Acta Botanica Brasilica
doi: 10.1590/0102-33062020abb0051

Toward a phylogenetic reclassification of the subfamily Ambavioideae (Annonaceae): establishment of a new subfamily and a new tribe

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Received: February 14, 2020
Accepted: June 12, 2020

ABSTRACT

A molecular phylogeny of the subfamily Ambavioideae (Annonaceae) was reconstructed using up to eight plastid DNA regions (*matK*, *ndhF*, and *rbcL* exons; *trnL* intron; *atpB-rbcL*, *psbA-trnH*, *trnL-trnF*, and *trnS-trnG* intergenic spacers). The results indicate that the subfamily is not monophyletic, with the monotypic genus *Meiocarpidium* resolved as the second diverging lineage of Annonaceae after *Anaxagorea* (the only genus of Anaxagoreoideae) and as the sister group of a large clade consisting of the rest of Annonaceae. Consequently, a new subfamily, Meiocarpidioideae, is established to accommodate the enigmatic African genus *Meiocarpidium*. In addition, the subfamily Ambavioideae is redefined to contain two major clades formally recognized as two tribes. The tribe Tetramerantheae consisting of only *Tetrameranthus* is enlarged to include *Ambavia*, *Cleistopholis*, and *Mezzettia*; and Canangeae, a new tribe comprising *Cananga*, *Cyathocalyx*, *Drepananthus*, and *Lettowianthus*, are erected. The two tribes are principally distinguishable from each other by differences in monoploid chromosome number, branching architecture, and average pollen size (monads). New relationships were retrieved within Tetramerantheae, with *Mezzettia* as the sister group of a clade containing *Ambavia* and *Cleistopholis*.

Keywords: Annonaceae, Ambavioideae, *Meiocarpidium*, molecular phylogeny, systematics, taxonomy

Introduction

Annonaceae, a pantropical family of flowering plants prominent in lowland rainforests, consist of 110 genera (Guo *et al.* 2017; Chaowasku *et al.* 2018a; b; Xue *et al.* 2018) and approximately 2430 species (Chatrou *et al.* 2018). The family has been classified into four subfamilies, *viz.*, Anaxagoreoideae, Ambavioideae, Annonoideae, and Malmeoideae; the last two subfamilies, which constitute the majority of generic and species diversity in the family, have each been further subdivided into tribes (Chatrou *et al.* 2012). Two additional tribes in Malmeoideae have been subsequently proposed (Guo *et al.* 2017; Couvreur *et al.* 2019). Every subfamily received unequivocally and consistently strong molecular support except the subfamily Ambavioideae, which is composed of nine genera: *Ambavia*, *Cananga*, *Cleistopholis*, *Cyathocalyx*, *Drepananthus*, *Lettowianthus*, *Meiocarpidium*, *Mezzettia*, and *Tetrameranthus* (*e.g.*, Surveswaran *et al.* 2010; Chatrou *et al.* 2012; Guo *et al.* 2017; Couvreur *et al.* 2019). The monotypic *Meiocarpidium* endemic to Africa is a phylogenetically problematic genus because it was identified as the sister group of a clade composed of the remaining genera of Ambavioideae, but with only moderate to no support in certain analyses (Guo *et al.* 2017), which is in contrast to the analyses presented by Surveswaran *et al.* (2010), Chatrou *et al.* (2012), and Xue

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et al. (2018), yielding rather strong support. Further, in an analysis using a targeted enrichment of nuclear genes by Couvreur *et al.* (2019), this genus was either weakly supported as the sister group of the remaining Ambavioideae genera or moderately supported as the sister group of Annonaceae excluding Anaxagoreoideae and other Ambavioideae members.

