RESEARCH ARTICLE

Fruit morphology helps identifying evolutionary groups in Alpinieae (Zingiberaceae): inferences from phylogenetic analysis of gingers in Sri Lanka

P Karunarathne^{1,2,3*}, D Yakandawala² and P Samaraweera^{2,3}

¹ Department of Life Sciences, Texas A&M University, Corpus Christi, Corpus Christi, Texas, USA.

² Department of Botany, Faculty of Science, University of Peradeniya, Peradeniya.

³ Postgraduate Institute of Science, University of Peradeniya, Peradeniya.

Submitted: 10 Mar 2020; Revised: 01 September 2020; Accepted: 26 March 2021

Abstract: To assess the systematics and the phylogenetic placement of the members of the two important genera *Alpinia* Roxb. and *Amomum* Roxb. in Sri Lanka, molecular data of twelve in-group species together with three out-group taxa of the family Zingiberaceae were extensively analysed for phylogenetic significance. The current analysis of the evolutionary relationships of the Sri Lankan members of the genera of interest, utilising DNA sequence data of the chloroplast genome regions *trn* L-F and *trn* S-fm, has resolved four groups of *Alpinia* and two major clades of *Amomum* with substantial parsimony analysis and Bayesian inferences consistency values. With new accessions from the entire native range of the family, this result points to the need for an inevitable re-circumscription of the genus *Alpinia*, and shows congruence to the recent reshuffling of the genus *Amomum* and the family Zingiberaceae, except for the placement of each genus as monophyletic groups in the context of our study. Here, we suggest swapping the group defining species of an *Alpinia* clade observed in previous studies. Finally, our study suggests the use of fruit morphology to distinguish among recognised groups for Sri Lankan species as they exhibit positive correspondence.

Keywords: *Alpinia*, *Amomum*, phylogeny, Sri Lanka, Zingiberaceae.

INTRODUCTION

The two largest genera *Alpinia* and *Amomum* of the family Zingiberaceae, not only show a wide geographical

distribution (Kiew, 1982; Smith, 1990; Larsen *et al.*, 1998; Wu & Larsen, 2000; Sirirugsa, 2001) but also possess an array of homoplasious and plesiomorphic characters that make the classification of the genera problematic (Kress *et al.*, 2002; Lamxay & Newman, 2012). Recent phylogenetic analyses of *Alpinia* and *Amomum* revealed the polyphyletic nature of the genera (Rangsiruji *et al.*, 2000a; Xia *et al.*, 2004; Kress *et al.* 2005; Boer *et al.*, 2018) rejecting the previous classification which was based solely on morphological characters (Schumann, 1904; Smith, 1990). Boer *et al.* (2018) presented a more thorough phylogenetic analysis of Alpinieae, concluding the presence of 10 *Amomum* clades and six *Alpinia* clades. Further, they proceeded with re-circumscription of the *Amomum* clade by resurrecting three genera (*Conamomum*, *Meistera*, and *Wurfbania*) and describing three new genera (*Epiamomum*, *Lanxangia*, and *Sundamomum*). However, the current phylogenetic and evolutionary analyses of the two genera are far from being completed as they lack many representative species (e.g. species from South and Southeast Asia). Furthermore, only a few of the Sri Lankan species have been used in the aforementioned studies [*Amomum pterocarpum*, *Meistera echinocarpa* (formerly *Amomum echinocarpum*), *Alpinia abundiflora* and *Alpinia fax*] despite the fact that there are several taxonomically paramount species occurring in Sri Lanka (Burt & Smith, 1983). In this paper, phylogenetic placement of

* Corresponding author (piyal.karunarathne@tamucc.edu; <https://orcid.org/0000-0002-1934-145X>)

This article is published under the Creative Commons CC-BY-ND License (<http://creativecommons.org/licenses/by-nd/4.0/>). This license permits use, distribution and reproduction, commercial and non-commercial, provided that the original work is properly cited and is not changed in anyway.

the Sri Lankan members of the two genera of interest is comparatively and comprehensively discussed, using the inferences drawn from the DNA sequence analysis.

With the recognition of the polyphyletic origin of the previously recognised tribes of the family Zingiberaceae (Sakai & Nagamasu, 1998; Rangsiruji *et al.*, 2000a; Kress *et al.*, 2002; Harris *et al.*, 2003; Kress & Specht, 2005), several generic-level phylogenetic studies addressed the evolutionary relationships of the genera *Alpinia* (Rangsiruji *et al.*, 2000b; Kress *et al.*, 2005) and *Amomum* (Kaewsri, 2006; Kaewsri *et al.*, 2007; Droop, 2012) as well as the tribe Alpinieae (Kaewsri *et al.*, 2007; Kress *et al.*, 2007; Boer *et al.*, 2018) nesting two genera in comparative assessments. A common conclusion of these studies was that the members of the tribe do not follow the previous classification system and instead they tend to show different inter- and intra-generic evolutionary relationships indicating the polyphyletic nature of *Alpinia* and *Amomum*.

Nuclear and plastid DNA sequence analysis of *Alpinia* indicated that taxa described under this genus are closer to other genera of Alpinieae than they are to each other making classification of the tribe difficult (Rangsiruji *et al.*, 2000a; Kress *et al.*, 2005) due to its polyphyletic origin. Rangsiruji *et al.* (2000a) identified four separate clades of *Alpinia* whereas Kress *et al.* (2005) and Boer *et al.* (2018) described the separation of the genus into six groups. However, none of these studies agreed with the comprehensive infra-generic classification of *Alpinia* by Smith (1990), where she identified two subgenera within the genus encompassing 221 species. Similarly, during an analysis to assess the species boundaries of *Aframomum*, Harris *et al.* (2000) suggested that *Amomum* might be paraphyletic. Later studies by Xia *et al.* (2004) demonstrated the segregation of the genus *Amomum* into three major clades and they used fruit morphology to explain the clades observed. In another study by Kaewsri *et al.* (2007) using amplified fragment length polymorphism (AFLP) markers to analyse the phylogenetic relationships of Thai *Amomum* showed the positive correspondence of fruit morphology to the clades observed. Droop (2012) explained the presence of at least seven groups of *Amomum*, inferred by the results of the phylogenetic analysis using Internal transcribed spacer (ITS) and *matK* data, whereas Boer *et al.* (2018) found 10 clades of *Amomum* using the same gene regions and they used both anther crest and fruit morphology in combination to discriminate the clades.

