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Evolution, Ecology and Population Biology

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Evolutionary Developmental Leaf Morphology of the Plant Family Araceae

by

Claudia Liliana Henriquez

A dissertation presented to the
Graduate School of Arts & Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2015
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Claudia Henriquez

Washington University in St. Louis

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Dedicated to Thomas B. Croat and Barbara A. Schaal
for making my dream project a reality.

ABSTRACT OF THE DISSERTATION

Evolutionary Developmental Leaf Morphology of the Plant Family Araceae

by

Claudia Liliana Henriquez

Doctor of Philosophy in Biology and Biomedical Sciences

Evolution, Ecology and Population Biology

Washington University in St. Louis

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Professor Allan Larson, Chair

Studying the evolutionary developmental morphology of leaves using next-generation phylogenetics, a candidate gene approach and comparative developmental studies in the plant family Araceae is the overarching theme of the dissertation.

The plant family Araceae is an ancient lineage from the Early Cretaceous and belongs to the monocotyledons. Members of Araceae display striking variation in leaf development; such variation contradicts traditional models of monocot leaf development. Additionally, dissected leaves, which are rare in monocots, seem to have evolved independently multiple times in Araceae by various developmental mechanisms.

Despite extensive efforts to elucidate the evolutionary history of Araceae, phylogenetic ambiguity in the backbone of the tree has precluded answering questions about the early evolution of the family. To depict the sequence of morphological and developmental modifications to leaf ontogeny over time, it is essential to have a strongly supported hypothesis of the evolutionary relationships among species in the family.

To resolve the remaining questions in the deep phylogeny of Araceae a phylogenomic analysis was carried out using next-generation sequencing technology and reference-based assembly of chloroplast and mitochondrial genomes for 37 genera representing 42 of the 44 major clades in the family. Chloroplast sequences produced strongly supported phylogenies in contrast to mitochondrial sequences, which produced poorly supported trees although smaller clades were recovered. The plastid phylogeny obtained from this study is the first for Araceae with a strongly supported backbone and was used for subsequent studies of evolutionary developmental leaf morphology in the family.

Studies of the genetic basis of dissected leaf morphology via blastozone fractionation in plants outside monocots have almost always implicated the action of class I *KNOX* (*KNOXI*) genes with one exception - in peas a homolog of the floral meristem gene *FLO/LFY* is implicated. However, studies of dissected leaf development in monocots, and an examination of the developmental genetics for those monocots that putatively share the blastozone fractionation mechanism are lacking. Two genera in Araceae, *Anthurium* and *Amorphophallus* were studied and confirmed to produce lobes and leaflets through blastozone fractionation. To test whether *KNOXI* genes are involved in leaf dissection in these genera, immunolocalizations using both a full-length and C-terminus anti-*KNI* antibodies were performed on histological sections of developing dissected leaves. *KNOXI* protein expression detected by the full-length anti-*KNI* antibody and by the C-terminus anti-*KNI* antibody was absent and present in developing dissected leaves, respectively. To resolve these conflicting results, an RT-PCR assay was designed to test for the presence of *KNOXI* mRNA transcripts during leaf development in *Anthurium*. Results of the RT-PCR assay support the *KNOXI* protein expression pattern seen in

immunolocalizations using the C-terminus anti-*KNI* antibody. This suggests that monocots share the same genetic mechanism for dissected leaf development with other angiosperms.

Historical models of leaf development posit that structural similarities between monocot and dicot leaves are the result of convergence, although this hypothesis has been contested. Araceae displays both dicot and monocot leaf characters. Previous researchers have remarked on the departure of leaf development in Araceae from traditional models of monocot leaf development. Araceae displays both dicot and monocot leaf characters. To test the hypothesis of a developmentally independent origin of dicot-like leaf characters in monocots, leaf primordium diversity was evaluated in 30 genera of Araceae, along with 36 taxa spanning the angiosperm phylogeny. Leaf primordia were scored for 14 developmental, morphological and anatomical leaf characters. Ancestral character state reconstruction was carried out using the phylogeny obtained from Chapter One, embedded in two contrasting phylogenetic hypotheses of angiosperm evolution. Taxa were plotted in morphospace constructed using the morphological matrix to test whether dicot and monocot leaves occupy similar or different parts of the morphospace. The results of ancestral character state reconstruction and morphospace plotting suggest that at the developmental morphological level, aroid and dicot leaves are homologous. However, at the molecular genetic level, a review of the literature suggests that statements of homology between monocot and dicot leaves must be tested within a framework of the hierarchically organized gene regulatory networks regulating leaf development.

The leaves of Araceae have historically been considered “odd” within monocots. However, the incredible morphological and developmental diversity of leaves in Araceae has provided a powerful study system with which to investigate the unifying aspects of leaf development across angiosperms.

Introduction of the Dissertation

Meet the Aroids

The plant family Araceae belongs to the monocotyledons, and is hypothesized to have shared its most recent common ancestor with other Alismatids in the Early Cretaceous, approximately 135 Mya (Nauheimer et al., 2012). Members of Araceae (called aroids) are recognized by their unique inflorescence type, composed of tiny flowers compacted onto a terminal structure called the spadix with a subtending leaf-like organ called the spathe (Mayo et al., 1997). Calcium oxalate crystal diversity in Araceae is unrivaled among plants, and includes such configurations as raphides, druses, prismatic and crystal sand (Keating, 2002, 2004b). The family consists of ca. 3,800 species in 118 genera, distributed mostly in the tropics but can range into temperate and, in the case of *Calla palustris*, circumboreal regions (Boyce and Croat, 2013; Ulrich et al., 2013). Members of Araceae occupy a wide array of ecological habitats from sea level to above 4000 m and range from submerged, emergent or free-floating aquatics, to epiphytic, climbing and terrestrial plants (Bown, 2000; Cabrera et al., 2008; Croat, 1988; Mayo et al., 1997).

Araceae is a member of the order Alismatales, which is characterized ancestrally by the aquatic habit of its members, including the only marine angiosperms (Les and Tippery, 2013). During the evolution of the family there have been several adaptive shifts in and out of aquatic habitats (Cusimano et al., 2011), which may have played an important role in its evolutionary morphology. The morphological diversity of Araceae is arguably the most striking in the plant kingdom, considering that it includes the smallest known angiosperms and one of the largest inflorescences in the world (Simpson, 2006). Among monocots, no other family boasts the vast diversity of leaf morphology seen in Araceae, which can be one of the most salient features of tropical vegetation.

The importance of Araceae in leaf evolutionary development studies

As photosynthetic structures, leaves of terrestrial plants are an essential source of food, oxygen and CO₂ sequestration (Field et al., 1998). This functional role imparts three fundamental properties to leaves: 1) lateral determinate growth from an indeterminate meristem, 2) dorsiventral asymmetry giving rise to a marginal meristem or blastozone (Hagemann and Gleissberg, 1996), and 3) simple to complex vasculature (Ambrose and Ferrándiz, 2013; Kaplan, 1997). Despite these unifying fundamental properties, vascular plants have evolved an astonishing diversity of leaf morphology within the bounds of maintaining functional photosynthetic machinery. Theories attempting to explain leaf shape diversity include thermoregulation, hydraulic constraints, biomechanical constraints, adaptations to optimize light interception or avoid herbivory, among others (Nictora et al., 2011). A major goal of plant evolutionary biology is to understand the various developmental programs plants have evolved that enable such tremendous leaf diversity, while still operating as an essential factory of primary productivity. The photosynthetic efficiency of leaves is mediated to a large extent by leaf shape (Nicotra et al., 2008). Understanding the connections between leaf development, efficient photosynthesis and productivity is of critical importance for improving crop performance in the field, where yield size is directly related to photosynthetic capacity (Zelitch, 1982).

The field of leaf evolutionary development has made great strides in revealing the molecular genetic mechanisms that give rise to many of the diverse leaf morphologies seen in the natural world. Many of these insights come from model species within a group of plants – the monocots. Monocots are the single most important plant group in terms of world food and biofuels production, including crops like rice, corn, wheat, sugarcane, bananas, yams, onions, taro and palms (Chase, 2004). Model crops such as corn and rice, which are members of a highly

derived clade, Poales, have been extensively investigated due to their unprecedented economic importance (Kellogg, 2001; Linder and Rudall, 2005). Monocot species outside Poales cultivated by smallholder farmers are staples for food security in developing countries and merit investigation. Among these, taro (*Colocasia esculenta*), giant swamp taro (*Cyrtosperma merkusii*), giant taro (*Alocasia macrorrhiza*), cocoyam (*Xanthosoma sagittifolium*) and elephant foot yam (*Amorphophallus paeonifolius*), are members of the plant family Araceae (Lebot, 2009).

There is general consensus that aroid crops are of extreme importance to the poor, occasionally accompanied by the societal stigma as a “poor man’s crop” (Lebot, 2009). According to the National Root Crops Research Institute (NRCRI), cocoyam ranks third in importance after cassava and yam among the root and tuber crops cultivated and consumed in Nigeria. Nutritionally it is superior to cassava and yam (<http://www.nrcri.gov.ng/pages/cocoyam.htm>). It is also the third most important starch food crop in Nicaragua and is cultivated countrywide (Lebot, 2009). Taro is a staple crop that an estimated 400 million people include in their diets and is the 14th most consumed vegetable worldwide (Singh et al., 2007). It has strong cultural ties and high market value in the Pacific Islands and Papua New Guinea, and is cultivated in many other countries including Egypt, Cuba, Southern China and Thailand (Lebot, 2009). Giant swamp taro is a major food in the Pacific atolls and elephant foot yam is grown in several parts of the Pacific, Asia and India (Lebot, 2009). Yet, in spite of the widespread cultivation and consumption of aroids, they are still regarded as orphan crops with untapped potential for further economic development (Lebot, 2009).

The need for genetically improved aroid crops is apparent in countries where yields per unit of area and time are clearly too low (Lebot, 2009). Factors directly influencing taro yield, such as growth vigor, photosynthesis and overall health, are tightly associated with leaf area and plant height (Lebot, 2006b; Simin et al., 1995). Currently there is no breeding program working on taro leaf quality, much less cocoyam or elephant foot yam (Lebot, 2009). Research efforts must take into account leaf traits if overall crop improvement is to be achieved. A first step is characterization of leaf development.

In addition to their importance as crops, aroids display leaf-developmental mechanisms found rarely across other monocots; including blastozone fractionation, plication and programmed cell death occur numerous times independently within Araceae (Gunawardena and Dengler, 2006). Aroids have been noted for their leaf characters that are more similar to dicots than monocots (Bharathan, 1996; Kaplan, 1973). Although Araceae are more closely related to Poales than to eudicots, they may share with dicots pleisiomorphic developmental genetic mechanisms subsequently lost by most other monocots. Thus, Araceae is an excellent system for studying the developmental and genetic evolutionary transitions between the two major angiosperms clades.

Leaf evolution before the split of dicots and monocots

The evolution of leaves in vascular plants is thought to have occurred independently numerous times; once in lycophytes (spikemosses, clubmosses and quillworts) and between two and seven times in euphyllophytes (ferns, gymnosperms and angiosperms) after their divergence from a common ancestor over 400 million years ago (Ambrose and Ferrándiz, 2013; Floyd and Bowman, 2006; Harrison et al., 2005; Langdale et al., 2002). Leaves of lycophytes (microphylls) have a single unbranched vascular strand and are not associated with a leaf gap in the stem,

whereas the leaves of euphyllophytes (megaphylls) have complex venation patterns, are associated with a leaf gap, and are extremely variable in shape and size (Ambrose and Ferrándiz, 2013; Langdale et al., 2002).

