



Phylogeny, molecular dating and floral evolution of Magnoliidae (Angiospermae)

Julien Massoni

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par

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Phylogeny, molecular dating, and floral evolution of Magnoliidae (Angiospermae)

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Abstract

Deep phylogenetic relationships in the angiosperms had long been uncertain. However, by the end of the 1990s, large-scale studies contributed to the current well resolved picture of the tree of flowering plants, in which eudicots, monocots, and magnoliids are the three largest clades. Whereas monocots and eudicots have been recognized since the very first phylogenetic analyses, the monophyly of magnoliids (Canellales, Laurales, Magnoliales, and Piperales) is a more recent result. Magnoliidae, as now circumscribed, consist of 20 families and ca. 10,000 species mostly distributed in the tropics (with a few exceptions extending into the temperate zone). Before the present thesis, several parts of the magnoliid tree had been well studied, but little was known about the evolutionary history of Magnoliidae as a whole. The first chapter of this thesis is a phylogenetic study conducted to clarify the phylogenetic relationships among families and orders of Magnoliidae. To do so, I sampled 199 species of Magnoliidae and 12 molecular markers from the three genomes (plastid *atpB*, *matK*, *trnL* intron, *trnL-trnF* spacer, *ndhF*, *rbcl*; mitochondrial *atp1*, *matR*, mtSSU, mtLSU; nuclear 18S rDNA, 26S rDNA) and conducted phylogenetic analyses using parsimony, maximum likelihood, and Bayesian methods. The results confirm, with a greater level of support, two clades in Magnoliidae: Canellale + Piperales, and Laurales + Magnoliales. In addition, the relationships among the 20 families are generally well supported, and Lactoridaceae, and Hydnoraceae are nested within Aristolochiaceae (Piperales). However, two parts of the tree remain uncertain: the relationships among three families of Laurales (Hernandiaceae, Lauraceae, and Monimiaceae), and the position of Magnoliaceae within Magnoliales. In the second chapter, the ages and phylogenetic positions of 10 fossils attributed to Magnoliidae were reviewed in detail. The goal of this study was to provide new reliable calibration points in order to conduct molecular dating analyses. These fossils were selected from the rich fossil record of the group because of their previous inclusion in phylogenetic analyses with extant taxa. The resulting calibration scheme provides six solid, internal minimum age constraints: crown Canellales (≥ 126.3 million years, Ma), stem *Saururus* (≥ 45 Ma), crown Laurales (≥ 107.7 Ma), stem *Calycanthus* (≥ 86.3 Ma), crown core Laurales (≥ 107.7 Ma), and crown Magnoliaceae (at least 113 Ma). The third chapter includes molecular dating analyses using the present calibration scheme and the same molecular dataset of Chapter 1. This study tends to push back in time the ages of the crown nodes of Magnoliidae (127.1-198.9 Ma), and of the four orders, Canellales (126.3-141.0 Ma), Piperales (88.2-157.7 Ma), Laurales (111.8-165.6 Ma), and Magnoliales (115.0-164.2 Ma). In the same chapter, I investigated the mode of diversification in the group. The strongly imbalanced distribution of species appears to be best explained by models of diversification with 6 to 14 diversification rate shifts. Significant increases are inferred within Piperaceae and Annonaceae, while the low species richness of Calycanthaceae, Degeneriaceae, and Himantandraceae appears to be the result of decreases in both speciation and extinction rates. Finally, in the last chapter, I traced the evolution of 26 floral characters to reconstruct the ancestral flowers in key nodes of Magnoliidae. I used the phylogeny of Chapter 1 and an exemplar approach. Our results show that the most recent common ancestor of all Magnoliidae was a tree bearing actinomorphic, bisexual flowers with a differentiated perianth of two alternate, trimerous whorls of free perianth parts (outer and inner tepals) and probably three free stamens. Although the optimization of several traits remain equivocal in the most recent common ancestors of the four orders, this study suggests that Canellales, Laurales, Magnoliales, and Piperales could have departed from their Magnoliidae ancestor each by the modification of one or two floral traits. This work provides key results on the evolution of Magnoliidae and raises several new questions such as the impact of geological crises on diversification of the group or the influence of pollinators and the environment on the evolution of floral morphology.

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Introduction

The advent of phylogenetic methods was a turning point in the apprehension of the evolutionary history of organisms. It disqualified the intuitive approach in favor of objective tests of hypotheses. In addition to providing a framework for a large variety of analyses addressing a wide range of questions, phylogenies are used as a basis to establish classifications that reflect the relationships among taxa. Probably due to the lack of a well established phylogeny for the angiosperms, botanical intuitive evolutionary classifications have been widely used until the end of the nineties. Although the monocot and eudicot clades had been recognized for several years, the relationships among these two groups and other lineages of flowering plants have long remained uncertain. In 1999 and 2000, a series of phylogenetic studies reduced this uncertainty by providing the sequence of divergence of the first lineages in the angiosperm tree: Amborellales, Nymphaeales, and Austrobaileyales (Mathews and Donoghue 1999; Qiu et al. 1999; Soltis et al. 1999; Graham and Olmstead 2000; Fig. 1). Since then, phylogenetic relationships in angiosperms have been refined, and a large number of studies have recognized a clade concentrating the great majority of non-monocot and non-eudicot species within four orders: Canellales, Laurales, Magnoliales, and Piperales (Qiu et al. 1999, 2000, 2005, 2006, 2010; Soltis et al. 1999, 2000a,b, 2007, 2011; Mathews and Donoghue 1999, 2000; Savolainen et al. 2000; Graham and Olmstead 2000; Zanis et al. 2002, 2003; Nickrent et al. 2002; Borsch et al. 2003; Hilu et al. 2003; Jansen et al. 2007; Moore et al. 2007, 2010; Burleigh et al. 2009; Fig. 1). The latest version of the phylogenetic classification by the Angiosperm Phylogeny Group (APG III 2009) referred to this clade informally as magnoliids, while Cantino et al. (2007) proposed to recircumscribe the name Magnoliidae to correspond to this clade, a convention followed in the present manuscript. Magnoliidae were initially often found as the sister group of eudicots (e.g., Qiu et al. 2000; Borsch et al. 2003; Zanis et al. 2003), but more recent studies have supported a position as the sister group of Chloranthaceae (e.g. Jansen et al. 2007; Moore et al. 2007; Soltis et al. 2011; Fig. 1).

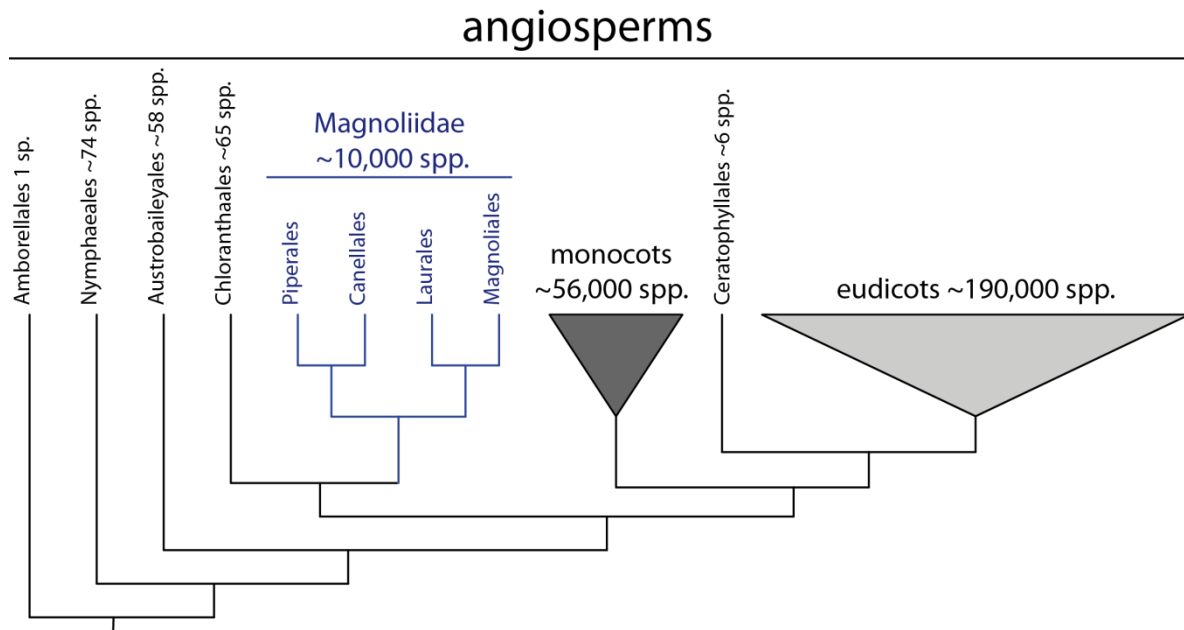


Figure 1: Simplified tree of angiosperms, based on APG III (2009). Abbreviations: sp./spp., species.

Magnoliidae consist of about 10,000 species grouped in 20 families. The clade has a global geographic distribution (Heywood et al. 2007; Fig. 2). Aristolochiaceae (Piperales) are the northernmost family, extending to northern Europe and Canada, while Monimiaceae (Laurales) are the southernmost families, with species in southern South America, Australia, and New Zealand. However, the great majority of species occur in tropical areas where they are an important part of rainforest floras (e.g., Ayyappan and Parthasarathy 1999; Pitman et al. 2001). Therefore, there is a latitudinal gradient in magnoliid diversity. As a consequence, a better understanding of the evolution of this group will provide key knowledge to reconstruct the history of tropical environments through time as well as their influence on the evolution of organisms inhabiting them. For instance, the evaluation of the model of diversification sustaining its higher diversity under tropics might help us understand the diversification processes in tropical environments and test the "cradle" vs. "museum" models of Stebbins (1974) or the more recent "out of tropics" model of Jablonski et al. (2006). This question of the latitudinal gradient has long fascinated scientists and remains at the center of a strong debate (e.g., Arita and Vázquez-Domínguez 2008; Rolland et al. 2014). Furthermore, the worldwide presence of Magnoliidae appears to be ancient, as suggested by a number of Cretaceous (145-66 Ma) fossils securely attributed to the group (Fig. 3).

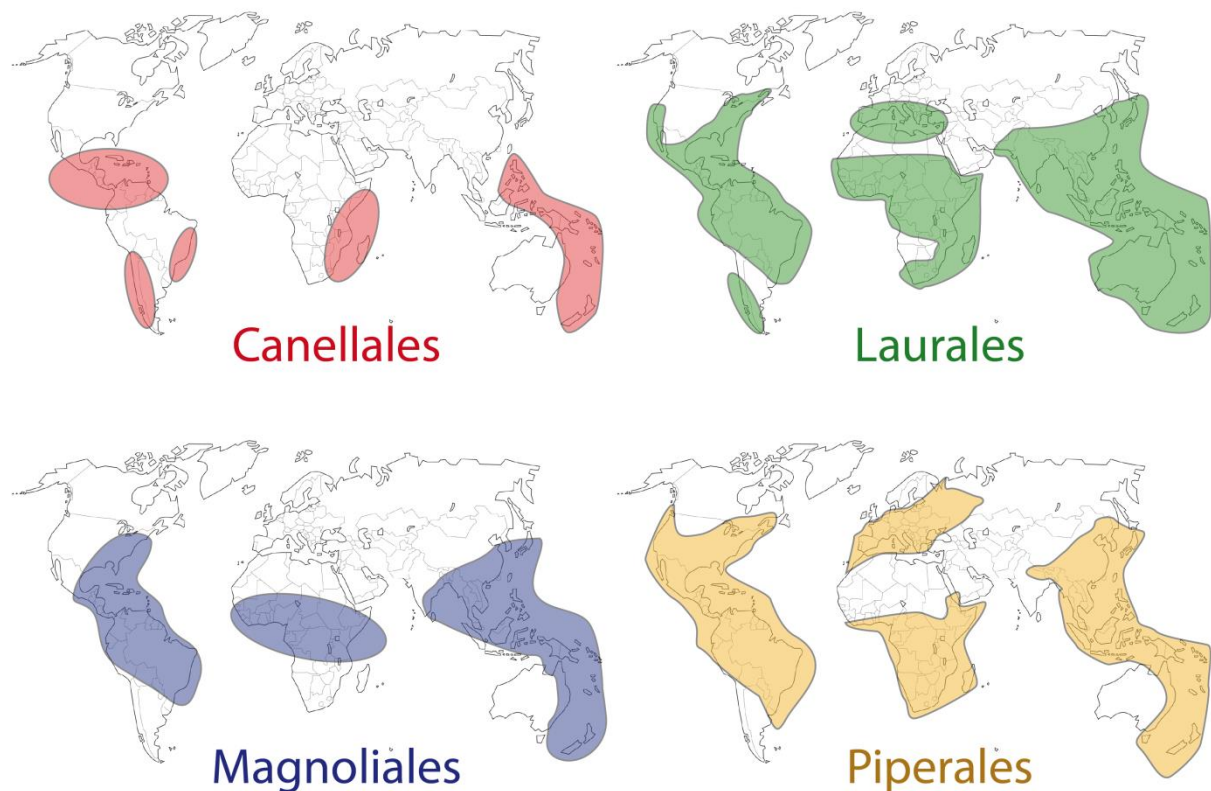


Figure 2: Distribution of the four orders of Magnoliidae according to Heywood et al. (2007).

Before this thesis, only studies focused on all angiosperms (or larger clades) had included the four orders together in the same phylogenetic analysis. Among them, a maximum of 43 genera out of 263, and 18 families, have been sampled (Qiu et al. 1999, 2000; Zanis et al. 2002, 2003). On the other hand, several phylogenetic studies have investigated relationships at the ordinal or family level (e.g., Renner 1999; Sauquet et al. 2003; Wanke et al. 2007). When I started working on this project, several parts of the tree were still poorly supported and the relationships among the four orders were debated. Most analyses had supported a clade of Canellales and Piperales and a clade of Laurales and Magnoliales (Fig. 1). However, many other studies found different sets of relationships (Soltis et al. 2000b, 2007, 1999, 2000a; Doyle and Endress 2000; Savolainen et al. 2000; Zanis et al. 2002; Nickrent et al. 2002; Hilu et al. 2003; Qiu et al. 2005; Moore et al. 2007, 2010; Burleigh et al. 2009). Therefore, reconstructing a well-supported phylogeny for the group, with higher taxonomic and molecular sampling, was the first step to achieve (chapter 1 of this thesis) before starting more investigations on the evolution of Magnoliidae.

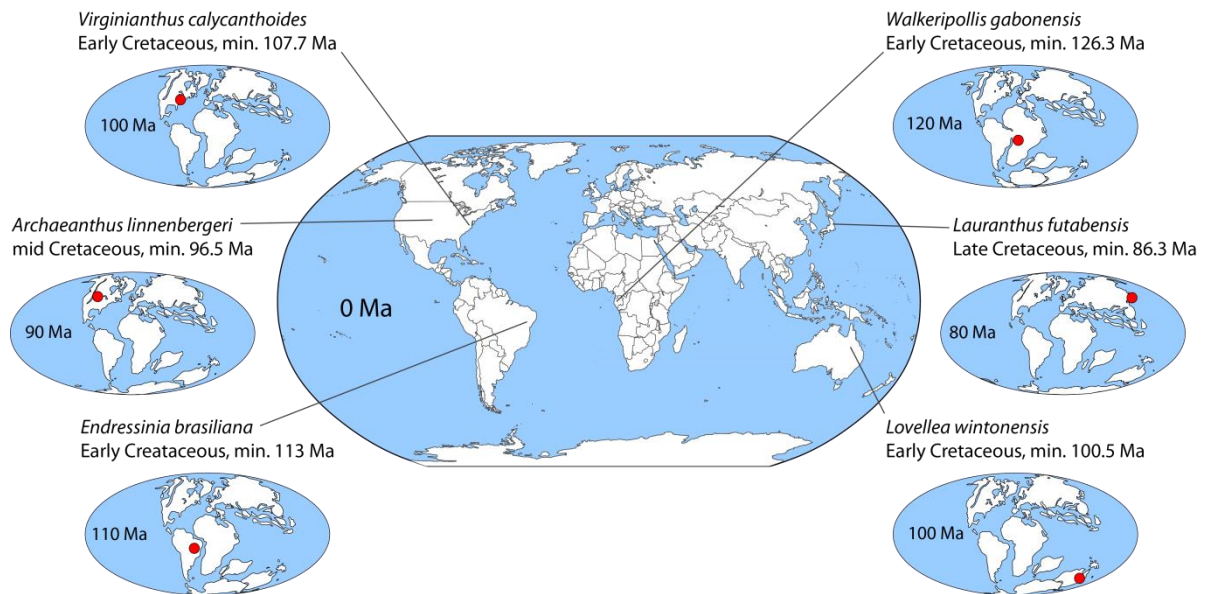


Figure 3: Fossil species attributed to Magnoliidae. Dates associated to the fossils are minimum ages of the species (upper bound of the stage where the fossil was collected) reviewed in Massoni et al. (2014) [see second chapter of this thesis] with the exception of *Laurantus futabensis* for which the age was taken from Takahashi et al. (2001). Boundaries of ages follow Ogg and Hinnov (2012). Paleo-planispheres are from Scotese (2004), red dots present the position of the fossil. Abbreviations: min., minimum age.

The six fossil species on Fig. 3 are only a small fraction of a rich paleontological record attributed to Magnoliidae (more than 100 species; Friis et al. 2011). These extinct taxa are fossilized in the form of wood, leaves, flowers, or pollen grains, including several exceptionally preserved fossils (e.g., Takahashi et al. 2001). This fossil record supports an old origin of the group at least back to the mid-Cretaceous. For instance, because the fossil *Walkeripollis gabonensis* has been securely placed within Canellales as the sister group of extant Winteraceae (Doyle and Endress 2010), it supports an age of the order at least as old as the Barremian-Aptian boundary, or 126.3 million years (Ma) (Doyle et al. 1990). In comparison, the oldest fossil pollen confirmed to be angiospermous (stem or crown relative) is dated to the Valanginian (139.4-133.9 Ma) or the Hauterivian (133.9-130.8 Ma) (Doyle 2012). Unlike many lineages of angiosperms with a poor fossil record, the diverse and well preserved fossil record of Magnoliidae offers the potential of using a solid paleontological basis to increase the reliability and accuracy of molecular dating methods, and, as a consequence, those of all subsequent studies based on dated trees. One of the critical points in estimating divergence times is to calibrate the analyses with reliable minimum and

maximum ages for several nodes of the phylogeny (Sanderson 1997; Inoue et al. 2010; Meredith et al. 2011; Sauquet et al. 2012). To do so, the most common and consensual practice is to use the fossil record, which requires knowing with some precision the age and phylogenetic position of fossil species. The age of the most recent common ancestor of the clade in which the fossil is nested is at least the age of the fossil. Unless there is additional evidence to support that this node cannot be much older, a strict minimum age constraint (equal to the age of the fossil) is typically applied to this node in the molecular dating analysis. Therefore, the relationships between fossil and living taxa is a crucial point and several recent studies have argued that such relationships need to be thoroughly addressed using phylogenetic or apomorphy-based approaches instead of relying on intuitive placements (Gandolfo et al. 2008; Parham et al. 2012; Sauquet et al. 2012). Because extinct species generally lack many characters (not fossilized), their phylogenetic placement can sometimes be difficult. However, the amount of missing data does not predict the precision of phylogenetic placement because, more than the numbers of observed characters available, it is the phylogenetic signal of characters which conditions this precision. Because floral features are generally more diagnostic than other organs, fossils flowers of Magnoliidae (e.g., Dilcher and Crane 1984; Takahashi et al. 2001; Crepet et al. 2005; von Balthazar et al. 2011) are good candidates to provide reliable calibration points. Until now, the fossil record of Magnoliidae has been sparsely used to estimate divergence times within the group. Before the present work (see chapter 2 of this thesis), a maximum of six magnoliid fossils had been used to calibrate divergence times of Magnoliidae (Magallón and Castillo 2009).

Previous large scale studies have suggested a much older age for the crown group of Magnoliidae than for the eudicots (e.g., Smith et al. 2010) and, to a lesser extent, for the monocots (e.g., Magallón et al. 2013). However, the general time scale of the group remained uncertain (Forest and Chase 2009). For instance, Schneider et al. (2004) found an age of 223.1 ± 15.6 Ma, whereas Soltis et al. (2008) supported an age between 98 and 162 Ma. In addition, because divergence times had been investigated either in large scale studies with a limited number of Magnoliidae terminal taxa (35 maximum in Magallón and Castillo 2009), or at the scale of families (e.g., Pirie and Doyle 2012), several deep nodes of the

magnoliid phylogeny had never been dated. New molecular dating analyses, using high taxonomic sampling and a robust and accurate set of calibration points, were needed in order to evaluate reliable ages for all nodes between the crown group of Magnoliidae and all extant families. These results (chapter 3 of this thesis) will allow to apprehend the evolutionary history of Magnoliidae in geological time, allowing a better perception of the context (paleo-environments) in which they have evolved and diversified, and allowing tests of evolutionary hypotheses using branch lengths proportional to time.

The reconstruction of a dated phylogeny including a great part of the genera of Magnoliidae offers the possibility of investigating additional evolutionary questions such as the mode of diversification of Magnoliidae. One of the key questions I wished to address is: did Magnoliidae constantly accumulate lineages through time or did diversification rates vary through time and across lineages (see chapter 3)? It is now possible to address this question using incomplete phylogenies (i.e., with only a fraction of the total species diversity) (Alfaro et al. 2009). Previous analyses using this approach investigated variation of accumulation of lineages through time in the monocots and eudicots (Arakaki et al. 2011; Xi et al. 2012; Beaulieu and Donoghue 2013; Escudero and Hipp 2013; Koenen et al. 2013), and so far, very little work has been conducted on diversification rates in Magnoliidae. For instance, Couvreur et al. (2011) supported a constant diversification in Annonaceae. Among extrinsic features probably influencing the mode of diversification, specialist pollination systems have often been cited (Vamosi and Vamosi 2011). However, our review (L. Rabeau, H. Sauquet, and J. Massoni, unpubl. data) of the pollination in Canellales and Piperales suggested that the great majority of species of Magnoliidae are generalist and pollinated by a wide range of flies, thrips, beetles. For this reason, pollination might not be the principal driver of diversification in Magnoliidae. Numerous intrinsic (e.g., herbaceous habit) and extrinsic (e.g., distribution) traits have been shown to be correlated with variation in diversification rates (Vamosi and Vamosi 2011). Therefore, the investigation of the evolution of these characters in the group will give material to raise hypotheses and design future tests about plausible correlations among different traits and variations of diversification.



Figure 4: A. *Glossocalyx longicuspis* (Siparunaceae, Laurales). B. *Magnolia* sp. (Magnoliaceae, Magnoliales).

Another fascinating aspect of the angiosperms is their extraordinary diversity of floral morphology, and Magnoliidae themselves exhibit a very wide range of floral architecture, shape, size, and color (Figs. 4-5). Thus, one of my goals was to investigate the changes which occurred from ancestral flowers to the diversity of forms observed in living species. Magnoliaceae were long seen as the most “primitive” family of the angiosperms, later joined by Winteraceae and Degeneriaceae (Nooteboom 1993). This led to the widespread view that characters present in these families were ancestral features in angiosperms. When basal relationships in flowering plants became better understood, several authors investigated the evolution of morphological traits in this new phylogenetic framework and contradicted the previously assumed polarizations of several characters (Doyle and Endress 2000, 2011; Ronse De Craene et al. 2003; Zanis et al. 2003; Soltis et al. 2005; Endress and Doyle 2009). For instance, their results were in conflict with the spiral insertion of the perianth as a primitive feature inherited from the ancestral angiosperms in Magnoliidae. They supported, at the crown node of Magnoliidae, an ancestrally whorled perianth, with subsequent transitions to spiral insertion in several clades nested in the group. before the present thesis, these previous studies at the scale of all basal angiosperms were the only ones to have investigated the evolution of characters in Magnoliidae as a whole. They have used a limited

number of Magnoliidae taxa and always represented them as supra-specific terminals (i.e., a small sample of genera, or families and subfamilies). Therefore, a study of the floral evolution focused on Magnoliidae with higher taxonomic sampling may help us to clarify several character states, which have remained ambiguous in the deepest nodes of the extant phylogeny (chapter 4). These results may also have a direct impact on reconstructions of the ancestral flower of angiosperms as a whole.



Figure 5: Illustration of perianth diversity in Magnoliidae. A, *Piper nigrum* (Piperaceae, Piperales): inflorescence, perianth absent. B, *Aristolochia gigantea* (Aristolochiaceae, Piperales): perianth parts completely fused in a tube with bilateral symmetry. C, *Asimina triloba* (Annonaceae,

Magnoliales): three alternate whorls of tepals (outer tepals sepaloid and barely visible here). D, *Chimonanthus praecox* (Calycanthaceae, Laurales): spiral of numerous tepals.

Objectives of this thesis

During the last three and a half years, I have concentrated my efforts on several questions presented in the introduction and I decided to achieve the following objectives: to reconstruct the phylogeny of Magnoliidae, to establish a new calibration scheme, to conduct a molecular dating study, to study the variation of diversification rates through time and lineages of Magnoliidae, and to study on the evolution of floral traits to reconstruct the portrait of ancestral flowers in the group. These choices were driven by my primary interests, a maximization of the diversity of topics related to the evolutionary history of the group, and my learning of new methods. Beyond the help of my supervisor (Hervé Sauquet) on all the aspects of this thesis, the different questions I have worked on have led me to collaborate with different specialists in each field.

First, I reconstructed a phylogeny focused on Magnoliidae as a whole, which I present in Chapter 1 of this thesis. To do so, I started to establish a list including all the valid genera in Magnoliidae (263) and a second list of species to represent them in my different datasets. For each genus, and among all its species, I chose one species according to the number of sequences available on GenBank for twelve molecular markers selected because of their previous use in the literature. I downloaded all the sequences and started to build a reliable molecular dataset. To do so, I conducted many successive phylogenetic analyses with each markers to detect misidentified sequences. In parallel, I completed my dataset with lab work using a collection of fresh and silicagel-dried material which I assembled through contacting many botanical gardens around the world (about 100 specimens). I also spent one month at the Jodrell Laboratory of the Royal Botanic Gardens in Kew (U.K.) to benefit from the expertise of László Csiba and Dion S. Devey on extraction and amplification of DNA and of Félix Forest and Sven Buerki on phylogenetic reconstruction. This part of my work has resulted in a new phylogeny, better supported than any previous higher-level study, and has now been published in *Molecular Phylogenetics and Evolution*.

The involvement of Hervé Sauquet in the promotion of good practices to calibrate molecular dating analyses has made me particularly interested in this question. I started a review of the fossil record with the help of James Doyle (University of California, Davis) to provide the new calibration scheme presented in Chapter 2. We selected all the fossils which had been previously included in phylogenetic analyses with living taxa. We did a survey of all the phylogenetic analyses and reviewed the ages of the geological formations where the fossils were collected. This part of my work has resulted in a manuscript that has now been accepted and is in press in *Palaeontologia Electronica*.

After these two first studies, I started to investigate the time scale of Magnoliidae and the presence of shifts of diversification in the phylogeny. To do so I used the phylogenetic results of Chapter 1 and the same molecular dataset. This part of my work has led to a manuscript, which is presented in Chapter 3 of this thesis and which I intend to submit to the new Tree of Life section of *Molecular Biology and Evolution*. This work had been motivated by the journal clubs organized by Hervé Sauquet and H  l  ne Morlon (Institut de Biologie de l'  cole Normale Sup  rieure de Paris) on the diversification methods during the first years of my PhD. In addition to the experience of Herv   Sauquet in molecular dating approaches, Thomas Couvreur (Institut de Recherche pour le D  veloppement) provided an expertise on Annonaceae (the largest family of Magnoliidae), which was essential for this study.

Finally, Chapter 4 of this thesis presents the preliminary results from a collaboration, which I initiated to involve different specialists of magnoliid families in the building of a large floral morphological dataset including all the species sampled in our phylogeny. Today, the resulting dataset includes 188 taxa and 6611 data. We based our work on the literature as well as some personal observations. Due to limited time, I have not been able to finish this part of my work entirely yet. In particular, the data set remains partly incomplete and there are many more analyses that I wish to do on these data. However, I have decided to present this part of my work here, focusing on some promising results I have obtained in reconstructing the portraits of ancestral flowers of Magnoliidae, Canellales, Laurales, Magnoliales, and Piperales.

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Chapter 1

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Increased sampling of both genes and taxa improves resolution of phylogenetic relationships within Magnoliidae, a large and early-diverging clade of angiosperms

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Supplementary information available at:

https://www.dropbox.com/sh/mxrommnod3i19xm/O_XkTwcjk

Abstract

Magnoliidae have been supported as a clade in the majority of large-scale molecular phylogenetic studies of angiosperms. This group consists of about 10,000 species assigned to 20 families and four orders, Canellales, Piperales, Laurales, and Magnoliales. Some relationships among the families are still largely debated. Here, we reconstruct the phylogenetic relationships of Magnoliidae as a whole, sampling 199 species (representing ca. 75% of genera) and 12 molecular markers from the three genomes (plastid *atpB*, *matK*, *trnL* intron, *trnL-trnF* spacer, *ndhF*, *rbcl*; mitochondrial *atp1*, *matR*, mtSSU, mtLSU; nuclear 18S rDNA, 26S rDNA). Maximum likelihood, Bayesian and maximum parsimony analyses yielded congruent trees, with good resolution and high support values for higher-level relationships. This study further confirms, with greater levels of support, two major clades in Magnoliidae: Canellales + Piperales and Laurales + Magnoliales. Relationships among the 20 families are, in general, well resolved and supported. Several previously ambiguous relationships are now well supported. For instance, the Aristolochiaceae *s.l.* (incl. Asaroideae, Aristolochioideae, and *Lactoris*) are monophyletic with high support when Hydnoraceae are excluded. The latter family was not included in most previous studies because of the lack of suitable plastid sequences, a consequence of the parasitic habit of its species. Here, we confirm that it belongs in Aristolochiaceae. Our analyses also provide moderate support for a sister group relationship between Lauraceae and Monimiaceae. Conversely, the exact position of Magnoliaceae remains very difficult to determine. This study provides a robust phylogenetic background to address the evolutionary history of an important and highly diverse clade of early-diverging angiosperms.

Keywords: Magnoliidae, Canellales, Laurales, Magnoliales, Piperales, phylogeny, taxonomic sampling, molecular sampling.

Footnote

Abbreviations: SI, supplementary information; ML, maximum likelihood; MP, maximum parsimony; BS, bootstrap; PP, posterior probability; I, invariant.

Introduction

During the last decade, while phylogenetic relationships among the earliest-diverging lineages of angiosperms were being resolved, the great majority of phylogenetic studies supported a clade consisting of orders Piperales, Laurales, Magnoliales, and Canellales (Qiu et al., 1999, 2000, 2005, 2006, 2010; Soltis et al., 1999, 2000a, 2000b, 2007, 2011; Mathews and Donoghue, 1999, 2000; Savolainen et al., 2000; Graham and Olmstead, 2000; Zanis et al., 2002, 2003; Nickrent et al., 2002; Borsch et al., 2003; Hilu et al., 2003; Jansen et al., 2007; Moore et al., 2007, 2010; Burleigh et al., 2009). While the Angiosperm Phylogeny Group (APG, 2003, 2009) referred to this clade informally as the magnoliids and Chase and Reveal (2009) as superorder Magnolianaes, Cantino et al. (2007) re-circumscribed the taxon Magnoliidae to correspond to this clade, a convention we follow in this paper.

Although the majority of species of Magnoliidae (ca 10,000 species assigned to 20 families) occur in tropical areas, the group has a global distribution. For instance, the Aristolochiaceae extend from Canada to Scandinavia and several species of Laurales occur in southern Chile (Heywood et al., 2007). Several species of Magnoliidae are of economical interest, such as avocado (*Persea americana*; Lauraceae), nutmeg (*Myristica fragrans*; Myristicaceae), cinnamon (*Cinnamomum verum*; Lauraceae) or black pepper (*Piper nigrum*; Piperaceae). Many studies have investigated phylogenetic relationships in Magnoliidae at the family level, especially within the larger families Annonaceae and Lauraceae (e.g., Chanderbali et al., 2001; Rohwer and Rudolph, 2005; Couvreur et al., 2011; Chatrou et al., 2012). However, only a very small fraction of the total diversity of the clade has been sampled in higher-level studies that included the four orders. A maximum of 43 out of 263 genera of Magnoliidae have been sampled in these studies (Qiu et al., 1999, 2000; Zanis et al., 2002, 2003). Although most analyses have supported two clades, Canellales + Piperales and Laurales + Magnoliales, other studies have suggested different relationships or left this question unresolved (SI Table 1) (Soltis et al., 2000b, 2007, 1999, 2000a; Doyle and Endress, 2000; Savolainen et al., 2000; Zanis et al., 2002; Nickrent et al., 2002; Hilu et al., 2003; Qiu et al., 2005; Moore et al., 2007, 2010; Burleigh et al., 2009). Relationships within each order of Magnoliidae are also debated (e.g., the monophyly of Aristolochiaceae; relationships within

Laurales and Magnoliales) and several taxa remain difficult to place (e.g., Hydnoraceae) (Renner and Chanderbali, 2000; Nickrent et al., 2002).

The present study was designed to address these questions and reconstruct the phylogeny of Magnoliidae as a whole, based on a comprehensive sample of representatives from all families and 12 molecular markers from the three genomes. Our results confirm, with greater support, the general backbone tree of Magnoliidae suggested by previous studies. The relationships among the four orders are very well supported with all approaches. Several relationships debated in the literature are resolved with high support in the present study: Aristolochiaceae *s.l.* are monophyletic with *Lactoris* sister to subfamily Aristolochioideae, Hydnoraceae are closely related to this family, and Monimiaceae appear to be the sister group of Lauraceae. This study also further demonstrates that including more genes can help resolve deep relationships, even if such genes are not sampled for all taxa.

Materials and methods

Taxonomic and molecular sampling

To reconstruct the phylogeny of Magnoliidae as a whole, we made a first selection of markers previously used in the literature at the suprafamilial and intrafamilial levels in the group. Sequences available for them on GenBank had to feature a good representation and distribution across the clade. We selected 12 coding and non-coding regions: *atpB*, *matK*, *trnL* intron, *trnL-trnF* spacer, *ndhF* and *rbcL* from the plastid; *atp1*, *matR*, mtSSU, mtLSU from the mitochondrion; 18S rDNA and 26S rDNA from the nucleus. For each of the 263 genera of Magnoliidae, we selected one representative species. Our choice was driven by the number of sequences available for the species on GenBank among the 12 markers used in the present study. When multiple sequences were available for the same species and marker, we gave priority to those linked to published papers and long sequences. In addition, we sampled 23 outgroup taxa from all other lineages of angiosperms (Amborellales, Nymphaeales, Austrobaileyales, Chloranthaceae, eudicots, and monocots) with the exception of Ceratophyllaceae, which we excluded to avoid long-branch attraction artifacts (Moore et al., 2007). GenBank accession numbers and voucher details of sequences

generated for this study are available in Supplementary information (Supplementary information (SI) Table 2).

In order to have at least one marker available for all species in our study, we sequenced the *trnLF* region (*trnL* intron and *trnL-trnF* spacer) and the *rbcL* gene from a collection of silica gel-dried leaves or pre-extracted genomic DNA. DNA extractions were made with a Macherey-Nagel NucleoSpin II kit (Düren, Germany) following the manufacturer's protocol. To amplify the *trnLF* region we used the c and f primers described in Taberlet et al. (1991). For *rbcL*, we modified the primers of Qiu et al. (2005) in order to avoid unnecessary degenerations in Magnoliidae (SI Table 3). Details on amplification and sequencing conditions are available from J. Massoni upon request. PCR products were sent to Beckman Coulter Genomics for sequencing (Takeley, Essex, UK). Editing and assembling of sequences were accomplished with CodonCode Aligner (CodonCode Corporation, Centerville, Massachusetts, USA).

Alignments for each of the 12 markers were made using MAFFT v6.850b (Katoh et al., 2005), then edited by hand in BioEdit 7.1.3 (Hall, 1999). Ambiguous regions were excluded using Gblocks 0.91b (Castresana, 2000). All parameters used in this program for the 12 alignments are available in Supplementary data (SI Table 4).

Datasets and Phylogenetic methods

Gaps were coded as missing data in all phylogenetic analyses. Maximum likelihood (ML) reconstructions were first carried out separately for each one of the 12 markers (corresponding phylograms available in SI Fig. 1). For these analyses we used the approach combining a rapid bootstrapping algorithm with a subsequent thorough ML search implemented in RAxML 7.3.2 (Stamatakis et al., 2008) on the CIPRES cluster (Miller et al., 2010). After this first step, to minimize problems linked with missing data, we reduced our taxonomic sample to include all taxa of Magnoliidae for which at least the *trnL-trnF* spacer was available. However, in order to test the position of all families of Magnoliidae, we added the following taxa to the latter reduction: *Aristolochia macrophylla* (Aristolochiaceae), *Degeneria vitiensis* (Degeneriaceae), and *Gomortega keule* (Gomortegaceae). After checking

for the lack of unsupported conflict among separate analyses, we concatenated the 12 markers within one supermatrix including 19,517 characters and 198 genera of Magnoliidae plus 23 outgroup taxa (12-marker data set). We did not incorporate the 26s rDNA sequence of *Degeneria vitiensis*, because of long-branch attraction issues (see discussion). *Hydnora africana* was also excluded because only few molecular data were available from the nuclear and mitochondrial genomes of this parasitic family, making its placement difficult. In order to investigate the phylogenetic signal supported by each genome, we also conducted analyses in ML and maximum parsimony (MP) for each genome (using the taxonomic sampling of the 12-marker data set): for Magnoliidae, 198 genera and 6,771 characters for the plastid markers; 43 genera and 8,044 characters for the mitochondrial markers; and 54 genera and 4,702 characters for the nuclear markers.

We partitioned our concatenated datasets according to the 12 markers. We used MrModeltest 2.3 (Nylander, 2004) to evaluate the best fitted model for each partition. For all partitions, GTR + Gamma was selected as the most appropriate model according to the Akaike Information Criterion and the evaluation of invariant sites (I) was recommended for all, except for the plastid spacer *trnL-trnF* (SI Table 5). RAxML does not allow the application of different models to different partitions, so we applied a GTR + Gamma + I model to each of the 12 partitions. For the 12-marker data set, we carried out 1,000 replicates of non-parametric ML bootstrapping and a ML search of the best tree using the hill-climbing algorithm starting from 10 distinct randomized maximum parsimony starting trees, using RAxML 7.3.2 installed on a HP Z800 workstation. ML analyses were carried out on all other concatenated matrices using RAxML 7.3.2 on the CIPRES cluster using the same search strategy as for separate marker analyses. In addition to ML analyses, we carried out 1,000 bootstrap replicates of MP using PAUP4.0b10 (Swofford, 2002) on all concatenated data sets. For each replicate of bootstrap, a heuristic search was conducted using a single replicate of the Random Addition Sequence, Tree-Bisection-Reconnection (TBR) branch swapping, and a maximum number of trees (“maxtrees”) set to 10. Finally, we conducted Bayesian analyses of the 12-marker data set with MrBayes 3.2.1 (Ronquist et al., 2012) on a desktop PC and on the Bioportal cluster (Kumar et al., 2009). For each analysis, we used two runs of four chains each. For the 12-markers dataset we carried out 14,209,000 generations.

At the end of the runs the average standard deviation was 0.0098. All potential scale reduction factors were equal to 1.00. We summarized results excluding the first 10^6 generations (effective sample size of the InL for the two runs combined = 1,251). For the 12-markers data set including *Hydnora africana*, we carried out an analysis with 15×10^6 generations. At the end of the runs the average standard deviation was 0.013. All potential scale reduction factors were equal to 1.00. We summarized results excluding the first 5×10^6 generations (effective sample size of the InL for the two runs combined = 1,548).

In the present paper, we consider bootstrap values between 70% and 80% and PP values between 0.90 and 0.95 as moderate branch support. Well-supported clades are characterized by greater values.

Results

12-marker concatenation

ML analyses of each marker taken separately yielded, in general, congruent higher-level relationships among Magnoliidae (SI Fig. 1). With the exception of a problematic 26S sequence (see discussion below), no supported conflict could be identified among separate analyses and therefore all markers were combined. At the interfamilial level, ML, MP, and Bayesian analyses of the 12-marker data set provided congruent trees (Fig. 1, SI Figs. 2-4). Our analyses support two main clades, Piperales + Canellales and Laurales + Magnoliales, with high MP and ML bootstrap support (BS) and high posterior probabilities (PP). All families of Magnoliidae are monophyletic with high support except for Aristolochiaceae and Lauraceae in the MP analysis. Within Piperales, Aristolochiaceae *s.l.* (including *Lactoris*) are monophyletic and sister to a well-supported clade including Saururaceae and Piperaceae. Within Laurales, Calycanthaceae are sister to the remaining families of the order. Siparunaceae are sister to a clade comprised of Gomortegaceae and Atherospermataceae. Hernandiaceae, Lauraceae and Monimiaceae form a well-supported monophyletic group, but the relationships among these three families are not resolved in the MP analysis and receive low support in the ML analysis. In the Bayesian analysis, Lauraceae and Monimiaceae form a well-supported clade. Finally, within Magnoliales, in all analyses, Myristicaceae are

sister to all remaining families with high support. Two additional clades are supported, Eupomatiaceae + Annonaceae and Degeneriaceae + Himantandraceae, but the relationships among these and Magnoliaceae are not resolved.

At the intrafamily level, relationships supported by ML, MP, and Bayesian analyses are congruent. Within Winteraceae and Canellaceae, *Takhtajania* and *Canella* are the earliest-diverging lineages in their respective families. In Saururaceae, *Gymnotheca* and *Saururus* form a clade, *Houttuynia* and *Anemopsis* another. *Verhuellia* is the sister group of all remaining Piperaceae. In Laurales, relationships within the three largest families (Lauraceae, Atherospermataceae, and Monimiaceae) are not well resolved. However, in Monimiaceae a clade consisting of *Peumus*, *Monimia*, and *Palmeria* is sister to all remaining genera of the family. Finally, in Magnoliales, relationships are only partially resolved within Myristicaceae, and within Annonaceae, *Anaxagorea* is sister to the rest of the family, which can be split in three well-supported clades.

Position of Hydnoraceae

When Hydnoraceae are included in our 12-marker dataset, the family is nested in Aristolochiaceae with high ML and Bayesian support (Fig. 2), and the relationships among the remaining families of Magnoliidae are unaffected. However, bootstrap values decrease for several nodes and the relationships among families of Piperales are no longer resolved in MP (except for the clade Piperaceae + Saururaceae, which is well supported).

Separate-genome analyses

The four orders of Magnoliidae appear to be monophyletic in the ML and MP analyses of the three genome datasets (SI Figs. 5-10). Canellales and Magnoliales receive low support in the MP analysis of the nuclear dataset. In the MP analysis of the mitochondrial dataset, relationships among the four orders of Magnoliidae and Chloranthaceae are not resolved. In the ML and MP analyses of the nuclear dataset the relationships among orders of Magnoliidae and other lineages of angiosperms (except *Amborella* and Nymphaeales) are also not resolved. Within orders, most relationships among families are congruent with

results obtained from the 12-markers concatenated dataset, except in two cases. First, the MP analysis of the nuclear data set weakly supports *Lactoris* as the sister group of Asaroideae (BS=67%; SI Fig. 10). Second, the same analysis weakly supports a clade of Annonaceae and Myristicaceae (BS=61%).

Discussion

The phylogenetic relationships obtained from the three different approaches (ML, MP, and Bayesian) are highly congruent. In order to investigate the position of the group within the extant phylogeny of angiosperms a greater taxonomic sampling outside Magnoliidae would be essential. The position of Magnoliidae outside a clade including eudicots and monocots (sometimes with *Ceratophyllum*) is the most widely accepted result (e.g., Jansen et al., 2007; Moore et al., 2007; Soltis et al., 2011). In addition, a sister-group relationship of Magnoliidae and Chloranthaceae was well supported in studies using large molecular datasets (Jansen et al., 2007; Moore et al., 2007, 2010; Soltis et al., 2011). In the present study, Magnoliidae are nested in a clade together with eudicots, monocots, and Chloranthaceae. In the Bayesian analyses, Magnoliidae are sister to a clade consisting of Chloranthaceae and monocots (SI Fig. 3), whereas the relationships among these four lineages are poorly supported in MP and ML analyses (SI Figs. 2, 4). Fortunately, the position of Magnoliidae does not seem to influence the deepest relationships within the group (e.g., Graham and Olmstead, 2000; Borsch et al., 2003; Moore et al., 2007; Soltis et al., 2011). In the present discussion, we focus on the relationships within the Magnoliidae. In the framework of this study, a considerable increase in taxonomic and gene sampling resulted in much improved branch support for the set of relationships among the four orders of Magnoliidae found in most recent higher-level analyses (Fig. 1, SI Table 1). No previous analysis had found simultaneously comparable support with ML, MP, and Bayesian approaches (SI Table 1) for the monophyly of the group and the two clades Canellales + Piperales and Laurales + Magnoliales (Qiu et al., 1999, 2000, 2005, 2006, 2010; Soltis et al., 1999, 2000a, 2000b, 2007, 2011; Mathews and Donoghue, 1999, 2000; Savolainen et al., 2000; Doyle and Endress, 2000; Graham and Olmstead, 2000; Nickrent et al., 2002; Zanis et al., 2002, 2003;

Borsch et al., 2003; Hilu et al., 2003; Jansen et al., 2007; Moore et al., 2007, 2010; Burleigh et al., 2009).

Relationships within Canellales

Winteraceae and Canellaceae are monophyletic with high support in ML, MP, and Bayesian analyses (Fig. 1, SI Figs. 2-4), consistent with previous studies (e.g. Chase et al., 1993; Zanis et al., 2002, 2003; Qiu et al., 2005, 2006; Marquínez et al., 2009). Within Winteraceae, the intrafamilial relationships supported in the present study are congruent with past analyses including more species for each genus (Karol et al., 2000; Doust and Drinnan, 2004; Marquínez et al., 2009). Within Canellaceae, relationships are also mostly congruent with previous studies (Karol et al., 2000; Salazar and Nixon, 2008).

Relationships within Piperales

The clade of Saururaceae and Piperaceae, well supported in the present study, was recovered in the great majority of previous phylogenetic studies (Qiu et al., 2000, 2005, 2006, 2010; Soltis et al., 1999, 2000a, 2000b, 2007, 2011; Mathews and Donoghue, 1999, 2000; Qiu et al., 1999; Doyle and Endress, 2000; Savolainen et al., 2000; Zanis et al., 2002, 2003; Nickrent et al., 2002; Borsch et al., 2003; Hilu et al., 2003; Kelly and González, 2003; Jaramillo et al., 2004; Neinhuis et al., 2005; Wanke et al., 2007a). The relationships between this group and the Aristolochiaceae *s.l.* (incl. *Lactoris*) have remained uncertain in the sense that the monophyly of the latter family has been debated in the literature. The family was found to be paraphyletic or received low support in several studies (Mathews and Donoghue, 1999; Soltis et al., 1999; Graham and Olmstead, 2000; Qiu et al., 2000; Savolainen et al., 2000; Borsch et al., 2003; Hilu et al., 2003; Kelly and González, 2003; Wanke et al., 2007a). Our 12-marker ML and Bayesian analyses present Aristolochiaceae *s.l.* as a well-supported clade (BS=99%, PP=1; Fig. 1). In previous analyses supporting *Lactoris*, Aristolochioideae, and Asaroideae as a monophyletic group, the position of *Lactoris* varied: either *Lactoris* was sister to Asaroideae with low support (Doyle and Endress, 2000; Savolainen et al., 2000; Jaramillo et al., 2004), or it was sister to Aristolochioideae (Soltis et al., 2000a, 2000b, 2007, 2011; Qiu et al., 1999, 2000, 2005, 2006; Soltis et al., 1999; Doyle

and Endress, 2000; Savolainen et al., 2000; Zanis et al., 2002, 2003; Borsch et al., 2003; Hilu et al., 2003; Neinhuis et al., 2005; Wanke et al., 2007a). Our 12-marker Bayesian analysis strongly supports the second alternative (PP=1; Fig. 1).

In the past, the placement of the parasitic family Hydnoraceae has been difficult. The unusual morphology of these plants does not allow straightforward hypotheses of homology with other angiosperms. The complete or semi-subterranean, flowers are composed of three or four fleshy tepals, fused with the staminal filaments to form a tepalostemon. In addition, these non-photosynthetic plants present gene losses and rate acceleration, causing potential artifacts linked with long-branch attraction and missing data that may hamper phylogenetic reconstruction (Nickrent and Starr, 1994; Nickrent et al., 1998). In our analyses, we incorporated the markers used in Nickrent et al. (2002) plus the mtSSU for Hydnoraceae. As in the latter study, our ML analysis places Hydnoraceae nested within Aristolochiaceae with high support (Fig. 2). Within Aristolochiaceae, our ML analysis and Bayesian analysis present *Hydnora* as the sister group of a clade including *Lactoris* and Aristolochioideae (ML bootstrap=76%, PP=0.94; Fig. 2), whereas Aristolochioideae were sister to a clade of *Lactoris* and Hydnoraceae in Nickrent et al. (2002) with low support in MP (BS=57%). The close relationship between Aristolochiaceae and Hydnoraceae is consistent with the single whorl of tepals and the inferior ovary shared by members of the two families (Nickrent et al., 2002).

With respect to the intrafamilial level (SI figs. 2-4), our results are highly congruent with studies including more species per genera. Within the Saururaceae, our analyses support two clades previously found with high support (Meng et al., 2002, 2003): *Saururus* + *Gymnotheca* and *Anemopsis* + *Houttuynia*. Within Piperaceae, we also recovered two previously supported clades: *Zippelia* + *Manekia* and *Piper* + *Peperomia* (Jaramillo et al., 2004; Wanke et al., 2007a, 2007b). When the genus *Verhuellia* was included in previous analyses it appeared to be the sister group of all remaining genera of Piperaceae (Wanke et al., 2007b). We recovered this position with ML and MP bootstrap values greater than 95% and PP=0.98.

