Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Biologie
Studijní obor: Cévnaté rostliny

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The evolution of *Elettariopsis* (*Zingiberaceae*)

Evoluce rodu *Elettariopsis* (*Zingiberaceae*)

Diplomová práce

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne 14. 08. 2014

Podpis

Abstract

This work attempts to offer an insight into the problematic of the genus *Elettariopsis* Baker, the last unrevised genus in the subfamily Alpinioideae (*Zingiberaceae*). Phylogenetic analyses are performed on ITS, *mat*K and DCS sequence data and correlated with absolute genome size and biogeographical distribution of the samples. *Elettariopsis* as a genus is found to be weakly supported and strongly supported only with the addition of some species of *Amomum* Roxb., including the type species *A. subulatum*. The absolute genome size in this group is greater than in the outgroup represented by members of the Zingiberoideae subfamily. The evidence given by sequence data further suggests that *Elettariopsis* is divided into two well-supported groups, the *"E. curtisii*" group and the *"E. triloba/E. unifolia*" group, each of which contains several well-supported clades. In the analysis of absolute genome size it is shown that the absolute genome size in the "*E. triloba/E.unifolia*" group is higher than in the "*E. curtisii*" group. These two groups also differ slightly in their biogeographical distribution, with group G being distributed in only in Vietnam, Laos, and Thailand, while members of group H are also occurring in Singapore and Indonesia (Borneo).

Keywords: *Zingiberaceae*, *Elettariopsis*, South-East Asia, ITS, *mat*K, DCS, absolute genome size

Abstrakt

Tato práce si klade za cíl podat komplexní vhled do problematiky rodu *Elettariopsis* Baker, posledního nerevidovaného rodu v podčeledi Alpinioideae (*Zingiberaceae*). Na sekvenčních datech z úseků ITS, *mat*K, a DCS jsou provedeny fylogenetické analýzy, které jsou korelovány s absolutní velikostí genomu a biogeografickým rozložením vzorků. Z výsledků je zřetelné, že *Elettariopsis* jakožto rod je jen slabě podpořen a silně podpořen je pouze po spojení s některými druhy rodu *Amomum* Roxb., včetně typového druhu *A. subulatum*. Absolutní velikost genomu je v této skupině větší, než v mimoskupině (outgroup) tvořené zástupci podčeledi Zingiberoideae. Ze sekvenčních dat dále vyplývá, že *Elettariopsis* je rozdělen do dvou silně podpořených skupin, skupiny F a skupiny G, z nichž každá zahrnuje několik silně podpořených skupin. Z analýzy absolutní velikosti genomu se ukazuje, že absolutní velikost genomu je vyšší ve skupině F než ve skupině G. Tyto dvě skupiny se rovněž liší svým biogeografickým rozložením; skupina "*E. triloba/E. unifolia*" se vyskytuje pouze ve Vietnamu, Laosu a Thajsku, zatímco zástupci skupiny "*E. curtisii*" se vyskytují rovněž v Singapuru a v Indonésii (na Borneu).

Klíčová slova: *Zingiberaceae*, *Elettariopsis*, jihovýchodní Asie, ITS, *mat*K, DCS, absolutní velikost genomu

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Abbreviations

BI	Bayesian inference
bp	Base pair
BS	Bootstrap value (in maximum parsimony and maximum likelihood)
DCS	Diketide CoA synthase
ILD	Incongruence length difference
ITS	Internal transcribed spacer
***mat*K**	Maturase K
ML	Maximum likelihood
MP	Maximum parsimony
NMNH	Smithsonian National Museum of Natural History, USA
PBG	Prague Botanic Garden in Trója
PP	Posterior probability (in Bayesian inference)
RBGE	Royal Botanic Gardens Edinburgh
SBG	Singapore Botanic Garden

Acknowledgement

I would like to extend my thanks to my supervisor Mgr. Tomáš Fér, Ph.D. for his kind guidance and patience, as well as for much appreciated help with statistical programs during the writing of this thesis; to my consultant, Dr Jana Leong-Škorničková, and Mgr. Otakar Šída for providing me with an insight into ginger fieldwork, advice, and data; to Mgr. Eliška Záveská for valuable explanations, help and perpetual good mood; to Ing. L. Flašková for valuable advice on lab work; to Dr Mark Newman for providing me with kindness and care during my stay in Edinburgh, and to Mgr. Monika Pospíšilová for her support and aid in the greenhouses and elsewhere. Furthermore, I would like to thank prof. RNDr. Tomáš Herben, CSc. who developed the script for parameter estimates that was used in this thesis; the botanic gardens (Royal Botanic Gardens Edinburgh, Singapore Botanic Garden, Prague Botanic Garden in Trója) for taking good care of their valuable ginger collections and letting us work with them; Helen Yates from the RBGE for her kind assistance in the greenhouses; my sister for her help with the graphic layout, and the rest of my family and colleagues for support.

2. Introduction

1.1 Introduction

Like many other tropical regions, South-East Asia has been intensively researched for decades, but to the present day much of its biodiversity remains undescribed or even hidden. This is caused by many factors. In the past, the main cause was the limited access to many areas, as the tropical biomes were physically demanding and often dangerous to the (mostly Western) researchers. Nowadays these physical obstructions are somewhat diminished, but new ones arise, such as the restrictions of access to some areas, or the lack of understanding and cooperation by the local government. In many areas, exploit for commercial reasons is prioritized over scientific research, which causes the loss of (often undescribed) species.

The taxonomic revision in many areas of South-East Asia is still very poor, largely due to the high numbers of species that are still being discovered in the more remote areas. Taxonomic revision is much needed not only for the purposes of science, but also to aid in determining the useful and endangered species and to suggest strategies for their effective conservation.

One of the important South-Asian plant families are the *Zingiberaceae* (gingers), the members of which have long been used by the locals as remedies, spices, and decorative plants. *Zingiberaceae* have been intensively researched in the past few decades (the most recent comprehensive phylogeny was made by Kress *et al.* in 2002); however, they still remain largely unresolved.

Elettariopsis is one of the less intensively researched genera in the *Zingiberaceae*, and the only genus in the subfamily Alpinioideae that is still lacking revision. It is a small genus counting around 36 recognized species (Newman, Škorničková and Pullen 2013), with more species still being discovered and published (e.g. Picheansoonthon and Yupparach 2012, Saensouk and Saensouk 2014).

Elettariopsis is very similar in morphology (especially in the flowers, which in the *Zingiberaceae* are an important determining character; e.g. Holttum 1950, Kam 1982) to another genus in the Alpinioideae, *Amomum* Roxb. Recent molecular and morphological analyses of the polyphyletic *Amomum* (Xia *et al.* 2004, Droop 2012, Šída *et al.* unpubl.) show that *Elettariopsis* forms a strongly supported clade with some species of *Amomum*, particularly those from the *A. maximum* group of Xia *et al.*, while other *Amomum* species are completely separated from this clade and cluster with *Etlingera* and *Hornstedtia* (Fig. 1). The monophyly of *Elettariopsis* is uncertain as the intrageneric relationships are also unresolved,

especially in the complex *E. curtisii* which acts as a collective species where species of uncertain determination are placed.

So far there has not yet been a phylogenetic study on *Elettariopsis* which would encompass its whole range of distribution, and the sampling in most analyses of the Alpinioideae has been very poor (the highest number being 8 samples in the revision of *Amomum* Roxb. by Jane Droop 2012). This work is the first to attempt a complex insight into the problematics of this genus with the use of taxonomic and molecular methods, partly as a preparation for the intended revision of *Elettariopsis.* It is also the first analysis to include *Amomum subulatum* Roxb., the type species of *Amomum*. The sampling is the most extensive so far; however, it covers only about a third of the whole scope of variability in *Elettariopsis* (only seven taxa are determined among the samples of the dataset, out of twenty four currently accepted species).

Molecular research carried out to determine the position of *Elettariopsis* within the Alpinioideae involved DNA sequencing and subsequent construction of phylogenetic trees. Species of *Amomum* which form a clade with *Elettariopsis* in previous analyses (especially Droop 2012) were included into the analysis to resolve the relationship between *Elettariopsis* and *Amomum*. In the molecular analyses, three independent markers were used to infer the phylogeny of the group. The popular ITS (internal transcribed spacer) and *mat*K (maturase K) marker have already been successfully used in many previous studies of the *Zingiberaceae*, including the major phylogenetic study by Kress *et al.* (2002) or
the phylogeny of the Alpinioideae (Pedersen 2004). Along them a single-copy nuclear gene, DCS (diketide CoA synthase) (Radhavakrishnan *et al.* 2010), was used; single-copy DNA has been proven to have higher rates of nucleotide substitution than chloroplast DNA (Wolfe *et al.* 1987) and is inherited biparentally, which is useful in resolving phylogenies both on the intra and interspecific level (Strand *et al.* 1997).

The absolute genome size (2C) was determined with the use of flow $_{c}$ytometry. The genome size has been used in many studies to investigate its relationship to phylogeny and ecology. A subsequent mapping of genome size on a Bayesian phylogeny of the investigated group and an analysis estimating the *lambda*, *kappa* and *delta* parameters was performed. The results of these analyses show that the absolute genome size in the group *Elettariopsis* s. l. is higher than in the outgroup, and that in group F of *Elettariopsis* it is higher than in the *A. maximum* group (defined by Droop, 2012) and in the other *Elettariopsis* group (group G). An estimation of ancestral states performed in the *phytools* package in R (Revell 2012; function

fastAnc) suggests an overall increasing trend in the absolute genome during the evolution of *Elettariopsis*, and a decreasing trend in the recently evolved groups.

Most of the samples used in this thesis have been collected on excursions to Vietnam, Thailand, and Laos by *Zingiberaceae* taxonomists Jana Leong-Škorničková, Otakar Šída, Mark Newman and their co-workers. Although current sampling covers most of the regions of the generic distribution, the sampling within these regions is not dense enough and still may lead to a bias in the assessment of biogeography. In future studies, more extensive sampling, especially more samples from individual determined genera, will be needed to enable a full understanding of the biogeographical patterns in the distribution of *Elettariopsis*.

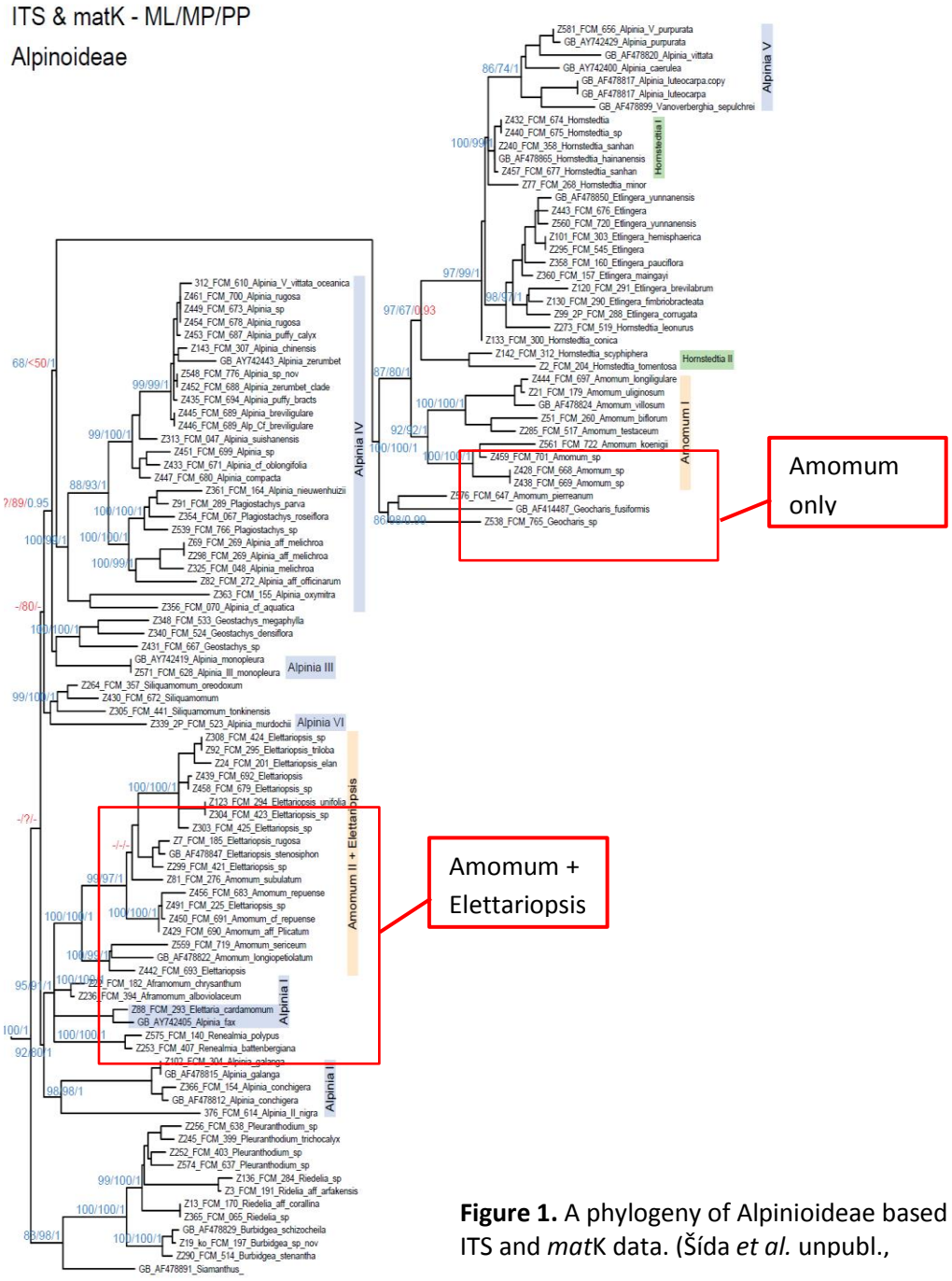

Figure 1. A phylogeny of Alpinioideae based on ITS and *mat*K data. (Šída *et al.* unpubl.,

1.2 Questions

1. Is *Elettariopsis* monophyletic?
    a. What is the relationship between genera *Amomum* and *Elettariopsis*?
    b. What is the support for *Elettariopsis* as a phylogenetic group?
    c. What is the infrageneric structure of *Elettariopsis*?
2. Is there any variability in genome size?
    a. How does the genome size correspond with phylogeny?
    b. Are there any trends in genome size evolution?
3. What is the relationship between the phylogeny of *Elettariopsis* and its geographical distribution?

2. Literature search

2.1 Family *Zingiberaceae*

The *Zingiberaceae* is a pantropical family belonging to the order Zingiberales. With around fifty genera and over 1200 species, it is the largest family of the order (Kress *et al.* 2002). The centre of diversity lies in South and South-East Asia, where most species are distributed; four genera (*Aframomum, Aulotandra, Renealmia,* and *Siphonochilus*) occur in Africa and the genus *Renealmia* is the sole member that is native to South America.

The members of this family are rhizomatous plants whose leaves form a pseudostem. They produce a terminal inflorescence, either on a leaf-shoot or on a separate shoot growing directly from the rhizome near the base of the leaf-shoot. The inflorescence comprises an axis which bears spirally arranged cincinni supported by primary bracts; in some groups the cincinni are reduced to a single flower (Holttum 1950). The zygomorphic flowers usually possess only one fertile stamen. The main identifying character is the labellum, formed by the fusion of two lateral staminodes of the inner whorl (Missouri Botannical Garden 1995-2002).

Most *Zingiberaceae* are evergreen (especially members of the Alpinoideae subfamily), as they are distributed in the warm tropical forests. Some, mostly representatives of Zingiberoideae, survive periods of drought in dormant state (Kress *et al.* 2002). The rhizome of these species (e.g. *Curcuma*, *Zingiber*) is usually fleshy and adapted as a resting organ; they are therefore able to adapt to a drier climate, although none are truly xerophytic (Holttum 1950). Epiphytic species are also present, though rarely, for example in the genus *Burbidgea* Hook. f.

In 1999, Sakai, Kato and Inoue observed the pollinators of 29 species of Bornean *Zingiberaceae* and *Costaceae* and discovered three pollination guilds: one was pollinated by spiderhunters (Nectariniidae), one by medium-sized *Amegilla* bees (Anthophoridae), and one by small hallictid bees. These guilds correlated with the flower morphology, especially in the formation of the floral tube and labellum (Sakai *et al.* 1999).

Zingiberaceae contain aromatic oils and are thus often used as spices or in traditional medicines, such as the commercially grown ginger (*Zingiber officinale* Roscoe) or the green cardamom (*Elettaria cardamomum* (L.) Maton). As their flowers are often large and brightly coloured, they are also grown as ornamental plants (e. g. species of *Etlingera* Giseke).

The *Zingiberaceae* are a difficult and still largely unresolved group. The main (and perhaps only) morphological character by which they can be securely distinguished is the

flower, and until the development of morphological analyses the taxonomy of *Zingiberaceae* had been largely based on their flower morphology. The most recent monography of *Zingiberaceae* remains that written by Schumann in 1904. *Zingiberaceae* have been intensively researched during the past few decades (Holttum 1950, Kress *et al.* 2001, Kress *et al.* 2002, Newman *et al.* 2004), and the research is continuing with the use of new methods in botany such as next-generation sequencing, while new species are still being discovered in the area of dispersal.

In 2002, Kress *et al.* divided *Zingiberaceae* into four subfamilies, Siphonochileae, Tamijioideae, Alpinoideae and Zingiberoideae (Fig. 2), on the basis of the ITS and *mat*K regions and morphological data. The subfamily Alpinioideae, which *Elettariopsis* is a member of, is characterised by the absence or reduction of the two lateral staminodes, a plane of distichy perpendicular to that of the growth of the rhizome, and the lack of a dormant period (Kress *et al.* 2002). It contains two tribes, Alpinieae and Riedeliae. The largest genus of Alpinioideae, *Alpinia* Roxb., is polyphyletic (Rangsiruji *et al.* 2000), and so are the remaining two large genera, *Amomum* Roxb. (Kress *et al.* 2002, Xia *et al.* 2004, Kress *et al.* 2007, Droop 2012) and *Etlingera* Giseke (Pedersen 2004).

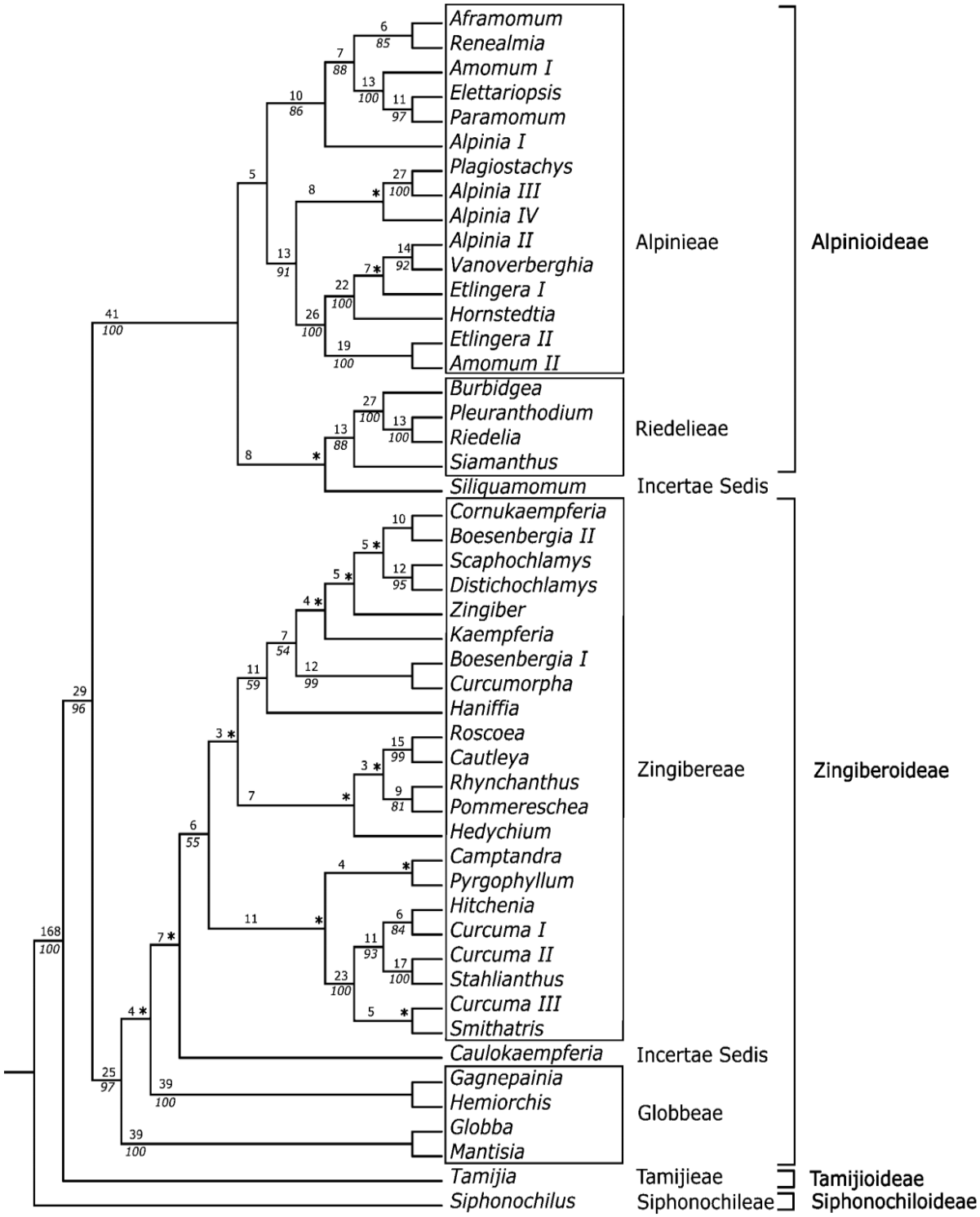

Figure 2. Subfamilies and tribes in the *Zingiberaceae*. (Kress *et al.* 2002).

