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Abstract
Trade and commercialization of non-timber forest products, like cycas palms, rattans, and orchids form a serious threat to biodiversity in South-East Asia. The intensity at which these resources are collected, as well as the techniques used, are unsustainable. To distinguish between common and endangered species is complicated, especially of related species within the same family or genus. Molecular barcoding applied to plants uses DNA-sequences to contribute to identification and distinction between species. In this paper we investigate the possibility of finding suitable barcodes for *Amomum* Roxb., a genus of well-known medicinal plants in South-East Asia, by comparing three genetic markers *matK*, *ITS* and *trnL*.

Keywords. *Amomum,* barcoding, medicinal plants

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1. *Amomum*

1.1 Distribution

Amomum Roxb. is the second largest genus after *Alpinia* in the family *Zingiberaceae* (the ginger family) in the order *Zingiberales*. The genus consist of approximately 170 species (Lamxay 2011) distributed mainly in tropical parts of Southeast Asia, but also widely spread in China, the Himalayas and northern Australia.

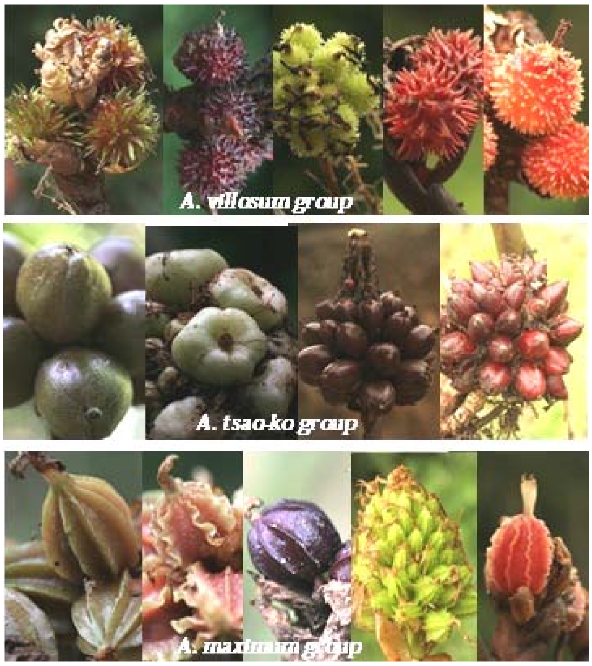


Figure 1. The different types of *Amomum* fruits. Pictures by V. Lamxay.

1.2 Taxonomy

The genus *Amomum* (Zingiberaceae) was first described by Linnaeus in 1753 and since then several changes with the placement of species and descriptions of new species have been made (Roxburgh 1819; Schumann 1904; Loesener 1930). Schumann and Loesner recognized three tribes in the family of *Zingiberaceae*: *Globbeae*, *Hadychieae* and *Zingibereae*, the latter including *Alpinieae* and *Amomum*. Recent phylogenetic studies of the *Zingiberaceae* family (Kress et al. 2002) show that *Amomum* is a paraphyletic group while earlier morphological studies (Schumann 1904; Tsai et al. 1981) have considered and supported *Amomum* as a monophyletic group in the *Zingiberaceae* family; the polyphyly is however confirmed by Xia et al. who made the most recent classification of the genus *Amomum* (Xia et al. 2004). The classification divides *Amomum* into three groups based on the difference of the fruit: 1) the *Amomum tsao-ko* clade which is distinguished by bi- or trilobed anther appendages and a smooth fruit, so called Tsao-ko type fruit; 2) the *Amomum villosum* clade distinguished by bi- or trilobed anther appendages, an elongated infructescense, various labellum shapes and a typical fruit covered in spines, the Villosum type; 3) the *Amomum maximum* clade distinguished by an intact anther appendage, partially elongate infructescence and a winged fruit, a typical Maximum or Sereicum fruit.

1.3 Economic importance

In South-East Asia, *Amomum* is locally known as Cardamom and is used as a medicinal herb. However the culinary spice which is well-known in Europe as Cardamom are the seed and seed capsules from a species in the genus *Elettaria*, species *Elettaria cardamomum* Maton. In South-East Asia and China many *Amomum* species are well known as medicinal herbs, in particular for their fruits, such as *Amomum tsao-ko* Crevost & Lemarié, *A. villosum* Lour. (known as "sha-ren"), *A. krervanh* Pierre ex Gagnep. and *A. xanthioides* Wall. These are mainly used to treat digestive and gastric disorders in China and some fruits are also considered to work as emmenagogues (stimulating menstrual flow, and possibly abortifacient) and have antipyretic (fever reducing) properties (De Padua et al. 1999). In the Malaysian region *Amomum* fruits are also used to cure coughs and colds (De Padua et al.

GLOSSARY

Clade: a group of organisms derived from a common evolutionary ancestor.

Phylogeny: the evolutionary history of species relationships, often visualized as phylogenetic tree.

Taxonomy: the science of finding, describing and classifying organism groups, generally also reflecting evolutionary relations. Biological taxonomy creates a hierarchical classification of biological taxa.

Monophyletic: A group of taxa consisting of all the descendants of a common ancestor.

Paraphyletic: A group of taxa consisting of all the descendants of a hypothetical closest common ancestor *minus* one or more monophyletic groups of descendants.

1999). The plants of the *Amomum* species are generally evergreen and are inhabitants of forest margins and light gaps in moist locations (Xia et al. 2004). *Amomum* grows near the forest floor with its characteristic basal compact cone-like inflorescence (Xia et al. 2004) and the fruit is berry-like with three valves packed with numerous angular seeds (De Padua et al. 1999). Flowering and fruiting of *Amomum* starts around 4-5 years after planting and the individual flowers usually last less than one day.

2. Medicinal plants

2.1 Meaning

Plants were the first material used to treat illness and diseases among humans, and even though most pharmaceutical today are synthetic compounds, medicinal plants still play an important part in many cultures. The World Health Organization (WHO) estimates that 80% of the world's population depends on different plants and herbs for medicinal causes (WHO 2011). In addition the market of traditional herb remedies is growing as an alternative or complement to chemical generated medicines. The same source tells us that in the United States, the number of people using herbal medicines has increased from 2.5% in 1990 to 37% in 2000.

2.2 Trade

Trade with non-timber forest products (NTFPs) has great significance in many countries in South-East Asia and many species of the tropical forests are raw-materials used in international *phytopharmaceuticals* (pharmaceuticals derived from botanicals instead of chemicals); and collection and trade of wild plant material is an important source of subsistence for many people in South-East Asia. The expansion of trade and commercialization of medicinal plants conveys increased exploitation of particular plant species and forms a serious threat to biodiversity if not properly monitored and regulated; the material is often exhaustively collected without respect for sustainability. *Amomum* seeds are often harvested indiscriminately and the marketed product has multiple species origins. As a result, many individual species are threatened or endangered through over-exploitation.
Cardamom is among the most important NTFP in South-East Asia and is collected both from natural forests and cultivated fields. In the Lao People's Democratic Republic medicinal Cardamom was the second biggest agricultural export product after coffee (Aubertin 2004). Lacks of control of the cross-border trade of medicinal plants, and the intensity at which these resources are collected, as well as the techniques used, have created an untenable situation.