The primary aim of the present study is, therefore, to re-elucidate the position of *Meiocarpidium* by sequencing two and three additional plastid regions of this genus compared to Chatrou *et al*. (2012) and Guo *et al.* (2017), respectively. In addition, more plastid regions are also sequenced for representatives of *Mezzettia*, *Cyathocalyx*, and *Drepananthus* in order to gain deeper insights into relationships of particular clades in Ambavioideae, *i.e.*, the *Ambavia*-*Cleistopholis*-*Mezzettia* and *Cananga*-*Cyathocalyx*-*Drepananthus* clades. It is worthy of notice that the relationships in the latter clade have been controversial since *Cyathocalyx* was found to be the sister group of *Drepananthus* in one study (Guo *et al*. 2017), whereas *Cananga* and *Drepananthus* were recovered as sister genera in another study (Xue *et al*. 2018).

Materials and methods

Character and taxon sampling (see List S1 in supplementary materials, for a list of taxa, voucher information, and GenBank accession number)

Twenty-four accessions comprised the ingroup, with three representatives of Annonoideae, four representatives of Malmeoideae, and 17 representatives of Ambavioideae covering all currently accepted genera in this subfamily. At least two accessions or species per genus were included for *Cananga*, *Cyathocalyx*, *Drepananthus*, *Meiocarpidium*, and *Mezzettia*. Two species of *Anaxagorea* (the only genus of Anaxagoreoideae) were assigned as outgroups because this subfamily has always been recovered as the sister group of a consistently strongly supported clade comprising the remaining subfamilies of Annonaceae (*e.g.*, Chatrou *et al*. 2012; Guo *et al*. 2017). Up to eight plastid regions (*matK*, *ndhF*, and *rbcL* exons; *trnL* intron; *atpB*-*rbcL*, *psbA*-*trnH*, *trnL*-*trnF*, and *trnS*-*trnG* intergenic spacers) were included in this study. Seventy-seven sequences were newly produced for the present study; the remaining sequences were taken from GenBank. There were some missing data in the following accessions (see List S1 in supplementary materials): *Ambavia gerrardii* (Baill.) Le Thomas, *Lettowianthus stellatus* Diels, *Tetrameranthus duckei* R.E.Fr., and one specimen of *Meiocarpidium oliverianum* (Baill.) D.M.Johnson & N.A.Murray (*M. oliverianum* is the same species as the well-known *M. lepidotum* (Oliv.) Engl. & Diels; the basionym of the former was published before the basionym of the latter; see Johnson & Murray 2018).

DNA extraction, amplification, and sequencing

All methods for DNA extraction, amplification, and sequencing used in the present study were the same as those described in Chaowasku *et al*. (2018a; b; 2020). For plastid regions not included in Chaowasku *et al*. (2018a; b; 2020), their primer sequences for the amplification and sequencing were obtained from Hoot *et al*. (1995) and Scharaschkin & Doyle (2005) for *atpB*-*rbcL* intergenic spacer, with internal primers newly designed for the present study: ATPB-RBCL-INT-A (5'- GGATGCTGAAATAAAGAACAACAGCC -3') and ATPB-RBCL-INT-B (5'- GGCTGTTGTTCTTTATTTCAGCATCC -3'); and Hamilton (1999) for *trnS*-*trnG* intergenic spacer, with internal primers newly designed for the present study: TRNSG-NEW-F-SHORT (5'- CCTCTTTGATTCCGTACGAAAGG -3'), TRNSG-NEW-R (5'- GTCGAATAAGCGAATGAGACG -3'), and TRNSG-INT-R-SHORT (5'- GGAATGGAAATAGCCTTTGTCAC -3').

Phylogenetic analyses

Sequences were edited using the Staden package [http://staden.sourceforge.net] (Staden *et al*. 2000) and then aligned by Multiple Alignment using Fast Fourier Transform (MAFFT; Katoh *et al*. 2002) via an online platform (Katoh *et al*. 2017), with default settings. The aligned matrix was subsequently checked and manually adjusted (where necessary) using the similarity criterion (Simmons 2004). In total, 7,149 aligned nucleotide plus twelve binary-coded indel characters were included. Following Simmons & Ochoterena (2000), the simple coding method for the binary indel characters was implemented, with the focus on non-autapomorphic and less homoplasious indel structures. A 15 base-pair inversion is present in certain accessions in the *psbA*-*trnH* intergenic spacer and was complementarily reversed to be homologically alignable to the remaining accessions, following Pirie *et al*. (2006). The phylogenetic trees were rooted by specifying the two *Anaxagorea* species as outgroups.