Although *Alpinia* and *Amomum* add the highest number of endemic species to the ginger family in Sri Lanka (Burt & Smith, 1983), taxonomy and phylogenetic relationships of the members of these two genera has been poorly studied. The last taxonomic revision of the family Zingiberaceae in Sri Lanka was in 1983 (Burt & Smith, 1983). Since then, several major revisions have taken place in the world utilising new phylogenetic tools, which resulted in major changes in the classification of the family (Rangsiruji *et al.*, 2000a; Xia *et al.*, 2004; Kress *et al.*, 2005; de Boer *et al.*, 2018). More importantly, none of the Sri Lankan endemic species had been used in any of the previous phylogenetic analyses. This observation justifies the need for a thorough phylogenetic account of Sri Lankan members of the family Zingiberaceae. As a pioneering step, the present study employed molecular data to determine evolutionary relationships among the members of the two genera *Alpinia* and *Amomum* in Sri Lanka and to compare the results with the current major revisions.

The goals of this study are: to construct phylogenetic hypothesis of interspecific and intergeneric relationships of the two genera primarily focusing on Sri Lankan species and compare the results with currently existing classifications; and to assess the congruence of several morphological characters with the molecular analysis results.

METHODOLOGY

Sampling

Ingroup taxa

Representative samples from 12 species of both genera were collected for the study (Figure 1 and 2). These included two native *Alpinia* species, four species of non-native *Alpinia*, four native *Amomum* species, one endemic *Amomum* species, and a new record of *Amomum* from Sri Lanka [*Wurfbainia villosa* var. *zeylanicus* (formerly *Amomum villosum zeylanicus*)] - Karunarathne *et al.* (2014). Unfortunately, four species of endemic *Amomum* (*A. nemorale*, *A. graminifolium*, *A. benthamianum*, and *A. trichostachyum*), one species of endemic *Alpinia* (*A. rufescens*) and one native *Amomum* species (*A. hypoleucum*) were not found during comprehensive field sampling. Most of these species have not been collected for more than 100 years and almost all types of localities are under intense human activities. Therefore, we present

Table 1: List of species of Sri Lankan *Alpinia* Roxb. and *Amomum* Roxb. showing the latest National Red List (2012) status of the native and endemic species and the GenBank accession numbers for the two sequences *trnL*-F and *trnS*-trnM of each studied species.

Species	Suggested infra-generic clade (Xia <i>et al.</i> , 2004; Kress <i>et al.</i> , 2005)	National Red List status (MOE, 2012)	Last collection	Collected in this study	GenBank accession numbers	
					<i>trnL</i> - <i>trnF</i>	<i>trnS</i> - <i>trnM</i>
<i>Alpinia abundiflora</i>	<i>A. fax</i> clade	LC	2008	Yes	KF748154	KF748169
<i>Alpinia fax</i>	<i>A. fax</i> clade	VU	1996	Yes	KF748153	KF748168
<i>Alpinia rufescens</i> ‡	<i>A. fax</i> clade	CR(PE)	1862	No		
<i>Amomum pterocarpum</i>	-	EN	1987	Yes	KF748162	KF748177
<i>Amomum masticatorium</i>	-	EN	1988	Yes	KF748160	KF748175
<i>Amomum fulviceps</i>	-	VU	1973	Yes	KF748159	KF748174
<i>Amomum hypoleucum</i>	-	CR(PE)	1861	No		
<i>Meistera echinocarpa</i>	-	VU	1989	Yes	KF748161	KF748176
<i>Amomum graminifolium</i> ‡	-	EN	1994	No		
<i>Amomum acuminatum</i> ‡	-	CR(PE)	1855	Yes	KF748157	KF748172
<i>Amomum trichostachyum</i> ‡	-	EN	1973	No		
<i>Amomum nemorale</i> ‡	-	CR(PE)	1861	No		
<i>Amomum benthamianum</i> ‡	-	CR(PE)	1864	No		
<i>Wurfbainia villosa zeylanicus</i> ‡	-	-	-		KF748158	KF748173
Non-native species						
<i>Alpinia galanga</i>	<i>A. galanga</i> clade	-	-	Yes	KF748152	KF748167
<i>Alpinia calcarata</i>	<i>A. zerumbet</i> clade	-	-	Yes	KF748151	KF748166
<i>Alpinia malaccensis</i>	<i>A. zerumbet</i> clade	-	-	Yes	KF748155	KF748170
<i>Alpinia zerumbet</i>	<i>A. zerumbet</i> clade	-	-	Yes	KF748156	KF748171
Out-group species						
<i>Zingiber cylindricum</i>	-	-	-	Yes	KF748148	KF748163
<i>Zingiber</i> sp.	-	-	-	Yes	KF748149	KF748164
<i>Hedychium coronarium</i>	-	-	-	Yes	KF748150	KF748165

LC - least concerned; VU - vulnerable; EN - endangered; CR (PE) - critically endangered (possibly extinct)

‡: endemic species

an adequate sampling of extant taxa of the two genera in Sri Lanka (Table 1).