2.2 Genus *Elettariopsis* Baker

2.2.1 Description and geography

Elettariopsis is a genus in the subfamily Alpinioideae whose representatives are tropical evergreen plants growing from rhizomes. The leaves grow directly from the rhizome, forming a pseudostem. In some species such as *E. curtisii* and *E. burttiana*, however, no distinct pseudostem is formed, as the leaves are only loosely clasping (Kam 1982). The inflorescence sprouts from the base of the pseudostem and remains rather short so that the flowers open directly above ground. The flowers are usually whitish or pinkish, often with a yellow stripe in the middle of the labellum which is bordered by two red stripes; the lateral staminodes are absent or reduced (Baker 1892, Holttum 1950; Fig. 3.).

Figure 3. Flowers of *E. curtisii*, the type species. (photo by J. Leong-Škorničková, 2014)

As is usual in *Zingiberaceae*, *Elettariopsis* plants contain essential oils and are often used in traditional Asian medicine and cuisine. The chemical constitution of the leaves differs between the species; as a result, each species has a distinct odour, which has been considered by some to serve as a useful aid in their determination (Lim 2003).

The species of *Elettariopsis* are distributed throughout China, Indonesia, Laos, Malaysia, Thailand and Vietnam (Wu *et al.* 1994+) and the centre of diversity seems to lie in Malaysia according to previous research (Hlavatá 2012; Fig. 4), but its location is still rather unclear as the whole range of occurrence has probably not yet been mapped.

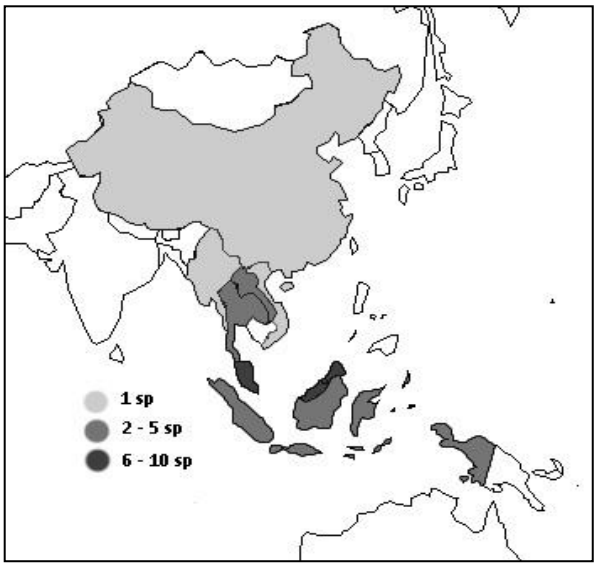

Figure 4. The distribution of *Elettariopsis.* (Hlavatá 2012)

2.2.2 Problems of studying **Elettariopsis**

Elettariopsis was established as a genus by Baker in 1892, comprising at the time three species: *E. exserta, E. curtisii* and *E. serpentina*, of which *Elettariopsis serpentina* is currently not accepted and considered a synonym of *E. curtisii* (Holttum 1950).

The Zingiberaceae Resource Centre (Newman, Škorničková and Pullen 2013) lists 36 recognized species of *Elettariopsis*, of which 24 are accepted (World Checklist of Selected Plant Families 2014, Saensouk and Saensouk 2014; Table 1). The type species is *E. curtisii* Baker. New species from South-East Asia are still being published (e.g. Picheansoonthon and Yupparach 2012, Saensouk and Saensouk 2014) and many more have been discovered, but as they are often found in the sterile state (and often will not flower easily in cultivation either) many of them still remain undetermined. At present, *Elettariopsis* remains the last genus in the Alpinioideae that has not yet been revised.

Determining *Elettariopsis* from herbarium material is also problematic as many diagnostic features such as differences in colour, or glossiness of the leaves are lost in herbarium specimens. Some characters such as the length of inflorescence axis, the relative lengths of corolla and calyx tubes or the size of floral parts are unusable in morphological analyses, as they are influenced by the thickness of debris layers above the inflorescence. Kam (1982) also notes that the type of inflorescence may change with the change of conditions; she notes that when a collection of *E. curtisii* was transplanted and cultivated in debris-free clayish ground, the plants produced terminal inflorescence.

The morphology of *Elettariopsis*, especially in the flowers, is very resemblant to that of *Amomum* Roxb (Holttum 1950, Kam 1982). Because of this, a number of species of *Elettariopsis* was in the past included in *Amomum* (e.g. *Elettariopsis stenosiphon* (K.Schum.) B.L.Burtt & R.M.Sm. or *Elettariopsis triloba* (Gagnep.) Loes.), and some *Amomum* species were previously included in *Elettariopsis* (e.g. *Amomum biflorum* Jack, renamed to *Elettariopsis pubescens* by Ridley). Droop (2012) notes that "*Elettariopsis* species usually have a tufted pseudostem, open bracteoles and a large, petaloid anther crest. The above characters are not unique to *Elettariopsis*, but are not found in combination in species of *Amomum*." This distinction, however, is applicable only to flowering specimens which make up only a small portion of the newly discovered species.

There were also some species previously described as *Amomum*, which were then transferred to *Elettariopsis* and later still into a different genus. An example is *E. stoloniflora* (K.Schum.) Ridl., which was first described as *Amomum stoloniflorum* by Schumann, then

transferred to *Elettariopsis* by Ridley and finally accepted as *Elettaria stoloniflora* as proposed by Sakai and Nagamasu in 2000 (Zingiberaceae Resource Centre 2014).

Table 1. An overview of accepted species in *Elettariopsis*.

Species name	Basionym	Protologue
Elettariopsis burttiana Y.K. Kam	*Elettariopsis burttiana* Y.K. Kam	Notes Roy. Bot. Gard. Edinburgh 40(1): 144. (1982)
Elettariopsis chayaniana Yupparach	*Elettariopsis chayaniana* Yupparach	Acta Bot. Yunnan. 30(5): 525. figs.1-5. (2008)
Elettariopsis curtisii Baker	*Elettariopsis curtisii* Baker	Fl. Brit. India 6: 252. (1892)
***Elettariopsis diphylla* (K.Schum.) Loes.**	*Cyphostigma diphyllum* K.Schum.	Pflanzenr. IV 46(Heft 20): 272. (1904)
Elettariopsis elan C.K.Lim	*Elettariopsis elan* C.K.Lim	Folia malaysiana 4(3+4): 218. (2003)
Elettariopsis exserta (Scort.) Baker	*Cyphostigma exsertum* Scort.	Nuovo Giorn. Bot. Ital. 18:310 (1886)
Elettariopsis kandariensis (K.Schum.) Loes.	*Amomum kandariense* K.Schum.	Bot. Jahrb. Syst. 27(3): 323. (1899)
Elettariopsis kerbyi R.M.Sm.	*Elettariopsis kerbyi* R.M.Sm.	Edinburgh J. Bot. 47(3): 371. (1990)
Elettariopsis latiflora Ridl.	*Elettariopsis latiflora* Ridl.	J. Straits Branch Roy. Asiat. Soc. 32: 154. (1899)
Elettariopsis monophylla (Gagnep.) Loes.	*Amomum monophyllum* Gagnep.	Bull. Soc. Bot. France 54: 163 1907
Elettariopsis procurrens (Gagnep.) Loes.	*Amomum procurrens* Gagnep.	Bull. Soc. Bot. France 49: 254. 1903 [1902 publ. 1903]
Elettariopsis puberula Ridl.	*Elettariopsis puberula* Ridl.	Bull. Misc. Inform. Kew 1926: 88. (1926)
Elettariopsis rugosa (Y.K.Kam) C.K.Lim	*Eletariopsis smithiae var. rugosa* Y.K. Kam	Notes Roy. Bot. Gard. Edinburgh 40(1): 150. (1982)
Elettariopsis serpentina Baker	*Elettariopsis serpentina* Baker	Fl. Brit. India 6: 252. (1892)
Elettariopsis slahmong C.K.Lim	*Elettariopsis slahmong* C.K.Lim	Folia malaysiana 4(3+4): 223. (2003)
Elettariopsis smithiae Y.K.Kam	*Elettariopsis smithiae* Y.K.Kam	Notes Roy. Bot. Gard. Edinburgh 40(1): 148. (1982)
Elettariopsis stenosiphon (K.Schum.) B.L.Burtt & R.M.Sm.	*Amomum stenosiphon* K. Schum.	Notes Roy. Bot. Gard. Edinburgh 31(2): 312. (1972)
Elettariopsis sumatrana Valeton	*Elettariopsis sumatrana* Valeton	Bull. Jard. Bot. Buitenzorg 3: 148. (1921)
Elettariopsis triloba (Gagnep.) Loes.	*Amomum trilobum* Gagnep.	Bull. Soc. Bot. France 51: 453. (1905)
Elettariopsis unifolia (Gagnep.) M.F.Newman	*Amomum unifolium* Gagnep.	Bull. Soc. Bot. France 54: 403. (1907)
Elettariopsis wandokthong Picheans. & Yupparach	*Elettariopsis wandokthong* Picheans. & Yupparach	Taiwania 55: 244 (2010)
Elettariopsis limiana Picheans. & Yupparach	*Elettariopsis limiana* Picheans. & Yupparach	J. Jpn. Bot. 87: 85-95 (2012)
Elettariopsis poonsakiana Picheans. & Yupparach	*Elettariopsis poonsakiana* Picheans. & Yupparach	J. Jpn. Bot. 87: 85-95 (2012)
Elettariopsis biphylla S. Saensouk & P. Saensouk	*Elettariopsis biphylla* S. Saensouk & P. Saensouk	Phytotaxa 159(1): 023-025

2.2.3 Phylogeny

The relationships within *Elettariopsis* are unresolved, mainly because of the lack of studies focusing on this particular group of species, or due to lack of proper sampling covering entire diversity of this genus. Especially regarding the *Elettariopsis curtisii* complex, which has so far been used as a collective taxon for species of uncertain placement. Therefore, the monophyly of *Elettariopsis* remains questionable. The relationship of *Elettariopsis* and *Amomum* Roxb. is also unclear, owing largely to their morphological similarity described above and their simultaneous distribution in South-East Asia.

In their comprehensive phylogeny of the *Zingiberaceae*, Kress *et al.* (2002) placed *Elettariopsis* into a clade including *Renealmia* L.f. and *Aframomum* K.Schum. in the Alpinieae tribe. This position was also confirmed in Pedersen's (2004) phylogeny of the Alpinioideae and in phylogenetic studies of *Amomum* done by Xia *et al.* (2004) and Jane Droop (2012).

In the analysis of Xia *et al.* (2004), *Elettariopsis*, together with some species of the *Amomum* maximum group, forms a moderately supported clade sister to the monotypic genus *Paramomum* S.Q. Tong (the recent *Amomum petaloideum* (S.Q.Tong) T.L.Wu; Wu 1998), while the *Elettariopsis* clade is weakly supported. They confirm the monophyly of *Elettariopsis* and speculate that *Elettariopsis* and *Paramomum* "both evolved from a core clade of *Amomum* through inflorescence and flower diversification" (Xia *et al.* 2004: 338). However, as this analysis was focused on *Amomum*, only four species of *Elettariopsis* were used, which is a rather low number to extrapolate from.

Results similar to those of Xia *et al.* were obtained by Droop (2012), who used eight accessions of *Elettariopsis*. In her analysis of the ITS marker (Fig. 5), *Elettariopsis* formed an unsupported clade on its own and a well-supported clade (PAUP* jackknife 97, Bayesian posterior probability 1) with some species of Amomum.

2.2.4 The problematics of genus **Amomum** *Roxb.*

The most recent molecular analyses, performed on ITS and mat*K* data (Šída *et al.* unpubl.) indicate that *Amomum* is divided into at least two major groups, one of which forms a strongly supported clade with *Elettariopsis* and the other is completely separated from this clade and clusters with *Etlingera* and *Hornstedtia* (Fig. 1.). These results are also supported by the findings of Droop (2012). While the intrageneric partition of *Amomum* is not the subject of this thesis and has been covered in detail in Droop's work, considering the close relationship of this genus and *Elettariopsis* it is necessary to describe it here as well.

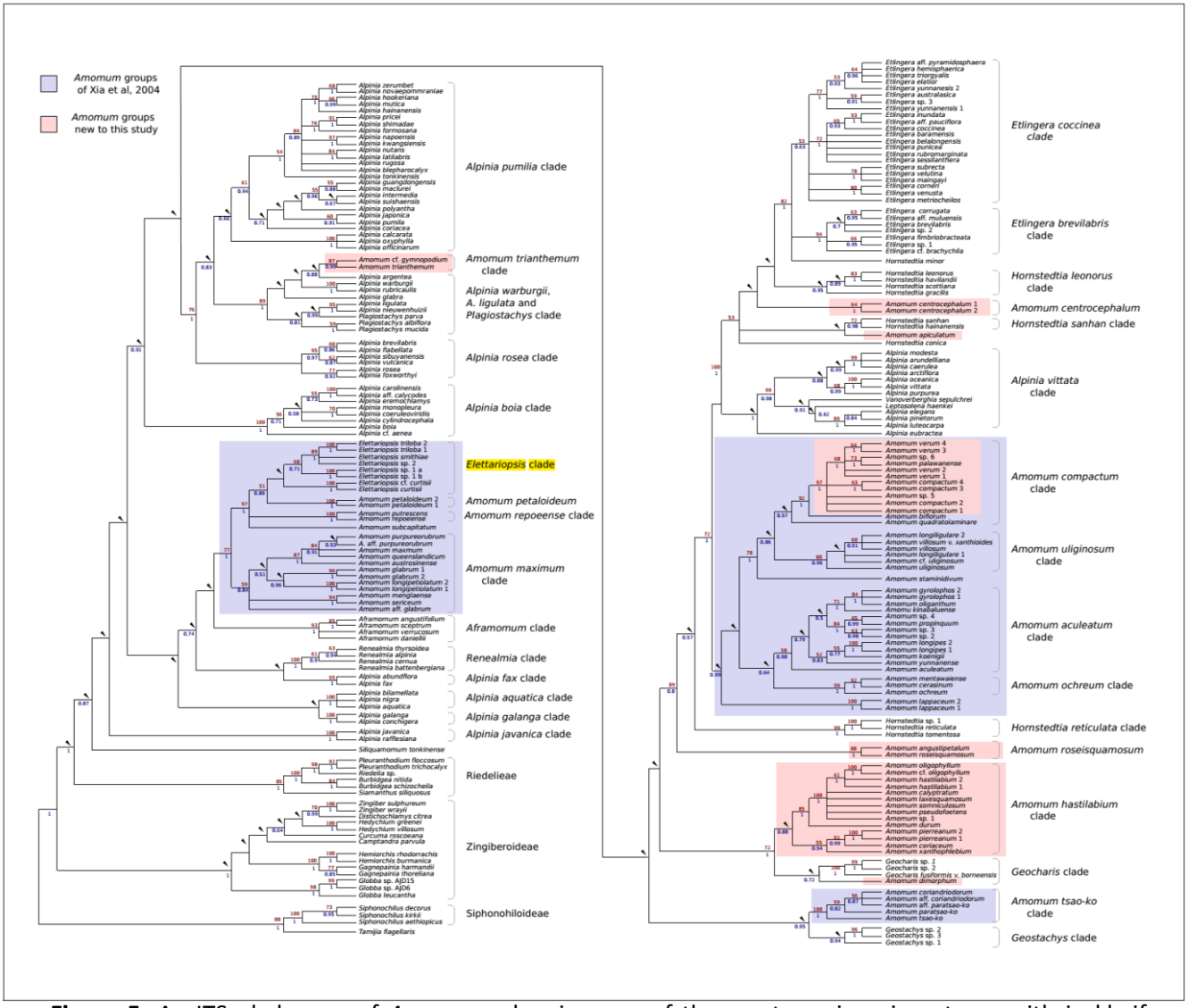

Figure 5. An ITS phylogeny of *Amomum* showing one of the most parsimonious trees with jackknife (red) and Bayesian (blue) support above and below branches. The position of *Elettariopsis* is marked in yellow. (Droop 2012, modified).

The genus *Amomum*, established in its current form by Roxburgh in 1820, is the second largest genus in the *Zingiberaceae* encompassing over 180 accepted species (WCSP 2014), while more are still being published. The name had already been used by Linnaeus in his *Species Plantarum* (1753; cited in Droop 2012); his *Amomum* consisted of only five species, which were later transferred into different genera.

The only revision of *Amomum* so far as a whole has been done by Schumann (1904), who placed its African species into a new genus *Aframomum* and divided the rest into two sections and four series, using the morphology of the anther crest as a distinguishing feature (Droop 2012). From that time, *Amomum* was revised only in limited areas of its occurrence,

for example the Malay Peninsula (Holttum 1950) or Borneo (Smith 1985, Sakai and Nagamasu 2003).

As has been proven by many previous studies (Kress *et al.* 2002, Xia *et al.* 2004, Kress *et al.* 2007, Droop 2012), *Amomum* is polyphyletic and consists of more groups scattered through the Alpinoideae subfamily. Kress Xia *et al.* (2004) divided it into three provisional groups based on ITS and mat*K* analysis: the *A. villosum* group, paraphyletic with *Etlingera, Vanoverberghia* and *Hornstedtia*; the *A. maximum* group, paraphyletic with *Elettariopsis* and *Paramomum*; and the monophyletic *A. tsao-ko* group. These groups were further supported by morphological evidence on the flowers and fruits (Fig. 6.).

In the past, the morphological characters used in the classification of *Amomum* were mostly those found on the flowers (the anther crest, labellum, or the shape of the bracteoles). The morphology of the fruit was often omitted from the studies and considered unimportant (Droop 2012). Holttum (1950) distinguishes between two types of fruit: "one smooth and thin-walled, dry at maturity, mostly ridged, the other a fleshy spiny berry, (…)" (Holttum 1950: 193). Another study which takes this character into account is that of Xia *et al.* (2004), who have done a phylogenetic study on *Amomum* and divided it into four types according to fruit morphology: "Tsao-ko" possessing smooth fruit, "Villosum" with echinate fruit, "Maximum" with winged fruit, and "Sericeum" with shallowly ridged fruit. Three of these groups corresponded with three strongly supported groups, the *A. tsao-ko, A. villosum* and *A. maximum* group (with the exception of *A. koenigii*, which had smooth fruits similar to the Tsao-ko type but was strongly supported as a member of the *A. villosum* group), while the fourth group "Sericeum", consisting of *A. sericeum* and *A. menglaense*, was a part of the *A. maximum* group.

The findings of Xia *et al.* are further supported by Jane Droop's analysis, with the addition of new fruit types found in Sumatra: the *A. compactum* type, which is lobed, dry, thin-walled and dehiscent; the *A. apiculatum* type, which is elongated and hard and resembling *Hornstedtia* fruits; and spherical fruits with up to 15 longitudinal ridges, which are found in some species such as *A. laxisquamosum* and *A. hastilabium* (Droop 2014).

The species of *Amomum* forming a strongly supported group with *Elettariopsis* all have winged fruits (Šída *et al.*, unpubl.). Those species belong to the *A. maximum* group of Xia *et al.*, and the *A. maximum* and *A. repoeense* clades of Droop, as seen in Fig. 6 and Fig. 5, respectively.