Parsimony analysis was performed in TNT version 1.5 (Goloboff & Catalano 2016). All characters were equally weighted and unordered. Incongruence among regions was assessed by performing an analysis for each region to see if there was any significant conflict in clade support (*e.g.*, Wiens 1998). Most parsimonious trees were generated by a heuristic search of the combined data, with 9,000 replicates of random sequence addition, saving 10 trees per replicate, and using the tree bisection and reconnection (TBR) branch-swapping algorithm. Clade support was evaluated by symmetric resampling (SR; Goloboff *et al.* 2003). A default change probability was used. One hundred thousand replicates were run, each with five replicates of random sequence addition, saving five trees per replicate. A clade with SR ≥ 85 %, 70–84 %, or 50–69 % was considered strongly, moderately, or weakly supported, respectively.

Maximum likelihood analysis was accomplished in IQ-TREE version 1.6.10 (Nguyen *et al*. 2015) under partition models (Chernomor *et al*. 2016) performed with the "-spp" command, whereas Bayesian Markov chain Monte Carlo (MCMC; Yang & Rannala 1997) phylogenetic analysis was implemented in MrBayes version 3.2.7a (Ronquist *et al*. 2012). Both methods were analyzed via the CIPRES Science Gateway version 3.3 (Miller *et al*. 2010). The data matrix was divided into seven partitions based on identity of DNA region (the *trnL* intron and adjacent *trnL*-*trnF* intergenic spacer were combined as a single partition) plus a binary-coded indel partition. The most suitable model of sequence evolution for each DNA partition was selected by Akaike Information Criterion (AIC; Akaike 1974) scores, using FindModel [https://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html] (Posada & Crandall 1998). The General Time Reversible (GTR; Tavaré 1986) nucleotide substitution model with a gamma distribution for among-site rate variation was selected for all seven DNA partitions (*atpB*-*rbcL*, *matK*, *ndhF*, *psbA*-*trnH*, *rbcL*, *trnLF* [= *trnL* intron + *trnL*-*trnF* intergenic spacer], and *trnS*-*trnG*). In the maximum likelihood analysis, the model "JC2+FQ+ASC" was selected by corrected AIC scores for the binary indel partition. Clade support was measured by non-parametric bootstrap resampling method (BS; Felsenstein 1985) with 2,000 replicates. A clade with BS ≥ 85 %, 70–84 %, or 50–69 % was considered strongly, moderately, or weakly supported, respectively.

In the Bayesian analysis, the "coding=variable" setting was implemented for the binary indel partition, which was performed under a simple F81-like model without a gamma distribution for among-site rate variation. Four independent analyses, each using four MCMC chains, were simultaneously run; each run was set for 10 million generations. The default prior settings were used except for the prior parameter of rate multiplier ("ratepr" [=variable]). The temperature parameter was set to 0.08. Trees and all parameter values were sampled every 1,000th generation. Convergence was assessed by checking the standard deviation of split frequencies of the runs with values < 0.01 interpreted as indicating a good convergence and by checking for adequate effective sample sizes (ESS > 200) using Tracer version 1.6 (Rambaut *et al*. 2013). The first 25 % of all trees sampled were discarded as burn-in, and the 50 % majority-rule consensus tree was built from the remaining trees. A clade with posterior probabilities (PP) ≥ 0.95, 0.9–0.94, or 0.5–0.89 was considered strongly supported, weakly supported, or unsupported, respectively.