Outgroup taxa

Two representative species of *Zingiber* (*Z. cylindricum* and *Zingiber* sp.) and one species of *Hedychium* (*H. coronarium*) were collected during the field sampling for out-group taxa. For a better comparison of the overall phylogeny and the geographical distribution of the phylogenetic clusters, DNA sequences of *trnL*-*trnF* intergenic spacer region of four *Aframomum*,

three *Renealmia*, and one *Elettariopsis* accessions were also downloaded, following a *nucleotide blast* from GeneBank for outgroup taxa (supplementary Table 1). These three genera are sister groups to the studied two genera in the present study (Kress & Specht, 2005; Kress *et al.*, 2007).

DNA extraction and sequencing

Fresh leaf samples dried with silica beads were used for DNA extractions. Genome DNA was extracted using a modified Doyle and Doyle (1987) CTAB protocol with

few additional purification steps: suspended DNA in TE was treated with ProteinaseK and RNase followed by phenol chloroform extraction and sodium acetate precipitation of DNA. PCR amplification was done for two chloroplast gene regions *trn* L-F and *trn* S-fM, using *trn* L-F (Taberlet *et al.*, 1991) and *trn* S-fM (Shaw *et al.*, 2005) primer pairs, respectively. The chloroplast genome regions *matK*, and *trn* L-F together with nuclear ribosomal ITS regions have been explicitly used in evolutionary studies of the family Zingiberaceae (Johnson & Soltis, 1994; Baldwin *et al.*, 1995; Kress *et al.*, 2002). However, only the chloroplast intergenic spacer *trn* L-F and the non-coding region *trn* S-fM were used because in the initial steps, the primers that were used for ITS and *matK* regions failed to amplify the desired region for several species (*A. echinocarpum*, *A. pterocarpum*, and *A. fax*) and two species produced multiple bands for ITS region (*A. acuminatum* and *A. fulviceps*). The region *trn* S-fM has been documented to be informative in phylogenetic studies (Shaw *et al.*, 2005; Minami *et al.*, 2009).

According to the manufacturers' recommendations all amplifications used Promega GoTaq® Flexi DNA polymerase (Madison, WI, USA). Eppendorf Mastercycler® thermal cycler (Hauppauge, NY, USA) with the following programs was used for PCR amplifications.

trn L-F region: preheat at 80 °C for 3 min, 2 cycles with 3 temperature segments (94 °C for 2 min, 50 °C for 40 s and 72 °C for 2 min), 30 cycles with 3 temperature segments (94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min) and final extension 72 °C for 8 min.

trn S-fM region: preheat at 94 °C for 3 min, 30 cycles with 3 temperature segments (94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min) final extension 72 °C 8 min.

PCR product purification and DNA sequencing were done by Macrogen Inc. Korea (Sanger sequencing: 96-capillary 3730XL DNA Analyzer: Applied Biosystems®). Same primer pairs as in PCR amplifications were used in sequencing for the respective gene region. Raw sequences were screened and assembled in SeqMan Pro, DNASTar® Lasergene 8.1 (DNASTAR, Inc., 2009). Assembled contigs were edited using BioEdit (ver. 7.1.9) (Tom Hall, Ibis Biosciences, California, USA). Edited sequences were then aligned using ClustalX 2.1 (Larkin *et al.*, 2007). Alignment files were further edited in BioEdit to process unaligned bases. This was repeated for all sequences of the studied two DNAs separately. A combined analysis of the two regions was also performed

to obtain better resolved trees and thereby to estimate phylogeny with the strongly supported results (Nixon & Carpenter, 1996).

Furthermore, to conduct a more inclusive sampling of the two genera, sequences of both *Alpinia* and *Amomum* were downloaded from GeneBank to analyse with the accessions from the present study. DNA sequences of *trn*L-*trn*F intergenic spacer region of six *Alpinia* and seven *Amomum* accessions were downloaded following a *nucleotide blast* from GeneBank (Supplementary Table 1).

Phylogenetic analysis

Maximum Parsimony Analysis (MPA)

MPA of the aligned sequence data was accomplished using PAUP* 4.0 (Swofford, 2003) on a power Macintosh G4. Heuristic search strategies were used in 1000 Tree Bisection Reconnection (TBR) branch swapping replicates of random taxon addition with MULPARS in effect. The majority-rule strict consensus tree assembly was used to build the most parsimonious trees. All positions were weighted equally; gaps were treated as missing values. The Consistency Index (CI) and Retention Index (RI) values for tree topologies were also calculated with PAUP. Bootstrap analyses (Felsenstein, 1985) were conducted using PAUP with a simple addition of 1000 fast-swap replicates to 100 tree topologies with a 50 % confidence.

Bayesian Inferences

Bayesian analysis was used to draw model based phylogenetic inferences (Yang & Rannala, 1997; Larget & Simon, 1999) from the DNA sequence data. MrBayes (Huelsenbeck & Ronquist, 2001) was used for the analysis to obtain the posterior probabilities (PP). Models for the sequence evolution was determined using *jModeltest* (Posada, 2008) and the following parameters were used in the analysis; GTR, nst = 6, rates = inverse gamma, statefreqpr = fixed (equal). The analysis comprised generations of 10,000,000 Markov Chain Monte Carlo (MCMC) chains with a sampling frequency of every 10,000 generations. The initial 25 % samples from each run were discarded as burn-in, after which the run was summarised as a majority rule consensus tree and the posterior probability values were saved in MrBayes. Separate analysis of the two regions and the combined analysis produced a similar tree topology for the strict consensus. Therefore, only the combined tree is presented here.

RESULTS AND DISCUSSION

Analysis of *trn* L-F intergenic spacer data

A total of 449 parsimony-informative characters were present in the *trn* L-F region, which produced 5473 equally parsimonious trees in more than 270,000 rearrangement steps in parsimony analysis. Strict consensus of these trees (Supplementary Figure 1) placed all the in-group taxa in a monophyletic clade (bootstrap 91 %) separating out-group taxa. *Alpinia* and *Amomum* were placed in two separate clades (*A* and *B* in Supplementary Figure 1) with moderate and strong bootstrap values: 65 and 100, respectively. Hence, genera *Alpinia* and *Amomum* in Sri Lanka are strongly supported as monophyletic. Bayesian inferences also supported this observation with a high PP value of one.