It is worth mentioning that none of the abovementioned works used a sample of the type species *A. subulatum*, but both Kress *et al.* (2007) and Droop (2012) presumed that as it has

winged fruits, it would probably fall into the *A. maximum* clade. That gives rise to a nomenclatorial problem, as this clade also encompasses the weakly supported *Elettariopsis* clade. It may therefore be necessary in the future to transfer *Elettariopsis* species to *Amomum* and solve future problems of nomenclature in regard to this genus. It is likely that the other *Amomum* groups, which are nested elsewhere in the Alpinioideae, would then probably require being assigned a new name entirely.

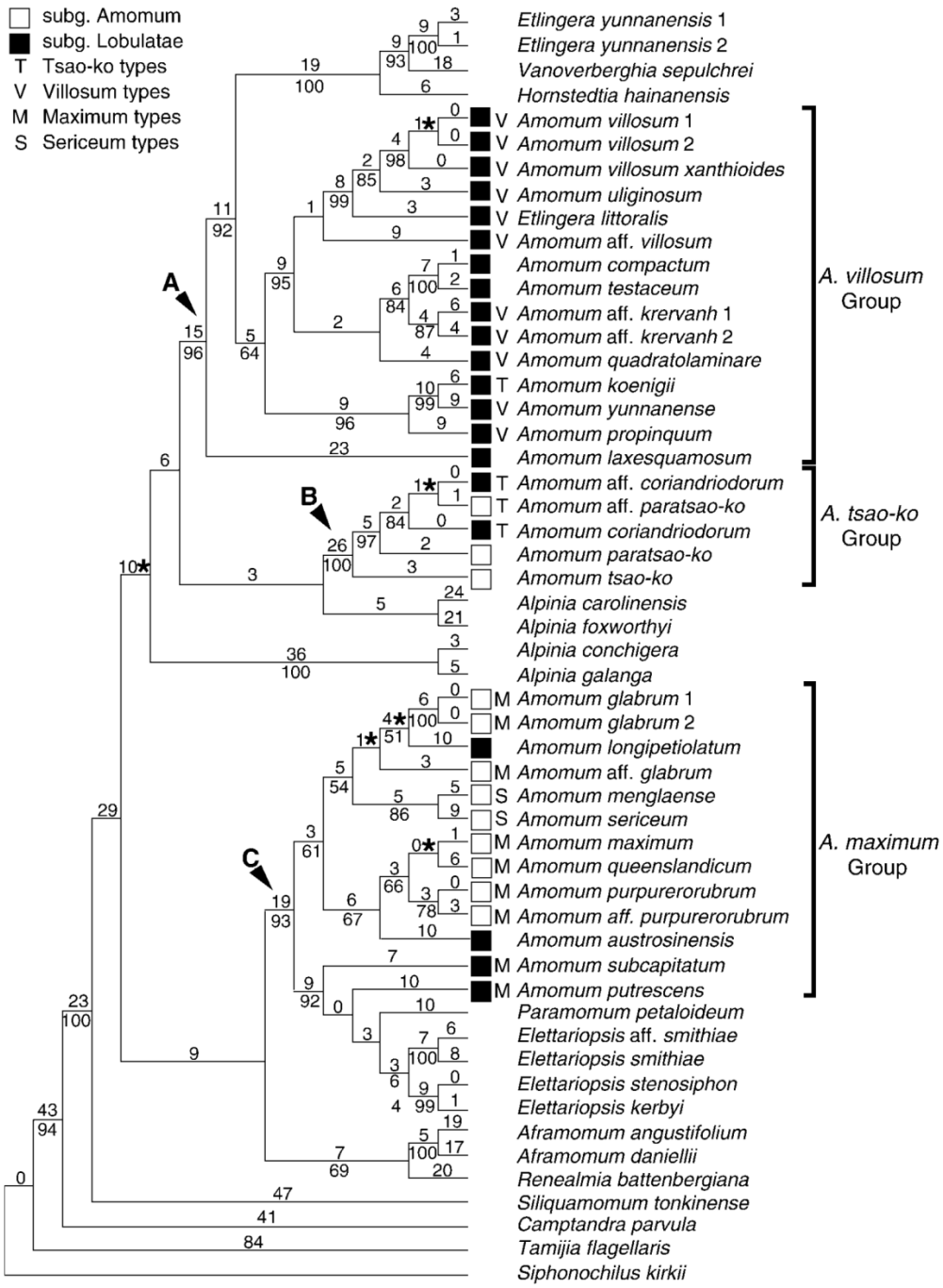

Figure 6. The division of *Amomum* by Xia *et al.* (2004).

2.3 Phylogenetic methods used in this study

2.3.1 DNA sequencing

2.3.1.1 Internal transcribed spacer (ITS)

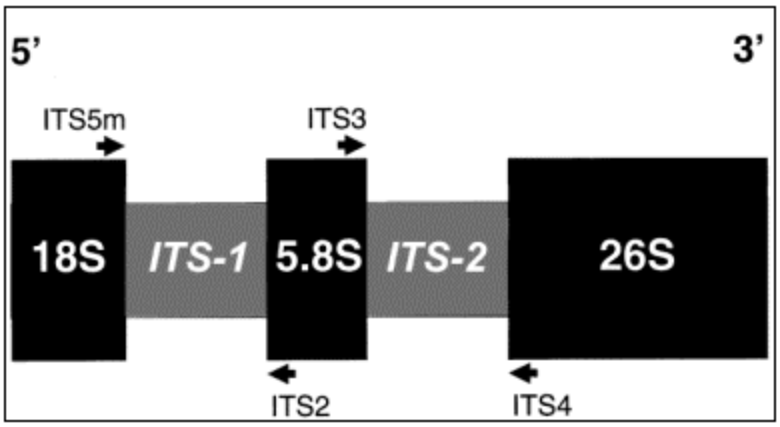

Figure 7. A scheme of the internal transcribed spacer and the primers used for its sequencing. (Saar and Polans 2000)

ITS (Fig. 3.1) is the 18S-5.8S-26S region of the nuclear ribosomal DNA (nrDNA) which plays a role in the formation of ribosomal subunits. The ITS marker is very popular in phylogenetic studies, owing to multiple factors. One of these is its biparental inheritance, where two copies are inherited from each of the parents. Phylogenetic analyses could be complicated by the fact that the two copies may be different, but due to concerted evolution (Elder and Turner 1995) these copies are with time homogenized within the individual. There are however also drawbacks, as concerted evolution may also produce chimeric sequences or eliminate the copy from one of the parents (Krak *et al.* 2013). Where the copies still differ from one another (such as in hybrid individuals), they may be used to determine the parents of hybrid taxa (Pospíšilová 2012).

In 1990, White *et al.* described a set of universal ITS primers, which greatly facilitated the use of this marker as there is no longer need to design specific primers and ITS data can be easily obtained from most plants and fungi. Moreover, the abundance (there are hundreds to thousands of nrDNA copies in the plant genome) and simplicity (the ITS is a small fragment, starting at approximately 500 bp length and not exceeding 3,700 bp; Alvárez and Wendel 2003) make this region easy to obtain even with less expertise and old material such as herbarium specimens.

*2.3.1.2 Chloroplast DNA (*mat*K*)

The *mat*K region (abot 1,500 bp in length) is a gene coding the enzyme maturase, which belongs to the DNA of the chloroplast genome (cpDNA). This is a circular molecule of haploid DNA, which is about 120 to 217 kbp in length (Petit and Vendramin 2007); the genome contains several tens of copies of cpDNA. Chloroplast DNA is inherited maternally in angiosperms and paternally in gymnosperms. There is no recombination in cpDNA and its mutation speed is low. Due to the haploidy of cpDNA, the allele fixation is faster in the chloroplast, which causes low variability on the intrageneric level. The intergenic regions of cpDNA are useful in interspecific analyses, while the coding regions are preferred for studies at the family level (Krak 2011, cited in Pospíšilová 2012).

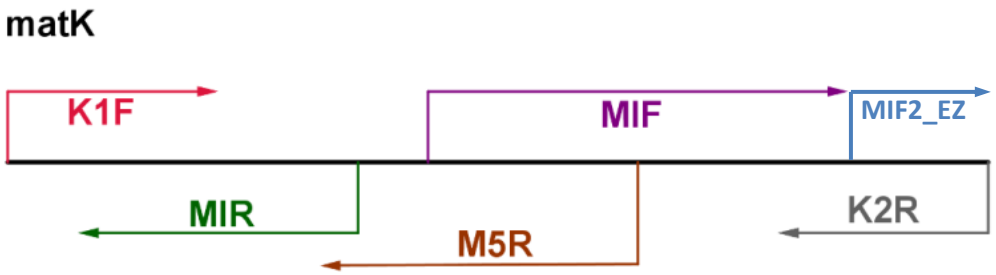

Figure 8. A schematic depiction of the matK primers. (Pospíšilová 2012, modified to show MIF2_EZ)

2.3.1.3 Diketide CoA synthase (DCS)

DCS is a gene from Type III polyketide synthase (PKS) gene family, which is closely related to Chalcone synthase (Radhavakrishnan *et al.* 2010). Chalcone synthase (CHS) is a single-copy gene in some plant families, for example in the *Brassicaceae*; Koch *et al.* 2000. A phylogeny of the PKS gene family published by Radhavakrishnan *et al.* (2010) includes several members of the Zingiberaceae, namely *Alpinia*, *Curcuma* and *Zingiber* species, and suggested single-copy character of DCS in some of these genera. Therefore, two pairs of specific DCS primers were designed for the *Zingiberaceae* and tested with satisfactory results (Záveská, in press).

The amount of variable characters in single-copy genes is much higher than in chloroplast DNA, as they evolve approximately twice as fast (Wolfe *et al.* 1987), and they are less susceptible to concerted evolution. However, these genes are often difficult to isolate and specific primers need to be designed in order to obtain them, and may show intraspecific, intrapopulational or even intraindividual polymorphism. These and other advantages and drawbacks of low-copy nuclear genes have been reviewed by Small *et al.* (2004).

2.3.2. Statistical methods used in phylogenetic analyses

2.3.2.1 Bayesian inference

This method is used to calculate the posterior probability of a tree topology from DNA sequence data (multiple alignment). The basis of this method is the Bayes' theorem, which states that

$$P(A|B) = \frac{P(B|A)\,P(A)}{P(B)}.$$

meaning that the conditional probability can be calculated from the reversed conditional probability. In inferring phylogeny, this means that we can calculate the probability of a tree topology on the condition of using sequence data *P(topology | data)* using the probability of the sequence data on the condition that the chosen topology is valid – *P(data | topology)*. The informative character in this type of analysis is the posterior probability (PP), the use of which in phylogeny is similar to the use of bootstrap values. Posterior probability usually reaches somewhat higher values than bootstrap.

To find the tree with the highest posterior probability, the *Markov chain Monte Carlo* (MCMC) method is used. This is an algorithm which searches for the best topology in an imaginary landscape of "hills" and "valleys", which represent the high and low posterior probabilities; the highest posterior probability is represented by the highest "hill". To increase the effectivity of this search, the *Metropolis coupling Markov chain Monte Carlo* (MCMCMC) method was invented, combining multiple Markov chains with different search criteria.

As the analysis starts by finding a random topology and the quality of the topologies further increases, a proportion of the first found topologies is usually excluded from the analysis (the default setting is 25 %) as *burn-in*.

The main criterion in the analysis is the *average standard deviation of split frequencies*. This value shows the similarity rate of two simultaneously running analyses. The smaller is the number, the more probable is the resulting topology. Ideall, the *average standard deviation of split frequencies* should be lesser than 0.01, but values under 0.05 are also acceptable (Ronquist *et al.* 2009).

2.3.2.2 Maximum parsimony

The aim of this method is to find a "maximally parsimonious" tree, i.e. a tree with the shortest length (the least evolutionary change to eplain the data). For exhaustive search, only

about 11 taxa can be used, otherwise the analysis is extremely time-consuming. To avoid this problem with larger sets of data, it is necessary to optimise the search with the use of heuristic methods such as branch swapping or the gradual adding of samples, which are often included in phylogenetic software. Two indexes are used in interpretation of the tree: the *consistency index* (CI), which measures the extent of homoplasy in the data, and the *retention index* (RI) which shows the amount of synapomorphies (Kluge and Farris 1969, 1989).

For interpretation of the resulting phylogeny, jackknifing (resampling without replacement) or bootstrapping (resampling with replacement) is usually performed. The result of bootstrap, which is used more often, is majority-rule consensus tree with percentages showing support on individual branches.

2.3.2.3 Maximum likelihood

This method searches for the most topology with the highest likelihood (L), which is the probability that the given DNA sequences will evolve with the given topology and model of evolution. The aim of this method is to find the topology which has the highest L. When using maximum likelihood, it is paramount to choose the correct model of evolution which correlates best with the given data. This can be done for example in the software *jModeltest* (Posada 2008).

2.3.3 Genome size evolution

2.3.3.1 Genome size studies in the Zingiberaceae

There have been many studies on genome size and its relationship to various other factors in plants such as ecology or phylogenetic relationships. Genome size is often similar in specific phylogenetic groups, as is proven e.g. by the studies on *Lupinus* (Naganowska *et al.* 2003) or *Echinops* (Sánchez-Jiménez *et al.* 2012).

Leitch *et al.* (2010) characterise genome evolution in monocots as rather continuous with the majority of changes happening in the shorter branches (in the longer branches changes happen at a slower rate and most of them in the early stages of evolution). They also estimated the ancestral genome size of monocotyledonous plants as 1C = 1,9 pg.

In *Zingiberacaeae*, genome size in relation to phylogeny has been so far investigated only on the genera *Curcuma* L. (Leong-Škorničková *et al.* 2007, Záveská *et al.* 2011) and *Globba* L. (Pospíšilová 2012). The former described two lineages within Indian *Curcuma* which corresponded with genome size and polyploidy levels. A major role was also played by the modes of reproduction

1.3.2.2 The estimation of ancestral characteristics

For estimating the ancestral values of a character, software comparing quantitative variable (here the absolute genome size) with topology can be used. The packages included in the statistical software R (R Core Team 2014) *caper* (Orme *et al.* 2013), *ape* (Paradis 2004) and *geiger* (Harmon *et al.* 2008, or software BayesTraits (Pagel *et al.* 2004), Mesquite (Maddison and Maddison 2007)

There are three parameters characterizing evolution (a maximum likelihood approach): lambda, kappa, and delta (Orme 2013).

The lambda (λ) parameter states how much the evolution of a character agrees with the phylogeny. Thus, if $\lambda = 1$, the topology plays an important role in the evolution of the character; if $\lambda = 0$, the taxa are independent, i.e. there is no phylogenetic structure in the data.

The kappa (κ) parameter estimates the gradualism of changes, i.e. the influence of branch length on the nature of the character evolution. When $\kappa > 1$, the longer branches influence the evolution of the character more than the shorter branches, and when $\kappa < 1$, more changes happen on the shorter branches. $\kappa = 0$ suggests that the evolution is punctualistic and does not depend on the branch length.

Delta (δ) measures the distance of the taxa from the root of the tree and suggests the acceleration or deceleration of the evolution in regard to the given topology. $\delta < 1$ indicates adaptive radiation (fast initial evolution, followed by slower changes), while $\delta > 1$ suggests adaptation and constant amount of changes in given time.

3. Materials and methods

3.1 Collection of material

Protologues were researched for all species of *Elettariopsis*. Some scanned protologues were available from the online databases Zingiberaceae Resource Centre (http://elmer.rbge.org.uk/zrc/) and Kew Monocot checklist (http://apps.kew.org/wcsp/) or online journals (e.g. Taiwania, Acta Botannica Yunnanica), others were kindly provided by Mgr. Otakar Šída, Head of the Botany Department of National Museum in Horní Počernice in scanned form. Locations of the individual species' holotypes, found in the protologues, were mapped in the Google Earth application.

Type specimens of all species were downloaded from online databases and herbaria (e.g. Musée National d'Historie Naturelle de Paris, Royal Botanic Gardens Edinburgh, Kew HerbWeb) and in online storages (*plants.jstor.org*). Spirit material (flowers and fruits) was examined in the herbarium in Royal Botanic Garden Edinburgh (further referred to as RBGE). Live material was provided by.

For flow cytometry, living material was collected from botanic gardens (RBGE, Singapore Botanic Garden [further SBG] and the Prague Botanic Garden in Trója [further PBG] or collected at sites of occurrence in South-East Asia by the researchers from Department of Botany (see Appendix 6 for localities).

To include the greatest number of samples possible in the analyses, sequences of the investigated regions (ITS, mat*K*, DCS) of chosen *Elettariopsis* and *Amomum* species were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/), added to the dataset and labelled by adding "GB" before the accession number from GenBank (Appendix 7). Some of these had previously been used in the dissertation thesis of Jane Droop (2012). Some of the sequences used (both *Elettariopsis* and *Amomum* and the sequences of other representatives of *Zingiberaceae* used in the analyses) were obtained from the work of O. Šída *et al.* (unpubl.). These samples are listed in Appendix 8.

3.2 Flow cytometry

Absolute genome size of the analysed plants was measured using flow cytometry according to the method described by Doležel *et al.* (2007). The measuring took place in the flow cytometry laboratory of the Department of Botany of the National Museum in Horní Počernice.

The used standard was *Bellis perennis* (2C = 3.42 pg; Pospíšilová 2012) the leaves of which were collected nearby a bush in front of the building of the Biological Department of the National Museum. Measurements were taken using *Partec CyFlow® ML* cytometer with green laser (532 nm) and further analysed with the software *Partec FloMax®*.

About 0.5-1 cm^2 of the sampled leaf and the same amount of the standard was chopped in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid monohydrate, 0.5% Tween 20; Otto 1990) in a small Petri dish using a new razor blade (each side of the razor was used only once). The resulting suspension was filtered through a nylon mesh (42 µ) into a test tube and left standing for 10-20 min in laboratory temperature. Then 970 µl of colouring medium consisting of Otto II buffer (0.4M Na_2HPO_4 .$12H_2O$; Otto 1990), 1 ml of propidium iodide, 1 ml RNase IIA and 40 µl of β-mercaptoethanol (all three by Sigma) was added to each test tube and left standing for about 8-10 min in laboratory temperature. Each sample was measured three times on three different days. If a value differed by 2% or more from the others, it was discarded and the sample was measured again on another day. Similarly, measurements where the CV (coefficient of variation) values exceeded 4.0 were discarded and the sample was measured again. An arithmetic mean was calculated from three acceptable results.

Samples were collected into re-sealable plastic bags, moistened, labelled with a permanent marker and stored in a refrigerator. Measurements were better if the sample was not chopped too finely before filtration. As the leaf tissue is rather firm, the necessity to use a fresh razor for each sample must be stressed to avoid crushing the nuclei.

After taking the final measurement, the remaining leaf tissue of each sample was cut into parts approximately 1x2 cm in size and put into a labelled teabag. The teabags were then placed in a re-sealable plastic bag with silica gel to dry.

3.3 DNA sequencing

3.3.1 DNA isolation

Samples of *Elettariopsis* and *Amomum* (Appendix 6) were used for DNA extraction. About 20 mg of dried material was placed in a 2ml Eppendorf tube and two tungsten carbide balls were added. The samples were handled by tweezers which were wiped with ethanol after each sample. The Eppendorf tubes were placed into a mill (Retsch *Mixer Mill MM400*) and ground for 3 minutes at 27,000 rpm.

Invisorb® Spin Plant Mini Kit (250) (Invitek) was used following a modification. To the pulverized material, 450 µl of Lysis Buffer P and 20 µl Proteinase K was added. After

adding 450 µl of Lysis Buffer P (as opposed to 400 in the original protocol) and Proteinase K and incubation, the tubes were centrifuged for 30 s at 13,200 rpm on a Sigma *4-16K* centrifuge. Instead of the recommended 40 µl, only 5 µl of RNase A (10 mg/ml, Fermentas) was added. For the first elution, 70 µl of Elution Buffer D was added and the mixture was left to stand for at least 30 minutes (instead of 15). For the second elution, 50 µl of the elution buffer was added and the samples were left to stand for at least 45 minutes.

Two elutions were obtained, of which the second one was usually diluted and used for PCR preparation. In cases when the concentration of the second elution was too low (habitually less than 20 ng/µl) or the DNA quality was bad, the first elution was used. The concentration of isolated DNA was measured using NanoDrop 1000 (Thermo Scientific) spectrophotometer.