2.3 Sustainable trade

Plant material on markets usually consists of leaves, seeds or essentials oils, and this makes them almost unidentifiable, as identification of plants in general usually requires complete and flowering material. Distinguishing species is complicated, especially of related species within the same family or genus, not only for amateurs but also professionals. The identification of species is not only an issue in consideration of sustainability of the species itself, but also of the safety and efficacy within trade, export and import of products. Proper identification facilitates for both the supplier and the receiver to follow the biological material in managing the products internationally. Definition and recognition of species from threatened or endemic populations can help a nation to enhance the ability to identify their unique genetic materials. To bring safety into the trade and usage of medicinal plants it demands a practical and strong tool for the identification of different species.

2.4 *Amomum*

Amomum species are not only important products within herbal medicine and trade; they are also significant in tropical forest ecosystems (Lamxay 2011), especially in South-East Asia where the genus has its largest distribution. The large number of species in *Amomum*, the lack of collections and the complex morphological characters make it complex to make an adequate study of all the *Amomum* species (Xia et al. 2004). Hence, *Amomum* has been investigated by numerous people in various regions; Tsai et al. (1981) in China, Smith (1989) and Sakai & Nagamasu (1998) in Borneo with traditional methods, distinguishing species by characters such as habits, inflorescence, capsule and phylogenetic analyses. The latest taxonomical revision of *Amomum* was made by Vichith Lamxay (2011) and covers the species in Lao PDR, Cambodia and Vietnam, and was based on morphological characters. Even though *Amomum* is of significant importance both in tropical ecosystems and an important trade product in several countries, scientific research concerning many species in the genus is lacking with regard to taxonomy (Lamxay 2011), and especially of collection of wild cardamoms (Aubertin 2004).

3. Identification methods

Different identification tools have been used throughout history: from the traditional methods using keys, counting and comparing each detail of a species, to molecular methods examining the plant's genetic variation. Here follows a short presentation of some methods for the identification of plants but since this project aims to develop the method of identifying plants investigating their proper DNA, the chapter about DNA-barcoding is deeper and wider in order to explain its background.

3.1 Southern blot

Also more formally called a DNA blot and also used for plant identification (McCabe et al. 1997; Mandolino et al. 1999). DNA from different organisms is isolated and fragmented with a particular combination of restriction enzymes. It is then loaded into an agarose gel and the fragments will be separated in a gel electrophoresis according to their size. The loaded particles move in different length in the gel according to their size and the smaller fragments will move faster than the large fragments. The gel is then transferred to a nylon filter. To bind complementary DNA segments a hybridization probe (radioactively nucleic acid) is added, this is the specific sequence of the target DNA. To detect the pattern of hybridization the filter is visualized under X-ray film. If the organism does not have the complementary DNA sequence, no probe will be visualized (McGraw-Hill).

3.2 DNA fingerprinting

There are various methods for DNA fingerprinting but they are all based on the fact that the chemical structure of DNA is the same and the only thing that differs them from each other is the order of base pairs. This method also assists to identify organisms by their DNA and the technique is often employed among scientists studying plants (Vosman et al. 1992; Khadari et al. 1994; Raina et al. 2001). Different methods of DNA fingerprinting are: Restriction fragment length polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Amplified fragment length polymorphism (AFLP) and Simple Sequence Repeats (SSRs) (Buzzle.com).

For example, the restriction fragment length polymorphisms (RFLPs) method is used to identify the origin of plants species by using the genetic polymorphism of individuals. Restriction enzymes are used to cut a particular DNA region with known variability. With gel electrophoresis the DNA is separated according to size and the pattern on the agarose gel will be different for each individual (Davidson 2001).

The randomly Amplified Polymorphic DNAs (RAPDs) is likely the most common method used for DNA fingerprinting and unlike PCR analysis it does not require any information about the actual DNA. By adding short fragments of primers these will or will not bind to the complementary fragments and amplify these (NCBI 2010). The method requires small amounts of DNA and includes no radioactivity (www.molecular-plant-biotechnology.info).

3.3 PCR

Although not an identification technique by itself, this technique is crucial to DNA sequencing. PCR (Polymerase Chain Reaction) is a technique well used in molecular biology to amplify sections of DNA using DNA primers creating millions of copies of the copied DNA sequence.

3.4 Barcoding

3.4.1 Introducing the method

A genetic barcoding library defines biological material with a certain barcode created from its genome. To form a universal barcode it demands a standardized region in the plants genome; and this region should make it possible to identify even a small piece of tissue from an unidentified organism (Kress et al. 2007). The aim is then to create a DNA library of reference sequences for comparing other species, or even unknown species, to species registered in a DNA library (Kress et al. 2007). Finding the perfect barcode in plants has appeared to be problematic, especially for two reasons (Chase et al. 2005): (i) the DNA regions used in algae, fungi and animals have low levels of variability in plants and (ii) the chloroplast markers typically used seem to have too little variation among plants. The criteria for the essential barcode are many and the qualifications high. An ideal barcode should; (i) be short enough to be able to detect even small or damaged plant material, (ii) allow a clear-cut species identification by having adequate variation among and within species and (iii) be robust and reliable for amplification and sequencing.

3.4.2 Plants vs. animals

Giving each species a DNA barcode for identification demands the assimilation in the genome of all individuals you wish to compare. Numerous plants attributes such as hybridization, asexual reproduction and polyploidy make them a less easily distinguished group than animals, and species discrimination is much more complex. In the animal kingdom genetic barcoding has had great success where one gene, the mitochondrial cytochrome c oxidase 1 (CO1) is mutual for nearly all species and this gene is used as a universal barcode for animals groups (Fazekas et al. 2008). For plants though, the nucleotide substitution of the mitochondrial DNA is lower and cannot be utilized to classify species. Mitochondrial DNA in animals evolves much faster than their nuclear DNA (Wolfe et al. 1987). The chloroplast DNA has a larger genetic variation, and therefore has more desirable capacities for species distinction (Seberg et al. 2009).

3.4.3 Challenging the method

The inception of DNA barcoding has been met with both relief and antagonism. For morphological taxonomists DNA barcoding can seem like a threat to their entire occupation and for an ecologist it can aid and reduce the time it takes to identify samples for the identification of plants. Packer's et al. study about DNA barcoding as an identification method (Packer et al. 2009) presents two major criticisms of DNA barcoding: (i) barcoding does not, or cannot, work for the identification of species or the discovery of new ones and (ii) barcoding ignores the rich legacy of traditional taxonomy. There are several cases when DNA barcoding has given a high rate of discrimination between animal species in different animal groups, e.g. fish and birds, (Ward et al. 2005; Kerr et al. 2007). It has also been used to actually identify groups of animal species in studies, and in this case among birds (Hebert et al. 2004).

The examination of taxonomy never stops and the re-examination of families and genera often leads to increasing or decreasing the number of species since the individual factor of the person identifying often has an impact on the outcome (Packer et al. 2009) . In Packer's own study of bee taxonomy, the different taxonomists identifying the same material came to different conclusions regarding the bee-species. Discrimination of species also rest on morphological polymorphisms that may be strongly influenced by environmental factors (Aras et al. 2003).