Figure 1: Floral pictures of some *Alpinia* species found in Sri Lanka; (A) *A. abundiflora*, (B) *A. fax*, (C) *A. galanga*, (D) *A. calcarata*, (E) *A. maleccensis*, (f) *A. zerumbet*

Two major clades were observed in the *Amomum* group: *Am I*, *A. pterocarpum* (bootstrap 100 %) and *Am II*, *A. echinocarpum*, *A. villosum zeylanicus*, *A. masticatorium*, *A. fulviceps* and *A. acuminatum* (bootstrap 93 %). Separation of these two clades were also observed in the Bayesian analysis with PP values of 1.00 and 0.98, respectively. Four clades were identified in the *Alpinia*

group: *Alp I*, *Alp II*, *Alp III*, and *Alp IV*. *A. abundiflora* and *A. fax* formed a separate group (*Alp I*) basal to the rest of the *Alpinia* with moderate bootstrap values (69 %) and with high Bayesian support (PP=0.98). The other three clades (*Alp II*- *A. galanga*; *Alp III*- *A. calcarata*; *Alp IV*- *A. malaccensis* and *A. zerumbet*), possess moderate support values.

Analysis of *trn* S-fM intergenic spacer data

This region resulted in 3673 equally parsimonious trees in more than 670,000 rearrangement steps in the MPA. This region harboured 140 parsimony-informative characters (Supplementary Figure 2). Strict consensus of these trees also produced two major groups of in-group species with moderate bootstrap values and high PP values: A) *Alpinia* clade (bootstrap = 69 %, PP = 1); B) *Amomum* clade (bootstrap = 50 %, PP = 1) (Supplementary Figure 2). However, all in-group species formed a single monophyletic clade providing higher support values (87 %, PP = 1). Same three clades that were observed with *trn* L-F for the *Amomum* group was also observed with this analysis but with different bootstrap values. *A. fax* and *A. abundiflora* formed the basal clade (*Alp I*) of the *Alpinia* group with strong support values (bootstrap 80 %, PP = 1). Despite the low PP value (0.58), the rest of the *Alpinia* further split into three distinctive groups separating *A. galanga* (*Alp II*); *A. calcarata* (*Alp III*); *A. zerumbet* and *A. malaccensis* (*Alp IV*).

Figure 2: Floral pictures of few Sri Lankan *Amomum* species; (A) *A. masticatorium*, (B) *A. echinocarpum*, (C) *A. pterocarpum*, (D) *A. fulviceps*

Figure 3: Best trees resulting from the parsimony analysis of the GeneBank accessions and the data from the present study of *trn L-F* intergenic spacer region (length = 510; consistency index = 0.901; retention index = 0.966; rescaled consistency index = 0.900); branch labels: above - bootstrap values, Al I, II, IV – *Alpinia* groups that corresponds to Kress *et al.* (2005), *A. villosum* group – *Amomum* group that corresponds to Xia *et al.* (2004) and *Wurfbainia* group in Boer *et al.* (2018).

Analysis of the combined dataset

Combined analysis resolved Sri Lankan *Alpinia* into three major clades (i.e. *Al I*, *Al II*, *Al IV*), and *Amomum* into one group (i.e. *A. villosum* group *sensu* Xia *et al.*, 2004 and *Wurfbainia* group *sensu* Boer *et al.*, 2018) (Figure 3, also see Supplementary Figure 3). *A. galanga* grouped with GeneBank accessions in a separate group while all the other *Alpinia* were resolved in sister clades. Interestingly, *A. fax* and *A. abundiflora* resolved into one clade in the latter while the rest of the species were separated in individual branches with strong statistical support (Figure 3). Within the *Amomum* clade, we see the same inner groups as were resolved in separate analyses. Nevertheless, all the clades were strongly supported by bootstrap values except for two branches within the *Amomum* clade.

Overall, *Alpinia* and *Amomum* in Sri Lanka form a monophyletic group with strong phylogenetic support values (bootstrap 100 %, PP = 1). All the *Amomum* species formed a monophyletic clade (bootstrap 100 %, PP = 1) where they were further divided into two monophyletic groups separating basal clade *Am I*: *A. pterocarpum* (bootstrap: 100 %, PP = 1) and the second clade *Am II* nesting all the other species (bootstrap 100, PP = 0.98) (Supplementary Figure 3). *A. fax* and *A. abundiflora* were placed separately (*Al I*) from other *Alpinia*s with high support values (bootstrap 97 %, PP = 1). Although the other three *Alpinia* accessions nested together with all the other Sri Lankan species, *A. galanga* clustered with other accessions of the species from the GeneBank (*Al II*). *Al IV* clade nested non-native - *A. zerumbet* and *A. malaccensis* (bootstrap 100, PP = 1).

Bayesian analysis of the *trn* L-F and *trn* S-fm sequence data resulted in cladograms similar to that of the MPA. All the clades observed in the maximum parsimony analysis were supported with higher posterior probability (PP) values in the Bayesian analysis. The two genera are monophyletic with strong PP value of 1.00. The *Alpinia* group formed two clades in the analysis separating *A. abundiflora* and *A. fax* from the rest with a PP value of 0.98. Overall, the Bayesian inferences of the phylogenetic trees is congruence with the results of MPA.