3.3.2 PCR amplification

The DNA was diluted by Mili-Q water to obtain the concentration of 5 ng/µl in a volume of 15 µl, and 10 ng/µl in a volume of 50 µl or 20 µl, depending on how much DNA was needed. The primers (ITS primers ITS4 and ITS5, ribosomal matK primers K1F and K2R_EZ, and nuclear DCS primers CHS-F1, CHS-R1 and CHS-R2) (Table 2) were used in the concentration 25 pmol/µl. The PCR mixes were prepared for individual markers as is shown in Appendix 1. The reaction was carried out in an *Eppendorf Mastercycler* and *Eppendorf Mastercycler ep S* (gradient cycler). Individual cycles are described in Appendix 2.

Quality of the PCR products was assessed using gel electrophoresis. 2 µl of PCR product with 1 µl of loading dye (or 3 µl of undyed product if Red polymerase was used) were loaded onto a 1% agarose gel with one drop of ethidium bromide in a 1xTAE buffer. *O'GeneRuler™ 100bp Ladder Plus* (Fermentas) (Fig. 9.) was used as a size standard for all samples. The gel was subsequently visualised in a computer using a UV transilluminator (Herolab UVT-20M) and *Gel Logic 100* imaging system and *1D Image Analysis* software (both by Kodak).

Table 2. Primer sequences used in PCR and DNA sequencing.

DNA region	Primer name	Primer sequence	Reference
ITS	**ITS4**	5‘- TCCTCCGCTTATTGATATGC - 3‘	White *et al.* 1990
	ITS5	5‘- GGAAGTAAAAGTCGTAACAAGG - 3‘	White *et al.* 1990
mat*K*	***trn*K1F**	5‘- CTCAACGGTAGAGTACTCG - 3‘	Manos and Steele 1997
	***trn*K2R_EZ**	5‘- AACTAGTCGGATGGAGTAG - 3‘	Záveská unpubl.
	mIF	5‘- GTTCAGTACTTGTGAAACGTT - 3‘	Kress *et al.* 2002
	m5R	5‘- AGGATCCTTGAAAATCCATAG - 3‘	Kress *et al.* 2002
	mIR	5‘- CGTTTCACAAGTACTGAACTA - 3'	Kress *et al.* 2002
	mIF2_EZ		Záveská unpubl.
DCS	**CHS-F1 (A)**	5' - CGACTTCTATTTCCGGGTCA - 3'	Záveská *et al.* in prep.
	CHS-F2 (A)	5' - CATCCCAAGTGATGCTTCC - 3'	Záveská *et al.* in prep.
	CHS-R1 (S)	5' - GCTCTTGAGGTGGAAGGTCA - 3'	Záveská *et al.* in prep.
	CHS-R2	5' - CAGGGACCTTCTTCTTTTCAGA - 3'	Záveská *et al.* in prep.

3.3.3 DNA purification

For the majority of samples, the *Gel/PCR DNA Fragments Extraction kit* (Geneaid) was used, following the enclosed protocol with some modifications: Milli-Q water prewarmed to 65°C was used instead of the elution buffer, and the column was left to stand for 10-15 minutes (the protocol states at least 2 minutes). Twenty to thirty microlitres of water was added depending on the amount of product needed for the sequencing reaction.

Larger numbers of PCR products were purified using sodium acetate (NaOAc). To each PCR product (16-20 µl), 2 µl of 3M sodium acetate and 50 µl of 96% ethanol was added using a dispenser. The solution was mixed by vortex and centrifuged for 1 minute at 40 RCF, then left to stand 10-15 minutes at laboratory temperature. Then the samples were centrifuged again for 30 minutes at maximum speed (3700 rpm) in an Eppendorf *5804R Centrifuge*, rotor diameter 9.5 cm. The strips (in a stand) containing the samples were then opened, covered by folded cellulose sheets, and a lid was placed over the cellulose. The stand was then placed upside down into the centrifuge and centrifuged for 1 min at 40 RCF to drain the ethanol. Then 100 µl of 70% ethanol was added to each sample and the samples were centrifuged for 10 minutes at maximum speed. The supernatant was then discarded the same way as previously, using folded cellulose sheets. The stand with the opened strips was then placed into a flowbox (turned off) and left standing for 5-10 minutes and finally the drying was

finished by placing the strips in a thermomixer (Eppendorf) for 5 minutes at 65ºC. The DNA pellet was subsequently resuspended in 30-50 µl Milli-Q water.

Some samples were purified using the *Agencourt® AMPure® XP* kit (Agencourt Bioscience), following the enclosed protocol for 96 well format. This kit works with a suspension of magnetic balls, to which the desired DNA is ligated and after purification it is washed off. Milli-Q water was used as an elution buffer.

Samples which showed more bands in gel electrophoresis were purified using *MinElute® Gel Extraction kit* (Qiagen), following the enclosed protocol with slight modifications. To elute DNA from the MinElute column, 15 µl (instead of 10 µl) of prewarmed water (65°C) was added to the membrane. The column was then left to stand for 10 minutes (instead of 1 minute).

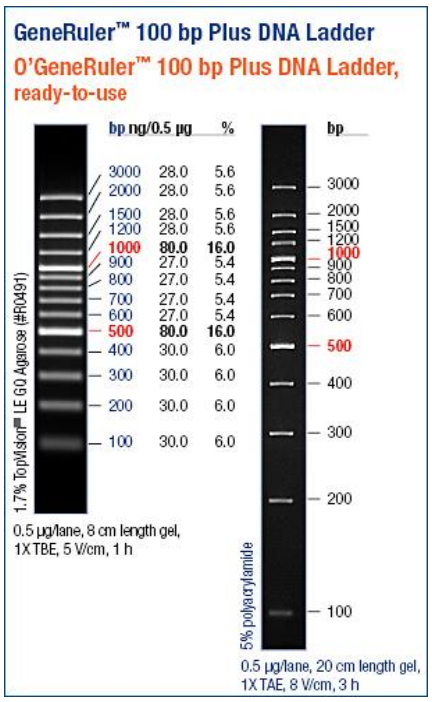

Figure 9. The O'GeneRuler 100bp Ladder Plus used in gel electrophoresis. (www.thermoscientificbio.com)

The concentration of the obtained DNA was measured on *Nanodrop 1000* (Thermo Scientific) spectrophotometer.

3.3.4 Sequencing reaction

The reaction was prepared to obtain a total volume of 8 µl. Each PCR product was diluted by Mili-Q water to the volume of 7 µl and 1 µl of the primer was added. For ITS, only the ITS4 primer was used; for *mat*K, five primers (K1F, mIR, m5R, mIF and mIF2_EZ) were used (see Fig. 8.); for DCS, the primers CHS-R1 and CHS-R2 were used (for the primer sequences, see Table 2.). For *mat*K, the K2R_EZ primer was used at first, but in most cases it did not produce good results, and was subsequently replaced by mIF2_EZ. However, in some sequences mIF2_EZ did not join with mIF, as quite a long part of the sequence at the end of mIF was bad quality/illegible and could not be used. The missing bases were replaced by Ns.

Sequencing was carried out in the DNA sequencing laboratory of Faculty of Science, Charles University in Prague on a *3130xl Genetic Analyzer* sequencer (Applied Biosystems) with 50 capillaries on POP-7 polymer (Applied Biosystems). The capillary electrophoresis was carried out in buffer with EDTA.

The *BigDye® Terminator v3.1 Cycle Sequencing Kit* was used with enhancing buffer BDX64 (MACLAB) and Sequencing buffer (Applied Biosystems). The samples were purified

by ethanol precipitation with 3 M sodium phosphate and resuspended in Hi-Di formamide (Applied Biosystems). The reaction consisted of 35 respective cycles: 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 3 min.

For *mat*K samples, the sequencing temperature was decreased, which remarkably improved the legibility of the sequences in most cases. Even so, the *mat*K sequences were often bad quality and the PCR and sequencing had to be repeated multiple times to achieve a legible sequence. Even so, some samples remain incomplete as some primers produced low quality sequences even after multiple attempts.

Some DCS sequences contained many double peaks. This was probably caused by multiallelic diversity. To be able to use those sequences, they would need to be cloned; unfortunately, due to time restrictions this was not possible and only a limited proportion of the dataset could be used in the analyses.

3. 4. Analysis of sequencing results

3.4.1. Sequence viewing, contig and alignment assembly

The sequences (obtained in *.ab1 and *.seq format) were viewed with the use of the program *FinchTV 1.4.0* (Geospiza, Inc.; http://www.geospiza.com/finchtv). Contigs were created from the forward and reverse sequences using the programs *SeqMan 5.06* (DNASTAR) and Geneious 7.0.6 (Biomatters Ltd.). The contigs (saved in *.fas, FASTA format) were then aligned using MAFFT algorithm (*Multiple Alignment using Fast Fourier Transform*; http://www.ebi.ac.uk/Tools/msa/mafft/) with default setting.

The alignment was further edited manually in *BioEdit 7.0.4.1* (http://www.mbio.ncsu.edu/bioedit/). Missing data was coded as N (any nucleotide) and discrepancies were coded in accordance with IUPAC coding (Appendix 3). Discrepancies which occurred in one sequence of the dataset only were considered to be polymerase errors and removed. Exceptions were made in some cases where sequences from GenBank or those acquired from Šída *et al.* (unpubl., Appendix 8) were used; discrepancies in these sequences were sometimes left unchanged after thorough consideration, on the premise that they originated from a reliable source. GenBank sequences were over 900 bp shorter than sequences of other samples; the missing part was coded the same way as missing data in other sequences, i.e. replaced with N.

3.4.2 Samples used in phylogenetic analyses

A Bayesian analysis was carried out using ITS sequences of the targeted group and all available samples of Alpinioideae to determine the position of *Amomum* and *Elettariopsis* within the subfamily. This included all samples of *Elettariopsis* and *Amomum*, and the samples downloaded from GenBank.

In the subsequent analyses, only samples belonging to the *Elettariopsis+Amomum* group as per Droop (2012) and Šída *et al.* (unpubl.) were used, along with some samples from Droop's *A. maximum* clade, which is sister to this group. The other *Amomum* group was not taken into consideration as it was not the objective of this thesis to resolve the relationships within *Amomum*.

Species used in the analyses are listed in Appendices 6.

3.4.3 Construction of phylogenetic trees

Alignments were converted from *.fas to *.nex and other formats using the online tool *Bugaco* (https://app.bugaco.com/converter/biology/sequences/).

Outgroup was chosen on the basis of the previous findings of Šída *et al.* (unpubl.) and contained representatives of the genera *Renealmia* and *Aframomum*. The species used are listed in Appendix 8 with other samples therefrom.

Program *jModelTest 0.1.1* (Posada 2008, Guindon and Gascuel 2003) was used to find the optimal evolution model for Bayesian and maximum likelihood analyses on the basis of the Akaike information criterion (AIC; Cavanaugh 2007).

For ITS, the chosen model was TPM3+G, which corresponds with the following commands in *PAUP*: Lset base=equal nst=6 rmat=(1.5015 7.4973 1.0000 1.5015 7.4973 1.0000) rates=gamma shape=0.3650 ncat=4 pinvar=0.

For *mat*K, the TPM1uf+G model was chosen and the set of commands is as follows: Lset base=(0.3452 0.1414 0.1468 0.3666) nst=6 rmat=(1.0000 1.5104 0.1437 0.1437 1.5104 1.0000) rates=gamma shape=0.0160 ncat=4 pinvar=0.

For DCS, the chosen model was HKY+G, and the commands are: Lset base=(0.1753 0.3217 0.3217 0.1813) nst=2 tratio=0.8906 rates=gamma shape=0.0160 ncat=4 pinvar=0.

PAUP 4.0b10* (Swofford 2003) was used to conduct incongruence length difference (ILD) tests (Farris *et al.* 1994) to determine whether it was possible to use concatenated datasets – ITS with DCS, and ITS with *mat*K. If the resulting *p-value* was lesser than 0.05, the datasets were considered incongruent (they prefer significantly different evolutionary

hypotheses). The concatenated datasets were also surveyed for incongruences in *SplitsTree 4.12.8* (Huson and Bryant 2006).

*PAUP** was also used to carry out maximum parsimony analyses. A heuristic search was done, with 100 random sequence additions and no more than 100 trees of length greater than or equal to 1 saved per replicate, TBR branch swapping, and the MulTrees option in effect. Support for the nodes was evaluated with bootstrap analyses (Felsenstein, 1985). A heuristic search was done, with the MulTrees option off, on 1000 bootstrap replicates with random addition of sequences (100 replicates for each bootstrap replicate) followed by TBR branch swapping.

For Bayesian inference, *MrBayes 3.2.2 x86* was used with 15 million generations, *nst* = 2, *rates* = gamma and *pinvar* = (all).

For maximum likelihood analyses, *GARLI 2.0 Web Service* (molecularrevolution.org Bazinet *et al.* 2014, Zwickl 2006) was used with 100 bootstrap replications, *ratematrix* = 2rate, *statefrequencies* = estimate, *ratehetmodel* = gamma and *invariantsites* = estimate.

For viewing the trees and for their further modifications (rooting, displaying bootstrap values, modyfing taxon names) *FigTree 1.4.0* (Rambaut 2006-2012, http://tree.bio.ed.ac.uk/software/figtree/) and *SplitsTree 4.12.8* (Huson and Bryant 2006) were used. The trees were then converted to *.pdf format and viewed in *Foxit Reader 6.2.0.0429* (Foxit Corporation 2004-2014), which was also used to map bootstrap values on maximum likelihood trees. Bootstrap values from MP and ML analyses were rounded to a whole number. Bootstrap values from ML analyses are given in decimal format, as they were in the bootstrap consensus tree.

Only the trees from Bayesian inference analyses are presented in the Results, on which the support from all three analyses (posterior probabilities from Bayesian inference, bootstrap values from maximum likelihood analysis and bootstrap values from maximum parsimony analysis) has been mapped.

3.4.4 ITS analysis including Alpinioideae

A Bayesian inference analysis was carried out on a dataset containing ITS sequence data of all samples of *Elettariopsis* and *Amomum* of the targeted group and all available Alpinioideae samples (Appendix 8; data and sequences obtained from Šída *et al.* unpubl.) to illustrate the position of the *Elettariopsis/Amomum* group in the subfamily. The analysis was done in *MrBayes* with the abovementioned setting on 158 taxa in total. Two samples of

Tamijia and two of *Siphonochilus* were chosen as outgroup. Posterior probability values higher than 0.95 were mapped above the branches.

3.4.5 Analyses of the individual markers

Three datasets of the individual marker sequences (ITS, *mat*K, and DCS) were analysed using Bayesian inference, maximum parsimony and maximum likelihood methods for ITS, *mat*K and DCS. There were 62 ITS, 58 *mat*K and 33 DCS samples analysed in total.

The ITS dataset contains the highest number of samples, as it was the easiest to obtain good quality sequences of this relatively short DNA Numerous accessions of *Elettariopsis* and *Amomum* ITS sequences are also found on GenBank, as this marker had been used in many of the major studies of the *Zingiberaceae* (e.g. Kress *et al.* 2002, Pedersen 2004, Xia *et al.* 2004, Droop 2012). Twelve accessions from GenBank which were also used in Jane Droop's thesis were included into the dataset (see Appendix 7 for a list of all GenBank sequences used in the analysis).

The number of samples in *mat*K was lower than in ITS, and some of the sequences remain partly incomplete. This is due to the difficulties experienced while sequencing the mat*K* primers (described above). Nine *mat*K sequences were downloaded from GenBank, namely those used by Droop (2012) which also had ITS counterparts.

There were no GenBank sequences used in the DCS dataset as no accessions of the DCS marker could be found on GenBank for *Elettariopsis* or the targeted *Amomum* species.

Two samples of *Renealmia* (*R. battenbergiana*, *R. polypus*) and two samples of *Aframomum* (*A. alboviolaceum*, *A. chrysanthum*) were used as outgroup. In the DCS analyses, only *Renealmia battenbergiana* and the two *Aframomum* samples were used, obtained from Šída *et al.* (unpubl.). Due to the low quality of many DCS sequences (see above), the number of samples is much lower than that in the ITS and *mat*K analyses.

*3.4.6 Analysis of the concatenated ITS and mat*K *dataset*

A test of incongruence (ILD; Farris *et al.* 1994) was carried out to determine if the ITS and mat*K* datasets could be analysed as a concatenated dataset. The resulting *p-value* was 0.003 at maximum capacity of 1000 trees, which signifies that the topologies are incongruent as it is lower than 0.05 (see above). However, when surveying the concatenated dataset in *SplitsTree* and the trees from the separate analyses of both datasets, no major incongruences in the clades were found; moreover, when the concatenated dataset was analysed the overall support was stronger than in separate analyses. With this taken into consideration, the

concatenated dataset was analysed by the three methods described above (Bayesian inference, maximum parsimony and maximum likelihood) to obtain a comprehensive phylogeny of the group.

For the modelling of genome size evolution (see below), sequences from GenBank were removed from the dataset as there was no genome size data available for those. A Bayesian inference analysis with the same setting was subsequently carried out on this dataset.

3.4.7 Modelling of genome size evolution

For the following analyses, program *R 3.1.1* (version for 64 bit Windows; R Core Team 2014) was used. A BI tree based on the concatenated ITS and *mat*K sequences and containing only sequences with known absolute genome size was used with a table of absolute genome size values matched to the names of the taxa in the tree in the exact same order.

3.4.7.1 Reconstruction of ancestral characteristics

For this analysis, a Bayesian inference tree containing only sequences with absolute genome size data was used. The reconstruction of ancestral genome size was carried out using the package *phytools* (Revell 2012) implemented in R. The function *contMap* (mapping the evolution of a continuous character on a tree) was used to plot a tree with mapped absolute genome size. It uses the function *fastAnc*, which estimates states at internal nodes of the phylogeny with the use of ML methods, and interpolates the states along each edge using Felsenstein's equation [2] (Felsenstein 1985, cited in Revell, in press.). Finally, the function *writeAncestors* was used to create a tree (*.tre) file with ancestral character states stored as node value (Revell, in press.). The set of commands used (developed by T. Fér) is enclosed in Appendix 4.

3.4.7.2 Estimation of lambda, kappa *and* delta *parameters*

The parameters for inferring evolutionary change were estimated using the *generalized least squares* method with the package *caper* (Orme *et al.* 2013) implemented in R. The functions *comparative.data* (*comparative dataset creation*; this function matches rows of data to the tips of the phylogeny) and *pgls* (*phylogenetic generalized linear models*; this incorporates the covariance between the taxa into the calculation of estimated coefficients) were used. A table of absolute genome size values for each taxon, in the same order as the taxa in the tree and saved in *.txt format was used along with the BI tree in *.nwk (newick) fomat. A conversion from newick to nexus (*.nex) format is implemented in the set of

commands (see Appendix 5; commands were developed by T. Herben, modified by T. Fér). The *pgls* function fits a linear model on phylogenetically non-independent data; the strength and type of the phylogenetic signal in the data matrix can also be accounted for by adjusting branch length transformations (lambda, delta and kappa) (Orme *et al.* 2013).

For estimating λ, κ and δ, a set of commands (Appendix 5) was used to estimate the parameters for the given data, plot the likelihood surface with a 95% confidence interval and save the outcome as a "lambda/kappa/delta.png" file.

3.4.7.3 Genome size distribution in Elettariopsis

The genome size of the two groups (Group G and Group H) in *Elettariopsis* was statistically compared using *PAST 2.17* software (Hammer *et al.* 2001). A boxplot diagram was constructed to show the variation in absolute genome size (2C) in the two groups.

3.4.8 Mapping the geographical distribution of **Elettariopsis** *s. l.*

The localities of samples belonging to the targeted group (all *Elettariopsis* with closely related *Amomum* species together with the *A. maximum* group as defined by Droop, 2012) were researched or obtained from J. Leong-Škorničková (SBG) and O. Šída. The aim was to obtain localities as exact as possible (ideally, GPS coordinates), but in some cases this was not possible and the sample was only localised on the country level. These samples were not mapped; all the samples that were mapped had at least the province specified.

Three major groups in the targeted group (chosen on the basis of phylogeny reconstruction; see Results Fig. 11) were visualised on a map of South-East Asia using the software *GoogleEarth 7.1.2.2041* (Google, Inc. 2013). The groups (defined in Results, chapter 3.3.) are coloured as follows: group C (*A. maximum*) – orange, *A. repoeense* – turquoise, *A. subulatum/A. petaloideum* – red, group F (*E. curtisii, E. kerbyi, E. stenosiphon*) – green, group G (*E. triloba*, *E. elan, E. unifolia*) – dark blue.

4. Results

4.1 Analyses and materials used

ITS, *mat*K and DCS sequence data from 62 taxa in total were analysed together with 13 sequences from GenBank used previously by Jane Droop (2012). 95 sequences were obtained from Šída *et al.* (unpubl.). For some samples, only ITS sequences existed; the *mat*K dataset was smaller due to obstructions which arose during sequencing (see Methods II.4.1). For the same reasons, only a sub-dataset could be used for analyses of the DCS region, and as such it may not be very informative. There were too few sequences in DCS, so concatenation was not reasonable as it would not bring any improvement in the results (also, the data proved incongruent upon performing an ILD test).

