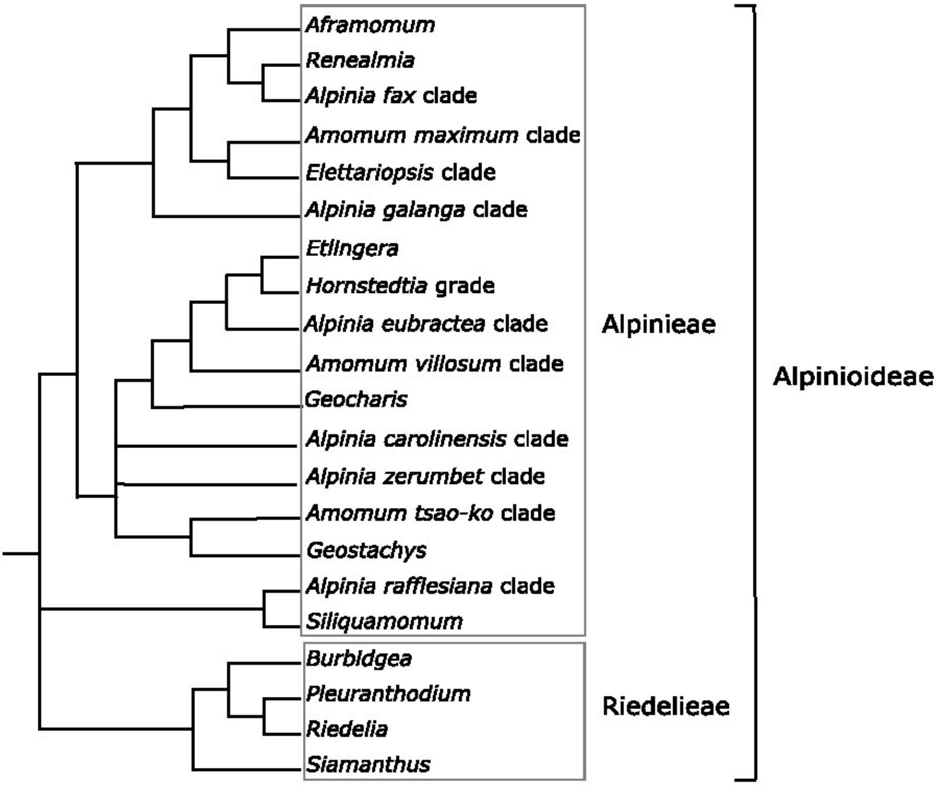

Figure 4. Condensed tree of the Alpinioideae resulting from the analysis of the combined ITS and *trn*K/*mat*K sequence data (see Fig. 3) in which the major clades/grades have been collapsed into single branches for clarity.

(1) Well-supported lineages that correspond to formerly recognized genera whose taxonomic names should be maintained.

Aframomum. As earlier demonstrated by Harris *et al.* (2000), this African genus is strongly supported as monophyletic by molecular data. The flask-shaped fruit is a distinct synapomorphy of the genus. The basal inflorescence radical to the leafy shoot is shared with its sister taxon *Renealmia* from which it is distinquished by the presence of scale-like trichomes on the vegetative structures in *Aframomum*.

Renealmia. This genus is one of the few genera in the order Zingiberales with an amphi-Atlantic distribution with species in the tropical forests of the Americas and Africa. The clade is well-supported as monophyletic, and although it shares the distinctive basal inflorescence with *Aframomum*, it is distinguished by stellate, rather than scale-like, trichomes. It should be noted that at least a few species on both sides of the Atlantic have inflorescence terminal on the leafy shoots (e.g., the neotropical *R. cernua* (Sw. ex. Roem. & Schult.) J. F. Macbr., *R. helenae* Maas, and *R. pyramidalis* (Lam.) Maas, and the African *R. battenbergiana* Cummins).

Alpinia galanga clade. The type species of the large conglomerate genus *Alpinia* is *A. galangal* (L.) Willd., which is placed in this small clade in the molecular analyses. For this reason, the generic name *Alpinia* is best applied to this group of four species. Both the placement of this clade in relationship to other genera and the monophyly of the four species have strong jackknife support (100%). Branched inflorescence with small flowers, open bracteoles, a clawed labellum, and thin-walled fruits are characteristic of the species in the *A. galanga* clade. Members of this clade are distributed primarily in continental Asia with the wide distribution of *A. galanga* most likely due to its important culinary use by local peoples.