The complexity of the classification of the two polyphyletic genera *Alpinia* and *Amomum* has been acknowledged by many authors (Smith 1990;

Larsen *et al.*, 1998; Kress *et al.*, 2002). As a result, many researchers who were interested in the family Zingiberaceae have dealt with taxonomy, phylogeny and the classification of these two genera. Remarkably, the phylogenetic relationships of the members of the family in Sri Lanka have never been assessed and none of the endemic species have been assessed in any of the recent phylogenetic studies of the family (e.g. Kress *et al.*, 2002, Xia *et al.*, 2005, Boer *et al.*, 2018, Droop 2019). Hence, the current study attempted phylogenetic analysis of the Sri Lankan members of the two genera *Alpinia* and *Amomum*. Results of the present analysis provides better insight to the evolutionary relationships of the studied species in Sri Lanka.

According to the latest phylogenetic analysis of *Amomum* group (de Boer *et al.*, 2018), Sri Lankan taxa of the *Amomum* group consist of three genera (i.e. *Amomum*, *Wurfbainia* and *Meistera*). However, the present study has not resulted in similar groups, and was rather congruent with the previous studies of the family Zingiberaceae (Rangsiruji *et al.*, 2000a; Kress *et al.*, 2002) by resolving only two clades. This may be due to the limited sampling in our study representing all the species of *Amomum* group. Although the present analysis does not include accessions representing all the genera of the family, our study highlighted two *Amomum* clades with strong statistical support (Figure 3). Therefore, in congruence with infra-generic relationships that were illuminated in recent studies (Kress *et al.*, 2007; Droop, 2012), it can be concluded that Sri Lankan members of the two genera of interest make two evolutionary groups. Reduced staminodes, capsule: globose, echinate or irregular shaped, and relatively short stamen can be listed as a combination of features that characterise the separation of the clade.

Alpinia

Although all the *Alpinia* species studied in this analysis formed a monophyletic group, the separation of the clades within the genus (i.e. *Alp I*, *Alp II*, *Alp III* and *Alp IV*) agrees with the broader studies of the family (Rangsiruji *et al.*, 2000a; Kress *et al.*, 2002). These groups are in congruence with the recent phylogenetic studies on the family (Rangsiruji *et al.*, 2000a; Kress *et al.*, 2002, 2005, 2007) and also confirm the incongruence with earlier morphological treatments (Schumann, 1904; Smith, 1990).

A. abundiflora and *A. fax* were placed as the basal group (*Alp I*), separately from all the other members of the genus with strong support values in both MPA and Bayesian analyses of the molecular data. Recognition of this clade was also described in phylogenetic analyses of *Alpinia* by Kress *et al.* (2005) and Rangsiruji *et al.* (2000b). As the latter study explained, these species (namely *A. fax* clade) are characterised by the presence of capitate, usually radical inflorescence surrounded by a world of sterile bracts; fertile bracts subtending a cincinnus of several flowers. Furthermore, this observation reinforces the suggestion by Kress *et al.* (2007) that these species require a new generic name. Kress *et al.* (2005) also suggested that the inclusion of *A. rufescens* in this clade is appropriate since this species also shares this distinctive morphological character combination. *A. rufescens* has been recorded only in Sri Lanka. It is only known from the type locality and has never been recollected since its type gathering (1862). During our extensive field sampling efforts (comprised of more than 25 locations) it was observed that the type locality of *A. rufescens* is no longer a potential area to support these species since the area is under intense human activities. This species was not found from any other location either. After close and careful observation of the characters of this clade, it could be suggested that *A. abundiflora* be used as the clade defining species instead of *A. fax* because *A. abundiflora* possesses typical characters such as capitate inflorescence, occurrence of the inflorescence either (rarely) on the vegetative stem or on a separate basal peduncle and typically a cincinnus of seven (7) flowers. These are also shared with *A. rufescens* (except for the number of flowers in a cincinnus). On the other hand, the inflorescence of *A. fax* rather elongates with age and it bears a lesser number of flowers in a cincinnus (seldom up to six flowers). Further, Karunarathne *et al.* (2015) reported the confusion of morphological characters of *A. fax* that was also presumably recorded in South India (Kumar *et al.*, 2002).

Although the inclusion of *Alpinia galanga* with the rest of Sri Lankan *Alpinia* (except *Alp I*) showed weak support values (bootstrap 56 %, PP = 0.58: combined analysis), the separation of groups *Alp I*, through *Alp IV* was strongly supported. The clade *Alp I* separated *A. abundiflora* and *A. fax* from the rest with strong support (bootstrap 75 %, PP = 0.98). Next to the *A. abundiflora* clade, *Alp II* isolated *A. galanga* basal to the *Al* group. *A. galanga* clade is characterised by higher number of flowers in the branched inflorescence, smaller size of flowers, clawed labellum and the glabrous ovary. Inclusion of additional species such as *A. nigra* (not found in our field observations) might better explain

these characters. Although *A. calcarata* was included as a member of the *A. zerumbet* clade in previous studies, the present results placed it as a separate clade (*Alp III*). Linear-lanceolate shape of the lamina and the reduced lamina width along with the smaller sized fruits can be listed as deviating characters. *A. zerumbet* and *A. malaccensis* formed the terminal clade with strong bootstrap support. Besides the robustness of the habit of the plant, relatively large, globose fruits are characteristic to this clade.

Amomum

All the studied Sri Lankan *Amomum* species resolved into two major clades (*Am I* and *Am II*). This exhibits congruence with recent evolutionary analyses of the genus (Xia *et al.*, 2004; Kaewsri *et al.*, 2007; Droop, 2012; Boer *et al.*, 2018). Results of the current analysis also do not correspond to earlier infra-generic classification of the genus (e.g. Schumann 1904; Smith 1990).