Most taxa were collected on research expeditions to Vietnam, Laos, and Thailand, while other countries of its distribution (such as Malaysia, Indonesia, China) were rather poorly sampled, which may have led to a bias in biogeographical conclusions. For some taxa, the exact localities were unknown (see Appendix 6); those were not mapped unless at least the province was specified.

The three separate datasets and a concatenated dataset of ITS and *mat*K were analysed using Bayesian inference (MrBayes), maximum parsimony (PAUP) and maximum likelihood (GARLI). The results of the analyses are presented here using Bayesian majority rule tree as a representative phylogeny with posterior probability (PP) mapped on the corresponding branches supported with PP>0.95. Bootstrap support values from MP and ML analyses are mapped on the corresponding branches of the Bayesian tree to enable direct comparison of performed analyses. The bootstrap values of MP analysis are rounded up to a whole number.

A dataset comprised only of own sequences and sequences obtained from Šída *et al.* was used in genome size evolution analyses, as no genome size data exists for the sequences downloaded from GenBank.

4.2 Phylogeny of the Alpinioideae based on ITS sequence data (Fig. 10)

The group of interest (*Elettariopsis* s. l.) which includes all *Elettariopsis* species together with three species of *Amomum* (*A. subulatum*, *A. petaloideum* and *A. repoeense*) is forming a well-supported monophyletic clade (further called **clade B**) within the bigger group involving the genera *Elettaria*, *Aframomum* and *Renealmia* and one of the *Alpinia* species in an unsupported relationship (**clade A**). The remaining species of *Amomum* form a well-

supported distinct group closely related to the genera *Hornstedtia* and *Etlingera*, making the genus *Amomum* paraphyletic.

4. 3 Phylogeny of *Elettariopsis* s. l. based on ITS, *mat*K and concatenated data

4.3.1 ITS phylogeny (Fig. 11)

All three analyses used for phylogenetic reconstruction based on ITS data (MP, ML and Bayesian analysis) resolved similar structures of basic relationships differing mainly in the BS and PP values for particular groups. The results of the analyses are presented in Fig. 11 using Bayesian majority rule tree as a representative phylogeny with posterior probability (PP) mapped on the corresponding branches supported with PP>0.95. Bootstrap support values from MP and ML analyses are mapped on the corresponding branches of the Bayesian tree to enable direct comparison of performed analyses. The bootstrap values of MP analysis are rounded up to a whole number.

An ingroup, strongly supported in relation to the outgroup (composed from the genera *Aframomum* and *Renealmia*) comprised all *Elettariopsis* species and all species of *Amomum* generally characterised by winged fruits and Indochinese origin (Vietnam, Laos, and Thailand) (100% BS, 1.00 PP). Within the ingroup, there are two well-supported main lineages sister to each other. The first (labelled as **C** in Fig. 11; PP 1.00, MP BS 96, ML BS 0.94) contains species belonging to the *A. maximum* clade (defined in Droop 2012) and the second lineage (labelled as **D** in Fig. 11; PP 1.00, MP BS 94, ML BS 0.94) includes *Amomum subulatum*, *A. petaloideum*, species from the *A. repoeense* clade (defined in Droop 2012), and all *Elettariopsis* species (*Elettariopsis* s. s., further referred to as **group E**).

The internal structure of lineage D is poorly resolved based on ITS data only (but see the concatenated dataset in Fig. 11) and four main groups can be recognized here (*A. repoeense*, *A. subulatum/petaloideum*, and two groups of *Elettariopsis* – **group F** and **group G**). Further structure within the particular groups will be described below based on the concatenated dataset.

The overall support is higher than in *mat*K analyses, and there are more well-supported groups. The *A. repoeense* group, which here is well-supported (PP 1.00) is in *mat*K only moderately supported (PP 0.87) as sister to *Elettariopsis* s. s. (group E). The *A. subulatum/petaloideum* group, which is here strongly supported (PP 1.00) as basal in

relation to group E is in *mat*K still strongly supported as a clade, but falls in between the *A. maximum* group as sister to its members.

Group C and Group D, which here are strongly supported (PP 1.00), do not form distinct groups in *mat*K. The *E. unifolia* group (containing also the sample *E. sp.* Z329), which here has support PP 1.00, is in *mat*K supported only moderately (PP 0.81). The well-supported clade formed by *E. sp.* samples Z641 and Z643, which here is sister to the rest of the species in *Elettariopsis* II group, are sister to each other in *mat*K.

4.3.2 mat*K phylogeny* (Fig. 12)

In the *mat*K Bayesian analysis, the ingroup is strongly supported in relation to the outgroup (PP 1.00) and contains both lineages of the ITS analysis (C and D). The *mat*K region was overall less variable than the ITS one.

The *Elettariopsis* s. l. (group E) is here supported only in BI (PP 0.96), while in the MP and ML analyses it is not supported as a separate group and forms a group with There is no clear distinction between groups G and H as defined in ITS, and the clades contained in those two groups are mostly sister to each other.

4.3.3 Phylogeny based on concatenated ITS and mat*K dataset* (Fig. 10)

Prior to using the concatenated dataset from ITS and *mat*K data, a test of incongruence (ILD; Farris *et al.* 1994) was run to determine if the ITS and mat*K* datasets could be analysed as a concatenated dataset. The resulting *p-value* was 0.003 at maximum capacity of 1000 trees. Therefore, $p < 0.05$, which signifies that the two regions prefer significantly different evolutionary hypotheses (are incongruent). However, as can be seen from the evidence written above, there are no incongruences which would have a strong influence on the major group level; therefore the dataset was joined and analysed together. Moreover, when the concatenated dataset was analysed the overall support was stronger. Three analyses of the concatenated dataset were carried out (Bayesian inference, maximum parsimony and maximum likelihood).

The major structure of this phylogeny (Fig. 10) is basically the same as in ITS and *mat*K, but some groups have better support. The detailed structure of this phylogeny is described below. The first well-supported group (PP 1.00, ML BS 1.00) is group D (*Elettariopsis* s. l.) which is sister to the *A. maximum* group. In the MP analysis, however, this Group Gas only poor support (BS 49.5). *Amomum* in group D forms three distinct groups which correspond with those described by Droop (2012): the most basal, weakly supported

A. maximum group, a well-supported (MP BS 06.1á, PP 1.00, ML BS 0.94) *A. repoeense* group and a well-supported (PP 1.00, MP BS 99.9, ML BS 1.00) *A. subulatum/petaloideum* group.

Figure 10. A BI phylogeny (major rule consensus tree) based on ITS sequence data and including the targeted group together with the Alpinioideae dataset. Posterior probabilities over 0.95 are mapped above branches.

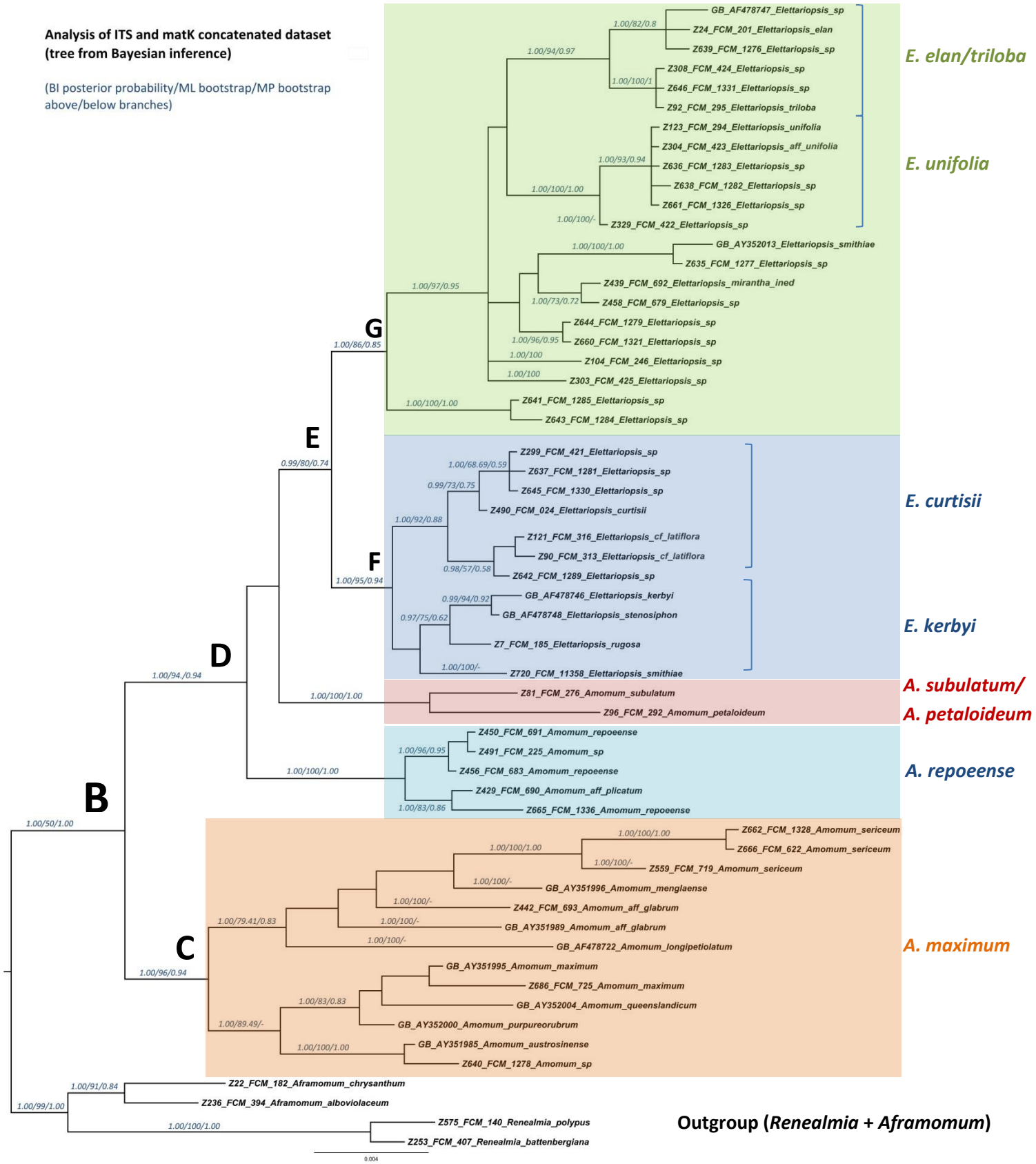

Figure 11. A phylogeny based on a concatenated dataset of ITS and *mat*K sequence data, showing the main groups in clade B The tree shown is the major rule consensus from BI. Mapped above/below branches are the values of posterior probability/MP bootstrap/ML bootstrap.

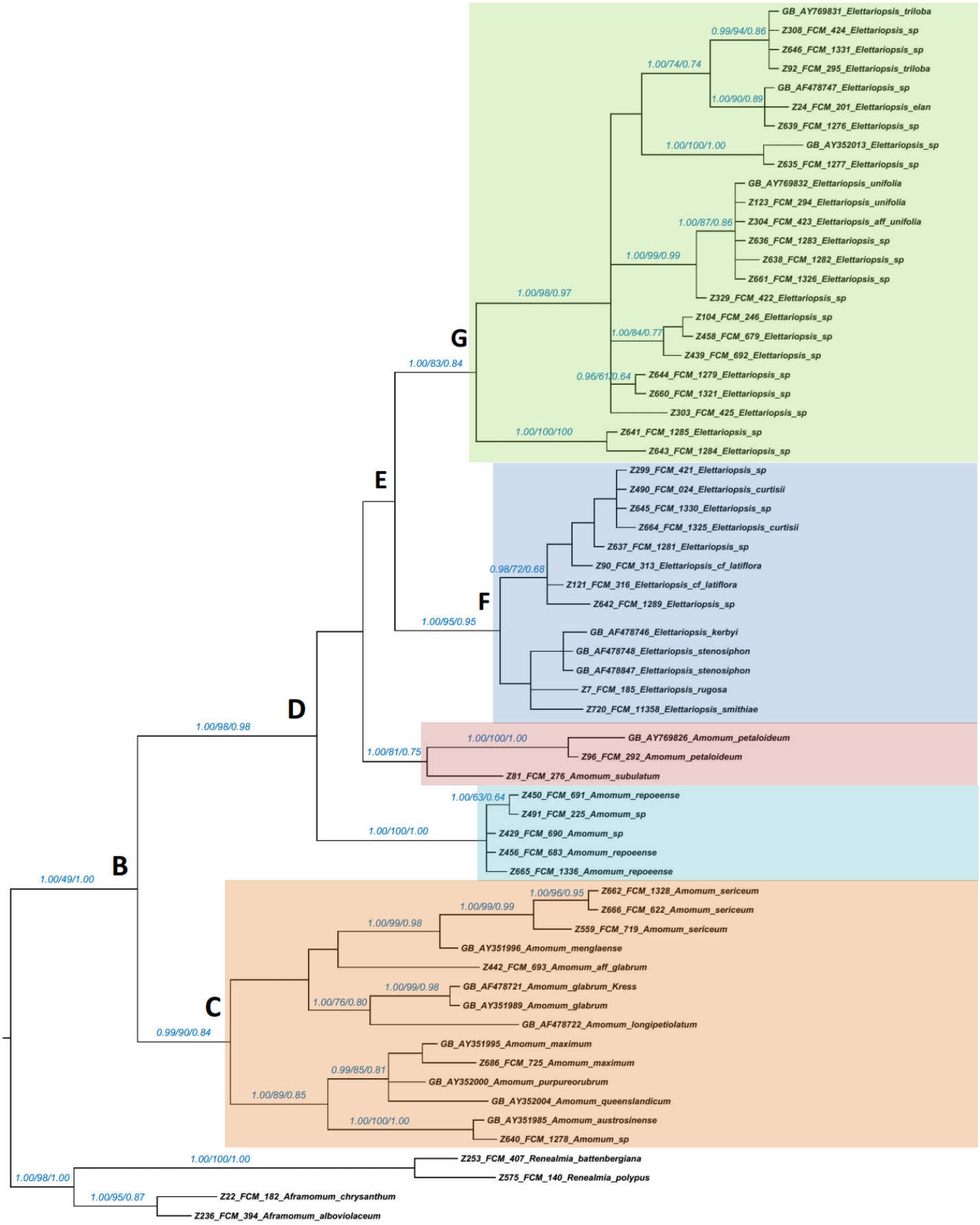

Figure 12. A phylogeny based on ITS sequence data. The tree shown is the major rule consensus from BI; PP/MP BS/ML BS values are mapped above branches. The groups are highlighted as defined in Fig. 10.

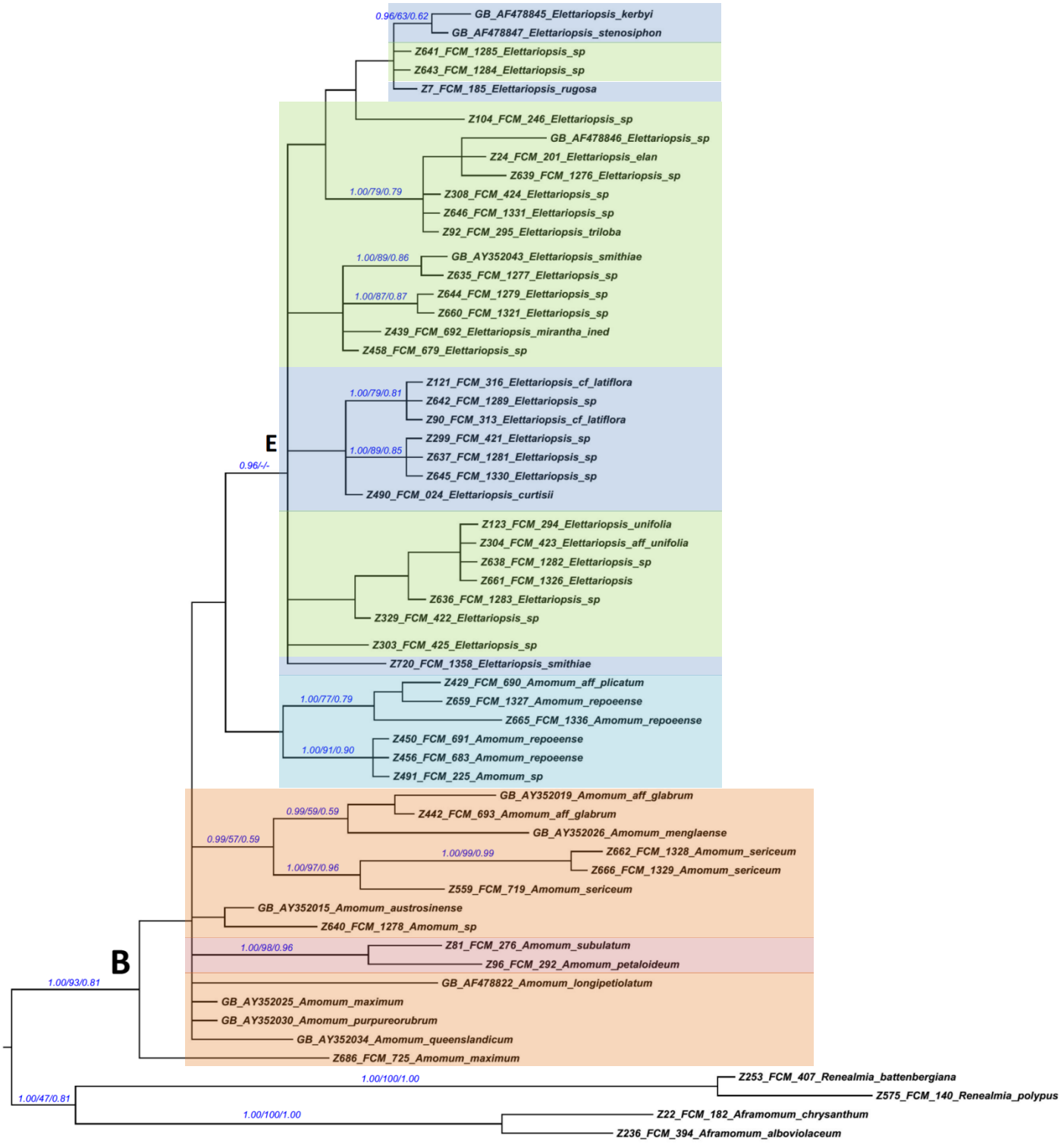

Figure 13. A phylogeny based on *mat*K sequence data. The tree shown is the major rule consensus from BI; PP/MP BS/ML BS values are mapped above branches. The groups are highlighted as defined in Fig. 10.