Results

The parsimony analysis resulted in six most parsimonious trees with 2,506 steps. The consistency (CI) and retention (RI) indices were 0.81 and 0.84, respectively. There were no strong conflicts (SR ≥ 85 %) among the analyses of different plastid regions. Figure 1 shows a 50 % majority-rule consensus tree derived from the Bayesian analysis, with corresponding support values from the other two analyses, parsimony and maximum likelihood, whereas Figure 2 depicts a phylogram derived from the maximum likelihood analysis and a strict consensus cladogram obtained from the parsimony analysis. The ingroup, comprising Ambavioideae, Annonoideae, and Malmeoideae, was recovered as a monophyletic group with maximum support. Two accessions of *Meiocarpidium oliverianum* were retrieved as a maximally supported clade, which was the sister group of a moderately to strongly supported clade (SR 99 %, BS 78 %, PP 0.97) composed of the remaining ingroup accessions: Annonoideae (three accessions), Malmeoideae (four accessions), and the rest of Ambavioideae (15 accessions). The three accessions of Annonoideae, four of Malmeoideae, and 15 of the remaining Ambavioideae were each recovered as a maximally supported clade, with the last one being the sister group of a strongly supported clade (SR 99 %, BS 98 %, PP 1) comprising Annonoideae and Malmeoideae accessions.

In the 15-accession clade of Ambavioideae, two major clades can be identified: 1) a maximally supported clade consisting of *Cananga*, *Cyathocalyx*, *Drepananthus*, and *Lettowianthus*; and 2) a strongly supported clade (SR 99 %, BS 100 %, PP 1) comprising *Ambavia*, *Cleistopholis*, *Mezzettia*, and *Tetrameranthus*. In the former clade, *Lettowianthus* was the sister group of a strongly supported clade (SR 99 %, BS 100 %, PP 1) embracing *Cananga*, *Cyathocalyx*, and *Drepananthus*. Each of these three genera was monophyletic with maximum support, but their relationships were completely unresolved. In the *Ambavia*-*Cleistopholis*-*Mezzettia*-*Tetrameranthus* clade, *Tetrameranthus* was retrieved as the sister group of a strongly supported clade (SR 96 %, BS 97 %, PP 1) comprising *Ambavia*, *Cleistopholis*, and *Mezzettia*. *Mezzettia* was then the sister group of a moderately to strongly supported clade (SR 94 %, BS 75 %, PP 0.98) consisting of *Ambavia* and *Cleistopholis*.

Discussion

With more plastid DNA sequenced, the Ambavioideae topology has changed. Apart from Anaxagoreoideae, *Meiocarpidium* is sister to a well-supported clade embracing all other members of Annonaceae (Fig. 1). Given this new topology and the previously reported negligible support for *Meiocarpidium* as the sister group of the remaining Ambavioideae (Guo *et al*. 2017; Couvreur *et al*. 2019), maintaining *Meiocarpidium* in Ambavioideae is inappropriate. In phylogenetic systematics, the most pivotal element for delimiting a taxon is well-supported monophyly and in this case *Meiocarpidium* should be reclassified in its own subfamily because all other genera in the family have been assigned to subfamilies. Therefore, a new subfamily accommodating this African monotypic genus is proposed below. *Meiocarpidium* becomes the second diverging lineage of Annonaceae after *Anaxagorea*. The genus is easily identifiable because of the possession of lepidote indumentum (*e.g.*, Heusden 1992; Keßler 1993), which is rarely found elsewhere in the family (*i.e.*, in *Duguetia*; Maas *et al*. 2003). Several palynological features, especially the obvious intine extrusion (Thomas 1980; 1981; Hesse *et al*. 1985), have suggested that *Meiocarpidium* may be placed phylogenetically somewhere near *Anaxagorea*. Apart from *Meiocarpidium* and *Anaxagorea*, the more or less bulging intine also occurs in several other genera of Malmeoideae exhibiting a monosulcate pollen, *e.g.*, *Bocageopsis*, *Malmea*, *Unonopsis* (Waha 1985); *Maasia* (Waha & Hesse 1988); *Mwasumbia* (Couvreur *et al*. 2009); and *Dendrokingstonia*, *Monocarpia* (Chaowasku *et al*. 2012). Further research is required to ascertain if the monosulcate pollen of all other genera of Annonaceae really does not exhibit a bulging intine. The lack of intine extrusion could be due to immature material or unsuitable methodology of pollen preparation.