Relationships within Laurales

Within the order, several long-established relationships are well supported in our analyses. First, Calycanthaceae are sister to all remaining families (ML BS=99%; MP BS=93%; PP=1) (Fig. 1), as in most previous studies (Soltis et al., 1999, 2000a, 2000b, 2007, 2011; Qiu et al., 1999, 2000, 2005, 2006, 2010; Renner, 1999, 2004; Doyle and Endress, 2000; Savolainen et al., 2000; Zanis et al., 2002, 2003; Nickrent et al., 2002; Hilu et al., 2003). Second, when Atherospermataceae, Gomortegaceae and Siparunaceae were included, they formed a clade (the AGS clade) (Qiu et al., 1999, 2000; Renner, 1999, 2004; Doyle and Endress, 2000; Soltis et al., 2000b, 2011; Zanis et al., 2002, 2003). Our 12-marker analyses support this clade with bootstrap values greater than 80% (Fig. 1). Within the AGS clade, Siparunaceae are sister to the two remaining families with bootstrap support values greater than 90% and PP=1 (Fig. 1), confirming previous studies (Qiu et al., 1999, 2000; Renner, 1999, 2004; Doyle and Endress, 2000; Soltis et al., 2000b, 2011; Zanis et al., 2003). Finally, Hernandiaceae, Lauraceae, and Monimiaceae form a clade (the HLM clade) that receives high support values in ML, MP, and Bayesian analyses (Fig. 1) and was previously supported in the literature (Qiu et al., 1999, 2000, 2005, 2006, 2010; Renner, 1999, 2004; Doyle and Endress, 2000; Savolainen et al., 2000; Renner and Chanderbali, 2000; Hilu et al., 2003; Zanis et al., 2003; Soltis et al., 2007, 2011).

Relationships within the HLM clade have been a matter of debate for some time. The first alternative, Monimiaceae as sister to the two other families, was well supported (MP BS=100%) in a combined analysis of morphological and molecular data (Doyle and Endress, 2000). In addition, based on the morphological dataset of Doyle and Endress (2010) the sister-group relationship of Lauraceae and Hernandiaceae is 16 steps more parsimonious than a relationship of Lauraceae and Monimiaceae, and 15 steps more parsimonious than a relationship of Hernandiaceae and Monimiaceae (Doyle and Endress, 2010). This topology (Monimiaceae sister to the other two families) was also recovered in the ML analysis of Renner and Chanderbali (2000). The second alternative, Lauraceae sister to the remaining families, was well supported only in Qiu et al. (2006) and Soltis et al. (2011), although it was also found in several other studies (Qiu et al., 1999, 2000, 2010; Renner and Chanderbali, 2000; Hilu et al., 2003; Zanis et al., 2003). The third alternative, Hernandiaceae sister to the

remaining families, received either low support (Renner and Chanderbali, 2000; Savolainen et al., 2000; Qiu et al., 2005) or moderate support (Renner, 1999, 2004). The study of Renner and Chanderbali (2000) focused specifically on the relationships among Hernandiaceae, Lauraceae, and Monimiaceae, but did not obtain good support for any of the three alternatives. The topology showing Hernandiaceae as sister group to the other two families was obtained in our 12-marker ML and Bayesian analyses (Fig. 1). The clade of Lauraceae + Monimiaceae was supported by 69% ML bootstrap and 0.97 posterior probability (Fig. 1). Given the uncertainty that remains regarding the relationships among Hernandiaceae, Lauraceae, and Monimiaceae, it would be necessary to conduct new phylogenetic analyses combining molecular and morphological data. If the results of such analyses support one of the two main alternatives obtained based on molecular data (Lauraceae or Hernandiaceae as sister to the other two families), this would illustrate either a morphological convergence in Lauraceae and Hernandiaceae or a morphological reversal in Monimiaceae.

Within Lauraceae, our analyses support with high bootstrap values two clades found in previous studies focusing on the family: the core Lauraceae and the *Mezilaurus* group (SI Figs. 2-4) (Rohwer, 2000; Chanderbali et al., 2001; Rohwer and Rudolph, 2005). The relationships outside these two clades are not well resolved in the present study (SI Figs. 2-4). The position of genera *Cassytha*, *Neocinnamomum*, *Caryodaphnopsis*, and *Hypodaphnis* are debated in the literature (Rohwer, 2000; Chanderbali et al., 2001). Only Rohwer and Rudolph (2005) found good support with a Bayesian approach for the position of these taxa. Within the Monimiaceae, in previous studies, the first split is between a clade consisting of *Monimia*, *Palmeria* and *Peumus* and a clade containing all remaining Monimiaceae (Renner, 1998; Renner et al., 2010). In addition, in the latter clade, *Hortonia* is sister to all other genera (Renner, 1998; Renner et al., 2010). Our analyses strongly support this topology (SI Figs. 2-4). The relationships among the remaining genera are not well resolved in the present study. In Hernandiaceae, our analyses strongly support two clades: *Sparattanthelium* + *Gyrocarpus* and *Hazolomania* + *Illigera*. These two groups are also well supported in previous studies including more species of the family (Renner and Chanderbali, 2000; Michalak et al., 2010). The relationships among genera of Atherospermataceae are not

resolved in the present study. Finally, in Calycanthaceae, as in Zhou et al. (2006), *Idiospermum* is sister to *Calycanthus* + *Chimonanthus*, with high support values (SI Figs. 2-4).

Relationships within Magnoliales

Our ML, MP, and Bayesian analyses of the 12-marker dataset strongly support a sister group relationship between Myristicaceae and all remaining Magnoliales (Fig. 1), confirming previous studies based on fewer taxa and markers (Soltis et al., 1999, 2000a, 2007; Qiu et al., 1999, 2000, 2005, 2006, 2010; Doyle and Endress, 2000; Savolainen et al., 2000; Zanis et al., 2002, 2003; Nickrent et al., 2002; Borsch et al., 2003; Hilu et al., 2003; Sauquet et al., 2003). A clade formed of Eupomatiaceae and Annonaceae is also strongly supported (Fig. 1), as found in most previous analyses (Soltis et al., 2000a, 2000b, 2007, 2011; Qiu et al., 1999, 2000, 2005, 2006, 2010; Soltis et al., 1999; Doyle and Endress, 2000; Savolainen et al., 2000; Nickrent et al., 2002; Zanis et al., 2002, 2003; Sauquet et al., 2003). Our ML and MP analyses also suggest, albeit with low support, a clade of Degeneriaceae and Himantandraceae (the DH clade) (Qiu et al., 1999; Soltis et al., 2007; Qiu et al., 2000, 2005, 2006; Soltis et al., 1999, 2000a, 2000b; Doyle and Endress, 2000; Savolainen et al., 2000; Zanis et al., 2002, 2003; Sauquet et al., 2003). In the literature, the position of the DH clade varies (Fig. 3). If we consider only relationships supported by more than 50% bootstrap values, the DH clade is either sister to Magnoliaceae (Fig. 3a) (Soltis et al., 1999, 2007; Qiu et al., 2000, 2005, 2006; Savolainen et al., 2000; Zanis et al., 2002, 2003) or to Eupomatiaceae + Annonaceae (Fig. 3c) (Doyle and Endress, 2000; Sauquet et al., 2003). Only the two latter studies provided high support for the position of the clade. Both used a combined molecular and morphological data set and Sauquet et al. (2003) used more variable markers (*trnK* intron and *trnT-trnL* intergenic spacer) than those used in the present study. Unfortunately, our analyses do not provide resolved relationships for this part of the tree, suggesting that many more genes or a detailed morphological data set will be required to address adequately this question.

As in Sauquet et al. (2003), the relationships in Myristicaceae are not entirely resolved in the present study (SI Figs. 2-4). Our results are congruent with this previous study. First we recover part of the mauloutchioid clade, consisting here in *Cephalosphaera*, *Brochoneura*, and *Mauloutchia*, with similar relationships among these three genera. However, contrary to

Sauquet et al. (2003), the relationships among *Staudtia* and the three other mauloutchioid genera included in the present study are not resolved. In addition, in all analyses *Coelocaryon* and *Pycnanthus* form a highly supported clade. Finally we recovered the clade *Knema* + *Myristica*. The relationships within Annonaceae are, by and large, well resolved in the present study (SI Figs. 2-4, 11). We follow here the nomenclature of Chatrou et al. (2012). In the literature and within the present study, *Anaxagorea* is the sister group of all remaining Annonaceae with high support (SI Figs. 2-4, 11; Doyle and Le Thomas, 1994, 1996; Doyle et al., 2000; Sauquet et al., 2003; Couvreur et al., 2008, 2011; Erkens et al., 2009; Chatrou et al., 2012). The monophyly of subfamily Ambavioideae is weakly supported in the MP analysis, as in Chatrou et al. (2012), but receives moderate support in ML and high support in the Bayesian analysis (SI Figs. 2-4, 11). This latter clade has been previously supported in the literature (Doyle and Le Thomas, 1994, 1996; Doyle et al., 2000; Sauquet et al., 2003; Erkens et al., 2009; Couvreur et al., 2011). Subfamily Annonoideae is monophyletic with high support (SI Figs. 2-4, 11). Within this subfamily, tribe Bocageae is sister to all remaining tribes with high support (SI Figs. 2-4, 11), as in other studies focused on the family (Sauquet et al., 2003; Couvreur et al., 2008, 2011; Erkens et al., 2009; Chatrou et al., 2012). In the present study, within the Annonoideae all tribes are monophyletic except the Monodoreae (SI Figs. 2-4, 11). The monophyly of this latter tribe was only weakly supported in previous studies (Couvreur et al., 2008; Erkens et al., 2009; Chatrou et al., 2012). Subfamily Malmeoideae, corresponding to the Short-Branch Clade, or malmeoid/piptostigmoid/miliusoid clade in the literature, receives high support as in previous studies (SI Figs. 2-4, 11; Doyle and Le Thomas, 1994, 1996; Doyle et al., 2000; Sauquet et al., 2003; Erkens et al., 2009; Couvreur et al., 2011; Chatrou et al., 2012). Tribe Piptostigmateae is sister to the remaining tribes of the subfamily (SI Figs. 2-4, 11). This position has been supported in other studies (Couvreur et al., 2011; Chatrou et al., 2012).

26s rDNA isolate of *Degeneria* leads to long-branch attraction

The introduction of the only 26S rDNA sequence available on GenBank for the species *Degeneria vitiensis* sparks a surprising phylogenetic result. The well-supported position of Myristicaceae as sister to all remaining families of Magnoliales is not recovered when the latter sequence is included in the 12-marker data set (Fig. 4). Rather, in this case

Degeneriaceae is sister to Myristicaceae both in MP (BS=100%) and ML analyses (BS=88%) (Fig. 4). This uncommon relationship between the two families was also found, with high support values, in two previous studies using, for *Degeneria*, either the same sequence of 26S rDNA (Qiu et al., 2005) or another sequence for this marker (Soltis et al., 2011) (Fig 3d, e). A tempting explanation would be that the sequence labeled as *Degeneria* belongs in fact to Myristicaceae, due to misidentification or contamination. However, this is unlikely for two reasons. First, this sequence was produced from the same voucher specimen (JM Miller 1189-63) as other *Degeneria* sequences included in this study, none of which showed a clear association with Myristicaceae (SI Figs. 1i-j). Second, if this were the case, we would expect the sequence to fit in the crown of short internal branches of Myristicaceae, not attached low on the long stem lineage of Myristicaceae as we observe (Fig. 4, SI Fig. 1l). Instead, we argue that this result is due to an artifact of attraction between the well-known long stem branch of Myristicaceae (Sauquet et al., 2003) and the unusually long branch of *Degeneria* for 26S rDNA. Importantly, *Degeneria* has a much shorter branch in all other 9 separate analyses in which the taxon was sampled and is the sister group of *Galbulimima* or nested within a clade excluding Myristicaceae in six of them (in the three remaining that include this species, the resolution is not sufficient; SI Figs. 1a, b, e-k).

Conclusion

For many years, a debate in the literature on the best way to improve the accuracy of phylogenies has not settled in favor of increasing the number of taxa or the number of characters. For several authors, using more markers with longer sequences would be the best way (e.g. Rosenberg and Kumar, 2001). On the contrary, other authors argue that the best strategy is to include more taxa in phylogenetic analyses (e.g. Pollock et al., 2002; Zwickl and Hillis, 2002). From our point of view, both approaches present their own advantages. For instance, with respect to long-branch attraction (LBA), one solution proposed by several authors is to incorporate new taxa in order to break up the problematic branches (e.g. Hillis, 1998; Poe, 2003). In the context of the probabilistic approaches (ML and Bayesian), reported to be less sensitive to LBA artifacts, the taxonomic sampling strategy is critical too. This is because these methods involve estimations of the parameters of evolutionary models. This

assessment of the model is improved with high taxonomic sampling (Pollock and Bruno, 2000). On the other hand, many empirical studies have shown that sampling more characters can help to resolve problematic parts of the Tree of Life (e.g. Jansen et al., 2007; Regier et al., 2010; Silberfeld et al., 2010). Here, focusing for the first time on Magnoliidae as a whole, we placed a similar effort on increasing both marker and taxon sampling relative to all previous estimates of the phylogeny of this clade. This combined strategy has resulted in a robust phylogenetic backbone for the entire clade. Results confirm topologies previously found in the literature at a more local level or with fewer representative taxa sampled at a higher level. Although relationships among families are, in general, well supported in the present study, two parts of the tree are still uncertain (relationships among the *Degeneria-Galbulimima* clade and Magnoliaceae and relationships in the HLM clade). Sampling new molecular markers or complete plastid genomes may lead to a better resolution and greater support. In particular, using low-copy nuclear genes may help increase the phylogenetic signal from the nuclear genome (Zhang et al., 2012). Finally, the fact that MP analyses provide similar results as ML and Bayesian analyses contradicts the widespread tendency to assume that parsimony is obsolete. This is quite reassuring in a context where this method is still the widely used to incorporate the paleontological diversity in extant phylogenetic contexts. This detailed phylogeny of Magnoliidae is a first step towards reconstructing the evolutionary history of an important and highly diverse clade of early-diverging angiosperms.

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Figures

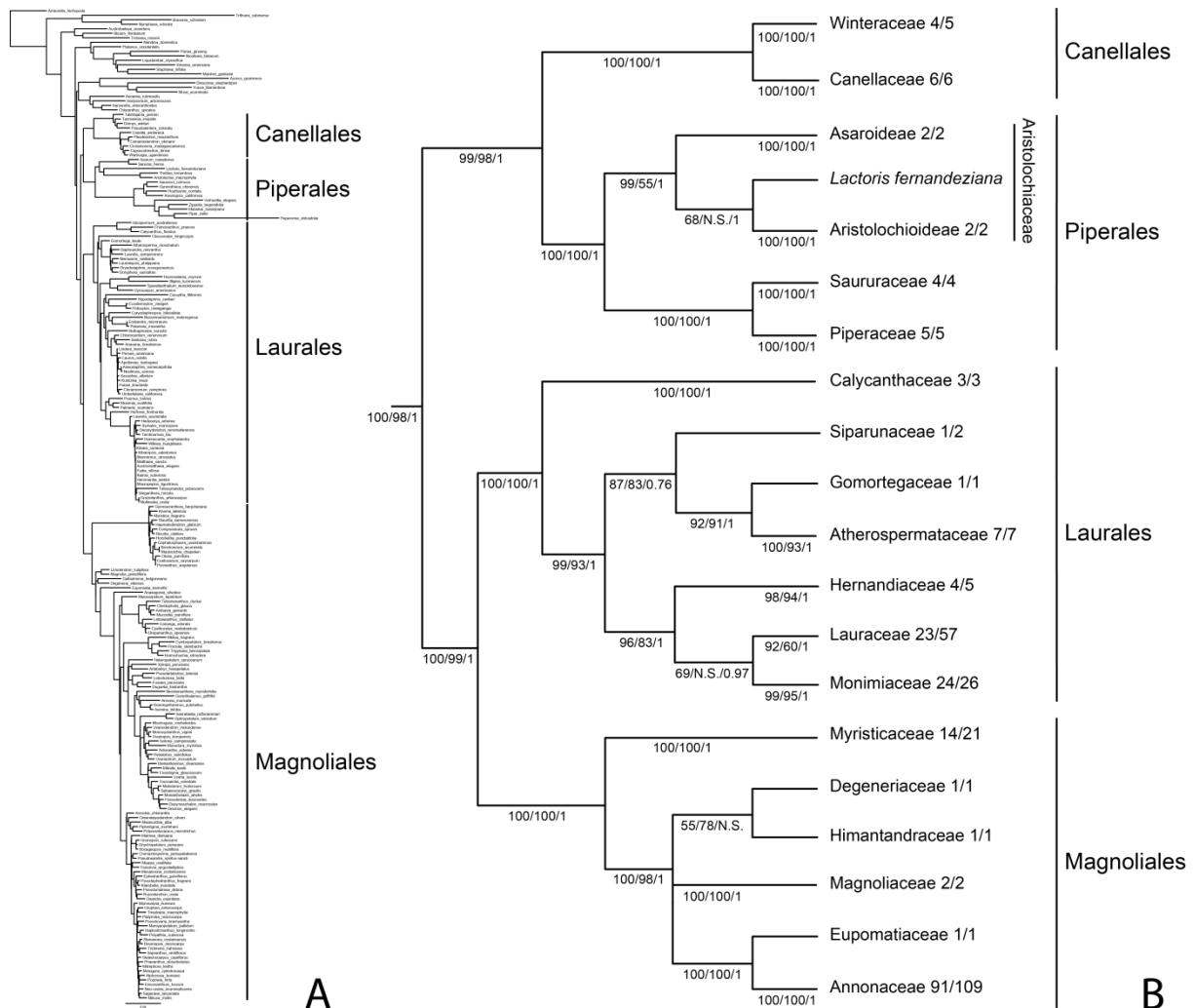


Figure 1: 12-marker data set. A, Maximum likelihood phylogram of the 12-marker data set with species as terminals (see SI Fig. 2 for full detail). B, Simplified tree of the relationships among families obtained in MP, ML and Bayesian analyses of the 12-marker dataset. Numbers below branches are maximum likelihood bootstrap values followed by maximum parsimony bootstrap values and posterior probabilities. Nodes supported by less than 50% bootstrap and 0.9 posterior probability have been collapsed. Family names are followed by the number of genera included in the data set out of the total number of genera recognized in the family. Abbreviation: N.S., not supported.

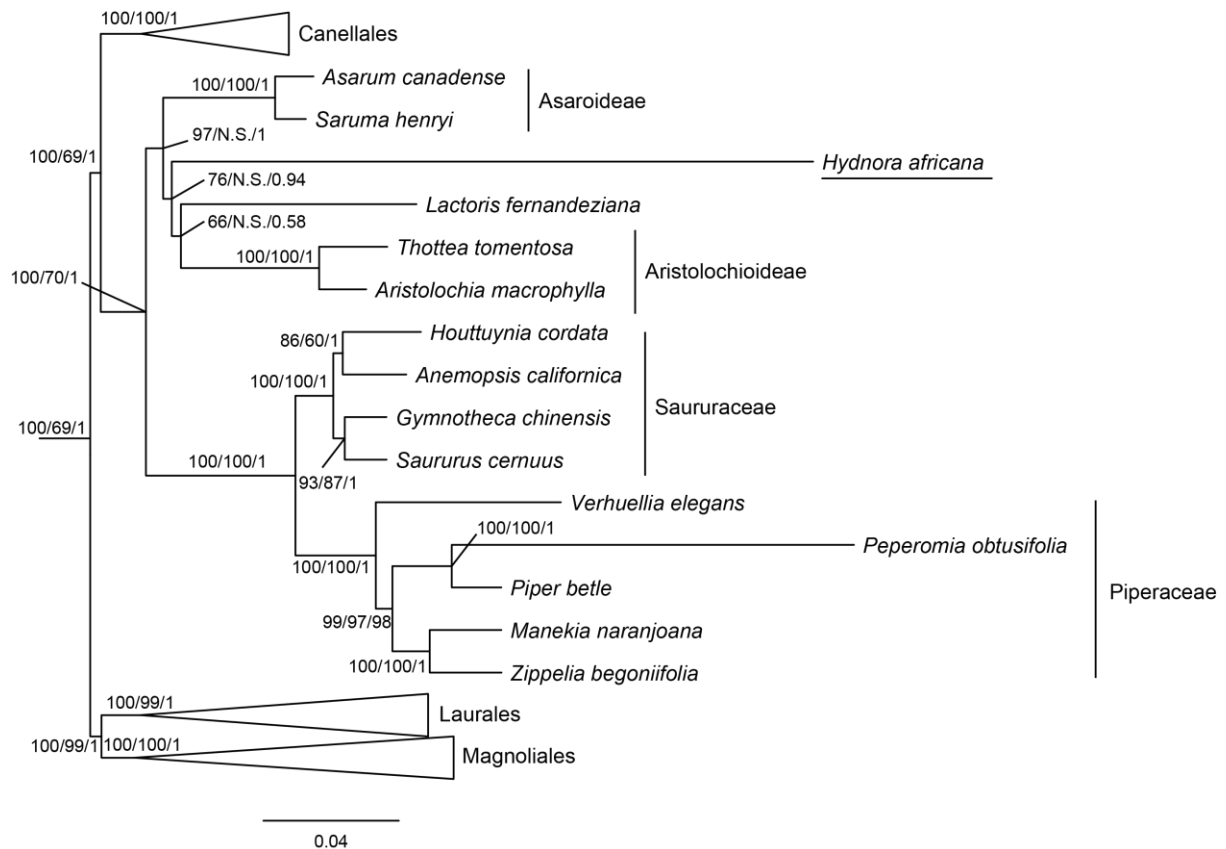


Figure 2: 12-marker data set including Hydnoraceae. Phylogram of the relationships among families of Piperales obtained in the ML analysis of the 12-marker dataset including Hydnoraceae. Numbers along branches are maximum likelihood, maximum parsimony bootstrap values, and posterior probabilities. Nodes supported by less than 50% bootstrap and 0.9 posterior probability have been collapsed. Triangles represent supraspecific clades. Abbreviation: N.S., not supported.

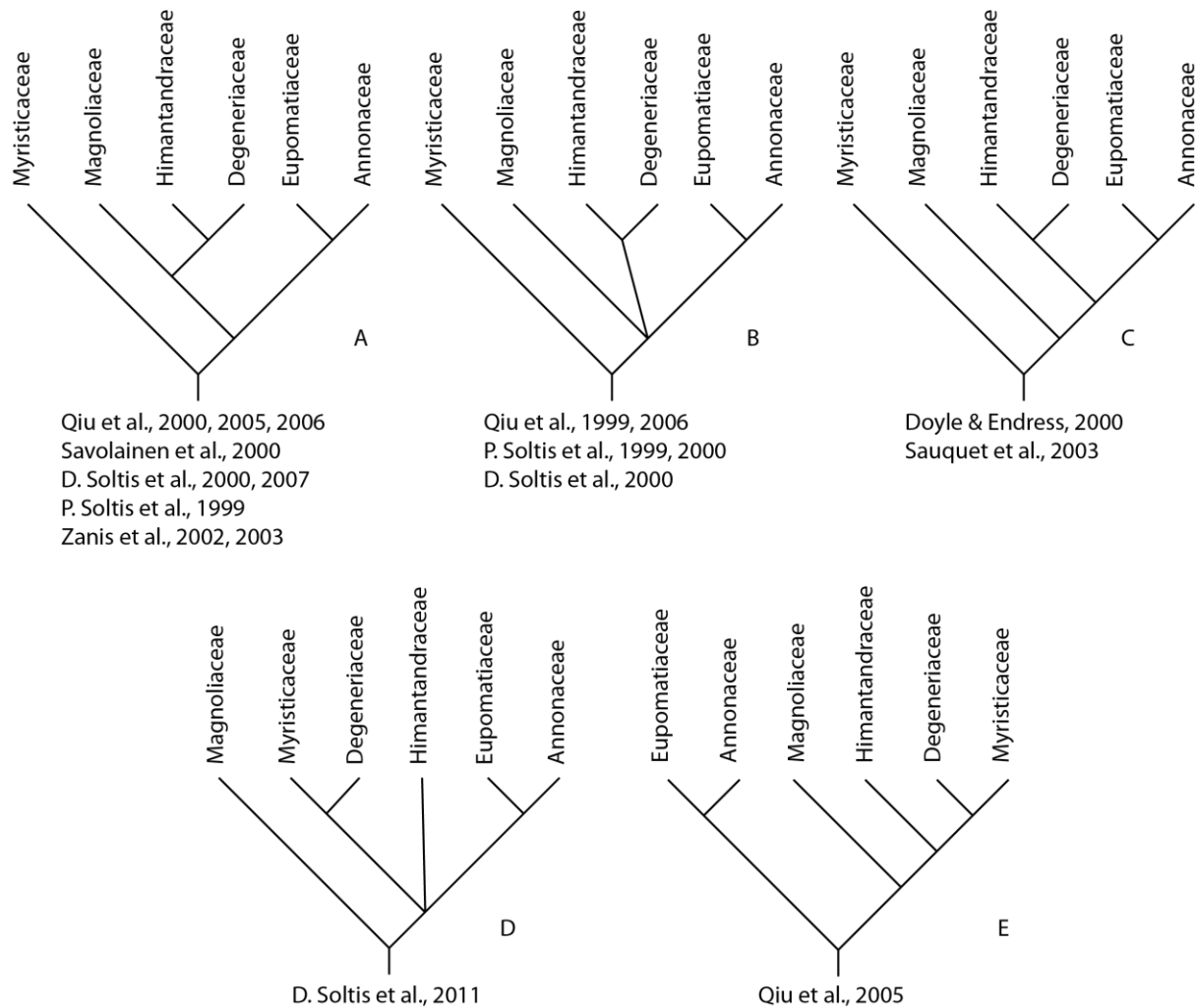


Figure 3: Simplified trees of the relationships among the families of Magnoliales reported in previous analyses. Nodes supported by less than 50% bootstrap values are collapsed.

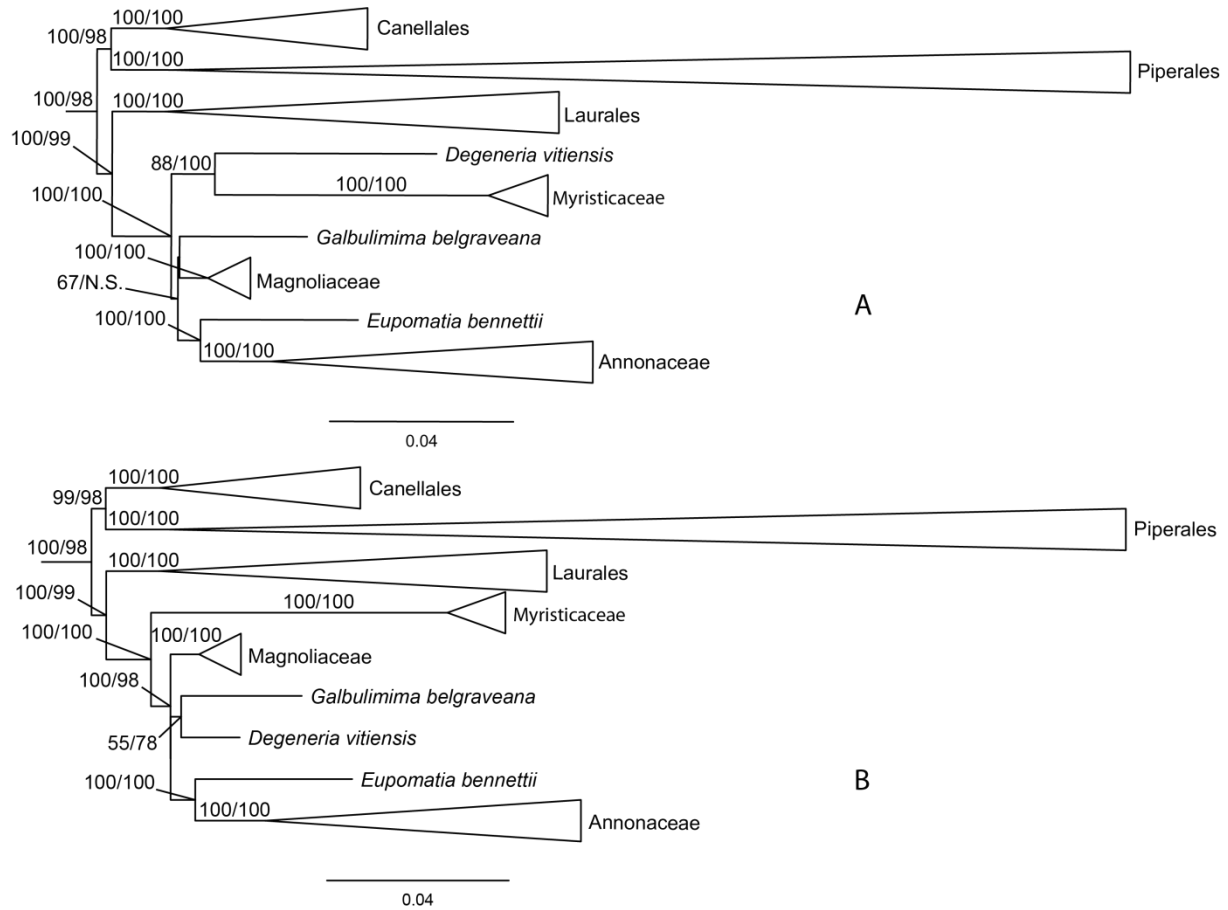


Figure 4: Impact of the 26S rDNA sequence of *Degeneria*. Simplified maximum likelihood phylograms with maximum likelihood bootstrap values followed by maximum parsimony bootstrap values. **A**, Phylogram obtained from the 12-marker data set including 26S rDNA of *Degeneria*. **B**, Phylogram obtained from the 12-marker data set excluding 26S rDNA of *Degeneria* (same tree as Fig. 1). Triangles represent supraspecific clades. Abbreviation: N.S., not supported.

Chapter 2

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Fossil calibration of Magnoliidae, an ancient lineage of angiosperms

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Abstract

In order to investigate the diversification of angiosperms, an accurate temporal framework is needed. Molecular dating methods thoroughly calibrated with the fossil record can provide estimates of this evolutionary time scale. Because of their position in the phylogenetic tree of angiosperms, Magnoliidae (10,000 species) are of primary importance for the investigation of the evolutionary history of flowering plants. The rich fossil record of the group, beginning in the Cretaceous, has a global distribution. Among the hundred extinct species of Magnoliidae described, several have been included in phylogenetic analyses alongside extant species, providing reliable calibration points for molecular dating studies. Until now, few fossils have been used as calibration points of Magnoliidae, and detailed justifications of their phylogenetic position and absolute age have been lacking. Here, we review the position and ages for 10 fossils of Magnoliidae, selected because of their previous inclusion in phylogenetic analyses of extant and fossil taxa. This study allows us to propose an updated calibration scheme for dating the evolutionary history of Magnoliidae.

Keywords: fossil calibration; fossil flowers; Canellales; Laurales; Magnoliales; Magnoliidae; Piperales.

Introduction

Among the first diverging lineages of angiosperms (outside monocots and eudicots), Magnoliidae are the largest clade, comprising about 10,000 species divided among 20 families and four orders: Magnoliales, Laurales, Canellales, and Piperales (APG III, 2009). Although Magnoliidae have a global distribution extending into the temperate zones of both hemispheres, most of their diversity occurs in tropical areas. This group has been supported as monophyletic by the great majority of molecular phylogenetic studies (Qiu et al., 1999, 2000, 2005, 2006, 2010; Soltis et al., 1999, 2000a, 2000b, 2007, 2011; Mathews and Donoghue, 1999, 2000; Savolainen et al., 2000; Graham and Olmstead, 2000; Zanis et al., 2002, 2003; Nickrent et al., 2002; Borsch et al., 2003; Hilu et al., 2003; Jansen et al., 2007; Moore et al., 2007, 2010; Burleigh et al., 2009). Although the majority of the relationships among families of Magnoliidae are well established, the exact positions of Hydnoraceae and Magnoliaceae and the relationships among Hernandiaceae, Lauraceae and Monimiaceae are still debated (Fig. 1; Massoni et al., 2014). This group has a rich fossil record starting in the Early Cretaceous. Cretaceous fossils assigned to Magnoliidae have been found in many parts of the world: South America (e.g., Mohr and Bernardes-de-Oliveira, 2004; Mohr et al., 2013), North America (e.g., Dilcher and Crane, 1984; Crepet et al., 2005), Europe (e.g., Friis et al., 2010, 2011), Asia (e.g., Takahashi et al., 2001, 2008), Africa (e.g., Doyle et al., 1990), Australasia (e.g., Dettmann et al., 2009), and Antarctica (e.g., Poole and Gottwald, 2001; Eklund, 2003). Fossil Magnoliidae come in a variety of forms, from wood (e.g., Herendeen, 1991; Poole and Gottwald, 2001; Schöning and Bandel, 2004) and leaves (e.g., Upchurch and Dilcher, 1990; Kvacek, 1992) to flowers (e.g., Dilcher and Crane, 1984; Drinnan et al., 1990; Takahashi et al., 2008; Friis et al., 2011), fruits (e.g., Friis et al., 2010, 2011), seeds (e.g., Knobloch and Mai, 1986; Frumin and Friis, 1996), and pollen (e.g., Doyle et al., 1990; Macphail et al., 1994). Because the number of characters observed in such fossils is often limited, establishing their phylogenetic relationships to extant taxa is not always straightforward. However, among fossils assigned to Magnoliidae, several are characterized by good to exceptional preservation and a large number of systematically useful characters, such as the lignitized and charcoalfied flowers considered here.

Investigating the evolutionary history of organisms often requires a temporal context, which until recently was based almost exclusively on the fossil record. Insofar as fossils can be placed in a phylogenetic tree, a paleontological approach can provide minimum ages and, into a lesser extent, maximum ages for clades. Over the past two decades, this method has been supplemented with molecular dating approaches, which generate estimates of absolute divergence times throughout the tree (i.e., not just in the neighborhood of a particular fossil). The first such studies were based on the concept of a molecular clock, but the development of new methods has helped to relax the assumption of a strict clock across the tree.

In molecular dating analyses, the fossil record provides minimum bounds to calibrate the trees. These calibration points are not only used to convert the time scale of relative ages into one of absolute ages, but they also serve as anchors for modeling molecular rate variation across the tree (e.g., Sanderson, 1997; Sauquet et al., 2012). In this context, calibration is being recognized as a critical point and can have a drastic influence on results (Inoue et al., 2010; Meredith et al., 2011; Sauquet et al., 2012). Because of re-evaluation of the boundaries of geological time units, and sometimes re-dating of fossil beds, the accepted age of a fossil can change through time (Gandolfo et al., 2008). Therefore, an exhaustive geological review is essential in order to provide an accurate minimum age (Parham et al., 2012). Except for recently developed total-evidence dating approaches that combine the phylogenetic analysis of extant and fossil taxa with the estimation of divergence times (Pyron 2011; Ronquist et al., 2012), a review of the phylogenetic position of a fossil is a second crucial point in the calibration process (Gandolfo et al., 2008; Parham et al., 2012; Sauquet et al., 2012). Molecular scaffold approaches (analyzing morphological data with the topology of living taxa fixed by a backbone constraint tree; Springer et al., 2001) or total evidence approaches (using both molecular and morphological data; Kluge, 1989) can provide a genuine test for the phylogenetic position of extinct taxa. A decision regarding the identity of the node calibrated by a fossil has to take into account the uncertainty of its phylogenetic position and the uncertainty of the relationships among extant species. A number of fossil taxa of Magnoliidae have already been included in morphological data matrices and analyzed with a phylogenetic approach taking into account both morphological

and molecular data (e.g., Doyle and Endress, 2010; von Balthazar et al., 2011). These previous studies should, in principle, allow firm calibration of a molecular time scale, which is currently lacking for Magnoliidae (Forest and Chase, 2009).

The purpose of the present study is to provide a reliable calibration scheme for the Magnoliidae, usable as a starting point for any molecular dating study of Magnoliidae or subgroups of this clade, as well as higher-level angiosperm divergence time studies. To do so, we have reviewed the geologic age and phylogenetic relationships of all fossils of Magnoliidae that have been included in at least one phylogenetic analysis (10 fossils, Table 1). For this paper, we did not re-examine the fossil specimens. Instead, our argumentation is based on previously published descriptive and phylogenetic studies. Nine of these are represented by fossil flowers, in four cases with associated vegetative parts, but one (*Walkeripollis gabonensis*) is based on dispersed pollen grains that have a particularly diagnostic combination of characters. Following a strict minimum age philosophy, we propose to calibrate the most recent common ancestor of all most parsimonious assignments of previous analyses, and we provide associated synapomorphies when they were mentioned in the original study.

Most of the fossils treated here are from continental deposits that contain neither marine fossils that would allow direct correlation with the marine-based relative time scale, nor minerals suitable for absolute radiometric dating. In most cases, their ages are based on indirect palynological correlations with pollen and spore sequences that are dated by marine fossils. For example, for the US Atlantic Coastal Plain, these reference sections are in the US Western Interior and Gulf Coast (Hedlund and Norris, 1968; Ward, 1986; Ludvigson et al., 2010) and western Europe (Góczán et al., 1967; Kemp, 1970; Laing, 1975; Hughes, 1994; Heimhofer et al., 2005, 2007; Hochuli et al., 2006), which belonged to the same Southern Laurasian phytogeographic province of Brenner (1976; see Doyle, 1969a, 1977, 1992; Wolfe and Pakiser, 1971; Doyle and Hickey, 1976; Doyle and Robbins, 1977; Christopher, 1979; Christopher et al., 1999; Christopher and Prowell, 2010). Some of these fossils, or others from the same localities, have been used for calibration in previous molecular dating studies (Doyle et al., 2004; Magallón and Castillo, 2009; Clarke et al., 2011; Pirie and Doyle, 2012). In some cases, Clarke et al. (2011) proposed much younger minimum ages than those

recommended here, because of conflicting conclusions of earlier authors on the phylogenetic position of fossils or on stratigraphic correlations. We argue that these minimum ages can be improved with critical examination. Our treatment includes a review of the age of units in the Atlantic Coastal Plain, the source of four of the fossils treated here and several non-magnoliid taxa used in other dating analyses, which has been a subject of recent discussion (Hochuli et al., 2006).

Absolute ages used here follow Ogg and Hinnov (2012). This chapter of the revised Geologic Time Scale of Gradstein (2012) is the most recent comprehensive synthesis of absolute dates for the Cretaceous, including estimated ages for each substage, which we derive here from the charts in Fig. 27.6 of Ogg and Hinnov (2012). When possible, use of substage boundaries is preferable to the use of stage boundaries only, which can be overly conservative. Many radiometric dates are available for the Late Cretaceous, thanks particularly to the abundance of bentonites. There are fewer radiometric dates for the Early Cretaceous, but there has been much progress in integrating these with sequence stratigraphy, magnetostratigraphy, isotope stratigraphy, and cyclostratigraphy (which relates cyclic sedimentation to astronomical cycles of the earth's orbit and tilt). Significantly younger ages and shorter durations for several stages were proposed by Fiet et al. (2006) based on K/Ar glauconite dates and cyclostratigraphy. Scott et al. (2009) suggested that these ages may be too young due to argon leakage, a problem in glauconites, and Fiet et al. (2006) recognized that they require testing with studies on bentonites. By contrast, Huang et al. (2010), also using cyclostratigraphy, obtained ages for the Aptian and Albian very close to those of Ogg and Hinnov (2012) and suggested that the shorter durations of Fiet et al. (2006) were due to incompleteness of the sections studied. The dates of Ogg and Hinnov (2012) still involve considerable extrapolation, but use of this single comprehensive scheme should make correction of the calibrations presented here easier if this becomes necessary in the future.

We organized the present text by sections on calibrated nodes. When several fossil species were available to calibrate the same node, we used the oldest (reported as the "preferred fossil" section in the text) to define the age associated with the calibration. The remaining extinct species, which could be used to calibrate the same node, are reported as "additional fossils". We organized the "additional fossil" sections following the same structure used for

the “preferred fossil” section. We proceeded in this way because future phylogenetic and stratigraphic studies may refine the position and the age of the fossils, leading to future improvement of the current scheme. We also believe that reporting the complete details of redundant fossils identified as suitable calibrations is useful to increase confidence in the proposed calibration of the corresponding nodes.

Node 1: crown-group Magnoliineae

Fossil Taxon 1 (preferred, given current knowledge): *Endressinia brasiliana* Mohr and Bernardes-de-Oliveira, 2004

Node Calibrated: crown-group Magnoliineae (Doyle and Endress, 2010; Mohr et al., 2013)

Reference Specimen: MB. PB. 2001/1455 in Museum of Natural History, Institute of Paleontology, Berlin, Germany (holotype; branching axis with attached leaves and flowers).

Phylogenetic Justification: A molecular scaffold analysis by Doyle and Endress (2010), including 64 extant taxa sampled across angiosperms and 142 morphological characters, placed *Endressinia* in seven different most parsimonious positions: all positions within the crown group of the clade Himantandraceae + Degeneriaceae + Eupomatiaceae + Annonaceae (each represented as one terminal), or as the sister group of this clade. These relationships were supported by one unequivocal synapomorphy, the presence of glands on the stamens or staminodes (Doyle and Endress, 2010). A more recent molecular scaffold analysis (Mohr et al., 2013), which used a modified version of the morphological data set of Doyle and Endress (2010) reduced to Magnoliales, Laurales, and Canellales (as outgroup), placed *Endressinia* as the sister group of *Schenkeriophyllum glanduliferum* (another fossil from the same deposit, discussed below), with the clade of the two fossils being the sister group of Magnoliaceae. *Endressinia* and *Schenkeriophyllum* were united by sessile leaf blade (a new character) and linked with Magnoliaceae by sheathing leaf base and dry fruit wall. As noted by Mohr et al. (2013), Doyle and Endress (2010) did not score *Endressinia* as having a sheathing leaf base. This was probably because the sheath was formed from the unusual sessile leaf blade, rather than a leaf base separated from the blade by a petiole, but this difference does not rule out homology of the character. We have not attempted to resolve

this conflict with a new analysis. The implications for dating are complicated by the fact that the position of Magnoliaceae within Magnoliales is still debated. Two alternative positions have been supported by most analyses: either as the sister group of a clade of Degeneriaceae + Himantandraceae (Soltis et al., 1999, 2007; Qiu et al., 2000, 2005, 2006; Savolainen et al., 2000; Zanis et al., 2002, 2003), or sister to a clade of Degeneriaceae + Himantandraceae + Eupomatiaceae + Annonaceae (Doyle and Endress, 2000; Sauquet et al., 2003). However, the results of both Doyle and Endress (2010) and Mohr et al. (2013) support a position of *Endressinia* within the crown group of Magnoliineae, the well-supported clade of five families that is sister to Myristicaceae (Sauquet et al., 2003), and each study alone leads to use of this fossil to calibrate the crown node of this clade. Therefore, *Endressinia* provides a safe minimum age for the crown node of Magnoliineae (Fig. 1).

Minimum Age: Aptian-Albian boundary, 113 Ma

Age Justification: The fossil considered here was collected from the Crato Formation in the Araripe sedimentary basin of northeastern Brazil (Mohr et al., 2013). Mohr and Bernardes-de-Oliveira (2004) assumed that the Crato Formation is late Aptian or early Albian in age, based on numerous previous estimates (e.g., Pons et al., 1996). Because of this uncertainty, Clarke et al. (2011) proposed a minimum age for the Crato of 98.7 Ma, the top of the Albian. However, evidence has been accumulating in favor of a late Aptian age (Coimbra et al., 2002). Most recently, using gymnosperm pollen and dinoflagellates to correlate with better-dated sections, Heimhofer and Hochuli (2010) concluded that the Aptian-Albian boundary lies above the Crato Formation, and this was accepted by Mohr et al. (2013). We therefore propose a minimum age of 113.0 Ma for *Endressinia*, the Aptian-Albian boundary (Ogg and Hinnov, 2012).

Previous Use as Calibration: *Endressinia* has been used in several molecular dating studies with different ages and as a calibration for different nodes than recommended in the present study. In order to estimate divergence times within angiosperms, Magallón and Castillo (2009) used *Endressinia* with a similar age (112 Ma) but applied this age to the stem lineage of Magnoliineae. The same age was used by Pirie and Doyle (2012) to fix the age of the stem node of the clade of Eupomatiaceae and Annonaceae, sister to Magnoliaceae in

their study. Surveswaran et al. (2010) used the fossil to provide a minimum age for the same stem lineage corresponding, in their study, to the same node recommended here (crown node Magnoliaceae). However, they used an age of 115 Ma. Finally, Couvreur et al. (2011) calibrated the same node (crown node Magnoliaceae) with the same age but using it as a fixed age constraint.

Fossil Taxon 2 (additional): *Schenkeriophyllum glanduliferum* Mohr, Coiffard and Bernardes-de-Oliveira, 2013

Node Calibrated: crown-group Magnoliaceae (Mohr et al., 2013)

Reference Specimen: MB. Pb. 1999/2356 in Museum of Natural History, Institute of Paleontology, Berlin, Germany (holotype; branching axis with leaves and flowers).

Additional Specimens: MB Pb. 2008/350. Paratype (branching axis with leaves and flowers; will be deposited at the Geosciences Institute of the University of São Paulo, Brazil and is being stored with the number GP/3T2442).

MB.Pb. 2002/1336 (specimen with poorly preserved flowers).

MB. Pb. 1997/1219 (twigs with attached leaves).

MB. Pb. 1999/575, 1999/577A plus 1999/577B (dispersed leaves).

Phylogenetic Justification: As discussed for *Endressinia* (Fossil 1), the molecular scaffold analysis of Mohr et al. (2013) placed *Schenkeriophyllum glanduliferum* in a single most parsimonious position, together with *Endressinia brasiliensis*, as sister to the Magnoliaceae. This result implies that *Schenkeriophyllum* provides a minimum age for the stem node of Magnoliaceae or the crown node of Magnoliaceae, which are the same in the reference backbone tree used by Mohr et al. (2013). Because the position of Magnoliaceae with respect to Himantandraceae + Degeneriaceae and Eupomatiaceae + Annonaceae remains unresolved, as discussed under *Endressinia* (Soltis et al., 1999, 2007; Doyle and Endress, 2000; Qiu et al., 2000, 2005, 2006; Savolainen et al., 2000; Zanis et al., 2002, 2003; Sauquet et al., 2003), and the position of *Endressinia* is debated (see phylogenetic justification for

Endressinia; Doyle and Endress, 2010; Mohr et al., 2013), we propose to use the age of *Schenkeriphyllum* conservatively as a minimum age for crown group Magnoliaceae (Fig. 1).

Minimum Age: Aptian-Albian boundary, 113 Ma

Age Justification: *Schenkeriphyllum* was collected from the same sedimentary unit as *Endressinia*, the Crato Formation in the Araripe basin of northeastern Brazil (Mohr et al., 2013). As discussed for *Endressinia* (Fossil 1), we propose a minimum age of 113.0 Ma, the Aptian-Albian boundary (Ogg and Hinnov, 2012), for this fossil, based especially on palynological correlations by Heimhofer and Hochuli (2010).

Previous Use as Calibration: None to our knowledge.

Fossil Taxon 3 (additional): *Archaeanthus linnenbergeri* Dilcher and Crane, 1984

Node Calibrated: stem Magnoliaceae (Doyle and Endress, 2010)

Reference Specimen: UF 15703-4152 in University of Florida, Gainesville, USA (holotype of *Archaeanthus linnenbergeri* Dilcher and Crane; multifollicular fruit and proximal reproductive and vegetative portions of the same axis).

Additional Specimens: UF 15703; 2300, 2317, 2318, 2590, 3022, 3837, 3907, 4105, 4112, 4134-4150, 4152, 4153, 4155-4158, 4163, 4164, 4166-4170, 4198, 4532-4534: other specimens of *Archaeanthus linnenbergeri* examined in Dilcher and Crane (1984).

UF 15703-3179 (holotype of *Archaeopetala beekeri* Dilcher and Crane; perianth parts).

UF 15703-3882. Other specimen of *Archaeopetala beekeri* examined in Dilcher and Crane (1984).

UF 15703-2266 (holotype of *Archaeopetala obscura* Dilcher and Crane; perianth parts).

UF 15703-2747 (holotype of *Kalymnanthus walkeri* Dilcher and Crane; bud-scales).

UF 15703-4114, UF 15703-4115. Other specimens of *Kalymnanthus walkeri* examined in Dilcher and Crane (1984).

UF 15703-2272 (holotype of *Liriophyllum kansense* Dilcher and Crane; leaves)

UF 15703; 2267, 2271-2277, 2309, 2456, 2463-2466, 2469-2471, 2473, 2475-2477, 2479, 2480, 2482, 2484, 2485, 2487, 2488, 2492, 2493, 2679, 2948, 3443, 2813, 3816-3818, 3823, 3826, 3827, 3836, 3839, 3859, 3885, 3886, 3890, 3894, 3895, 3992, 4028, 4029, 4051, 4120. Other specimens of *Liriophyllum kansense* examined in Dilcher and Crane (1984).

Phylogenetic Justification: The position of *Archaeanthus linnenbergeri* was investigated by Doyle and Endress (2010), in a molecular scaffold analysis in which the family Magnoliaceae (ca. 227 species) was split into two taxa: Magnolioideae, often treated by recent authors as the single genus *Magnolia*, and *Liriodendron*. This analysis placed *Archaeanthus* in three different most parsimonious positions: one as the sister group of Magnoliaceae as a whole, and two within crown-group Magnoliaceae, either sister to *Liriodendron* or sister to Magnolioideae. The clade of *Archaeanthus* and Magnoliaceae was supported by three unambiguous synapomorphies: sheathing leaf base, bilobed stipules, and elongate receptacle, while the positions within the family were supported by bilobed leaf apex (shared with *Liriodendron*) or dehiscent fruit (shared with Magnolioideae). By contrast, a recent cladistic analysis by Romanov and Dilcher (2013) positioned *Archaeanthus* sister to the Late Cretaceous seed genus *Liriodendroidea* (Frumin and Friis, 1996) and identified the clade made up of these two extinct genera as the sister group of *Liriodendron*, supported by four synapomorphies. This would imply that *Archaeanthus* provides a minimum age for crown-group Magnoliaceae. However, the taxonomic sampling of this analysis was very limited, as the extant taxa included only Magnoliaceae s.s. (= Magnolioideae) as a supra-specific terminal, *Liriodendron*, and *Illicium* (Austrobaileyales), which is many nodes more distant from Magnoliaceae than are other members of the order Magnoliales. In addition, one of the four proposed synapomorphies of *Archaeanthus* and *Liriodendron*, whorled perianth phyllotaxis, vs. spiral in *Illicium* and *Magnolia*, appears to be a symplesiomorphy in Magnoliales, where the perianth is basically whorled and trimerous (Endress and Doyle, 2009). Furthermore, although the perianth is spiral in some species of *Magnolia* s.l., in many

species it is whorled (e.g., *M. denudata*: Erbar and Leins, 1981). Two other proposed synapomorphies, bilobed leaf apex and leaf lobation, are not independent characters, since the only lobation in the leaf of *Archaeanthus* is that of the apex; its origin requires only one change, not two. The status of the fourth synapomorphy, fruitlets shed from the receptacle, is uncertain, since *Degeneria* and most Annonaceae also have this feature (van Setten and Koek-Noorman, 1992). These observations imply that there are no more acceptable synapomorphies of *Archaeanthus* and *Liriodendron* in the Romanov and Dilcher (2013) data set than in Doyle and Endress (2010).