3.4.4 Barcoding and traditional taxonomy

Packer et al. (2009) suggest that when DNA barcoding is compared to traditional identification through taxonomy and morphological characters, DNA barcoding nearly always outperforms morphology. The traditional methods are particularly problematic when applied to cryptic species recognition, which is rarely the case working in areas with endangered species. It has also shown that in some cases traditional morphological identification of species does not work, not even in animals; see Wong and Hanner´s study about fish-identification of market samples (Wong et al. 2008).

As one single barcode is difficult to find for the entire plant kingdom, an alternative is to use more than one marker of the plant genome; multiple markers or combining markers that can be used for identification. Another alternative is to find a barcode for each group of plants, for example a family or a genus, to aid the identification process. Barcoding does not have to displace taxonomic work but can serve as a first attempt to roughly identify species in taxonomic analyses when variation within species is complex. This method could entirely democratize the taxonomic process; more people would be able to identify an organism from a mere fragment and the taxonomy hopefully become more effective (Packer et al. 2009). Creating a barcode system would allow a bigger group of persons to work in the field of plants and ecology when identification is available through relatively easy applicable lab work. Barcoding today is still not developed entirely and is still at a high-cost level, but several groups are working trying to develop an operating method (Erickson et al. 2008; CBOL et al. 2009; Dunning et al. 2010) and the interest in finding a barcode for plants is growing.

4. Aim

This project aims to build up a DNA-laboratory for barcoding of commercially traded plant material from endemic populations in particularly endangered species. The laboratory is being formed at the National University in Vientiane, Lao PDR with colleges from Vietnam and Cambodia. The main goal in this specific study is to evaluate genetic barcodes with regard to *Amomum* specimens collected from Lao PDR, Vietnam and Cambodia. Included in the process are also nine unknown samples collected in Vietnam. With these samples we attempt to identify unknown *Amomum* species by comparison with the genetic information from known *Amomum* species.

Three genetic markers have been chosen to try to find a suitable barcode for the *Amomum* species, nuclear ribosomal Internal Transcribed Spacer (*ITS*) and two genes from the chloroplast genome; *matK* and *trnL*. Several studies have shown that *ITS* is a good representative for land plants; (Chen et al. 2010) study to identify a barcode for medicinal plants presents *ITS* as a strong barcode for medicinal plants and studies of the *Zingiberaceae* (Harris et al. 2000; Rangsiruji et al. 2000; Searle et al. 2000; Wood et al. 2000; Kress et al. 2002) shows that both *ITS* and *matK* both have good qualities for investigating phylogenetic relationships within this family. Three different gene prospects with separate or combined analyses may increase the probability to find common patterns of the *Amomum* species.

5. Material and methods

All laboratory work was carried out in the lab of Dr. Hien Le Thu, at the Institute for Biotechnology at the Vietnamese Academy of Sciences, Hanoi, Vietnam.

Collection and identification of samples. The samples were sourced from Vichith Lamxay´s collection of *Amomum* silica samples of herbarium vouchers, see *Appendix 1*. Index of exsiccate can be found in his "A revision of *Amomum* (*Zingiberaceae*) in Cambodia, Laos and Vietnam" (Lamxay 2011). Additional sequences were downloaded from GenBank (Benson et al. 2000) and later were used for the phylogenetic analyses. The list of GenBank species and references can be found in *Appendix 2*.

DNA-extraction. Total DNA was extracted from ~0.05 g of leaf tissue from *Amomum* leaves dried in silica-gel. DNA was extracted with a CTAB buffer method, the Carlson & Yoon DNA isolation procedure (Yoon et al. 1991) (addition of 750 μl of Carlson lysis buffer and incubated at 60°C for 60 min). The samples were grinded with metal beads or manually grinded with plastic pestle in liquid nitrogen. The purification from the aqueous phase was made twice with chloroform-isoamyl alcohol (24:1) solution. After purification the DNA pellet was resuspended in 200 μl of DNasefree water without discarding RNAs. The total DNA was then purified with Fermentas® GeneJET™ Genomic DNA Purification Kit.

DNA amplification and sequencing. Template DNA, with no dilution, were amplified by PCR (machine PTC-100™ Programmable Thermal Controller by MJ Research Inc.), thermal cycle (95°Cx3min (94°Cx1min, 50°Cx1min, 74°Cx1min)x35, - 72°Cx10min, 4°C∞) using three pairs of primers: *matK-A_F&R* (unpublished under development: 'F';5'- ACY GTA CTT TTA TGT TTA CGA GC -3','R'; 5'- TCC ATH TDG AAA TCT TGG TTC A -3'), *trnLc & trnLf* (Taberlet, P., Gielly, L., Pautou, G., and Bouvet, J.,1991'c': 5'- CGAAATCGGTAGACGCTACG -3', 'f': 5'- ATTTGAACTGGTGACACGAG -3'), and *ITS_AB101* & *ITS_AB 102* (extensively used and quoted however with unknown formal publisher: AB101: 5'- ACGAATTCATGGTCCGGTGAAGTGTTCG -3', AB102: 5'- TAGAATTCCCCGGTTCGCTCGCC-GTTAC -3'). An annealing temperature of 50°C was used. The amplicons were approximately 800 bp in length. Purification of the PCR products was subsequently made with the GeneJET™ Gel Extraction Kit. The complete purified PCR product was sent to Macrogen Inc., Seoul, Korea for sequencing. Sequencing was made with PCR primers.

Sequence analysis. *Siphonchilus kirkii* was chosen as outgroup. Species from the genera; *Alpinia, Etlingera, Vanoverberghia, Hornstedtia, Paramomum, Elettariopsis, Aframomum*, and *Renealmia* were added to the phylogenetic analyses. These genera accompany *Amomum* in its paraphyletic group.

Sequence trace files were compiled into contigs with the program Gap4 and edited using Pregap4 (Bonfield et al. 1995), both modules in the Staden package (Staden 1996). Sequences were aligned manually in Se-Al (Rambaut 1996). Sequence data was available for trnL (56 % of taxa), matK (50 %), ITS (65 %). The analysis was made with three markers, *matK* and *trnL* from the chloroplast genome and *ITS* from the nuclear. In order to learn which marker is the most representable to discover affinity and changes among the *Amomum* species, we divide the data into three sets: chloroplast (a concatenation of both *matK* and *trnL*), nuclear (*ITS*) and combined set (all markers). Data was gapcoded using the Simmons & Ochoterena simple method (Simmons 2000) implemented in SeqState (Müller 2005).

Phylogenetic analysis. Bayesian inference used the GTR + G model (with the default four rate categories) plus a proportion of invariable sites and was computed using MrBayes (Huelsenbeck et al. 2001) on the CIPRES cluster (Miller 2010). The combined dataset was analyzed using three partitions (nuclear, plastid, gap data), allowing partition models to vary by unlinking gamma shapes, transition matrices, and proportions of invariable sites. Markov chain Monte Carlo (MCMC) runs started from independent random trees, were repeated twice, and extended for one million generations, with trees sampled every 1000th generation. We used the default priors in MrBayes, namely a flat Dirichlet prior for the relative nucleotide frequencies and rate parameters, a discrete uniform prior for topologies, and an exponential distribution (mean 1.0) for the gamma-shape parameter and branch lengths. Convergence was assessed by checking that the standard deviations of split frequencies were <0.01; that the log probabilities of the data given the parameter values fluctuated within narrow limits; that the convergence diagnostic (the potential scale reduction factor given by MrBayes) approached one; and by examining the plot provided by MrBayes of the generation number versus the log probability of the data. Trees saved prior to convergence were discarded as burn-in (100 trees) and a consensus tree was constructed from the remaining trees.