Meiocarpidioideae Chaowasku ***subfam. nov.***
Type genus: *Meiocarpidium* Engl. & Diels

Trees or shrubs, with distichous arrangement of both leaves and lateral branches; indumentum of lepidote (sometimes stellate) hairs; inflorescences 1- or few-flowered, terminal; flowers bisexual, both petal whorls of ± equal size; staminal connective apex truncate and dilated; carpels free in flower and fruit; ovules many, with lateral placentation; monocarps subsessile, monocarp abscission basal; aril absent; endosperm ruminations lamelliform; middle seed integument present.

Genus included: *Meiocarpidium*

The maximally supported Ambavioideae excluding *Meiocarpidium* are recircumscribed herein as Ambavioideae *sensu stricto*, containing eight genera (Fig. 1): *Cananga*, *Cyathocalyx*, *Drepananthus*, and *Lettowianthus* in a maximally supported clade; *Ambavia*, *Cleistopholis*, *Mezzettia*, and *Tetrameranthus* in another clade with strong support. The latter clade corresponds to the "ambavioid" clade previously coined and defined by Doyle & Thomas (1996) and Thomas & Doyle (1996), which differs greatly from the former clade ("canangoid" clade *sensu* Surveswaran *et al*. 2010), *e.g.*, in monoploid chromosome number ($x = 7$ in members of the "ambavioid" clade *vs*. $x = 8$ in members of the "canangoid" clade [karyological data of *Lettowianthus* are unknown]; Okada & Ueda 1984; Morawetz 1986; Morawetz & Thomas 1988), branching architecture (distichous arrangement of lateral branches in members of the "ambavioid" clade [except *Tetrameranthus*, which has spirally arranged leaves and lateral branches unique