Within euphyllophytes, a prevailing hypothesis known as the ‘Telome theory’ proposes that megaphylls evolved by planation, webbing and determinacy of the lateral branches on a simple dichotomously branching system (Zimmerman, 1965). The presence of intermediate forms between highly branched lateral projections and leaves in the fossil record makes it a plausible hypothesis (Kenrick, 2002). This intrinsic relationship between the shoot and leaf was noted by early botanists and led them to propose the ‘Leaf-skin’ theory (Saunders, 1922) and the ‘Partial-shoot’ theory (Arber, 1950).

The Leaf-skin theory was based on evidence from the distribution of hairs and other surface features and proposed that the superficial layers of the whole shoot axis in seed plants are of foliar origin (Saunders, 1922; Arber 1925). Sachs (1887) also expressed that distinctions between the stem and leaf were correlative, and merely parts of a whole – the shoot (Arber, 1925). The ‘Partial-shoot theory (Arber, 1950), although highly metaphysical in its explanation, noted that radially of the shoot and dorsiventrality of the leaf were interrelated. Interestingly, studies of the Gene Regulatory Networks (GRNs) involved in leaf development, particularly with respect to dorsiventrality and determinacy, lend support to the Partial-shoot theory. These concepts are discussed in further detail in Chapter 4.

Gene Regulatory Networks

The concept of modularity is a paradigm for describing the levels and kinds of functional and structural heterogeneity in organisms (Wagner et al., 2007). Modules are units that are highly connected into subcircuits (Erwin and Davidson, 2009). In a biological organism, those

units can be RNAs, genes, proteins, metabolites, hormones, cells or morphological characters and their connections can be physical, dynamical or statistical (Townesley and Sinha, 2012; Wagner et al., 2007). Unique combinations of units and connections create modules that vary in the biological processes in which they are involved. For example, a module can be functional if the units work together to perform a specific physiological function; they can be variational if the units co-vary independently of other units; or they can be developmental as in an autonomous developmental signalling cascade (Wagner et al., 2007). The connectedness of units into modules and of modules to one another creates a network organization. In the case of ontogenetic development, regulatory units that interact to coordinate development spatially and temporally are called Gene Regulatory Networks (GRNs) (Erwin and Davidson, 2009). In GRNs, the basic unit is the functional linkage between a transcription factor and a *cis*-regulatory element (Carroll, 2008). Rewiring of GRNs requires the evolution of *cis*-regulatory elements governing the spatio-temporal expression of transcription factors (Carroll, 2008).

The role of natural selection in the origin of modularity is still unclear, but studies have revealed that GRNs are highly plastic and that many of the changes in GRN architecture are largely nonadaptive (Wagner et al., 2007; Townesley and Sinha, 2012; True and Haag, 2001). This neutral process of GRN re-wiring has been termed ‘developmental systems drift’ or DSD (True and Haag, 2001). DSD has large implications for the study of evolutionary developmental biology, requiring studies of homology to deepen the scope of comparison beyond morphology to include GRN architecture.

The action of GRNs has been described as hierarchical where portions controlling the initial stages of development are at the core, portions controlling patterning are intermediate, and portions controlling morphogenesis are at the periphery (Erwind and Davidson, 2009).

However, modules that serve a core function can also be co-opted for patterning or morphogenesis, and are thus not restricted to a certain level in the hierarchy. Hierarchy, like modularity, is a fundamental property of biological organization (Wagner et al., 2007).

Morphology, which is produced by the action of hierarchically organized GRNs, is thus also hierarchically organized.

Blastozone fractionation – a developmental mechanism shared by monocots and dicots

One of the best-studied examples highlighting the dynamic nature of GRNs across the morphological hierarchy of shoot and leaf in angiosperms is dissected leaf development through blastozone fractionation. As mentioned earlier, one of the fundamental properties of leaves is determinate growth. Leaves arise as lateral projections on the shoot apical meristem (SAM) at the location of incumbent leaf inception, or p0, where auxin concentration has reached a maximum (Byrne, 2012; Hay et al., 2006). Auxin is antagonistic to the meristematic fate of cells in the SAM, a fate maintained by a class of transcription factors containing a conserved motif called the homeobox (Kerstetter et al., 1997; Vollbrecht et al., 1991). The homeobox is a sequence motif encoding 61 amino acids that act as a sequence-specific DNA-binding domain (Reiser et al., 2000). This homeodomain was found encoded in genes that produce homeotic mutations and was originally discovered in *Drosophila* (Hake et al., 2004). Since then, homeobox genes have been shown to play a pivotal role in regulating development across all eukaryotic lineages (Hake et al., 2004).

In plants, homeobox genes were first discovered in a maize dominant gain-of-function mutation in the gene *knotted-1*, so called for the knots of displaced tissue in the developing leaf (Vollbrecht et al., 1991). *kn-1* and related *knotted1*-like homeobox (*KNOX*) genes fall into two classes as a result of a gene duplication that occurred before the divergence of bryophytes from

euphyllophytes approximately 400 Mya (Hake et al., 2004; Kerstetter et al., 1994; Reiser et al., 2000). In plants, class I *KNOX* (*KNOXI*) genes are required for the maintenance of meristem identity, or totipotency, and are downregulated at the site of leaf initiation by another class of genes, the *ARP* genes (Bertolino et al., 1995). It is the down-regulation of *KNOXI* genes at p0 and the ongoing suppression of *KNOXI* gene expression during leaf development that promotes determinacy (Janssen et al., 1998).

Terminated development in leaves at a given stage, however, is better called ‘pseudo-determinacy’ since it has been shown that the GRN responsible for leaf inception can be reactivated within the developing leaf to produce a variety of morphological alterations, as is the case in dissected leaf development (Bharathan et al., 2002; Gleissberg et al., 2005; Johnston et al., 2014; Kim et al., 2003a; Townsley and Sinha, 2012).

During dissected leaf development, *KNOXI* gene expression is upregulated at regular intervals along the blastozone, alternating with regions of *KNOXI* gene suppression. The regions along the blastozone where *KNOXI* gene expression is present have reinstated meristematic potential and repeat the developmental pathway of leaf formation that initially occurred in the SAM, producing leaflets (Kimura et al., 2008; Lincoln et al., 1994; Sinha et al., 1993). The alternating pattern of expansion and suppression along the leaf margin is called blastozone fractionation (Gunawardena and Dengler, 2006).

Studies of the effects of altered *KNOXI* gene expression patterns in developing leaves have been undertaken in a broad sampling across the angiosperm phylogeny; however, major lineages of plants have yet to be characterized (Bharathan et al., 2002). This is especially true of basal monocots. Chapters 2 and 3 will explore the roles of blastozone fractionation and *KNOXI* gene expression in dissected leaf development in Araceae.

The unique leaf development modes of monocots

A broad distinction between monocot and dicot leaves permeates botanical literature. The appearance of unique leaf morphologies in monocots has been used along with roots, anatomy and seed structure/cotyledonary condition as support for the monophyly of monocots (Arber, 1918; Chase, 2004; Kaplan, 1973). Many monocot leaves have closed, parallel venation, sheathing leaf bases and a linear blade; however, the more typical dicot condition of petiolate leaves with an expanded lamina and reticulate venation occurs also among monocots, particularly in Araceae (Arber, 1918; Chase, 2004; Kaplan, 1973; Rudall and Buzgo, 2002). The ‘dicot leaf type’ in monocots is thought to be non-homologous with dicot leaves (Kaplan, 1973; Keating, 2002; Knoll, 1948; Troll, 1955). In the early 20th century botanists proposed various hypotheses as to the evolutionary origin of monocots and their particular leaf morphology (Arber, 1918; Henslow, 1911; Sargent, 1904). Most notable among these were the ‘Phyllode theory’ (Arber, 1918; de Candolle, 1827; Henslow, 1911) and the ‘Leaf-base theory’ (Hagemann, 1970; Kaplan, 1973, 1975; Knoll, 1948; Troll, 1955), which is discussed in further detail in Chapter 4.

Aside from the gross morphological differences between monocot and dicot leaves, there are several modes of leaf development unique to monocots. These include plication, plication followed by schizogeny, programmed cell death, and abaxialization leading to the unifacial/ensiform leaf (Gunawardena and Dengler; Kaplan, 1973, 1975; Nowak et al., 2011; Yamaguchi et al., 2010). Plication followed by schizogeny and cell death are mechanisms by which monocots achieve lobed and dissected leaves in addition to the mechanism of blastozone fractionation shared with other angiosperms. Dissected leaves are rare in monocots, making the existence of alternative developmental routes to dissected leaf morphology within the clade

interesting. Not only do members of Araceae vary widely in cell-death patterns, dissected and lobed leaf morphology via blastozone fractionation is extremely varied throughout the group as well (Mayo et al., 1997).

The amazing diversity of vegetative morphology in Araceae has been the subject of several developmental and morphological studies; however, much work is still needed (Blanc, 1977a,b; Engler, 1877; Madison, 1978; Murata, 1990; Ray, 1987b,c). Leaf developmental mechanisms in Araceae need to be addressed in a phylogenetic context to be able to investigate the contributions of robustness and evolvability of leaf GRNs in the evolution of leaf morphology within the family (Pigliucci, 2010). For this task, a strongly supported phylogeny is needed. Current family-wide phylogenies based on a small number of chloroplast and nuclear loci have not been able to resolve the early evolution of Araceae (Chartier et al., 2014, Cusimano et al., 2011). Obtaining a well-resolved phylogeny of the family in order to have an evolutionary framework with which to explore the diversity of leaf morphology and development is the goal of Chapter 1.

Finally, as early-diverging members of monocots that possess both monocot and dicot-like traits, Araceae are in an excellent position to help understand the evolutionary events that resulted in a clade with such unique leaf traits. A strongly-supported phylogeny of Araceae must be put in the context of broader angiosperm evolution in order to 1) gauge just how unusual Araceae and monocot leaf characters are, and 2) understand how ancestral leaf GRNs have been maintained and/or modified through time.

Is leaf development in Araceae non-homologous with dicot leaf development? By which mechanism(s) are the highly dissected and unique leaves of *Anthurium* and *Amorphophallus* produced? If produced by blastozone fractionation, are *KNOX1* genes involved? What are the

evolutionary relationships in Araceae among genera with such highly different developmental modes? These are the pressing questions in the evolution of leaf morphology in Araceae that will be addressed in this dissertation using next-generation phylogenetics, a candidate gene approach, and comparative developmental studies.

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Chapter 1

Phylogenomics of the Plant Family Araceae

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1.1 Introduction

Araceae, or the Arum family, is a large and ancient monocot plant family most notable for its impressive morphological diversity, including the smallest known angiosperm and some of the largest vegetative and reproductive structures in the world (Simpson, 2006). The family consists of c. 3,800 species in 118 genera, distributed mostly in the tropics but can range into temperate and, in the case of *Calla palustris*, circumboreal regions (Boyce and Croat, 2013, Ulrich et al., 2013). Members of Araceae occupy a wide array of ecological habitats from sea level to above 3000 m and range from submerged, emergent or free-floating aquatics, to epiphytic, climbing and terrestrial plants (Bown, 2000, Cabrera et al., 2008, Croat, 1988, Gonçalves, 2004, Gonçalves et al., 2007). Stems can be rhizomatous, cormose, tuberous or reduced to a thallus-like structure and leaves can be simple, highly divided or fenestrate (Mayo et al., 1997, Simpson, 2006). Araceae are distinguished from closely related families in having a great diversity of calcium oxalate crystals (raphides, druses, crystal sand, styloids and prismatic), possessing a spadix of small, bisexual or unisexual flowers, subtended by a spathe, and they lack ethereal oil cells (Grayum, 1990, Keating, 2003, Stevens, 2001 onwards).