A. pterocarpum was placed as the basal most lineage (clade *Am I*) of the *Amomum* group. The clade is characterised by the presence of ribbed capsule with wings, sub-globose inflorescence with quickly deciduous bract and bracteoles. *Am II* clade resembles the *A. maximum* clade which was observed in the study by Xia *et al.* (2004) and other studies (Kress *et al.*, 2007; Droop, 2012). The main characteristic feature of this group is the presence of smooth or winged fruit coat (Xia *et al.*, 2004). *Am II* placed all the other members of the genus (*A. echinocarpum*, *A. masticatorium*, *A. fulviceps* and *A. acuminatum*, *A. villosum zeylanicus*) in a single clade with strong branch support values. The most conspicuous morphological characters that shared among these species are slender basal peduncle covered with scaly sheaths, ovoid to elongated inflorescence, brown-red bracts and bracteoles, tri-lobed anther appendage, bifid labellum and echinate fruits. This is in congruence with the recent evolutionary analysis of the genus. Xia *et al.* (2004) identified this group as *A. villosum* clade with echinate fruits being a distinctive character and later studies confirmed this placement (Xia *et al.*, 2004; Kress *et al.*, 2007; Droop, 2012). The new record of species *Amomum villosum* var. *zeylanicus* shows the typical characters that Xia *et al.* (2004) highlighted for the group. In contrast to this, Boer *et al.* (2018) separated these species into two separate genera: i) *Meistera* – *M. echinocarpa*, *M. masticatorium*; ii) *Wurfbainia* – *W. villosa* (var. *zeylanicus*), respectively. They described echinate fruits and semilunar anther crest for *Meistera* and echinate fruits and eared anther crest for *Wurfbainia* as conspicuous characters of the two genera. However,

Figure 4: Tree depicting the fruit morphology attribution for the characterisation of the *Alpinia* and *Amomum* clades observed in Sri Lanka, obtained from the molecular data analysis; A. *Alpinia* clade, B. *Amomum* clade; *. *Am II* clade: *Amomum* group of species bearing echinate fruits (*A. echinocarpum*, *A. masticatorium*, *A. fulviceps*, *A. acuminatum*, and *A. villosum zeylanicus*.) (Drawings are according to their relative size)

this separation of polyphyletic clades was not observed in the present study.

Moreover, after comprehensive observation of the morphological characters of each clade resulted in the current phylogenetic analysis, besides the above-mentioned group specific characters, fruit morphology of the studied species can be effectively utilised to separate different clades of the Sri Lankan members of *Alpinia* and *Amomum*. Figure 4 shows a detailed attribution of the fruit morphology of the groups for this context. However, we admit that we are not the first to use the fruit morphology to separate phylogenetic clades in the family Zingiberaceae (Kress *et al.*, 2002; Xia *et al.*, 2005; Droop *et al.*, 2012; Boer *et al.*, 2018). Nevertheless, the use of the fruit morphology for Sri Lankan species presents an efficient strategy.

Sri Lankan gingers in a global phylogeny

The three *Alpinia* groups observed in our analysis are in close congruence with the most recent thorough analysis of the genus (Kress *et al.*, 2005). Most importantly the analysis shows that *Alpinias* are not monophyletic, contrary to our analysis only with Sri Lankan species (Figure 4). This is expected as several other evolutionary groups in the family included in the analysis, and as observed in the phylogenetic analysis of the family (Kress *et al.*, 2002; 2005; de Boer 2018). In contrast, the *Amomum* species in Sri Lanka formed a monophyletic group with strong bootstrap support (100 %). Furthermore, this resolution of one *Amomum* clade in Sri Lanka corresponds to the same *Amomum villosum* clade as in Xia *et al.* (2004) of the *Amomum* phylogeny. This suggests that all the Sri Lankan species of the genus

evolved from one common ancestor although the genus itself in the region is not monophyletic. However, none of the other species of *Amomum* in this group were included in the previous evolutionary analyses of the genus. Therefore, a comparison of the results of this study is not appropriate. Most importantly, even though the evolutionary groups within the two genera slightly changed with the global phylogeny, our use of the fruit morphology still applies to the evolutionary groups found in Sri Lanka (Figure 4). Moreover, this observation can further be extended to identify corresponding clades of the two genera in other regions as well, as they show high congruence to previous studies by Xia *et al.* (2004), Kress *et al.* (2005) and Boer *et al.* (2018).

Overall, the current analysis of the evolutionary relationships of Sri Lankan *Alpinia* and *Amomum* utilising DNA sequence data of the chloroplast genome regions *trn L-F* and *trn S-M* and the combined dataset resolved four groups of *Alpinia* and two major clades of *Amomum*. This result is in congruence and consistent with recent phylogenetic analyses of the two genera using molecular data to a certain extent, except for the placement of each genus as monophyletic groups in the context in the present analysis. This discrepancy in comparison to other recent studies seems to arise from the fact that none of the endemic Sri Lankan species were used in previous studies. Finally, the present study suggests using the fruit morphology to distinguish the observed groups for Sri Lankan species because they exhibit positive correspondence with the fruit morphology, which is also consistent.

Acknowledgement

This research project was supported by the National Science Foundation of Sri Lanka (Grant SIDA/2007/BT/02). The support and services provided by the Forest Department of Sri Lanka, Department of Wildlife Conservation Sri Lanka and the National Herbarium of Sri Lanka is also appreciated.

Conflict of interest

All the authors of this manuscript declare that no conflict of interest has occurred among authors pertaining to this study.