Even though we cannot dismiss the possibility that future analyses, based on denser taxon sampling and better knowledge of the phylogenetic position of Magnoliaceae, may eventually support a position of *Archaeanthus* within crown-group Magnoliaceae, we prefer to be conservative and recommend the use of *Archaeanthus* to serve as a minimum age constraint for the stem node of Magnoliaceae. In the current consensus tree presented in Fig. 1, this is the same node as crown-group Magnoliinae (the larger clade of five families found by Doyle and Endress, 2010, and Sauquet et al., 2003). However, this is not the case in one of the resolved trees of Massoni et al. (2014) on which this consensus tree is based, in which Magnoliaceae are the sister group of the clade consisting of *Degeneria* (Degeneriaceae) and *Galbulimima* (Himantandraceae). Furthermore, maintaining a distinction between the two nodes may be useful because the association of *Archaeanthus* with Magnoliaceae appears to be more strongly supported than that of *Endressinia* (and *Schenkeriphyllum*) and less likely to change in future analyses.

Minimum Age: early-middle Cenomanian boundary, 96.5 Ma

Age Justification: All the specimens used to describe *Archaeanthus linnenbergeri* come from the Dakota Formation at the Linnenberger Ranch in Russell County, central Kansas, USA (Dilcher and Crane, 1984). This formation lies between the underlying Kiowa Shale of Albian age and the overlying Graneros Shale (Retallack and Dilcher, 2012) of Cenomanian age. It has been traditionally divided into two members, the Terra Cotta Clay Member below and the Jansen Clay Member above (Plummer and Romary, 1942). The beds containing the specimens considered here were assigned to the Jansen Clay Member (Dilcher and Crane,

1984). Dilcher and Crane (1984) considered the age of this locality to be latest Albian to earliest Cenomanian. However, D. L. Dilcher (pers. comm. in Doyle and Endress, 2010) argued that it is more likely latest Albian, based on a carbon isotope and sequence stratigraphic study by Gröcke et al. (2006) at the Rose Creek locality in Nebraska, where a flora described by Upchurch and Dilcher (1990) lies just below the Albian-Cenomanian boundary, plus the fact that the Dakota is transgressive toward the east and sites such as Russell County are among its most western exposures. A latest Albian age was accepted by Doyle and Endress (2010) and reaffirmed without discussion by Romanov and Dilcher (2013). However, although the Rose Creek and Linnenberger Ranch floras were both assigned to the Jansen Clay Member and considered roughly coeval by Farley and Dilcher (1986), their equivalence needs reexamination in light of detailed sequence stratigraphic and palynological analyses of the Dakota Formation in Kansas, Nebraska, and Iowa by Ludvigson et al. (2010). This study showed that the Dakota does not represent a simple transgressive sequence but rather three transgressive-regressive cycles. The first two cycles (equivalent to Palynostratigraphic Units 1 and 2) are late Albian, while the third (Units 3 and 4) is early and middle Cenomanian; the boundary recognized by Gröcke et al. (2006), between the second and third cycles, falls within beds formerly assigned to the Jansen Clay Member. Unfortunately, this analysis did not extend as far west as Russell County, although in Lincoln County, just to the east, the lower part of the third cycle is represented by Dakota continental beds that interfinger with marine rocks to the west. Because we cannot exclude the possibility that the Linnenberger flora is from the lower part of the third cycle, which Ludvigson et al. (2010) dated as early Cenomanian, we propose a conservative minimum age of 96.5 Ma for *Archaeanthus*, the early-middle Cenomanian boundary (Ogg and Hinnov 2012).

Previous Use as Calibration: *Archaeanthus* has been used to provide calibration points within Magnoliidae in studies focused on angiosperms (Magallón and Castillo, 2009) and on Annonaceae (Doyle et al., 2004; Richardson et al., 2004; Pirie et al., 2006; Couvreur et al., 2008; Erkens et al., 2009; Su and Saunders, 2009; Pirie and Doyle, 2012). The great majority of these analyses used this fossil as a minimum age constraint of 98 Ma for the node recommended in the present study (stem Magnoliaceae; Doyle et al., 2004; Richardson et

al., 2004; Pirie et al., 2006; Couvreur et al., 2008; Erkens et al., 2009; Su and Saunders, 2009). Only Magallón and Castillo (2009) used the age we recommend (96.5 Ma), whereas Pirie and Doyle (2012) used an age of 100 Ma.

Node 2: crown-group Laurales

Fossil Taxon 4 (preferred, given current knowledge): *Virginianthus calycanthoides* Friis, Eklund, Pedersen and Crane, 1994

Node Calibrated: crown-group Laurales (Doyle et al., 2008)

Reference Specimen: PP43703 in Field Museum of Natural History, Chicago (holotype; flower).

Phylogenetic Justification: Friis et al. (1994) assigned *Virginianthus calycanthoides* to the stem lineage of Calycanthaceae because it resembles extant Calycanthaceae (including *Idiospermum*) but is more plesiomorphic in characters such as monosulcate rather than disulcate pollen. This assignment was questioned by Crepet et al. (2005) based on a combined (total evidence) analysis of a data set of Renner (1999), which included 15 morphological characters, sequences of six molecular markers, 25 taxa of Laurales, and three outgroups. This analysis identified *Virginianthus* as the sister group of either Laurales as a whole or all Laurales other than Calycanthaceae. A molecular scaffold analysis by Doyle et al. (2008), incorporating 65 morphological characters and using the same backbone trees as Doyle and Endress (2010), found two alternative most parsimonious positions for this fossil, one sister to Calycanthaceae, the other sister to the clade formed by all remaining Laurales. The first position was supported by extended anther connective, the second by embedded pollen sacs. Positions sister to Laurales as a whole and nested within Calycanthaceae were one step less parsimonious. Here we follow the result of Doyle et al. (2008) because it is based on a data set that included far more characters than Crepet et al. (2005), many derived from in-depth analyses of gynoecial morphology (e.g., Igersheim and Endress, 1997). Both most parsimonious positions imply that *Virginianthus* provides a minimum age for the crown node of Laurales (Fig. 1).

Minimum Age: middle-late Albian boundary, 107.7 Ma

Age Justification: The fossil flower described by Friis et al. (1994) comes from the Potomac Group at the Puddledock locality in the Tarmac Lone Star Industries sand and gravel pit 9 km southwest of Hopewell, Prince George County, Virginia, USA. Friis et al. (1994, 1995) and von Balthazar et al. (2011) considered this locality early or middle Albian, based on palynological correlation by R. A. Christopher (in Dischinger, 1987) with the basal part of Potomac Subzone II-B of Brenner (1963) and Doyle and Robbins (1977) and the suggestion of Doyle (1992) that Subzone II-B may begin in the early Albian. However, an early Albian age for Subzone II-B (and II-A) now appears unlikely in light of palynological correlations by Hochuli et al. (2006) with the well-dated marine Lower Cretaceous of Portugal and earlier work of Kemp (1970) on the marine Albian of England (cf. Doyle et al., 2008). These studies support correlation of upper Zone I with the basal early Albian of Portugal and the early Albian of England, based on the appearance in all these intervals of reticulate tricolpate pollen and *Clavatipollenites rotundus* (aff. *Retimonocolpites dividuus* of Doyle and Robbins, 1977), as argued by Doyle and Robbins (1977), but not striate tricolpates, which appear later in the early Albian of Portugal (Hochuli et al., 2006). Consistent with this, the Zone II index spore species *Apiculatisporis babsae* of Brenner (1963) appears at the base of the middle Albian in England (Kemp 1970). The conclusion of Doyle (1992) that the Zone I/II boundary lies well down in the Aptian was based largely on comparisons with *Pennipollis* (*Brenneripollis*) species and *Schrankipollis* in Africa that appear to have involved too indirect correlations and incompletely controlled species ranges, as argued by Hochuli et al. (2006).

Clarke et al. (2011) proposed a much younger minimum age for Puddledock, 92.7 Ma, or the top of the Cenomanian, based on the suggestion of Hochuli et al. (2006) that Zone II extends into the Cenomanian and the presence of late Cenomanian ammonites in the next younger unit, the Raritan Formation of New Jersey (Cobban and Kennedy, 1990). Hochuli et al. (2006) argued convincingly that there is a significant break between Zones I and II in the Potomac sequence, since the early to middle Albian interval in Portugal shows continuing higher diversity of angiosperm monosulcates than tricolpates, whereas tricolpates are already more diverse at the base of Zone II. They argued that Subzone II-B is late rather than middle Albian, based on the higher diversity of tricolpates than in the Portuguese middle Albian and

the presence through Subzone II-B of the smooth tricolpate species *Cupuliferoidapollenites* (*Tricolpopollenites*) *parvulus*, which they noted has not been reported in dated sequences until the late Albian. However, the reliability of *C. parvulus* is uncertain, since this species is rare in Subzone II-B and easy to overlook (for example, it was not reported by Brenner, 1963). Furthermore, its first occurrences cited by Hochuli et al. (2006) are in Canada, in the Northern Laurasia province of Brenner (1976), where angiosperms were less abundant than in Southern Laurasia, and in deep sea cores. It is also possible that the higher diversity of angiosperms observed in the Potomac is partly a facies effect of comparing continental and marginal marine sequences. If angiosperms were locally dominant in some lowland habitats but subordinate to ferns and gymnosperms at the regional scale (cf. Pierce, 1961; Doyle and Hickey, 1976), more angiosperms (including rare species) might be detected in a fluvial sequence such as the Potomac Group than in marine deposits like those in Portugal, where they would be diluted by the higher regional production of fern spores and gymnosperm pollen. It is also likely that the contrast between diversity curves from the two sequences is exaggerated by the fact that the Portuguese curves were based on number of species per sample, whereas the Potomac curves were based on a range chart (Doyle and Robbins, 1977), so that species whose ranges pass through the horizon of a given sample but were not found in that sample were treated as present.

More positive evidence that much of Subzone II-B is middle Albian comes from palynological correlations with well-dated sequences in the US Gulf Coast and Western Interior, which were not considered by Hochuli et al. (2006). Doyle (1977) showed that the diverse angiosperm flora in the middle of Subzone II-B is especially similar at the species level to that described by Hedlund and Norris (1968) in the “Walnut” Clay and Antlers Sand (Fredericksburgian) of Oklahoma, which lies below the middle-late Albian boundary defined by ammonites in the overlying Goodland Limestone (Hedlund and Norris, 1968; Mancini and Puckett, 2005). Doyle and Robbins (1977) dated Subzone II-C as latest Albian, but Hochuli et al. (2006) argued that it is Cenomanian, based on the psilate tricolporate species *Tricolporoidites* (*Tricolporopollenites*) *distinctus* and *Tricolporoidites* (*Tricolporopollenites*) *triangulus*, which they stated first appear in the Cenomanian. However, most of the studies that Hochuli et al. (2006) cited considered only Cenomanian beds, not the latest Albian.

More important, Ludvigson et al. (2010) listed psilate tricolporates (as *Psilatricolporites* sp.) in the latest Albian (Palynostratigraphic Unit 2) of the Dakota Formation (see discussion of *Archaeanthus*, Fossil 3), and Laing (1975) recorded triangular grains similar to *T. triangulus* as *Psilatricolpites rectilatibus* in the marine upper Albian of France (Laing distinguished *P. rectilatibus* from *T. triangulus* on lack of pores, but the bent shape of the colpi in the Cenomanian grain illustrated in his pl. 90, figs. 11-12 suggests that rudimentary pores were present). Together, these correlations lead us to consider that *Virginianthus* is of middle Albian age. Therefore, we use the middle-late Albian boundary, 107.7 Ma (Ogg and Hinnov, 2012), as a minimum age for *Virginianthus*.

Previous Use as Calibration: *Virginianthus* has been used in several large-scale studies (Magallón and Sanderson, 2005; Moore et al., 2007; Soltis et al., 2008; Bell et al., 2010). With the exception of Magallón and Sanderson (2005), who used this fossil to calibrate the crown node of Laurales with a minimum age of 110 Ma, all these analyses used it to calibrate the most recent common ancestor of Laurales and Magnoliales (i.e., the stem node of Laurales). Moore et al. (2007) used it to define a minimum age of 113 Ma, Soltis et al. (2008) an age fixed between 98 and 113 Ma, and Bell et al. (2010) a minimum age of 98 Ma. In order to date divergence times within Calycanthaceae, Zhou et al. (2006) used this species to calibrate the age of the stem node of the family as at least 112 Ma.

Fossil Taxon 5 (additional): *Lovellea wintonensis* Dettmann, Clifford and Peters, 2009

Node Calibrated: crown-group Laurales (Dettmann et al., 2009)

Reference Specimen: QMF51133 in the Palaeontological Collection of the Queensland Museum, Queensland, Australia (Holotype, originally a complete specimen, now consisting of portions of a permineralized (silicified) flower/fruit in rock matrix cut longitudinally into two slices and two thin sections: QMF51133 a-d).

Additional Specimens: QMF51134, QMF51135, QMF51132. Other specimens used for the description (flowers/fruits).

Phylogenetic Justification: A morphological parsimony analysis using the matrix of Doyle and Endress (2000), with the exclusion of several taxa (eudicots, Piperales, Nymphaeales, monocots, *Austrobaileya*, Schisandraceae, and *Illicium*), placed *Lovellea wintonensis* in one most parsimonious position sister to all Laurales excluding Calycanthaceae (Dettmann et al., 2009). This “core Laurales” clade was well supported in previous studies (Soltis et al., 1999, 2000a, 2000b, 2007, 2011; Qiu et al., 1999, 2000, 2005, 2006, 2010; Renner, 1999, 2004; Doyle and Endress, 2000; Savolainen et al., 2000; Zanis et al., 2002, 2003; Nickrent et al., 2002; Hilu et al., 2003). Relationships within the clade based on the morphological analysis were not identical to those found in molecular or combined morphological and molecular analyses (Doyle and Endress, 2000), but they are consistent in supporting the monophyly of the Hernandiaceae-Lauraceae-Monimiaceae clade (though with the addition of Siparunaceae) and the position of Atherospermataceae and Gomortegaceae as outgroups to this clade (though as two successive branches rather than a clade). We consider *Lovellea wintonensis* to provide a minimum age for crown-group Laurales, or the stem node of all Laurales except Calycanthaceae (Fig. 1).

Minimum Age: Albian-Cenomanian boundary, 100.5 Ma

Age Justification: *Lovellea wintonensis* comes from the basal part of the the Winton Formation, 48 km WNW of Winton, western Queensland, Australia (Dettmann et al., 2009). Dettmann et al. (2009) placed the sediments containing these fossils in the *Coptospora paradoxa* or *Phimopollenites pannosus* spore-pollen Zones of Helby et al. (1987) based on the co-occurrence of *Cicatricosisporites*, *Crybelosporites*, *Clavatipollenites*, and *Phimopollenites*, indicating that they are no older than middle Albian. Because the Winton Formation overlies the late Albian Mackunda Formation but no palynomorph taxa indicative of a Cenomanian or younger age are present, Dettmann et al. (2009) suggested a latest Albian age. Here we accept this age for *Lovellea wintonensis* and therefore use the upper boundary of the Albian, 100.5 Ma (Ogg and Hinnov, 2012) as a safe minimum age for this fossil.

Previous Use as Calibration: None to our knowledge.

Node 3: crown-group Calycanthoideae

Fossil Taxon 6: *Jerseyanthus calycanthoides* Crepet, Nixon and Gandolfo, 2005

Node Calibrated: crown-group Calycanthoideae (Crepet et al., 2005)

Reference Specimen: CUPC 1483 in the Paleobotany Collection of the L.H. Bailey Hortorium, Cornell University (holotype / flower).

Additional Specimens: CUPC 1484–1502. Paratypes (flowers).

Phylogenetic Justification: Using the combined morphological and molecular data set described for *Virginianthus* (Fossil 4), in which Calycanthaceae were represented by *Idiospermum*, *Chimonanthus*, and *Calycanthus*, Crepet et al. (2005) found one most parsimonious position for *Jerseyanthus calycanthoides*, as the sister group of *Calycanthus*. Addition of the fossil *Virginianthus calycanthoides* did not influence the position of *Jerseyanthus*. The relationships among the three extant genera of Calycanthaceae are well supported in the literature, with *Idiospermum* sister to *Chimonanthus* and *Calycanthus* (Renner, 1998, 1999; Zhou et al., 2006; Massoni et al., 2014). *Jerseyanthus calycanthoides* therefore provides a minimum age for crown-group Calycanthoideae, the clade that is sister to *Idiospermum* and contains *Chimonanthus* and *Calycanthus* (Fig. 1).

Minimum Age: Coniacian-Santonian boundary, 86.3 Ma

Age Justification: These fossils were collected from the South Amboy Fire Clay Member of the Raritan Formation at the Old Crossman clay pit in Sayreville, New Jersey, USA (Crepet et al., 2005). This unit was first studied palynologically by Groot et al. (1961), who considered it Turonian based on preliminary studies on European sequences, and subsequently by Doyle (1969b), Wolfe and Pakiser (1971), Doyle and Robbins (1977), and Christopher (1979). Building on the palynological zonation of the Potomac Group by Brenner (1963), to which Doyle (1969a) added Zone III (uppermost Potomac) and Zone IV (lower Raritan), Sirkin (1974) assigned South Amboy palynofloras to a new Zone V. This unit was renamed the *Complexiopollis exigua-Santalacites minor* Zone by Christopher (1979) and redefined by

Christopher et al. (1999) as the lowest of three subzones of the *Sohlipollis* Taxon Range Zone. Wolfe and Pakiser (1971) and Sirkin (1974) considered the South Amboy late Cenomanian, not much younger than underlying Woodbridge Clay Member (Zone IV), but Doyle (1969b) and Doyle and Robbins (1977) argued that it is no older than middle Turonian, based on the presence of Normapolles genera that appear at that level in Europe (Góczán et al., 1967). Doyle and Robbins (1977) and Christopher (1979) allowed that it was “possibly Coniacian,” but Crepet and Nixon (1994) and Crepet et al. (2005) accepted a late Turonian age. By contrast, Clarke et al. (2011) suggested a minimum age of the Santonian-Campanian boundary, 82.8 Ma. However, correlations by Christopher et al. (1999) and Christopher and Prowell (2010) with better-dated rocks in South Carolina imply that the Crossman locality is not this young; they correlate the *C. exigua*-*S. minor* Zone with calcareous nannofossil zones CC13 and CC14, which extend from late Turonian through Coniacian (Burnett, 1998; Ogg and Hinnov, 2012). We therefore believe there is enough evidence to consider that *Jerseyanthus* was at least of Coniacian age, which translates into a conservative minimum age of 86.3 Ma, the Coniacian-Santonian boundary (Ogg and Hinnov, 2012).

Previous Use as Calibration: None to our knowledge.

Node 4: crown-group core Laurales

Fossil Taxon 7 (preferred, given current knowledge): *Cohongarootonia hispida* von Balthazar, Crane, Pedersen and Friis, 2011

Node Calibrated: crown-group core Laurales (the clade consisting of Laurales except Calycanthaceae) (von Balthazar et al., 2011)

Reference Specimen: PP53716 in the Field Museum of Natural History, Chicago (holotype, flower).

Phylogenetic Justification: A molecular scaffold analysis by von Balthazar et al. (2011), using the morphological data set from Doyle and Endress (2010) and one of the same backbone trees, in which Lauraceae and Hernandiaceae form a clade sister to Monimiaceae, placed *Cohongarootonia hispida* in a single most parsimonious position as the sister group of

Lauraceae + Hernandiaceae. Synapomorphies of the three taxa were whorled tepals, whorled stamens, and one carpel. As a result, von Balthazar et al. (2011) unequivocally assigned the fossil to the order Laurales. However, although all recent analyses agree that Lauraceae, Hernandiaceae, and Monimiaceae form a well-supported clade within Laurales, the relationships among these three families are still debated. In analyses by Doyle and Endress (2000), a sister group relationship of Lauraceae and Hernandiaceae was strongly supported by morphological data and by combined morphological and molecular data, but analyses of molecular data alone have linked either Monimiaceae and Lauraceae or Monimiaceae and Hernandiaceae (Qiu et al., 1999, 2000, 2005, 2006, 2010; Renner, 1999; Doyle and Endress, 2000; Savolainen et al., 2000; Renner and Chanderbali, 2000; Hilu et al., 2003; Zanis et al., 2003; Soltis et al., 2011; Massoni et al., 2014). Until this conflict is resolved, we consider that *Cohongarootonia hispida* provides a minimum age for the stem node of the clade including Lauraceae, Monimiaceae, and Hernandiaceae, in other words the crown node of the clade of Laurales excluding Calycanthaceae (Fig. 1).

Minimum Age: middle-late Albian boundary, 107.7 Ma.

Age Justification: *Cohongarootonia* was collected from the Potomac Group at the same Puddledock locality, 9 km southwest of Hopewell, Virginia, as *Virginianthus calycanthoides* (Fossil 4). As discussed for that species, this locality has been correlated palynologically by R. A. Christopher (in Dischinger, 1987) with the lower part of Subzone II-B of Brenner (1963), which we argue is of middle Albian age. Therefore, we use the middle-late Albian boundary, 107.7 Ma (Ogg and Hinnov, 2012), as a minimum age for *Cohongarootonia*.

Previous Use as Calibration: None to our knowledge.

Fossil Taxon 8 (additional): *Mauldinia mirabilis* Drinnan, Crane, Friis and Pedersen, 1990

Node Calibrated: crown-group core Laurales (the clade consisting of Laurales except Calycanthaceae) (Doyle and Endress, 2010)

Reference Specimen: PP35297 in Field Museum of Natural History, Chicago, USA (holotype of *Mauldinia mirabilis*; inflorescence).

Additional Specimens: PP34733, PP34794, PP34796, PP34797, PP35002-PP35006, PP35008, PP35056, PP35061, PP35141, PP35295-PP35305, PP35338-PP35340. Other specimens of *Mauldinia mirabilis* cited in Drinnan et al. (1990) (inflorescence fragments with flowers).

PP34709-PP34715, PP34728-PP34732, PP34779, PP34780, PP34926, PP34927, PP34929, PP35007, PP35016-PP35019, PP35051-PP35055, PP35057-PP35060, PP35140, PP35150, PP35151, PP35306-PP35309, PP35315-PP35319. Other specimens of *Mauldinia mirabilis* cited in Drinnan et al. (1990) (flowers).

PP34903-PP34925, PP35009, PP35050, PP35144. Other specimens of *Mauldinia mirabilis* cited in Drinnan et al. (1990) (dispersed stamens).

PP34781-PP34783, PP34795, PP34928, PP34930, PP34931, PP35010-PP35012, PP35142, PP35143. Other specimens of *Mauldinia mirabilis* cited in Drinnan et al. (1990) (dispersed carpels).

PP34932, PP34933, PP35024, PP35025, PP35026, PP42982, PP42983. Other specimens of *Mauldinia mirabilis* cited in Drinnan et al. (1990) (cuticle preparations).

PP42981. Other specimens of *Mauldinia mirabilis* cited in Drinnan et al. (1990) (unsorted fragments).

PP35023 (holotype of *Paraphyllanthoxylon marylandense* Herendeen, 1991; mature wood).

PP43591, PP43592, PP43617, PP43619, PP43620, PP43621, PP43622, PP43624, PP43625, PP43627, PP43629, PP43630, PP43631, PP43632, PP43636. Paratypes of *Paraphyllanthoxylon marylandense* Herendeen, 1991 (mature wood).

Phylogenetic Justification: Because of identical features in the first formed wood of *Paraphyllanthoxylon marylandense* (Herendeen 1991) and inflorescence axes of *Mauldinia mirabilis* (Drinnan et al., 1990), Doyle and Endress (2010) combined these two taxa in their analyses. Their molecular scaffold analysis, which used a backbone tree in which Lauraceae and Hernandiaceae formed a clade sister to Monimiaceae, placed this fossil in a single most parsimonious position, as the sister group of Lauraceae + Hernandiaceae. The three taxa

were united by the following unequivocal synapomorphies: solitary vessels, inflorescences with lateral cymes, whorled tepals, whorled stamens, and one carpel. The basal position of *Mauldinia* relative to the two living taxa was supported by the absence of well-developed paratracheal parenchyma in the wood, the superior position of the ovary, and the presence of endosperm in the seed, while Lauraceae and Hernandiaceae are united by paratracheal parenchyma, an inferior ovary (reversed within Lauraceae: Rohwer and Rudolph, 2005), and lack of endosperm in the mature seed. A position sister to Lauraceae alone was four steps less parsimonious. However, as discussed for *Cohongarootonia* (Fossil 7), Lauraceae and Hernandiaceae are included together with Monimiaceae in a well-supported clade, but different analyses have found all possible relationships among the three families. When Doyle and Endress (2010) used a molecular backbone in which Monimiaceae were sister to Lauraceae, the single most parsimonious position of *Mauldinia* was sister to the whole clade of Hernandiaceae + Lauraceae + Monimiaceae. Doyle and Endress (2010) did not test the third alternative present in the literature (Lauraceae sister to the remaining two families), but using their data set we find that the most parsimonious position for *Mauldinia* under this arrangement is also sister to the three living taxa. Until this conflict is resolved, we consider *Mauldinia mirabilis* to provide a minimum age for the stem node of the clade of Lauraceae, Hernandiaceae, and Monimiaceae, in other words the crown node of the clade of Laurales excluding Calycanthaceae (Fig. 1).

Minimum Age: middle-late Cenomanian boundary, 95.5 Ma

Age Justification: *Mauldinia mirabilis* and *Paraphyllanthoxylon marylandense* were described from the Mauldin Mountain locality in the upper Potomac Group (“Maryland Raritan”) of northeastern Maryland, USA (Drinnan et al., 1990; Herendeen, 1991). These beds contain a palynoflora assigned to the lower part of Zone III of the Potomac sequence, which Doyle and Robbins (1977) dated as early Cenomanian. The age of Zone III is bracketed above by the appearance of triporate Normapolles pollen (*Complexiopollis* spp.) in the lower Raritan Formation of New Jersey (Zone IV) and the upper part of the Peruc Formation of Bohemia (Pačtová, 1971; Doyle and Robbins, 1977), and by late Cenomanian ammonites in the lower Raritan (Cobban and Kennedy, 1990). The Peruc Formation underlies marine sediments with late Cenomanian mollusks, and its upper part was correlated palynologically

by Pacltová (1977) with late middle Cenomanian marine beds that contain the first Normapolles in England and France (Azéma et al., 1972; Laing, 1975); this agrees with studies of sequence stratigraphy in the Bohemian section by Uličný et al. (1997). The probable latest Albian age of Potomac Subzone II-C is discussed under *Virginanthus*. Because these data imply that the Zone III-IV boundary may lie within the middle Cenomanian, and the length of time between the base and top of Zone III is uncertain, it appears safest to conclude that *Mauldinia* could be of either early or middle Cenomanian age. Therefore, we propose the middle-late Cenomanian boundary, 95.5 Ma (Ogg and Hinnov, 2012), as a conservative minimum age for *Mauldinia*.

Previous Use as Calibration: None to our knowledge.

Node 5: crown-group Canellales

Fossil Taxon 9: *Walkeripollis gabonensis* Doyle, Hottin and Ward, 1990

Node Calibrated: crown-group Canellales (Doyle and Endress, 2010)

Reference Specimen: Single-pollen grain preparation 2963-27 (holotype of *Walkeripollis gabonensis*). Doyle et al. (1990) stated that this specimen is deposited in the Elf-Aquitaine collection, but it is on loan to J. A. Doyle at the University of California, Davis. Because the company Elf-Aquitaine no longer exists, it will be deposited at the University of California (Berkeley) Museum of Paleontology (UCMP).

Additional Specimens: Sections, uncut block, and negatives, from Elf-Aquitaine preparation 2963, TM.1 (N'Toum No. 1) well, core 8, 939-944 m, Subzone C-VIIc, Gabon.

Phylogenetic Justification: Use of fossil pollen for calibration can be questioned because it usually lacks sufficient characters for secure phylogenetic placement, but this taxon has such a unique combination of features that it could be unambiguously placed in a large-scale phylogenetic analysis. A molecular scaffold analysis placed *Walkeripollis gabonensis* in a single most parsimonious position as sister to Winteraceae (Doyle and Endress, 2010), one of the two families of Canellales. Synapomorphies supporting this sister-group relationship

were permanent tetrads and round aperture shape. The sculpture on the pore, forming a ring around a central thin area, also suggests a close relationship between the fossil species and Winteraceae (Doyle and Endress, 2010). However, this character was not included in the Doyle and Endress (2010) data set because it was not applicable to most taxa. The sister-group relationship between living Winteraceae and Canellaceae is very well supported in the literature (e.g., Chase et al., 1993; Zanis et al., 2002, 2003; Qiu et al., 2005, 2006; Marquinez et al., 2009). We therefore consider *Walkeripollis gabonensis* to provide a minimum age for crown-group Canellales (Fig. 1)

Minimum Age: Barremian-Aptian boundary, 126.3 Ma

Age Justification: *Walkeripollis gabonensis* comes from the upper part of Elf-Aquitaine palynological Zone C-VII (Subzone C-VIIc) in the Cocobeach sequence (Doyle et al. 1990), near the town N'Toum in northern Gabon. The age of Zone C-VII is bracketed by late Aptian marine fossils in overlying units (Doyle et al., 1977, 1990). Doyle et al. (1977, 1982) dated Zone C-VII as early Aptian, but Doyle et al. (1990) and Doyle (1992) suggested it may be late Barremian, based on the occurrence of other taxa that appear in Zone C-VII, correlative rocks in Brazil, and better-dated Barremian rocks elsewhere, notably *Afropollis* and the first reticulate tricolpates (Doyle et al., 1982; Gübeli et al., 1984; Penny, 1989; Regali and Viana, 1989; Doyle, 1992). Additional evidence that favors a pre-Aptian age is the absence in Zone C-VII of two groups that appear in the overlying Zones C-VIII and C-IX and the Aptian of Egypt, namely striate tricolpates, which are not known until the Albian in Southern Laurasia but occur earlier in Northern Gondwana (Penny, 1988a; Hochuli et al., 2006; Heimhofer et al., 2007; Heimhofer and Hochuli, 2010), and the non-columellar reticulate monosulcate genus *Pennipollis* ("*Retimonocolpites*" *peroreticulatus*, etc.), which appears just above the base of the marine Aptian of England and has never been reported from well-dated pre-Aptian rocks (Penny, 1988b; Doyle, 1992; Hughes, 1994; Hochuli et al., 2006). We therefore believe it is safe to accept a late Barremian age for *Walkeripollis gabonensis* and thus propose 126.3 Ma, the upper boundary of the Barremian (Ogg and Hinnov, 2012), as a minimum age for this fossil.

Previous Use as Calibration: Magallón (2010) and Magallón et al. (2013) used *Walkeripollis* to provide a minimum age of 125 Ma for the same node recommended here (crown-group Canellales). Marquínez et al. (2009) used this fossil to fix the age of the crown node of Winteraceae at 120 Ma.

Node 6: stem node of extant *Saururus*

Fossil Taxon 10: *Saururus tuckerae* Smith and Stockey, 2007

Node Calibrated: stem node of extant *Saururus* (Smith and Stockey, 2007)

Reference Specimen: P1631 Bbot a in the University of Alberta (Edmonton) Paleobotanical Collections (UAPC) (holotype; inflorescence).

Additional Specimens: P1631 Btop a, Btop b, Btop f, Btop h, Bbot c, Cbot e; P5831 Bbot; P5839 A; P5937 Gbot b; P5991 B. Paratypes (isolated flowers).

Phylogenetic Justification: A morphological parsimony analysis using 24 morphological characters modified from matrices of Tucker et al. (1993), Tucker and Douglas (1996), and Meng et al. (2003), placed *Saururus tuckerae* in a single most parsimonious position within the family Saururaceae, as the sister group of a clade formed by the two extant species of *Saururus* (Smith and Stockey, 2007). The relationship of the fossil with extant *Saururus*, one of four genera in Saururaceae, was supported by the following synapomorphies: basally connate carpels, 1-2 ovules per carpel, and marginal placentation (Smith and Stockey, 2007). This study indicated that the genus *Saururus* was sister to *Gymnotheca*, and *Anemopsis* was sister to *Houttuynia*, relationships supported by other molecular and morphological studies (Meng et al., 2002, 2003; Jaramillo et al., 2004; Neinhuis et al., 2005; Wanke et al., 2007b; Massoni et al., 2014). Outside the Saururaceae, the relationships are compatible with molecular studies (e.g. Qiu et al., 2005, 2006; Soltis et al., 1999, 2000a, 2000b, 2007, 2011; Mathews and Donoghue, 1999, 2000; Qiu et al., 1999, 2000; Doyle and Endress, 2000; Savolainen et al., 2000; Zanis et al., 2002, 2003; Borsch et al., 2003; Hilu et al., 2003; Kelly and González, 2003; Jaramillo et al., 2004; Wanke et al., 2007a, 2007b; Massoni et al., 2014).

We thus consider *Saururus tuckerae* to provide a minimum age for the stem node of the extant genus *Saururus*, which is also the crown node of *Gymnotheca* + *Saururus* (Fig. 1).

Minimum Age: 45 Ma

Age Justification: Fossils described by Smith and Stockey (2007) come from the Princeton Chert, 8.4 km south of Princeton, British Columbia, Canada, which is part of the Princeton Group, Allenby Formation (Boneham, 1968). The paleontological record supports a middle Eocene age, such as an amiid fish correlated with the occurrence of comparable fossils in British Columbia and in the Klondike Mountain Formation of Washington State (Wilson, 1982), and teeth of the mammal group Tillodontia (Russell, 1935). In addition, potassium-argon dating studies have provided comparable ages for the Allenby Formation: 48 ± 2 Ma (Rouse and Mathews, 1961; Mathews, 1964) and 48 ± 3 and 51 ± 3 Ma (Hills and Baadsgaard, 1967). Finally, Smith and Stockey (2007) report a personal communication from H. Baadsgaard (University of Alberta, 1999) that supports an age of 48.7 Ma for the ash of Layer #22. We consider here that *Saururus tuckerae* provides a safe minimum age of 45 Ma (the youngest age given by potassium-argon dating taking the error of 3 Ma into account).

Previous Use of this Fossil: González et al. (2014) used *Saururus tuckerae* to constrain the same node as recommended here, but with a minimum age of 48.5 Ma.

Discussion

The 10 fossils reviewed in this study provide minimum age constraints on six internal nodes in the phylogeny of Magnoliidae (Table 1, Figure 1). Considered together, one of these age constraints (the crown node of Laurales) is uninformative because it is implied by an equal minimum age constraint nested higher in the tree. Six of these fossils have not yet been used to provide calibration points in any molecular dating studies. The four remaining have been used in several previous studies, often with different ages and to calibrate different nodes (see “Previous Use as Calibration” sections). Several other fossils have been used in the literature to set minimum age calibrations, some of which are different from those recommended here. In the majority of these papers the justification for a specific age and position used in association with these fossils was not provided. The present review is

intended to be a reliable source of information for readers about the bases underlying the use of these calibration points.

The positions of *Archaeanthus linnenbergeri*, *Endressinia brasiliana*, *Virginianthus calycanthoides*, *Mauldinia mirabilis*, *Cohongarootonia hispida*, and *Walkeripollis gabonensis* have been evaluated using similar backbone trees and either the data set of Endress and Doyle (2009) or the data set of Doyle and Endress (2010) (see fossil sections 1, 3, 4, 7, 8, and 9 for details). These studies investigated the position of the fossils in a broad context of basal angiosperms compatible with current knowledge of angiosperm phylogeny, and using a compromise between ‘exemplar’ and ‘compartmentalized’ approaches to taxon representation. When a supra-specific group did not have a homogeneous morphology, the authors of these studies filled the matrix with ancestral states deduced from knowledge of basal relationships in the taxon supported by other studies. Relationships within the compartments of Magnoliidae used by Endress and Doyle (2009) and Doyle and Endress (2010) to deduce plesiomorphic traits of these supra-specific terminals have not been contradicted by subsequent studies focused on particular families (Marquínez et al., 2009; Michalak et al., 2010; Renner et al., 2010; Chatrou et al., 2012). For all these reasons, we argue that the positions of the fossils supported in these studies are up-to-date, and can be used with some confidence. Regarding other calibration points, the phylogenetic analyses we have used as a reference for fossil relationships in this paper represent solid advances. However, future analyses with denser taxon sampling and updated morphological and molecular data sets will be required to challenge further, and hopefully confirm, the relationships summarized in this paper. For instance, the analysis of *Saururus tuckerae* by Smith and Stockey (2007) could be improved by adding key taxa of Piperales not included in their analysis (Hydnoraceae, *Thottea*, *Saruma*, *Manekia*, and *Verhuellia*).

All the fossil species incorporated in our calibration scheme presented the advantage of having enough informative characters to allow accurate phylogenetic placement. However, because such fossils are not well represented in the fossil record, the delay between the time of divergence of the taxon and the fossilization event of the specimen used in the present study is difficult to estimate. Bayesian relaxed clock methods allow calibrations to be modeled with parametric distributions (uniform, exponential, lognormal, and gamma priors).

These various priors are often used to model the probability that the node calibrated is older than the fossil used. However, given the difficulty in objectively setting parameters for these models in many empirical situations, as well as the phylogenetic uncertainty often associated with fossil relationships (leading to conservative calibration of the lowest safe node on the tree), Sauquet et al. (2012) recommended the use of uniform priors. For the present calibration scheme we agree with this point of view and recommend that our age constraints be used as strict minimum ages only. This issue, resulting from the sporadic preservation of extinct species in the fossil record, is exacerbated by the fact that different events of fossilization do not necessarily conserve the same part of the plant. Fortunately, in some cases, such as *Archaeanthus linnenbergeri*, morphological features have allowed safe identification of separate organs as parts of the same plant species. The association of different parts could lead to an older age associated with a fossil taxon. For instance, it is likely that *Mauldinia* or related plants extend down below the Albian-Cenomanian boundary, as leaves described as *Pabiania* from the latest Albian Rose Creek locality in the Dakota Formation of Nebraska (Upchurch and Dilcher, 1990; Gröcke et al., 2006) may represent plants like those that produced fossils described as *Prisca* by Retallack and Dilcher (1981) from the Hoisington and Linnenberger Ranch localities in Kansas (see *Archaeanthus*), which are probably inflorescences of *Mauldinia* (Drinnan et al., 1990). In other cases, there are slightly younger fossils that are even more like the putative extant relatives of the fossils used here. This is true for *Walkeripollis*, where tetrads with similar aperture structure but more open reticulate sculpture, approaching modern Winteraceae, are known from Aptian-Albian beds of Israel (Walker et al., 1983; Schrank, 2013) and the late Albian-Cenomanian of Argentina (Barreda and Archangelsky, 2006).

In addition to the 10 fossils listed here many more fossil taxa have been described as belonging to Magnoliidae, and a number of them could complement the current calibration set, pending further work on their phylogenetic relationships. There are species that are or bear flowers preserved in the form of compressions (e.g., Mohr and Eklund, 2003; Frumin et al., 2004), in three dimensions as charcoal (e.g., Kvaček and Eklund, 2003b; Viehofen et al., 2008), or in amber (e.g., Poinar and Chambers, 2005). In addition to fossil flowers, there are fossil woods (e.g., Poole and Gottwald, 2001) and leaves (e.g., Rüffle and Knappe, 1988).

Finally, pollen grains, seeds, and fruits referred to the group are also common in the fossil record (e.g., Friis, 1985; Carpenter et al., 1994; Friis et al., 1995). Among these extinct species several could be very useful to supplement our set of calibration points; those based on isolated organs may have too few characters for unambiguous placement, but the situation might improve with better understanding of character distributions in the living flora or association with other organs. In our scheme, the Piperales have only one minimum age constraint, in contrast to several for Laurales and Magnoliales. For Aristolochiaceae there are several fossil leaf and wood taxa described from the Late Cretaceous (e.g., Kulkarni and Patil, 1977) and the Cenozoic (e.g., MacGinitie, 1974), which if confirmed could provide a minimum age of Late Cretaceous for the Piperales. *Lactoripollenites africanus*, a fossil pollen type of monoporate tetrads from Turonian-Campanian sediments of the southern coast of southern Africa, was associated with the monotypic extant genus *Lactoris* by Zavada and Benson (1987). This fossil could also support the origin of the Aristolochiaceae in the Late Cretaceous. The 10 fossils reviewed in the present paper are generally close to the first reports of their taxonomic groups in the fossil record. However, *Saururus nipponensis* (Stopes and Fujii, 1911), interpreted as a fossilized stem of Saururaceae, from the Upper Cretaceous of Hokkaido (Japan), could provide much an older minimum age for the stem node of Saururaceae than the one used in the present review. Several fossils containing pollen grains in situ and sometimes branching axes bearing floral organs and leaves (e.g., Crepet and Nixon, 1998; Mohr and Eklund, 2003) would be good candidates for accurate phylogenetic placement. The latter cases are very interesting because they provide characters from different parts of the plant, without the uncertainty of association of two separate structures found in the same fossil bed. Several other fossils that are exceptionally well preserved could provide enough characters for an accurate phylogenetic placement within families. For instance, *Lauranthus futabensis* is a complete flower described from the lower Coniacian of the Futaba Group in northeastern Japan that has been interpreted as a member of Lauraceae (Takahashi et al., 2001).

Our new set of calibration points is a first step toward investigating the time scale of evolution of Magnoliidae as a whole more accurately than has been done before. Previous molecular dating analyses of Magnoliidae have been carried out either at an intra-ordinal

level (e.g., Chanderbali et al., 2001; Doyle et al., 2004; Pirie et al., 2006; Smith et al., 2008; Marquínez et al., 2009; Pirie and Doyle, 2012) or at the level of angiosperms and higher (e.g., Bell et al., 2005, 2010; Moore et al., 2007; Soltis et al., 2008; Magallón and Castillo, 2009; Magallón, 2010; Smith et al., 2010). A maximum of six calibration points have been used so far within the group (Magallón and Castillo, 2009). In terms of taxonomic sampling, the most complete molecular dating studies including Magnoliidae as a whole incorporated about 11 percent of the generic diversity of Magnoliidae (Wikström et al., 2001; Moore et al., 2007; Soltis et al., 2008). Previous dating analyses have provided dates for the origin of the Magnoliidae ranging from the Early Jurassic to the Early Cretaceous (Wikström et al., 2001; Bell et al., 2005, 2010; Moore et al., 2007, 2010; Soltis et al., 2008; Magallón and Castillo, 2009; Magallón, 2010; Smith et al., 2010). Among subgroups of Magnoliidae, several published ages are not compatible with minimum calibration points provided here. For instance, Bell et al. (2010) found younger ages for the diversification of Canellales (50-111 Ma) and Magnoliales (50-96 Ma), which are dated as at least 126.3 Ma and 113 Ma, respectively, by the present calibration scheme. A new molecular dating study of Magnoliidae, using this calibration scheme and denser taxonomic sampling, will certainly provide new insights on the tempo of the evolutionary history of this important group of angiosperms. In the future, a new morphological data set capturing the whole diversity of Magnoliidae would be very useful for filling in and improving the present calibration scheme. Such a data set, which we are currently assembling, will allow us to test and refine the phylogenetic placements of the fossils presented here and to evaluate the phylogenetic position of other described fossils.

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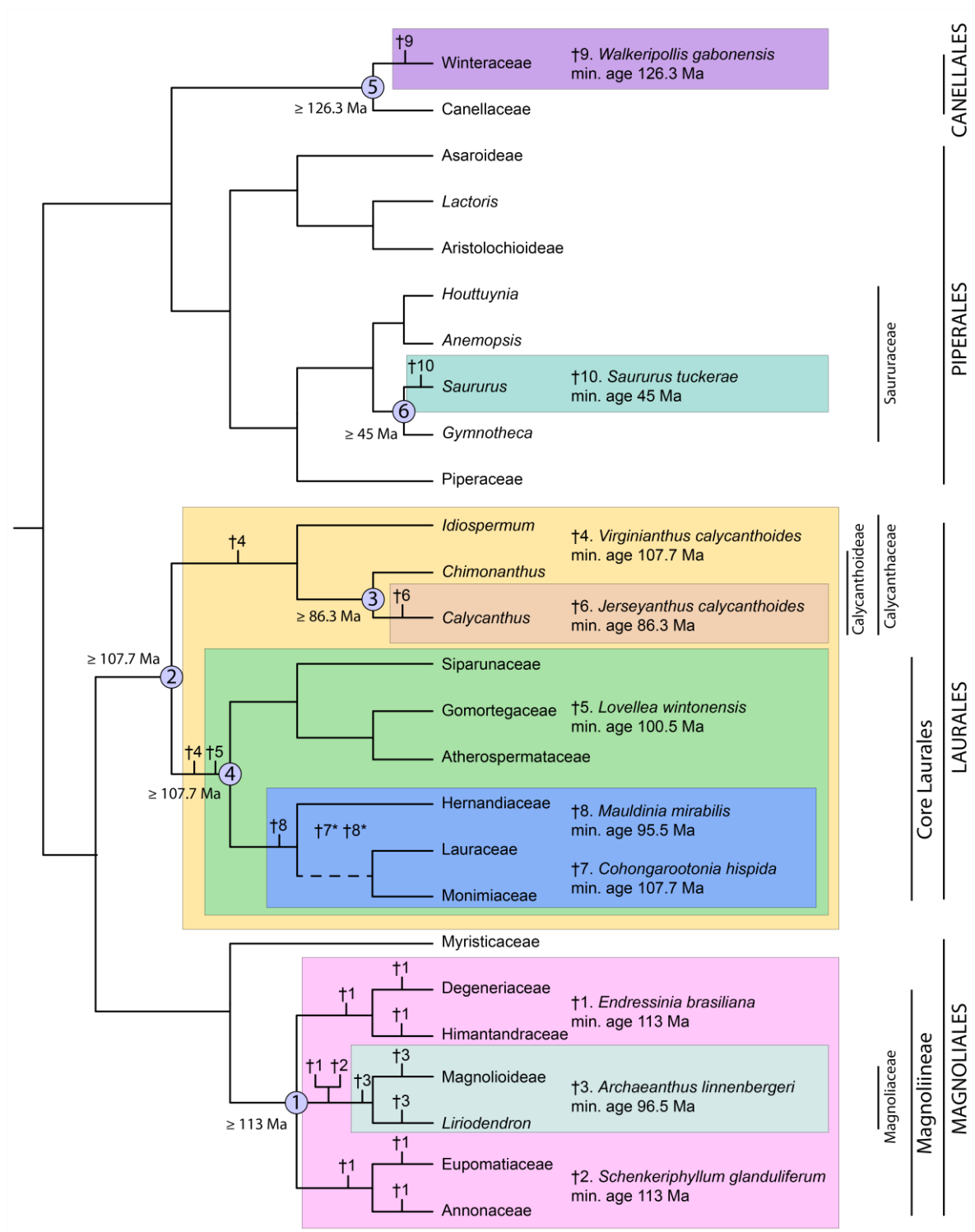
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Figure



(Continued overleaf)

Figure 1: Simplified phylogenetic tree of Magnoliidae, after Massoni et al. (2014). Hydnoraceae (Piperales) are excluded, because the family was not included in original publications positioning the 10 fossils considered here. Colored boxes summarize the positions of fossils reviewed in the present paper. Their specific positions are figured by small branches with the number of the corresponding fossil at the tip. These correspond to the most parsimonious position(s) found for each fossil in previous phylogenetic analyses (see text for details). The minimum ages provided by the fossils are presented at the nodes they calibrate. The dashed branch refers to the phylogenetic uncertainty about relationships among Hernandiaceae, Lauraceae, and Monimiaceae (the position of Fossil 7 is on a branch not represented here, corresponding to a different set of relationships among these three families). Fossils are numbered following their order in the text. Abbreviations: Ma, million anni.

Table

Table 1: Summary of the calibration points provided in the present paper. Abbreviations: Ma, million anni.

Fossil Taxon	Node	Minimum Age
<i>Endressinia brasiliana</i>	Crown-group Magnoliineae (Doyle and Endress, 2010; Mohr et al., 2013)	113 Ma
<i>Schenkeriophyllum glanduliferum</i>	Crown-group Magnoliineae (Mohr et al., 2013)	113 Ma
<i>Archaeanthus linnenbergeri</i>	Stem Magnoliaceae (Doyle and Endress, 2010)	96.5 Ma
<i>Virginianthus calycanthoides</i>	Crown-group Laurales (Doyle et al., 2008)	107.7 Ma
<i>Lovellea wintonensis</i>	Crown-group Laurales (Dettmann et al., 2009)	100.5 Ma
<i>Jerseyanthus calycanthoides</i>	Crown-group Calycanthoideae (Crepet et al., 2005)	86.3 Ma
<i>Cohongarootonia hispida</i>	Crown-group core Laurales (von Balthazar et al., 2011)	107.7 Ma
<i>Mauldinia mirabilis</i>	Crown-group core Laurales (Doyle and Endress, 2010)	95.5 Ma
<i>Walkeripollis gabonensis</i>	Crown-group Canellales (Doyle and Endress, 2010)	126.3 Ma
<i>Saururus tuckerae</i>	Stem node of extant <i>Saururus</i> (Smith and Stockey, 2007)	45 Ma

Chapter 3

Coming submission in Molecular Biology and Systematic

The mode and tempo of diversification in Magnoliidae

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Appendix S1 included in the present manuscript.

Supplementary information:

https://www.dropbox.com/sh/mxrommnod3i19xm/O_XkTwcjk

Abstract

With 10,000 species, Magnoliidae are the largest clade of flowering plants outside monocots and eudicots. Despite an ancient and rich fossil history, the tempo and mode of diversification of Magnoliidae remains poorly known. Using a molecular data set of 12 markers and 220 species (representing >75% of genera in Magnoliidae) and six robust, internal fossil age constraints, we estimate divergence times and significant shifts of diversification across the clade. In addition, we test the sensitivity of magnoliid divergence times to the choice of relaxed clock model and various maximum age constraints for the angiosperms. Compared with previous work, our study tends to push back in time the age of the crown node of Magnoliidae (127.1-178.9 million years, Ma), and of the four orders, Canellales (126.3-141.0 Ma), Piperales (88.2-157.7 Ma), Laurales (111.8-165.6 Ma), and Magnoliales (115.0-164.2 Ma). Although families vary in crown ages, Magnoliidae appear to have diversified into most of its extant families by the end of the Cretaceous. The strongly imbalanced distribution of extant diversity within Magnoliidae appears to be best explained by models of diversification with 6 to 14 shifts in net diversification rates. Significant increases are inferred within Piperaceae and Annonaceae, while the low species richness of Calycanthaceae, Degeneriaceae, and Himantandraceae appears to be the result of decreases in both speciation and extinction rates. This study provides a new robust framework for future studies of morphological evolution as well as future tests of determinants of diversification rates in an important clade of angiosperms.