Detailed classification of Araceae, established as a family in 1789 (Jussieu, 1789), began in the nineteenth century with the work of Heinrich Wilhelm Schott (1794-1865) and Adolf Gustav Engler (1844-1930). Schott's pre-Darwinian classification grouped genera based on inflorescences, flowers and fruits (Mayo et al., 1997, Nicolson, 1987). A modified version of this classification was used by Hooker (1883) who divided Araceae into 11 tribes, and later by Hutchinson (1973) who divided the family into 18 tribes (Grayum, 1990, Hooker, 1883, Hutchinson, 1973). Engler's new system of classification, which included hypotheses of evolutionary transitions of not only floral, but also of vegetative morphological and anatomical

characters (Engler, 1920, Mayo et al., 2007, Nicolson, 1987), has been the framework for much subsequent work (Bogner, 1978, Bogner and Nicolson, 1991, Grayum, 1990, Hotta, 1970, Mayo et al., 2007, Nakai, 1943). Grayum's 1990 revision, based on a large survey of palynological characters, is notable in recognizing *Acorus* as separate from all other Araceae (Grayum, 1987, 1990).

Since the chloroplast restriction site data of French et al. (1995), molecular data have been used to infer evolutionary relationships at all levels in the family (Barabé et al., 2002, Cabrera et al., 2008, Chartier et al., 2013, Cusimano et al., 2011, Gauthier et al., 2008, Goncalves et al., 2007, Nauheimer et al., 2012, Renner et al., 2004, Renner and Zhang 2004, Rothwell et al., 2004, Tam et al., 2004). To date, the most comprehensive family-wide molecular data set consists of six chloroplast (*rbcL*, *matK*, partial *trnK* intron, partial *tRNA-Leu* gene, *trnL-trnF* spacer, and partial *tRNA-Phe* gene) and one nuclear (*PhyC*) markers (Cabrera et al., 2008, Chartier et al., 2013), and has been used to clarify the evolutionary history, biogeography, pollination biology and chromosomal evolution of Araceae (see also Cusimano et al., 2011, Nauheimer et al., 2012). Araceae has an inferred ancestral haploid chromosome number of $n=16$ or $n=18$, and began to diversify in the Early Cretaceous, approximately 122 Mya, as the break up of Pangea was finalizing (Cusimano et al., 2012, Nauheimer et al., 2012). By the Cretaceous/Paleogene boundary all eight of the currently recognized subfamilies, including the duckweed subfamily Lemnoideae, were present and form a clade that is sister to a clade comprising all other members of the order Alismatales (Cabrera et al., 2008, Nauheimer et al., 2012, Tobe & Kadokawa, 2010). Evolutionary relationships among six of the eight subfamilies (Gymnostachyoideae, Orontioideae, Lemnoideae, Pothoideae, Monsteroideae and Lasioideae), all of which contain bisexually-flowered members, are well-supported (Cabrera et al., 2008,

Cusimano et al., 2011, Nauheimer et al., 2012). The Unisexual Flowers clade, containing subfamilies Zamioducadoideae and the highly diverse Aroideae (1573 species, 75 genera), diverged from the bisexual-flowered lineage during the Late Cretaceous approximately 90 Mya (Nauheimer et al., 2012). Low resolution of several deep nodes in the phylogeny of the Unisexual Flowers clade leaves open several important questions, including the position of the highly autapomorphic, bisexually-flowered genus *Calla* (Cabrera et al., 2008, Chartier et al., 2013, Cusimano et al., 2011, Ulrich et al., 2013). Although *Calla* is well-supported in a clade with two unisexually-flowered genera (*Montrichardia* and *Anubias*) in the nuclear tree from Chartier et al. (2013), the position of that clade at the base of Aroideae is not strongly supported and biogeographical and morphological features make this grouping dubious. *Calla* has spirally arranged perfect flowers that emerge acropetally, disulcate pollen, an inferred ancestral haploid chromosome number of $n=18$ and a circumboreal, mainly European geographical distribution (Chartier et al., 2013, Stevens, 2001 onwards, Ulrich et al., 2013). *Anubias* is an African genus and *Montrichardia* is South American, but both share an inferred ancestral haploid chromosome number of $n=12$. The only feature shared by all three is a helophytic habit, which occurs elsewhere in the family (Chartier et al., 2013, Cusimano et al., 2011, Grayum, 1990). Another generic placement that warrants further investigation is the weakly-supported sister relationship of the South African genus *Zantedeschia* ($n=16$) with the strictly South American tribe Spathicarpeae ($n=17$) (Cabrera et al., 2008, Chartier et al., 2013, Cusimano et al., 2011, Nauheimer et al., 2012). In addition, weakly-supported relationships among the smaller clades within the *Zantedeschia* clade are in need of further clarification.

With the advent of massively parallel sequencing, phylogenetic analyses can now be based on tens of thousands of nucleotides, which can greatly enhance our confidence in the

resulting evolutionary hypotheses (Givnish et al., 2010, Steele et al., 2012, Xi et al., 2012). Sequencing of plastomes and mitogenomes includes de-novo assemblies using complete genomic DNA and reference-based assemblies using DNA enriched for chloroplasts, and combinations thereof (Givnish et al., 2010, Steele et al., 2012). In addition to variation in the proportion of organellar and nuclear DNA used in creating libraries for sequencing, the suite of genomic tools now available to process and analyze the resulting deluge of genomic data permits the use of multiple software programs to corroborate results.

Phylogenomic studies in plants have generally focused on the chloroplast genome, whereas the mitochondrial genome, due to its complicated mutational dynamics, has been more commonly used in studies of structural variation, nucleotide substitution rates and horizontal gene transfer (Knoop et al., 2011, Mower et al., 2007, Palmer et al., 2000, Richards et al., 2009, Richardson et al., 2013, Xi et al., 2012). The low silent-site substitution rate of plant mitochondrial DNA, which has been shown to be one-third less than that of plant chloroplast DNA, plus the extensive RNA-editing and retroprocessing that occurs in this genome perhaps explain why, in plant phylogenetic studies, mitochondrial regions have typically been used in combination with plastid regions (Renner and Zhang, 2004, Seberg and Petersen, 2006, Seberg et al., 2012, Steele et al., 2012, Wolfe et al., 1987). In addition, previous studies have shown that phylogenies reconstructed from mitochondrial data are less resolved and incongruent with plastid data (Petersen, et al., 2006, Petersen et al., 2013). However, slow silent substitution rates are not consistent across the entire mitochondrial genome or among all plant lineages; mitochondrial genes from highly divergent plant genera have been shown to have substitution rates similar to that of the rapidly evolving mammalian mitochondrial genome (Mower et al., 2007, Palmer et al., 2000). The question remains whether large-scale datasets based on tens to hundreds of

thousands of aligned nucleotides, representing both coding and non-coding regions, from the mitochondrial genome possess enough phylogenetic signal to resolve evolutionary relationships in plants at the family level.

Here we use Illumina sequencing technology with total genomic DNA and reference-based assembly of the chloroplast, using the programs Geneious 6.0.3 and Bowtie2, to resolve some of the major remaining questions in the current phylogeny of Araceae. A subsequent phylogenomic analysis of mitochondrial sequences obtained from reference-based assembly was performed to compare congruence of the mitochondrial phylogeny with the plastid phylogeny.

1.2 Materials and Methods

1.2.1 *Taxon sampling*

For the chloroplast analysis, we sampled 32 genera of Araceae and obtained from GenBank the complete, annotated chloroplast genomes of 5 additional genera: *Colocasia esculenta* (Ahmed et al., 2012), *Lemna minor* (Mardanov et al., 2008), *Wolffiella lingulata*, *Wolffia australiana* and *Spirodela polyrhiza* (Wang and Messing, 2011). We included at least one representative from 42 of the 44 clades of Araceae named in Cusimano et al. (2011). For a list of genera included in this study and the higher taxa they represent refer to Table 1.1. The two taxa not sampled were Cryptocoryneae and Culcasieae, although larger clades within which they are nested were sampled; these are the Rheophytes clade and the *Homalomena* clade, respectively. Of the 11 phylogenetically isolated genera in Cusimano et al. (2011) (*Calla*, *Calloopsis*, *Montrichardia*, *Anubias*, *Zantedeschia*, *Philonotion*, *Protarum*, *Pistia*, *Alocasia*, *Pinellia* and *Arisaema*), 4 genera (*Calloopsis*, *Philonotion*, *Protarum*, *Pistia*) were not sampled. *Gymnostachys anceps*, the sole member of subfamily Gymnostachyoideae, was not sampled but

Table 1.1 List of genera, taxa represented from Cusimano et al. (2011) and putative synapomorphic indels.

Clade	Clade name	Representative genera and clades	Synapomorphic indels in plastid genes		
			Type	bp	gene
1	Orontioideae	<i>Orontium</i>			
2	Lemnoideae	<i>Spirodela, Lemna, Wolffia, Wolfiella</i>			
3	Potheae	<i>Pothos</i>			
4	<i>Heteropsis</i> clade	<i>Stenospermation</i>			
5	Spathiphyllae	<i>Spathiphyllum</i>			
6	<i>Rhaphidophora</i> clade	<i>Monstera, Rhaphidophora</i>			
7	Lasioideae	<i>Lasia</i>	deletion	2,145	<i>rpoC2</i>
8	Zamioculcadoideae	<i>Zamioculcas</i>			
9	Aglaonemateae	<i>Aglaonema</i>			
10	Nephtyhtideae	<i>Anchomanes</i>			
11	Culcasieae				
12	<i>Philodendron</i> clade	<i>Philodendron</i>	insertion	14	<i>matK</i>
13	Spathicarpeae	<i>Dieffenbachia, Tacca</i>	insertion	15	<i>matK</i>
14	Cryptocoryneae				
15	Schismatoglottideae	<i>Schismatoglottis</i>			
16	Thomsonieae	<i>Amorphophallus</i>			
17	Caladieae	<i>Syngonium, Ulearum, Xanthosoma, Zomicarpella</i>			
18	Arisareae	<i>Arisarum</i>			
19	Arophyteae	<i>Carlephyton</i>			
20	<i>Colocasia</i> clade	<i>Colocasia, Steudnera</i>			
21	Areae	<i>Typhonium</i>			
22	Proto-Araceae	<i>Orontium</i>			
23	Pothoideae	<i>Anthurium</i> , clade 3			
24	Monsteroideae	clades 4,5,6			
25	<i>Stylochaeton</i> clade	<i>Stylochaeton</i> , clade 8			
26	<i>Anchomanes</i> clade	clades 9, 10	insertion	6	<i>petA</i>
27	<i>Homalomena</i> clade	clades 11, 12			
28	Rheophytes clade	clades 14, 15			
29	<i>Typhonodorum</i> clade	clade 19			
30	<i>Alocasia</i> clade	<i>Alocasia, Arisaema, Pinellia</i> , clade 21			
31	Bisexual Climbers <i>clade</i>	clades 23, 24			
32	<i>Zantedeschia</i> clade	<i>Zantedeschia</i> , clades 13, 26, 27			
33	<i>Colletogyne</i> clade	clades 18, 29			
34	<i>Pistia</i> clade	clades 20, 30	insertion	3	<i>atpE</i>
35	<i>Amorphophallus</i> clade	clades 16, 17			
36	<i>Ambrosina</i> clade	clades 33, 34			
37	<i>Dracunculus</i> clade	clades 35, 36			
38	<i>Philonotion</i> clade	clades 28, 37			
39	Aroideae	<i>Anubias, Montrichardia</i> clades 32, 38			
40	Unisexual Flowers clade	clades 25, 39			
41	<i>Podolasia</i> clade	clades 7, 40			
42	True Araceae <i>clade</i>	clades 31, 41			
43	<i>Spirodela</i> clade	clades 2, 42	deletion	9	<i>atpF</i>
44	Araceae	clades 22, 43	insertion	22	<i>rpoC1</i>
			deletion	36	<i>rpoB</i>
			insertion	69	<i>ndhK</i>
			deletion	214	<i>cemA</i>