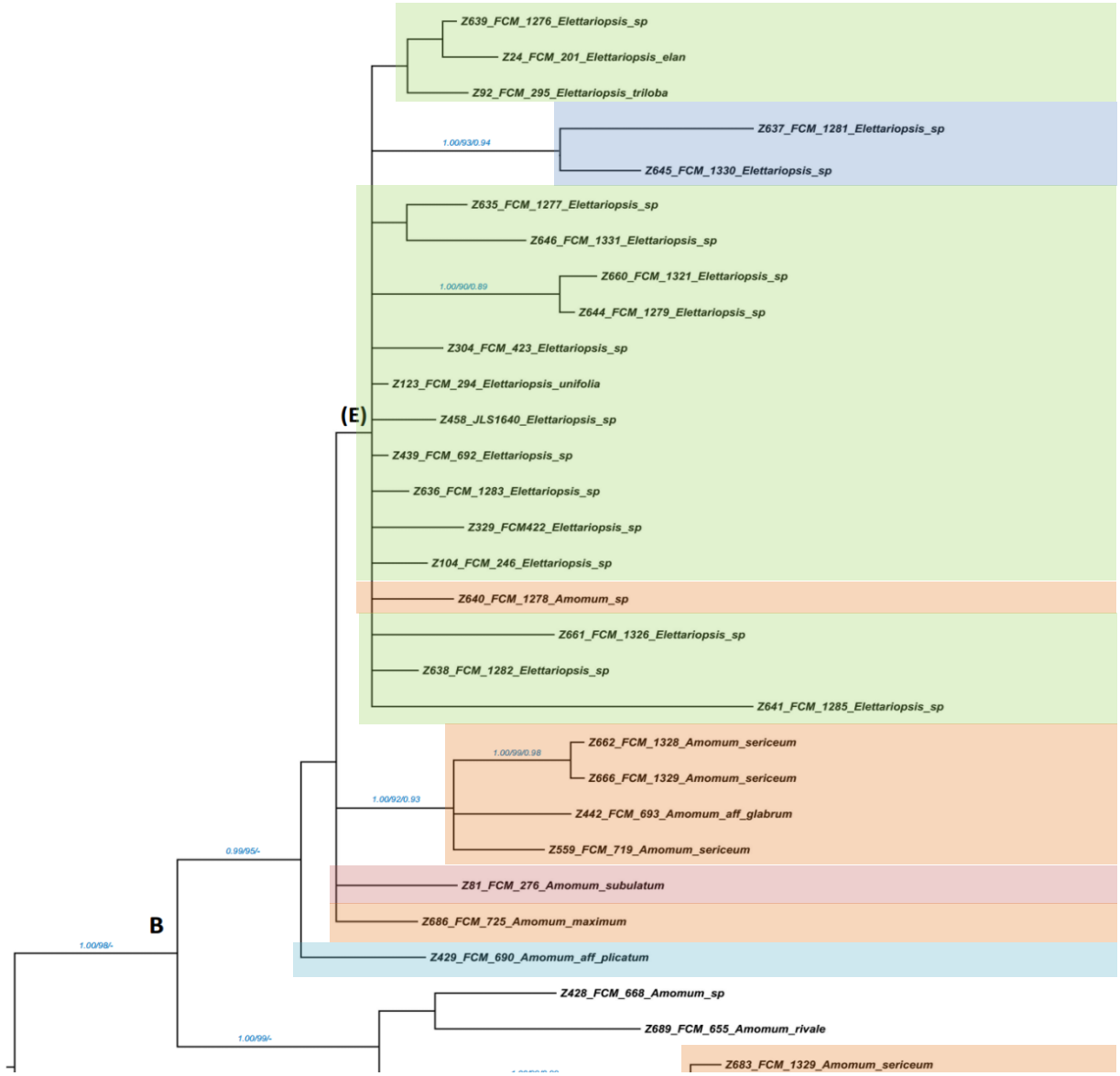

Figure 14. A phylogeny based on DCS sequence data. The tree shown is the major rule consensus from BI; PP/MP BS/ML BS values are mapped above branches. The groups are highlighted as defined in Fig. 10.

Elettariopsis s. l. is weakly supported in BI and MP (PP 0.52, MP BS 52.26), whereas the support in ML is quite strong (BS 0.94). *Elettariopsis* s. s. (group E) forms a strongly to moderately supported group (PP 0.99, MP BS 79.92, ML BS 0.74), which is not supported in the MP and ML analyses of *mat*K (Fig. 13), nor in the analysis of DCS (Fig. 14).

The *Elettariopsis* s s. group divides into two groups (group F, group G) with strong support (PP 1.00 and 1.00, MP BS 86.1 and 94.66, ML BS 0.85 and 0.94), which contain several seemingly monophyletic, strongly supported lineages. The more basal of these groups (further referred to as **group F**) is well-supported (PP 1.00, MP BS 95, ML BS 0.95) and contains *E. curtisii, E. kerbyi, E.* stenosiphon, *E. rugosa* and *E. smithiae*. The other group (further referred to as **group G**) contains two lineages: a moderately supported (PP 1.00, MP BS 94, ML BS 0.97) group formed by *E. elan, E. triloba, E. unifolia* and several *E. sp.* samples, and a strongly supported (PP 1.00, MP BS 100, ML BS 1.00) group formed by the two *E. sp.* samples Z641 and Z643.

These two groups are well-supported in the analysis of ITS of both the *Elettariopsis/Amomum* group and the Alpinioideae (in ITS, the support is high for group F an moderate for group G; Figs. 10 and 12) but in *mat*K they fall apart into several unsupported groups (Fig. 13). The DCS analyses produced results similar to those of *mat*K, but due to the insufficient sampling these results are not very reliable.

4.3.3.1 Group F

This group contains a moderately supported group of *E. curtisii* and affiliated *E. sp.* samples (PP 1.00, MP BS 92, ML BS 0.88), and an unsupported group formed by *E. kerbyi*, *E. stenosiphon*, *E. rugosa*, and *E. smithiae*.

The *E. curtisii* lineage consists of two groups. The first, with moderate support (MP bstp 71.58) contains two sister *E. curtisii* samples from Trầng Province in Thailand and a small, weakly supported (PP 1.00, but MP BS 69, ML BS 0.59) group formed by three *E. sp* samples (Z299, Z637 and Z645), all originating from Lâm Đông Province, Vietnam.

The second group is unsupported and contains two samples labelled as *E. cf. latiflora* (Z121 and Z90) and the sample *E. sp.* Z642. In the *mat*K analysis (Fig. 13) they form a moderately supported (PP 1.00, MP BS 79, ML BS 0.81) group with Z642, whereas in ITS Bayesian analysis (Fig. 12) Z90 is a part of a moderately supported (PP 0.98, MP BS 72, ML BS 0.68) group with *E. curtisii* and Z121 and Z642 are sister to each other. It should be noted here that the only about half of the sequences using sequencing primer of ITS4 of both Z90 and Z121 samples is good quality, and that both sequences become illegible at the same

position. All three samples originate from Singapore, where Z90 grows at Bukit Timah, while Z121 and Z642 are found at the Nature Trail near MacRitchie Reservoir. According to J. Leong-Škorničková (pers. comm. May 2014), samples Z121 and Z90 supposedly belong to the species *E. latiflora* Ridl. This species is currently not accepted and considered a synonym of *E. curtisii*, and it may be necessary to restore it. Before doing this, however, it is necessary to analyse the DNA of a specimen from the type locality of *E. curtisii* first; unfortunately this could not yet be done in these analyses, but it will be a part of future research.

The *E. kerbyi* lineage is composed of two groups. The sample Z720 (*E. smithiae*) alone is strongly supported (PP 1.00, MP BS 100) as basal to the remaining samples, while a weakly supported (PP 0.97, but MP BS 75, ML BS 0.62) group is formed by the remaining three species. *E. kerbyi* and *E. stenosiphon* are moderately supported as a group (PP 0.99, MP BS 94, ML BS 0.92). Most samples in group F had been collected in Malaysia; *E. stenosiphon* originates from Indonesia (Droop 2012).

In the ITS analysis (Fig. 12) the *E. kerbyi* lineage is moderately supported (PP 0.98, MP BS 82, ML BS 0.68), but the overall internal pattern does not differ much from the combined ITS and *mat*K analysis. In *mat*K (Fig. 13), however, this lineage is not supported. In the separate analyses, the support for *E. kerbyi* and *E. stenosiphon* is much stronger (ITS pp 0.95, matK pp 0.96).

4.3.3.2 Group G

This group contains a strongly supported basal lineage formed by *E. sp* samples Z641 and Z643, and several moderately to strongly supported groups formed by *E. elan*, *E. triloba*, *E. unifolia* and several *E. sp.* samples.

In the ITS analysis the overall pattern in this group remains very similar with a few slight variations, and the support is slightly higher, while in the *mat*K analysis the lineages are still strongly to moderately supported by themselves but do not cluster together.

The Z641+Z643 lineage is strongly supported (PP 1.00, MP BS 100, ML BS 1.00) and consists of two undescribed samples, both originating from Malaysia. In the ITS analysis this group is fully supported in BI, MP and ML, while in the *mat*K analysis both samples are sister as part of an unsupported group with *E. kerbyi*.

The *E. elan/triloba/unifolia* lineage is well-supported (PP 1.00, MP BS 97, ML BS 0.95) and consists of three strongly supported groups and several *E. sp.* samples which do not cluster together. The sub-groups of this group are described in detail below.

The E. elan/E. triloba subgroup. This group is moderatelz supported (PP 1.00, MP BS 94, ML BS 0.97) and consists of two smaller groups formed by *E. elan* and *E. triloba*. The group formed by *E. triloba* is well-supported (PP 1.00, MP BS 100, ML BS 1.00) and contains a sample of *E. triloba* together with two *E. sp.* samples. All three samples had been collected in Đông Nai Province in Vietnam. The second small group is less supported (PP 1.00 but MP BS 94, ML BS 0.80) and contains an *E. elan* sample and two *E. sp.* samples (Z639 and GB_AF478747). *E. elan* (Z24) and *E. sp.* Z639 were collected in Malaysia. The third sample is listed in Jane Droop's work (2012) as originating from Myanmar, but in the NMNH collection database (http://collections.nmnh.si.edu) its origin is Singapore. Unfortunately, no excact location is known, so it is unclear if this is a sample from the collections or if it was collected in the wild.

In the ITS analysis (Fig. 12) the groups and their composition do not change, but the support is higher; in the *mat*K analysis the *E. elan* group is maintained but unsupported, and the samples of the *E. triloba* group remain sister to each other.

The E. unifolia subgroup. This is also a well-supported group (PP 1.00, MP BS 100, ML BS 1.00) containing a moderately supported (PP 1.00, MP BS 93, ML BS 0.94) group consisting of *E. unifolia* and affiliated *E. sp.* samples, and the sample *E. sp.* Z329, which is strongly supported as basal to this group. The same arrangement is maintained in the ITS analysis. In the *mat*K Bayesian analysis a group is formed by the same species, but with no support.

Z644 and Z660. A well-supported (PP 1.00, MP BS 96, ML BS 0.95) group is formed by *E. sp.* samples Z644 and Z660. These two samples form a well-supported group also in the ITS and *mat*K analyses, and also have similar genome sizes (12.7 pg and 13.01 pg). These are probably two specimens from the same population or even two clones of the same plant, as one of these samples comes from SBG and one from RBGE, but both were collected by Trần, H.Đ in the Bolikhamsai province in Laos.

Other samples: E. mirantha, ined. (Z439) and *E. sp.* samples Z104, Z303, Z458 and Z635 are each strongly supported as sister to the groups described above. Most of these specimens were collected in Vietnam, in provinces Quảng Nam, Khánh Hòa, Kon Tum and Thừa Thiên - Huế. *E. sp.* Z635 originates from Laos. In the ITS analysis, "*E. mirantha*", ined. forms a well-supported group with a lineage consisting of Z458 and Z104, which are considered to be a new species (J. Leong-Škorničková, pers. comm. May 2014). Z104 (which has flowered in the PBG) resembles "*E. mirantha*" morphologically (Leong-Škorničková, pers. comm. July 2014). Both Z104 and Z458 have genome size of approximately 8 pg. In

*mat*K Bayesian analysis Z104 falls with weak support as sister to the *E. kerbyi/E. stenosiphon* group, while Z439 and Z458 are sister to the Z644+Z660 group.

4.3.4 Phylogeny based on DCS

As was already mentioned in Methods (chapter 2.3.4.), the sequencing of DCS was problematic and only a limited dataset could be obtained. To obtain a better resolved phylogeny, some samples from the other group of *Amomum* (as defined by the ITS analysis of the Alpinioideae) were also included. These samples form a distinct basal clade in the phylogeny, while the resolution of clade B (*Elettariopsis* s. l. together with *A. maximum* clade) is similar to that of *mat*K, but less supported. Most samples of the targeted group included in the dataset belong to Group G.

A test of incongruence (ILD; Farris *et al.* 1994) was run with ITS and *mat*K datasets to determine whether the sequences could be used in a concatenated dataset. The resulting values were p = 0.003 for ITS and p = 0.001 for *mat*K, which in both cases indicates that the two data sets are incongruent. Here the data was not further considered for concatenation as the sampling of DCS was poor and it would probably not improve the topology in any way.

4.4 Modelling of genome size evolution

The absolute genome size (2C) was reconstructed on a BI phylogeny from concatenated ITS and *mat*K data without GenBank accessions, as there was no absolute genome size data available for those. Subsequently, the parameters of character evolution were estimated and a boxplot was generated in *PAST 2.17* (Hammer *et al.* 2001) to show the differences in genome size in groups F and G.

The reconstruction of absolute genome size evolution (Fig. 15; created in the *phytools* package in R; Revell 2012) shows that group B (*Elettariopsis* s. l. with the *A. maximum* clade) has greater absolute genome size than the outgroup. Group E (*Elettariopsis* s.s.) is clearly divided as group F has overall smaller genome size than group G.

In group B, the sample with the largest genome size was *E. sp.* Z303 (2C = 15.52 pg) and the sample with the smallest genome size was *A. subulatum* (2C = 3.50). The median was 2C = 6.28 pg and mean 2C = 6.76 pg (values calculated in MS Excel 2010).

In group E, the sample with the smallest genome size was *E. sp.* Z637 (5.39 pg), the median was 2C = 6.42 pg and the mean was 2C = 7.38 pg.

For better illustration of absolute genome size variation in the two groups, a boxplot diagram created in *PAST 2.17* is shown below (Fig. 16).

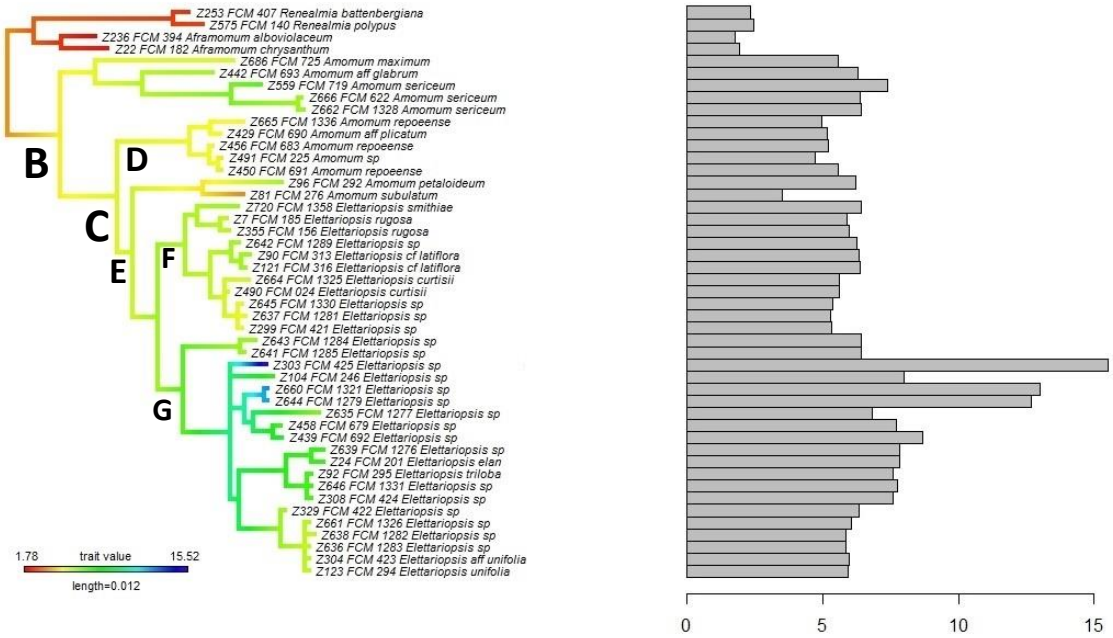

Figure 15. A reconstruction of absolute genome size (2C) evolution, visualised on a Bayesian inference phylogeny in R with the use of ML methods. Barplots on the right represent absolute genome size in individual taxa.

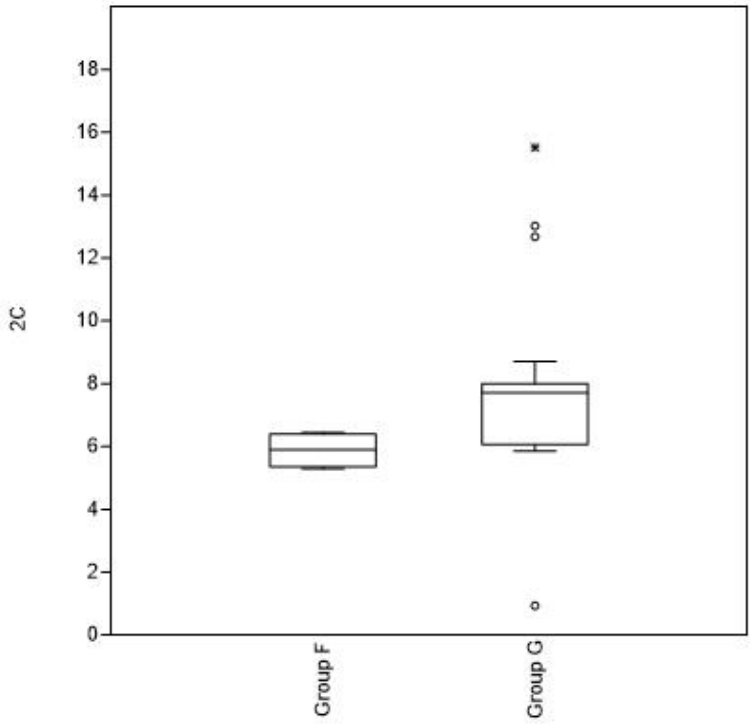

Figure 16. A boxplot made in PAST showing the absolute genome size in group F and group G.

The *lambda*, *kappa* and *delta* parameters were estimated in *caper* (Orme *et al.* 2013; Fig. 17) as can be seen in Table 3 below. The estimate of *lambda* (λ) was equal to 1, which

suggests that topology plays an important role in the evolution of absolute genome size in group B. The estimate of *kappa* (κ) was higher than 1, indicating that the longer branches have more influence on evolution. *Delta* (δ) was also estimated as higher than one, which suggests adaptation and constant change in absolute genome size over time rather than saltation or adaptive radiation.

Table 3. Estimates of the *lambda*, *kappa* and *delta* parameters and their confidence intervals in *caper* package.

Parameter	Estimate by *caper*	95% confidence interval
λ	1.000	0.993-NA
κ	1.777	1.315-2190
δ	1.462	0.491-2.524

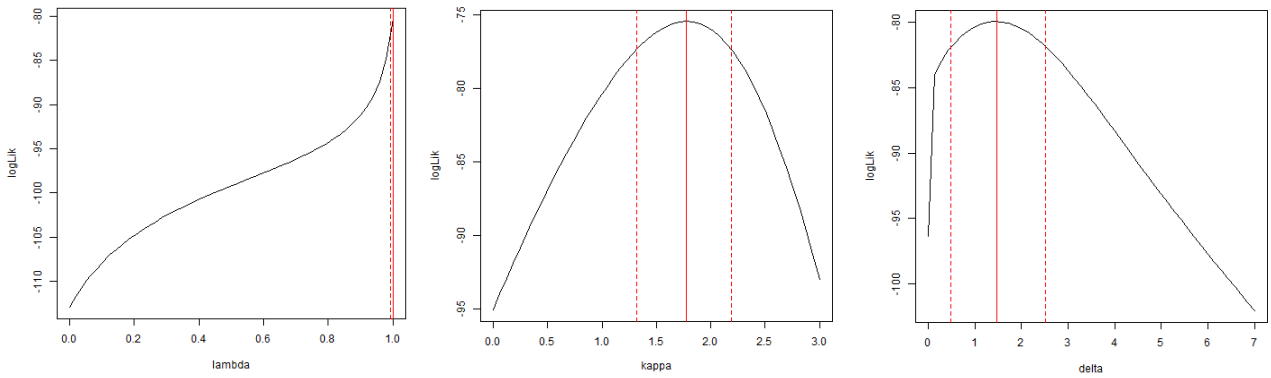

Figure 17. Plots of the parameters *lambda*, *kappa* and *delta* respectively, generated using the *caper* packege in R, for absolute genome size (2C). The 95% confidence interval is represented by red dotted lines.

The ancestral values of absolute genome size were estimated also in *phytools* (Revell 2012) and mapped on a BI phylogeny from concatenated ITS and *mat*K regions (the same as the one used for visualising genome size) using the command *writeAncestors*. The resulting tree can be seen in Fig. 18. As can be seen from this estimation, there is an increasing trend in absolute genome size evolution.

The ancestral absolute genome size is estimated to be 2C = 4.9 pg for group B (*Elettariopsis* s. l. and *A. maximum* clade), 2C = 5.51 pg for group D (*Elettariopsis* s. l.) and 2C = 5.74 pg for group E (*Elettariopsis* s. s.).

Within group D, there is a decrease in the *A. subulatum*/*A. petaloideum* group and increase in group E, which agrees with the absolute genome size data in Fig. 15 and suggests that the genome size in *Elettariopsis* has increased.

The evolution of absolute genome size has clearly been different in groups F and G, as group F shows a decrease in the estimated ancestral values (from 7.36 to 6.47) and group G shows an increase (from 7.36 to 9.87). Group G, where the sample with the highest absolute genome size (*E. sp.* Z303) is found, shows further increase in some lineages (such as the one formed by *E. sp.* species Z644 and Z660) and decrease in others (such as the *E. unifolia* lineage), but the overall trend is decreasing.