Introduction

With about 350,000 known species (Joppa et al., 2011), flowering plants are a rich clade in comparison with older living lineages such as Mammalia (about 5,400 spp., Meredith et al., 2011), or Squamata (about 8200 spp., Hedges and Vidal 2009). Because many organisms are highly dependent on this group (e.g. predation, symbiosis, or parasitism) and its species are components of all terrestrial ecosystems, investigating the tempo and mode of diversification in all parts of the tree will provide key knowledge to understand not only the evolutionary history of angiosperms as a whole, but also that of these related groups (e.g. McKenna et al. 2009) and of the environments they live in (e.g. Arakaki et al. 2011). Until now, the great majority of studies have estimated divergence times and diversification rates within monocots, eudicots, and at the scale of angiosperms as a whole (e.g., Wikström et al. 2001; Janssen and Bremer 2004; Magallón and Castillo 2009; Escudero and Hipp 2013; Koenen et al. 2013). However, an important part of the tree remains insufficiently explored. Among the eight orders of flowering plants outside the monocots and eudicots, Canellales, Laurales, Magnoliales, and Piperales concentrate more than 98% (about 10,000 spp.) of the species diversity. These four orders together form the larger clade Magnoliidae, *sensu* Cantino et al. (2007). The time scale of the evolutionary history of Magnoliidae remains largely uncertain as several important nodes have never been dated (Forest and Chase, 2009). However, previous studies at either very large (e.g., angiosperms) or narrow (e.g., a particular family) taxonomic scale have suggested that the age of the crown node of Magnoliidae to be older than the crown nodes of eudicots and monocots, and at least several of the 19 families appear to have diversified before the end of the Cretaceous (e.g. Wikström et al. 2001; Doyle et al. 2004; Renner 2004; Zhou et al. 2006; Magallón and Castillo 2009; Magallón 2010; Smith et al. 2010; Pirie and Doyle 2012; Magallón et al. 2013; Naumann et al. 2013). In addition, the biology of this clade differs from that of eudicots and monocots in several respects. First, Magnoliidae are predominantly restricted to tropical rainforests and most of the species are trees or shrubs, with the exception of Piperales, which contain many herbaceous species. Then, most members of Magnoliidae are pollinated by beetles, flies, and thrips, and bee or wind pollination are rare in the clade (Thien et al., 2000). Therefore, it is possible that Magnoliidae have experienced a different tempo and

mode of diversification than monocots or eudicots. Diversification in the group has been investigated in studies at either the scale of all angiosperms (e.g., Magallón and Sanderson 2001) or within families (e.g., Erkens et al. 2012). In addition, Magnoliidae are an important part of tropical rainforest floras. Therefore, a better understanding of their evolutionary history is important to reconstruct the history of tropical environments. In the present study, we estimate a new time scale for Magnoliidae as a whole and test the presence of shifts in diversification rates during their evolutionary history. To do so, we took advantage of a recent improvement in the phylogeny of the group (e.g., Renner 1999; Graham and Olmstead 2000; Zanis et al. 2002; Hilu et al. 2003; Sauquet et al. 2003; Qiu et al. 2005; Moore et al. 2010; Soltis et al. 2011; Naumann et al. 2013; Massoni et al. 2014b) and a revision of the rich fossil record of Magnoliidae providing reliable minimum-age calibration points (Massoni et al., 2014a). In order to conduct these analyses for nodes above and below the family level, we used a dataset including more than 75% of the existing genera and 12 molecular markers from the three genomes (6 plastid, 4 mitochondrial, 2 nuclear).

Material and methods

Molecular dataset

In order to conduct the molecular dating analyses we used the same 12-marker molecular dataset of Massoni et al. (2014b). This matrix includes 12 coding and non-coding markers from the three genomes: *atpB*, *matK*, *trnL* intron, *trnL-trnF* spacer, *ndhF*, *rbcl* from the chloroplast; *atp1*, *matR*, *mtSSU*, *mtLSU* from the mitochondrion; and 18S rDNA and 26S rDNA from the nucleus. In this dataset, we used an exemplar approach, in which each genus was represented by one species and the different markers used were from this species. The problematic parasitic family Hydnoraceae was excluded because it is a very long branch and its exact position within Piperales remains uncertain (Massoni et al., 2014b; Naumann et al., 2013). All remaining 19 families of Magnoliidae are represented, and more than 75% of the genera were sampled (198 genera out of 262). The same selection of 23 outgroup taxa was included (Amborellales, Nymphaeales, Austrobaileyales, Chloranthales, eudicots, and monocots).

Molecular dating analyses

Calibration scheme

All the geological ages presented in this study follow the revised Geological Time Scale of Gradstein et al. (2012). To calibrate the molecular phylogeny we used the calibration scheme proposed by Massoni et al. (2014a). This scheme consists in 10 fossils reviewed for both their phylogenetic positions (based exclusively on explicit phylogenetic analyses) and their absolute age based on the latest stratigraphic and geochronological literature (Tab. 1), resulting in solid minimum age constraints on six nodes of our tree (Fig. 1). In addition to these six minimum age constraints, we used two maximum ages. First, the crown node of eudicots was set to a maximum of 125 Ma, based on the appearance of tricolpate pollen grains near the late Barremian-early Aptian boundary (Friis et al., 2006). Second, the crown node of angiosperms was set to five different maximum ages in order to test the sensitivity of the Magnoliidae divergence time scale to this temporal constraint. The age of the crown node of angiosperms is a matter of debate (Doyle, 2012). Several pre-Cretaceous fossils for the angiosperms have been described but their ages and their phylogenetic affinities with the angiosperms (stem relatives of angiosperms, crown members of angiosperm, or gymnosperms) are controversial (Doyle, 2012). The oldest fossil pollen confirmed to be angiospermous (either crown or stem), based on columellar exine structure, is dated to the Hauterivian or Valanginian (i.e. 129.4-139.8 Ma; Doyle 2012). On the other hand, molecular dating studies have generally converged to an age for the crown node of angiosperms between 140 and 200 Ma (Bell et al., 2010, 2005; Magallón et al., 2013; Moore et al., 2007; Soltis et al., 2008; Wikström et al., 2001) with few of them inferring older ages (e.g. Schneider et al. 2004; Magallón 2010). In order to take into account this uncertainty on the crown age of angiosperms, we used five different age constraints: 130 Ma (angio-130 analyses), 140 Ma (angio-140 analyses), 150 Ma (angio-150 analyses), 170 Ma (angio-170 analyses) and 200 Ma (angio-200 analyses) for the maximum age constraint applied to the crown node of angiosperms in all molecular dating analyses.

Divergence time estimation

Uncorrelated lognormal clock (UCLN) analyses: In order to evaluate divergence times within Magnoliidae while taking into account phylogenetic uncertainty, we used BEAST v1.7.5 (Drummond et al., 2012) without fixing the tree. We partitioned our molecular dataset into 12 partitions (one per marker) as in Massoni et al. (2014b). A Birth-Death incomplete sampling prior was specified for the trees (Stadler, 2009), and rate heterogeneity was modeled using the UCLN relaxed clock of Drummond et al. (2006). All age constraints were applied using uniform priors (hard minimum and maximum ages) because of the difficulty of parameterizing non-uniform priors, which may involve subjectivity (Sauquet et al., 2012). We used a penalized likelihood (PL) chronogram as the starting tree, and because of branch lengths were rounded to a finite number of decimals in this starting tree, we had to set the maximum ages constraints to 125.1, 130.1, 140.1, 150.1, 170.1, and 200.1 Ma. For all analyses, we ran four independent chains of a Markov Chain Monte Carlo procedure of 100 million generations each, sampling parameters and trees every 1000 generations (Tab. S1). All analyses were performed on the CIPRES cluster (Miller et al., 2010). We evaluated the size of the burnin phase for each run using Tracer v1.5 (Rambaut and Drummond, 2007). The post-burnin posteriors of the four runs of each analysis were then combined using LogCombiner v1.7.5, sampling trees and parameters every 10,000 generations (because of computational limitations). We then used TreeAnnotator v1.7 to select the maximum clade credibility (MCC) tree of each analysis.

Penalized likelihood (PL) analyses: We also conducted PL analyses for each set of age constraints using r8s v1.8 (Sanderson, 2003). The PL relaxed clock assumes some degree of autocorrelation of molecular substitution rates between a parent and its immediate descendants (Sanderson, 2002). Although autocorrelated relaxed clock models are also available in Bayesian frameworks, our rationale for using r8s was to test the sensitivity of our age estimates to a fundamentally different approach to molecular dating. In all analyses, we used the best scoring maximum likelihood (ML) phylogram of Massoni et al. (2014b) obtained with the 12-marker RAxML analysis excluding *Hydnora*. In order to determine the optimal level of autocorrelation across the tree (smoothing parameter), we conducted cross validation procedures testing 17 different values of smoothing in a range between 0.1 and

10,000,000 for each calibration scheme, using the penalty additive function. For angio-130 the optimal value was 32, for angio-140 and angio-150 it was 3.2, and for angio-170 and angio-200 it was 10. In order to provide confidence intervals on age estimates with PL, we conducted non-parametric maximum likelihood bootstrapping of 1000 replicates on the original dataset of Massoni et al. (2014b) using RAxML v7.3.2 (Stamatakis et al., 2008) while fixing the tree topology to the best scoring ML tree obtained in this previous study. As RAxML requires the application of the same model to all partitions, we used the GTR + GAMMA + I model for each partition. Within the resulting 1000-tree collection, only branch lengths varied. We reconstructed the chronograms for both the best ML tree and the 1000 bootstrapped phylograms using the optimal smoothing parameter obtained for the best ML tree and the TN algorithm of r8s. All results were summarized using the software TreeAnnotator v1.7 with the 1000 PL trees as the input file and the ML-PL tree as the target tree.

Diversity dynamics

In the present study, not all extant species of Magnoliidae have been sampled, and our sample of species cannot be assumed to be random (instead, it was taxonomically biased to sample all families except one, and most of the genera). For this reason, we used the MEDUSA model of Alfaro et al. (2009) to test for significant diversification shifts in an incompletely sampled phylogeny, where each terminal can be assigned the number of extant species in the clade it represents. This procedure detects, with a stepwise AIC approach, shifts of diversification rates by evaluating the fit of different piecewise birth-death models to the data. In order to select the best model, we used the AICc criterion, a birth-death model of diversification, and we allowed the placement of shifts either on stem or on crown nodes. In order to test the sensitivity of the method to age estimates, we conducted our analysis on two collections of 1000 trees randomly sampled from the post-burnin phase of our collections of angio-140 and angio-200 BEAST trees. Prior to the analyses, each randomly sampled chronogram was transformed in two ways, using functions in the ape package of R (Paradis et al., 2004). First, all outgroups of Magnoliidae were pruned. Second, the chronograms were simplified by pruning selected terminal taxa so that each remaining terminal taxon would represent a monophyletic compartment with known

extant diversity (see below). All analyses were performed in R using the multiMEDUSA procedure from package MEDUSA v0.93 4.33 available on the website authored by Joseph W. Brown (<https://github.com/josephwb/turboMEDUSA>).

Extant taxonomic richness data

For the diversification analysis using the MEDUSA approach, we had to distribute the entire species richness of Magnoliidae among the tips of our trees. In several instances, this required us to merge tips into a single terminal to represent larger monophyletic groups with all of their diversity. Hereafter, we refer to these monophyletic groups as compartments. We proceeded in two steps. First, we maintained genera as tips or created larger compartments, depending on the monophyletic status of genera as tested in previous phylogenetic studies: if the literature supported the monophyly of a genus, this taxon was used as a terminal compartment; if the monophyly of a genus had never been tested before, a compartment including this genus and its sister group was defined (or a larger compartment if the neighborhood relationships were not well supported); if the monophyly of a genus had been challenged by previous studies, we defined a larger compartment to include this genus and all other genera potentially involved in the paraphyly or polyphyly. After this first step, we modified our compartmentalization scheme to take into account the species numbers of genera not sampled in our trees (less than 25% of the total number of accepted genera). If there was enough information in the literature to support an accurate placement (phylogenetic or apomorphy-based) of a missing genus, we added its number of species to that of earlier defined compartment (generic or supra-generic). In some cases, the missing genus could not be precisely placed within a larger clade of two or more previously defined compartments. As a guideline, we accepted to merge these compartments into a larger one if the number of species would represent more than three percent of the total number of species in the resulting compartment. Otherwise, the missing diversity was ignored in order to maintain enough compartments for conducting a meaningful analysis. In all cases, we defined these supra-generic compartments in such a way that they were present in all 1000 BEAST trees used to conduct the MEDUSA analyses (posterior probabilities [PP] equal to 100%). The resulting compartmentalization scheme consisted in 85 terminal taxa (Appendix S1). In total, 31 species were ignored due to unknown

phylogenetic placement, representing ca. 0.3% of the 10,209 species of Magnoliidae counted in the present study. For 15 genera (out of 262) the number of species was not clearly indicated in previous publications. In those cases, The Plant List (<http://www.theplantlist.org/>) was used to estimate the number of extant species currently accepted in the genus. The definition of supra-generic clades and the incorporation of the missing diversity are justified in detail in Appendix S1.

Results

Phylogenetic relationships within Magnoliidae

Each of the five BEAST analyses converged effectively and the final effective sampling sizes of the general likelihood were always superior to 3900 (Tab. S1). Although the great majority of the relationships among the families received high support values and are the same in all analyses, the positions of Siparunaceae (Laurales) and Magnoliaceae (Magnoliales) remained uncertain.

Influence of the angiosperm maximum age constraint on estimated divergence times

The 95% credibility intervals obtained with BEAST are larger than those obtained with PL (Fig. 2). In the BEAST analyses, only the ages estimated for the crown node of Magnoliidae, and the splits between Canellales and Piperales, and between Laurales and Magnoliales, are significantly different when the maximum age of the root varied (Fig. 2a). On the contrary, in the PL analyses, the majority of age estimates are significantly different when the maximum age of the root changed (Fig. 2b).

Age estimates

In general, the ages estimated in BEAST and r8s are compatible (Fig. 2, Tab. 2). In the present paper, we take into account the overlapping credibility intervals of all 10 analyses (see discussion). Our results tend to support an origin of extant Magnoliidae (crown node) between 127.1 and 178.9 Ma, that is, somewhere in the Lower Cretaceous or the Jurassic.

The crown nodes of Canellales, Laurales, Magnoliales, and Piperales are dated to be somewhere in these two geological periods (126.3-141 Ma, 111.8-165.6 Ma, 115-164.2 Ma, and 88.2-157.7 Ma, respectively). The time elapsed between the first split within Magnoliidae and the two following splits among the four orders seems to be very short (Fig. 2, Tab. 2). By the end of the Cretaceous (66.0 Ma), most families (crown nodes) were probably present, with the exception of Myristicaceae. The oldest families in terms of crown age appear to be either Aristolochiaceae and Calycanthaceae (BEAST: 38.6-135.6 Ma, 92.3-123.1 Ma, respectively) or Aristolochiaceae and Lauraceae (PL: 103.2-147.3 Ma, 92.2-147.2 Ma, respectively), depending on the relaxed clock method used (Fig. 2, Tab. 2).

Diversification analysis

Our MEDUSA analyses on 1000 trees sampled from the posterior of the BEAST angio-140 and angio-200 analyses present very similar scenarios of the mode of diversification within Magnoliidae (Fig. 3a, b). The two topologies used to summarize results are identical in topology except within the subclade of Annonaceae that includes the compartments *Dielsothamnus*, FM, TMSMFDD and *Uvaria*, where the relationships are weakly supported. We identified six to 14 significant diversification rate shifts. In both cases, models including nine shifts were the most often selected among the 1000 trees sampled (Fig. 3). The mean of the initial net diversification rate (speciation minus extinction), at the crown node of Magnoliidae, is estimated to 0.0384 ± 0.0148 species per million years (sp.myr^{-1}) in angio-140, and 0.0354 ± 0.0135 sp.myr^{-1} in angio-200. If we only consider the shifts present in more than 50% of the trees (Fig. 3), in both analyses, there are five main shifts in net diversification rates within Magnoliidae (the corresponding nodes were all supported by 1.0 posterior probabilities and numbered on Fig. 3). Three are negative and two are positive: 1) at the crown node of the clade of Piperaceae and Saururaceae (magnitude of shift: -0.0021 ± 0.0151 sp.myr^{-1} in 81% of trees angio-140; -0.0034 ± 0.0128 sp.myr^{-1} in 63% of trees angio-200); 2) at the crown node of Piperaceae excluding *Verhuellia* ($+0.0202 \pm 0.0376$ sp.myr^{-1} in 91% of trees angio-140, $+0.0328 \pm 0.0289$ sp.myr^{-1} in 81% of trees angio-200); 3) at the crown node of Calycanthaceae (-0.0286 ± 0.0158 sp.myr^{-1} in 87% of trees angio-140, -0.0243 ± 0.0141 sp.myr^{-1} in 88% of trees angio-200); 4) at the crown node of the clade of *Degeneria* and *Galbulimima* (-0.0356 ± 0.0147 sp.myr^{-1} in 79% of trees angio-140, -0.0337 ± 0.0133

sp.myr⁻¹ in 93% of trees angio-200); 5) at the crown node of tribe Miliuseae of Annonaceae (+0.1275 ±0.0336 sp.myr⁻¹ in 70% trees angio-140, +0.1173 ±0.0300 sp.myr⁻¹ in 83% trees angio-200). In addition to those five main shifts, the net diversification rate averaged over the sample of 1000 trees show a general increase both within the Laurales and within the Magnoliales (Fig. 3). Our estimates of the relative extinction rate (ratio of extinction over speciation) at the crown node of Magnoliidae is 0,9000 ±0.1533 (angio-140) and 0,8857 ±0.1603 (angio-200) (Fig. S2). This rate remained relatively high (more than 0.5) in Magnoliidae except in Laurales and part of Piperales.

Discussion

Age estimates

The use of BEAST to estimate divergence times yielded a new phylogeny that is entirely compatible with our previous one obtained with the same molecular dataset but different phylogenetic methods (Massoni et al., 2014b). The application of different maximum age constraints for the crown node of angiosperms highlighted the sensitivity of the age estimated for the deepest nodes of Magnoliidae to this restriction on the age of the root (Fig. 2). Although previous work has highlighted a long gap in the fossil record of the angiosperm stem lineage, there is no indisputable argument in favor of one particular maximum age constraint for the crown node of angiosperms. Therefore, in the rest of this paper, we will systematically consider the whole range of estimates obtained across our five maximum age calibration schemes.

The efforts to incorporate in the most appropriate way (Parham et al., 2012) all the most reliable knowledge in the field of paleobotany of Magnoliidae (Massoni et al., 2014a) and chrono-stratigraphy (Gradstein et al., 2012), as well as a comprehensive sample of taxa and molecular markers, support the presence of extant lineages of Magnoliidae earlier than suggested in the literature. Thus, the present study supports older ages for the crown node of Magnoliidae (Bell et al., 2010, 2005; Magallón, 2010; Magallón et al., 2013; Moore et al., 2007; Smith et al., 2010; Soltis et al., 2008; Wikström et al., 2001), of Canellales (Bell et al., 2010; Magallón, 2010; Magallón et al., 2013; Wikström et al., 2001), of Piperales (Bell et al.,

2010; Magallón, 2010; Magallón et al., 2013; Naumann et al., 2013; Wikström et al., 2001), of Laurales (Bell et al., 2010; Magallón et al., 2013; Wikström et al., 2001), and of Magnoliales (Wikström et al. 2001; Bell et al. 2010; Fig. 2). However, few studies have supported much older ages within the angiosperms (Magallón and Castillo, 2009; Magallón, 2010; Schneider et al., 2004; Smith et al., 2010). At the familial level, in addition to providing the first estimates for the ages of crown-group Canellaceae (12.3-69.9 Ma), Winteraceae (13.5-70.4 Ma), and Saururaceae (48.4-84.3 Ma) (Tab. 1), the crown nodes of families are also generally dated to an older range of dates (Couvreur et al., 2011; Doyle et al., 2004; Naumann et al., 2013; Pirie and Doyle, 2012; Renner, 2004; Renner et al., 2000; Su and Saunders, 2009; Zhou et al., 2006). The large amount of combinations of parameters influencing age estimates limits straightforward explanations of these differences with previous studies (e.g., topology used, Sanderson and Doyle 2001; fossil species and priors used to define and model age constraints, Sauquet et al. 2012; molecular dating method, Magallón 2010). Despite variation among the ages obtained for the crown groups of families, Magnoliidae had probably diversified into morphologically distinct clades (now identified as families) by the end of the Cretaceous (66 Ma, Fig. 2). The present older estimates for divergence times in this clade provide arguments for an earlier diversification of angiosperms. We support the presence of more lineages of flowering plants in the Cretaceous than thought before.

Several of our older estimates are not compatible with previous biogeographic scenarios. The time scale of the tropical family Annonaceae has been the most extensively studied in Magnoliidae (e.g., Su and Saunders 2009; Couvreur et al. 2011; Pirie and Doyle 2012), but our results imply that some of the conclusions of these studies may need to be revised. For instance, we dated the crown node of the family between 74.9-126.7 Ma, whereas Su and Saunders (2009), Couvreur et al. (2011), and Pirie and Doyle (2012) supported younger ages (89-90.4 Ma, 89-93 Ma, and 89-98 Ma, respectively). The use of a younger age constraint for the crown node of Annonaceae in these studies could explain this difference. Within Annonaceae, subfamily Malmeoideae presents a clear geographic clustering in four major subclades: tribe Piptostigmatheae in Africa, tribe Malmeae in South and Central America, the miliusoid clade (Miliuseae + Monocarpieae of Chatrou et al. 2012) mainly restricted to

Southeast Asia, and the Central American clade nested within Miliuseae. Richardson et al. (2004) estimated the age of the crown node of Malmeoideae between 53.1–62.5 Ma. Based on this age, Richardson et al. (2004) and Pirie et al. (2006) suggested that the distribution pattern within the subfamily resulted from boreotropical migrations during the Early Eocene Climatic Optimum (optimum at 52–50 Ma followed by a 17 My-long trend toward cooler conditions, Zachos et al. 2001). Because our older estimates are not preceding the Gondwanian break-up (which could suggest vicariance scenarios to explain disjunction in tropical distribution), they are compatible with the boreotropical hypothesis. For the disjunction between Africa and Southeast Asia, Su and Saunders (2009) proposed an alternative scenario in which Annonaceae inhabiting the Deccan plate (India) rafted during the Cretaceous and subsequently dispersed into Southeast Asia after the collision between both plates (India, Asia). Even though the maximum likelihood analysis of Couvreur et al. (2011) provided significant support for the boreotropical hypothesis over the rafting hypothesis, their estimate of the timing of dispersal of the miliusoid clade into Southern Asia postdates the collision between India and Asia (c. 35 Ma, Ali and Aitchison 2008). This observation potentially supports the rafting hypothesis. In the present study, the older estimate for the age of the stem (22.6–76.8 Ma) and crown (18.0–65.3 Ma) nodes of the miliusoids could indicate the presence of Annonaceae in Southeast Asia before the collision between India and Asia. However, our estimated ages are also compatible with a migration from the Indian plate to Southeast Asia, but a few million years before the final collision, when the north-east of the Indian plate reached Sumatra followed on its northern end by Burma around 57 Ma ago (Ali and Aitchison, 2008). Under the Boreotropical model, Couvreur et al. (2011) explained the apparent geographical clustering within Malmeoideae by the fact that the crown node of this subfamily was dated between 26.1–40.1 Ma, suggesting that boreotropical migration routes were cut off quickly after their initiation due to the drastic Oligocene global temperature drop, which affected rainforest vegetation worldwide. Our older estimates of dates do not suggest this latter explanation.

Previous estimates of the crown age of Myristicaceae have been problematic because they were difficult to interpret from a biogeographic point of view (Doyle et al., 2004) and because they would be drastically changed if a fossil calibration could securely be placed

within the crown group (Doyle et al., 2008). Doyle et al. (2004) suggested that the ancestral area for the clade was Africa-Madagascar with a Late Cretaceous dispersal in both directions across the South Atlantic, and one or more dispersal events from Africa (or Madagascar) to Asia. Their estimate of the age of the crown node, between 15-21 Ma implied an implausible transoceanic dispersal of large, animal dispersed-seeds across the South Atlantic. The present older estimate range (12.0-52.2 Ma) for crown-group Myristicaceae does not change this interpretation nor resolve this biological contradiction, since the shortest distance between Africa and South America was already more than 1400 kilometers 52.2 Ma (Ford and Golonka, 2003). Doyle et al. (2008) described a fossil seed from the early Eocene of southern England with ruminations typical of Myristicaceae, which could push back in time the age of the crown group. However, the position of this fossil as a stem or crown relative has not been elucidated. In the present study, the oldest estimate for crown-group Myristicaceae is compatible with either a stem or crown placement for this extinct species.

Within Laurales, molecular dating analyses have been used to investigate the biogeographic history of Atherospermataceae (Renner et al., 2000), Calycanthaceae (Zhou et al., 2006), Hernandiaceae (Michalak et al., 2010), Lauraceae (Chanderbali et al., 2001), and Monimiaceae (Renner et al., 2010). Unfortunately, the relationships within Atherospermataceae, Lauraceae, and Monimiaceae are poorly resolved in the present study, making any direct comparison with these previous studies difficult. Regarding the Calycanthaceae, Zhou et al. (2006) argued that *Idiospermum*, restricted to Australia, and the two remaining genera *Chimonanthus* and *Calycanthus*, present in China and North America, are the surviving lineages of a widely distributed stem group of Calycanthaceae. They based their argumentation on the presence of fossil species probably related to the family in the Cretaceous of South America and North America and the fact that the dispersal biology of *Idiospermum* is not compatible with a dispersal from Laurasia to Australia about 72 Ma ago. Our estimate for the split between *Idiospermum* and the remaining Calycanthaceae is between 92.3-113.9 Ma, an age also implying an unlikely dispersal event as an alternative to the explanation of the ancestral wide distribution of the family by Zhou et al. (2006). The geographical history of the disjunction between East Asia and North America in the clade of *Chimonanthus* and *Calycanthus* is more ambiguous (Zhou et al., 2006). Last, the geographical

origin of Hernandiaceae is uncertain and the current distribution of the family could have involved long-distance dispersal events (Michalak et al., 2010). In this context, all of our estimates are compatible with the scenario proposed in Michalak et al. (2010). However, as in this previous study, because the movement of Madagascar relative to Africa began in the Jurassic (Jokat et al., 2003), our date estimates for the stem lineage of *Hazolomania* (11.1–102.7 Ma) are not compatible with the idea that the isolation of the genus on Madagascar is due to vicariance.

Here, we restricted ourselves to conservative fossil age constraints based on phylogenetic analyses of fossil and extant taxa (Massoni et al., 2014a). This led us to consider only 10 out of more than 100 described fossils putatively belonging to Magnoliidae (Friis et al., 2011). However, our new age estimates for families and orders of Magnoliidae appear, in general, to be compatible with the putative fossil record attributed to each of these taxa. Future investigations of the phylogenetic placement of other magnoliid fossils will likely provide additional minimum age constraints, which could push back in time several young estimates supported in the present study, and decrease the size of the credibility intervals. This study further illustrates that, just as paleontological dating, molecular dating estimates are often associated with large uncertainty. The latter approach has to be seen as an attempt to reduce the range of the most likely ages for nodes constrained by the age of the fossil record securely placed and dated, and to evaluate the probability of the ages of nodes not directly influenced by this extinct diversity. Because all the ambiguity of our current knowledge has to be taken into account, the molecular dating approach cannot provide exact secure ages, except for exceptionally fossil-rich clades.

Diversification

The new timetree obtained for Magnoliidae in this study allowed us for the first time to test whether diversification has been a homogeneous process throughout the history of the clade and across lineages. Using the MEDUSA approach, we detected significant variations in the tempo of diversification across lineages, with an average of nine shifts to explain the distribution of extant diversity across Magnoliidae (Fig. 3). Previous studies using the same statistical approach in other angiosperm clades found, in general, fewer shifts than in the

present analysis. Within eudicots, Beaulieu and Donoghue (2013) supported six shifts in Campanulidae (35,000 spp.), Koenen et al. (2013) supported nine shifts in Fabaceae (19,500 spp.), Xi et al. (2012) found five significant shifts in Malpighiales (16,000 spp.), and Arakaki et al. (2011) found seven shifts in Cactaceae (1850 spp.). Within monocots, Escudero and Hipp (2013) supported three shifts in the family Cyperaceae (5480 spp.). Despite the dependence of the number of shifts detected on the taxonomic level of tips in the chronograms used (MEDUSA cannot detect shifts within supraspecific terminal compartments), it seems that Magnoliidae experienced an unusually high variation in diversification rates. Because of their longer evolutionary history in comparison with these other clades and because their fossil record supports a global distribution during the Cretaceous and the Paleogene (Friis et al., 2011), Magnoliidae as a whole may have been affected by more events potentially influencing the speciation and extinction rates (e.g. climatic shifts, Jansson and Dynesius, 2002; variation in amount of available areas, Vamosi and Vamosi, 2011; variation in geographic distribution, Payne and Finnegan, 2007; dynamic of diversification of associated groups such as pollinator, Cardinal and Danforth, 2013).

Establishing the causes sustaining diversification-rate variations is a very complex problem. There is a large diversity of intrinsic and extrinsic traits which could influence speciation and extinction (Vamosi and Vamosi, 2011), all of them being potentially correlated to each other (e.g., Vamosi et al. 2003). The influence of a character on birth and death of species is dependent on other taxa, other traits of the same organism, and the physical environment (de Queiroz, 2002), implying that a character could have different effects on different clades. Finally, the actual shift of diversification could happen several nodes after the evolution of an influencing trait. One explanation could be that an isolated trait will influence diversification in combination with other characters, the effect appearing when the entire set of traits needed is present (Beaulieu and Donoghue, 2013). For these reasons the investigation of the correlation between traits and diversification rates will need to take into account as many potential factors as possible (e.g., Leslie et al., 2013).

The older estimates of the ages in angio-200 lead to slightly lower rates than in angio-140. Because the general pattern is the same in both analyses, in the present paragraph we refer only to rates of the angio-140 analysis. The net diversification rates obtained across the tree

of Magnoliidae (mean = $0.0458 \text{ sp.myr}^{-1}$) are comparable to those found in angiosperms (e.g., Magallón and Sanderson 2001; Magallón and Castillo 2009) and lower than the highest rates found outside Magnoliidae (for a review, see Valente et al. 2010). In the present study, the lowest net diversification rates, resulting from the shifts 3 and 4 (Fig. 3), were found in the crown group of Calycanthaceae (mean = $0.0169 \text{ sp.myr}^{-1}$), and in the clade of Degeneriaceae and Himantandraceae (mean = $0.0047 \text{ sp.myr}^{-1}$). These three families share distinctive floral features not or seldom found in other parts of the Magnoliidae tree, such as the presence of inner staminodes that cover the stigma at the end of the female phase (Endress, 2010), and a spiral phyllotaxy of perianth parts (Endress and Doyle, 2007; Endress, 1993). However, it is difficult to link these two characters with mechanisms sustaining a low diversification rate (influence on speciation and/or extinction). Within Calycanthaceae, the low rate of net diversification is associated with a low speciation rate in the entire clade (mean = $0.0177 \text{ sp.myr}^{-1}$) and near-zero extinction (mean = $0.0007 \text{ sp.myr}^{-1}$). The absence of extinction in this clade would seem contradictory with the presence of several fossils in the Cretaceous (Zhou et al., 2006) and could be an artefact of the method which does not take into account the extinct diversity (see last paragraph). However, because their phylogenetic positions within the family have not been tested (except for *Virginianthus calycanthoides*, Crepet et al. 2005), it is difficult to draw conclusions from these fossils. The pollination systems within Calycanthaceae involve trapping of a large variety of beetles and thrips (Grant, 1950; Worboys and Jackes, 2005). In comparison with specific pollination systems involving a unique relationship for the two partners, this generalist interaction is not favorable for genetic isolation promoting diversification (Kay and Sargent, 2009). In addition, Calycanthaceae have a temperate distribution, except for the monospecific genus *Idiospermum* growing in a restricted area in north-east Australia. The low ecological limits on species richness in temperate areas (Vamosi and Vamosi, 2011) could provide an explanation for the low diversification of the lineage leading to the genera *Calycanthus* and *Chimonanthus*. On the other hand, the apparent lack of diversification of the lineage leading to *Idiospermum* could be explained by different causes. The species is a long-lived tree (Jones et al., 2010) involving low fixation rates in diverging populations. The genus is restricted to very wet humid tropical lowland rainforests (Goosem, 2002), which could have been a refuge area during the decrease of the surface covered by rainforests during the

Cenozoic in Australia (Martin, 2006). *Idiospermum* presents one of the heaviest seeds among Australian plants and is therefore probably not dispersed by animals (Edwards et al., 2001; Jones et al., 2010). In addition, the capacity of dispersion of the lineage leading to the extant species of the genus could have been limited, thereby restraining the number of available areas promoting diversification (Vamosi and Vamosi, 2011). This latter explanation might be generalized for all Calycanthaceae as the seeds of the family contain secondary metabolites that are toxic at least for mammals (Kubitzki, 1993). On the other hand, the highest diversification rate supported in the present study was found on the branch leading to the terminal compartment Miliuseae within Annonaceae (0.1590 ± 0.0597 sp.myr⁻¹). A shift of $+0.1275 \pm 0.0336$ sp.myr⁻¹ at the node sustaining the branch of the tribe is present in 70% of the trees tested and the associated rate of speciation on the branch is on average equal to 0.4903 sp.myr⁻¹. Erkens et al. (2012) found a shift at the crown node of this clade and mentioned the presence of distinctive pollen features, compared with other Malmeoideae. In addition, the tribe may have become particularly species-rich as a result of radiation following founder events during their biogeography history (Erkens et al., 2012). Within Magnoliidae, the studies of Erkens et al. (2012) and Couvreur et al. (2011) on Annonaceae are the only previous diversification studies we are aware of. Using a different approach (whole-tree tests of diversification models), Couvreur et al. (2011) supported the absence of shifts in diversification rates within the family. On the contrary, Erkens et al. (2012), and the present results, support the presence of significant variations (Fig. 3), confirming that the diversification of an important part of rainforest tree diversity was not the result of gradual accumulation of lineages during the Cretaceous and the Cenozoic as suggested by Couvreur et al. (2011) (museum model of tropical diversification).

More generally, Canellales and Piperales have, on average, higher speciation rates than Magnoliales and Laurales (means = 0.8279, 0.4659, 0.2931, 0.1029 sp.myr⁻¹, respectively). Within Piperales, this may be explained by widespread herbaceous habits, resulting in faster life history and higher fixation rates in divergent populations. In addition, within this order, Aristolochiaceae typically have zygomorphic flowers, which has been shown to have a positive impact on diversification (Kay and Sargent, 2009). However, Canellales and Piperales also share the highest extinction rates (means = 0.8075 and 0.4408 sp myr⁻¹ respectively),

making Laurales and Magnoliales the most productive orders in terms of net diversification (0.0451 and 0.0554 sp.myr⁻¹ respectively). Differences in extinction rates are difficult to explain. For instance, there are no major differences in pollination systems among the four orders (Endress, 2010), and the current geographic distributions of the four orders are similar (Heywood et al., 2007). However, Doyle and Endress (2011) found several synapomorphies for the clade of Laurales and Magnoliales, specifically the presence of more than two whorls of stamens and more than one whorl of carpels. These characters might have played a role in relation with pollination, but the underlying mechanisms remain unknown.

Last, even though the MEDUSA approach represents a significant conceptual improvement over previous models, it still requires several important assumptions that may have influenced our results. First, MEDUSA does not implement diversification models in which the extinction rate can be higher than speciation rates. Second, the rates are assumed to be constant in each part of the tree, and only abrupt variations are evaluated, probably not in accordance with the biological reality in which rates could gradually fluctuate. Third, because the fossil record is not taken into account, very low rates of speciation and extremely low extinction rates are always allocated in the old lineages with few species (long branches in chronograms; Alfaro et al. 2009), which have probably been more diverse in the past (e.g., *Idiospermum* in the present study).

Conclusion

The present study suggests that Magnoliidae began to diversify somewhere between the Toarcian (Early Jurassic) and the Barremian (Early Cretaceous), or 127.1-178.9 Ma. Several key nodes within Magnoliidae are dated here for the first time. In general, our age estimates tend to support an older diversification of the group than previously suggested. The rich fossil record of Magnoliidae may eventually provide additional calibration points to refine the time scale proposed here. However, considerable work remains to be done to securely relate this extinct diversity to the extant one, a task for which an integrated morphological dataset will be essential. The tempo of diversification within this clade of angiosperms appears to be characterized by several shifts of diversification toward both higher and lower

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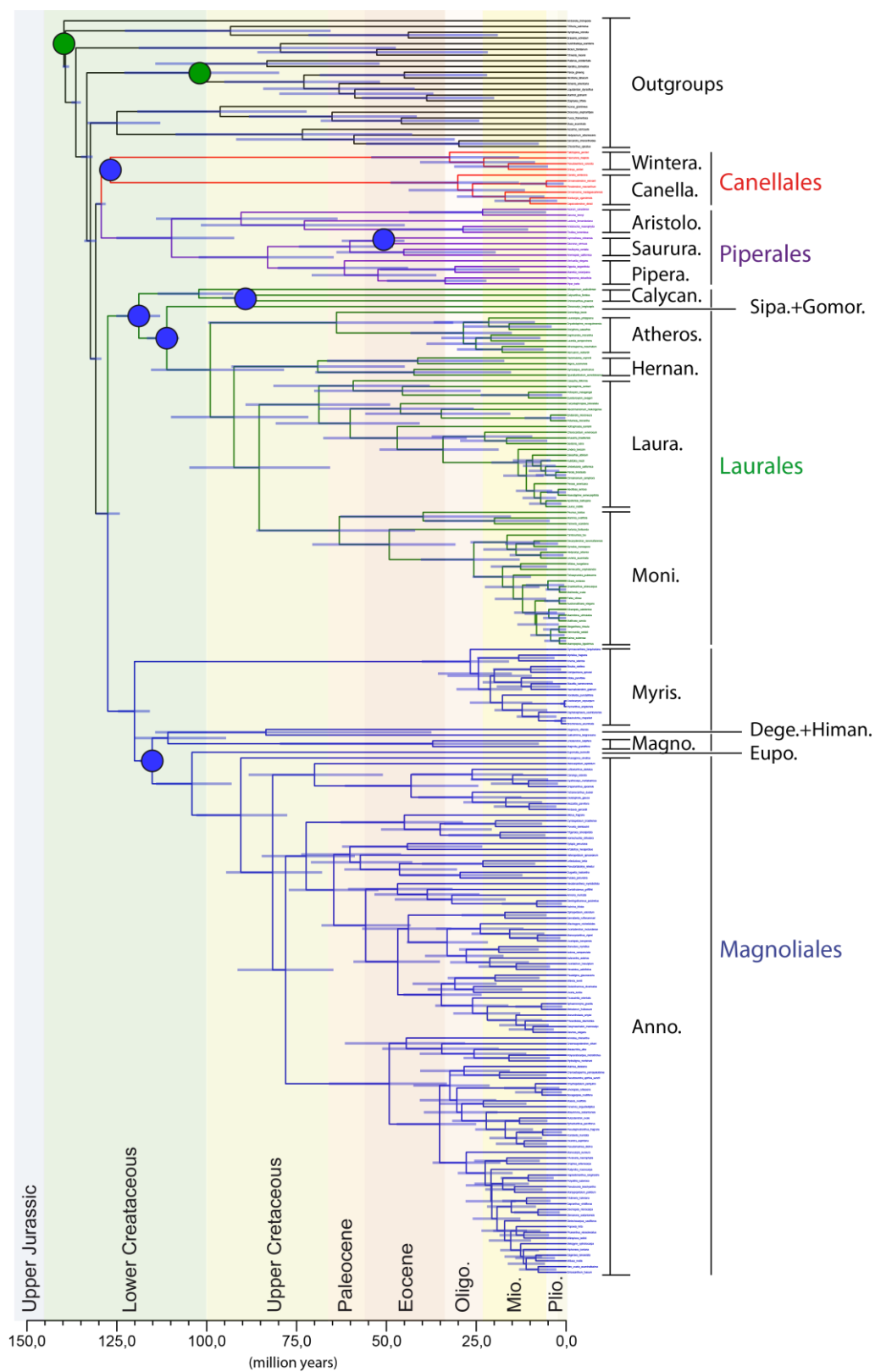
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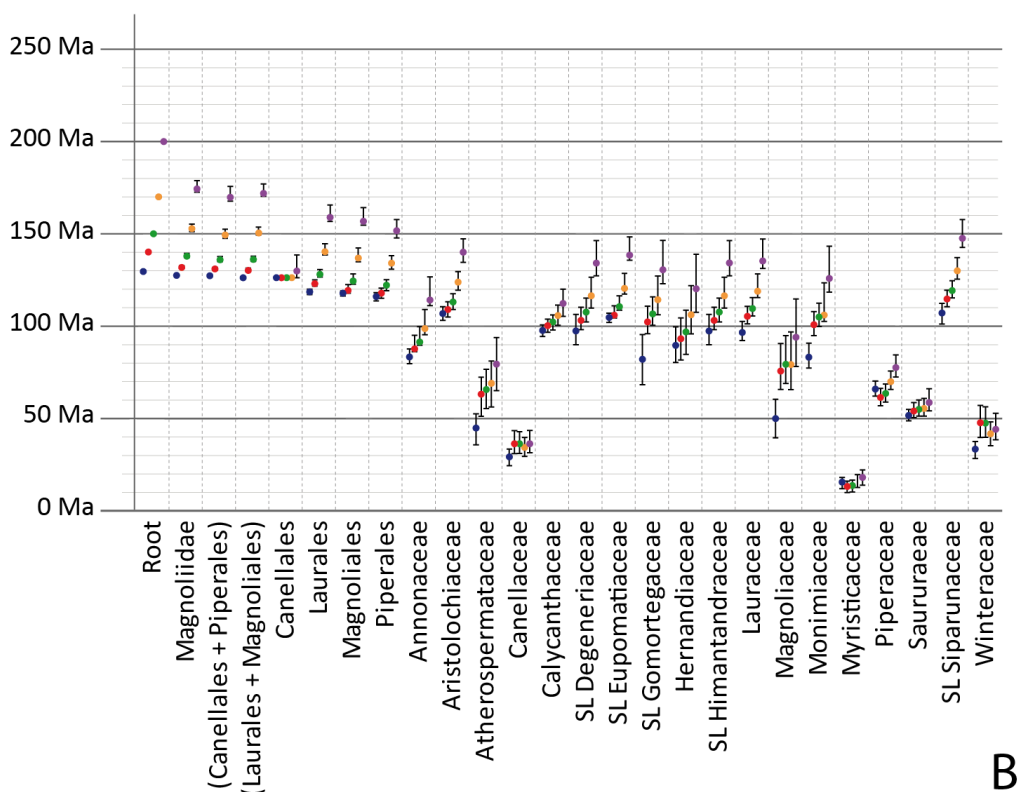
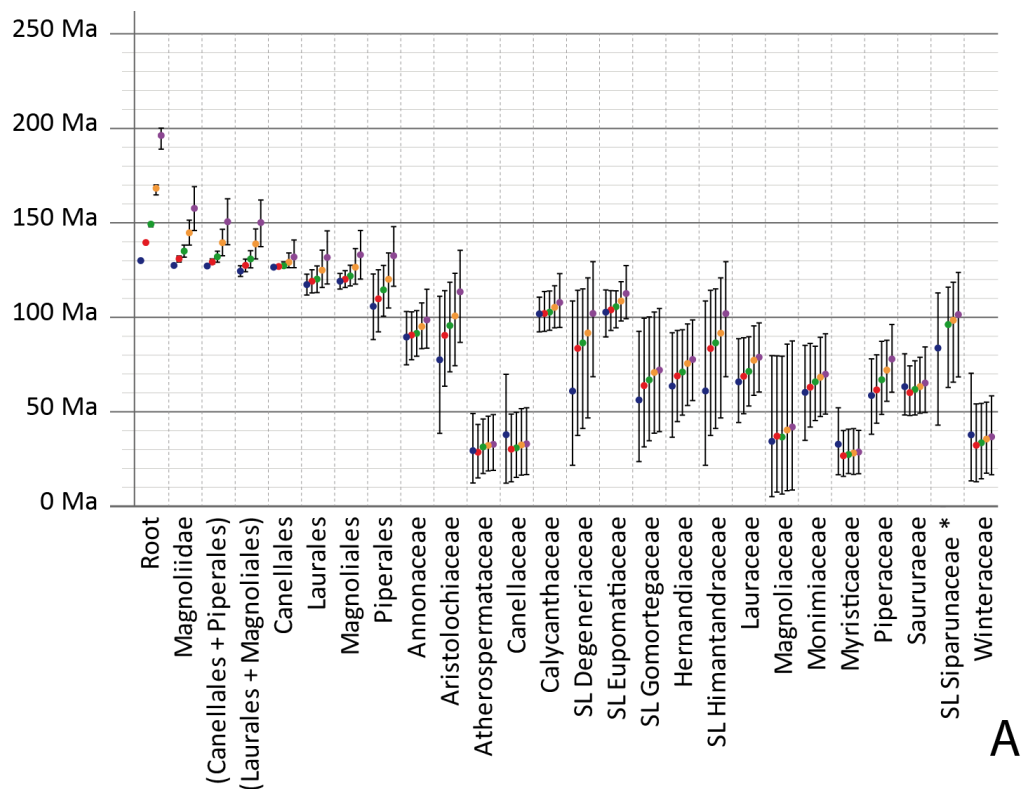
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Figures



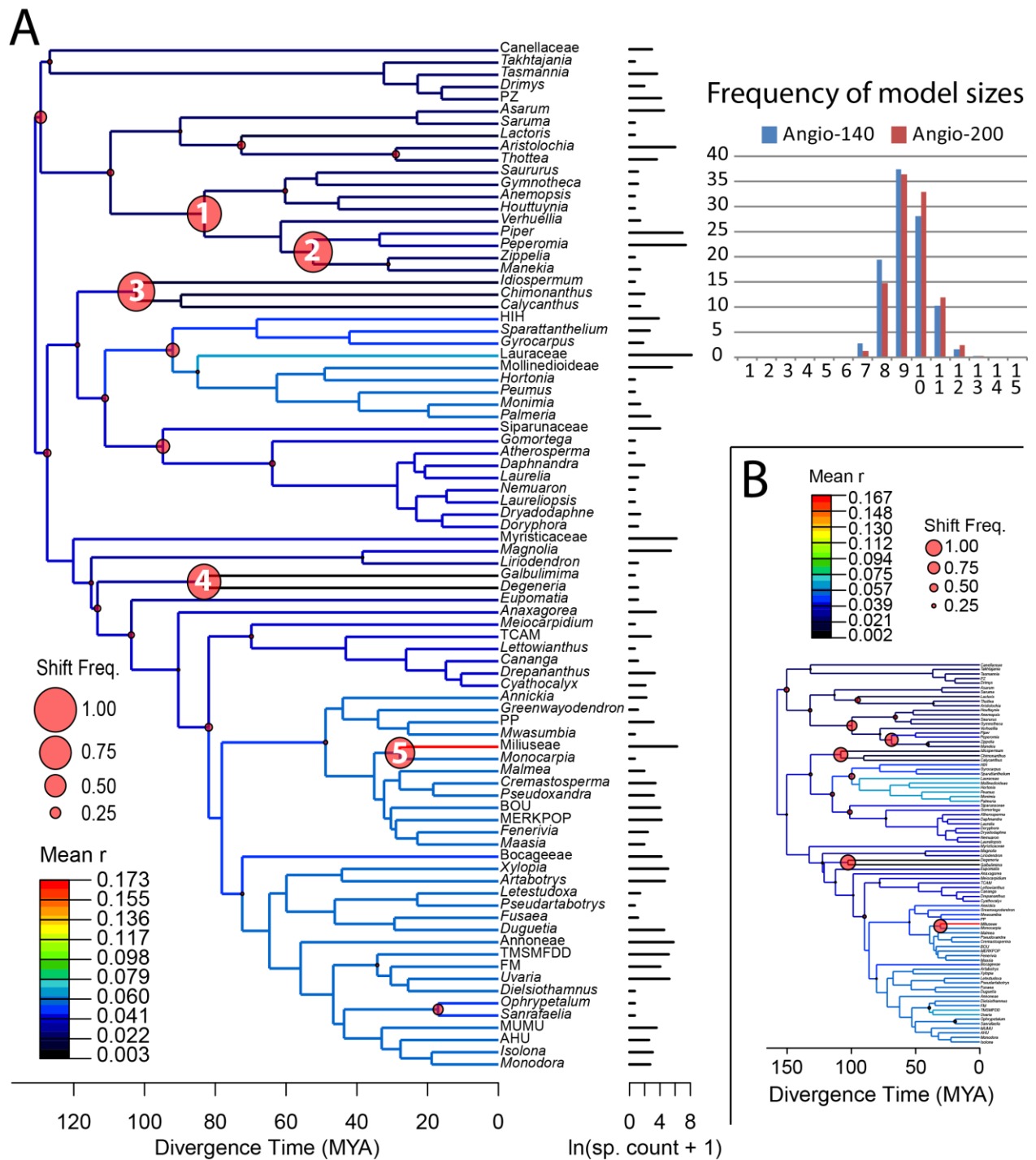
(Continued overleaf)

Figure 1: Maximum clade credibility tree obtained with BEAST and the maximum age constraint for the crown node angiosperms set to 140 million years. Node bars are 95% credibility intervals. Blue dots symbolize minimum age constraints, and green dots the maximum age constraints applied to crown eudicots and crown angiosperms. The geologic time scale follows Gradstein et al. (2012). Abbreviations: Oligo., Oligocene; Mio., Miocene; Plio., Pliocene; Wintera., Winteraceae; Canella., Canellaceae; Aristolo., Aristolochiaceae (incl. *Lactoris*); Saurura., Saururaceae; Pipera., Piperaceae; Calycan., Calycanthaceae; Atheros., Atherospermataceae; Hernan., Hernandiaceae; Laura., Lauraceae; Moni., Monimiaceae; Myris., Myristicaceae; Dege., Degeneriaceae; Himan., Himantandraceae; Magno., Magnoliaceae; Eupo., Eupomatiaceae; Anno., Annonaceae.