6. Results

Combined markers tree (Tree 1). *Tree 1* (see page 9): Four clades are represented with *Amomum* species but not all of them showed monophyletic linages. There are two larger groups (*Amomum I and Amomum II*) and two smaller groups (*Amomum III and Amomum IV*). In the *Amomum II* group, two sets of species are found: 1. Nine *Amomum* species (*A. maximum VN05, A. repoeense var pinnate blade*

1191, *A. putrescens GB1*, *A. subcapitatum 2060*, *A. subcapitatum 1145*, *A. repoeense 2072*, *A. repoeense VN01*, *A. petaloideum GB1* and A. subcapitatum *GB1*) are paraphyletic with one species from *Paramomum* (*P. petaloideum GB1*) and two from *Elettariopsis* (*E. smithiae GB1* and *E. unifolia GB1*). The branch that *AmosubGB1* shares with the two species of *Elettariopsis* species, however has only very limited support (0.54) and the rest of the *Amomum* species which are paraphyletic with *Paramomum* have higher but not full support (0.87), and 2. The other set of *Amomum* species in the *Amomum II* (Red) group have 15 *Amomum* species that are divided into two monophyletic groups with almost full support (0.90 and 1.00). One species from the samples is found outside of these four large clades; *A. truncatum VN06* ended up on a branch among the other *Alpinia* species with full support (1.00), which indicates that this sample probably is an *Alpinia*, not *Amomum*.

The other three groups: *I*, *III* and *IV*, all have monophyletic linages. The *Amomum III* (pink) group contains totally eight species where six of them are *A. tsao-ko* or *A. paratsao-ko* with 1.00 support. The last and smallest group in *Tree 1* is *Amomum IV* set, which contains only two species, *A. laxesquamosum* and *A. pierreanum*. *Amomum I, III* and *IV* are together paraphyletic with another set of species from other genera: *Etlingera yunnanensis, Vanoverberghia sepulchrei and Hornstedtia hainanensis.*

Chloroplast and nuclear trees.

The Nuclear tree (Tree 2). *Tree 2* (see page 10) resembles *Tree 1* in almost all cases. But since it has added more sequence, some changes occur. In the bottom of the tree, in the *Amomum II* group, where there are several *Amomum* species in a paraphyletic group, we also find *Amospe1171* (*A. chryseum*) as a sister to the paraphyletic group.

The Chloroplast tree (Tree 3). The chloroplast (see page 11) and nuclear sets are broadly the same as *Tree 1* (which consist of all markers combined). All trees have the same position of the four groups we found in *Tree 1; Amomum I, II, III* and *IV* with similar consistence of species. *Tree 2,* the nuclear sequences (*ITS* marker) tree, has additional species in the *Amomum IV* group, where the combined tree only had two species (*A. pierranum* and *A. laxesquamosum*). The chloroplast tree (*Tree 3*), though, does not have an *Amomum IV* group similar to the others; instead *A. pierranum* is on a sister branch to the large *Amomum I* group with species from other genera, e.g. *Etlingera*, *Hornstedtia* and *Vanoverberghia. A. laxesquamosum* was unfortunately not successfully amplified with any of the chloroplast primers. The chloroplast tree also differs from the other three trees in the *Amomum II* group: this group is divided into two sets of *Amomum* where one set is paraphyletic and contains species other than *Amomum* (two species from *Elettaria*) and the other set in the *Amomum II* group is monophyletic.

7. Discussion

Combined trees. *Tree 1* is divided into four larger sets which resemble the phylogenetic analyses of *Amomum* that (Xia et al. 2004) made. The results divide the *Amomum* clade into three groups; *A. maximum, A. tsao-ko* and *A. villosum*. In *Tree 1* the monophyletic *Amomum I* group has a strong likeness with the *A. villosum* group. Both contain all *A. villosum* species from the different sets and equally include species: *A. krervanh, A. quadratomalinare, A. koenigii, A. yunnaense, A. propinquum* and *A. compactum.* Included in Xia's et al. divisions in the *A. villosum* group is also *A. laxesquamosum* which is paraphyletic with the rest of the species. *Amomum IV* set is analogous in *Tree 1*, containing *A. laxesquamosum* and *A. pierreanum*, and can therefore also be counted in the *A. villosum* group.

The *Amomum II* group resembles Xia et al.'s *A. maximum* group with analogous species; *A. maximum*, *A. glabrum, A. austrosinensis, A. purpurerorubrum, A. queenslandicum, A. sericeum, A. putrescens, A. subcapitatum* and *A. menglaense*. In the *Amomum II* group there are two *Amomum* clades, one is monophyletic and supported (0.90), whereas the other is paraphyletic and has little statistical support (0.54). This second clade includes ElesmiGB1 (*E. smithiae*) and *EleuniGB1* (*E. unifolia*), as