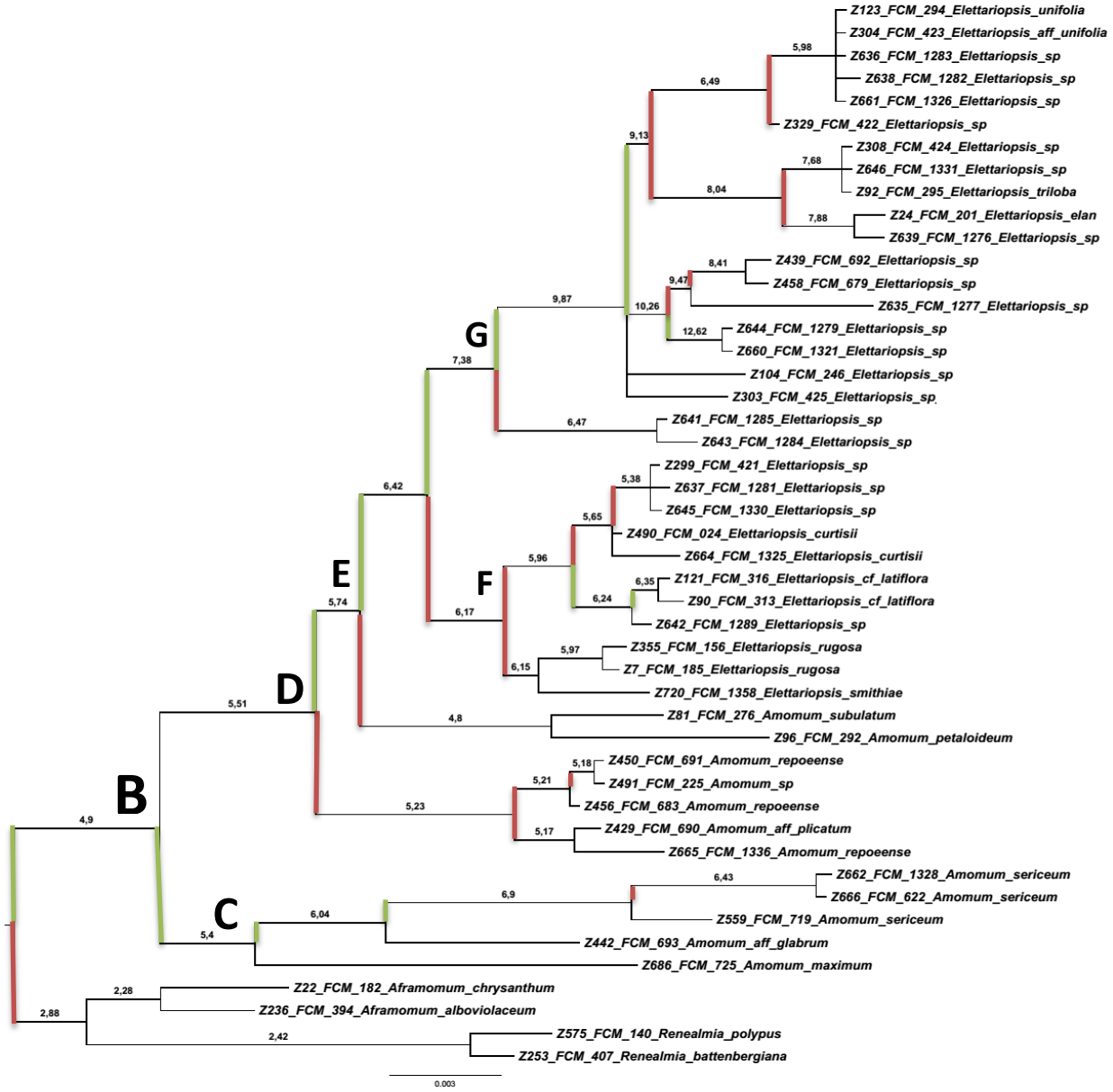

Figure 18. A Bayesian inference phylogeny with estimates of the ancestral states of the absolute genome size (2C) mapped on the branches using the package *phytools* in R. Green and red lines on branches show increase and decrease in the absolute genome size, respectively.

4.5 Geography

The localities of samples used in the analyses (see Appendix 6) were recorded onto a map of South-East Asia (Fig. 19). It can be seen that the representatives of the *A. maximum* clade (orange) together with *A. petaloideum* and the type species of *Amomum*, *A. subulatum* (red) are the northernmost distributed groups (*A. subulatum* being the only species found in India), while the representatives of *Elettariopsis* (dark blue) together with the *A. repoeense* subgroup (turquoise) are distributed more in the south-east. Samples of *Elettariopsis* form two distinct groups in terms of geography: group F (dark blue) has a more northern distribution and does not occur in southern Thailand and peninsular Malaysia, while group G (green) is distributed chiefly in the south and reaches to Singapore and Borneo.

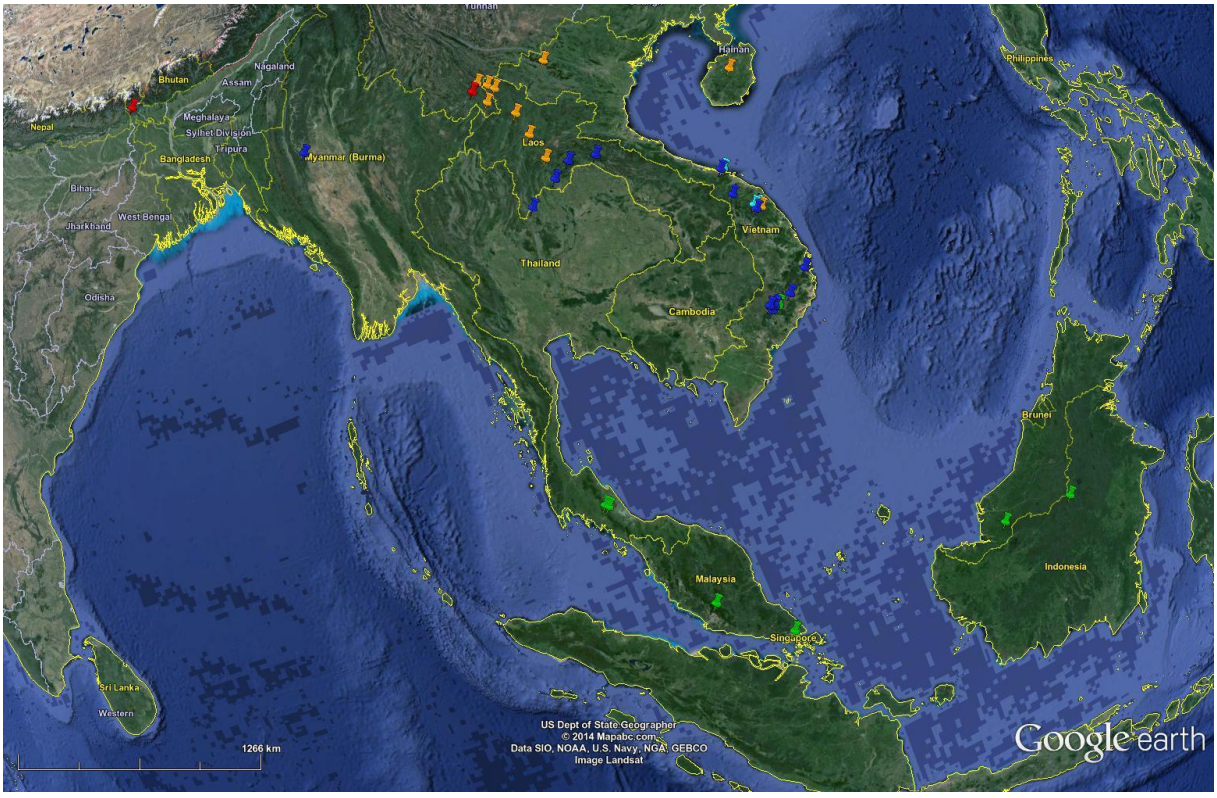

Figure 19. The geographical distribution of Group B (*Elettariopsis* s. l. and *A. maximum*). The legend is as follows: group C – orange, *A. repoeense* group – light blue, *A. petaloideum* + *A. subulatum* – red, group F – dark blue, group G – green.

5. Discussion

A dataset containing samples of *Elettariopsis* and several *Amomum* species was analysed with the use of flow cytometry, molecular, phylogenetic, and statistical methods. For the first time, *Amomum subulatum* Roxb., the type species of the genus *Amomum*, has been included into the analysis to elucidate its position in the one or another main lineages of *Amomum*. A well-resolved phylogeny was obtained based on the concatenated dataset of the ITS and *mat*K regions. Phylogenetic relationships were compared with patterns of absolute genome size and its ancestral states and with the geographical distribution of the targeted group. The results of these analyses are discussed below.

Unfortunately, many specimens used in this thesis are undetermined or are determined only provisionally. Therefore, the main objective of the molecular phylogeny reconstructions presented here is to observe the structure in the group of interest to see the relationships between these "undetermined species", i.e. how they cluster together. The next step then would be a classification of these specimens and their sorting into different species using the results of molecular analyses. Of course, morphological characters should not be overlooked during this determination; however, for this it is necessary to have a specimen available in a greenhouse and observe it in flower (and, ideally, in fruit), which is often difficult to achieve. Many specimens have not yet been seen in flower, and the number of these increases with new field expeditions, which further complicates the matter.

5.1 Position of *Elettariopsis* within the Alpinioideae and the polyphyly of *Amomum*

A set of ITS sequences for 157 taxa containing the *Elettariopsis* s. l. dataset and all available sequences of the Alpinioideae (obtained from Šída *et al.* unpubl.; Appendix 8) was analysed to determine the position of *Elettariopsis* and the examined *Amomum* specimens in the subfamily. The resulting phylogeny (Fig. 10) clearly shows that *Amomum* is polyphyletic and divided into two separate groups of which one can be found in the "*Renealmia* clade" of Kress *et al.* (2002) together with *Elettariopsis*, *Aframomum* and one species of *Alpinia* and the other one is separated and sister to *Etlingera* and *Hornstedtia.*

Within the "*Renealmia* clade", **clade B** (*Elettariopsis* together with *A. subulatum, A. petaloideum* and *A. repoeense*) forms a strongly supported monophyletic clade together with the *A. maximum* group (defined by Jane Droop, 2012; **clade A**). These results agree with the

previous findings of Kress *et al.* (2002), Xia *et al.* (2004), Droop (2012), and Šída *et al.* (unpubl.) and confirm the position of *Elettariopsis* within the *Renealmia* clade of Kress *et al.* (2012). *Amomum subulatum* Roxb., the type species of *Amomum*, has not been used in the previous studies; however, it was expected by Jane Droop (2012) to fall in the *A. maximum* clade, as it has winged fruits. This is confirmed here, as *A. subulatum* forms a well-supported group with *A. petaloideum* which, like in the previous studies, is placed sister to *Elettariopsis*.

In accordance with the results of Xia *et al.* (2004), all specimens of *Amomum* grouping with clade B have winged or ridged fruits, corresponding with their "Maximum" and "Sericeum" type, while *Amomum* species grouping with *Etlingera* and *Hornstedtia* have echinate fruits (Droop 2012; Otakar Šída, pers. comm. May 2014).

5.2 Relationships among *Elettariopsis*, *Amomum* and *A. subulatum* (group B)

This clade, which includes all *Elettariopsis* accessions together with three species of *Amomum* (*A. subulatum*, *A. petaloideum* and *A. repoeense*), is a well-supported monophyletic clade. In the present analysis it is grouped with the *A. maximum* group (defined by Jane Droop, 2012), here labelled as group C) with good support in BI and ML (1.00, 1.00). This grouping corresponds with the results of Droop (2012) and Xia *et al.* (2004).

All species in group C have winged (Xia's Maximum type) fruits or ridged (Sericeum type) fruits. The determined *Elettariopsis* species which were included in the analysis have ridged fruits (Kam 1982), belonging to the Sericeum type of Xia, and according to Otakar Šída (pers. comm. May 2014), there are no other types of fruits present in this group than ridged or winged. Therefore, it could be speculated that all species of *Elettariopsis* will have ridged or winged fruits as they fall into the same group. The entire group covering both *Amomum* and all *Elettariopsis* species is very morphologically diverse and in many cases it is hard decide if the species is a representative of the genus *Amomum* or *Elettariopsis* based on morphology only. For example, *A. biflorum* has inflorescences with only few flowers, while this is one of the main distinguishing characters of *Elettariopsis* (Droop 2012).

5.3 Relationships within Group D – *Elettariopsis*, *A. repoeense*, *A. petaloideum* and *A. subulatum*

In the analysis of Kress *et al.* (2002), *Elettariopsis* was supported with BS 99, but only three accessions were used. In previous analyses, *Elettariopsis* was only weakly supported as

a clade (Xia *et al.* 2004) or collapsed in the strict consensus (Droop 2012). However, Droop notes that with the addition of more samples, the support for *Elettariopsis* may increase; this is indeed observed in the concatenated analysis of ITS and *mat*K regions, where *Elettariopsis* s. s. (group E; Fig. 11) is moderately supported (PP 1.00, MP BS 80, ML BS 0.74), but in the individual datasets the support is not as high. It is possible, however, that with more thorough sampling and the use of more genes the support of *Elettariopsis* might increase further.

The fact that *A. subulatum* falls into the strongly supported clade which also contains *Elettariopsis* gives rise to a question if *Elettariopsis* should be maintained as a genus in the future. With more extensive sampling and the use of more genes in the phylogeny, it is possible that *Elettariopsis* will be strongly supported as a genus; in that case, only *A. subulatum* and *A. petaloideum* will belong to the genus *Amomum*. The other solution is the inclusion of *Elettariopsis* in *Amomum*, in case of which the other group of *Amomum* would probably need to be given a new name as it is completely separated from the clade containing *A. subulatum*.

5.4 Relationships within *Elettariopsis* s. s. (group E)

In Jane Droop's work (2012), *Elettariopsis* is represented only by eight accessions; however, even these accessions form clades corresponding to their taxonomic names, namely *E. triloba* and *E. curtisii*. Out of these eight samples six were used in this thesis, of which only three could be used in the concatenated dataset as there were no *mat*K sequences available for them in GenBank.

Both the ITS analysis and the concatenated analysis of ITS and *mat*K regions (Figs. 12 and 13) support the partition of *Elettariopsis* into two distinct groups (here called group G and group H) which correspond well with absolute genome size patterns (Fig. 15). Group G contains the species *E. curtisii, E. kerbyi, E. stenosiphon, E. rugosa, E. smithiae* and *E. cf. latiflora* together with some undetermined *Elettariopsis* samples. Group H comprises *E. elan, E. triloba, E. unifolia* and *E. mirantha*, ined, with other undetermined *Elettariopsis* samples and an accession of *E. smithiae* from Jane Droop's work which will be discussed later. The groups are discussed in further detail below.

In the *mat*K analyses the *Elettariopsis* clade is much less resolved. This is probably due to the overall lower variability of chloroplast DNA, but it is probably also influenced by the fact that parts of some sequences are missing. This was caused by from the problematic sequencing of *mat*K (see Methods 3.3.4) and, in some cases, by the impossibility of creating

a full consensus sequence from primers mIF and mIF2_EZ as the end of the mIF primer was illegible. It is curious that this happened only in some of the analysed samples; in future analyses the primers may need to be modified to be able to obtain a full *mat*K sequence in all samples.

Despite these restrictions, the *mat*K analyses confirm the topology of some of the groups, such as the *A. repoeense* group, the *E. triloba* group or the *E. unifolia* group (Fig. 11).

In the analysis of the nuclear low-copy gene DCS, *Elettariopsis* is poorly resolved mainly due to poor sampling, which was caused by problems that arose during sequencing (see Methods 3.3.4). Only a reduced dataset could be used as other sequences contained many double peaks, signifying intraindividual allelic variability. This was also observed in a study on *Curcuma* L. conducted by Záveská *et al.* (in prep.), where multiple alleles were detected in some individuals. In future analyses the DCS sequences will need to be cloned and the cloned sequences analysed separately, to inspect the sources of this variation. Such patterns may indicate either the presence of more than one copy of the gene in several *Elettariopsis* genomes, or past events of hybridization (as reviewed by Knowles and Kubatko 2010). It is noteworthy that more occurrences of multiallelic variability were observed in group F of *Elettariopsis* (comprising *E. curtisii* and *E. kerbyi* groups) than in group G (comprising *E. triloba*, *E. elan* and *E. unifolia*). As a result, most samples present in the analysis belong to group H.

The two groups F and G seem to correspond to C. K. Lim's (2003) division by inflorescence type. He notes that *E. triloba*, *E. elan* and *E. slahmong* have clustered flowers in cincinni, and *E. burttiana*, *E. curtisii*, *E. exserta*, *E. latiflora*, *E. smithiae*, and *E. rugosa* have "single flowers along a basal rhizome and scape" (Lim 2003: 209). Only some species from his list have been used in this analysis; however, it is evident that species with the same inflorescence type form two distinct groups, i.e. group F comprises species with single flowers and group G those with clustered flowers in cincinni. Therefore, the inflorescence type could be a useful distinguishing character within *Elettariopsis*; an analysis comprising more accessions of determined species would be in order to examine this in more depth.

The samples *E. triloba* GB_ AY769831 and *E. sp.* GB_AF478747 used by Jane Droop fell into group M in both the ITS and concatenated analyses. The sample GB_ AY769831 was used only in the ITS analysis (no sequence was available on GenBank for *mat*K) and grouped with *E. triloba* as expected, while GB_AF478747 grouped in all three analyses with *E. elan*. It can therefore be assumed that this sample is an accession of *E. elan*.

Jane Droop's sample GB_AY352013, labelled as *E. smithiae*, fell into the less resolved part of group H and formed a strongly supported clade with *E. sp.* Z635. However, a determined species of *E. smithiae* (Z720) fell into group G of *Elettariopsis*. While Z720 was collected in Malaysia, where lies the type locality, GB_AY352013 was collected in Loei, Thailand. According to Jana Leong-Škorničková (pers. comm. May 2014), *E. smithiae* does not occur so far north in Thailand; other sources, (Kam 1982 or Picheansoonthon and Yupparach 2007) also list only collections from JW Thailand. A photograph of the flower in the NMNH collections database (http://collections.nmnh.si.edu) and compared to the drawing in the protologue (Kam 1982). The flower in the photograph has a differently shaped anther crest than that in the protologue, which was a further confirmation of the fact that GB_AY352013 is indeed not *E. smithiae*, but rather a different species belonging to group H.

The *E. sp.* samples Z641 and Z643, which are strongly supported as a group within group G in all analyses, are a possible new species. Similarly, *E. sp.* samples Z644 and Z660 form a strongly supported clade within the same group.

5.5 Patterns in genome size and its evolution

The absolute genome size (2C) of samples belonging to the targeted group was measured using flow cytometry and its evolution was reconstructed with the use of maximum likelihood methods. Estimates of the *lambda*, *kappa* and *delta* parameters were computed and ancestral states of the absolute genome size were estimated and mapped onto a Bayesian inference phylogeny.

The *lambda* parameter was estimated to be equal to 1, which confirmed a strong relationship between the phylogeny and the evolution of absolute genome size. *Kappa* was estimated to be higher than 1 (1.777; see Table 3), suggesting a stronger influence on the genome size evolution by the longer branches. Finally, *delta* also exceeded 1 (1.462), which implies that the genome size in *Elettariopsis* did not undergo adaptive radiation, but changed constantly over time.

Upon comparation of these results with the phylogeny, it was observed that group B (*Elettariopsis* s. l. together with the *A. maximum* clade) has higher absolute genome size than the outgroup (*Aframomum* and *Renealmia*). Within group B, the smallest genome size was found in *A. subulatum*, while the greatest was present in *Elettariopsis*. The high values of absolute genome size probably indicate polyploidy, but as little is known about the

chromosomes of *Elettariopsis*, this has yet to be confirmed by a study involving the counting of chromosomes.

The evolution of the absolute genome size confirmed the division of *Elettariopsis* into two groups, and the estimates of ancestral characteristics in these groups showed that while in one (group F) genome size decreased over time, in the other (group G) it increased. However, the overall trend in ancestral genome size was decreasing.

A study with larger sampling, exploring the genome size and chromosome numbers in *Elettariopsis* is in order to better understand and explain these patterns.

5.6 Geographical distribution of *Elettariopsis* and its correlation with phylogeny

Mapping the geographical locations of the samples on a map of South-East Asia showed that like the absolute genome size, the biogeography of *Elettariopsis* corresponds with the main phylogenetic patterns within the group. The *A. maximum* clade occurs in the north, including China, but does not occur further south than the north of Vietnam. Contrastingly, no species of *Elettariopsis* occur in China, and the distribution of the genus encompasses also Singapore and Indonesia (Borneo).