its sister relationship with subfamily Orontioideae, here represented by *Orontium*, has been strongly supported in other studies (Cabrera et al., 2008, Cusimano et al., 2011, Nauheimer et al., 2012). Two species of *Acorus* were used as an outgroup: *Acorus americanus* (Peery et al., 2007) and *Acorus calamus* (Goremykin et al., 2005). For the mitochondrial analysis, the complete, annotated mitochondrial genome of *Spirodela polyrhiza* (Wang et al., 2012) was taken from GenBank and the 32 genera sampled (above) were included. Silica samples of *Calla palustris* were obtained from the Nancy Botanical Garden in France. All remaining genera were collected as fresh samples from the Araceae Greenhouse and Temperate Greenhouse at the Missouri Botanical Garden in St. Louis, Missouri. Although 31.4% of the total genera in the family were sampled (37 of 118), they represent 95.5% of the major named taxa (42 of 44) in Araceae. The list of species used in this study with GenBank accession numbers and herbarium voucher numbers appears in Table 1.2.

1.2.2 *Illumina sequencing*

Total genomic DNA was extracted from 100 mg tissue for all fresh samples, 20 mg for the silica sample of *Calla palustris*, using Qiagen DNeasy Minikit (Qiagen, Germantown, Maryland, USA). Two extractions per taxon were performed eluting DNA with 125 uL elution buffer for each extraction, which were then combined for a total of 250 uL, or alternatively each extraction was eluted with 75 uL for a total of 150 uL. The quality and concentration of DNA samples were quantified with Nanodrop (ThermoScientific, Delaware, USA) and gel electrophoresis. Illumina TruSeq kits recommend 55 uL of DNA at a concentration of 20 ng/uL; samples that were below this concentration threshold were concentrated using ethanol precipitation, while those that were over-concentrated were diluted with autoclaved H₂O or

Table 1.2 Voucher and Genbank accession numbers for included taxa

Species	Voucher	Genbank
<i>Acorus americanus</i> Raf.		NC_010093
<i>Acorus calamus</i> L.		NC_007407
<i>Aglaonema costatum</i> N.E.Br.	T. Croat 101495b (MO)	
<i>Aglaonema modestum</i> Schott ex Engl.	T. Croat 79477 (MO)	
<i>Aglaonema nitidum</i> Kunth	T. Croat 53507b (MO)	
<i>Alocasia fornicata</i> Schott	T. Croat 74063d (MO)	
<i>Alocasia navicularis</i> K.Koch & C.D.Bouché	T. Croat 78014b (MO)	
<i>Amorphophallus titanum</i> (Becc.) Becc. ex Arcang.	T.Croat 103059 (MO)	
<i>Anchomanes hookeri</i> Schott	T. Croat 75213 (MO)	
<i>Anthurium huixtlense</i> Matuda	T. Croat 63309 (MO)	
<i>Anubias heterophylla</i> Engl.	T. Croat 95582 (MO)	
<i>Arisaema franchetianum</i> Engl.	T. Croat 78435 (MO)	
<i>Arisarum simorrhinum</i> Durieu	T. Croat 101519 (MO)	
<i>Calla palustris</i> L.	BotGardNb 1970.8.001 (N)	
<i>Carlephyton glaucophyllum</i> Bogner	T. Croat 101527 (MO)	
<i>Colocasia esculenta</i> (L.) Schott		NC_016753
<i>Dieffenbachia parlatoresii</i> Linden & André	T. Croat 56557 (MO)	
<i>Lasia spinosa</i> Thwaites	T. Croat 71753 (MO)	
<i>Lemna minor</i> L.		NC_010109
<i>Monstera adansonii</i> Schott	T. Croat 103052 (MO)	
<i>Montrichardia arborescens</i> Schott	T. Croat 101645 (MO)	
<i>Orontium aquaticum</i> L.	T. Croat 103050 (MO)	
<i>Philodendron lanceolatum</i> Schott	T. Croat 71917 (MO)	
<i>Pinellia pedatisecta</i> Schott	T. Croat 81511 (MO)	
<i>Pinellia tripartita</i> Schott	T. Croat 103060 (MO)	
<i>Pothos scandens</i> L.	T. Croat 95634 (MO)	
<i>Rhaphidophora amplissima</i> Schott	T. Croat 69749 (MO)	
<i>Schismatoglottis calypttrata</i> Zoll. & Moritzi	T. Croat 103051 (MO)	
<i>Spathiphyllum patulinervum</i> G.S. Bunting	T. Croat 75478 (MO)	
<i>Spirodela polyrhiza</i> (L.) Schleid.		NC_015891, NC_017840
<i>Stenospermation multiovulatum</i> N.E.Br.	T. Croat 82903b (MO)	
<i>Stuednera colocasiifolia</i> K.Koch	T. Croat 77954a (MO)	
<i>Stylochaeton bogneri</i> Mayo	T. Croat 87579 (MO)	
<i>Syngonium angustatum</i> Schott	T. Croat 69812 (MO)	
<i>Taccarum caudatum</i> Rusby	T. Croat 95539c (MO)	
<i>Typhonium blumei</i> Nicolson & Sivad.	T. Croat 103053 (MO)	
<i>Ulearum donburnsii</i> Croat & Feuerstein	T. Croat 84834a (MO)	
<i>Wolffia australiana</i> (Benth.) Hartog & Plas		NC_015899
<i>Wolffiella lingulata</i> Hegelm		NC_015894
<i>Xanthosoma helleborifolium</i> Schott	T. Croat 103054 (MO)	
<i>Zamioculcas zamiifolia</i> (Lodd.) Engl.	T. Croat 97755 (MO)	
<i>Zantedeschia aethiopica</i> (L.) Spreng.	T. Croat 103049 (MO)	
<i>Zomicarpella amazonica</i> Bogner	T. Croat 71763b (MO)	

elution buffer from Qiagen DNeasy Minikit. Library preparation in the Pires lab at the University of Missouri, Columbia, followed the TruSeq DNA Sample Preparation Guide protocol (Illumina, Inc. 2010), except where noted. Sonication to shear total genomic DNA was performed for a total of 15-24 min. using a Bioruptor (Diagenode, Inc., New Jersey, USA). Gel extractions of size-selected samples (200-400 bp) were performed using x-tracta disposable gel extraction tools (USA Scientific, Ocala, Florida, USA) and purified with the Gel Extraction kit (Qiagen) for the end repair, adenylation of 3' ends, ligation, and enrichment steps. All gels for electrophoresis were 2% low-melt agarose stained with ethidium bromide and run at 120 volts for 1 hour with a 100 bp ladder to visualize sheared DNA for size. Prepared DNA libraries were sent to the University of Missouri DNA Core for quantification and fragment-size verification with an ABI 3730xl DNA Analyzer (Life Technologies Corp., Carlsbad, California). Sequencing was performed with the Illumina HiSeq 2000 (Illumina, Inc., San Diego, California) for single-end reads of a length of 101 bps. For the first sequencing run, we multiplexed 8 samples per lane using adapters 1-8. For the second and final sequencing run we multiplexed 12 samples using adapters 1-12. Illumina HiSeq 2000 automatically removes adapter ends and parses reads based on adapter ends into separate files. Raw fastq reads for each taxon were concatenated when presented in multiple files. The total number of reads generated for each taxon is listed in Table 1.3.

1.2.3 Data quality-trimming and filtering

Raw fastq reads were quality trimmed using DynamicTrim (Cox et al., 2011), which uses the Burrows-Wheeler Alignment trimming algorithm to crop reads that are below a quality cutoff ($p = 0.05$, Phred score $Q = 13$). Based on the increased mutational complexities of mitochondrial

Table 1.3 Raw data information for each of the alignments used in phylogenetic analysis.

Taxa	Raw reads	Filtered reads	Mean coverage plastid protein-coding genes	Mean coverage mitochondrial genes, tRNAs, rRNAs	Mean coverage entire chloroplast sequence	Mean coverage entire mitochondrial sequence
<i>Aglaonema costatum</i>	19,455,075	16,097,991	89.4			
<i>Aglaonema modestum</i>	13,771,620		61.8			
<i>Aglaonema nitidum</i>	23,037,033	17,535,145	103.0	3,247.9	88.1	1,643.0
<i>Alocasia fornicata</i>	23,100,492		136.1			
<i>Alocasia navicularis</i>	12,580,339	10,496,587	75.6	381.1	64.9	197.6
<i>Amorphophallus titanum</i>	18,021,330	14,882,635	146.6	210.4	123.5	528.5
<i>Anchomanes hookeri</i>	28,743,540	22,307,306	1,303.2	521.0	1,123.8	389.1
<i>Anthurium huixtlense</i>	14,129,239	11,677,966	108.9	23.3	97.6	1,612.3
<i>Anubias heterophylla</i>	24,011,002	20,266,562	191.8	362.3	180.8	346.7
<i>Arisaema franchetianum</i>	24,341,409	17,693,614	387.1	469.4	329.3	701.1
<i>Arisarum simorrhinum</i>	14,646,430	12,811,273	228.5	300.4	202.7	320.2
<i>Calla palustris</i>	16,715,852	14,608,316	938.3	275.9	832.1	469.4
<i>Carlephyton glaucophyllum</i>	17,921,059	15,258,012	410.2	49.5	367.1	566.3
<i>Dieffenbachia parlatorei</i>	11,306,711	9,358,638	173.4	539.1	162.5	273.1
<i>Lasia spinosa</i>	38,056,690	31,722,071	973.6	1,515.0	816.4	1,353.1
<i>Monstera adansonii</i>	36,278,235	27,002,232	774.9	761.1	727.0	1,000.6
<i>Montrichardia arborescens</i>	12,493,655	11,108,076	192.7	204.9	183.8	220.6
<i>Orontium aquaticum</i>	20,860,738	18,136,283	99.2	145.1	81.8	299.2
<i>Philodendron lanceolatum</i>	21,202,424	17,651,991	291.0	404.3	276.6	339.2
<i>Pinellia pedatisecta</i>	8,856,861	7,309,388	1,150.3	594.6	1,064.5	482.0
<i>Pinellia tripartita</i>	20,898,685	15,048,528	526.7			
<i>Pothos scandens</i>	22,207,465	18,417,735	353.2	56.6	328.0	305.0
<i>Rhaphidophora amplissima</i>	12,870,626	11,122,937	115.8	136.6	130.2	213.8
<i>Schismatoglottis calyptata</i>	13,869,294	12,054,112	62.2	77.4	53.3	95.4
<i>Spathiphyllum patulinervum</i>	13,898,684	12,465,700	34.4	534.0	29.7	544.8
<i>Stenospermation multiovulatum</i>	42,788,539	35,975,487	343.8	2,388.5	321.0	2,258.7
<i>Studnera colocasiifolia</i>	13,038,295	11,332,048	100.5	116.0	93.2	233.0
<i>Stylochaeton bognerii</i>	12,709,376	11,211,182	67.7	477.6	65.7	2,418.4
<i>Syngonium angustatum</i>	24,674,957	18,265,606	483.6	595.5	450.6	486.5
<i>Taccarum caudatum</i>	18,998,555	15,611,970	449.1	114.7	417.5	361.7
<i>Typhonium blumei</i>	21,868,376	15,754,513	955.2	1,188.1	849.3	604.5
<i>Ulearum donburnsii</i>	24,551,954	20,392,535	159.7	808.7	151.0	834.4
<i>Xanthosoma helleborifolium</i>	11,667,350	9,823,510	492.6	145.8	458.0	303.7
<i>Zamioculcas zamiifolia</i>	43,288,898	32,819,219	540.0	2,883.0	496.3	3,808.8
<i>Zantedeschia aethiopica</i>	14,776,054	12,956,453	1,553.4	221.4	1,021.4	295.2
<i>Zomicarpella amazonica</i>	17,421,774	15,066,253	101.9	31.8	92.6	125.2
		Mean Coverage Total	393.8	618.2	365.0	738.5
		Total bases	61,716	113,181	211,614	318,210
		Constant bases	45,615	95,153		
		Parsimony uninformative bases	6,335	11,871		
		Parsimony informative bases	9,766	6,157		