(Continued overleaf)

Figure 2: Mean ages and 95% credibility intervals estimated for the root (constrained with a maximum age) and different nodes within the tree of Magnoliidae. Names of families refer to the crown node ages except if SL (stem lineage) is mentioned. Colors correspond to the five different maximum age constraints applied to the root of trees: blue, 130 million years; red, 140 million years; green, 150 million years; orange, 170 million years; violet, 200 million years. A, BEAST analyses. B, r8s analyses. *The stem lineage of Siparunaceae, in the BEAST analysis with the maximum age of angiosperms set to 140 million years, did not correspond to the same node in other analyses. Abbreviation: Ma, megaannum; SL, stem lineage.



(Continued overleaf)

Figure 3: Results of the MEDUSA analyses obtained using 1000 chronograms randomly sampled from the BEAST posterior: A, angio-140, B, angio-200. Topologies are the maximum clade credibility tree of the 1000 trees used, the relationships between both are identical. Names of leaves refer to terminal compartments defined to conduct these analyses. Branch colors illustrate the mean of net diversification rate (r). Red dots denote significant shifts in r , their size being proportional to their frequency among the 1000 trees tested. Numbered shifts in Figure 3a: 1, crown node of the clade of Piperaceae and Saururaceae; 2, crown node of the clade of Piperaceae excluding *Verhuellia*; 3, crown node of Calycanthaceae; 4, crown node of the clade of Himantandraceae and Degeneriaceae; 5, crown node of the clade of Miliuseae and *Monocarpia*. The diagram in the top-right corner represents the frequency of the different model sizes in the 1000-model collections for the two analyses (angio-140 and angio-200). Abbreviation: sp., species.

Tables

Table 1: Fossil species used to define the calibration scheme presented in Massoni et al. (2014a).

Fossil	Age	Node
<i>Archaeanthus linnenbergeri</i>	96.5 Ma	Crown-group Magnoliineae
<i>Cohongarootonia hispida</i>	107.7 Ma*	crown-group core Laurales
<i>Endressinia brasiliana</i>	113 Ma*	crown-group Magnoliineae
<i>Jerseyanthus calycanthoides</i>	86.3 Ma*	crown-group Calycanthoideae
<i>Lovellea wintonensis</i>	100.5 Ma	crown-group Laurales
<i>Mauldinia mirabilis</i>	95.5 Ma	crown-group core Laurales
<i>Saururus tuckerae</i>	45 Ma*	stem node of extant <i>Saururus</i>
<i>Schenkeriphyllum glanduliferum</i>	113 Ma*	crown-group Magnoliineae
<i>Virginianthus calycanthoides</i>	107.7 Ma*	crown-group Laurales
<i>Walkeripollis gabonensis</i>	126.3 Ma*	crown-group Canellales

*Date effectively used as calibration (several fossils with different ages could calibrate the same node). Abbreviation: Ma, million anni.

Table 2: Age estimates for several nodes of Magnoliidae.

All the age estimates are in million years. Estimates presented are 95% credibility intervals, followed by mean estimates in brackets. Bold numbers are from the BEAST analyses, and plain numbers are from the r8s analyses. Abbreviations: angio-130, angio-140, angio-150, angio-170, and angio-200 correspond to the different maximum age constraints applied to the root (130, 140, 150, 170, and 200 million years respectively). The names of taxa refer to their crown node except if SL (stem lineage) is mentioned. *The stem lineage of Siparunaceae, in the BEAST analysis with the maximum age of angiosperms set to 140 million years, did not correspond to the same node in other analyses.

Node	Angio 130	Angio 140	Angio 150	Angio 170	Angio 200
	95% CI (mean)	95% IC (mean)	95% CI (mean)	95% CI (mean)	95% CI (mean)
Root	129.83-130.10 (130.01) 130.00-130.00 (130.00)	138.94-140.10 (139.7) 140.00-140.00 (140.00)	147.88-150.10 (149.33) 150.00-150.00 (150.00)	164.78-170.10 (168.28) 170.00-170.00 (170.00)	188.96-200.10 (196.22) 200.00-200.00 (200.00)
Magnoliidae	127.14-127.82 (127.45) 127.30-127.67 (127.48)	129.29-132.50 (130.89) 131.32-132.80 (131.89)	131.81-138.21 (134.93) 137.01-139.45 (137.88)	138.22-151.45 (144.65) 151.16-155.23 (152.71)	145.93-169.14 (157.76) 172.56-178.89 (174.51)
(Canellales + Piperales)	126.87-127.45 (127.14) 127.06-127.44 (127.25)	128.09-130.90 (129.34) 130.20-131.80 (130.89)	129.09-135.03 (131.96) 134.91-137.73 (136.05)	132.56-146.55 (139.46) 147.51-152.55 (149.45)	138.50-162.73 (150.52) 167.68-175.70 (169.74)
(Laurales + Magnoliales)	121.70-127.26 (124.65) 125.50-126.64 (126.08)	124.18-130.78 (127.53) 129.46-131.41 (130.28)	126.20-135.23 (130.77) 135.11-137.90 (136.10)	130.96-146.82 (138.84) 149.36-153.68 (150.54)	137.40-162.10 (150.20) 170.33-176.97 (171.85)
Canellales	126.30-126.56 (126.39) 126.30-126.30 (126.30)	126.30-127.68 (126.78) 126.30-126.30 (126.30)	126.30-129.32 (127.37) 126.30-126.3 (126.30)	126.30-134.07 (129.09) 126.30-126.30 (126.30)	126.30-141 (132) 126.30-138.54 (129.77)
Laurales	111.80-122.82 (117.27) 117.08-120.09 (118.48)	112.94-125.15 (118.89) 121.72-125.02 (123.06)	113.09-127.22 (120.35) 126.59-130.68 (128.05)	115.75-135.63 (125.00) 138.53-144.67 (140.30)	117.66-145.79 (131.66) 156.68-165.59 (158.95)
Magnoliales	114.98-123.24 (119.13) 116.29-119.09 (117.68)	115.82-124.67 (120.08) 117.91-122.47 (119.30)	116.56-127.6 (121.85) 122.73-128.36 (124.51)	117.52-136.41 (126.52) 134.86-142.28 (136.98)	120.18-145.96 (133.01) 154.65-164.21 (156.79)
Piperales	88.24-122.91 (105.84) 113.69-118.15 (115.90)	92.35-125.12 (109.77) 115.13-120.61 (117.79)	100.53-127.52 (114.55) 119.23-125.23 (122.29)	104.93-134.06 (119.99) 130.86-138.24 (134.13)	116.43-147.94 (132.50) 147.81-157.72 (151.68)
Annonaceae	74.92-103.06 (89.57) 79.71-87.71 (83.26)	77.58-102.88 (90.52) 86.12-95.11 (87.71)	79.44-103.55 (91.53) 89.63-99.73 (91.48)	83.4-107.58 (95.09) 95.37-109.04 (98.59)	83.75-114.89 (98.61) 111.08-126.71 (114.03)
Aristolochiaceae	38.57-111.18 (77.65) 103.16-110.51 (106.84)	63.57-114.1 (90.42) 105.01-113.28 (109.00)	71.17-118.55 (95.57) 108.99-117.55 (113.20)	74.42-123.27 (100.76) 119.43-129.59 (123.75)	86.79-135.45 (113.46) 134.51-147.31 (140.03)

Node	Angio 130	Angio 140	Angio 150	Angio 170	Angio 200
	95% CI (mean)	95% IC (mean)	95% CI (mean)	95% CI (mean)	95% CI (mean)
Atherospermataceae	12.41-49.07 (29.45) 35.80-52.56 (44.75)	15.11-43.29 (28.56) 51.23-72.41 (63.06)	17.37-46.2 (31.43) 55.39-76.63 (65.72)	18.79-47.75 (32.30) 56.26-81.15 (69.10)	18.98-48.54 (32.84) 65.06-93.82 (79.43)
Canellaceae	12.32-69.87 (38.02) 24.44-33.44 (29.05)	13.07-48.86 (30.11) 31.00-43.46 (36.23)	15.35-49.73 (30.92) 31.09-42.86 (36.30)	16.49-51.71 (32.41) 29.49-39.70 (34.36)	16.77-52.13 (32.99) 31.55-43.53 (36.23)
Calycanthaceae	92.28-110.69 (101.85) 94.37-100.61 (97.58)	92.57-113.63 (102.18) 96.71-103.89 (100.27)	93.20-113.94 (102.73) 97.95-106.14 (102.19)	94.48-116.71 (105.15) 100.51-111.40 (105.74)	94.68-123.14 (107.86) 105.32-120.05 (112.28)
SL Degeneriaceae	21.72-108.61 (61.04) 89.94-106.44 (97.36)	37.5-114.34 (83.51) 98.13-110.27 (103.12)	41.19-115.04 (86.51) 102.26-115.16 (107.61)	46.74-120.85 (91.67) 109.78-126.49 (116.46)	68.56-129.44 (102.01) 127.33-146.37 (134.17)
SL Eupomatiaceae	89.64-114.43 (102.7) 102.00-107.03 (104.66)	93.06-114.09 (104.09) 104.40-110.97 (105.92)	94.53-114.02 (105.48) 108.66-116.45 (110.46)	98.07-118.88 (108.63) 117.32-128.56 (120.42)	99.27-127.35 (112.58) 135.75-148.38 (138.49)
SL Gomortegaceae	23.72-92.55 (56.25) 68.29-95.47 (82.03)	31.42-99.6 (63.89) 95.91-110.84 (102.37)	34.69-100.19 (66.72) 100.49-115.94 (106.59)	38.77-102.8 (70.88) 106.22-127.16 (114.32)	39.52-104.64 (72.11) 123.00-146.42 (130.51)
Hernandiaceae	36.52-91.83 (63.67) 80.36-99.62 (89.65)	44.88-93.13 (69.1) 81.68-104.5 (93.19)	48.24-93.40 (71.09) 84.74-108.65 (96.92)	53.42-96.52 (75.55) 95.81-121.90 (106.12)	55.98-98.64 (77.75) 107.53-138.93 (120.24)
SL Himantandraceae	21.72-108.61 (61.04) 89.94-106.44 (97.36)	37.5-114.34 (83.51) 98.13-110.27 (103.12)	41.19-115.04 (86.51) 102.26-115.16 (107.61)	46.74-120.85 (91.67) 109.78-126.49 (116.46)	68.56-129.44 (102.01) 127.33-146.37 (134.17)
Lauraceae	44.34-88.68 (65.87) 92.21-102.54 (96.65)	48.95-89.21 (68.78) 101.27-110.75 (105.32)	53.1-89.69 (71.45) 105.58-115.53 (109.55)	58.79-95.42 (77.3) 115.52-128.31 (118.94)	60.45-97.11 (78.78) 131.25-147.2 (135.27)
Magnoliaceae	5.17-79.66 (34.38) 39.54-60.41 (50.02)	7.59-79.64 (37.11) 65.68-90.57 (75.70)	6.59-79.40 (36.71) 69.00-94.87 (79.37)	8.25-85.81 (40.44) 65.66-96.85 (79.31)	8.66-87.51 (41.90) 78.19-114.76 (94.02)
Monimiaceae	34.93-85.11 (60.32) 77.32-90.74 (83.22)	42.00-86.21 (63.11) 94.90-107.87 (100.81)	45.36-84.72 (65.67) 99.88-112.43 (104.99)	47.48-89.50 (68.35) 102.59-123.44 (106.12)	48.87-91.30 (69.90) 118.37-143.31 (125.75)
Myristicaceae	16.71-52.16 (32.88) 12.00-18.12 (15.53)	15.89-40.16 (26.66) 9.92-16.10 (13.22)	17.39-40.88 (27.58) 10.23-16.68 (13.78)	16.89-41.09 (28.14) 12.61-19.60 (16.12)	17.33-40.23 (28.75) 13.95-22.20 (18.03)
Piperaceae	38.14-78.09 (58.57) 62.19-70.33 (65.88)	43.96-80.24 (61.66) 56.92-66.35 (61.50)	48.64-87.38 (66.97) 58.90-68.72 (63.59)	55.60-87.87 (71.98) 65.71-75.64 (69.94)	60.41-96.19 (78.08) 72.45-84.43 (77.53)
Saururaee	48.39-80.74 (63.33) 48.81-54.98 (51.65)	48.12-74.33 (60.17) 50.47-58.64 (54.09)	48.41-77.02 (61.82) 51.31-60.02 (54.94)	49.28-78.89 (63.35) 51.32-60.89 (55.47)	49.68-84.34 (65.18) 54.19-66.08 (58.65)

	Angio 130	Angio 140	Angio 150	Angio 170	Angio 200
Node	95% CI (mean)	95% IC (mean)	95% CI (mean)	95% CI (mean)	95% CI (mean)
	43.02-112.92	X-X	62.92-115.96	65.67-118.55	68.52-123.83
	(83.64)	(X)*	(96.29)	(98.46)	(101.53)
SL Siparunaceae	101.16-112.35	110.63-119.41	115.37-124.66	125.48-137.19	142.68-157.71
	(107.06)	(114.63)	(119.27)	(130.05)	(147.59)
	13.52-70.38	13.06-54.20	14.59-54.50	17.63-55.13	16.73-58.49
	(37.81)	(32.43)	(33.70)	(35.48)	(36.70)
Winteraceae	28.30-37.53	39.82-57.09	39.90-56.34	35.30-48.18	38.50-52.83
	(33.35)	(47.68)	(47.55)	(41.73)	(44.15)

Appendix S1

This appendix provides tables justifying the monophyly and the number of species within compartments. In addition, a text provides all justifications for the definition of supra-generic compartments, for incorporation in the diversification analyses of the missing diversity not sampled in our chronograms, and for ignoring a part of this diversity. Figure S1 illustrates the present appendix. The posterior probabilities presented in this figure are similar in all other BEAST analyses.

Annonaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
AHU	<i>Asteranthe</i>	2	(Chatrou et al. 2012)	12	(Couvreur et al. 2008)/ (Chatrou et al. 2012) / (Massoni et al. 2014)
	<i>Hexalobus</i>	5	(Chatrou et al. 2012)		
	<i>Uvariastrum</i>	5	(Couvreur 2014)		
<i>Anaxagorea</i>	-	30	(Chatrou et al. 2012)	30	(Scharaschkin and Doyle 2005)
<i>Annickia</i>	-	8	(Chatrou et al. 2012)	8	(Pirie et al. 2005) / (Couvreur et al. 2009)
Annoneae	<i>Annona</i>	162	(Chatrou et al. 2012)	321	(Richardson et al. 2004)/ (Chatrou et al. 2012) / (Massoni et al. 2014)
	<i>Anonidium</i>	4	(Chatrou et al. 2012)		

	<i>Asimina</i> (including <i>Deeringothamus</i>)	7	(Chatrou et al. 2012)		
	<i>Disepalum</i>	9	(Chatrou et al. 2012)		
	<i>Goniothalamus</i>	134	(Chatrou et al. 2012)		
	<i>Neostenanthera</i>	5	(Fero et al. 2014)		
<i>Artabotrys</i>	-	102	(Chatrou et al. 2012)	102	(Thongpairaj 2008)
Bocageae	<i>Bocagea</i>	2	(Chatrou et al. 2012)	65	(Chatrou et al. 2012) / (Massoni et al. 2014)
	<i>Cardiopetalum</i>	3	(Chatrou et al. 2012)		
	<i>Cymbopetalum</i>	27	(Chatrou et al. 2012)		
	<i>Froesiodendron</i>	3	(Chatrou et al. 2012)		
	<i>Hornschuchia</i>	10	(Chatrou et al. 2012)		
	<i>Mkilua</i>	1	(Chatrou et al. 2012)		
	<i>Porcelia</i>	7	(Chatrou et al. 2012)		
	<i>Trigynaea</i>	12	(Chatrou et al. 2012)		
BOU	<i>Bocageopsis</i>	4	(Chatrou et al. 2012)	54	(Richardson et al. 2004)/ (Chatrou et al. 2012) / (Xue et al. 2012)/

					(Massoni et al. 2014)
	<i>Onychopetalum</i>	2	(Chatrou et al. 2012)		
	<i>Unonopsis</i>	48	(Chatrou et al. 2012)		
<i>Cananga</i>	-	2	(Chatrou et al. 2012)	2	(Surveswaran et al. 2010)
<i>Crematosperma</i>	-	29	(Chatrou et al. 2012)	29	(Pirie et al. 2005)
<i>Cyathocalyx</i>	-	7	(Chatrou et al. 2012)	7	(Surveswaran et al. 2010)
<i>Dielsiothamnus</i>	-	1	(Chatrou et al. 2012)	1	-
<i>Drepananthus</i>	-	26	(Chatrou et al. 2012)	26	(Surveswaran et al. 2010)
<i>Duguetia</i>	-	93	(Chatrou et al. 2012)	93	(Pirie et al. 2005)
<i>Fenerivia</i>	-	10	(Chatrou et al. 2012)	10	(Saunders et al. 2011)
FM	<i>Fissistigma</i>	48	(Chatrou et al. 2012)	56	(Chatrou et al. 2012) / (Massoni et al. 2014)
	<i>Mitrella</i>	8	(Chatrou et al. 2012)		
<i>Fusaea</i>	-	2	(Chatrou et al. 2012)	2	(Chatrou et al. 2012)
<i>Greenwayodendron</i>	-	2	(Chatrou et al. 2012)	2	(Pirie et al. 2006)
<i>Guatteria</i>	-	210	(Chatrou et al. 2012)	210	(Erkens et al. 2007)

<i>Isolona</i>	-	20	(Chatrou et al. 2012)	20	(Couvreur 2009)
<i>Letestudoxa</i>	-	3	(Chatrou et al. 2012)	3	(Richardson et al. 2004)
<i>Lettowianthus</i>	-	1	(Chatrou et al. 2012)	1	-
<i>Maasia</i>	-	6	(Chatrou et al. 2012)	6	(Saunders et al. 2011)
<i>Malmea</i>	-	6	(Chatrou et al. 2012)	6	(Pirie et al. 2006)
<i>Meiocarpidium</i>	-	1	(Chatrou et al. 2012)	1	-
MERKPOP	<i>Ephedranthus</i>	6	(Chatrou et al. 2012)	66	(Couvreur et al. 2009) / (Pirie and Doyle 2012) / (Xue et al. 2012) / (Massoni et al. 2014)
	<i>Klarobelia</i>	12	(Chatrou et al. 2012)		
	<i>Mosannonna</i>	14	(Chatrou et al. 2012)		
	<i>Oxandra</i>	28	(Chatrou et al. 2012)		
	<i>Pseudephedranthus</i>	1	(Chatrou et al. 2012)		
	<i>Pseudomalmea</i>	4	(Chatrou et al. 2012)		
	<i>Ruizodendron</i>	1	(Chatrou et al. 2012)		
Miliuseae	<i>Alphonsea</i>	25	(Chatrou et al.	510	(Chatrou et al. 2012) /

		2012)	(Xue et al. 2012) / (Massoni et al. 2014)
<i>Desmopsis</i>	14	(Chatrou et al. 2012)	
<i>Enicosanthum</i>	18	(Chatrou et al. 2012)	
<i>Haplostichanthus</i>	11	(Chatrou et al. 2012)	
<i>Marsypopetalum</i>	6	(Chatrou et al. 2012)	
<i>Meiogyne</i> (including <i>Fitzalania</i>)	17	(Thomas et al. 2012)	
<i>Miliusa</i>	50	(Chatrou et al. 2012)	
<i>Mitrephora</i>	47	(Chatrou et al. 2012)	
<i>Neo-uvaria</i>	5	(Chatrou et al. 2012)	
<i>Orophea</i>	50	(Chatrou et al. 2012)	
<i>Phaeanthus</i>	9	(Chatrou et al. 2012)	
<i>Platymitra</i>	2	(Chatrou et al. 2012)	
<i>Polyalthia</i>	135	(Chatrou et al. 2012)	
<i>Popowia</i>	26	(Chatrou et al. 2012)	
<i>Pseuduvaria</i>	57	(Chatrou et al. 2012)	

	<i>Sageraea</i>	9	(Chatrou et al. 2012)		
	<i>Sapranthus</i>	6	(Chatrou et al. 2012)		
	<i>Stelechocarpus</i>	3	(Chatrou et al. 2012)		
	<i>Stenanona</i>	14	(Chatrou et al. 2012)		
	<i>Tridimeris</i>	1	(Chatrou et al. 2012)		
	<i>Trivalvaria</i>	4	(Chatrou et al. 2012)		
	<i>Woodiellantha</i>	1	Chatrou et al., 2012		
<i>Monocarpia</i>	-	1	(Chatrou et al. 2012)	1	-
<i>Monodora</i>	-	14*	(Chatrou et al. 2012)	14*	(Couvreur 2009)
MUMU	<i>Mischogyne</i>	2	(Chatrou et al. 2012)	34	(Couvreur et al. 2008) / (Chatrou et al. 2012) / (Massoni et al. 2014)
	<i>Monocyclanthus</i>	1	(Chatrou et al. 2012)		
	<i>Uvariadendron</i>	15	(Chatrou et al. 2012)		
	<i>Uvariopsis</i>	16	(Chatrou et al. 2012)		
<i>Mwasumbia</i>	-	1	(Chatrou et al. 2012)	1	-
<i>Ophrypetalum</i>	-	1	(Chatrou et al. 2012)	1	-

				et al. 2012)	
PP	<i>Piptostigma</i>	14	(Chatrou et al. 2012)	22	(Couvreur et al. 2009)
	<i>Polyceratocarpus</i>	8	(Chatrou et al. 2012)		
<i>Pseudartabotrys</i>	-	1	(Chatrou et al. 2012)	1	-
<i>Pseudoxandra</i>	-	23	(Chatrou et al. 2012)	23	(Pirie et al. 2006)
<i>Sanrafaelia</i>	-	1	Chatrou et al., 2012	1	-
TCAM	<i>Ambavia</i>	2	(Chatrou et al. 2012)	15	(Chatrou et al. 2012) / (Massoni et al. 2014)
	<i>Cleistopholis</i>	4	(Chatrou et al. 2012)		
	<i>Mezzettia</i>	3	(Chatrou et al. 2012)		
	<i>Tetrameranthus</i>	6	(Chatrou et al. 2012)		
TMSMFDD	<i>Dasymaschalon</i>	21	(Chatrou et al. 2012)	171	(Chatrou et al. 2012) / (Massoni et al. 2014)
	<i>Desmos</i>	26	(Chatrou et al. 2012)		
	<i>Friesodielsia</i>	51	(Chatrou et al. 2012)		
	<i>Melodorum</i>	10	(Chatrou et al. 2012)		
	<i>Monanthotaxis</i>	56	(Chatrou et al. 2012)		

	<i>Sphaerocoryne</i>	3	(Chatrou et al. 2012)		
	<i>Toussaintia</i>	4	(Chatrou et al. 2012)		
<i>Uvaria</i>	-	187	(Chatrou et al. 2012)	187	(Zhou et al. 2012)
<i>Xylopia</i>	-	157	(Chatrou et al. 2012)	157	(Richardson et al. 2004)

* This is a mistake there are 16 species. Not corrected for the present manuscript but must be for the submission.

Ignored diversity

Ignored genus	Nb. of sp. in genus	Ref. for sp. content
<i>Duckeanthus</i>	1	(Chatrou et al. 2012)
<i>Boutiquea</i>	1	(Chatrou et al. 2012)
<i>Diclinanona</i>	3	(Chatrou et al. 2012)
<i>Afroguatteria</i>	2	(Chatrou et al. 2012)
<i>Cleistochlamys</i>	1	(Chatrou et al. 2012)
<i>Exellia</i>	1	(Chatrou et al. 2012)
<i>Gilbertiella</i>	1	(Chatrou et al. 2012)
<i>Pyramidanthe</i>	1	(Chatrou et al. 2012)
<i>Schefferomitra</i>	1	(Chatrou et al. 2012)
<i>Dendrokingstonia</i>	2	(Chatrou et al. 2012)
<i>Oncodostigma</i>	2	(Chatrou et al. 2012)
<i>Phoenicanthus</i>	2	(Chatrou et al. 2012)

Among the 108 putatively monophyletic genera of Annonaceae recognized in the recent phylogenetic classification of Chatrou et al. (2012), 19 were not sampled in our dataset, and for several their monophyly has not been tested yet. In tribe Ambavioideae, the monophyly of the *Ambavia*, *Cleistopholis*, and *Mezzetia* has not been tested yet. For this reason, we defined the compartment TCAM to include these three genera as well as *Tetrameranthus*. The monophyly of the resulting compartment is well supported in the literature (Richardson et al. 2004; Chatrou et al. 2012; Massoni et al. 2014) and is supported by 100% of PP in our BEAST analyses (Figure S1).

Within tribe Bocageae, genera *Bocagea*, *Cardiopetalum*, and *Froesiodendron* have never been included in phylogenetic analyses. However, synapomorphies of tribe Bocageae occur in these three genera, which led Chatrou et al. (2012) to argue that they could be securely placed in this monophyletic group. We followed their point of view by incorporating these three genera within one compartment corresponding to the entire tribe Bocageae. We did not exclude this diversity because it represents 12 % of the diversity of the defined compartment.

In tribe Duguetieae, *Duckeanthus* has been only included in a morphological cladistic analysis (Chatrou et al. 2000). In their results, the relationships among the remaining genera of the tribe were not in accordance with those supported in recent molecular analyses (Chatrou et al. 2012; Massoni et al. 2014). Because this genus incorporates only one species, more investigations are needed to confirm its position within the tribe, and a conservative compartment would incorporate more than 99 species (the number of species in Duguetieae), we decided to ignore this single, unplaced species in our analyses.

In tribe Annoneae, the monophyly of *Neostenanthera* and *Asimina* has not been tested before. Consequently, we treated Annoneae as a single compartment sampled in our analyses. This supra-generic compartment is well supported in the literature (Richardson et al. 2004; Chatrou et al. 2012; Massoni et al. 2014) and in our BEAST analyses (Figure S1). The genus *Disepalum* was not sampled in our dataset used for molecular dating. The position of this genus as a sister group to *Asimina* is well supported in the literature (Chatrou et al. 2012). For this reason we have included the species of this genus in the diversity of our compartment Annoneae. *Boutiquea* was included in tribe Annoneae by Chatrou et al. (2012) based on palynological characters, but its phylogenetic position has not been tested in the literature. Because there is only one species in this genus, and its placement has never been tested, we preferred to exclude it. The genus *Diclinanona*, also placed in Annoneae, is not included in our molecular dating analyses. Its phylogenetic position is debated in the literature (Richardson et al. 2004; Erkens et al. 2009). Chatrou et al. (2012) has adhered to the results of Richardson et al. (2004). According to this latter study, a conservative definition for a compartment incorporating this genus would be all Annonoideae except Bocageae. Because this compartment would include 1393 species and *Diclinanona* presents

only three species, we have decided to exclude this genus from our analyses. Because the genus *Deeringothamnus* is considered in the study of Chatrou et al. (2012) as a synonym of *Asimina*, its diversity is included in the count of species of this latter genus.

In tribe Monodoreae, the monophyly of the genus *Mischogyne* has not been previously tested. For that reason we defined a compartment, MUMU (Figure S1) to include this genus and its sister group made up of *Uvariadendron*, *Monocyclanthus*, and *Uvariopsis*. This clade is well supported in the literature (Couvreur et al. 2008; Chatrou et al. 2012; Massoni et al. 2014) and received a support value of 100 % of PP in BEAST analyses (Figure S1). In addition, the monophyly of *Asteranthe* has never been tested either. We defined a supra-generic compartment, AHU, to accommodate this genus and its sister group, the clade of *Hexalobus* plus *Uvariastrum* (Figure S1). This monophyletic group is well supported in the literature (Couvreur et al. 2008; Chatrou et al. 2012; Massoni et al. 2014) and in the present study (Figure S1).

In tribe Uvarieae, the monophyly of *Mitrella* and *Toussaintia* has not been tested yet. For this reason, we created two compartments, one including *Mitrella* and its sister group *Fissistigma* (FM), and another including *Toussaintia* and its sister group made up of *Melodorum*, *Sphaerocoryne*, *Monanthotaxis*, *Friesodielsia*, *Dasymaschalon*, *Desmos* (TMSMFDD, Figure S1). Both clades received 100 % of posterior probability in BEAST analyses (Figure S1) and have been well supported in the literature (Chatrou et al. 2012; Massoni et al. 2014). *Afroguatteria*, including two species has never been included in a molecular phylogenetic analysis (Chatrou et al. 2012). Doyle & Le Thomas (1996) placed this genus as the sister group to *Uvaria* in a morphological cladistic analysis in which relationships among genera of Annonaceae were not compatible with the current phylogeny of the group. However, because a secure placement of the two species of this genus needs more investigation and the genus *Uvaria* contains 187 species, we have ignored *Afroguatteria* from our analyses. *Cleistochlamys* and *Gilbertiellia* have never been included in a phylogenetic analysis and their placement within Annonoideae is based on an intuitive approach (Chatrou et al. 2012). Because both of them are monotypic genus and represent less than three percent of any secure compartments in which we could incorporate their diversity, we have ignored them from our analyses. Because the monotypic genus

Schefferomitra has not been placed in a published phylogeny, we have preferred to exclude this genus from our analyses. *Exellia*, not included in our molecular dating analyses, has been placed in an unresolved position in Uvarieae (Chatrou et al. 2012). Because this is a monospecific genus we decided to exclude it. *Pyramidanthe* has been placed with a phylogenetic approach in a clade with *Dasymaschalon*, *Desmos*, *Dielsiothalamnus*, *Fissistigma*, *Friesodielsia*, *Mitrella*, *Monanthotaxis*, *Sphaerocoryne*, *Toussaintia* and *Uvaria*, but the relationships within the clade remain poorly resolved in their study (Zhou et al. 2010). Because the incorporation of this monospecific genus would need a conservative compartment consisting of all Uvarieae (at least 415 species), we ignored it.

In tribe Piptostigmateae, *Piptostigma* was shown to be paraphyletic with respect to *Polyceratocarpus* (Couvreur et al. 2009), even though Chatrou et al. (2012) provisionally maintained the two genera. We defined a compartment including the diversity of the two genera (PP, Figure S1).

In tribe Malmeeae the three genera *Bocageopsis*, *Onychopetalum*, and *Unonopsis* form a clade (PP=100 % in the present study, Figure S1), but the relationships among them are not well established (Richardson et al. 2004; Chatrou et al. 2012; Xue et al. 2012; Massoni et al. 2014). We defined a compartment including these three genera (BOU, Figure S1) because the monophyly of *Onychopetalum* has never been tested. In the same tribe, the monophyly of *Pseudomalmea* has never been tested either and its relationships are not clear in the present study. In addition, *Oxandra* has been shown to be polyphyletic even though the support values associated are low (Pirie et al. 2006; Chatrou et al. 2012). The least inclusive clade containing all species of *Oxandra* that is well supported includes *Mosannona*, *Ruizodendron*, *Ephedranthus*, *Klarobelia*, *Pseudephedranthus*, and *Pseudomalmea* (Couvreur et al. 2009; Chatrou et al. 2012; Xue et al. 2012; Massoni et al. 2014; Figure S1). For this reason, we decided to define a compartment including these seven genera (MERKPOP).

Tribe Dendrokingstonieae consists in one genus, *Dendrokingstonia* (Chatrou et al. 2012), not sampled in our dataset. Although Chatrou et al. (2012) referred to a phylogenetic placement by Chaowasku et al. (2012), the methodology used in the latter paper is not clear enough to

evaluate the quality of this placement. For this reason and because this genus only includes two species, we decided to exclude it from our analyses.

In tribe Miliuseae, *Fitzalania* was recognized by Chatrou et al. (2012), however a molecular phylogenetic study by Thomas et al. (2012) supports a position for this genus nested in *Meiogyne*. Consequently, we included the diversity of *Fitzalania* in *Meiogyne*. In Xue et al. (2012), *Polyalthia*, *Enicosanthum*, and *Haplostignanthes* are not monophyletic. The smallest, well supported clade including all the species of these genera also includes all genera of Miliuseae (Xue et al. 2012). This tribe is well supported in the literature (Chatrou et al. 2012; Massoni et al. 2014) and in the present study (Figure S1). We defined a compartment including all the genera of tribe Miliuseae. The monotypic genus *Woodiellantha*, not sampled in our chronograms, was found to be nested in *Orophea* with good support by Chatrou et al. (2012) and Richardson et al. (2004). In a more recent study, this taxon was placed in a well-supported clade including several species of *Polyalthia* and *Enicosanthum* (Xue et al. 2012). We have counted this species in the compartment Miliuseae (Figure S1). The taxonomic status of *Oncodostigma* as an accepted genus or a synonym of *Meiogyne* is not clear and needs further clarification (Chatrou et al. 2012). In their classification, Chatrou et al. (2012) did not specify whether the species diversity of this genus was included or not in the count of species of *Meiogyne*. For this reason and because the genus includes only two species (out of 510 species in our compartment Miliuseae), we have ignored this genus from our analyses. Last, *Phoenicanthus* has never been included in any phylogenetic analysis. For this reason and because there are only two species in this genus, we have also ignored it from our analyses.

Aristolochiaceae + Hydnoraceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Aristolochia</i>	-	400	(Ohi-Toma et al. 2006)	400	(Neinhuis et al. 2005)
<i>Asarum</i>	-	90	(Jiang et al. 2011)	90	(Neinhuis et al. 2005)

<i>Lactoris</i>	-	1	(González and Rudall 2001)	1	-
<i>Saruma</i>	-	1	(González and Rudall 2003)	1	-
<i>Thottea</i>	-	35	(Oelschlägel et al. 2011)	35	(Oelschlägel et al. 2011)

Ignored diversity

Ignored genus	Nb. of sp. in genus	Ref. for sp. content
<i>Hydnora</i>	9	(Bolin et al. 2011)
<i>Prosopanche</i>	2	(Ferreira Machado and Paganucci de Queiroz 2012)

All the genera of Aristolochiaceae (incl. *Lactoris*) were sampled in our study. However, Hydnoraceae were excluded (see Molecular dataset section in the materials and methods). Previous studies have placed this parasitic family within Aristolochiaceae (Nickrent et al. 2002; Massoni et al. 2014) and, more recently, Naumann et al. (2013) refined its position as sister to subfamily Aristolochioideae. In order to incorporate the nine species of *Hydnora* (Bolin et al. 2011) and the three species of *Prosopanche* (Ferreira Machado and Paganucci de Queiroz 2012) in our analyses of diversification we would need to define a broader compartment to include Hydnoraceae and Aristolochioideae. The species richness of Hydnoraceae represents less than three percent of this compartment. Consequently, we have decided to exclude the family.

Atherospermataceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Atherosperma</i>	-	1	(Schodde 1969)	1	-
<i>Daphnandra</i>	-	6	(Schodde 1969)	6	(Renner et al. 2000)
<i>Doryphora</i>	-	2	(Schodde 1969)	2	(Renner et al. 2000)
<i>Dryadodaphne</i>	-	3	(Schodde 1969)	3	(Renner et al. 2000)
<i>Laurelia</i>	-	2	(Schodde 1969)	2	(Renner et al. 2000)

					2000)
<i>Laureliopsis</i>	-	1	(Schodde 1969)	1	-
<i>Nemuaron</i>	-	1	(Schodde 1969)	1	-

Calycanthaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Calycanthus</i>	-	3	(Zhou et al. 2006)	3	(Zhou et al. 2006)
<i>Chimonanthus</i>	-	6	(Zhou et al. 2006)	6	(Zhou et al. 2006)
<i>Idiospermum</i>	-	1	(Zhou et al. 2006)	1	-

Canellaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
Canellaceae	<i>Canella</i>	1	(Salazar and Nixon 2008)	18	(Salazar and Nixon 2008) / (Massoni et al. 2014)
	<i>Capsicodendron</i>	1	(Salazar and Nixon 2008)		
	<i>Cinnamodendron</i>	6	(de Barros and Salazar 2009)		
	<i>Cinnamosma</i>	3	(The Plant List 2013)		
	<i>Pleodendron</i>	3	(Hammel and Zamora 2005)		
	<i>Warburgia</i>	4	(The Plant List 2013)		

All genera of the family were sampled in our chronograms. In the phylogenetic analysis of Salazar and Nixon (2008), *Cinnamodendron* was paraphyletic and the monophyly of *Cinnasmoma* was not well supported. In addition, deeper relationships in this family were not well supported in their tree and in present analyses (Figure S1). Therefore we defined a compartment to include all genera of Canellaceae.

Degeneriaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Degeneria</i>	-	2	(Kubitzki 1993a)	2	Present study

Degeneriaceae include a single genus, *Degeneria*, with two species (Kubitzki 1993a). The monophyly of this taxon has been tested with *matK* and *ndhF* markers with the separate molecular datasets of Massoni et al. (2014). Because the genus appears to be monophyletic (J. Massoni, unpubl. data), we have defined *Degeneria* as a terminal compartment.

Eupomatiaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Eupomatia</i>	-	2*	(Endress 2003)	2*	(Kim et al. 2004)

* This is a mistake there are three species. Not corrected for the present manuscript but must be for the submission.

Gomortegaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Gomortega</i>	-	1	(Kubitzki 1993b)	1	-

Hernandiaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
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HIH	<i>Hazolomania</i>	1	(Michalak et al. 2010)	45	(Michalak et al. 2010)
	<i>Hernandia</i>	22	(Michalak et al. 2010)		
	<i>Illigera</i>	22	(Michalak et al. 2010)		
<i>Gyrocarpus</i>	-	5	(Michalak et al. 2010)	5	(Michalak et al. 2010)
<i>Sparattanthelium</i>	-	13	(Michalak et al. 2010)	13	(Michalak et al. 2010)

Hernandiaceae comprise 62 species in five genera (Michalak et al. 2010). The monophyly of the four non-monospecific genera has been well supported in the literature (Michalak et al. 2010). *Hernandia* (22 spp.) was not sampled in our chronograms. In the previous phylogenetic study, the genera *Hernandia*, *Hazolomania*, and *Illigera* were found in a clade, but the relationships among them have not been resolved. In order to include the diversity of *Hernandia*, we defined a compartment including both *Hazolomania* and *Illigera* in which we counted the species diversity of the first genus (HIH).

Himantandraceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. Monophyly compartment
<i>Galbulimima</i>	-	1	(Doweld and Shevryyova 1998)	1	-

Lauraceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
Lauraceae	<i>Actinodaphne</i>	100	(Zhi-Ming et al. 2006)	3469	(Renner 1999) / (Renner and Chanderbali 2000) /

			(Massoni et al. 2014)
<i>Adenodaphne</i>	5	(The Plant List 2013)	
<i>Aiouea</i>	19	(Kubitzki and Renner 1982)	
<i>Alseodaphne</i>	50	(Li et al. 2011)	
<i>Anaueria</i>	1	(van der Werff 1987)	
<i>Aniba</i>	41	(Kubitzki and Renner 1982)	
<i>Apollonias</i>	2	(Li et al. 2011)	
<i>Aspidostemon</i>	28	(van der Werff 2006)	
<i>Beilschmiedia</i>	250	(Nishida 1999)	
<i>Caryodaphnopsis</i>	15	(Liu et al. 2013)	
<i>Cassytha</i>	20	(Rohwer 1993)	
<i>Chlorocardium</i>	2	(Rohwer et al. 1991)	
<i>Cinnadenia</i>	2	(The Plant List 2013)	
<i>Cinnamomum</i>	250	(Ho and Hung 2011)	
<i>Clinostemon</i>	2	(van der Werff 1987)	
<i>Cryptocarya</i>	350	(van der Werff 2008)	
<i>Dehaasia</i>	35	(Li et al. 2011)	
<i>Dicypellium</i>	2	(van der Werff 1991a)	
<i>Dodecadenia</i>	2	(The Plant List 2013)	
<i>Endiandra</i>	129	(The Plant List 2013)	
<i>Endlicheria</i>	60	(Chanderbali 2004)	
<i>Eusideroxylon</i>	1	(Kimoto et al. 2006)	
<i>Gamanthera</i>	1	(van der Werff 1991a)	
<i>Hexapora</i>	1	(Chanderbali et al. 2001)	
<i>Hypodaphnis</i>	1	(Rohwer and	

<i>Iteadaphne</i>	2	Rudolph 2005) (Li and Christophel 2000)
<i>Kubitzkia</i>	2	(van der Werff 1991a)
<i>Laurus</i>	203	(The Plant List 2013)
<i>Licaria</i>	69	(van der Werff 2009)
<i>Lindera</i>	121	(The Plant List 2013)
<i>Litsea</i>	562	(The Plant List 2013)
<i>Machilus</i>	100	(Li et al. 2011)
<i>Mezilaurus</i>	21	(Alves et al. 2012)
<i>Mocinnodaphne</i>	1	(Hernandez 1995)
<i>Nectandra</i>	175	(Wagner de Oliveira et al. 2001)
<i>Neocinnamomum</i>	6	(Wang et al. 2010)
<i>Neolitsea</i>	100	(Li et al. 2007)
<i>Nothaphoebe</i>	40	(Li et al. 2011)
<i>Ocotea</i>	375	Henk van der Werff (personal communication, 2013: 350- 400 species)
<i>Paraia</i>	1	(Rohwer et al. 1991)
<i>Parasassafras</i>	1	(The Plant List 2013)
<i>Persea</i>	90	(Rohwer et al. 2009)
<i>Phoebe</i>	100	(Li et al. 2011)
<i>Phyllostemonodaphne</i>	1	(van der Werff 1991a)
<i>Pleurothyrium</i>	39	(van der Werff 1993)
<i>Potameia</i>	21	(van der Werff 1996)

<i>Potoxylon</i>	1	(Chanderbali et al. 2001)
<i>Povedadaphne</i>	1	(Burger 1988)
<i>Rhodostemona daphne</i>	41	(Madriñán 2004)
<i>Sassafras</i>	3	(Nie et al. 2007)
<i>Sextonia</i>	2	(van der Werff 1997)
<i>Sinapora</i>	1	(Li et al. 2008)
<i>Syndiclis</i>	9	(The Plant List 2013)
<i>Triadodaphne</i>	3	(Chanderbali et al. 2001)
<i>Umbellularia</i>	1	(van der Werff 1991a)
<i>Urbanodendron</i>	3	(van der Werff 1991a)
<i>Williamodendron</i>	3	(van der Werff 1991b)
<i>Yasunia</i>	2	(van der Werff and Nishida 2010)

Lauraceae is the largest family within Magnoliidae, with 2,500 to 3,500 species (Rohwer 1993). The relationships within this clade are still debated in the literature (Rohwer 2000; Chanderbali et al. 2001; Li et al. 2011). However, several clades are well supported. This is the case for the Perseeae-Laureae clade (Chanderbali et al. 2001), and named core Lauraceae by Rohwer and Rudolph (2005), an informal name we adopt here for practical reasons. Within the core Lauraceae, Li et al. (2011) focused on the phylogeny of the *Persea* group as defined by Rohwer et al. (2009). In addition to the paraphyly of *Alseodaphne* and *Dehaasia*, they showed that *Persea* is paraphyletic, with *Apollonias* nested in. In our chronograms, these two genera were sampled and we find them to be nested in a well-supported core Lauraceae clade. However, there is a well-supported conflict among the present study and the literature requiring a larger compartment than core Lauraceae. *Nothaphoebe*, which was nested in this clade in previous studies (Rohwer et al. 2009; Li et al. 2011), was found in a different position in the present study (see also Massoni et al. 2014b). This genus, consisting in about 40 species (Li et al. 2011), has never been included in a phylogenetic analysis of Lauraceae as a whole, and a maximum of two species have been

included together in the same phylogenetic analysis. The phylogenetic placement of *Nothaphoebe umbellifera* and *N. semecarpifolia* were investigated by Rohwer et al. (2009) and Li et al. (2011) sampled the second species. In their results, these two taxa were nested in the core Lauraceae. In the present study and in Massoni et al. (2014b), we used a different species in order to represent the genus (*N. konishii*). Because these were the first introduction of this species in a phylogenetic study and because more than 95 % of the diversity of this genus has never been included in a phylogenetic approach, it is difficult to affirm or disconfirm a misidentification of the taxa used to generate the sequence. Because more investigations are needed to elucidate this question, we preferred to define a conservative compartment including all of the Lauraceae. Indeed, because an infinitesimal part of the species diversity of the Lauraceae has been sampled in previous phylogenetic studies, its distribution in smaller compartments will be problematic.

Magnoliaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Magnolia</i>	-	223	(Kim and Suh 2013)	223	(Kim and Suh 2013)
<i>Liriodendron</i>	-	2	(Sauquet et al. 2003)	2	(Kim and Suh 2013)

Monimiaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Hortonia</i>	-	1	(Renner 2005) / (Renner and Takeuchi 2009)	1	-
Mollinedioideae	<i>Austromatthaea</i>	1	(Sampson 1997) / (Renner and Takeuchi	256	(Renner 1999) / (Renner et al. 2010) /

		2009)	(Massoni et al. 2014)
<i>Decarydendron</i>	4	(Renner et al. 2010)	
<i>Ehippiandra</i>	7	(Renner et al. 2010)	
<i>Faika</i>	1	(Renner and Takeuchi 2009)	
<i>Graziellanthus</i>	1	(Peixoto and Pereira-Moura 2008)	
<i>Hedycarya</i>	15	(Sampson 1997)	
<i>Hemmantia</i>	1	(The Plant List 2013)	
<i>Hennecartia</i>	1	(Peixoto and Pereira-Moura 2008)	
<i>Kairoa</i>	3	(Renner and Takeuchi 2009)	
<i>Kibara</i>	40	(Renner 2005)	
<i>Kibaropsis</i>	1	(Renner 1998)	
<i>Levieria</i>	7	(Renner et al. 2010)	
<i>Macropeplus</i>	4	(Costa et al. 2010)	
<i>Macrotorus</i>	1	(Renner 1998)	
<i>Matthaea</i>	13	(The Plant List 2013)	
<i>Mollinedia</i>	70	(Peixoto and Pereira-Moura 2008)	
<i>Steganotheca</i>	17	Takeuchi, 2001 / (Sampson 1997)	
<i>Tambourissa</i>	51	(The Plant List 2013)	
<i>Tetrasynandra</i>	3	(Sampson 1997)	
<i>Wilkiea</i>	14	(The Plant List 2013)	
<i>Xymalos</i>	1	(Renner and Takeuchi 2009)	

<i>Monimia</i>	-	3	(Renner 2005)	3	(Renner et al. 2010)
<i>Palmeria</i>	-	14	(Renner 2005)	14	(Renner et al. 2010)
<i>Peumus</i>	-	1	(Renner et al. 2010)	1	-

Ignored diversity

Ignored genus	Nb. of sp. in genus	Ref. for sp. content
<i>Lauterbachia</i>	1	(Renner et al. 2010)
<i>Parakibara</i>	1	(Renner et al. 2010)

Monimiaceae consist of about 280 species in 28 genera. Renner et al. (2010) challenged the monophyly of four genera in the family. *Tetrasynandra* was nested within *Steganthera* in their maximum likelihood analysis with moderate support values. *Mollinedia* was included in a clade with the monotypic genus *Graziellanthus*, the first being paraphyletic, but the relationships among the species of both genera were not supported. Finally, *Hedycarya* and *Wilkiea* are paraphyletic with good support values involving *kibaropsis*, *Levieria*, and *Kairoa*, *kibara* respectively. In order to take into account the paraphyly of *Wilkiea*, we needed to define a well-supported compartment (PP=1 in the present study) that includes at least *Wilkiea*, *Kibara*, and *Kairoa* (Figure S1). This compartment corresponds to subfamily Mollinedioideae (Figure S1). This clade is well supported in the literature (Renner 1999; Renner et al. 2010; Massoni et al. 2014). The monotypic genus *Lauterbachia* discovered in 1899 has not been sampled since this date and the type seems to have been destroyed (Renner et al. 2010). Because it has never been included in a phylogenetic approach, and its diversity is negligible, we have decided to ignore this genus. The placement of *Parakibara* (one species) has never been investigated with a phylogenetic approach either. This genus, known only from the type collection, is ignored in the present study. Finally, the last genus not sampled in our chronograms is *Ephippiandra*. In their maximum likelihood phylogeny, Renner et al. (2010) placed this taxon with high support within the core Monimiaceae compartment we have defined. Consequently we have counted its species diversity (seven species) in this supra-generic terminal.

Myristicaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. Content	Nb. of sp. in compartment	Ref. monophyly compartment
Myristicaceae	<i>Bicuiba</i>	1	(Sauquet et al. 2003)	476	(Sauquet et al. 2003) / (Massoni et al. 2014)
	<i>Brochoneura</i>	3	(Doyle et al. 2004)		
	<i>Cephalosphaera</i>	1	(Sauquet et al. 2003)		
	<i>Coelocaryon</i>	4	(Sauquet et al. 2003)		
	<i>Compsoneura</i>	12	(Sauquet et al. 2003)		
	<i>Doyleanthus</i>	1	(Sauquet et al. 2003)		
	<i>Endocomia</i>	4	(Sauquet et al. 2003)		
	<i>Gymnacranthera</i>	7	(Sauquet et al. 2003)		
	<i>Haematodendron</i>	1	(Sauquet et al. 2003)		
	<i>Horsfieldia</i>	104	(Sauquet et al. 2003)		
	<i>Iryanthera</i>	25	(Sauquet et al. 2003)		
	<i>Knema</i>	95	(Sauquet et al. 2003)		
	<i>Mauloutchia</i>	10	(Doyle et al. 2004)		
	<i>Myristica</i>	144	(Sauquet et al. 2003)		
	<i>Osteophloeum</i>	2	(Sauquet et al. 2003)		
	<i>Otoba</i>	1	(Sauquet et al. 2003)		
	<i>Paramyristica</i>	1	(Sauquet et al. 2003)		
	<i>Pycnanthus</i>	3	(Sauquet et al. 2003)		
	<i>Scyphocephalum</i>	2	(Sauquet et al.		

	<i>Staudtia</i>	1	2003) (Sauquet et al. 2003)
	<i>Virola</i>	54	(Sauquet et al. 2003)

21 genera are currently recognized in Myristicaceae (Sauquet et al. 2003). Phylogenetic relationships among and within genera of this family remain poorly known. In their combined morphological and molecular analysis, Sauquet et al. (2003) sampled all 21 genera but only the monophyly of the two genera *Brochoneura* and *Mauloutchia* was tested. Because the monophyly of all other genera incorporating more than one species has not been tested in the literature and the positions of genera not sampled in our chronograms are unclear within the family, we defined one compartment to include all the species of Myristicaceae (Figure S1).

Piperaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Manekia</i>	-	3	(Schubert et al. 2012)	3	(Wanke et al. 2007a)
<i>Peperomia</i>	-	1600	(Wanke et al. 2006) (1500-1750 spp)	1600	(Wanke et al. 2007a)
<i>Piper</i>	-	1050	(Mabberley 2008)	1050	(Wanke et al. 2007a)
<i>Verhuellia</i>	-	3	(Samain et al. 2008)	3	(Wanke et al. 2007b)
<i>Zippelia</i>	-	1	(Wanke et al. 2007a)	1	-

Saururaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Anemopsis</i>	-	1	(Meng et al.	1	-

			2003)		
<i>Gymnotheca</i>	-	2	(Meng et al. 2003)	2	(Meng et al. 2003)
<i>Houttuynia</i>	-	1	(Meng et al. 2003)	1	-
<i>Saururus</i>	-	2	(Meng et al. 2003)	2	(Meng et al. 2003)

Siparunaceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
Siparunaceae	<i>Glossocalyx</i>	1	(Renner and Hausner 2005)	54	(Renner 1999) / (Renner and Won 2001)
	<i>Siparuna</i>	53	(Renner and Hausner 2005)		

Winteraceae

Compartments	Compartment content	Nb. of sp. in genus	Ref. for sp. content	Nb. of sp. in compartment	Ref. monophyly compartment
<i>Drimys</i>	-	6	(Ruiz et al. 2008) (between 5 and 7) / (Mabberley 2008)	6	(Marquínez et al. 2009) / (Pratt 2013)
PZ	<i>Pseudowintera</i>	3	(Lloyd and Wells 1992)	62	(Marquínez et al. 2009) / (Pratt 2013)
	<i>Zygogynum</i>	59	(Marquínez et al. 2009)		
<i>Takhtajania</i>	-	1	(Suh et al. 1993)	1	(Marquínez et al. 2009) / (Pratt 2013)
<i>Tasmania</i>	-	36	(Marquínez et al. 2009)	36	(Marquínez et al. 2009)

The taxonomy within the family varies among authors. The number of genera recognized varies between eight and five. In the present study, we followed the taxonomic revisions of Vink (1985, 1988), in which five genera of Winteraceae are recognized. The monophyly of these taxa have been tested and confirmed in Marquínez et al. (2009) and Pratt (2013) *Zygogynum* was not sampled in our chronograms. Because in the literature this genus is sister to *Pseudowintera* (Karol et al. 2000; Doust and Drinnan 2004; Marquínez et al. 2009; Pratt 2013), we combined the two genera in a single compartment.

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Chapter 4

Tracing floral evolution with higher taxonomic resolution leads to new portraits of the ancestral flowers of Magnoliidae

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Figures S2-S4 included in the present manuscript.

Remaining supplementary information available at:

https://www.dropbox.com/sh/mxrommnod3i19xm/O_XkTwcjk

Abstract

The morphology of the ancestral flowers of angiosperms have long been a matter of debate. With the resolution of relationships among early-diverging angiosperms and the recognition of Magnoliidae as a clade (Canellales, Laurales, Magnoliales, and Piperales), our understanding of floral character evolution at the base of angiosperms have dramatically changed. However, the exact architecture of flowers at the crown nodes of Magnoliidae, Canellales, Laurales, Magnoliales, and Piperales have remained partly ambiguous. The present study investigated the evolution of 26 floral characters in order to reconstruct portraits of the flowers at key nodes of the clade. We used an exemplar approach and a recently published phylogeny of 12 molecular markers and 198 species of Magnoliidae. Our results show that the most recent common ancestor of all Magnoliidae was a tree bearing actinomorphic, bisexual flowers with a differentiated perianth of two alternate, trimerous whorls of free perianth parts (outer and inner tepals) and probably three free stamens. Although the optimization of several traits remain equivocal in the most recent common ancestors of the four orders, this study suggests that Canellales, Laurales, Magnoliales, and Piperales could have departed from their Magnoliidae ancestor each by the modification of one or two floral traits.

Introduction

For a long time, botanists have studied the morphology of flowers in order to address a wide range of questions (e.g., delimitation of species, classifications, phylogenetic analyses, evolutionary history). In addition to highlighting the sequence of transformations, investigating the evolution of floral traits is essential to address several questions about the evolutionary history of angiosperms. Within the angiosperms floral diversity is variable among clades. Outside monocots and eudicots, the great majority of species is concentrated within Magnoliidae, which consist of four orders: Canellales, Laurales, Magnoliales, and Piperales (Cantino et al. 2007; APG III 2009; Massoni et al. 2014b). With about 10,000 species, this clade presents a wide diversity of floral morphologies. For instance, the perianth is absent in at least three different families of Magnoliidae: Saururaceae, Piperaceae, Eupomatiaceae (Endress 2003), and possibly Himantandraceae. Perianth parts can be free or variably fused (e.g., Myristicaceae), zygomorphic (*Aristolochia* in Aristolochiaceae and *Glossocalyx* in Laurales), and arranged either spirally (e.g., Calycanthaceae) or as whorls. The stamens also exhibit variable morphology with a filament present or absent (e.g., Myristicaceae), extrorse or introrse (sometimes within the same flower as in Lauraceae), and with different modes of dehiscence. Finally, the number of carpels is variable, the ovaries may be fused or not, and the style is present or absent (e.g., several Annonaceae). Until now, the evolution of floral characters within Magnoliidae has been investigated in studies focusing on angiosperms as a whole, with a special emphasis on the basal part of the tree (Doyle and Endress 2000, 2011; Ronse De Craene et al. 2003; Zanis et al. 2003; Soltis et al. 2005; Endress and Doyle 2009). Most of these previous studies used topologies which did not necessarily reflect our current knowledge of the position of Magnoliidae in the angiosperm tree. In addition, they used several supra-specific terminals in the group (genus and families) with different coding strategies. For instance, when supra-specific compartments did not have an homogeneous morphology, Zanis et al. (2003) coded them as polymorphic, whereas Doyle and Endress (2000), Endress and Doyle (2009), and Doyle and Endress (2011) filled the matrix with the presumed plesiomorphic states based on the phylogeny of these taxa obtained in previous phylogenetic studies. As suggested by previous studies on Magnoliales (Sauquet and Le Thomas 2003; Sauquet et al. 2003)

investigating the evolution of morphological traits in Magnoliidae with more terminal taxa can change our understanding of character evolution. Therefore, in the present study, we propose to investigate, with a higher taxonomic resolution and an exemplar approach (species used as terminals), the evolution of 22 floral characters within Magnoliidae. We use this framework to describe the morphology of the ancestral flower of extant Magnoliidae, and of the four orders (Canellales, Laurales, Magnoliales, and Piperales). To do so, we take advantage of a recent phylogeny of Magnoliidae, including both high taxonomic and molecular sampling (Massoni et al. 2014a,b), as well as a large morphological dataset of 26 floral characters scored for representative species of magnoliid genera.

Material and Methods

Taxonomic sampling

In the present study we did not use a supra-specific approach to code the morphology of genera sampled in our phylogeny because their monophyly has been sometimes challenged (e.g., Li et al. 2004; Thomas et al. 2012) or never tested (Massoni et al. 2014a). In addition, we used the same species of the molecular dataset in order to avoid the problem of the current and potentially future paraphyly of the two species according to other species used in the analysis. The exemplar approach involves no more assumption than the observation of a character on an individual of the species. On the contrary, supra-specific coding requires knowing all the diversity of morphologies within terminals, or previous knowledge of their internal phylogenetic relationships to score their plesiomorphic states. Finally, our coding will not be impacted by future inclusion of new species presenting new morphological features in magnoliid genera, whereas a supra-specific approach might be. To summarize, this choice ensures durability of the results in time (e.g., low impact of future variations of intrageneric phylogenies), repeatability of analyses (e.g., assumption of ancestral states not always explicit), and transparency of the data. One could argue that the sampling of exemplar species should be guided by specific criteria (basal position, maximally diverse, multiple exemplars; Prendini 2001). Instead, the choice of species was guided here by their presence in our molecular phylogenetic trees. However, future improvements of the current data set will just require adding more species, without having to change the current coding.

Phylogenetic background

We used two different topologies to reconstruct the evolution of characters. First, the maximum clade credibility (MCC) tree from the angio-140 analysis of Massoni et al. (2014a), obtained with the software BEAST v1.7.5 (Drummond et al. 2012). Second, the majority-rule consensus tree from the Bayesian analysis of the 12-markers dataset of Massoni et al. (2014b), obtained with MrBayes (Ronquist et al. 2012). We used these two topologies because the BEAST analysis supported relationships within Magnoliales better resolved than in the MrBayes analysis. In the first topology, Himantandraceae and Degeneriaceae are sister groups, as previously supported in the literature (e.g., Sauquet et al. 2003; Zanis et al. 2003; Qiu et al. 2005). In the second topology, the relationships of these two families are not resolved. However, the MCC tree is an entirely resolved tree with several relationships not supported and by using the majority rule consensus of MrBayes we evaluated their potential influence on ancestral state reconstruction. Both analyses used the same molecular dataset in which each genus was represented by a unique species for which 12 molecular markers were sampled (six plastidial, four mitochondrial, and two nuclear). They included 198 genera of Magnoliidae plus 23 outgroups. All the families of Magnoliidae were included except for the parasitic family Hydnoraceae because of its very long branch and its uncertain phylogenetic position (Naumann et al. 2013; Massoni et al. 2014b). Figure S1 provides the posterior probabilities of the two topologies used in the present study. Because the relationships outside Magnoliidae are not in accordance with previous phylogenetic studies (see discussion of Massoni et al. 2014b) we modified our trees by hand according to recent studies, which have consistently supported Chloranthaceae as the sister group of Magnoliidae (Jansen et al. 2007; Moore et al. 2007, 2010; Soltis et al. 2011).

Morphological dataset

In order to build our morphological dataset, we used the Access database PROTEUS (Sauquet 2013). We scored 24 different primary characters to describe the flower in 178 species of Magnoliidae and outgroups, resulting in 3701 unique data records, each of them linked to a bibliographic reference. A detailed extraction from PROTEUS (with explicit references for

each morphological record) and the matrix of analysis are available in supplementary information online: https://www.dropbox.com/sh/mxrommnod3i19xm/O_XkTwcjk

From this general dataset, we generated a matrix of analysis including the 26 characters presented in this study (Appendix 1). To do so, we used characters automatically calculated after the definition by the user of the character states used in each of them and their correspondence with one or more states of characters of the general dataset. Let a discrete character present in the general dataset incorporating three different states: deep pink, pale pink, and red. One would like to make a matrix of analysis in which deep pink and pale pink will be treated as pink and red as red. We can ask PROTEUS to treat the two different character states of pink in a unique one "pink" and include it in the matrix of analysis. For continuous characters of the general dataset, the user may define any classes of variation to transform it into a discrete character with character states. Appendix 1 presents all the data used from the general dataset and associated bibliographic references, the mode of definition of the characters used in the matrix of analysis, and this matrix.

We traced the evolution of floral traits with a maximum parsimony approach using Mesquite 2.75 (Maddison and Maddison 2011). All characters were treated as unordered.

Results

The ancestral reconstructions using the two topologies are entirely compatible, except for the number of perianth parts and fertile stamens, two characters not taken into account in the description of ancestral flowers (see discussion below). Instead, the number of parts is deduced from the merism and the number of whorls in the perianth and androecium.

Ancestral flower of Magnoliidae

The present study unambiguously supports the most recent common ancestor of Magnoliidae to be a tree with actinomorphic, bisexual flowers. The perianth was differentiated in two alternate, trimerous whorls with free parts (Figs. 1, S2).

The androecium was either spiral or whorled. In the second alternative, our reconstruction supports the presence of one whorl of three extrorse stamens with a wide filament and a connective extension overtopping basifixed thecae with longitudinal dehiscence (Fig. S3).

The gynoecium was superior. Both the number and degree of fusion of carpels remain uncertain, but carpels had a differentiated style (Fig. S4).

Ancestral flower of Canellales

The most recent common ancestor of Canellales was also a tree with actinomorphic bisexual flowers. However, contrary to the ancestor of all Magnoliidae, its perianth was tetramerous, again with two differentiated, alternate whorls of free parts (Fig. S2).

The androecium had unambiguously one whorl of three extrorse stamens with a wide filament and a connective extension overtopping the basifixed thecae with longitudinal dehiscence (Fig. S3).

As in the ancestor of Magnoliidae, the gynoecium was superior. Both the number and degree of fusion of carpels remain uncertain, but carpels had a differentiated style (Fig. S4).

Ancestral flower of Piperales

Contrary to other deep ancestors of Magnoliidae discussed here, the most recent common ancestor of Piperales was a herbaceous plant. The flowers were actinomorphic and bisexual. The perianth had two alternate, differentiated and trimerous whorls of free or fused parts (Fig. S2).

The androecium was whorled, with one whorl of three or six extrorse stamens with a wide filament and a connective extension overtopping the basifixed thecae with longitudinal dehiscence (Fig. S3).

As in the ancestor of Magnoliidae, the gynoecium was superior. Both the number and degree of fusion of carpels remain uncertain, but carpels had a differentiated style (Fig. S4).

Ancestral flower of Laurales

The present study unambiguously supports the most recent common ancestor of Laurales to be a tree with actinomorphic, bisexual flowers. The perianth had one or two differentiated, alternate, trimerous or tetramerous whorls with free parts (Fig. S2).

The androecium was either spiral or whorled. In the second alternative, our reconstruction supports the presence of one whorl of three extrorse stamens with a wide filament and a connective extension overtopping basifixed thecae with longitudinal dehiscence (Fig. S3).

As in the ancestor of Magnoliidae, the gynoecium was superior. Both the number and degree of fusion of carpels remain uncertain, but carpels had a differentiated style (Fig. S4).

Ancestral flower of Magnoliales

The present study unambiguously supports the more recent common ancestor of Magnoliales to be a tree with actinomorphic, bisexual flowers. The perianth had one, two or three differentiated, alternate, trimerous whorls with free parts (Fig. S2).

The androecium was either spiral or whorled. In the second alternative, our reconstruction supports the presence of one whorl of three extrorse stamens with a wide filament and a connective extension overtopping the basifixed thecae with longitudinal dehiscence (Fig. S3).

As in the ancestor of Magnoliidae, the gynoecium was superior. Both the number and degree of fusion of carpels remain uncertain, but carpels had a differentiated style (Fig. S4).

Discussion

The use of a higher taxonomic resolution and an exemplar approach provide new portraits for the ancestral flowers of Magnoliidae, Canellales, Piperales, Laurales, and Magnoliales (Doyle and Endress 2000, 2011; Ronse De Craene et al. 2003; Zanis et al. 2003; Soltis et al. 2005; Endress and Doyle 2009). We obtained clear results for the perianths and androecia, whereas the gynoecia remain poorly described. Endress and Doyle (2009) and Doyle and Endress (2011) have produced the most significant analyses so far relevant to the discussion

of our new results. Because both these studies are updates of the original paper by Doyle and Endress (2000), we compare our findings to these two more recent studies rather than the original one in the discussion that follows.

Ancestral flower of Magnoliidae

Our results supporting the ancestor of Magnoliidae to be a tree bearing bisexual flowers with a whorled, trimerous, actinomorphic, and differentiated perianth (Figs. 1, S2), are broadly consistent with those of other recent studies (Ronse De Craene et al. 2003; Zanis et al. 2003; Soltis et al. 2005; Endress and Doyle 2009; Doyle and Endress 2011). These results further reject the older view that spiral phyllotaxy of the perianth was ancestral in Magnoliidae. The present reconstructions support at least four transitions to a spiral perianth within Magnoliidae, one of them being a synapomorphy of Calycanthaceae (Laurales). Ronse De Craene et al. (2003) and Soltis et al. (2005) supported an undifferentiated perianth. This difference with our results is explained by the fact that authors scored the character for genera of Magnoliidae without a perianth (e.g., *Eupomatia*) or with a single perianth whorl (e.g., Myristicaceae), whereas we considered it inapplicable in such taxa. In the present study, the ancestral magnoliid flower possessed two alternate perianth whorls (Figs. 1, S2), whereas previous authors supported more than two perianth whorls (Endress and Doyle 2009; Doyle and Endress 2011) or an ambiguous state (Ronse De Craene et al. 2003; Zanis et al. 2003). Endress and Doyle (2009) and Doyle and Endress (2011) coded the number of whorls for both whorled and spiral perianths. On the contrary, in the present study we did not apply this character when the insertion of perianth parts was spiral. If we code our character for non-whorled species (number of series), and modify by hand our topology to represent the same relationships as in Endress and Doyle (2009) and Doyle and Endress (2011) (Lauraceae sister group to Hernandiaceae; Siparunaceae sister to Atherospermataceae and Gomortegaceae), we obtain a result compatible with the presence of more than two whorl in the branch leading to Laurales and Magnoliales. However, the ancestral state for all Magnoliidae unambiguously remains two whorls. This could be explained by the high amount of missing data in Canellales in our current dataset. If future addition of data in Winteraceae and Canellaceae supported an ancestral state of "more than two whorls or series" in Canellales, our results in Magnoliidae would then be entirely

compatible with those of Endress and Doyle (2009) and Doyle and Endress (2011). We investigated the evolution of the number of perianth parts with two different ways to discretize this number. In the first we defined a characters state corresponding to the absence of perianth, whereas in the second the absence of perianth was treated as missing data (question mark). We obtained in both cases an ancestral flower of extant Magnoliidae with three or six perianth parts (Fig. S2). These two results are consistent with a trimerous flower, and the presence of two trimerous whorls of perianth support the presence of six tepals (Fig. 1). However, when we used the majority-rule consensus of the MrBayes analysis, the results were different: the ancestral states were two and more than ten, depending on the coding. Consequently, more investigation is needed to treat this continuous character in a more appropriate way. Finally, we investigated the evolution of the fusion of the perianth. The ancestral flower of Magnoliidae is reconstructed to have free perianth parts (Figure 1, Figure S2).

In our analyses, the androecium of the ancestral flower of Magnoliidae was either whorled or spiral (Figs 1, S3) as in Doyle and Endress (2011), whereas it was unambiguously reconstructed as whorled by Endress and Doyle (2009) and Ronse De Craene et al. (2003). In the study by Endress and Doyle (2009), Magnoliidae were the sister group of monocots, which presented a whorled insertion of stamens at their crown node. If we make Magnoliidae the sister group of monocots in our tree, the ancestral state at the crown node of Magnoliidae becomes unambiguously whorled. When Endress and Doyle (2009) used an alternative topology, in which *Ceratophyllum* + Chloranthaceae were sister to Magnoliidae, and Doyle and Endress (2011) used an alternative coding, the androecium phyllotaxy also became ambiguous in their analyses. In the study by Ronse De Craene et al. (2003), Magnoliidae were sister to eudicots. As in the case of monocots, manual moving of Magnoliidae to this position leads to unambiguous whorled phyllotaxy for their ancestral flower. If the androecium proves to be whorled, the present study supports a trimerous organization of stamens in the ancestral flower of Magnoliidae, as in Endress and Doyle (2009) (Figs. 1, S3). Ronse De Craene et al. (2003) found the merism of the androecium to be ambiguous at this node. As for the perianth, in our analysis, the character "number of androecium whorls" was treated as inapplicable in the case of a spiral phyllotaxy.

Furthermore, if the phyllotaxy of the androecium is whorled at the crown node of Magnoliidae, the ancestral flower presented one whorl of stamens (Figs. 1, S3). Endress and Doyle (2009) and Doyle and Endress (2011) found the number of stamen whorls (or series when the phyllotaxy is spiral) to be two in the ancestral flower of Magnoliidae, whereas it was ambiguous in Ronse De Craene et al. (2003). If we apply the same mode of coding as in Endress and Doyle (2009) and Doyle and Endress (2011), we find more than two whorls or series in the branch leading to the clade of Laurales and Magnoliales, which is consistent with their results for this branch. However, in these two studies, authors supported the presence of two whorls or series of stamens in the branch leading to the clade Canellales and Piperales (similar results were found by Ronse De Craene et al. 2003). Within this clade, Endress and Doyle (2009) and Doyle and Endress (2011) coded the ancestral state of Canellaceae as two and Ronse De Craene et al. (2003) sampled the two genera *Canella* and *Cinnamodendron*, which they coded "dicyclic". For this family, we sampled all genera and all the species used to represent them (except *Cinnamodendron ekmanii* for which this character could not be scored) shared one whorl of stamens (Perrier de la Bâthie 1954; Wilson 1966). Within Piperales, the large amount of missing data avoids a secure argumentation for a different ancestral state than those used by Endress and Doyle (2009) and Ronse De Craene et al. (2003). We attempted to reconstruct the number of fertile stamens in the ancestral flower of extant Magnoliidae. Because the ancestral state is highly sensitive to the different coding strategies (Fig. S3), we do not discuss this character for the moment. The presence of laminar stamens had often been assumed to be ancestral within the angiosperms (Cronquist 1988). However, Endress and Doyle (2009) showed that the situation is not clear in the ancestral flower of all angiosperms. In our tree, the ancestral state within Magnoliidae was unambiguously laminar (coded in the character filament, Figure 1, Figure S3). With a topology in which Magnoliidae were sister to monocots, Endress and Doyle (2009) found an ancestral flower of Magnoliidae with a long and narrow filament. On the contrary when they used a topology with the clade of Magnoliidae plus Chloranthaceae sister to the clade of monocots plus eudicots, the ancestral state (laminar or classic filament) was ambiguous. In our reconstructions, the unambiguous state at the base of the Magnoliidae is due to the ancestral state of Canellales and Piperales, being laminar stamens (see discussion below). The evolutionary history of long connective extensions in

the angiosperms has so far been unclear (Endress and Doyle 2009). In the present study, our reconstruction supports the presence of a connective extension (short or long) on the stamens of the ancestral flower of all extant Magnoliidae (Fig. S3) with subsequent losses across the tree. The coding of Endress and Doyle (2009) was different in not distinguishing between the presence or the absence of this extension, but rather whether the extension is long versus truncated or short rounded. With this coding they found that the ancestral state within Magnoliidae was truncated or smoothly rounded. Finally, the ancestral flower of Magnoliidae had basifixed, extrorse anthers with longitudinal dehiscence (Figs. 1, S3), consistent with the results of Endress and Doyle (2009).

Our results unambiguously support a superior in the ancestral flower of extant Magnoliidae (Fig. S4). Ronse De Craene et al. (2003) traced the evolution of the number of carpels in a character that also recorded their relative position (in the case of several carpels), the phyllotaxy of the gynoecium, and the number of whorls in the case of a cyclic insertion of carpels. They found an ambiguous result (no detail provided). Doyle and Endress (2011) also investigated the number of carpels and the number of whorls in a unique character. In the present study, we do not discuss the number of carpels as it is very sensitive to the mode of discretization (the number of carpels in the ancestral flower of all Magnoliidae varies between 1 and more than 10; Fig. S4). In the present analysis, it remains ambiguous whether ovaries were free or fused in the ancestral flower of Magnoliidae (Fig. S4). In their analysis, Endress and Doyle (2009) supported an ancestral flower with free carpels. In the present study, the ancestral state for Magnoliidae is ambiguous as the ancestral flowers of Canellales and Piperales were unambiguously fused, whereas the ancestral state for the clade of Canellales and Piperales was ambiguous in Endress and Doyle (2009). This ambiguity found in their study is due to the uncertain state of the terminal Winteraceae and the equivocal reconstruction for the ancestor of Aristolochiaceae. In the present study, the ancestral flower of Magnoliidae unambiguously presented a differentiated style (Fig. S4) whereas the state was ambiguous when Endress and Doyle (2009) used the topology in which Magnoliidae were sister to Chloranthaceae.

Ancestral flowers of Canellales, Laurales, Magnoliales, and Piperales

Between 178.9 and 88.2 Ma the ancestral lineage of Magnoliidae split into the four lineages that led to extant Canellales, Laurales, Magnoliales and Piperales (Massoni et al. 2014a). However, our results suggest that floral structure remained relatively conserved during this period, as our reconstructions for the ancestral flowers of each order do not significantly differ from that of the Magnoliidae ancestor described above (Figs. 1, S2-S4).

Canellales and Piperales

Along the branches leading to crown-group Canellales and Piperales, several transitions occurred (Figure 1). The present study suggests that the perianth may have become fused in the ancestral flower of Piperales but this remains ambiguous (Fig. 1). In addition, the differentiation of the two perianth whorls also becomes equivocal (as in Ronse De Craene et al. 2003; Zanis et al. 2003). However, this ambiguity is between differentiated and non-applicable. In Piperales the character is not applicable because many species have either one perianth whorl or no perianth. In our analysis, the number of whorls reconstructed for this node is two, supporting the presence of a differentiated perianth. Endress and Doyle (2009) presented an unambiguous differentiated perianth. In their study, Piperales were coded with missing or uncertain data. In the branch leading to the ancestral flower of Canellales, the perianth merism changed from trimerous to tetramerous (Figs. 1, S2). This result is not consistent with those of Ronse De Craene et al. (2003), Soltis et al. (2005), Endress and Doyle (2009) and Doyle and Endress (2011), who found that the ancestral flower of Canellales was trimerous. On the contrary, Zanis et al. (2003) found the state to be equivocal. This difference between our results and previous studies could be due to the difficulty in obtaining reliable data to record these characters in Canellales. Generally speaking, the apprehension of the number and organization of parts in the floral anatomy of the Canellales is difficult because of high variations (Doust 2000), even within individuals (Doust 2001). Therefore, before providing conclusions on the ancestral states of these two families we will need to reduce the number of missing data. If the androecium phyllotaxy was spiral in the ancestral flower of Magnoliidae, there was one transition to a whorled insertion of stamens in the branch leading to the last common ancestor of Canellales and Piperales, and the state

was retained in the two ancestral flowers of the orders. This result is consistent with Endress and Doyle (2009) and Ronse De Craene et al. (2003). The reconstruction of androecium merism is ambiguous in the branch leading to the ancestral flower of Piperales, either trimerous, as in Endress and Doyle (2009), or hexamerous (Fig. 1). The large amount of missing data in our dataset precludes any secure conclusions. For the ancestral flower of Canellales, Endress and Doyle (2009) supported more than three stamens per whorl. In our dataset, there are many missing data within Winteraceae. *Takhtajania perrieri* is the only species with data and it is polymorphic (either dimerous, trimerous, or pentamerous) (Endress et al. 2000). Trimery was reconstructed as the ancestral state of the family, but new data might change this ancestral state. Within Canellaceae all the species sampled share an androecium with more than seven parts per whorl, consistent with the polymeric state of Endress and Doyle (2009). Finally, in the branches leading to Canellales and Piperales, the ovaries are fused. Endress and Doyle (2009) instead found the state to be equivocal in this clade.

Laurales and Magnoliales

Along the branches leading to Laurales and Magnoliales, several transitions of floral traits occurred as well. In the present study, the merism of the perianth may have become tetramerous on the branch leading to the ancestral flower of all Laurales but this remains ambiguous since a trimerous perianth is equally parsimonious at this node (Fig. 1). This result is consistent with those of Endress and Doyle (2009) and Zanis et al. (2003). Instead, Ronse De Craene et al. (2003) found the merism of the perianth to be "indefinite" (i.e., non-fixed number) at this node. As in Zanis et al. (2003), the number of perianth whorls is ambiguous for the ancestral nodes of both Laurales and Magnoliales (one/two, and one/two/three whorls, respectively). However, in the Laurales if we move *Glossocalyx longicuspis* to a position where it is sister to Gomortegaceae and Atherospermataceae as several previous phylogenetic studies have suggested (e.g., Qiu et al. 1999, 2000; Renner 1999, 2004; Zanis et al. 2003), the ancestral state in Laurales becomes unambiguously two whorls. Because of this well supported position of *Glossocalyx* in the literature we decided to present only this alternative on Figure 1. Endress and Doyle (2009) reconstructed a perianth of more than two whorls for the ancestral nodes of both Laurales and Magnoliales. These

differences are explained by the alternative strategies of coding and topology, as discussed above. In the study by Ronse De Craene et al. (2003), the ancestral flower of Magnoliales was reconstructed to consist of one whorl only. There are two main differences between their dataset and ours. First, they coded the perianth of *Galbulimima* with one perianth whorl. Because the identity of the outer series of sterile organs in the genus is not well established (either tepals or outer staminodes; Peter Endress, pers. comm.), we preferred not to assume either way and thus did not score the perianth in *Galbulimima*. Second, the authors coded one whorl for *Eupomatia* whereas it seems that the perianth is absent in this genus (Endress 2003). In the same study, in the ancestral flower of Laurales, the number of perianth whorls was reconstructed as "indefinite", by which the authors meant a spirally inserted perianth at the crown node of Laurales. Ronse De Craene et al. (2003), Soltis et al. (2005), and Endress and Doyle (2009), reconstructed the perianth phyllotaxy at the crown node of Laurales to be either whorled or spiral. Endress and Doyle (2009) suggested that a spiral ancestral state for the order would imply several reversions, supporting the fact that this character is highly labile (Endress 1987). Doyle and Endress (2011) scored perianth merism in the same character as perianth phyllotaxy. In their result, the ancestral flower of Laurales unambiguously presented a spiral insertion of the perianth. In the present study as well as in the study by Zanis et al. (2003), the ancestral state for Laurales is unambiguously whorled (Fig. 1), rejecting the idea that the ancestral flower of Laurales would be generally similar to that of living Calycanthaceae (Doyle and Endress 2011). In the dataset of Endress and Doyle (2009) and Doyle and Endress (2011), the perianth phyllotaxy in Siparunaceae was coded as missing data and this family was sister to the clade of Gomortegaceae and Atherospermataceae. Ronse De Craene et al. (2003) and Soltis et al. (2005) did not sample the family. In our dataset, *Glossocalyx* was used to represent Siparunaceae and perianth phyllotaxy was coded as whorled (Staedler and Endress 2009). If we move the branch of *Glossocalyx* to the same position as in Endress and Doyle (2009), the ancestral state of Laurales remains unambiguously whorled. Another important difference is the coding of Atherospermataceae. Endress and Doyle (2009) and Doyle and Endress (2011) coded the putative ancestral state of the family to be spiral. In our exemplar approach we sampled all genera of the family and the seven representative species present a whorled perianth (Schodde 1969; Staedler and Endress 2009). Finally, in these two studies, they coded

Monimiaceae with the putative ancestral state spiral insertion of the perianth whereas in our study, where the basal relationships within the family are well supported, the ancestral state is ambiguous. Ronse De Craene et al. (2003) sampled the genera *Daphnandra* and *Doyphora* and coded them as spiral whereas they are coded as whorled in our dataset (Staedler and Endress 2009). Androecium merism was either trimerous or tetramerous in the ancestral flower of all Laurales (Fig. 1, S3). Endress and Doyle (2009) found the character to be equivocal at the crown node of Laurales and Ronse De Craene et al. (2003) reconstructed it as "indefinite". In the ancestral flower of Laurales the ovaries became free, which is consistent with the results of Endress and Doyle (2009).

Evolution of the filament of stamens

Our study allows a more detailed investigation of stamen evolution in Magnoliidae than has ever been done before. Stamens are particularly interesting in the clade because they vary not only in dehiscence and orientation but different taxa have either wide or thin filaments, two characters probably correlated with the nature of their pollinators. The presence of laminar stamens, characterized by a wide filament, was long seen as a primitive feature of Magnoliidae (Cronquist 1988). Endress and Doyle (2009) and Doyle and Endress (2011) investigated the evolution of the filament using three states: short, long wide, and long narrow. They considered that laminar stamens corresponded to either the first or the second state. Even though their study did not resolve the ancestral state for angiosperms as a whole, they supported that laminar stamens within Magnoliidae were derived features because the ancestral flower of the clade had typical long and narrow filaments. Later, Sauquet et al. (2003) confirmed the derived condition of these laminar filaments in Magnoliales. In the present study, we treated the character in a slightly different way: we did not consider all short filaments to be laminar. If a short filament was narrow, we treated it as typical (thin). In other words, we considered as laminar only stamens with a wide filament (whether long or short). Our results unambiguously support an ancestral flower of Magnoliidae with laminar stamens (Fig. 2). This result is robust to pruning all outgroups or rearranging them according to alternative relationships (e.g., Endress and Doyle 2009 and Doyle and Endress 2011). However, data are missing for most species of Canellaceae and Piperaleae in our dataset. The character of filament shape is not applicable in Canellaceae,

since the stamens are fused in a tube (Wilson 1966). This is also the case in Aristolochiaceae, where the gynoecium and androecium are often typically fused into a gynostenium (Ronse De Craene 2010). The evolution of this character might be correlated with the different modes of pollination and the nature of insects involved. Many species of Magnoliidae are pollinated by beetles (Thien et al. 2000). The attraction of these insects often involves edible resources in the form of food bodies, which are sometimes placed on staminodes or stamens (Himantandraceae, Eupomatiaceae, Annonaceae, and Calycanthaceae; Endress 2010). In other species of Laurales, the stamens and staminodes bear nectaries (Endress 2010). Stout filaments in the laminar stamens of these taxa might be directly related to and be advantageous for the evolution of these particular structures. The pollination by insects with destructive buccal organs (incl. beetles) requires protection of the gynoecium. The selection of an inferior ovary could have been one of the solutions to achieve this protection in angiosperms (Endress 2010). In Magnoliidae the great majority of species present a superior ovary. The presence of large filaments could be involved in the protection of the female part of the flower. For instance in most Annonaceae, a large number of stamens with a stout filament are typically tightly arranged in a compact crown around the carpels, which may be seen as an alternative protection. In the present study, we found convergent evolution to a typical filament (thin) in Canellales (*Tasmania insipida*), Piperales (Saururaceae), Laurales (Hernandiaceae, Atherospermataceae, Lauraceae, and Monimiaceae), and Magnoliales (some Annonaceae). Doyle and Endress (2011) suggested that the transitions in Laurales could be related to beetle pollination, but did not elaborate on the subject. However, in this group the reward is in the form of secretions by glands at the base of stamens and staminodes (Endress 2010). Therefore, it probably involves non-destructive beetles which do not consume floral tissues. Because the selective pressure of chewing beetles is absent it may be more advantageous to develop thin filaments (less energetic consuming during development). Within Piperales, the presence of typical filaments in Saururaceae might be related to the fact that the family is probably the only one partly pollinated by wind in Magnoliidae (Thien et al. 2000). The presence of laminar stamen limits the exposure of the thecae to wind and probably represents an obstacle to the take-off of the pollen grains, whereas a thin filament does not. Other species of the family are characterized by a clonal reproduction mode (Schroeder and Weller 1997), sometimes

without sexual reproduction at all (Takahashi 1986). The transition from laminar filament to typical filament in species where sexual reproduction is limited or absent provides arguments to that the idea that laminar stamens are selected by pollination systems (biotic or abiotic).

Conclusion

The present study supports new portraits of the bisexual flowers of the most recent common ancestors of extant Magnoliidae and of its four orders. The magnoliid ancestor was probably a tree, bearing flowers with a differentiated perianth of two whorls of three tepals. If stamen phyllotaxy was whorled, the androecium consisted in one whorl of three laminar, extrorse stamens with longitudinal dehiscence. On the other hand, the ancestral states of the gynoecium remain uncertain or equivocal. Future improvement of this study will include new data and species in our matrix. In addition, the investigation of the evolution of characters, using model-based alternative approaches to parsimony, may provide interesting, complementary results. Probabilistic methods take time into account (branch length taken into account in the estimation of the likelihood), and Monte Carlo Markov Chain procedures may help us to better quantify the uncertainty in the results (incl. uncertainty in model selection and parameter estimation, phylogenetic relationships and branch lengths). In addition, methods exist to treat continuous data as such and reconstruct ancestral states without going through the step of transformation into a discrete character.

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Figures

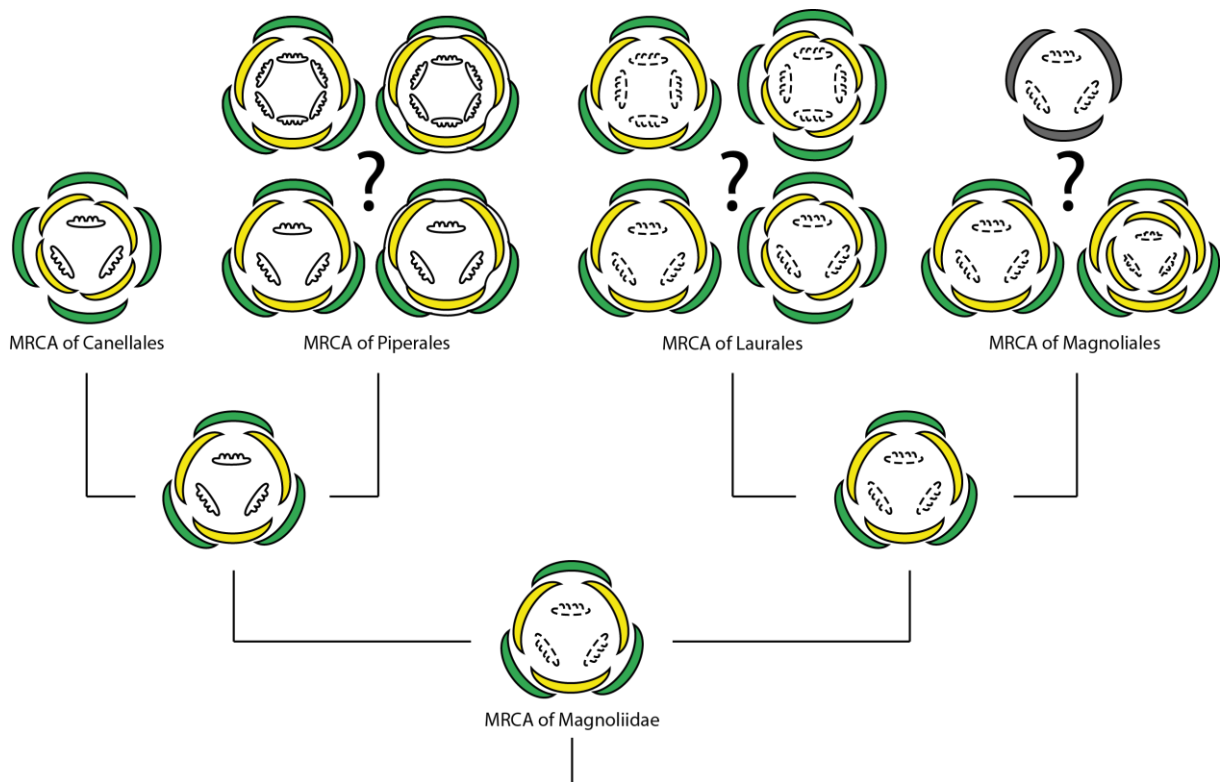


Figure 1: Floral diagrams summarizing ancestral states for the perianth and the androecium in Magnoliidae, Canellales, Piperales, Laurales and Magnoliales. Because of the ambiguity for several ancestral state reconstructions, several alternatives are possible for the ancestral flowers of Piperales, Laurales and Magnoliales. Because of the ambiguity in its phyllotaxy, the ancestral androecia of Magnoliidae, Laurales, and Magnoliales are presented with a dashed margin. We did not present the alternative with a spiral insertion because the number of stamen is two ambiguous in this case. Abbreviation: MRCA, most recent common ancestor.



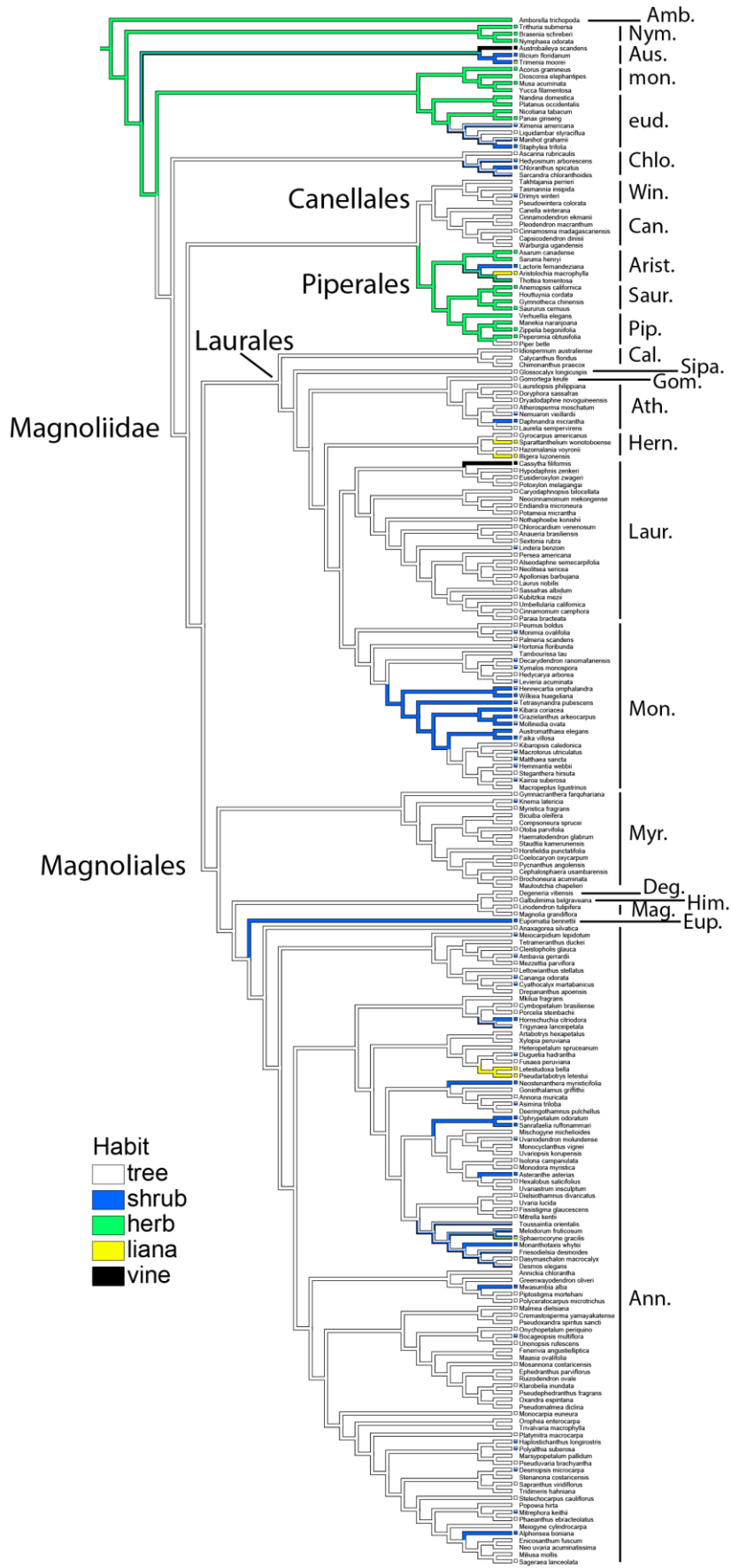
Figure 2: Evolution of the filament. Parsimony reconstruction on the majority-rule consensus of the Bayesian analysis of the 12-marker dataset of Massoni et al. (2014b). The relationships among Magnoliidae and other angiosperms have been modified by hand to better reflect results from recent, large-scale phylogenomic studies. Abbreviations: Amb., *Amborella*; Nym., Nymphaeales; Aus. Austrobaileyales; mon., monocots; eud., eudicots; Chlo., Chloranthaceae; Win., Winteraceae; Can., Canellaceae; Arist., Aristolochiaceae; Saur., Saururaceae; Pip., Piperaceae; Cal., Calycanthaceae; Sipa., Siparunaceae; Gom., Gomortegaceae; Ath. Atherospermataceae; Hern., Hernandiaceae; Laur., Lauraceae; Mon., Monimiaceae; Myr., Myristicaceae; Deg., Degeneriaceae; Him., Himantandraceae; Mag., Magnoliaceae; Eup., Eupomatiaceae; Ann., Annonaceae.

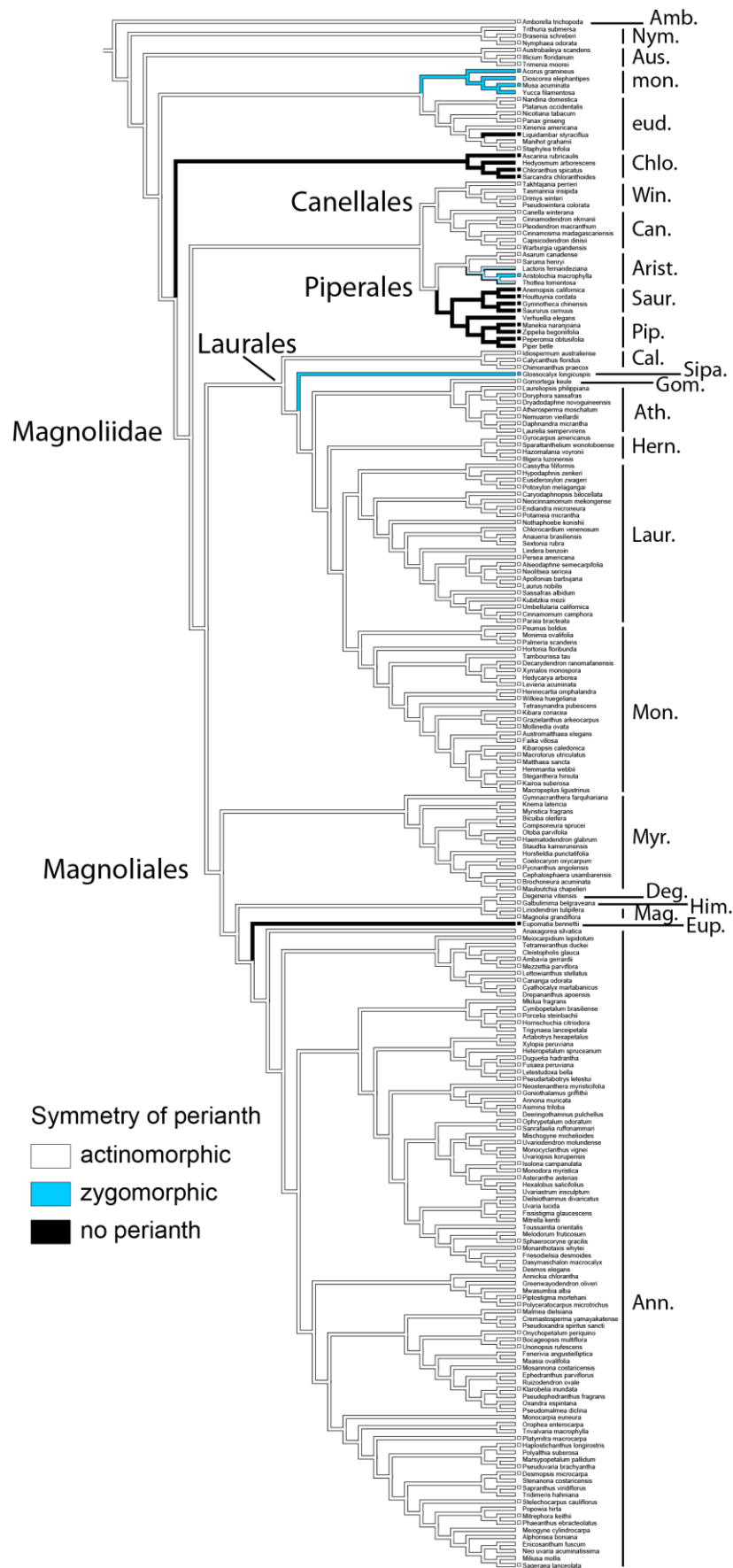
Supplementary information

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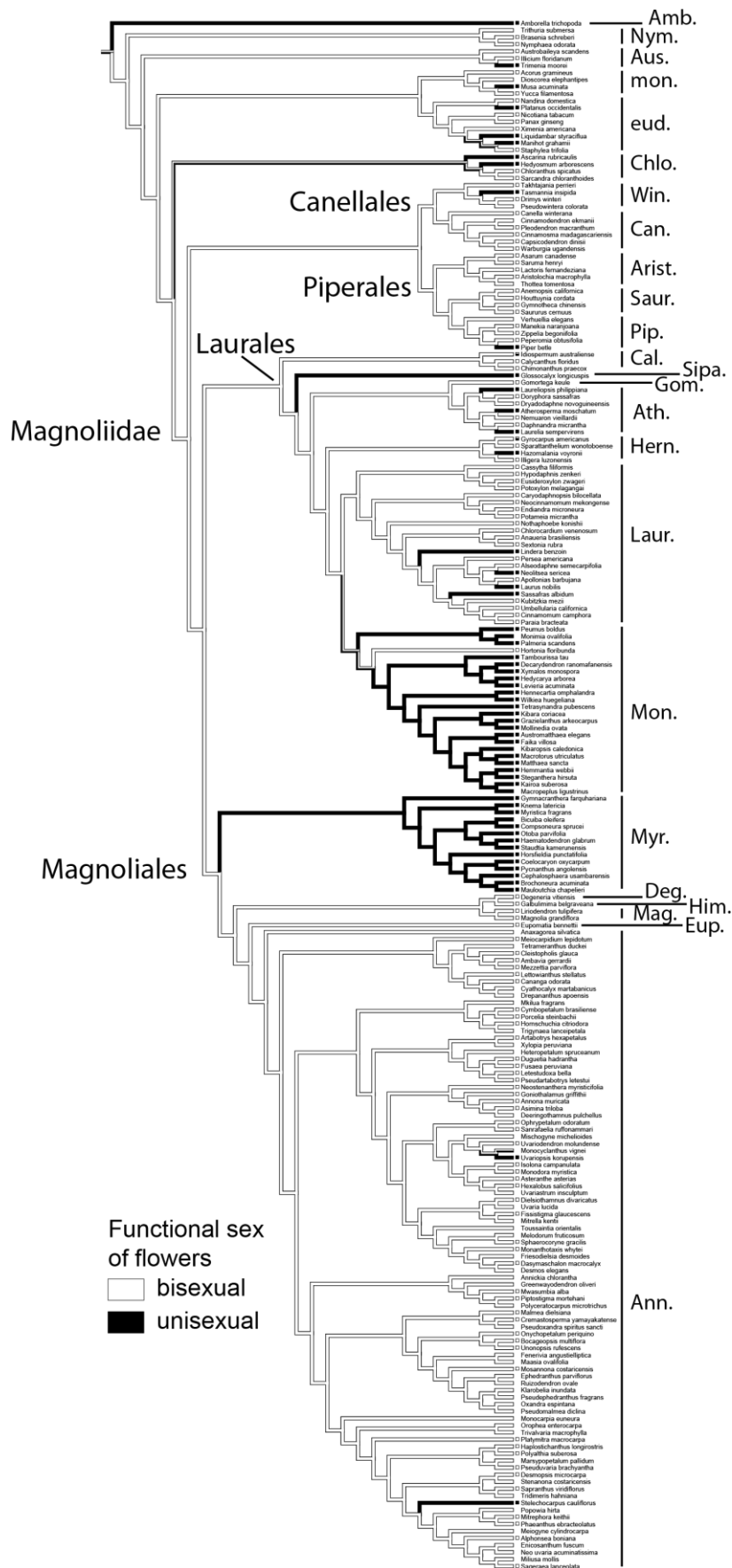
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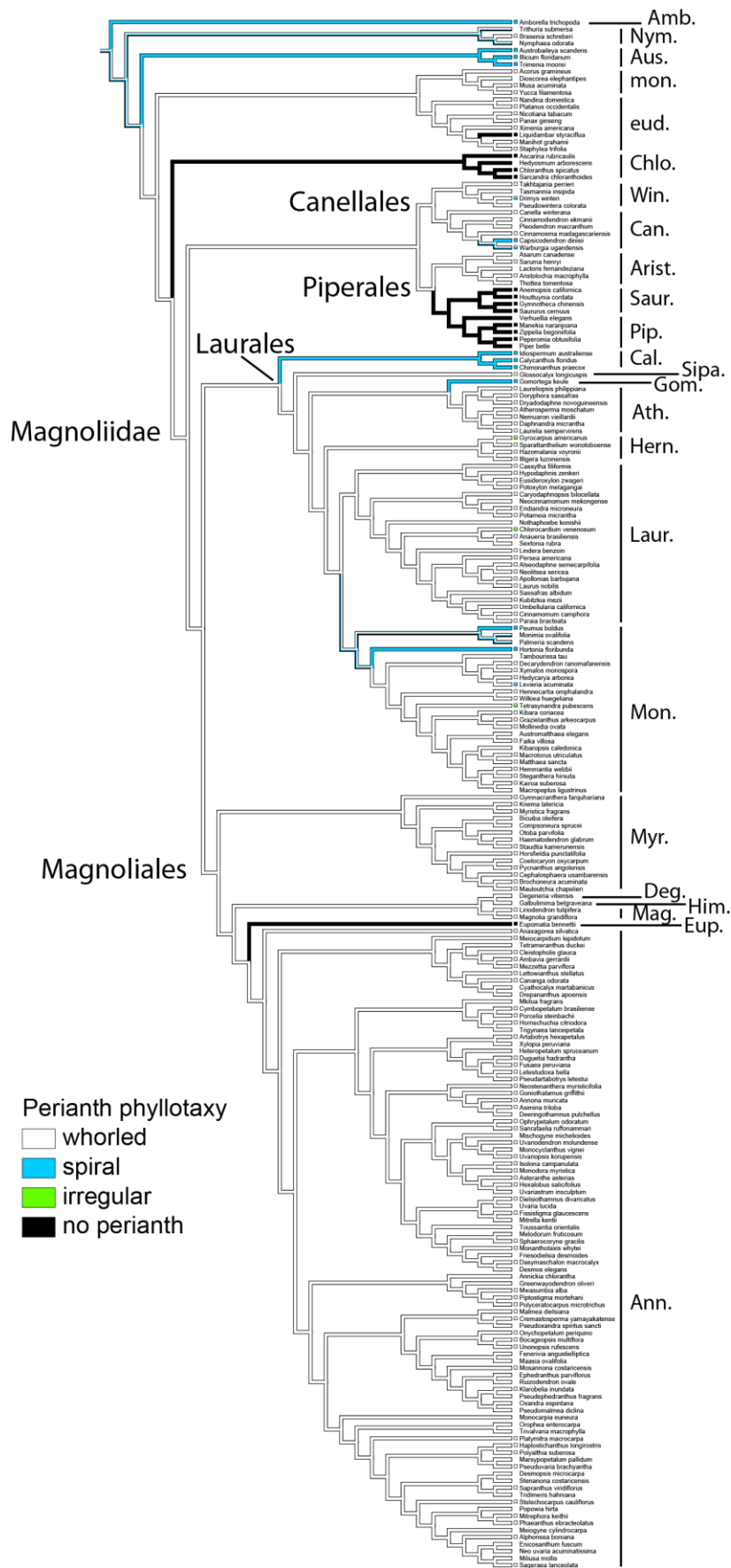
Figure S2: Evolution of the perianth. Topology: maximum clade credibility tree of angio-140 of Massoni et al. (2014a). The relationships among Magnoliidae and other angiosperms have been modified by hand according to the literature. Ancestral states are reconstructed using a parsimony approach and unordered characters. Abbreviations: Amb., *Amborella*; Nym., Nymphaeales; Aus. Austrobaileyales; mon., monocots; eud., eudicots; Chlo., Chloranthaceae; Win., Winteraceae; Can., Canellaceae; Arist., Aristolochiaceae; Saur., Saururaceae; Pip., Piperaceae; Cal., Calycanthaceae; Sipa., Siparunaceae; Gom., Gomortegaceae; Ath. Atherospermataceae; Hern., Hernandiaceae; Laur., Lauraceae; Mon., Monimiaceae; Myr., Myristicaceae; Deg., Degeneriaceae; Him., Himantandraceae; Mag., Magnoliaceae; Eup., Eupomatiaceae; Ann., Annonaceae.