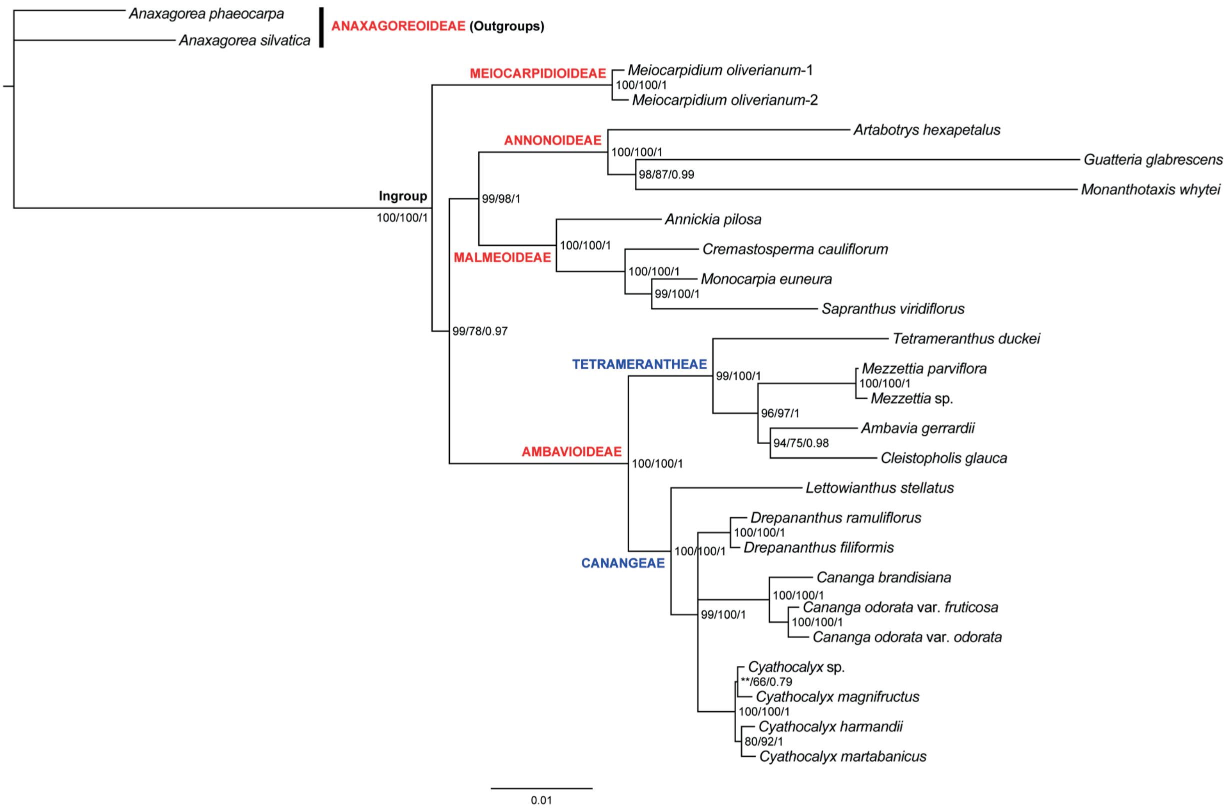

Figure 1. 50 % majority-rule consensus phylogram derived from Bayesian inference of combined eight plastid DNA regions. Bayesian posterior probabilities (PP) indicated on the right; maximum likelihood bootstrap (BS) percentages in the middle; parsimony symmetric resampling (SR) percentages on the left; scale bar unit = substitutions per site.

in the family] *vs*. spiral arrangement of lateral branches in members of the "canangoid" clade; Johnson 2003; Westra & Maas 2012; pers. obs. for *Ambavia*, *Drepananthus*, and *Mezzettia*), and average pollen size (monads: small [< 45 µm] in members of the "ambavioid" clade *vs*. medium [45–90 µm] to large [> 90 µm] in members of the "canangoid" clade; Surveswaran *et al*. 2010; Doyle & Thomas 2012). Consequently, each clade deserves formal recognition and a new tribe is proposed for the "canangoid" clade, whereas the monotypic tribe Tetramerantheae is enlarged to include