Figure 2. *Tree 1, Amomum* combined markers

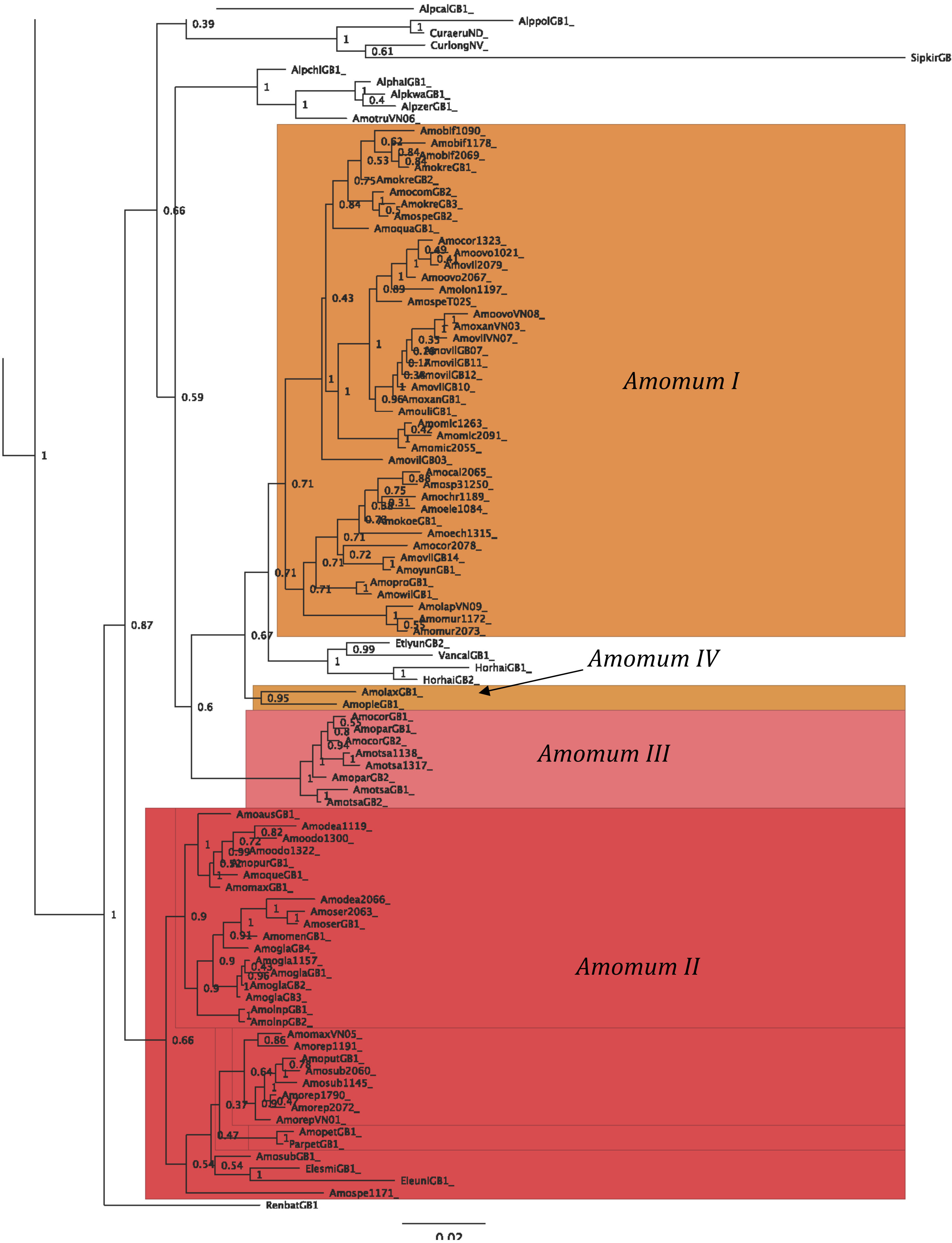


Figure 3. *Tree 2, Amomum* nuclear markers

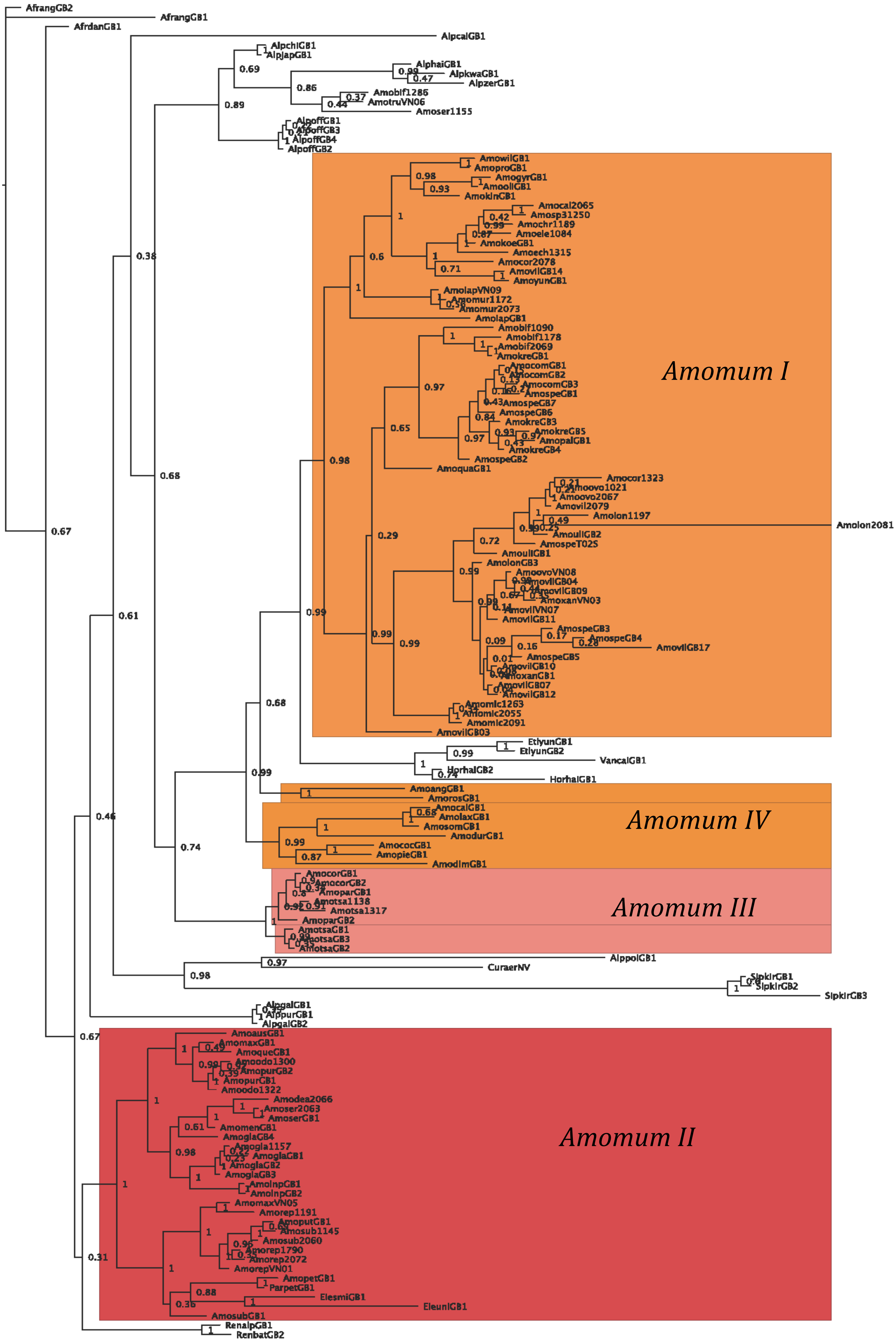
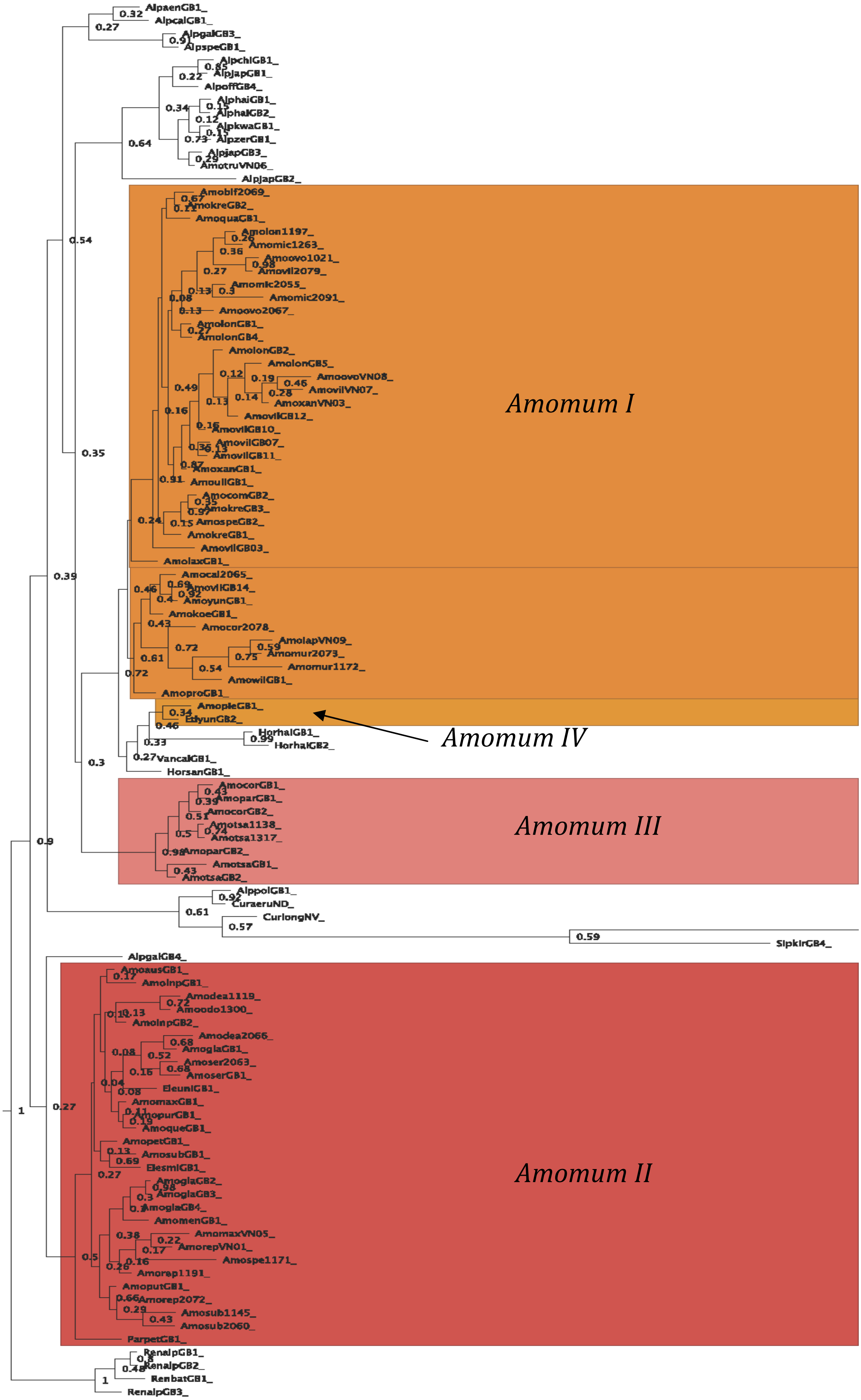