Phylogeny, molecular dating, and floral evolution of Magnoliidae (Angiospermae)
Chapter 4

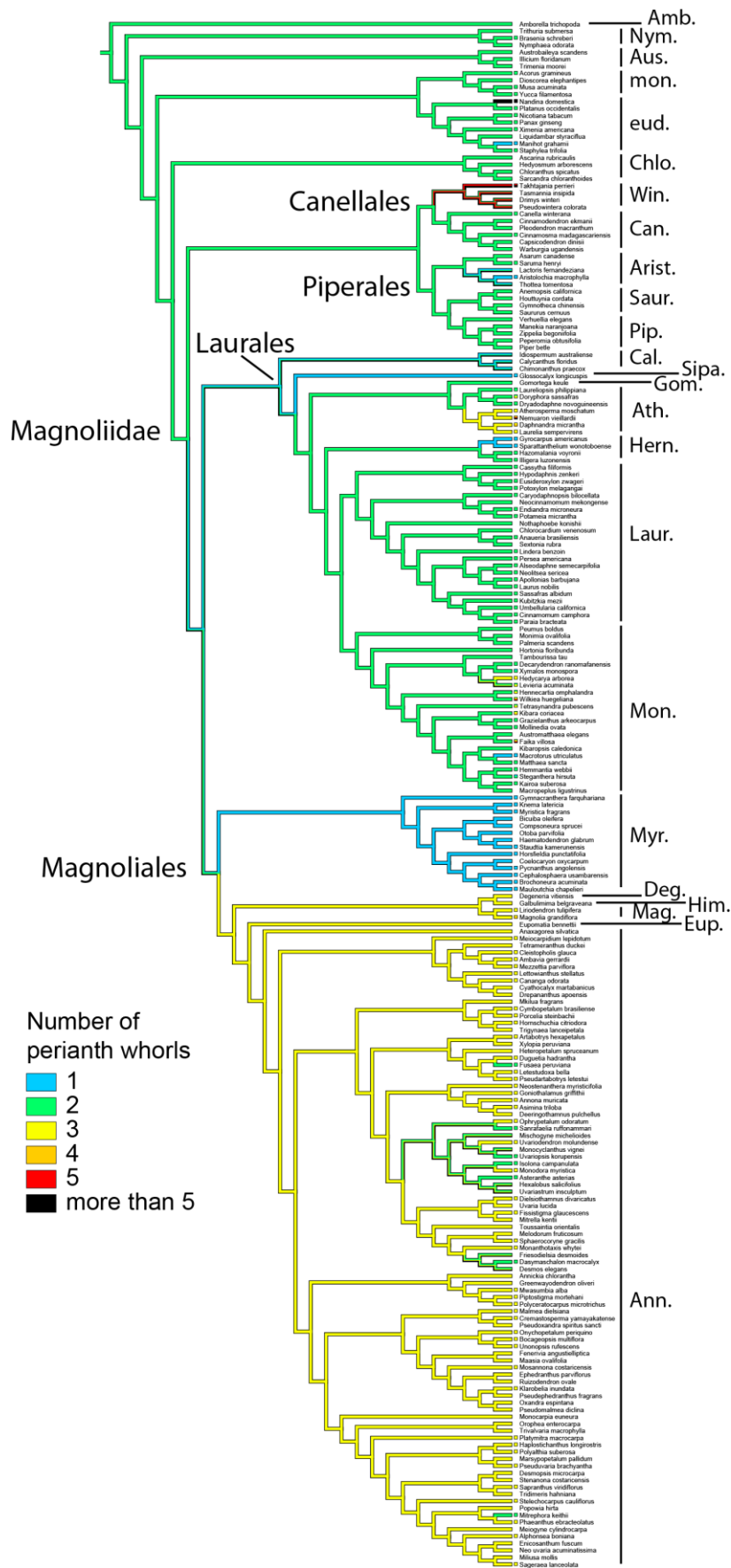


Phylogeny, molecular dating, and floral evolution of Magnoliidae (Angiospermae) Chapter 4

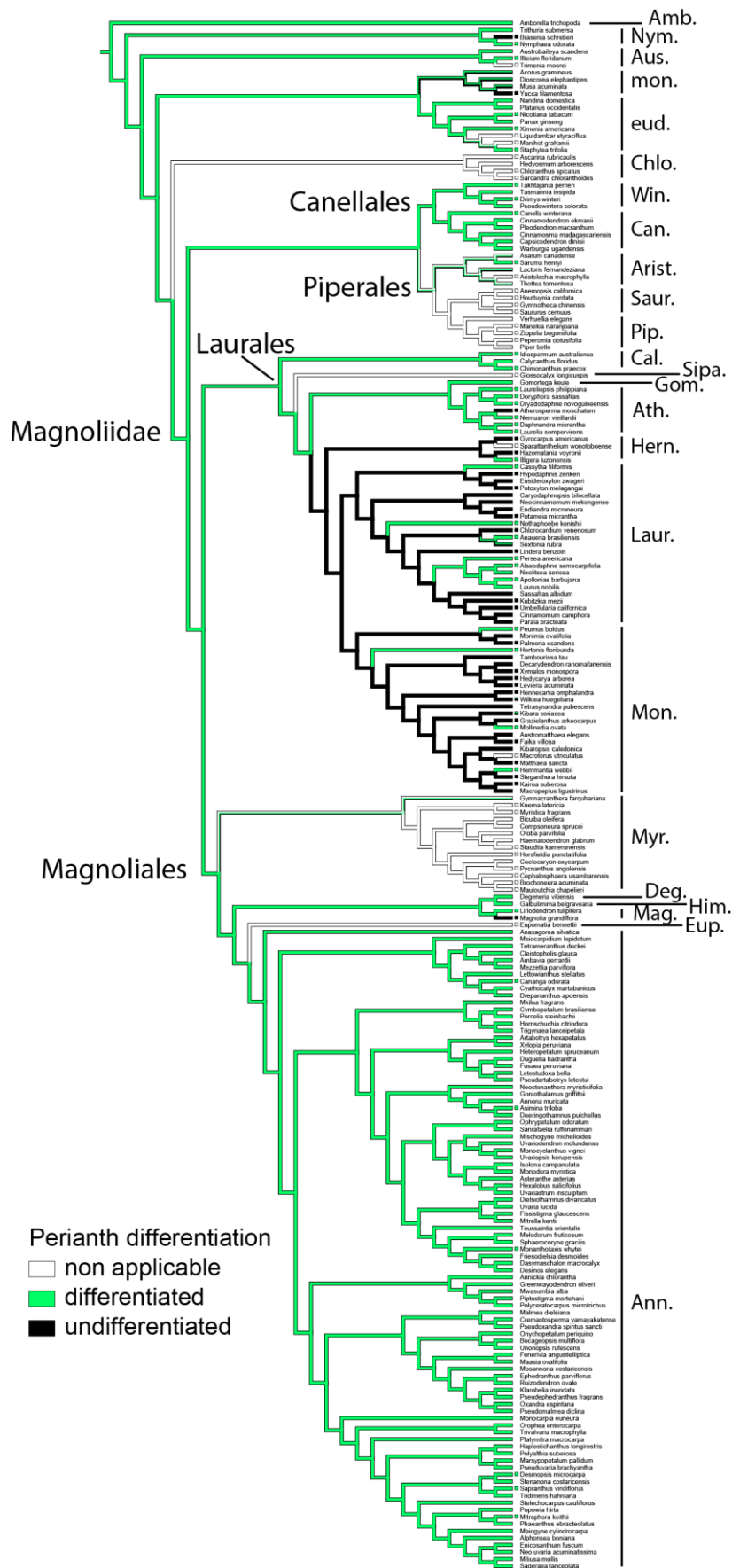


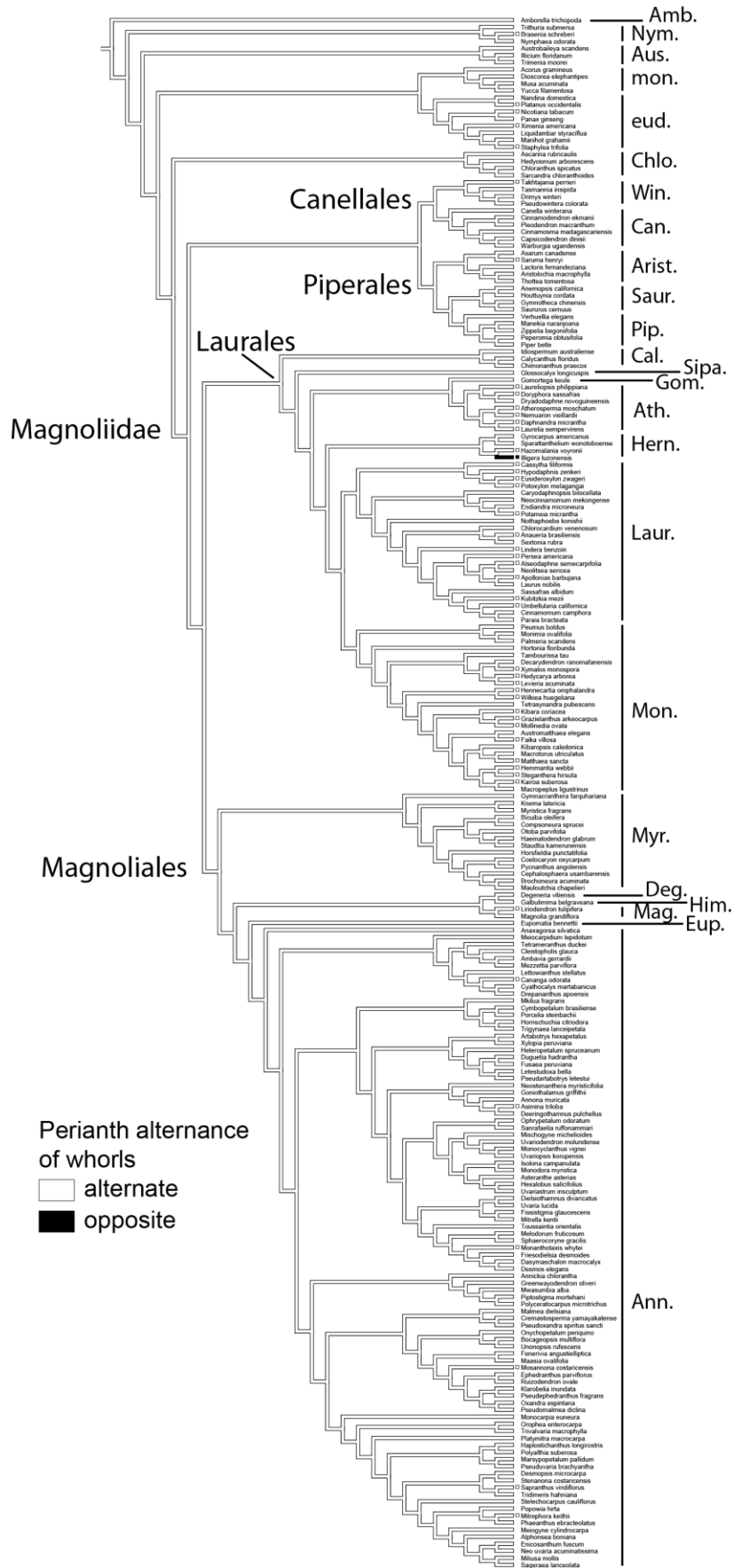


Phylogeny, molecular dating, and floral evolution of Magnoliidae (Angiospermae)
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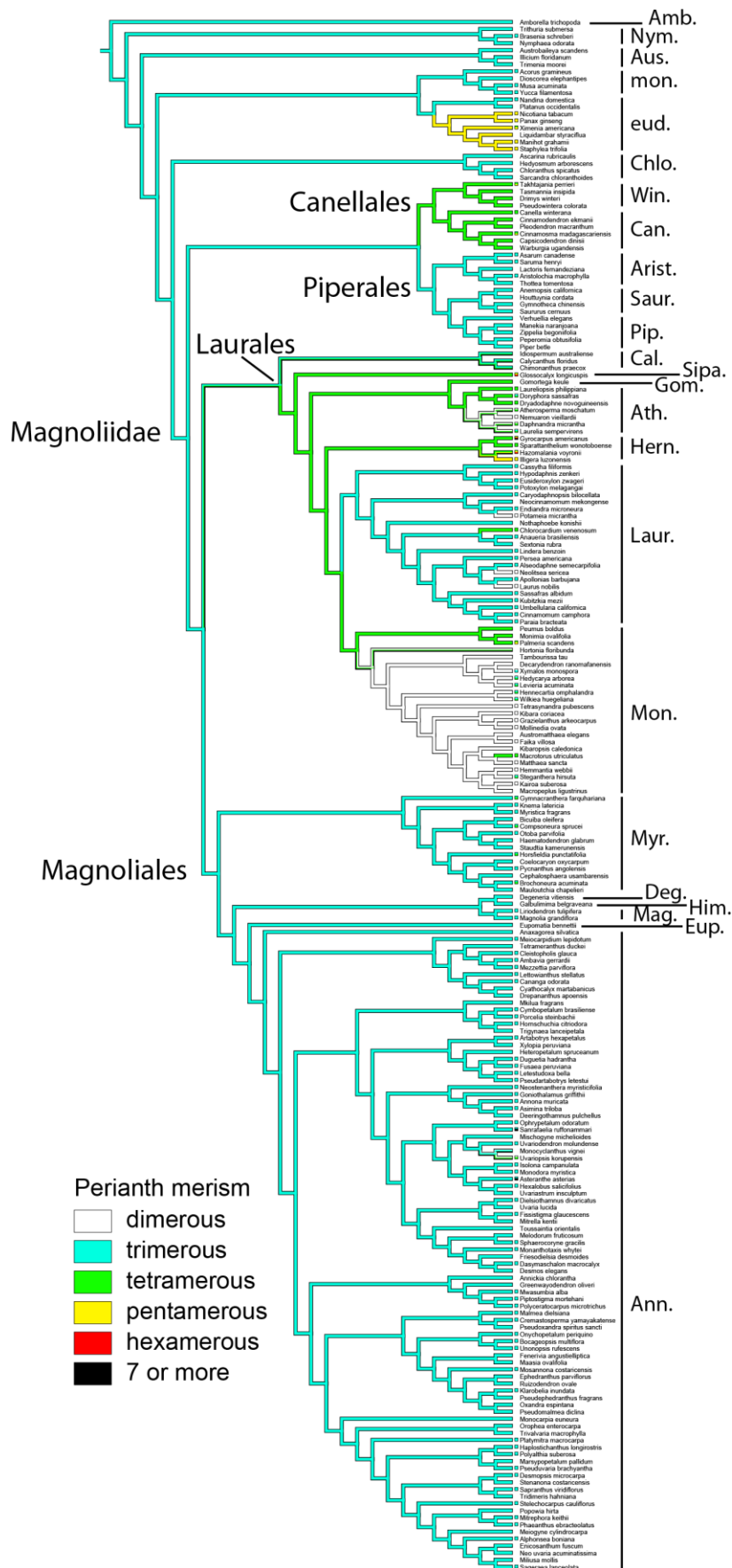


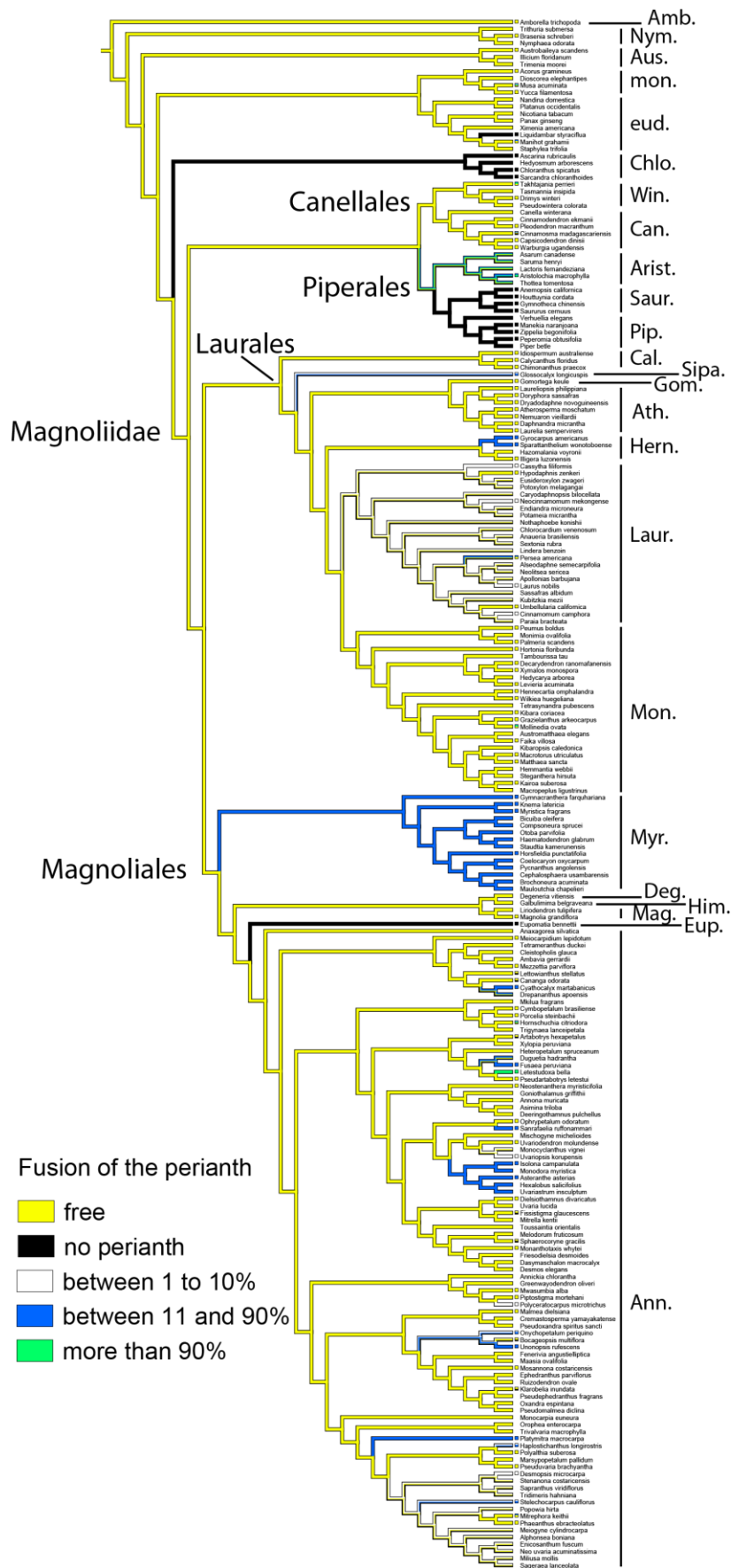
Phylogeny, molecular dating, and floral evolution of Magnoliidae (Angiospermae) Chapter 4



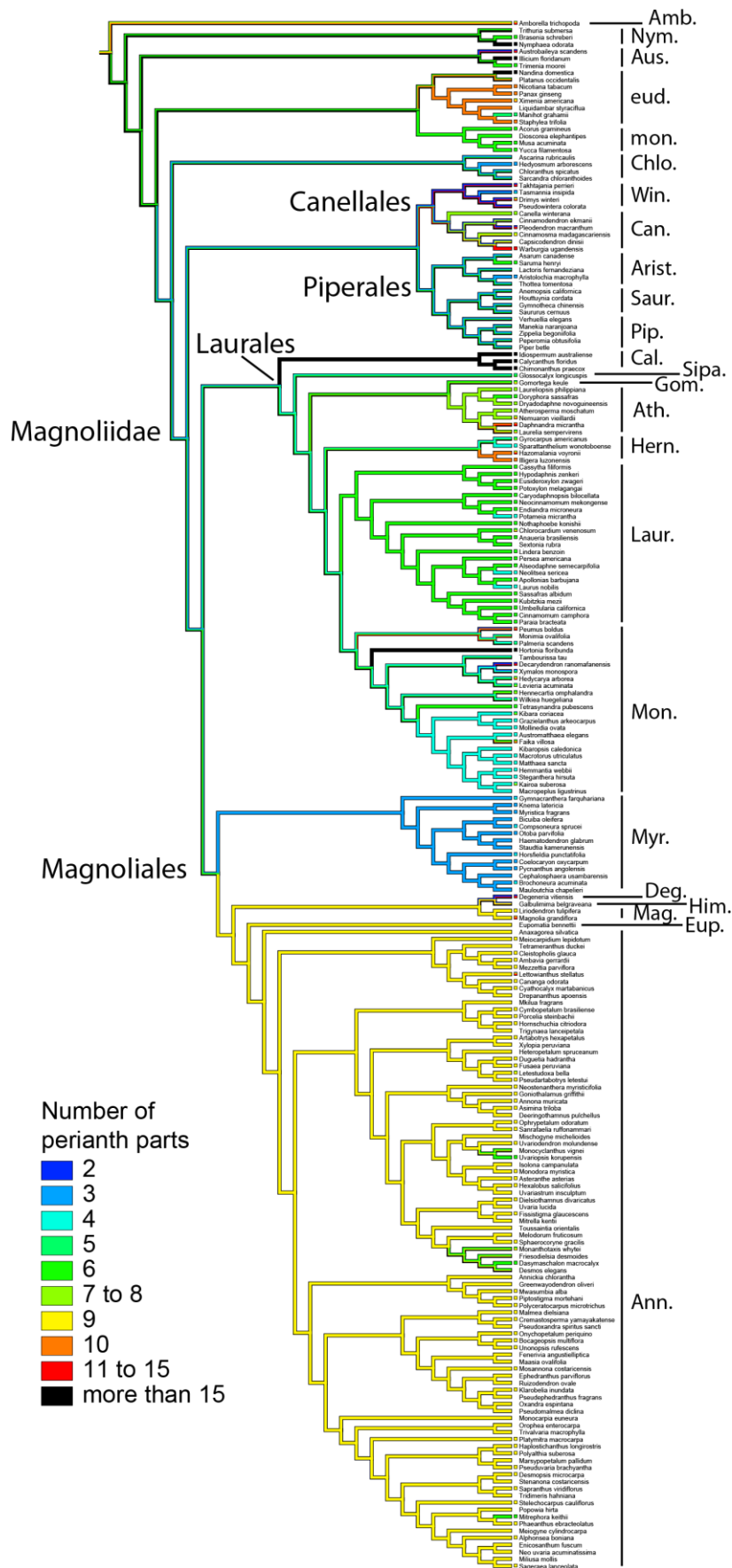


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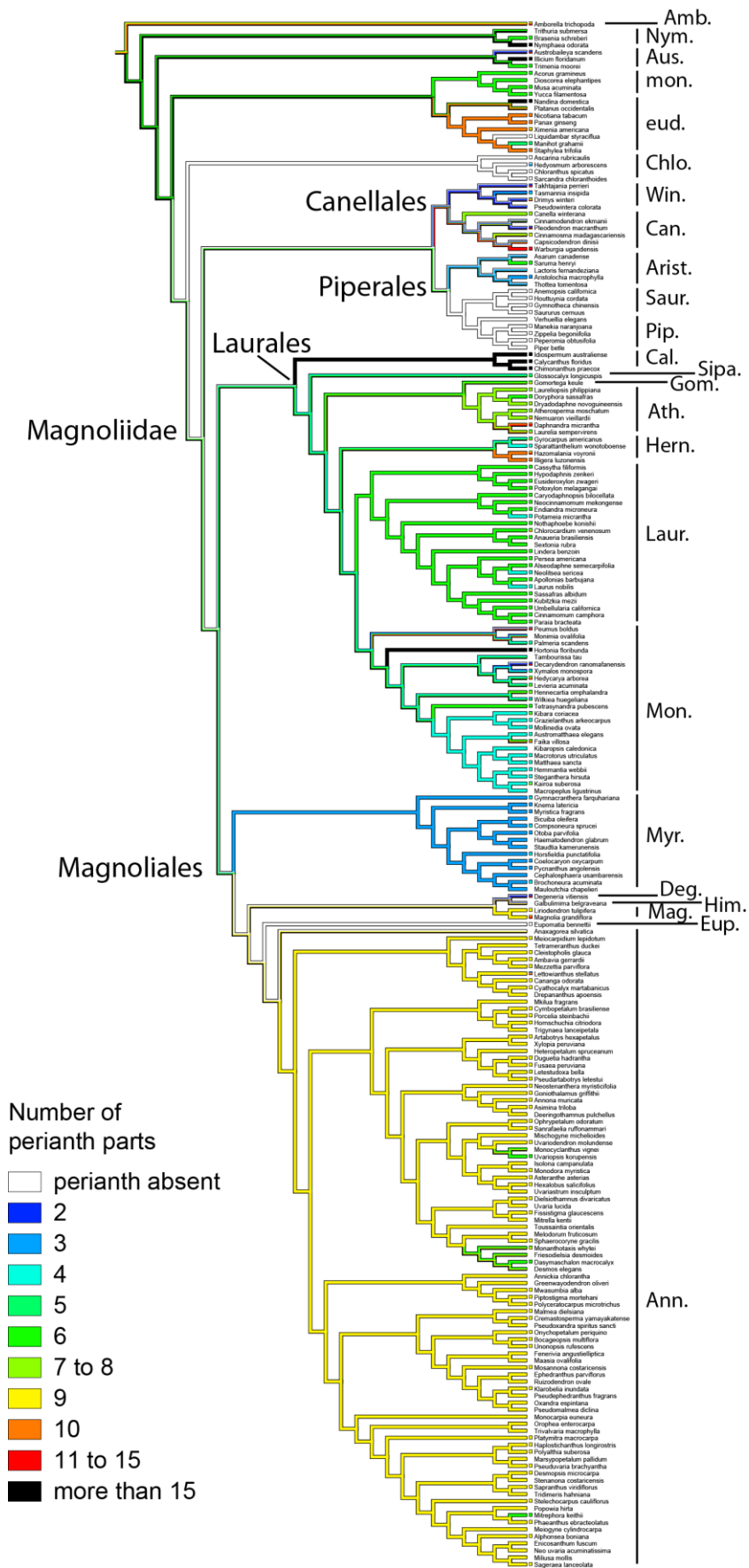
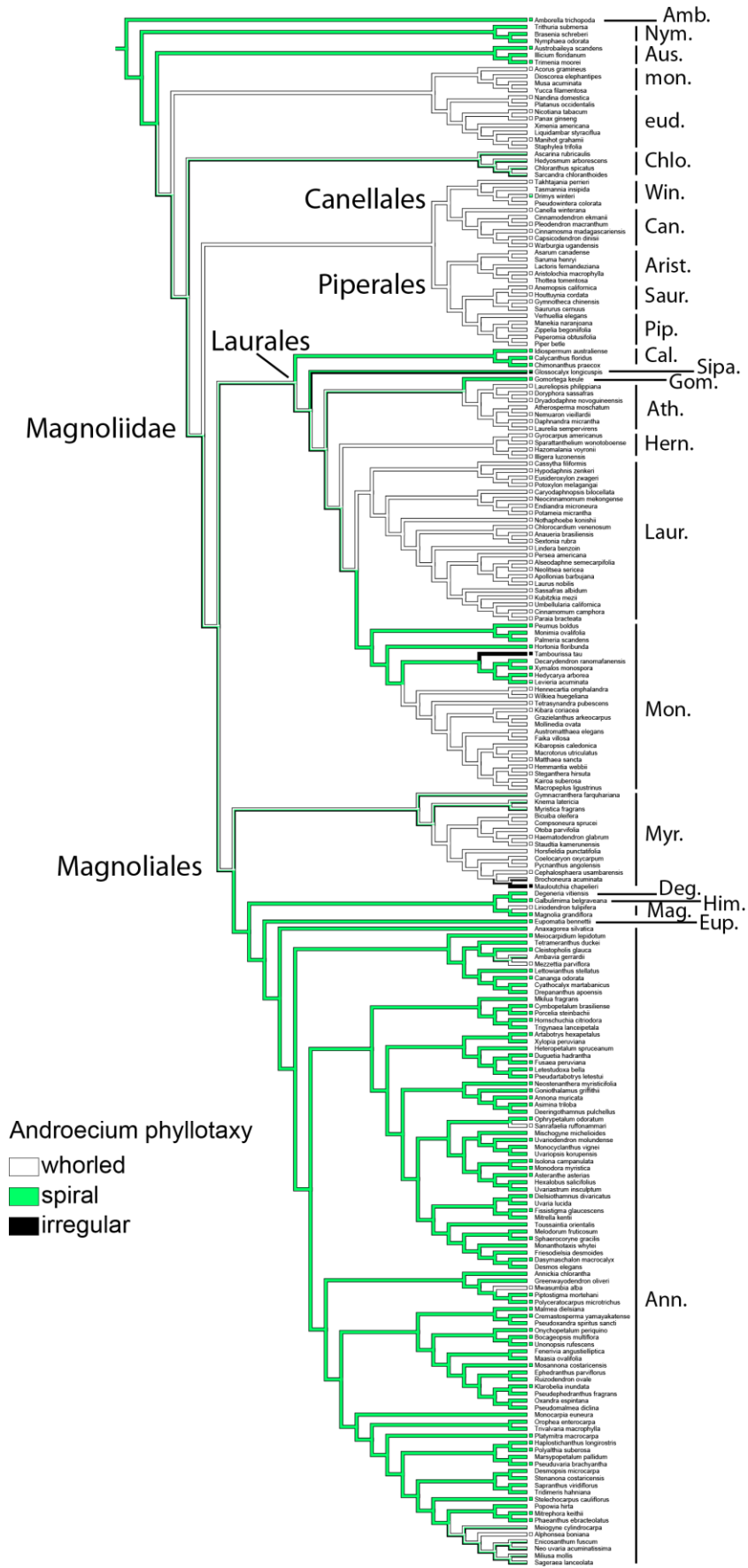
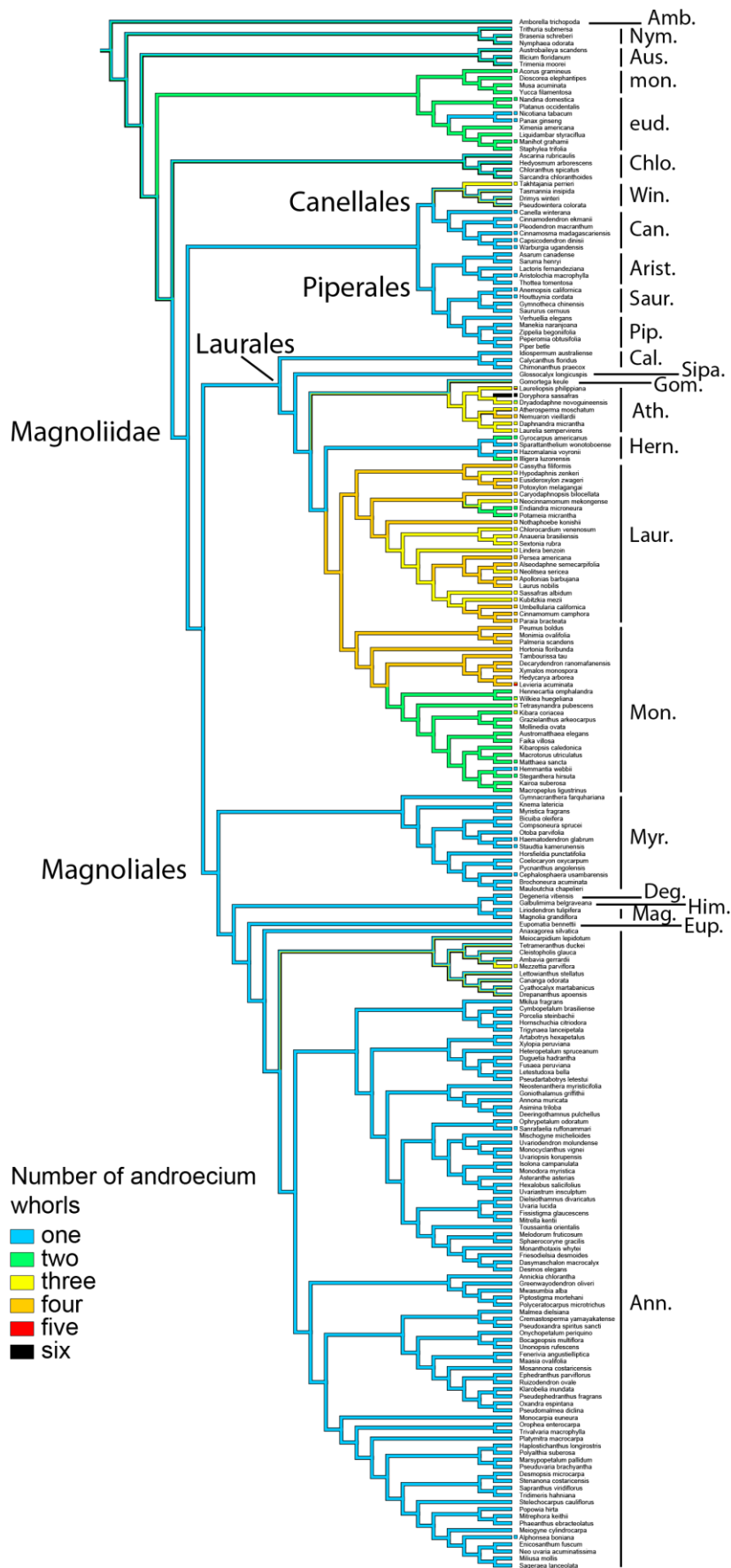
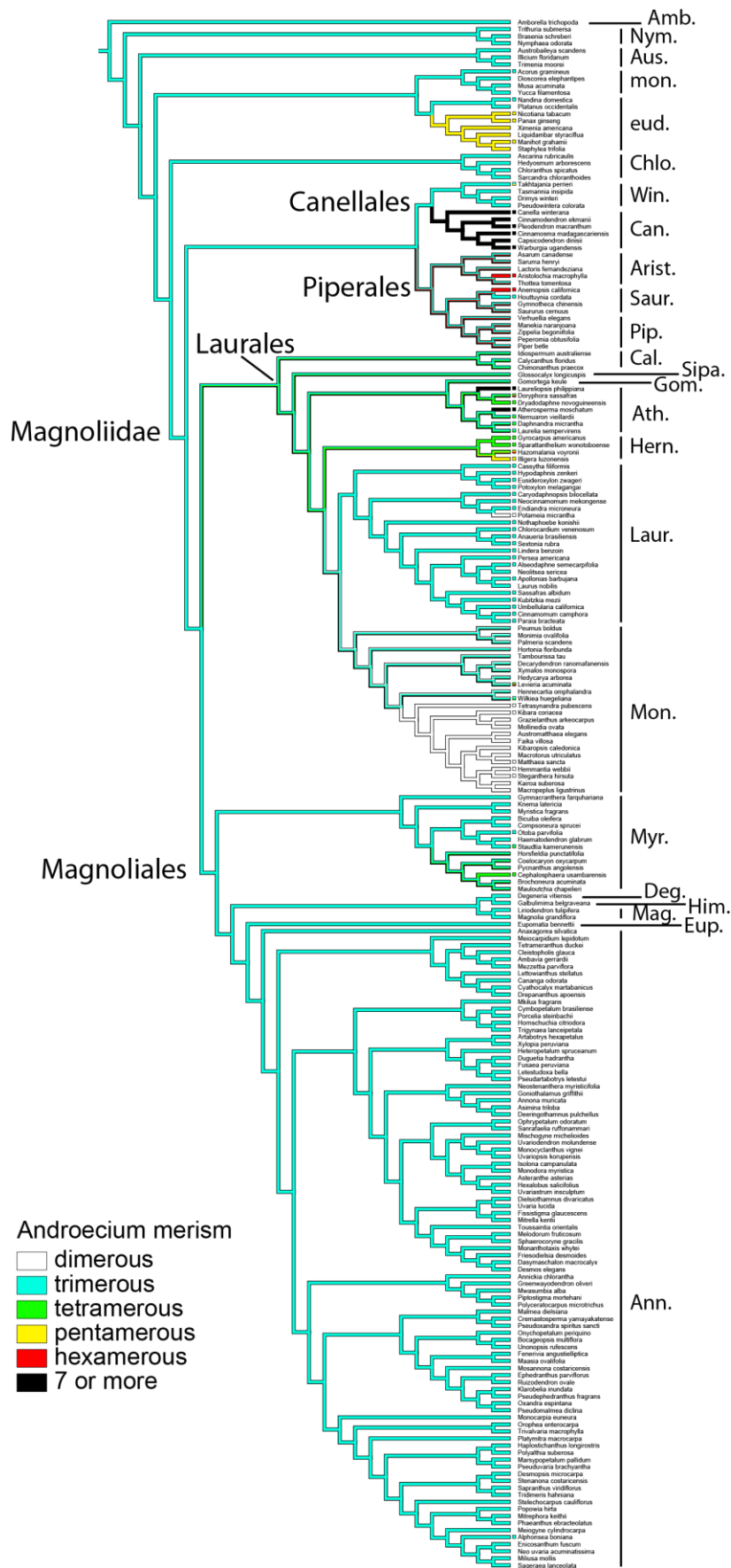


Figure S3: Evolution of the androecium. Topology: maximum clade credibility tree of angio-140 of Massoni et al. (2014a). The relationships among Magnoliidae and other angiosperms have been modified by hand according to the literature. Ancestral states are reconstructed using a parsimony approach and unordered characters. Abbreviations: Amb., *Amborella*; Nym., Nymphaeales; Aus. Austrobaileyales; mon., monocots; eud., eudicots; Chlo., Chloranthaceae; Win., Winteraceae; Can., Canellaceae; Arist., Aristolochiaceae; Saur., Saururaceae; Pip., Piperaceae; Cal., Calycanthaceae; Sipa., Siparunaceae; Gom., Gomortegaceae; Ath. Atherospermataceae; Hern., Hernandiaceae; Laur., Lauraceae; Mon., Monimiaceae; Myr., Myristicaceae; Deg., Degeneriaceae; Him., Himantandraceae; Mag., Magnoliaceae; Eup., Eupomatiaceae; Ann., Annonaceae.

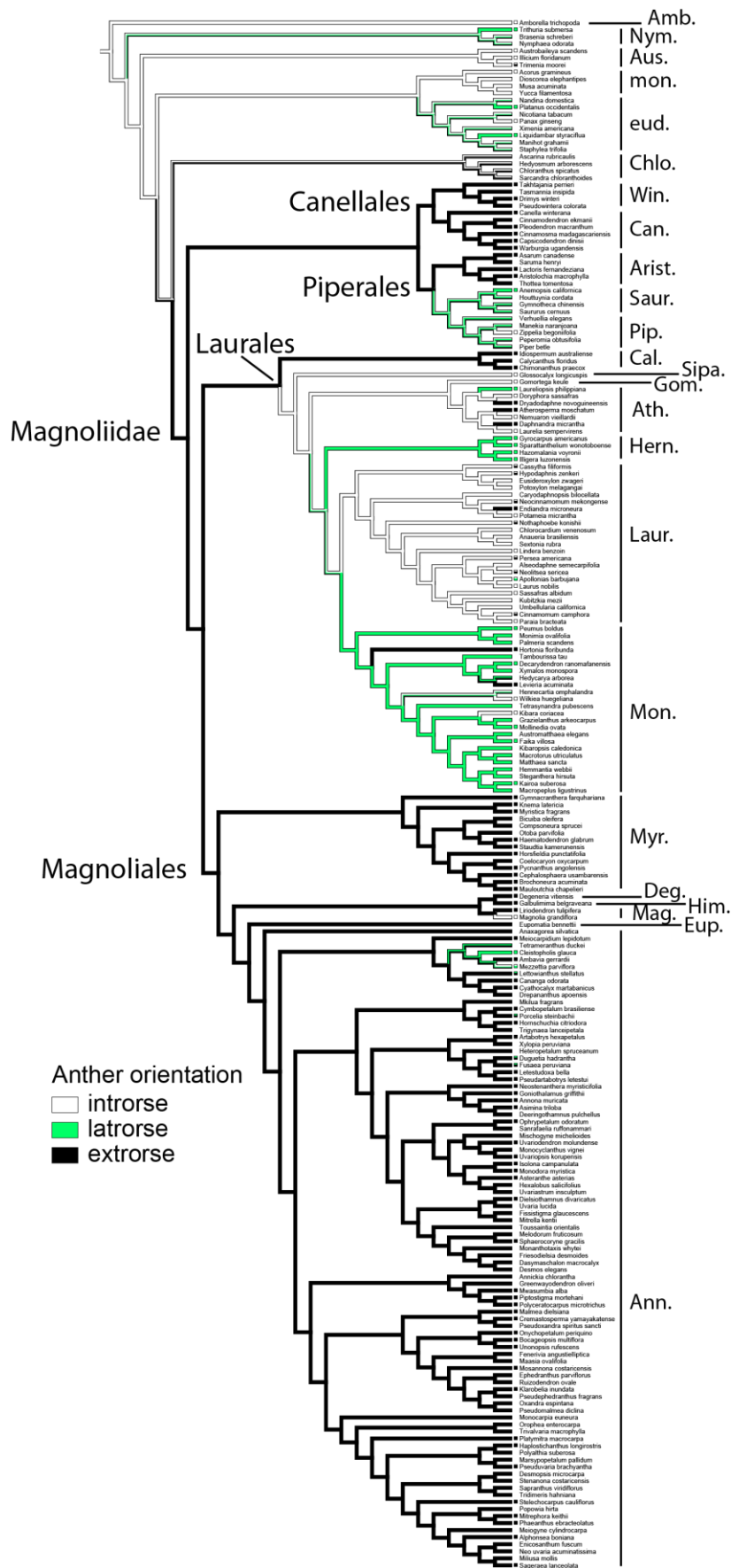


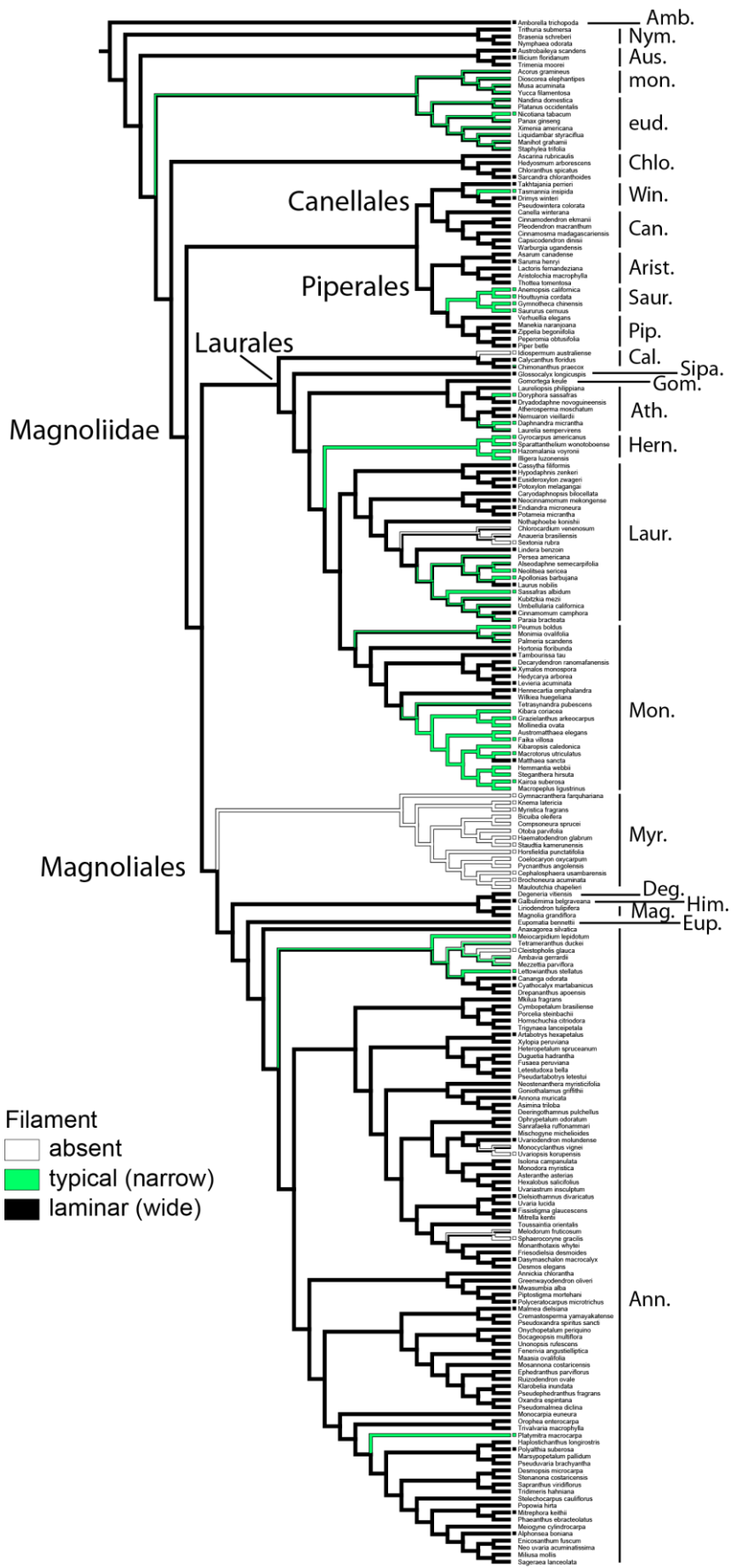


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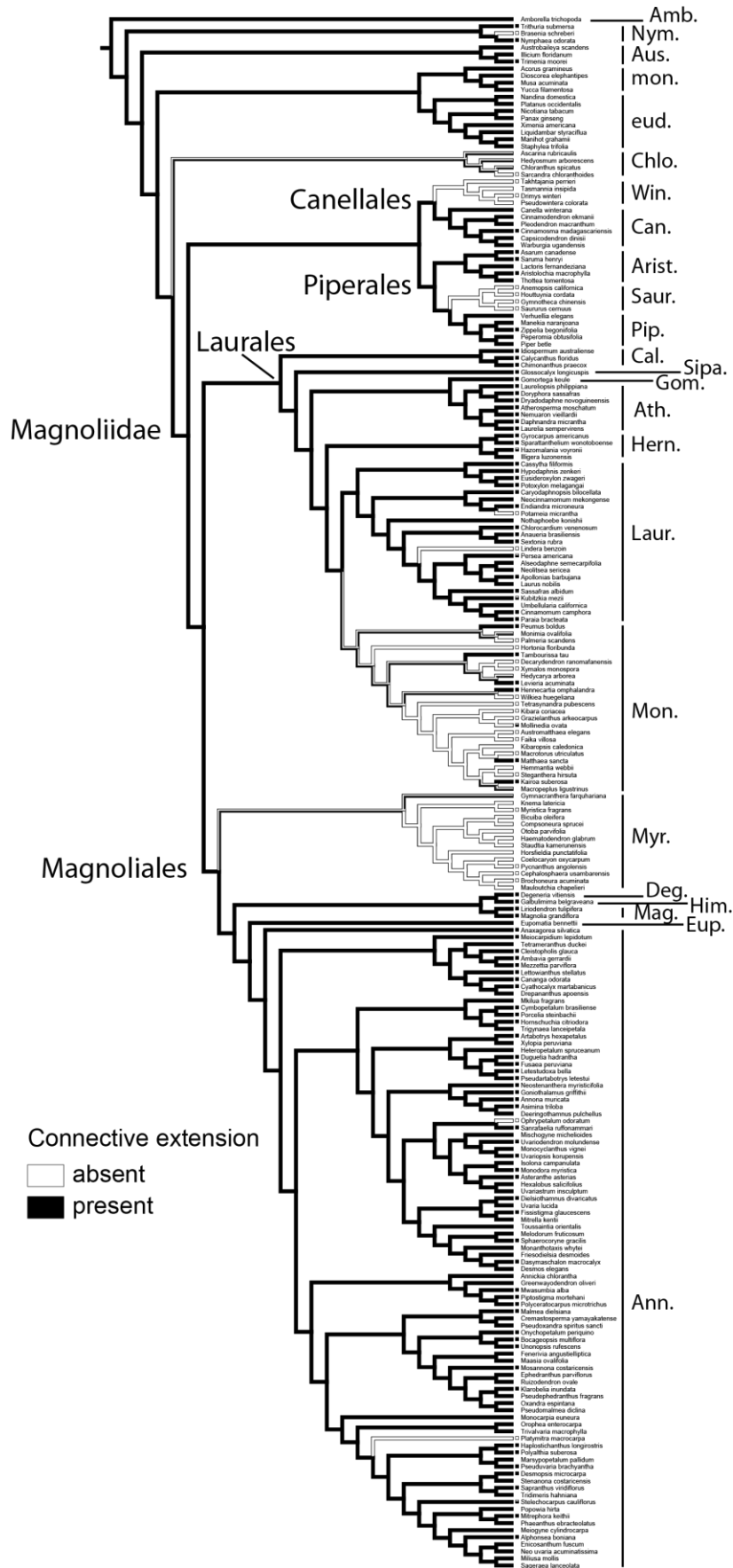