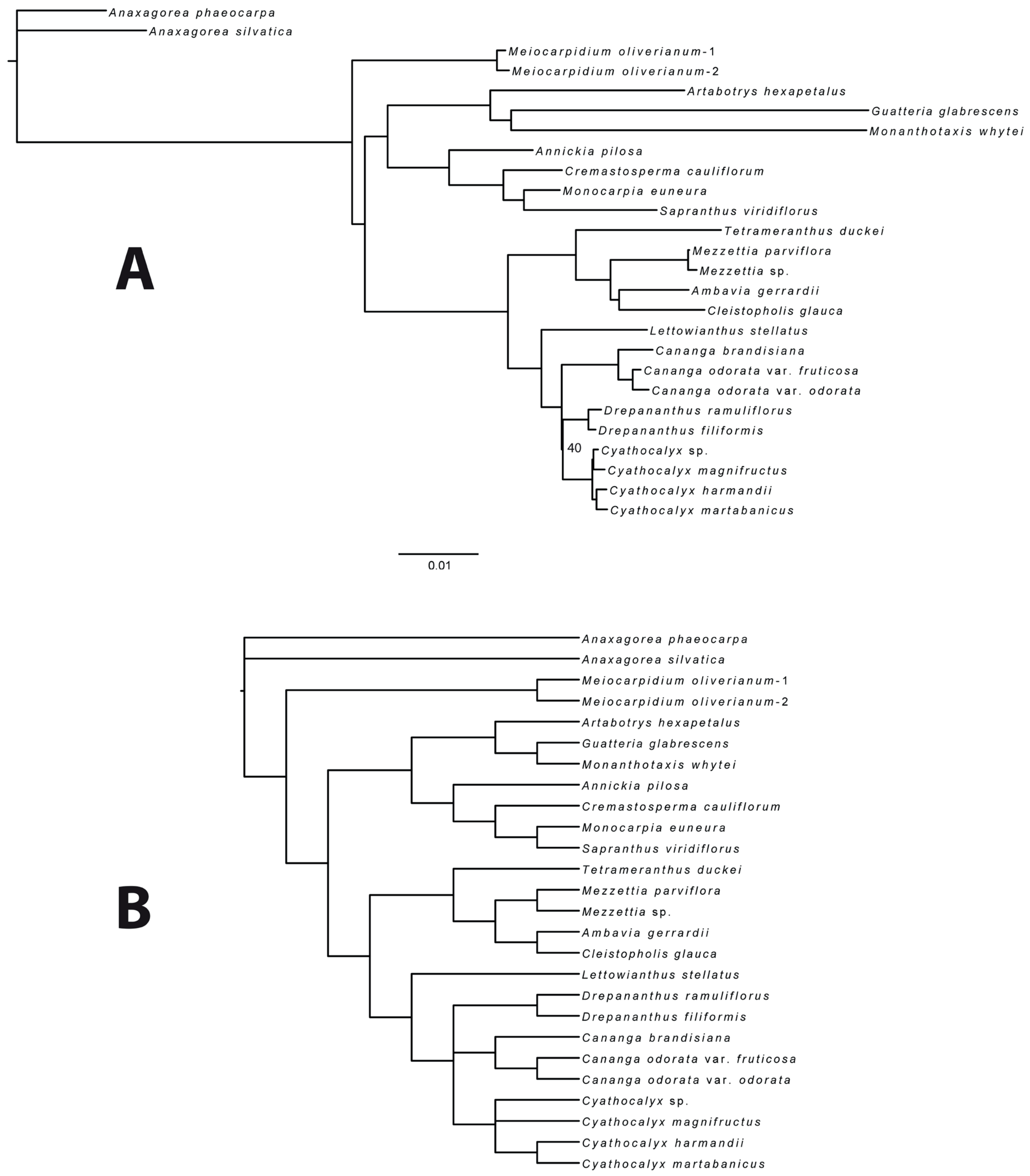

Figure 2. A. Phylogram derived from maximum likelihood analysis, with maximum likelihood bootstrap (BS) percentages shown for a clade absent in Figure 1; scale bar unit = substitutions per site. **B.** Strict consensus cladogram obtained from parsimony analysis.

the other three genera in the "ambavioid" clade. Table 1 summarizes the chief differences between the two tribes of the recircumscribed Ambavioideae.

Ambavioideae Chatrou, Pirie, Erkens & Couvreur ***descr. emend.***
Type genus: *Ambavia* Le Thomas

Trees or shrubs, with distichous or spiral arrangement of both leaves and lateral branches, or with distichous arrangement of leaves and spiral arrangement of lateral branches; indumentum of simple or stellate hairs; inflorescences 1- to many-flowered, terminal or axillary; flowers bisexual, both petal whorls of ± equal size or inner one being (much) smaller; staminal connective apex truncate and dilated, sometimes tongue-shaped, ± conical, or apiculate; carpels free in flower and fruit; ovules 2 to many, with lateral placentation; monocarps (sub)sessile or stipitate, monocarp abscission basal or apical; aril sometimes present; endosperm ruminations irregular to ± flattened peg-like, stout or not; middle seed integument present (unknown in *Ambavia*).

Genera included: *Ambavia*, *Cananga*, *Cleistopholis*, *Cyathocalyx*, *Drepananthus*, *Lettowianthus*, *Mezzettia*, and *Tetrameranthus*

Tetramerantheae R.E.Fr. ex Reveal ***descr. emend.***
Type genus: *Tetrameranthus* R.E.Fr.

Trees or shrubs, with distichous or spiral arrangement of both leaves and lateral branches; indumentum of simple or stellate hairs; inflorescences 1- to several-flowered, usually umbel-like when multi-flowered, axillary; flowers bisexual, both petal whorls of ± equal size or inner one being (much) smaller; staminal connective apex truncate and dilated, sometimes tongue-shaped or ± conical; carpels free in flower and fruit; ovules 2(–3), with lateral placentation; monocarps (sub)sessile or stipitate, monocarp abscission basal or apical; aril absent; endosperm ruminations irregular, stout; monoploid chromosome number $x = 7$; with small (< 45 µm) average pollen size [monads].