Figure 4. *Tree 3, Amomum* chloroplast markers



well as *ParpetGB1* (*Paramomum petaloideum*). The latter is sister to *AmopetGB1*(*Amomum petaloideum*), and closer inspection revealed that *P. petaloideum* is a synonym of *A. petaloideum* (cf. The Plant List, 2011).The set of *Amomum III* also resembles a group in Xia's et al. analysis; the *A. tsao-ko* group contented of *A. tsao-ko, A. paratsao-ko* and *A. coriandriodorum. AmokreGB1* and *GB2* (*A. aff. krervahn*) and *AmovilGB1* (*A. aff. villosum*) are all *Species Affinis* (a case when the species is unknown but has a strong similarity to a certain species) and in the tree they can be found in places where they might not fit in. Both *A. krervanh* accessions end up in a monophyletic cluster of species in the *Amomum I* group. One of them (*AmokreGB1*) is placed on a branch together with an *A. biflorum* and the other (*AmokreGB2*) is placed as a sister to all these *A. biflorum*. The "normal" *A. krervanh* is placed close to the *Affinis species* but in another monophyletic group within the *Amomum I* group among *A. compactum, A. qudratusquamosum* and *A. species*.

Chloroplast and nuclear trees. Comparing the trees we discover that the nuclear tree is one of the individual marker trees that have most likeliness with the combined trees. This suggests that the variation in the ITS data has a strong influence on the topology of the combined tree. Combining ITS data with other markers is probably the best choice.

The unknown *Amomum* species. The unknown species, collected in South-East Asia, have been studied by several experts and results from various people are not the same. This illustrates the difficulty in identifying species in this genus. See *Appendix 3*.

When unknown species are added in the analysis as in this case, we can use the final trees as reference trees. *Tree 1* (all markers combined), *Tree 2* (the nuclear sequences) and *Tree 3* (chloroplast sequences) are considered in this analysis. By studying the placement of the unknown species we can speculate what species it is, or at least to which species the unknown one is closely related. The unknown species are all collected in Vietnam and are therefore named with the initials *VN*. For some of the unknown species, samples of the extraction unfortunately failed, thus they are excluded from the analysis.

In *Tree 1*, with all three combined markers, *VN01* ended up on a branch as sister to two *A. repoeense* with 0.9 in support (that was placed on the same branch, though only with 0.47 support). *VN05* in the same monophyletic group together with an *A. plicatum* (0.86 support). *VN09* can be found in the *Amomum I* group (*A. villosum* group) as a sister to two *A. muricarpum* samples with 1.0 support. *VN03, VN07* and *VN08* are found in the so called *A. villosum* group as sisters to several *A. villosum* accessions. All three unknown species have 1.0 in support and are possibly *A. villosum*. *VN06* ended up outside of our *Amomum*-group among the *Alpinia* species which suggests this material probably belongs to the genus *Alpinia*.

In the tree with the nuclear sequences (see *Tree 2*) the unknown material has quite a similar placement as in *Tree 1. VN09* ends up in the *A. villosum* group among two *A. muricarpum* and *VN03*, *07* and *08* but here in the middle of several *A. villosum* species. *VN05* also here ends up together with the same *A. plicatum* (*Amorep1191*) as in *Tree 1*, and *VN01* is equally placed next to *A. repoeense* (*Amorep2072*), both in the *A. maximum* group. The chloroplast sequences (see *Tree 3*) almost have the same placement of the unknown species except for *VN01* and *05*. In this tree they end up on a branch together and the closest related is *A. chryseum* (*Amospe1171*). As a summary of this, it seems like the nuclear sequences or a combination of several sequences once again improved on the chloroplast solitary.

This study is merely a component of a larger ongoing study, thus the results are not definitive.