REFERENCES

Baldwin B.G., Sanderson M.J., Porter J.M., Wojciechowski M.F., Campbell C.S. & Donoghue M.J. (1995). The ITS region of nuclear ribosomal DNA: a valuable source of

- evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82**(2): 247–277.
DOI: <https://doi.org/10.2307/2399880>
- de Boer H. *et al.* (11 authors) (2018). Convergent morphology in Alpinieae (Zingiberaceae): recircumscribing *Amomum* as a monophyletic genus. *TAXON* **67**(1): 6–36.
DOI: <https://doi.org/10.12705/671.2>
- Burt B.L. & Smith R.M. (1983). Zingiberaceae. In: *Revised Handbook to the Flora of Ceylon* (eds. M.D. Dassanayake & E.R. Fossberg), pp. 488–532. Amerind Publication Co. Pvt. Ltd., Colombo.
- Droop A.J. (2012). Systematic and biogeographic studies in the genus *Amomum* Roxb. (Zingiberaceae) in Sumatra. *PhD thesis*, University of Aberdeen, UK.
- Felsenstein J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**(4): 783–791.
DOI: <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>
- Harris D.J., Newman M.F., Hollingsworth M.L., Möller M. & Clark A. (2003). The phylogenetic position of *Aulotandra* (Zingiberaceae). *Nordic Journal of Botany* **23**(6): 725–734.
DOI: <https://doi.org/10.1111/j.1756-1051.2003.tb00451.x>
- Harris D.J., Poulsen A.D., Fridtjof-Møller C., Preston J. & Cronk Q.C.B. (2000). Rapid radiation in *Aframomum* (Zingiberaceae): evidence from nuclear ribosomal DNA internal transcribed spacer (ITS) sequences. *Edinburgh Journal of Botany* **57**(03): 377–395.
DOI: <https://doi.org/10.1017/S0960428600000378>
- Huelsenbeck J.P. & Ronquist F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**(8): 754–755.
DOI: <https://doi.org/10.1093/bioinformatics/17.8.754>
- Johnson L.A. & Soltis D.E. (1994). matK DNA sequences and phylogenetic reconstruction in Saxifragaceae s. str. *Systematic Botany* **19**(1): 143–156.
DOI: <https://doi.org/10.2307/2419718>
- Kaewsri K. (2006). Systematic studies of the genus *Amomum* Roxb. (Zingiberaceae) in Thailand. *PhD thesis*, Kasetsart University, Bangkok, Thailand.
- Kaewsri W., Paisooksantivatana Y., Veasommai U., Eiadthong W. & Vajrodaya S. (2007). Phylogenetic analysis of Thai *Amomum* (Alpinioideae: Zingiberaceae) using AFLP markers. *Kasetsart Journal (Natural Science)* **41**: 213 – 226.
- Karunaratne P., Yakandawala D. & Samaraweera P. (2015). Important notes on the identity of *Alpinia fax* (Thwaites) B.L. Burt & R.M. Sm. *Taiwania* **60**(4): P204–207.
DOI: <https://doi.org/10.6165/tai.2015.60.204>
- Karunaratne P., Yakandawala D. & Samaraweera P. (2014). On the occurrence of a new variety of *Amomum villosum* (Family Zingiberaceae) in Central Hills of Sri Lanka. *Phytotaxa* **172**(2): 129.
DOI: <https://doi.org/10.11646/phytotaxa.172.2.9>
- Kiew K.Y. (1982). The genus *Elettariopsis* (Zingiberaceae) in Malaya. *Notes from the Royal Botanic Garden, Edinburgh* **40**(1): 139–152.
- Kress W.J., Liu A.-Z., Newman M. & Li Q.-J. (2005). The molecular phylogeny of *Alpinia* (Zingiberaceae): a

- complex and polyphyletic genus of gingers. *American Journal of Botany* **92**(1): 167–178.
DOI: <https://doi.org/10.3732/ajb.92.1.167>
- Kress W.J., Newman M.F., Poulsen A.D. & Specht C. (2007). An analysis of generic circumscriptions in tribe Alpinieae (Alpinioideae: Zingiberaceae). *The Gardens' Bulletin Singapore* **59**(1 & 2): 113–128.
- Kress W.J., Prince L.M. & Williams K.J. (2002). The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data. *American Journal of Botany* **89**(10): 1682–1696.
DOI: <https://doi.org/10.3732/ajb.89.10.1682>
- Kress W.J. & Specht C.D. (2005). Between cancer and capricorn: phylogeny, evolution and ecology of the primarily tropical Zingiberales. *Biologische Skrifter* **55**: 459–478.
- Kumar P., Sabu M. & Jayasree S. (2002). *Alpinia fax* Burt & Smith. A new record for South India. *Rheedea* **12**(2): 179–183.
- Lamxay V. & Newman M.F. (2012). A revision of *Amomum* (Zingiberaceae) in cambodia, laos and vietnam. *Edinburgh Journal of Botany* **69**(1): 99–206.
DOI: <https://doi.org/10.1017/S0960428611000436>.
- Larget B. & Simon D.L. (1999). Markov Chasin Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* **16**(6): 750–759.
DOI: <https://doi.org/10.1093/oxfordjournals.molbev.a026160>.
- Larkin M.A. *et al.* (13 authors) (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**(21): 2947–2948.
DOI: <https://doi.org/10.1093/bioinformatics/btm404>.
- Larsen K., Lock J.M., Maas H. & Maas P.J.M. (1998). Zingiberaceae. In: *Flowering Plants · Monocotyledons* (ed. K. Kubitzki), pp. 474–495. Springer Berlin Heidelberg, Berlin, Germany.
DOI: https://doi.org/10.1007/978-3-662-03531-3_49
- Minami M., Nishio K., Ajioka Y., Kyushima H., Shigeki K., Kinjo K., Yamada K., Nagai M., Satoh K. & Sakurai Y. (2009). Identification of Curcuma plants and curcumin content level by DNA polymorphisms in the trnS-trnFM intergenic spacer in chloroplast DNA. *Journal of Natural Medicines* **63**(1): 75–79.
- Ministry of Environment (MOE) (2012). *Threatened List. National Red List 2012 of Sri Lanka. Conservation Status of the Fauna and Flora*, pp. viii + 476. Ministry of Environment, Colombo, Sri Lanka.
- Nixon K. C. & Carpenter J. M. (1996). On simultaneous analysis. *Cladistics* **12**(3): 221–241.
DOI: <https://doi.org/10.1111/j.1096-0031.1996.tb00010.x>.
- Posada D. (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25**(7): 1253–1256.
DOI: <https://doi.org/10.1093/molbev/msn083>
- Rangsiruji A., Newman M. F. & Cronk Q. C. B. (2000a). A study of the infrageneric classification of *Alpinia* (Zingiberaceae) based on the ITS region of nuclear rDNA and the trnL-F spacer of chloroplast DNA. Monocots: systematics and evolution, pp 695–709. CSIRO, Collingwood, Australia.
- Rangsiruji A., Newman M. F. & Cronk Q.C.B. (2000b). Origin and relationships of *Alpinia galanga* (Zingiberaceae) based on molecular data. *Edinburgh Journal of Botany* **57**(1): 9–37.
DOI: <https://doi.org/10.1017/S0960428600000020>.
- Sakai S. & Nagamasu H. (1998). Systematic studies of Bornean Zingiberaceae I. *Amomum* in Lambir Hills, Sarawak. *Edinburgh Journal of Botany* **55**(01): 45.
DOI: <https://doi.org/10.1017/S0960428600004352>
- Schumann K. (1904). Zingiberaceae. In: *Das Pflanzenreich* (ed A. Engler.), pp. 1–458. Wilhelm Engelmann, Leipzig, Germany.
- Shaw J. *et al.* (10 authors) (2005). The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* **92**(1): 142–166.
DOI: <https://doi.org/10.3732/ajb.92.1.142>
- Siriruga P. (2001). *Zingiberaceae of Thailand*. Biodiversity Research and Training Program, Bangkok, Thailand, pp 63–77. Jirawat Express Co., Ltd, Bangkok, Thailand.
- Smith R.M. (1990). *Alpinia* (Zingiberaceae): a proposed new infrageneric classification. *Edinburgh Journal of Botany* **47**(1): 1–75.
DOI: <https://doi.org/10.1017/S0960428600003140>
- Swofford D.L. (2003). {PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4.}.
- Taberlet P., Gielly L., Pautou G. & Bouvet J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**(5): 1105–1109.
DOI: <https://doi.org/10.1007/BF00037152>
- Wu T.-L. & Larsen K. (2000). Zingiberaceae. In: *Flora of China* (eds Z.-Y. Wu & P.H. Raven), pp. 322–377. Sci. Press, Beijing, China.
- Xia Y.-M., Kress W.J. & Prince L.M. (2004). Phylogenetic analyses of *Amomum* (Alpinioideae: Zingiberaceae) Using ITS and matK DNA Sequence Data. *Systematic Botany* **29**(2): 334–344.
DOI: <https://doi.org/10.1600/036364404774195520>
- Yang Z. & Rannala B. (1997). Bayesian phylogenetic inference using DNA sequences: a Markov Chain Monte Carlo method. *Molecular Biology and Evolution* **14**(7): 717–724.
DOI: <https://doi.org/10.1093/oxfordjournals.molbev.a025811>