The distribution also supports the two groups found in *Elettariopsis* (F and G), as group F, which includes *E. curtisii*, *E. kerbyi*, *E. stenosiphon*, *E. smithiae* and *E. rugosa* is distributed mainly in Vietnam, Laos, Thailand and Myanmar, while group G, comprising *E. triloba, E. elan, E. unifolia* and *E. mirantha*, ined. occurs chiefly in Peninsular Malaysia, Singapore, and Borneo.

More samples with exact localities (ideall, GPS coordinates) are needed for a more thorough examination of this distribution; however, this is problematic as the origin of many specimens is unclear and so is their nativity to the given area.

6. Conclusion

Analyses were performed on *Elettariopsis* Baker and closely related species of *Amomum* Roxb. using molecular (ITS, *mat*K and DCS) and phylogenetic (Bayesian inference, maximum parsimony, maximum likelihood) methods and observing the absolute genome size (2C) and geographical distribution in the group.

Amomum subulatum Roxb., the type species of *Amomum* which has been sampled for the first time, is strongly supported together with *A. petaloideum* (S.Q. Tong) T.L. Wu as basal to *Elettariopsis.* This gives rise to a nomenclatural question whether *Elettariopsis* will be maintained as a genus, or if it will be included in *Amomum*. More extensive sampling and a phylogeny based on more genes is probably needed to resolve this question, but it is possible that the support of *Elettariopsis* as a genus will increase with the number of accessions used.

Two distinct groups were recognized within *Elettariopsis*, one comprising *E. curtisii*, *E. kerbyi*, *E. stenosiphon*, *E. smithiae* and *E. rugosa* (group F) and the other including *E triloba, E. elan, E. unifolia* and *E. mirantha*, ined. (group G). These two groups are clearly distinguished not only in the molecular analyses, but also by the reconstruction of evolution of the absolute genome size in the genus (the members of group G have larger genomes) and by their biogeographical distribution (group F occurs mainly in the northern part of the distribution of *Elettariopsis*, while group G is distributed chiefly in the south, including Indonesia).

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Appendices

Appendix 1. The PCR mixes used for individual markers.

Region	Mix contents (for 1 sample)	Concentration	Amount (µl)
ITS	Mili-Q H_2O		10.35
	5x MyTaq™ Reaction Buffer Red (Bioline)		3.0
	Forward primer (ITS4)	25 pmol/µl	0.375
	Reverse primer (ITS5)	25 pmol/µl	0.375
	MyTaq™ polymerase (Bioline)	5 U/µl	0.15
	Total		**14.2**
	DNA added		0.8
***mat*K**	Mili-Q H_2O		11.2
	5x Hi-Fi Buffer		4.0
	dNTP		2.0
	Forward primer (*trn*K1F)	25 pmol/µl	0.4
	Reverse primer (*trn*K2R_EZ)	25 pmol/µl	0.4
	DMSO		0.6
	Velocity™ polymerase (Bioline)		0.4
	Total		**19.0**
	DNA added		1.0
DCS	Mili-Q H_2O		10.35
	5x MyTaq™ Reaction Buffer Red (Bioline)		3.0
	Forward primer (ZingF)	25 pmol/µl	0.375
	Reverse primer (ZingR)	25 pmol/µl	0.375
	MyTaq™ polymerase (Bioline)		0.15
	Total		**14.25**
	DNA added		0.8

Appendix 2. PCR cycles used.

Region	Polymerase	Temperature (°C)	Duration	Repeats
ITS	MyTaq™ (Bioline)	95	1 min	
		95	20 s/30 s	35x
		55	50 s/1 min	
		72	1 min	
		72	15 min	
		10	∞	
mat*K*	Velocity™ (Bioline)	98	2 min	
		98	30 s	35x
		61	30 s	
		72	1 min 30 s	
		72	10 min	
		10	∞	
DCS	MyTaq™ (Bioline)	95	1 min	
		95	1 min	35x
		60	1 min	
		72	1 min 30 s	
		72	10 min	
		10	∞	

Appendix 3. IUPAC codes used in alignment edition.
(http://www.bioinformatics.org/sms/iupac.html)

IUPAC nucleotide code	Base
A	Adenine
C	Cytosine
G	Guanine
T (or U)	Thymine (or Uracil)
R	A or G
Y	C or T
S	G or C
W	A or T
K	G or T
M	A or C
B	C or G or T
D	A or G or T
H	A or C or T
V	A or C or G
N	any base
. or -	gap

Appendix 4. A set of commands used to map absolute genome size and estimate ancestral states in the package *phytools* implemented in R. Developed by T. Fér, 2014.

```
library (phytools)

tree = read.newick("noGB.nwk")
data = read.csv("noGB.csv")
data1<-as.vector(data$cvalue)
names(data1)<-data$taxon

layout(matrix(c(1,2),1,2),c(0.7,0.3))
xx<-contMap(tree,data1,fsize=0.7,mar=c(4.1,1.1,1.1,0),res=200,plot=FALSE)
plot(xx,legend=FALSE,fsize=0.7,outline=FALSE,mar=c(4.1,1.1,1.1,0))
## click to add legend interactively
add.color.bar(1,cols=xx$cols,lims=xx$lims,title="")
par(mar=c(4.1,0,1.1,1.1))
barplot(data1[tree$tip.label],horiz=TRUE,width=1,space=0,ylim=c(1,length(tree$tip.label))-
0.5,names="")
anc<-fastAnc(tree, data1)
writeAncestors(tree, Anc=anc, file="treeanc.tre", format="nexus")
```

Appendix 5. A set of commands used to estimate the evolution parameters lambda, kappa and delta in the package *caper* implemented in R. Developed by T. Herben, modified by T. Fér, 2014.

```
library("caper")
library("ape")

tr = read.nexus ("s.nex")
write.tree (tr, file = "tree.tre")
tr1 = read.tree ("tree.tre")
a <- read.table ("AEgroup.txt",sep = "\t", header = TRUE)

sumdata <- comparative.data (phy = tr1, data = a, names.col = taxon, vcv = TRUE, na.omit = FALSE,
warn.dropped = TRUE)
model.lambda <- pgls (cvalue~1, data = sumdata, lambda= "ML")
summary (model.lambda)
mod.l <- pgls.profile (model.lambda, "lambda")
plot (mod.l)
dev.copy (png, "lambda.png")
dev.off()
```

Appendix 6. Own samples used in the analyses. Samples belonging to the second *Amomum* group are marked with an asterisk. GS = abolute genome size.

Z no.	FCM	Other ID	Taxon	Collector(s)	Country	Locality	Alt. (m)	GPS (if available)	GS (pg)
Z7	FCM 185	S20002475	*Elettariopsis rugosa*	Leong- Škorničková , J.	Malaysia				5.90
Z24	FCM 201	S20050912	*Elettariopsis elan*	Leong- Škorničková , J.	Malaysia				7.83
Z81	FCM 276	JŠ 71468	*Amomum subulatum*	Leong- Škorničková , J., Fér, T.	India	West Bengal, Daarjeeling, Mirik	1577	26°53.251‘ N 88°11.288’ E	3.51
Z90	FCM 313	SNG-13	*Elettariopsis cf. latiflora*	Leong-Škorničková, J., Leong, P., Chua, Y.K. *et al.*	Singapore	Bukit Timah. Growing below the pathway in shadier conditions.			6.36
Z92	FCM 295	E19901444	*Elettariopsis triloba*	Newman, M.F.	Vietnam	Đông Nai Province			7.60
Z96	FCM 292	E19981704	*Amomum petaloideum*	Kress, W.John; Wood, T. & Li	China	Yunnan Province			6.24
Z104	FCM 246	Trần 383	*Elettariopsis sp.*	Trần H.Đ., Lê C.K., Nguyễn D.L., Šída O., Vũ H.Đ.	Vietnam	Quảng Nam Province	686	15°18'00.8" N, 107°43'44.1" E	8.01
Z121	FCM 297	SNG-46	*Elettariopsis cf. latiflora*	Trần H.Đ., Ng W. & Lee S.	Singapore	MacRitchie Reservoir, Nature Trail	66	1°21'06.5"N, 103°49'03.8"E	6.40
Z123	FCM 294	E19901449	*Elettariopsis unifolia*	Newman, M.F.	Vietnam	Đông Nai Province			5.93
Z275*	FCM 518	SNG-22	*Amomum sp.*	Leong- Škorničková , J. *et al.*	Singapore	About 50m from Kruing Hut, Bukit Timah Nature Reserve. Shade.			4.33
Z299	FCM 421	JLS 522	*Elettariopsis sp.*	Trần *et al.*	Vietnam	Viet Nam: Lâm Đông Prov., Đạ Huoai district, Đèo Chuối .			5.35
Z303	FCM 425	JLS 521	*Elettariopsis sp. “cinnamomeum”*	Trần, H.D.	Vietnam	Khánh Hòa prov., Ba Hồ			15.52
Z304	FCM 423	JLS 519	*Elettariopsis sp.*	Trần *et al.*	Vietnam	Đông Nai Prov., Tân Phú forest, Ngã 3 Quân Y.			5.99
Z308	FCM 424	JLS 518	*Elettariopsis sp. “bronze leaf”*	Trần *et al.*	Vietnam	Đông Nai Prov., Tân Phú forest, Ngã 3 Quân Y.			7.62
Z329	FCM 422	JLS 515	*Elettariopsis sp.*		Vietnam	Khánh Hòa Prov., Ba Hồ			6.34
Z429	FCM 690	JLS 1557	*Amomum aff. plicatum*		Vietnam	Kon Tum, Kon Plông Dist., Xã Hiếu; disturbed forest along road side.	1222	14°38'56.5"N, 108°25'03.1"E	5.19
Z439	FCM 692	JLS 1589	*“Elettariopsis mirantha”, ined.*		Vietnam	Kon Tum, Kon Plông Dist., Xã Hiếu	1231	14°40'48.7"N, 108°24'05.5"E	8.70
Z442	FCM 693	JLS 1598	*Amomum aff. glabrum*	Leong-Škorničková, j., Nguyễn Q.B., Trần,	Vietnam	Kon Tum, Kon Plông Dist., Xã Hiếu, way to Ka Bang	1197	14°33'18.6"N, 108°26'41.9"E	6.31

				H.Đ., Záveská E.					
Z450	FCM 691	JLS 1619	*Amomum repoeense*		Vietnam	Thừa Thiên - Huế Prov., Bạch Mã N.P., Pheasant Trail	469	16°13'44.86"N, 107°51'4.45"E	5.60
Z456	FCM 683	JLS 1637	*Amomum repoeense*		Vietnam	Thừa Thiên - Huế, Bạch Mã N.P., Đồng Truồi	272	16°13'4.84"N, 107°43'47.16"E	5.23
Z458	FCM 679	JLS 1640	*Elettariopsis sp.*		Vietnam	Thừa Thiên - Huế, Bạch Mã N.P., Đồng Truồi	241	16°13'13.51"N, 107°43'36.21"E	7.71
Z490	FCM 024	E20001425	*Elettariopsis curtisii*	Newman, M.F.	Thailand	Trẳng Province			5.64
Z491	FCM 225	Trần 366	Amomum sp.	Trần, H.Đ., Lê C.K., Nguyễn D.L., Šída O., Vũ H.Đ.		Kon Tum Prov., Kon Plông Dist., way to Măng La	1257 m	14°42'34.3"N 108°14'35.9"E	4.74
Z559	FCM 719	JLS 1675	*Amomum sericeum*		Laos	Luang Prabang Prov., Tad Se waterfall. Broad leaves forest near waterfall.	332	19°50'35.6"N, 102°13'14.9"E	7.39
Z635	FCM 1277	JLS s.n.	*Elettariopsis*		Laos				6.82
Z636	FCM 1283	JLS 1946	*Elettariopsis sp.*	Haager, J.R., Rybková, R.	Vietnam	Lâm Đông Prov., Di Linh, forest nearby the village	950	11°34'38.72"N, 108° 4'0.24"E	5.88
Z637	FCM 1281	JLS 520	*Elettariopsis sp. "unifolia, plicata"*	Trần *et al.*	Vietnam	Lâm Đông Prov., Đạ Huoai district, Madaguoi resort.			5.30
Z638	FCM 1282	JLS 730	*Elettariopsis sp.*	Leong-Škorničková, J.	Vietnam	Kon Tum Prov., road to Kon Plong.	891	14°32'33.4"N, 108°15'35.9"E	5.90
Z639	FCM 1276	JLS 1489	*Elettariopsis sp.*		probably Malaysia	Exact locality unknown			7.86
Z640	FCM 1278	JLS 1795	*Elettariopsis sp.*		Laos	Vientian Prov., Vang Vieng Dist., Ban Na Khun	238	18°52'32.9"N, 102°24'41.1"E	4.84
Z641	FCM 1285	S20050385	*Elettariopsis sp.*		Malaysia				6.42
Z642	FCM 1289	SNG 113	*Elettariopsis*	Ibrahim, H.	Singapore	MacRitchie Reservoir, Nature Trail	66	1°21'06.5"N, 103°49'18.0"E	6.26
Z643	FCM 1284	SBG20060610	*Elettariopsis sp.*	Leong-Škorničková, J.	Malaysia				6.42
Z644	FCM 1279	JLS 1200	*Elettariopis sp.*	Lamxay, V., Larnorsavanh, S., Souvannakoummal, K.	Laos	Bolikhamxai province, Pakkading district, Bane phonesai, Phoungou NPA, Phoungoungai area	120	N18° 18" 53.7" E104° 01' 21.7"	12.68
Z645	FCM 1330	E20081070	*Elettariopsis sp.*	Trần, H.D.	Vietnam	Lâm Đông Province			5.39
Z646	FCM 1331	E20081100	*Elettariopsis sp.*	Trần, H.D.	Vietnam	Đông Nai Province			7.77
Z659	FCM 1327	E20111045	*Amomum repoeense*	Lamxay, V.	Laos	Bolikhamxay Province			4.75

Z660	FCM 1321	E20111048	*Elettariopsis*	Lamxay, V.	Laos	Bolikhamxay Province			13.02
Z661	FCM 1326	E20081123	*Elettariopsis*	Trần, H.D.	Vietnam	Đông Nai Province			6.07
Z662	FCM 1328	E20100770	*Amomum sericeum*	Newman, M.F.	Cambodia				6.43
Z664	FCM 1325	E20001425	*Elettariopsis curtisii*	Newman, M.F.	Thailand	Trằng Province			5.68
Z665	FCM 1336	E20081047	*Amomum repoeense*	Trần, H.D.	Vietnam	Lâm Đông Province			5.00
Z666	FCM 1329	E20100766	*Amomum sericeum*	Newman, M.F.	Cambodia				6.41
Z683*	FCM 622	T-11-08	*Amomum sp.*		Thailand				3.51
Z684*	FCM 1280	S20121937	*Amomum sp.*	Leong-Škorničková, j., Nguyễn Q.B., Trần H.Đ., Záveská E.					3.52
Z686	FCM 725	JLS 1726	*Amomum maximum*	Leong-Škorničková J., Trần H.Đ., Šída O., Phoutthavong K., Oudomsack S.					5.57
Z689*	FCM 655	E20051592	*Amomum rivale*	Middleton, D.J.	Thailand	Phetchaburi			4.68
Z720	FCM 1358	S20001092	*Elettariopsis smithiae "silver leaf"*		Malaysia	close to type locality (Selangor, Ulu Gombak)			6.44

Appendix 7. A table of GenBank accessions used. In the concatenated analysis, only the ITS GenBank code was used. (after Droop 2012)

Name in ITS+*mat*K concatenated dataset	Taxon	Collection no. (herbarium)	Origin	Locality (if available)	GenBank accession no.	
					ITS	*mat*K
GB_AF478746	*E. kerbyi* R.M. Sm.	Kress 96-5746 (US)	Indonesia	Borneo	AF478746	AF478845
GB_AY352013	*E. smithiae* Y.K. Kam	Kress 99-6313 (US)	Thailand	Loei, Phu Rua District, 750 m alt.	AY352013	AY352043
GB_AF478747	*E. sp.*	Kress 00-6720 (US)	Myanmar/ Singapore*		AF478747	AF478846
GB_ AF478847	*E. stenosiphon* (K. Schum.) B.L. Burtt & R.M. Sm.	Kress 01-6847 (US)	Malaysia	Sarawak	AF478847	-
GB_AY769831	*E. triloba* Loes.	Rang.&New. s.n. (E)	Vietnam		AY769831	-
GB_AY769832	*E. unifolia* (Gagnep.) M.F. Newman	Newman 747 (E)	Vietnam	Đông Nai Province, Nam Bai Cát Tiên NP, c. 100 m alt.	AY769832	-
GB_AY351989	*A. aff. glabrum*	Xia 73 (HITBC)	China		AY351989	AY352019
GB_AY351985	*A. austrosinense* D. Fang	Xia 719 (HITBC)	China		AY351985	AY352015
GB_ AF478722	*A. longipetiolatum* Merr.	Kress 99-6353 (US)	China		AF478722	AF478822
GB_ AY351995	*A. maximum* Roxb.	Xia 725 (HITBC)	China		AY351995	AY352025
GB_AY351996	*A. menglaense* S.Q. Tong	Xia 726 (HITBC)	China		AY351996	AY352026
GB_AY352000	*A. purpureorubrum* S.Q. Tong et Y.M.Xia	Xia 727 (HITBC)	China		AY352000	AY352030
GB_AY352004	*A. queenslandicum* R.M. Sm.	Kmn 1428 (HITBC)	Australia		AY352004	AY352034

* This accession is listed by J. Droop as from Myanmar, but in NMNH database (http://collections.nmnh.si.edu) the origin is Singapore.

Appendix 8. An alignment of sequences used in the BI analysis including the Alpinioideae. Samples of the Alpinioideae species (excluding the targeted group of *Elettariopsis* and *Amomum* species) were obtained from Šída *et al.* (unpubl.)

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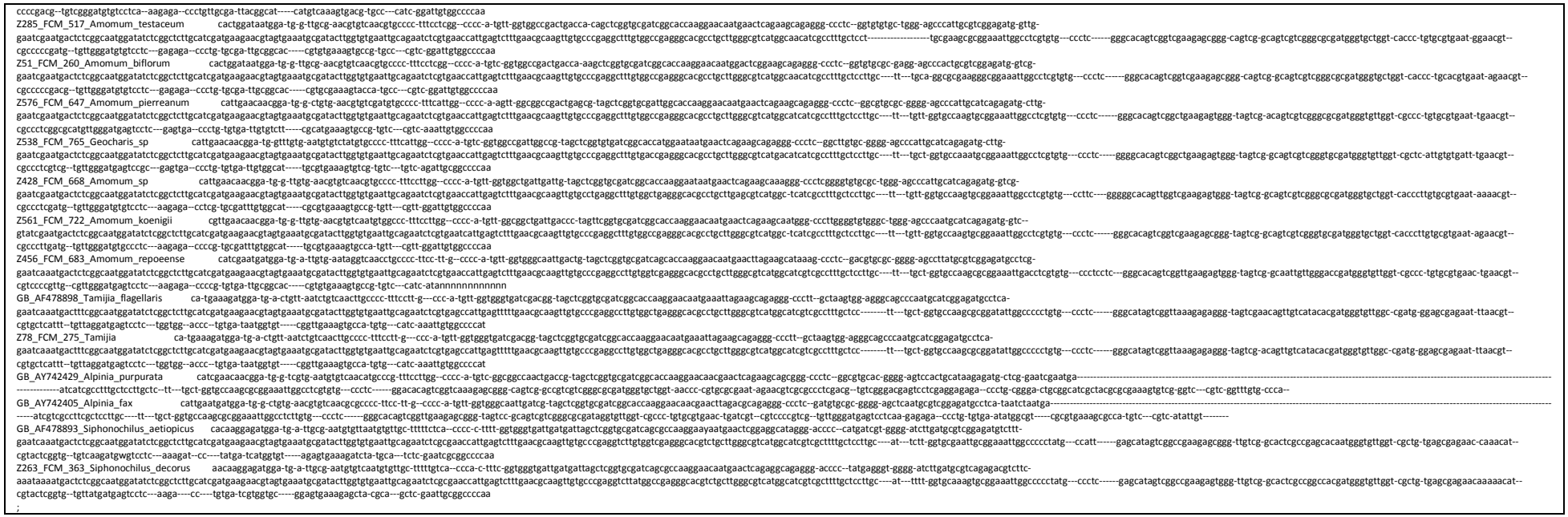
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