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Appendix 1: *Amomum* Lab samples

Species	**Name**	***Reference***
Amomum ovoideum	*Amoovo1021*	*Lamxay, V. 1021*
Amomum elephantorum	*Amoele1084*	*Lamxay, V. 1084*
Amomum chinense	*Amochi1089*	*Lamxay, V. & Bounlop 1089*
Amomum biflorum	*Amobif1090*	*Lamxay, V. 1090*
Amomum tomrey	*Amotom1114*	*Lamxay, V. 1114*
Amomum repoense	*Amorep1117*	*Lamxay, V. 1117*
Amomum dealbatum	*Amodea1119*	*Lamxay, V. 1119*
Amomum villosum	Amovil1120	*Lamxay, V. 1120*
Amomum dealbatum	Amodea1129	*Lamxay, V. & Phaphouampheng, P. 1129*
Amomum sp1	Amosp1131	*Lamxay V. et al. 1131*
Amomum glabrum	Amogla1137	*Lamxay, V. & Phounsimouang, S. 1137*
Amomum t-sao-ko	Amotsa1138	*Lamxay, V. & Phounsimouang, S. 1138*
Amomum subcapitatum	Amosub1145	*Lamxay, V. 1145*
Amomum petaloideum	Amopet1154	*Lamxay, V. 1154*
Amomum sericeum	Amoser1155	*Lamxay, V. & Phaphouampheng, P. 1155*
Amomum glabrum	Amogla1157	*Lamxay, V. & Phaphouampheng, P. 1157*
Amomum ***chryseum***	Amospe1171	*Lamxay V. et al. 1171*
Amomum muricarpum	Amomur1172	*Lamxay V. et al. 1172*
Amomum ***glabrifolium***	Amobif1178	*Lamxay, V. 1178*
Amomum ***celsum***	Amochr1189	*Lamxay, V. & Bounlop 1189*
Amomum ***plicatum***	Amorep1191	*Lamxay, V. & Bounlop 1191*
Amomum longiligulare	Amolon1197	*Lamxay, V. 1197*
Amomum chinense	Amochi1222	*Lamxay V. et al. 1222*
Amomum ***stephanocoleum***	Amosp31250	*Lamxay V. et al. 1250*
Amomum tomrey	Amotom1252	*Lamxay V. et al. 1252*
Amomum celseum	Amocel1253	*Lamxay V. et al. 1253*
Amomum staminidivum	Amosta1255	*Lamxay V. et al. 1255*
Amomum microcarpum	Amomic1263	*Lamxay V. et al. 1263*
Amomum elephantorum	Amoele1277	*Lamxay, V. & Newman,M.F. 1277*
Amomum ***glabrifolium***	Amobif1286	*Lamxay, V. 1286*
Amomum sp4	Amosp1290	*Lamxay V. et al. 1290*
Amomum calcicolum	Amocal1291	*Lamxay V. et al. 1291*
Amomum odontocarpum	Amoodo1300	*Lamxay, V. & Newman,M.F. 1300*
Amomum sp1	Amospe1303	*Lamxay V. et al. 1303*
Amomum sp5	Amospe1306	*Lamxay, V. & Newman,M.F. 1306*
Amomum echinocarpum	Amoech1315	*Lamxay, V. & Newman,M.F. 1315*
Amomum tsao-ko	Amotsa1317	*Lamxay, V. & Newman,M.F. 1317*
Amomum odontocarpum	Amoodo1322	*Lamxay, V. & Newman,M.F. 1322*
Amomum corynostachyum	Amocor1323	*Lamxay, V. & Newman,M.F. 1323*
Amomum ***plicatum***	Amorep1790	*Lamxay, V. 1790*
Amomum repoense var pinnetely blade	Amorep1880	*Lamxay, V. 1880*
Amomum dealbatum	Amodea2050	*Lamxay, V. 2050*
Amomum microcarpum	Amomic2055	*Lamxay, V. 2055*
Amomum subcapitatum	Amosub2060	*Lamxay, V. 2060*
Amomum sericeum	Amoser2063	*Lamxay, V. 2063*
Amomum calcaratum	Amocal2065	*Lamxay, V. 2065*
Amomum ***calcicolum***	Amodea2066	*Lamxay, V. 2066*
Amomum ovoideum	Amoovo2067	*Lamxay, V. 2067*
Amomum biflorum	Amobif2069	*Missing ref.*
Amomum repoeense	Amorep2072	*Lamxay, V. 2072*
Amomum muricarpum	Amomur2073	*Lamxay, V. 2073*

Amomum corynostachyum	Amocor2078	*Lamxay, V. 2078*
Amomum villosum	Amovil2079	*Lamxay, V. 2079*
Amomum longiligulare	Amolon2081	*Lamxay, V. 2081*
Amomum microcarpum	Amomic2091	*Lamxay, V. 2091*

Identifications of species in* ***bold* *have been changed from the field determinations. Mainly due to the description of new taxa.*

Appendix 2: GenBank samples

Species	Name	GenBank Accession number trnL	matK	ITS
Aframomum angustifolium isolate EDNA0800237	Afrang_GB	FJ848632		FJ848587
Aframomum angustifolium	Afrang_GB1		AF478804	AF478704
Aframomum daniellii	Afrdan_GB		AF478805	AF478705
Alpinia aff. calycodes Baker 1051	Alpcal_GB	AY769797		AY769834
Alpinia cf. aenea Argent et al. 0016	Alpaen_GB	AY769796		
Alpinia chinensis voucher LH	Alpchi_GB		EU586175	EU909426
Alpinia galanga	Alpgal_GB4	AY424775		
Alpinia galanga voucher DD	Alpgal_GB2			EU909429
Alpinia galanga voucher PS0515MT05	Alpgal_GB3		GU180388	
Alpinia galanga voucher XD	Alpgal_GB1			EU909428
Alpinia hainanensis voucher PS0511MT01	Alphai_GB1		GQ434102	GU180354
Alpinia hainanensis voucher PS0511MT02	Alphai_GB2		GQ434103	
Alpinia japonica	Alpjap_GB	AB111727		
Alpinia japonica	Alpjap_GB1	AB111720		
Alpinia japonica voucher LM	Alpjap_GB		EU586176	EU909427
Alpinia kwangsiensis voucher PS0513MT01	Alpkwa_GB		GU180382	GU180355
Alpinia officinarum voucher Xn	Alpoff_GB1			EU909414
Alpinia officinarum voucher GX	Alpoff_GB2			EU909422
Alpinia officinarum voucher HN	Alpoff_GB3			EU909424
Alpinia officinarum voucher PS0519MT04	Alpoff_GB4		GU180392	
Alpinia officinarum voucher SX	Alpoff_GB4			EU909425
Alpinia polyantha voucher PS0517MT01	Alppol_GB		GU180389	GU180373
Alpinia zerumbet voucher PS0532MT04	Alpzer_GB		GU180415	GU180368
Amomum aff. coriandriodorum Kress 99-6305	Amocor_GB1		AY352018	AY351988
Amomum aff. glabrum Xia-73	Amogla_GB4		AY352020	AY351990
Amomum aff. krervahn Xia-724	Amokre_GB1		AY352022	AY351992
Amomum aff. krervahn Xia-732	Amokre_GB2		AY352023	AY351993
Amomum aff. paratsao-ko Kress 98-6197	Amopar_GB1		AY352028	AY351998
Amomum aff. purpureorubrum Kress 98-6187	Amopur_GB1		AY352031	AY352001
Amomum aff. villosum Kress 00-6680	AmovilGB03		AY352040	AY352010
Amomum angustipetalum	Amoang_GB			AB097245
Amomum austrosinensis	Amoaus_GB		AY352015	AY351985
Amomum calyptratum	Amocal_GB			AB097239
Amomum compactum	Amocom_GB2		AY352016	AY351986
Amomum compactum voucher PS0535MT01	Amocom_GB3			GQ118672
Amomum compactum voucher PS0535MT01	Amocom_GB1			FJ972782
Amomum coriaceum	Amococ_GB			AB097240
Amomum coriandriodorum	Amocor_GB2		AY352017	AY351987