Supplementary data

Supplementary Table 1: GeneBank accessions used in the global phylogeny analysis of gingers in comparison with Sri Lankan species.

Species	Accession no.	Author(s)
<i>Amomum stenosiphon</i>	MH603415.1	Givnish <i>et al.</i> , 2018
<i>Elettariopsis triloba</i>	AY769794.1	Harris <i>et al.</i> , 2005
<i>Amomum longipetiolatum</i>	AY769788.1	Harris <i>et al.</i> , 2005
<i>Alpinia</i> aff. <i>calycodes</i>	AY769797.1	Harris <i>et al.</i> , 2005
<i>Amomum petaloideum</i>	AY769789.1	Harris <i>et al.</i> , 2005
<i>Alpinia</i> cf. <i>aenea</i>	AY769796.1	Harris <i>et al.</i> , 2005
<i>Alpinia galanga</i>	KY412470.1	Anju & Yusuf, 2016
<i>Amomum uliginosum</i>	AY769790.1	Harris <i>et al.</i> , 2005
<i>Zingiber zerumbet</i>	KY412469.1	Anju & Yusuf, 2016
<i>Amomum</i> sp.	AY769793.1	Harris <i>et al.</i> , 2005
<i>Alpinia purpurata</i>	MH603399.1	Givnish <i>et al.</i> , 2018
<i>Zingiber spectabile</i>	MH603449.1	Givnish <i>et al.</i> , 2018
<i>Amomum compactum</i>	NC_036992.1	Wu, 2018
<i>Alpinia oxyphylla</i>	KY985237.1	Gao <i>et al.</i> , 2017
<i>Alpinia galanga</i>	KJ609030.1	Mathew <i>et al.</i> , 2014
<i>Amomum glabrum</i>	FJ848631.1	Auvray <i>et al.</i> , 2009
<i>Alpinia zerumbet</i>	MH603400.1	Givnish <i>et al.</i> , 2018
<i>Hedychium coronarium</i>	MH603422.1	Givnish <i>et al.</i> , 2018
<i>Aframomum limbatum</i>	FJ848663.1	Auvray <i>et al.</i> , 2009
<i>Aframomum pilosum</i>	FJ848652.1	Auvray <i>et al.</i> , 2009
<i>Aframomum angustifolium</i>	MH603398.1	Givnish <i>et al.</i> , 2018
<i>Aframomum longiligulatum</i>	FJ848639.1	Auvray <i>et al.</i> , 2009
<i>Renealmia battenbergiana</i>	AY769802.1	Harris <i>et al.</i> , 2004
<i>Renealmia occidentalis</i>	AY769801.1	Harris <i>et al.</i> , 2004
<i>Renealmia alpinia</i>	DQ444491.1	Sarkinen <i>et al.</i> , 2006

Supplementary Figure 3: One of the best trees resulted from the parsimony analysis of the combined sequence data of *trn L-F* and *trn S-fM* (length = 1289; consistency index = 0.887; retention index = 0.939; rescaled consistency index = 0.833); branch labels: above - bootstrap values, below - posterior probability; A - *Alpinia* group, B - *Amomum* group, A1- *Alpinia* group without the basal clade of the A group.