Blank boxes denote genera that were excluded from the alignment due to redundancy.

sequences, an additional filtering step was performed for the mitochondrial analysis using Prinseq-lite-0.20.3 (Schmieder and Edwards, 2011), in which sequences under a length of 40 base pairs with a quality score lower than 30, and having more than 1% Ns were removed. For the number of filtered reads passing quality control for each taxon, refer to Table 1.3.

1.2.4 *Sequence assembly, validation and alignment of chloroplast coding sequences*

Quality-trimmed reads for each taxon were assembled to the chloroplast genome of *Lemna minor* (165,955 bases), used here as the reference sequence. Assembly was performed using Bowtie2-2.0.0-beta6 (Langmead and Salzberg, 2012) with parameters set to default. Assembly was also performed using Geneious versions 5.6-6.0.3 (created by Biomatters) using the Custom Sensitivity setting. The Custom sensitivity values were chosen to have a more stringent minimum overlap length than Bowtie2 and the maximum gap size was changed from 15 to 3 based on the need to minimize computing requirements. Fine-tuning was iterated up to five times. The consensus sequences from Bowtie2 and Geneious for each taxon were extracted and aligned to the reference genome using the mauveAligner algorithm plugin in Geneious. All discrepancies in consensus sequences from Bowtie2, Geneious and the chloroplast reference genome, excluding the second inverted repeat, were viewed with the ‘highlight disagreements to the reference’ setting in Geneious.

Validation of sequences for each taxon, based on highlighted differences, was performed using the assemblies of raw mapped reads from both Bowtie2 and Geneious to ensure appropriate SNP calling and indel mapping. This combinatorial approach for validation using the consensus sequences and mapped reads from both programs was used only on those regions of the genome that were homologous with the annotated protein-coding sequences of *Lemna minor*. The restriction of sequence validation to these regions was due to the high level of conservation

among plastid protein-coding sequences. Intergenic regions were too many and too variable to validate manually. This first round of assembly and validation permitted incorporation of many SNPs and indels that were lost in each single assembly, but ambiguities in more variable genes such as *ndhF* still remained. Therefore, the consensus sequences from the first round of assembly and validation for each taxon were then used as reference sequences in a subsequent round. After this second round, taxa still containing ambiguous sequences were assembled and validated reiteratively only in Geneious. Protein-coding sequences for each taxon were extracted and concatenated using the ‘extract annotations’ tool and were checked for start and stop codons using the ‘translation’ tool in Geneious. The concatenated protein-coding sequences for all taxa were aligned using the mauveAligner algorithm plugin in Geneious.

The focus on protein-coding sequences notwithstanding, certain genes proved to be too variable and labor intensive to validate even after several rounds (up to 10) of assembly and validation and were discarded from all taxa in the final alignment. Problematic genes, the species in which they occur, and a description of the issue are listed in Table 1.4. In total, 10 protein-coding genes (*infA*, *ycf68*, *rpl20*, *rps12*, *accD*, *clpP*, *rps19*, *rpl23*, *ycf1* and *rps15*) were removed from the final alignment, which consisted of 70 plastid protein-coding genes for 37 genera of Araceae and two species of *Acorus*. The final alignment can be accessed in TreeBASE at <http://purl.org/phylo/treebase/phylows/study/TB2:S15395>.

1.2.5 *Sequence assembly and alignment of the entire chloroplast*

We were interested in comparing the phylogenetic potential of a shotgun approach to obtaining complete chloroplast sequences versus the detailed validation approach (above). For this, the consensus sequences spanning the entire chloroplast from the second round of assembly and validation of coding sequences (above) were extracted for each taxon. Intergenic regions

Table 1.4 Genes removed from the alignment of plastid protein-coding sequences.

Plastid gene	Species	Problem
<i>accD</i>	<i>Anchomanes hookeri</i> , <i>Anthurium huixtlense</i> , <i>Zantedeschia aethiopica</i>	assembly toward beginning, no start/stop codon
<i>clpP</i>	<i>Stylochaeton bognerii</i>	assembly throughout
<i>infA</i>	<i>Acorus americanus</i> , <i>Acorus calamus</i>	present only in these species, a pseudo-copy in <i>Colocasia esculenta</i> (Ahmed et al., 2012)
<i>rpl20</i>	<i>Spirodela polyrhiza</i>	absent from this genus
<i>rpl23</i>	<i>Anchomanes hookeri</i>	no start/stop codon
<i>rps12</i>	<i>Spirodela polyrhiza</i> , <i>Wolffia australiana</i> , <i>Wolffiella lingulata</i>	absent from these genera
<i>rps15</i>	<i>Colocasia esculenta</i> , duckweeds	in IR ^a region in duckweed, in SSC ^b in <i>Colocasia esculenta</i> (Ahmed et al., 2012)
<i>rps19</i>	<i>Anchomanes hookeri</i> , <i>Zantedeschia aethiopica</i>	no start/stop codon
<i>ycf1</i>	<i>Aglaonema costatum</i> , <i>Aglaonema modestum</i> , <i>Aglaonema nitidum</i> , <i>Alocasia fornicata</i> , <i>Alocasia navicularis</i> , <i>Amorphophallus titanum</i> , <i>Anchomanes hookeri</i> , <i>Anthurium huixtlense</i> , <i>Calla palustris</i> , <i>Dieffenbachia parlatoresi</i> , <i>Orontium aquaticum</i> , <i>Pinellia tripartita</i> , <i>Pothos scandens</i> , <i>Rhaphidophora amplissima</i> , <i>Stenospermation multiovulatum</i> , <i>Stylochaeton bognerii</i> , <i>Taccarum caudatum</i> , <i>Typhonium blumei</i> , <i>Ulearum donburnsii</i> , <i>Zantedeschia aethiopica</i> , <i>Zomicarpella amazonica</i>	assembly, indels, no start/stop codons, in IR region in duckweed, in SSC in <i>Colocasia esculenta</i> (Ahmed et al., 2012)
<i>ycf68</i>	<i>Colocasia esculenta</i>	a pseudo-copy of <i>ycf68</i> reported in duckweed (Ahmed et al., 2012)

^a Inverted Repeat

^b Small Single Copy region

and introns spanning the entire genome, and coding sequences from the second inverted repeat were not validated. All consensus sequences were aligned using the mauveAligner algorithm plugin in Geneious.

1.2.6 Sequence assembly and alignment of mitochondrial sequences

Based on the well-supported phylogenies resulting from the shotgun approach to obtaining complete chloroplast sequences (refer to Results), we wanted to test whether large mitochondrial datasets obtained using this method could produce well-resolved phylogenies at the family level that are congruent with plastid data. For this, quality-trimmed and filtered reads were assembled to the *Spirodela polyrhiza* mitochondrial genome, which is the most compact

monocot mitochondrial genome known to date (228,493 bases) (Wang et al., 2012). It contains a total of 57 genes encoding 35 proteins, 3 ribosomal RNAs and 19 transfer RNAs (Wang et al., 2012). Assembly was performed in Geneious with the custom sensitivity settings similar to those above except that the maximum gap size allowed was increased to 70 base pairs. We assembled reads to the mitochondrial genome three times reiteratively, taking the consensus sequence from each assembly as the reference for the subsequent assembly.

Assembly of raw reads to the mitochondrial reference genome averaged over all genera was 2.11% compared to 3.58% for the chloroplast. In general, assembly to the mitochondrial genome was notably more sporadic than assembly to the chloroplast genome even though mean coverage values are on par with those in the chloroplast genome (Table 1.3). A consistent theme among most genera was two islands of extreme depth coverage in the *nad4* and *nad2* genes (i.e. up to 8,000x in a non-coding region of *nad2* in *Amorphophallus*), which may explain the high average coverage among mitochondrial assemblies. All genes encoding proteins, rRNAs and tRNAs for each taxon were extracted and concatenated using the ‘extract annotations’ tool in Geneious. Concatenated genes were aligned using the mauveAligner algorithm plugin in Geneious. For complete mitochondrial sequences, consensus sequences spanning the entire mitochondria were extracted for each taxon and aligned using the mauveAligner algorithm plugin in Geneious.

Concatenated plastid and mitochondrial gene alignments were analyzed using PAUPrat (Sikes et al., 2001) in CIPRES to obtain the number of constant, parsimony-uninformative and parsimony-informative bases. For the mean coverage of raw reads for each taxon in each alignment, the mean coverage of raw reads for all taxa in each alignment and the number of total bases in each alignment refer to Table 1.3. For the number of constant bases, parsimony

uninformative bases and parsimony informative bases in the concatenated gene alignments refer to Table 1.3.

1.2.7 *Phylogenomic analyses*

Phylogenetic analyses consisted of Maximum Likelihood and Bayesian analytical methods using Mr Bayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) and PhyloBench (Stamatakis et al., 2008). Maximum likelihood analysis, performed using RAxML HPC Black Box (Stamatakis, 2006a) consisted of 1000 rapid bootstrap inferences and a thorough ML search thereafter. The likelihood of the final tree was evaluated and optimized under the General Time Reversible substitution model with gamma-distributed rate variation across sites, and a proportion of invariant sites GTR + Γ + I (Stamatakis, 2006a; Yang, 1993). RAxML HPC Black Box uses the GTRCAT approximation of the GTR + Γ model with 25 per-site rate categories (Stamatakis, 2006b). The congruence between the ML phylogenetic trees based on complete plastid and complete mitochondrial sequences was compared using the Templeton test in PAUP (Swofford, 1991, Templeton, 1983).