Amomum dimorphum	Amodim_GB			AB097244
Amomum durum	Amodur_GB			AB097241
Amomum glabrum	Amogla_GB2		AF478821	AF478721
Amomum glabrum	Amogla_GB3		AY352019	AY351989
Amomum glabrum isolate EDNA0800236	Amogla_GB1	FJ848631		FJ848586
Amomum gyrolophos	Amogyr_GB			AB097242
Amomum kinabaluense	Amokin_GB			AF414489
Amomum koenigii	Amokoe_GB		AY352021	AY351991
Amomum krervanh voucher PS0516MT01	Amokre_GB3		FJ972783	GQ118669
Amomum krervanh voucher PS0516MT01	Amokre_GB4			FJ972779
Amomum krervanh voucher PS0516MT01	Amokre_GB5			GQ434442
Amomum lappaceum	Amolap_GB			AF414488
Amomum laxesquamosum	Amolax_GB		AY352024	AY351994
Amomum longiligulare	Amolon_GB4		GQ404378	
Amomum longiligulare voucher PS0522MT01	Amolon_GB1		FJ972784	
Amomum longiligulare voucher PS0522MT02	Amolon_GB2		GQ118673	
Amomum longiligulare voucher PS0522MT03	Amolon_GB3			GU180362
Amomum longiopetiolatum	Amolnp_GB1		AF478822	AF478722
Amomum longipetiolatum	Amolnp_GB2	AY769788		AY769825
Amomum maximum	Amomax_GB		AY352025	AY351995
Amomum menglaense	Amomen_GB		AY352026	AY351996
Amomum oliganthum	Amooli_GB			AB097243
Amomum palawanense isolate Cronk25351	Amopal_GB			FJ883009
Amomum paratsao-ko	Amopar_GB2		AY352027	AY351997
Amomum petaloideum	Amopet_GB	AY769789		AY769826
Amomum pierreanum	Amopie_GB	AY769792		AY769829
Amomum propinquum	Amopro_GB		AY352029	AY351999
Amomum purpureorubrum	Amopur_GB1		AY352030	AY352000
Amomum putrescens	Amoput_GB		AY352032	AY352002
Amomum quadratolaminare	Amoqua_GB		AY352033	AY352003
Amomum queenslandicum	Amoque_GB		AY352034	AY352004
Amomum roseisquamosum	Amoros_GB			AB097246
Amomum sericeum	Amoser_GB		AY352035	AY352005
Amomum somniculosum	Amosom_GB			AB097247
Amomum sp. CD-2009 voucher EDQM 25818	Amospe_GB3			FJ528293
Amomum sp. CD-2009 voucher EDQM 25819	Amospe_GB6			FJ528296
Amomum sp. CD-2009 voucher EDQM 32513	Amospe_GB5			FJ528295
Amomum sp. CD-2009 voucher EDQM 32539	Amospe_GB7			FJ528297
Amomum sp. CD-2009 voucher EDQM 32954	Amospe_GB1			FJ528298
Amomum sp. CD-2009 voucher EDQM 33015	Amospe_GB4			FJ528294
Amomum sp. Kress 99-6373	Amospe_GB2		AF478823	AF478723

Amomum sp. Wilkie et al. 29016	AmoWil_GB	AY769793		AY769830
Amomum subcapitatum	Amosub_GB		AY352036	AY352006
Amomum tsao-ko	Amotsa_GB2		AY352037	AY352007
Amomum tsaoko voucher PS0512MT01	Amotsa_GB1		FJ972785	GQ118666
Amomum tsaoko voucher PS0512MT01	Amotsa_GB3			FJ972776
Amomum uliginosum	Amouli_GB1	AY769790	AY352038	AY352008
Amomum uliginosum	Amouli_GB2			AY769827
Amomum villosum	AmovilGB07		AF478824	AF478724
Amomum villosum	AmovilGB10	AY769791	AY352039	AY769828
Amomum villosum	AmovilGB17			EF488008
Amomum villosum isolate Chenxiang	AmovilGB04			JF292430
Amomum villosum isolate Mayangxi	AmovilGB09			JF292431
Amomum villosum var. xanthioides	Amoxan_GB		AY352041	AY352011
Amomum villosum var. xanthioides voucher PS0526MT01	AmovilGB14		FJ972787	GQ118671
Amomum villosum voucher PS0514MT01	AmovilGB11		FJ972786	GQ118667
Amomum villosum voucher PS0514MT02	AmovilGB12		GU180383	GQ118668
Amomum yunnanense	Amoyun_GB		AY352042	AY352012
Cornus amomum voucher Xiang 01-127	Coramo_GB		DQ340453	
Elettariopsis smithiae	Elesmi_GB		AY352043	AY352013
Elettariopsis unifolia	Eleuni_GB	AY769795		AY769832
Etlingera yunnanensis	Etlyun_GB1			AF478751
Etlingera yunnanensis	Etlyun_GB2	AY769809	AY352044	AY352014
Hornstedtia hainanensis	Horhai_GB		AF478865	AF478766
Hornstedtia hainanensis voucher PS2000MT02	Horhai_GB1		GU180420	GU180371
Hornstedtia sanhan	Horsan_GB	AY769807		
Paramomum petaloideum	Parpet_GB		AF478872	AF478771
Renealmia alpinia isolate HN39	Renalp_GB1	DQ444497		
Renealmia alpinia isolate HN40	Renalp_GB2	DQ444498		
Renealmia alpinia	Renalp_GB3		AF478879	
Renealmia alpinia voucher Nagata 2338 (E)	Renalp_GB			DQ427030
Renealmia battenbergiana isolate TS23	Renbat_GB1	DQ444515	AF478880	
Renealmia battenbergiana voucher AN 044 (HLA)	Renbat_GB2			DQ427031
Siphonochilus kirkii	Sipkir_GB1+2		AF478895	AF478794
Siphonochilus kirkii	Sipkir_GB3			AF202417
Siphonochilus kirkii voucher Kress 94-3692	Sipkir_GB4	AY140429		
Vanoverberghia sepulchrei	Vancal_GB		AF478899	AF478798

Appendix 3: Unknown *Amomum* samples

Sample	**Det. Dr. Binh**	**Det Dr. Newman and Dr. Lamxay**	**Name**
VN01	*Amomum repoeense*	*Amomum subcapitatum*	AmorepVN01
VN02	*Amomum sp.*	*Amomum chryseum*	AmospVN02
VN03	*Amomum xanthioides*	*Amomum villosum var. xanthioides*	AmoxanVN03
VN04	*Amomum muricarpum*	*Amomum muricarpum*	AmomurVN04
VN05	*Amomum maximum*	*Amomum repoeense*	AmomaxVN05
VN06	*Amomum truncatum*	-	AmotruVN06
VN07	*Amomum villosum*	*Amomum villosum*	AmovilVN07
VN08	*Amomum ovoideum*	-	AmoovoVN08
VN09	*Amomum lappaceum*	*Amomum muricarpum*	AmolapVN09