Etlingera. The often large involucre of sterile inflorescence bracts and the fusion of the corolla tube to the labellum and single stamen filament, forming a staminal tube beyond the insertion of the corolla lobes, are characteristic of this monophyletic genus. Species are spread throughout the wet lowland tropics of Southeast Asia. The genus *Etlingera* is closely related and apparently paraphyletic with *Hornstedtia*.

Geocharis. Although only one of the six species of *Geocharis* was sampled in our molecular analysis, this genus appears to be distinctive in the tribe being differentiated by the radical, sometimes lax, inflorescence and the stem venation marked by prominent white hairs between major veins. However, increased sampling of species of this genus and other taxa placed in the genus *Amomum* are needed to determine the monophyly of *Geocharis*. Species are found in the Philippines, Peninsular Malaysia, Sumatra, and Borneo.

Geostachys. As in *Geocharis*, only one of the 20-25 species of this genus was included in our analysis. The stilt roots, lax inflorescence, non-imbricate bracts, and two or more flowers per cincinnus are characteristic of species of *Geostachys* and suggest that the genus is monophyletic. Many endemic species are found in this genus distributed in peninsular Malaysia, Sumatra, northwestern Borneo, and Thailand to Cambodia.

Siliquamomum. With only a single species found in northern Vietnam and bordering regions of tropical China, this genus has unique cylindrical torulose fruits resembling siliques. The phylogenetic placement of *Siliquamomum* has been problematic and often unresolved within the Alpinioideae (see Kress *et al.*, 2002, 2005). Our current analysis allies it to the *Alpinia rafflesiana* clade although this placement may change in future analysis. It is best maintained as a distinctive monotypic genus in the subfamily.

(2) Well-supported lineages in need of new (or previously used) taxonomic names.

Alpinia fax clade. The three species placed in this clade (only two sampled in the molecular study; the third species, *A. rufescens* (Thw.) K. Schum., is only known from the type specimen) form a well-supported monophyletic group characterized by a radical capitate inflorescence often borne on a long leafless peduncle with conspicuous sterile bracts (Sabu, 2006). This clade is distributed in Sri Lanka and southern India and is sister to the clade containing the genera *Renealmia* and *Aframomum* (see above). Together with these two genera the *A. fax* clade constitutes a strongly supported monophyletic lineage stretching from tropical America through Africa to south Asia, which may represent both vicariant and long-distance dispersal events. All three species of this clade were originally described in the genus *Elettaria*, which has not been included in the present analysis. A new generic name is required for this clade.

Alpinia eubractea clade. This clade is made up of species found primarily in the Pacific Ocean, including the Philippines, Oceania, and Australia, and includes taxa earlier described in three genera (*Alpinia*, *Leptosolena*, and *Vanoverberghia*). The *A. eubractea* clade is strongly supported (bootstrap = 100%) in the molecular analysis, but the morphological apomorphies of this group of species are not immediately obvious. This clade will require a new generic name after additional taxa are added to the phylogenetic analysis, especially species of *Alpinia* from New Guinea.

Amomum villosum clade. Although it may be premature to recognize segregate genera for species that were earlier described in the genus *Amomum*, the results of our analyses as well as earlier investigations by Xia *et al.* (2004) support at least three distinct lineages of taxa formerly placed in *Amomum*. The *A. villosum* clade is characterized by echinate fruits and a trilobed anther appendage. Species in this clade are distributed primarily in Indochina, peninsular Malaysia, and Borneo. Additional species samples throughout this distribution will provide a more complete picture of the taxonomic breadth of this clade.

Alpinia carolinensis clade. Species in this clade tend to be plants large in stature, with a caducous primary inflorescence bract, and a narrow fleshy labellum adpressed to the stamen. Members of the *A. carolinensis* clade are concentrated in Sulawesi and generally east of Wallace's Line in the Pacific Ocean. Additional species from Sulawesi and New Guinea should be added to the molecular analysis in anticipation of applying a new generic name to this clade.

Alpinia zerumbet clade. The great bulk of species named in the genus *Alpinia* are found in this well-supported clade with a broad geographic distribution in tropical Asia. The absence of a primary inflorescence bract and the presence of short one-three flowered cincinni characterize most of the species included in the *A. zerumbet* clade. At least four subclades with strong (= 100%) jackknife support are apparent within the *A. zerumbet* clade, including the *A. aquatica* subclade, the *A. nutans* subclade, the *A. calcarata* subclade, and the *Plagiostachys* subclade. It may be appropriate to recognize each of these well-supported subclades at the subgeneric level. However, if subgenera are to be established, at this time we advocate for the purpose of simplicity the recognition of only two subgenera corresponding to the *A. zerumbet* subclade and the *Plagiostachys* subclade as circumscribed in Fig. 3.