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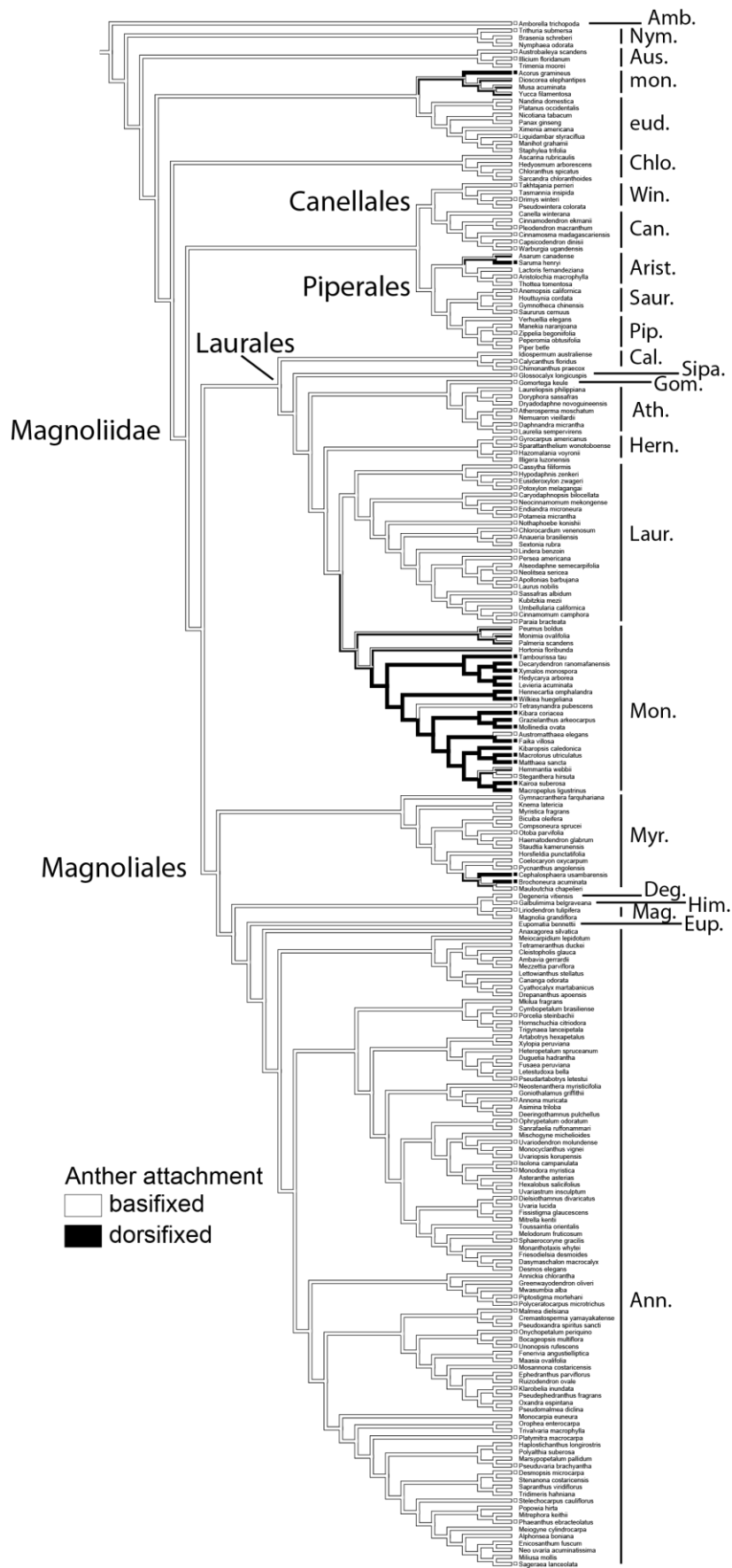


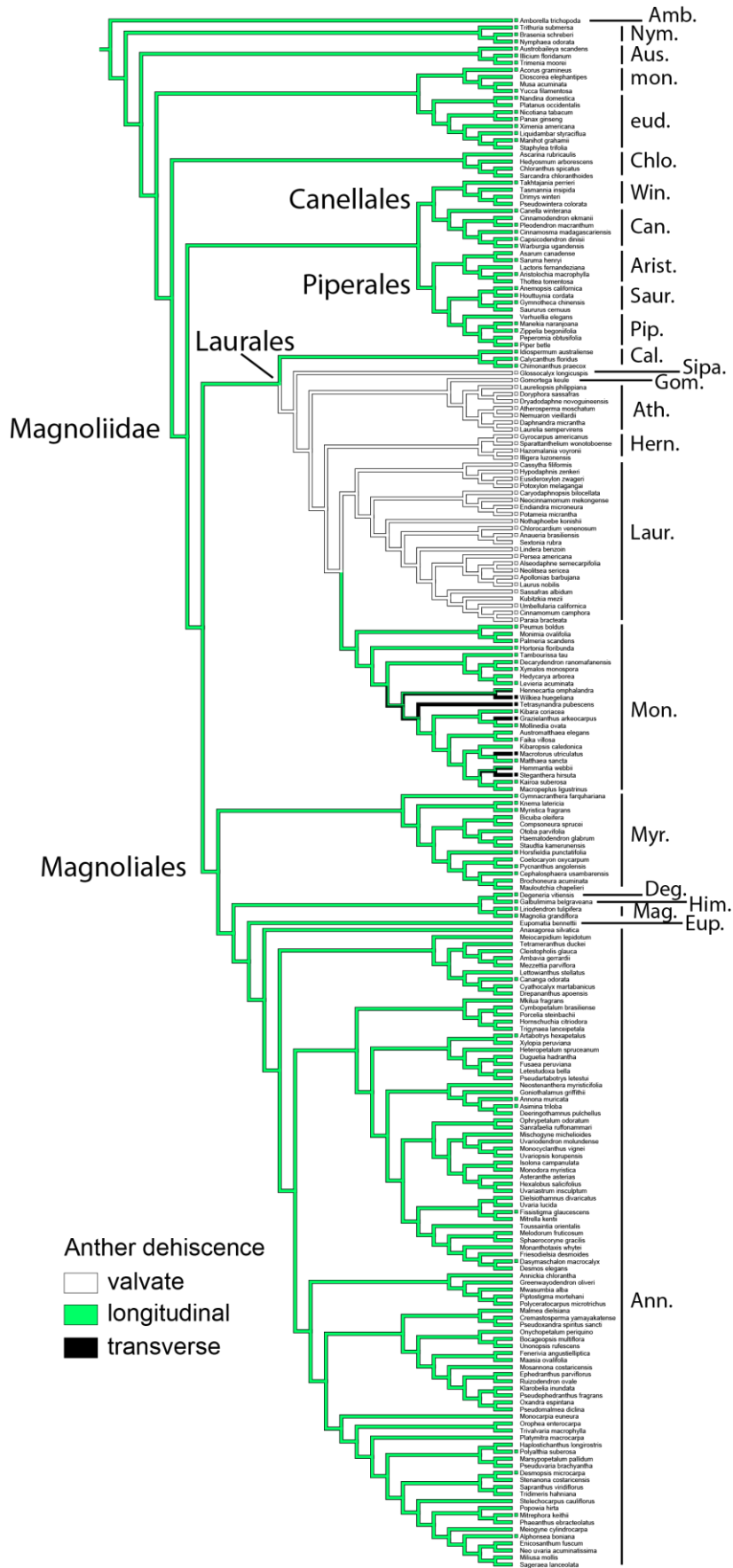


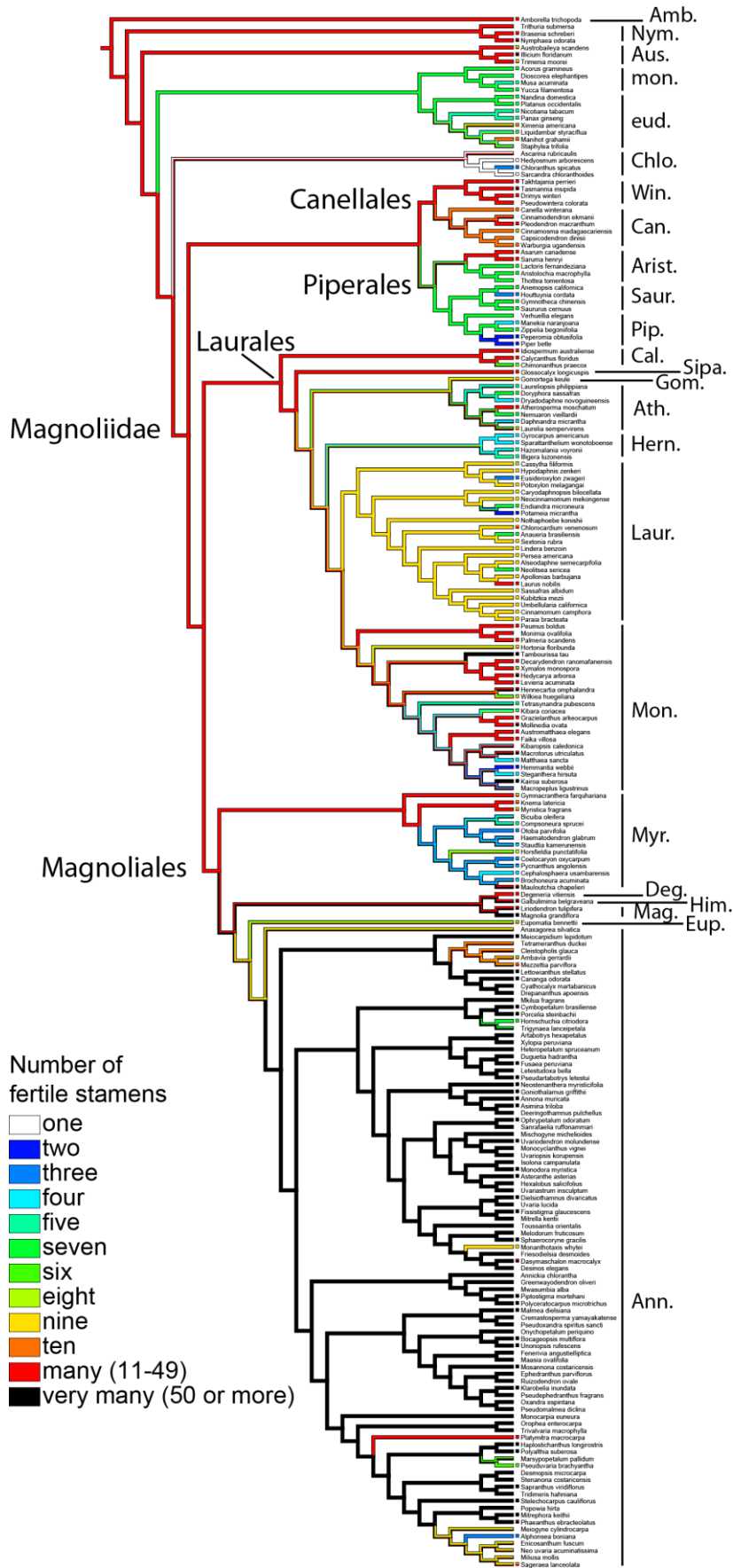
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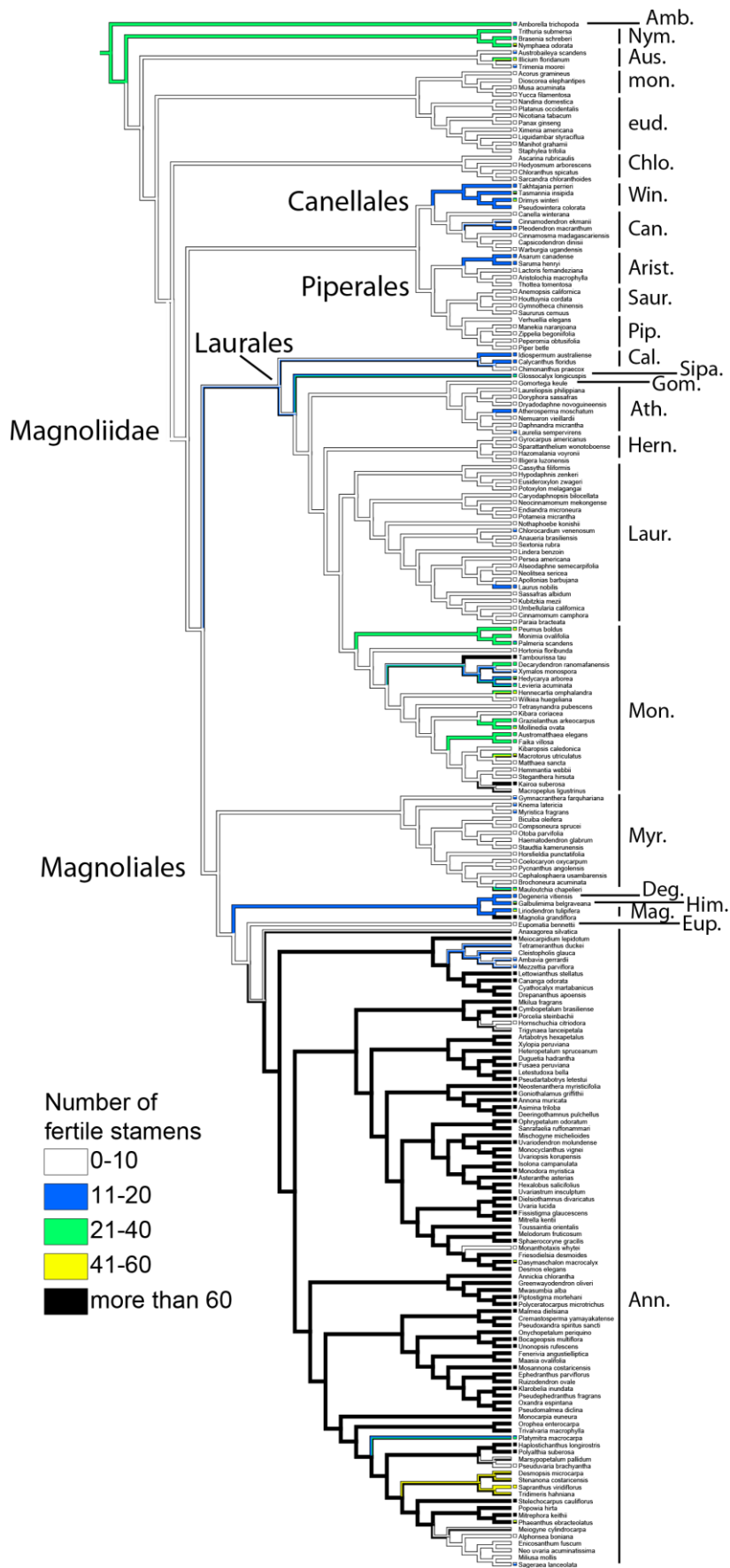
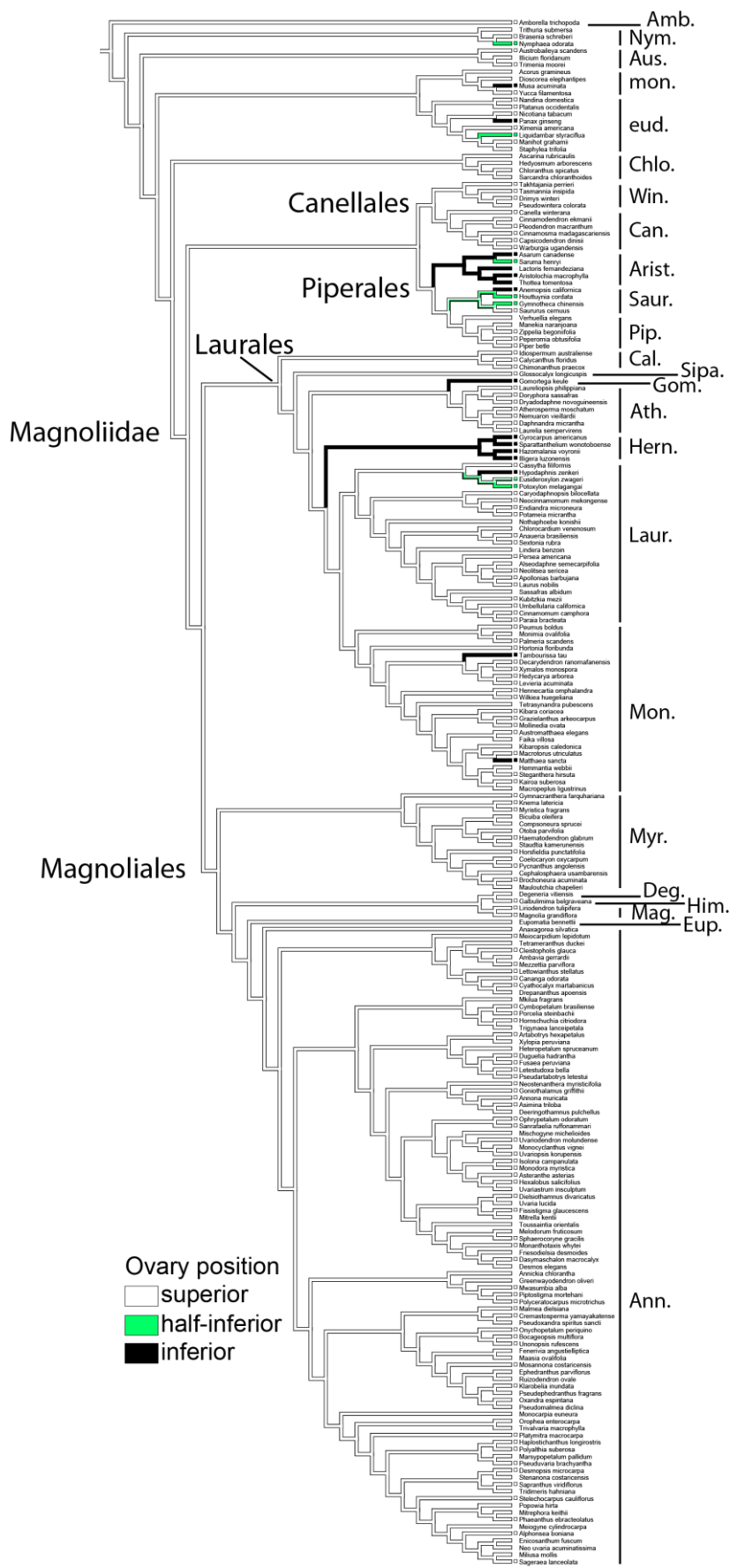
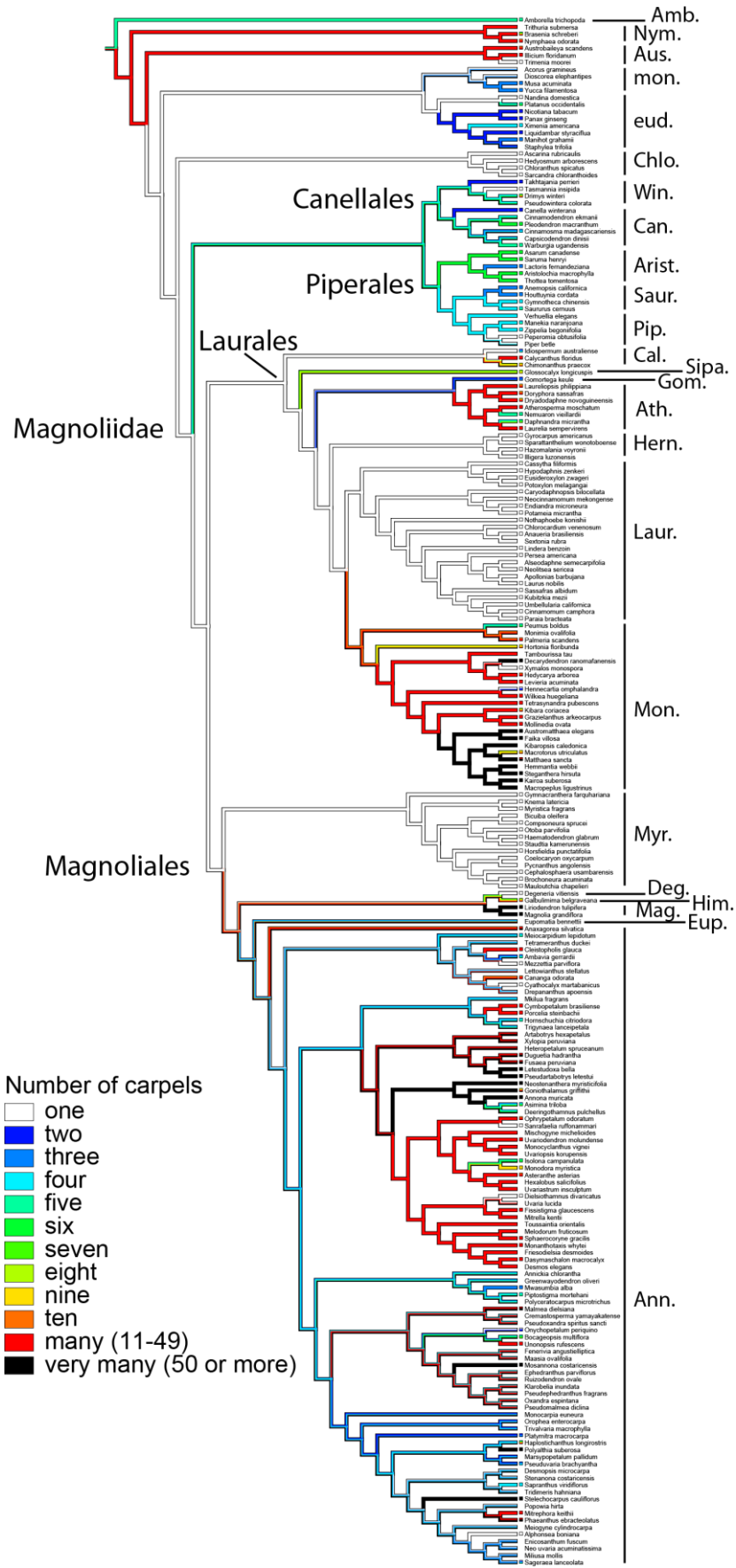
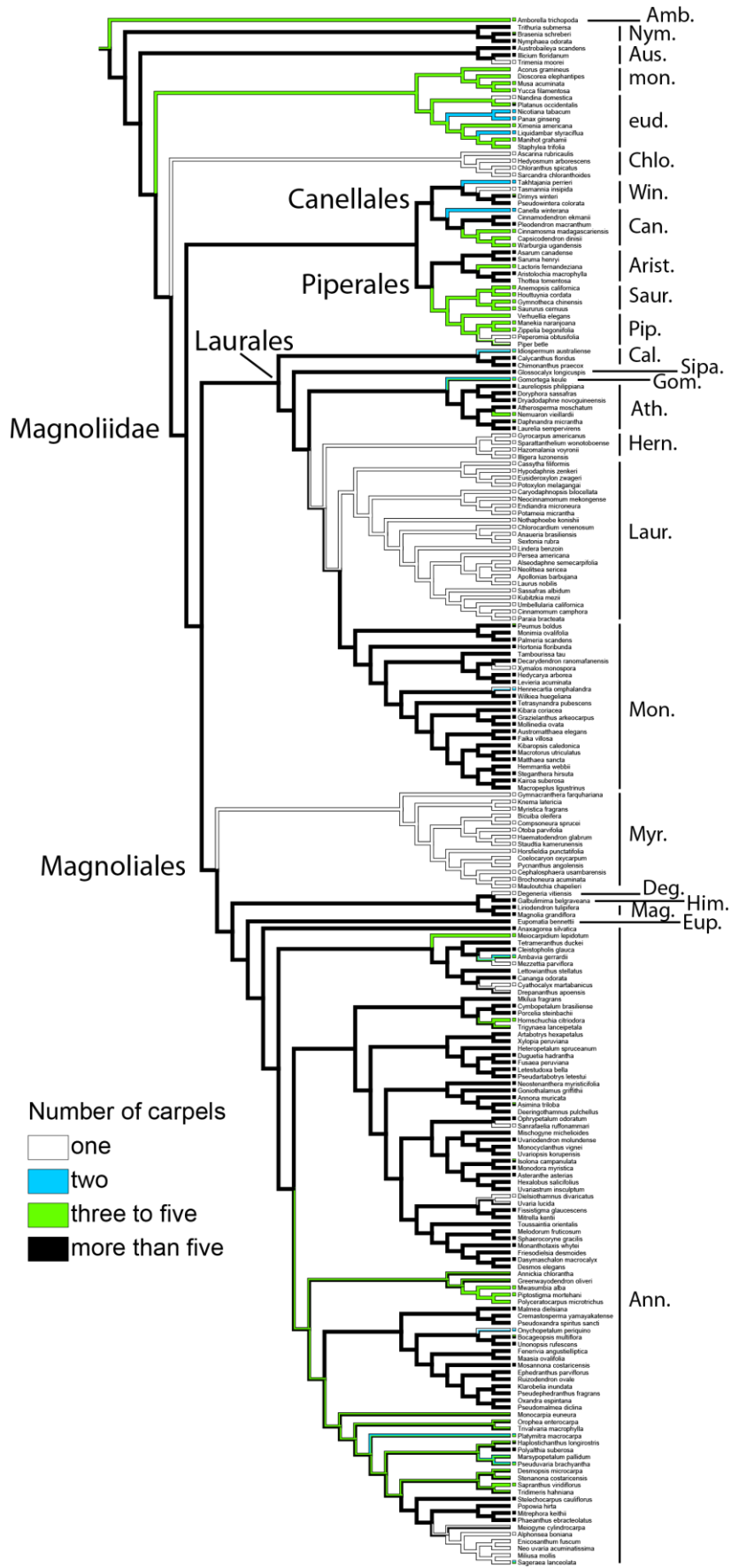


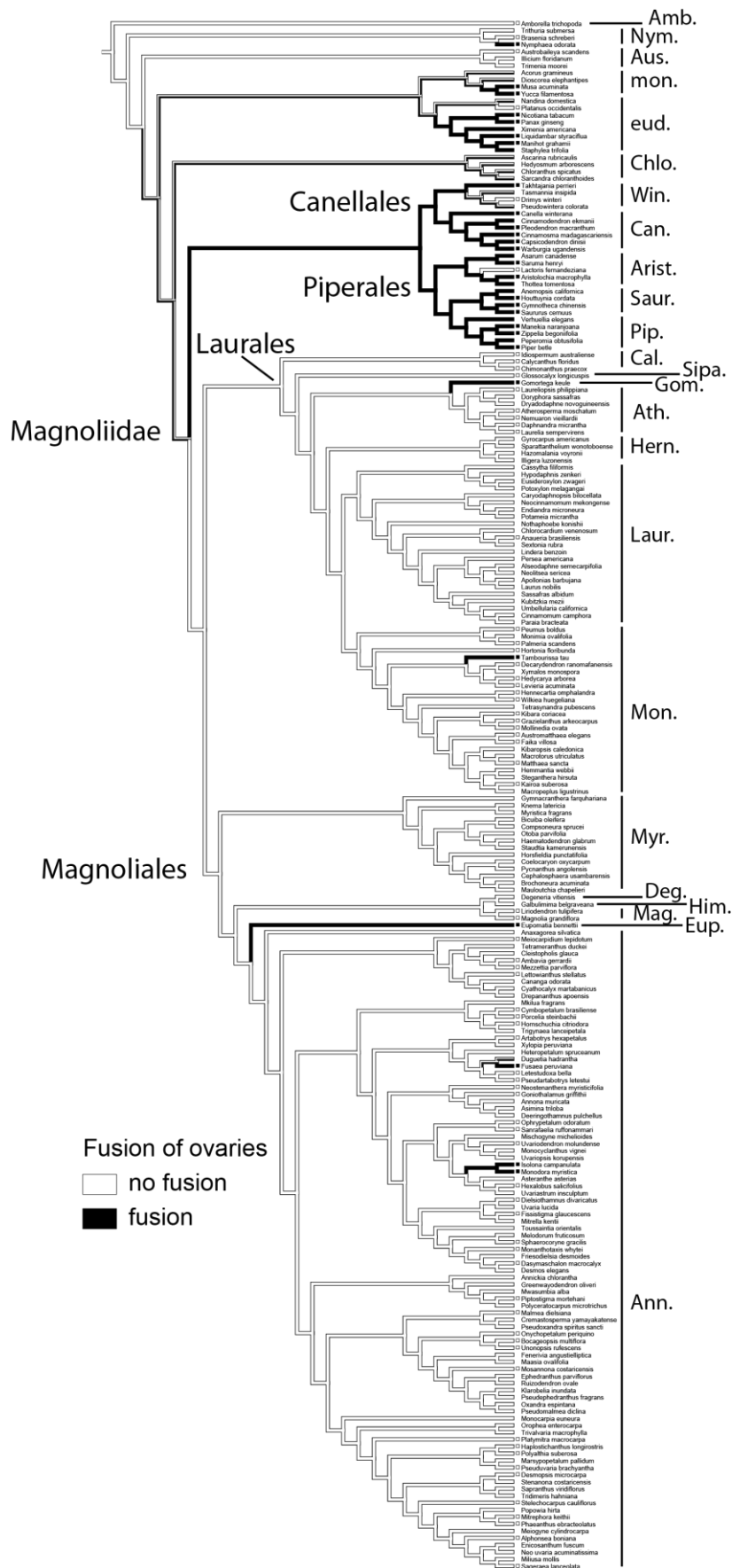
Figure S4: Evolution of the Gynoecium. Topology: maximum clade credibility tree of angio-140 of Massoni et al. (2014a). The relationships among Magnoliidae and other angiosperms have been modified by hand according to the literature. Ancestral states are reconstructed using a parsimony approach and unordered characters. Abbreviations: Amb., *Amborella*; Nym., Nymphaeales; Aus. Austrobaileyales; mon., monocots; eud., eudicots; Chlo., Chloranthaceae; Win., Winteraceae; Can., Canellaceae; Arist., Aristolochiaceae; Saur., Saururaceae; Pip., Piperaceae; Cal., Calycanthaceae; Sipa., Siparunaceae; Gom., Gomortegaceae; Ath. Atherospermataceae; Hern., Hernandiaceae; Laur., Lauraceae; Mon., Monimiaceae; Myr., Myristicaceae; Deg., Degeneriaceae; Him., Himantandraceae; Mag., Magnoliaceae; Eup., Eupomatiaceae; Ann., Annonaceae.

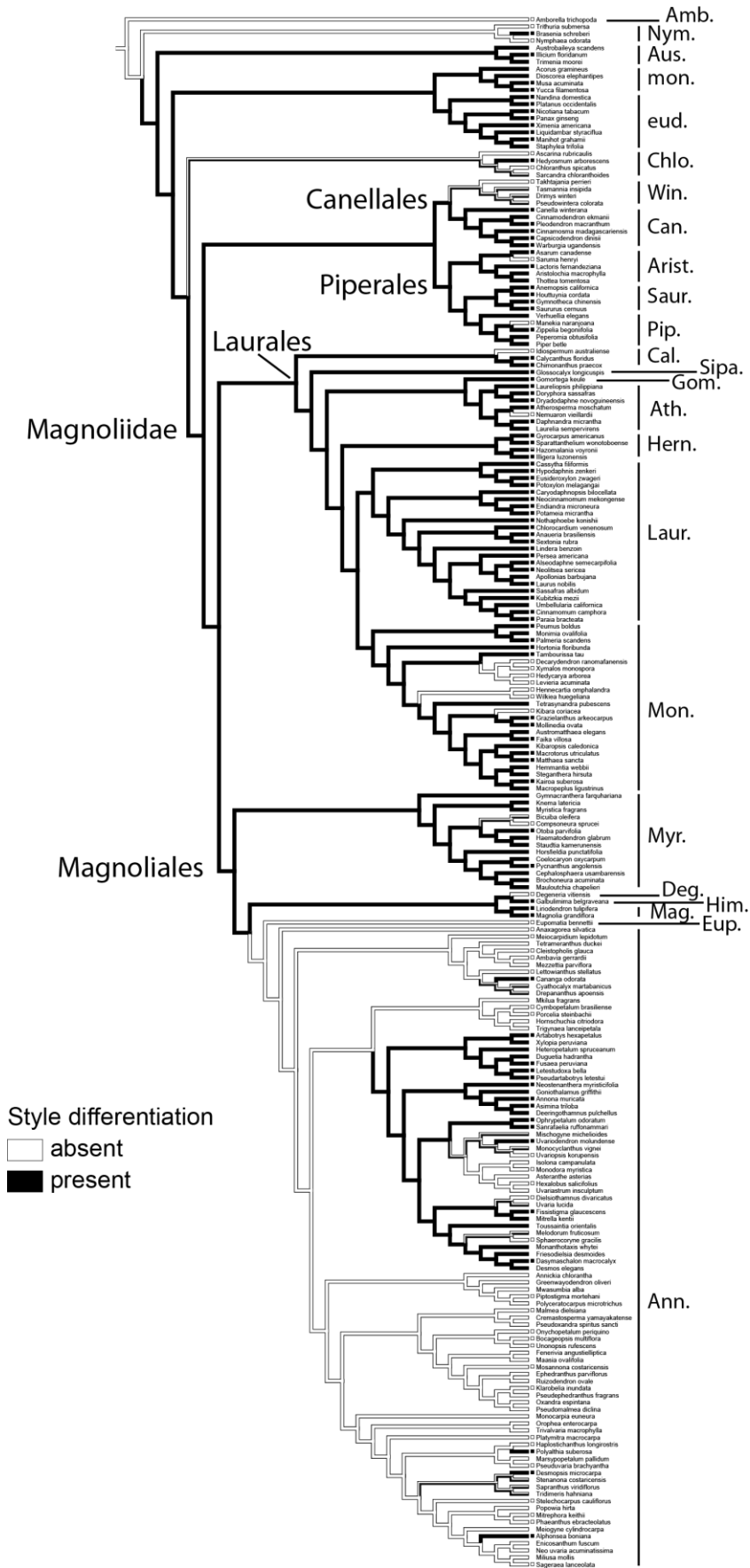
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Conclusion

The present thesis provides a new phylogenetic framework for Magnoliidae, which represents a significant improvement on the one that had emerged through previous large-scale studies (e.g., Mathews and Donoghue 1999; Soltis et al. 2000a,b, 2011; Zanis et al. 2002; Borsch et al. 2003; Qiu et al. 2005, 2010; Jansen et al. 2007). It further confirms, with greater levels of support, the presence of two clades in Magnoliidae: Canellales + Piperales, and Laurales + Magnoliales. Within each order, the relationships are also generally well established. In Piperales, we confirm *Lactoris* to be nested within Aristolochiaceae and our results suggest Hydnoraceae to be included in the same family. In Laurales, there are three well supported clades: Calycanthaceae, Gomortegaceae + Atherospermataceae, and Hernandiaceae + Lauraceae + Monimiaceae. In Magnoliales, even though the exact position of Magnoliaceae is still uncertain, the remaining relationships are well supported (Myristicaceae sister to the rest of the families, Eupomatiaceae sister to Annonaceae). The addition of about 100 new sequences, generated during my thesis but not yet used in our analyses, will likely further improve resolution and support of these relationships. These results show that good support for the deep relationships within Magnoliidae is generally obtained with datasets including more than five molecular markers (Qiu et al. 2005, 2006; Jansen et al. 2007; Moore et al. 2010; Soltis et al. 2011). The position of Magnoliaceae, and the relationships among Hernandiaceae, Lauraceae, and Monimiaceae remain poorly supported, probably because of very short branches in this part of the trees. By increasing the number of sampled characters, and therefore the number of variable nucleotide positions, next-generation sequencing approaches are a promising way to resolve these relationships. However, within Laurales, a faster and more cost-effective solution may be to take advantage of much more variable markers, already available on GenBank for numerous species of Laurales. For instance, preliminary experiments with the Internal Transcribed Spacer (ITS) sequence data, provided promising results. Consequently, using such markers at a higher taxonomic level than where they are typically used, in combination with less

variable ones used in my previous dataset, may allow us to obtain a much better supported phylogeny of Laurales.

The addition of low copy nuclear markers in our molecular dataset could also be very interesting. Such markers are still sparsely used to reconstruct deep relationships within angiosperms (e.g., Mathews and Donoghue 2000). However, Lee et al. (2011) reconstructed a phylogeny for the angiosperms with a phylogenomic approach including 22,833 sets of orthologs from the nuclear genome. They found well supported results that are partly congruent with the phylogenetic signal from the chloroplast and the mitochondrion. However, in their results, Magnoliidae were sister to eudicots with high support values, contrary to studies using large plastid datasets (Jansen et al. 2007; Moore et al. 2007). In the present analyses, we have included two ribosomal nuclear markers (18S and 26S rDNA) and the phylogenetic signal obtained from them was poorly supported. Because these two ribosomal DNA are involved in the constitution of subunits of the ribosome, a large amount of positions may not be independent. It could be of primary interest to include more nuclear data in our molecular matrix and compare their respective phylogenetic signals. Because the nuclear genome is inherited from both parents, this procedure could potentially detect ancient hybridization events.

Another improvement of this phylogeny will consist in increasing the number of taxa. Several genera of Magnoliidae could not be sampled during my PhD because I could not find suitable material for DNA amplification. These genera are typically not cultivated in botanical gardens and have not been sequenced yet. Field work will be required to obtain DNA material and add them to our phylogeny. In addition, for numerous other species of the same genera already included in our phylogeny, sequences for the 12 markers used in this thesis are available on GenBank.

The present molecular dating study supports older ages for numerous lineages of Magnoliidae than was previously thought. The crown node of the group is dated between 127.1 and 178.9 million years (MA). Laurales (111.8-165.6 Ma), Magnoliales (115.0-164.2 Ma), Canellales (126.3-141.0 Ma), Piperales (88.2-157.7 Ma), and most of the families were present before the end of the Cretaceous. Lauraceae, Aristolochiaceae, and Calycanthaceae

appear to be the oldest families in terms of crown ages. With our morphological dataset including the same list of species as in the molecular matrix, it will now be possible to conduct phylogenetic analyses for several additional fossils flowers not considered in this study. These new calibration points will likely help to reduce the uncertainty (confidence intervals) around some of our age estimates. In addition, the combination of the two datasets will now allows us to experiment with molecular dating studies using a total evidence approach (Pyron 2011; Ronquist et al. 2012). Such analyses would have the advantage to take into account the uncertainty on the placement of fossils. To my knowledge, no published study has so far taken this approach in angiosperms. Future comparisons with the results obtained from classical approaches (position of the fossil defined a priori in the form of an age constraint) will be interesting from a methodological standpoint.

Our new dated tree of Magnoliidae has highlighted among-lineage heterogeneity in speciation and extinction rates, by supporting at least six significant shifts in these rates across the tree. However, this study, and in particular the old origin of numerous lineages of Magnoliidae, raises new questions: How did geological crises such as the Cretaceous-Paleogene extinction, or more local events, affect the group? Could we detect an increase of the extinction rate during these periods with current diversification methods? Did the diversification rates of surviving lineages increase after these periods? These events had long been seen to be of low influence on the evolution of land plants, a view that is challenged today (McElwain and Punyasena 2007). Recent developments in models of diversification allow extinction rates to be higher than speciation rates (e.g., Morlon et al. 2011), allowing a decrease of species diversity through time, unlike most diversification models commonly used. In addition, we are now at an exciting turning point, where neontologists and paleontologists are joining forces to develop new statistical frameworks that take explicitly the paleontological record into account in the estimation of diversification rates. These new developments will probably be more suitable to address such questions. Because of their rich fossil record and old families, Magnoliidae may represent a model of choice to work on this topic.

The classic way to detect extinction events is to investigate variations of composition in an abundant and continuous paleontological record of the group of interest through geological periods where we suspect these crises occurred (e.g., Wilf and Johnson 2004). Because of the large uncertainty on the phylogenetic relationships of most plant fossils, this approach will require important background work to securely confirm the position of potential magnoliid fossils. However, because Magnoliidae share several characters seldom found in other angiosperms, new morphological datasets describing a maximum of characters of vegetative and floral organs would allow to securely place many fossils.

In the last chapter of my thesis, I have reconstructed the ancestral flowers of Magnoliidae. The results support an ancestral flower of all Magnoliidae with a differentiated perianth consisting of two trimerous whorls of free perianth parts. These preliminary results are promising. However, before submitting a manuscript, I would like to investigate the evolution of the same characters with probabilistic models. This approach will allow us not only to take time into account, but also phylogenetic uncertainty (relationships and branch lengths, in a Bayesian framework), and it will allow me to experiment with different models of evolution and the treatment of continuous characters. In addition, I would like to test the correlation among several characters. In the PROTEUS database, we have scored 75 different morphological characters to describe the magnoliid flowers, many of which remain to document in many taxa. Therefore, the matrix used in the last chapter (22 characters) will grow in the future. Tracing the evolution of these remaining features will allow us to draw a more complete reconstruction of the ancestral flowers than presented here, especially for the female parts, which are poorly described. The approach used in the present thesis, consisting in using the literature as the source for morphological characters, is very efficient for structures generally present in all angiosperms, but not for rare structures frequently omitted in descriptions. It is not clear if this lack of information is due to an absence of the structure or a lack of description by the author. This is often the case for characters specific of Magnoliidae (Endress 2010). Therefore, future new observations on fresh material will be vital to investigate the evolution of typical structures of the group that may have played an important role in their evolution and diversification.

Because non-biotic transfer of pollen is rare in Magnoliidae, it is of primary interest to study floral evolution in the light of the different modes of pollination. This context allows us to put forward hypotheses of correlations, which may then be tested. In the last chapter of my thesis, I have discussed the probable relationship between the nature of pollinators and the thickness of the filament in relation to their role in the protection of the gynoecium. However, pollination systems may also be correlated with other morphological features. For instance, the number of parts might be correlated with the presence of tissues consumed by insects (food bodies). Pollinators of *Calycanthus* (Calycanthaceae, Laurales) feed on the inner perianth parts (Staedler et al. 2007), and the genus has the highest number of tepals in Magnoliidae. The presence of sterile stamens (staminodes) is also widespread and both Himantandraceae and Eupomatiaceae have food bodies born on numerous staminodes (Endress 1984) (Figure 1).

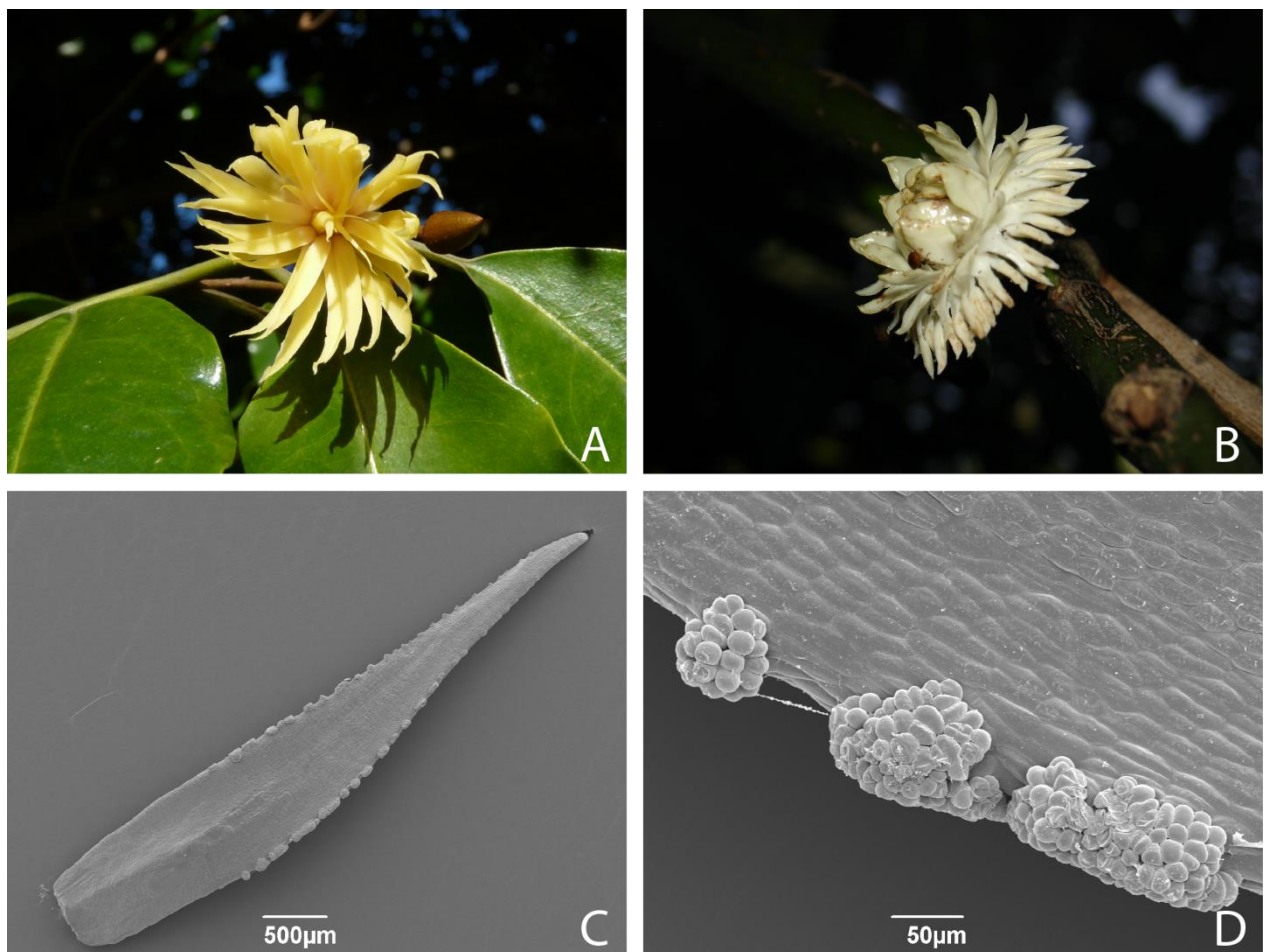


Figure 1: Flowers with sterile stamens (staminodes) that resemble perianth parts. In both species, no perianth is visible. A, *Galbulimima baccata* (Himantandraceae). B, *Eupomatia laurina*

(Eupomatiaceae). C, SEM picture of a sterile stamen (inner staminode) of *Galbulimima baccata* with food bodies on margins. D, SEM detail of these edible tissues.

The investigation of the influence of pollination on floral characters will require many new descriptions, such as floral scent, which appears to play an important role. However, in my opinion, it should already be possible to conduct some interesting analyses. For instance, it would be possible to investigate the correlation between different morphological characters and two classes of pollinators/visitors: destructive and non-destructive. If the literature lacks precise data on which species effectively pollinate the flowers, data are already available on visitors and their destructive nature or not (Endress 2010). Thus, we could test the correlation between these two classes of insects and various traits that may be involved in the protection of sexual parts of the flowers (e.g., androecium, movement of parts, staminodes, receptacle shape). Laurales, and to a lesser extent Annonaceae, may represent two models of choice to investigate these correlations. Both groups are known to be visited by the two types of pollinators and exhibit a diversity of protection strategies for the gynoecium.

The present thesis has improved our understanding of the evolutionary history of Magnoliidae. In addition to providing a new well-supported phylogenetic background, my work has highlighted an old origin for the majority of families, before the end of the Cretaceous, and heterogeneity in the rate of accumulation of lineages through time. The ancestral flower of the most recent common ancestor of Magnoliidae probably had a differentiated perianth of two trimerous whorls. This work will now represent a solid basis to put forward and test numerous hypotheses.

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