Bayesian analysis was performed on plastid and mitochondrial concatenated gene sequences using Mr. Bayes version 3.1.2 using the GTR + Γ substitution model with the number of gamma categories set to 4. The GTR + Γ substitution model was chosen using the Akaike information criterion (Akaike, 1974) in jmodeltest2 (Darriba, et al., 2012). The prior probability distribution for the substitution rates of the GTR model and the state frequencies was a flat Dirichlet. The prior for the shape parameter of the gamma distribution of rate variation was set to uniform. The prior for branch lengths was unconstrained and exponential. The analyses were run two times independently for 10,000,000 generations, sampling trees every 1,000 generations. Three heated chains (temp = 0.200) and one cold chain were used. The first 25% of samples

were discarded from the cold chain as burnin. Graphical exploration of MCMC convergence was performed using AWTY (Nylander et al., 2008, Wilgenbusch, et al., 2004). The Bayesian consensus phylogenetic tree inferred from concatenated plastid protein-coding sequences can be accessed in TreeBASE at <http://purl.org/phylo/treebase/phyloids/study/TB2:S15395>.

1.3 Results

1.3.1 *Phylogenomic analyses of chloroplast sequences*

Phylogenomic analyses based on chloroplast sequences, both complete and concatenated protein-coding, yielded similar strongly-supported family-wide phylogenies except for the placement of *Calla* and *Schismatoglottis* (Figure 1.1, Figure 1.2). The following are novel strongly-supported evolutionary relationships. *Anubias* and *Montrichardia* form a clade (BS=99%, PP=0.99) that is the sister group to the *Zantedeschia* clade (BS=100%, PP=0.99) based on concatenated protein-coding (PC) sequences (Figure 1). Although this topology does not change in the ML phylogeny inferred from complete chloroplast (C) sequences, the addition of the (*Calla*, *Schismatoglottis*) clade at its base decreases bootstrap support to 63% and 77%, respectively (Figure 1.2). The *Zantedeschia* clade consists of a grade, with *Philodendron* (representing the *Homalomena* clade) as sister to the rest (PC: BS=100%, PP=0.99; C: BS=100%), followed by Spathicarpeae (PC: BS=67%, PP=0.89; C: BS=93%), then followed by the South African genus *Zantedeschia* as the sister taxon to the Old World *Anchomanes* clade (PC: BS=73%, PP=0.99; C: BS=81% (Figure 1.1, Figure 1.2).

The placement of *Calla* is consistently the only node in all phylogenies with bootstrap support less than 85% and a posterior probability less than 0.95 (Figure 1.1, Figure 1.2). *Calla* is

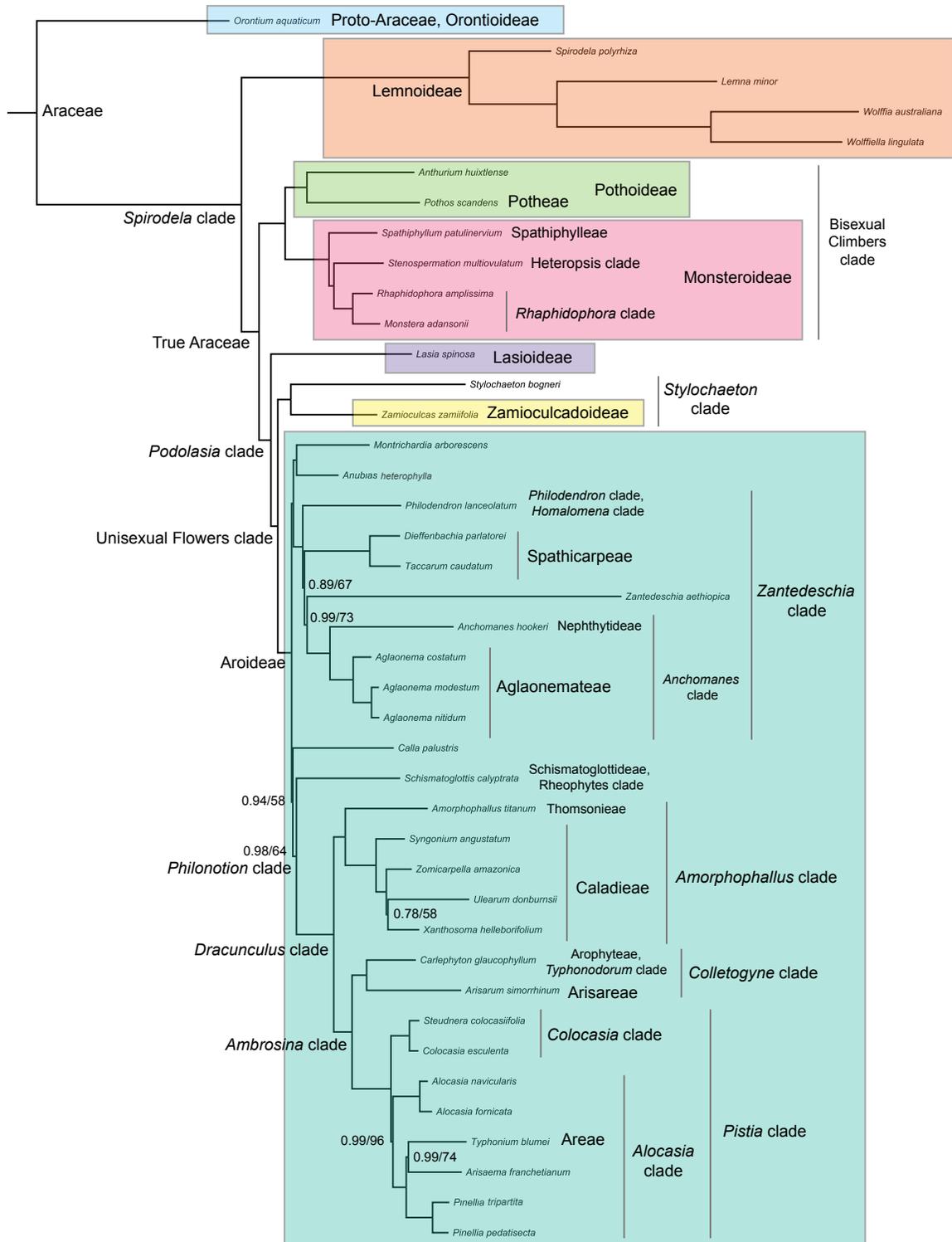


Figure 1.1 Phylogenetic tree of Araceae obtained from ML and Bayesian analysis of 70 plastid protein-coding genes for 37 genera of Araceae and two species of *Acorus*, used as the outgroup (not shown). Subfamilies are boxed (excluding Gymnostachydoideae). Nodes with no values have a posterior probability ≥ 0.98 and bootstrap support $\geq 99\%$.

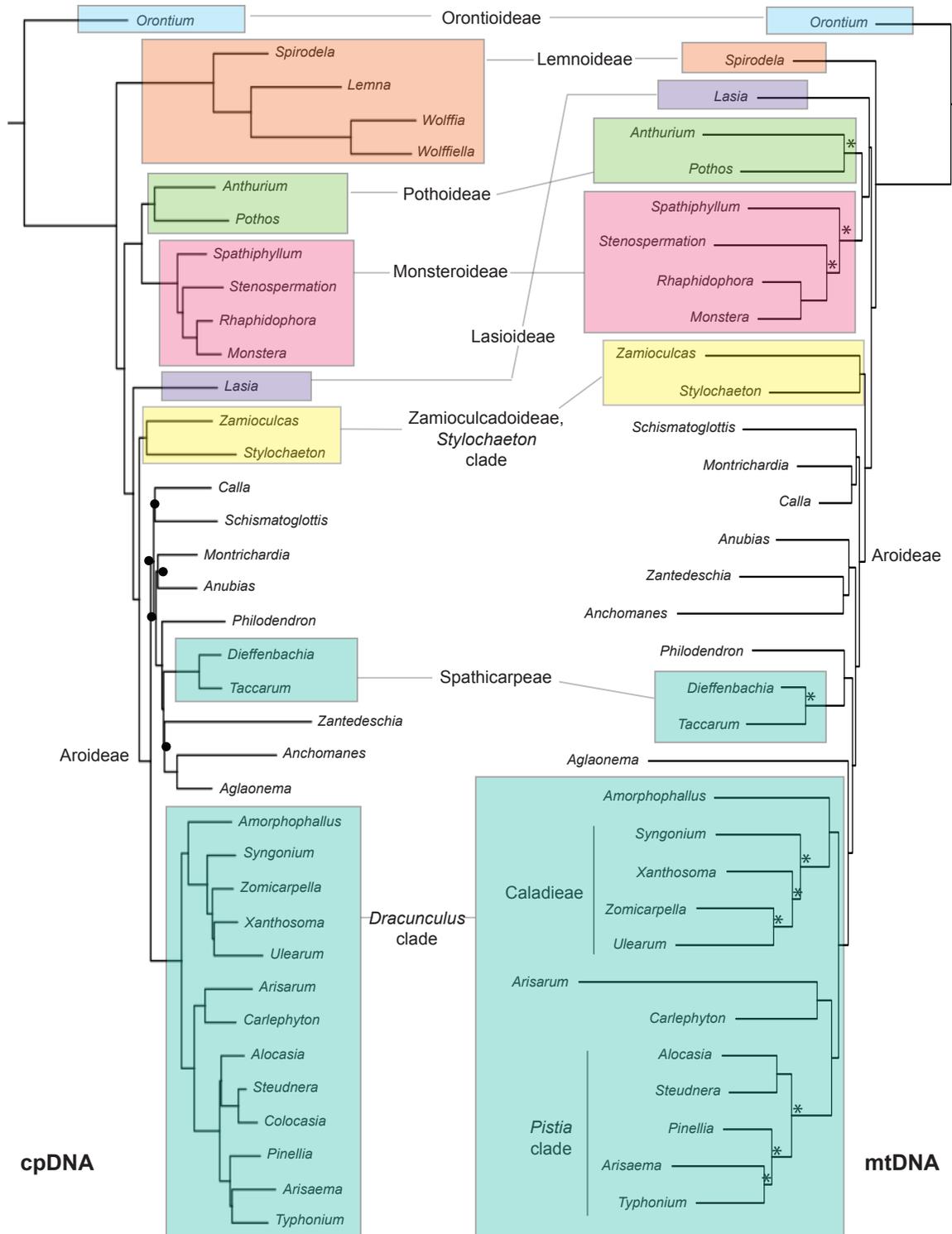


Figure 1.2 Best-scoring RAxML phylogenies based on complete chloroplast and mitochondrial sequences. Closed circles mark nodes with <85% bootstrap support in the plastid tree, asterisks nodes with $\geq 85\%$ bootstrap support in the mitochondrial tree. Boxes mark clades recovered in both analyses, colors correspond to subfamilies.

seen as either the sister taxon to the *Philonotion* clade (PC: BS=58%, PP=0.94)(Table 1.1), which includes *Schismatoglottis* (PC: BS=64%, PP=0.98) (Figure 1.1), or forms a clade with *Schismatoglottis* (C: BS=40%) that is sister to the ((*Montrichardia*, *Anubias*)*Zantedeschia* clade) (C: BS=58%) (Figure 1.2). The position of *Schismatoglottis* as sister to the other members of the *Philonotion* clade, seen in the phylogenies based on concatenated protein-coding sequences, is weakly supported in the ML analysis (BS=64%) but strongly supported in the Bayesian analysis (PP=0.98).

One strongly-supported clade presented here that was not seen in previous studies based on chloroplast sequences is tribe Spathiphyllae forms the sister taxon to the rest of subfamily Monsteroideae (PC: BS=100%, PP=0.99; C: BS=100%) (Figure 1.1, Figure 1.2). The Unisexual Flowers clade, containing the bisexually-flowered genus *Calla*, is also strongly supported for the first time using plastid data alone (PC: BS=100%, PP=0.99; C: 100%) (Figure 1.2).