Genera included: *Ambavia*, *Cleistopholis*, *Mezzettia*, and *Tetrameranthus*

Canangeae Chaowasku ***tribus nov.***
Type genus: *Cananga* (Dunal) Hook.f. & Thomson

Trees or shrubs, with distichous arrangement of leaves and spiral arrangement of lateral branches; indumentum of simple or stellate hairs; inflorescences 1- to many-flowered, terminal or axillary; flowers bisexual, both petal whorls of ± equal size; staminal connective apex truncate and dilated, sometimes apiculate; carpels free in flower and fruit; ovules 2 to many, with lateral placentation; monocarps (sub)sessile or stipitate, monocarp abscission basal or apical; aril sometimes present; endosperm ruminations ± irregular to ± flattened peg-like, sometimes stout; monoploid chromosome number $x = 8$ (unknown in *Lettowianthus*); with medium (45–90 µm) to large (> 90 µm) average pollen size [monads].

Genera included: *Cananga*, *Cyathocalyx*, *Drepananthus*, and *Lettowianthus*

It is noteworthy that all genera in Tetramerantheae possess irregular and stout endosperm ruminations (Setten & Koek-Noorman 1992). However, this trait is present in *Lettowianthus* of Canangeae as well, but there are also elements exhibiting more or less flattened peg-like with a dilated apex as observed in *Cananga*, *Cyathocalyx*, and *Drepananthus* (Setten & Koek-Noorman 1992). With more plastid regions sequenced, the phylogenetic relationships within the *Cananga-Cyathocalyx-Drepananthus* clade of Canangeae have become unresolved (Fig. 1), clearly necessitating further inclusion of more variable plastid and/or nuclear DNA sequences.

The presence of lamelliform endosperm ruminations in Meiocarpidioideae has some implications on the evolution of this trait in Annonaceae as discussed in Pirie & Doyle (2012); however, detailed comparisons with the lamelliform endosperm ruminations of Malmeoideae and Annonoideae genera should be conducted to verify whether they are homologous before performing any character evolutionary analyses. It would also be interesting to understand the evolution of a middle seed integument, whether it has originated independently several times (in Meiocarpidioideae, Ambavioideae, some genera and species of Malmeoideae, and a species of *Artabotrys* [Annonoideae]) or has originated in the common ancestor of the Meiocarpidioideae-Ambavioideae-Malmeoideae-Annonoideae clade, been lost in the Malmeoideae-Annonoideae clade, and then gained in some genera and species of Malmeoideae, plus a species of *Artabotrys* (Christmann 1989).

Table 1. Chief differences between two tribes, Tetramerantheae *descr. emend.* and Canangeae *tribus nov.*, of recircumscribed Ambavioideae.

Feature	Tetramerantheae *descr. emend.*	Canangeae *tribus nov.*
Monoploid chromosome number	$x = 7$	$x = 8$ (unknown in *Lettowianthus*)
Branching architecture	Distichous arrangement of lateral branches (except *Tetrameranthus*, which exhibits spirally arranged leaves and lateral branches)	Spiral arrangement of lateral branches
Average pollen size (monads, µm)	Small (< 45)	Medium (45–90) to large (> 90)

The well-supported phylogenetic relationships of Tetramerantheae in the present study reveal that *Ambavia* is the sister group of *Cleistopholis*, and a clade comprising *Ambavia* and *Cleistopholis* is then the sister group of *Mezzettia* (Fig. 1). This is in contrast to the previously reported phylogenetic hypotheses, *i.e.*, *Mezzettia* was the sister group of *Ambavia*, and a clade uniting these two genera was the sister group of *Cleistopholis* (*e.g.*, Surveswaran *et al*. 2010; Guo *et al*. 2017). As a consequence, there definitely are biogeographic implications, especially in the likely older split of Asian (*Mezzettia*) and Afro-Malagasy (*Cleistopholis*-*Ambavia*) lineages (see Thomas *et al*. 2015 for more details).

Acknowledgements

I would like to thank the curators/directors of CMUB and WAG (now under Naturalis, The Netherlands) herbaria for the material studied. Kithisak Aongyong and Maxim Nuraliev provided useful specimens for this study. Anissara Damthongdee and Hathaichanok Jongsook assisted with some laboratory works. This study was financially supported by the Thailand Research Fund (TRG5880118), IAPT Research Grants Program in Plant Systematics 2013, and Chiang Mai University. James Doyle and an anonymous reviewer considerably improved an earlier draft of this article.

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