Amomum tsao-ko clade. The species included in this clade are primarily Chinese in distribution. They are characterized by leaves with pleasant and distinctive aromatic oils, a bi- or tri-lobed anther appendage, and smooth fruits. In the molecular analysis this clade of *Amomum* is only moderated supported as sister to *Geostachys*, which is morphologically distrinct. As more species of *Amomum* are added to the overall analsyses, additional species may be included in the *A. tsao-ko* clade.

Alpinia rafflesiana clade. Only two of the over 200 species in tribe Alpinieae that we sampled are contained in this well-supported clade found in peninsular Malaysia and southern Thailand. The two species are characterized by a broadly spread labellum and/or drooping inflorescence. It is possible that after additional sampling other species will be included in this clade, such as *A. capitellata* Jack from Borneo, but the molecular distinctiveness of the lineage suggests that a separate generic name is warranted. Our analysis has placed the *A. rafflesiana* clade sister to the unique monotypic *Siliquamomum*, which should be maintained as a separate genus (see above).

(3) Problematic lineages in need of additional data prior to making final taxonomic decisions.

Amomum maximum clade. Species possessing an entire anther appendage, orange and yellow labellum, and winged fruits are contained within this clade, which was also recognized by Xia *et al*. (2004) in their molecular analysis of *Amomum*. Most of the taxa are distributed in China, India, and Australia. In our current sample of taxa the molecular data only moderately support the monophyly of the *A. maximum* clade. However, jackknife support is strong (100%) for the node joining the *A. maximum* clade with the *Elettariopsis* clade (see below). Although we have not yet obtained sequence data for *A. subulatum* Roxb., the type of the genus *Amomum* with winged fruits, we expect this species to be included in one of these clades and not in the *A. tsao-ko* clade or *A. villosum* clade. More sampling of species of *Amomum* with winged fruits, including the type species, is needed before a final decision can be made on taxonomic alignments.

Elettariopsis clade. This clade contains species of *Elettariopsis*, *Amomum,* and the monotypic *Paramomum* and, similar to the *A. maximum* clade, it is only moderately supported by our molecular data (jk = 89%). *Elettariopsis* has long been recognized as a genus because of the distinctive two-to-three-leaved shoots with characteristic aromatic oils, an elongated underground inflorescence with subterranean fruits, and a trilobed anther crest. Some of these features are also shared with the species of *Amomum* and *Paramomum* contained in the same clade. If sampling additional taxa does not provide better support for this clade, it may be justified to recognize the *Elettariopsis* clade together with the *A. maximum* clade as a single genus.

Hornstedtia grade. The characteristic stilt roots and elongated corolla tubes are found in most species of the widespread tropical Asian genus *Hornstedtia*. Although the close relationship between this genus and *Etlingera* has been recognized for over a half century (Holttum, 1950; Smith, 1985), the monophyly of *Hornstedtia* had not been challenged until recently. Pedersen (2004) in her analysis of *Etlingera* demonstrated that the species of *Hornstedtia* that she sampled formed a paraphyletic group. Our analyses with broader sampling confirm her result. At this point we suggest that *Hornstedtia* and *Etlingera* remain as separate genera until further analyses clarify the issue.

Several interesting genera in the Alpinieae were not sampled in our molecular analyses because of the unavailability of sufficient tissue samples or problems encountered in generating DNA sequences. *Cyphostigma*, a monotypic genus endemic to Sri Lanka and distinctive in its lax inflorescence, resupinate flowers, and large petaloid anther crest, may be related to *Elettariopsis* or the *Amomum maximum* clade because of similarities in inflorescence and floral features (Holttum, 1950). *Elettaria*, which has a broad distribution in India, peninsular Malaysia, Java, and Borneo and contains the economically important spice cardamon (*E. cardamomum* (L.) Maton), was also not sampled in our analysis. Its position in the Alpinieae is as yet unclear.

Conclusions

As DNA sequence data for additional taxa have been added to the phylogenetic analysis of the Alpinioideae, a more detailed and refined understanding of evolutionary relationships and generic boundaries in the subfamily has been obtained (Figs. 1-4). As outlined above, our investigations are centered on three goals, two of which are addressed in this paper. We will address the third goal (to provide new circumscriptions of genera and apply new names where necessary) in a future publication that will include a more complete sampling of critical taxa necessary to make final decisions on meaningful taxonomic units in the Alpinioideae and especially the tribe Alpinieae.

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