1.3.2 Comparison of chloroplast and mitochondrial phylogenies

In contrast to the strongly supported phylogenies obtained from chloroplast data, phylogenies based on mitochondrial sequences did not have strong statistical support, with the exception of several small clades (Figure 1.2). In the phylogenies inferred from concatenated mitochondrial genes (data not shown), strongly-supported clades were limited to the *Rhaphidophora* clade (BS=100%, PP=1.0), Spathicarpeae (BS=100, PP=1.0), Pothoideae (BS=33%, PP=1.0), the (*Pinellia*(*Arisaema*, *Typhonium*)) clade (BS=100%, 100%, PP=1.0, 1.0, respectively) and a highly morphologically incongruent clade composed of *Aglaonema* and *Spirodela* (BS=100%, PP=1.0). The Maximum Likelihood analysis based on complete mitochondrial sequences produced a greater number of clades with strong bootstrap support than did the analysis based on mitochondrial genes, but still not many (Figure 1.2). These include

Pothoideae, Monsteroideae, Spathicarpeae, Caladieae, the *Pistia* clade, and the (*Pinellia*(*Arisaema*, *Typhonium*)) clade. All have a bootstrap support value of 100%, except Pothoideae with BS=85%. No other node in the phylogeny is statistically well supported, but the relationships among the seven subfamilies mirror those in the chloroplast phylogeny with one exception. Lasioideae, containing bisexually-flowered taxa, is sister to the Bisexual Climbers clade, composed of Pothoideae and Monsteroideae, instead of sister to the Unisexual Flowers clade. This placement of Lasioideae is interesting in that it makes all bisexually-flowered taxa (with the exception of *Calla*) within True Araceae monophyletic.

Within subfamily Aroideae, the *Dracunculus* clade remains wholly intact except for the rearrangement of *Xanthosoma* and *Zomicarpella*. Relationships among the *Zantedeschia* clade, *Montrichardia*, *Anubias*, *Calla* and *Schismatoglottis* do not match those in either of the chloroplast phylogenies (Figure 1.1, Figure 1.2). *Calla* and *Schismatoglottis* (with the addition of *Montrichardia*) form a clade that is sister to the rest of Aroideae. Although smaller clades found in the complete chloroplast phylogeny are recovered in the complete mitochondrial phylogeny, and many relationships among genera and clades appear superficially similar, the results of the Templeton test reject congruency between the two topologies ($p = <0.0001$).

1.3.3 Synapomorphic indels in chloroplast protein-coding genes for major named taxa

Various indels notable among the clades in the alignment of chloroplast protein-coding sequences of Araceae and *Acorus* are listed in Table 1.1. As more genera are sequenced and added to the phylogeny, it will be interesting to discover if these indels hold as synapomorphies.

Synapomorphic indels for Araceae include a 22 bp insertion at the beginning of *rpoC1* (*Anchomanes hookeri* has an additional 5 bp insertion), a 36 bp deletion at the beginning of *rpoB*, a 69 bp insertion at the beginning of *ndhK* (*Stylochaeton bogneri* has an additional 3 bp

insertion) and a 214 bp deletion at the beginning of *cemA*. Members of the *Spirodela* clade, formed by Lemnoideae and the True Araceae clade, have no morphological synapomorphies, but all have a 9 bp deletion at the end of the second exon of *atpF*. In Lasioideae, *Lasia spinosa* has a 2,145 bp or 715 amino acid deletion at the end of *rpoC2*. The *Anchomanes* clade, formed by *Anchomanes hookeri* and three species of *Aglaonema*, has a 6 bp insertion toward the beginning of *petA*. The *Pistia* clade, containing *Colocasia*, *Staudnera*, *Alocasia*, *Typhonium*, *Pinellia* and *Arisaema*, has a 3 bp insertion in *atpE* followed closely by a transition SNP from A to G. Tribe Spathicarpeae, formed by *Dieffenbachia* and *Taccarum*, has a 15 bp insertion at the end of *matK*. *Philodendron*, representing the *Philodendron* and *Homalomena* clades, has a 14 bp insertion toward the end of *matK*.

1.4 Discussion

1.4.1 Evolutionary relationships of Araceae

This study provides the first well-supported phylogeny based on chloroplast sequences for the early evolution of Araceae, particularly the early evolution of the generically rich subfamily Aroideae. It is also the first glimpse at a mitochondrial phylogeny for the family. Most of our results corroborate previously established phylogenetic relationships, however, several key findings pertaining to the early evolution of Aroideae differ greatly from previous studies.

Our results support the current circumscription of Araceae into eight subfamilies: Gymnostachydoideae, Orotioideae, Lemnoideae, Pothoideae, Monsteroideae, Lasioideae, Zamiculcadoideae and Aroideae (for Gymnostachydoideae refer to Cabrera et al., 2008, Chartier et al., 2013). Relationships among the subfamilies containing bisexually-flowered

genera, Orontioideae, Lemnoideae, Pothoideae and Monsteroideae are strongly supported. Although there are no morphological synapomorphies for the *Spirodela* clade, this study suggests a 9 base pair deletion in the plastid gene *atpF* may be a diagnostic synapomorphic indel for the group. The sister relationship of Lemnoideae and True Araceae (Table 1.1) within the *Spirodela* clade is well established in this and previous studies, as is the sister relationship of the Bisexual Climbers and *Podolasia* clades (Figure 1.1).

Within the Bisexual Climbers clade, the placement of Spathiphyllae in subfamily Monsteroideae as sister to a clade containing the *Rhaphidophora* and *Heteropsis* clades has not been observed previously in chloroplast phylogenies (Figure 1.1, Figure 1.2). This result, distancing Spathiphyllae from other members of Monsteroideae evolutionarily, is not surprising given the tribe's unique morphological features such as lacking calcium oxalate prisms, vessels, a stem endodermis and cortical vascular system, and in having pollen and trichosclereids unlike any others in Araceae (Grayum, 1990).

The Unisexual Flowers clade, containing a sister group relationship between the *Stylochaeton* clade and subfamily Aroideae, is here strongly supported. In our chloroplast phylogeny (Figure 1.1), *Stylochaeton bogneri* is the only taxon not included in one of the eight subfamilies. We agree with previous workers that subfamily Zamiculcadoideae should be expanded to include *Stylochaeton*, thus characterizing the former as consisting of geophytic, sub-Saharan African plants that have perigoniate, unisexual flowers and lack laticifers.

In Aroideae, within the *Zantedeschia* clade, *Philodendron* is sister to all other genera. *Philodendron* here represents the *Homalomena* clade, which have the morphological synapomorphies of the occurrence of sclerotic hypodermis and resin canals in the roots and absence of endothecial thickenings in the anthers (Cusimano et al., 2011). The South African

genus *Zantedeschia* is sister to the *Anchomanes* clade formed by African Nephthytideae (tuberous or rhizomatous, seasonally dormant to evergreen) and Asian Agalonemateae (entirely evergreen), which share an inferred ancestral haploid chromosome number of twenty (Cusimano, et al., 2012, Mayo et al., 1997). *Zantedeschia* has an inferred ancestral haploid chromosome number of $n=16$, and the species are seasonally dormant, occasionally evergreen, tuberous herbs. This arrangement, seen here for the first time, makes the whole group strictly Old World and is biogeographically more congruent than previous studies in which the genus *Zantedeschia* is sister to the strictly South American Spathicarpeae. All members of Spathicarpeae have an inferred ancestral haploid chromosome number of seventeen (Cusimano, et al., 2012).

The phylogenetic position of *Calla*, a genus with a unique character combination in the family, has shifted dramatically among the various studies attempting to resolve its evolutionary history (Ulrich et al., 2013). Morpho-anatomical and palynological data suggest that *Calla* belongs in a lineage by itself in a transition zone between bisexually- and unisexually-flowered clades, while previous molecular data suggests that *Calla* is embedded in the Unisexual-Flowers clade but its placement therein is unresolved (Ulrich et al., 2013). This study unequivocally supports the inclusion of *Calla* in the Unisexual Flowers clade, but presents yet another hypothesis, albeit poorly-supported, of its evolutionary relationship to other unisexually-flowered genera (Figure 1.2). Interestingly, the phylogeny based on complete chloroplast sequences in this study is similar to the strict consensus tree from the combined parsimony analysis of chloroplast data by Cabrera et al. (2008) in that *Calla* and the Rheophytes clade (represented by *Schismatoglottis*) form a sister relationship. In Cabrera et al. (2008) the (*Calla*, Rheophytes clade) clade is at the base of what is now accepted as Aroideae, whereas in this study that clade is at the base of one of the two major clades forming Aroideae. The sister relationship

between *Anubias*, *Montrichardia* and *Calla* at the base of Aroideae, as seen in the nuclear data (Chartier et al., 2013), was not recovered in either of our chloroplast phylogenies, but a variation of it was seen with low statistical support in the mitochondrial phylogeny (Figure 1.2). Although the addition of the (*Calla*, Rheophytes clade) clade at the base of the ((*Montrichardia*, *Anubias*) *Zantedeschia* clade)) clade weakens the statistical support for the latter, this topology is supported by morphological and cytological features. Rare modifications of the leaf sheath into “ligule-“ or “stipulelike” structures, in which the leaf sheath is free at the tips, are shared by *Calla*, several Schismatoglottideae (Table 1.1), and some *Philodendron* species (Grayum, 1990). *Calla* also shares the morphological characters of unilocular ovules and basal placentation with *Nephtytis* (Nephtytideae, here represented by *Anchomanes*) and *Calloopsis* (Grayum, 1990). Furthermore, *Calla*, *Philodendron*, and members of the Rheophytes clade (*Lagenandra* and *Cryptocoryne*) share an inferred ancestral haploid chromosome number of $n=18$. In fact, the larger ((*Calla*, Rheophytes clade)((*Montrichardia*, *Anubias*) *Zantedeschia* clade)) clade is reminiscent of Grayum’s morphology-based circumscription of subfamily Calloideae Schott, including 14 of his 17 tribes (only tribes Peltandreae, Arophyteae and Callopsideae fall out elsewhere).

The inclusion of *Calla* in the Unisexual Flowers clade implies a return from unisexual to bisexual flowers - a transition that is exceedingly rare (Barrett, 2013). However, the multiple developmental pathways leading to unisexual flowers and the retention of sexual bipotency in many unisexual flower primordia suggest that sex determination in floral organs is a much more labile process than previously recognized (Dellaporta and Calderon-Urrea, 1993, Mitchell and Diggle, 2005). The presence of male flowers at the tips of spadices above the normal bisexual flowers in *Calla* and *Orontium* (Grayum, 1990) attest to this notion of ‘sex flexibility’ and make

the placement of *Calla* within the Unisexual Flowers clade more tenable. A closer look into the developmental pathways of floral organ evolution in Araceae is highly desired.

1.4.2 Phylogenomics: *chloroplasts vs. mitochondria*

The structural and mutational complexities of land-plant mitochondrial genomes and the difficulties they present for phylogenetic analyses have long been recognized (Petersen et al, 2006, Seberg and Petersen, 2006, Seberg et al., 2012). The results of this study show that indeed, even when using tens of millions of Illumina sequencing reads from total genomic DNA, the shotgun approach has vastly different potential for phylogenomics in plastid versus mitochondrial genomes. The shotgun approach, using the entire chloroplast genome as a reference, mainly had results as strongly supported as those of the carefully validated concatenated protein-coding sequences, with few inconsistencies between the two. In contrast, although many of the clades seen in the chloroplast phylogeny were recovered in the phylogeny based on complete mitochondrial sequences, the statistical support was too low in the latter to draw any meaningful conclusions about most generic relationships in the family. In addition, what appears at face value to be considerable similarity between two organellar phylogenies, is strongly rejected when scrutinized methodically.

In this study, the whole suite of mitochondrial genes was, *on average*, less informative than chloroplast protein-coding genes for a family-level phylogeny in Araceae. It is interesting to note that in spite of the questionable homology of intergenic regions in plant mitochondria the alignment including these genomic regions yielded a better-supported topology than did the combined-gene alignment alone; a possible explanation could be the small size of the *Spirodela* mitochondrion. Reference assemblies based on mitochondria are, in general, more problematic than chloroplast reference assemblies (Argelia Cuenca pers. comm.). Previous workers have

shown that excluding predicted RNA-edited sites in mitochondrial genes increases congruence between mitochondrial and plastid phylogenies (Petersen, 2013). Comparison of reference-based and de novo assemblies, and a look into the role of RNA-edited sites and processed paralogs in the mitochondrial phylogeny of Araceae are yet to be studied.

1.4.3 Problematic genes and *Zantedeschia*

Ten genes (*infA*, *ycf68*, *rpl20*, *rps12*, *accD*, *clpP*, *rps19*, *rpl23*, *ycf1* and *rps15*) were removed from the final concatenated plastid protein-coding sequence alignment because of either problematic assembly of raw reads and/or uncertainty of their presence or absence. In reference-based assembly, structural changes between the reference and target genomes are not captured and regions of exceptionally high variation are difficult to assemble. Several genes listed above have already been confirmed as pseudogenes in other angiosperms (*rpl23* in spinach, *ycf1* in rice and maize, *infA* in tobacco, *Arabidopsis* and *Oenothera*, *accD* in grasses) (Millen et al., 2001). In the case of *accD*, a study of mutation rates in eudicot legume chloroplast genomes showed that the *accD-psal-ycf4-cemA* region was hypermutable and that *accD* was transferred to the nucleus in *Trifolium* (Magee et al., 2010). Here, the hypermutability of the gene *accD* was observed in phylogenetically independent genera of Araceae (*Anthurium*, *Zantedeschia* and *Anchomanes*) and based on evidence in legumes, the transfer of *accD* to the nucleus in these genera may be a possibility. A similar pattern is seen in the *clpP* gene of *Stylochaeton*. Interestingly, *clpP* and *accD* were found to be essential for shoot and leaf development, respectively, in the eudicot tobacco, yet *accD* is unnecessary during development in grasses, a highly derived monocot group (Kode et al., 2005). These contradictory roles make the study of *accD* in Araceae especially intriguing, considering the leaf developmental patterns in the family that are transitional between ‘dicots’ and monocots (Bharathan, 1996).

The gene *infA* is among the most easily lost in the chloroplast genomes of land plants (Millen et al., 2001). Both species of *Acorus* used in this study possess the gene but not one of the duckweed genera obtained from GenBank has it. Using the sequence of *infA* from *Acorus americanus* as a reference, we found varying levels of coverage of the gene in phylogenetically disparate genera of Araceae. Coverage ranged from complete (*Orontium*), intermediate (*Calla*, *Lasia*, and *Anubias*) to absent (*Anthurium* and *Spathiphyllum*) and suggests that the mutational dynamics of *infA* have not stabilized within Araceae. The list of ten genes omitted from the final concatenated plastid protein-coding sequence alignment serves as a basis for future work into the structural and functional properties of these plastid genes in Araceae.

Of all the chloroplast genomes assembled in this study, *Anchomanes* and *Zantedeschia* proved to be the most problematic. The amount of autapomorphic substitutions in the chloroplast genome of *Zantedeschia* was surprisingly high, with the associated branch length almost as long as those in the highly morphologically derived duckweed subfamily Lemnoideae. Interestingly, the species of *Zantedeschia* used in this study (*Z. aethiopica*) is morphologically distinct from the other seven species in the genus (Mayo et al., 1997).

1.5 Conclusion

This study presents the first well-supported phylogeny for deep branches of the plant family Araceae using strictly chloroplast data, and the first glimpse at a family-wide phylogeny based on mitochondrial sequences. New evolutionary relationships seen in this study, the mutational dynamics of several plastid protein-coding genes and a comparison of chloroplast and mitochondrial sequences for phylogenomics are discussed. Although sampling was sufficient to resolve the relationships between the major clades in the family, the lack of sampling of several

key genera including *Pistia*, *Protarum*, *Philonotion* and especially *Calloopsis* leaves room for future work. As more nuclear data become available for the family, it will be interesting to see the ultimate placement of *Calla* and *Schismatoglottis* (Rheophytes clade).

1.6 References

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Chapter 2

Development of Dissected Leaf Morphology in *Anthurium* and *Amorphophallus*

2.1 Introduction

The leaves of flowering land plants (angiosperms) are highly variable in shape and complexity despite their common dorsiventral inception from the shoot apex and end fate as determinate structures (Kaplan, 1997). Dorsiventral asymmetry in leaves is the result of a juxtaposition of upper and lower, or adaxial and abaxial, cell identities within the leaf (Kaplan, 1997; Leyser and Day, 2003). This juxtaposition directs expansion of the leaf to the margin, a region called the blastozone (Hagemann and Gleissberg, 1996). Although the morphological variation of leaves is seemingly endless, a broad distinction can be made between leaves with a simple blade and those with a dissected blade. Simple leaves are those in which the blade is unsegmented, or has a continuous margin. In dissected leaves, on the other hand, the blade is segmented into leaflets so that the margin is disrupted in a reiterative fashion, called blastozone fractionation (Hagemann and Gleissberg, 1996). Even along the continuous margin of simple leaves, blastozone fractionation can give rise to serrations and lobes.

Dissected and deeply lobed leaf morphology is generally rare among monocots occurring in only four orders: Alismatales, Dioscoreales, Pandanales and Arecales (Gunawardena and Dengler, 2006). Blastozone fractionation is the most common mechanism among angiosperms for achieving dissected and lobed leaves (Bharathan et al., 2002); however, two additional mechanisms occur only in monocots – plication followed by schizogeny, and programmed cell death.

Plication followed by schizogeny occurs in palms (Arecales) and Cyclanthaceae (Pandanales). In these species what appears to be dissected leaf morphology is actually achieved by folding or plication of the young leaf primordium, with an abscission zone forming along the length of each fold. As the leaf matures and expands, the abscission zones tear by mechanical

force, or schizogeny, and give rise to ‘leaflets’. The palm leaf, then, is actually a simple leaf that has been torn into individual segments.

The mechanism of programmed cell death produces lobes and leaflets by cell necrosis in the intervening tissues (Kaplan, 1984; Gunawardena and Dengler, 2006). This mode appears only in the order Alismatales, and only in two families – Aponogetonaceae and Araceae (Gunawardena and Dengler, 2006). Aponogetonaceae is a monogeneric family, with cell death occurring in only one species *Aponogeton madagascariensis*. In Araceae, on the other hand, leaflet and fenestration formation putatively via cell death occurs in many genera that are phylogenetically distantly related (Cusimano et al., 2011; Mayo et al., 1997).

The plant family Araceae (Alismatales) is unique among monocots in that roughly one quarter of the ca. 3,800 species have pinnately, palmately or pedately dissected leaves (Mayo et al., 1997; Gunawardena and Dengler, 2006). Members of Araceae, or aroids, vary widely in cell death patterns, and dissected and lobed leaf morphology is extremely varied throughout the group as well (Mayo et al., 1997).

In addition to the diversity of developmental mechanisms and mature leaf shape, vegetative morphology in Araceae is further elaborated by the presence not only of different leaf types in each successive metamer (i.e., internode, leaf/leaves and vegetative/floral buds), but also of heteroblasty (i.e., a change in the leaf morphology of successive leaves over the course of ontogeny of an individual plant) (Goebel, 1889; Zotz et al., 2011).

Ray (1987b,c) refined a descriptive framework laid out by previous authors in order to describe the various leaf types and shoot organization of the vegetatively complex Araceae using highly specific terminology and diagrams (Arber, 1925; Blanc, 1977a,b; Engler, 1877; Engler et al., 1990; Ritterbusch, 1971; Tomlinson, 1970). Although this system is extremely useful for

highlighting homologies among the various leaf types and understanding overall growth patterns in Araceae, subsequent studies implementing it are lacking. Furthermore, studies investigating the development of the vegetative structures described by Ray are altogether absent.

Engler (1876, 1877), Ray (1987b,c, 1988) and Engler et al. (1990) have described shoot organization in Araceae. Engler divided the genera of Araceae into seven series based on leaf divergence angle, overall growth habit (i.e., subterranean, creeping, climbing, erect, etc.), and on “dromicity” (direction of the rolling of successive leaves) (Engler, 1877, Engler et al., 1990). *Anthurium* and *Amorphophallus* together fall into the seventh series, which is further divided into seven groups. Among the groups *Anthurium* and *Amorphophallus* fall separately based on overall growth habit. For diagrammatic representation of shoot organization in Araceae based on the Englerian system, refer to Engler et al. (1990) and Ray (1987c, 1988).

The vegetative morphology of the neotropical genus *Anthurium* is the most strikingly variable of all genera within Araceae, and arguably within all monocots (Mayo et al., 1997). Leaf shapes include ovate, cordate, lanceolate, peltate, trifid, trisect, pedatifid, pedatisect, palmatifid and palmatisect (Croat, 1983; Mayo, 1997). Before the advent of molecular phylogenetics, *Anthurium* species with dissected or deeply lobed leaves were divided into two groups. Section *Dactylophyllium* (Schott) Engler (Engler, 1879) contained species with three or more leaflets, while section *Schizoplacium* (Schott) Engler (Engler, 1879) contained species with five or more lobes (Croat and Carlsen, 2013; Engler, 1879, 1905; Madison, 1978; Schott, 1860). However, the current phylogenetic tree of *Anthurium*, based on a combined chloroplast and nuclear dataset, shows that section *Schizoplacium* is polyphyletic (Carlsen and Croat, 2013; Croat and Carlsen, 2013). Biogeographic and reproductive characters support most clades in the current phylogeny; however, one clade is composed entirely of species with palmately lobed or

palmatisect leaf morphology (Carlsen and Croat, 2013). This clade, termed Clade 3 (Carlsen and Croat, 2013) represents the current circumscription of section *Dactylophyllum* (Croat and Carlsen, 2013) (Figure 2.1). Species of *Anthurium* with deeply lobed pedatifid leaves fall into two independent clades. These are Clade 16, characterized by species from northern Central America with bright orange berries, and Clade 14 containing species with hooded spathes and pendent spadices (Carlsen and Croat, 2013) (Figure 2.1). Studies are needed to test whether dissected and deeply lobed leaves of independent origin in *Anthurium* share a common developmental mechanism.

Amorphophallus Blume is a paleotropical genus belonging to the morphologically derived subfamily Aroideae, and contains species that all possess a decomposed, or highly dissected, trisect lamina (for a further discussion of decomposed see Results) (Hay and Mabberley, 1991; Hetterscheid and Ittenbach, 1996). Decomposed leaves putatively arise through blastozone fractionation but this has not been confirmed by developmental studies. The close resemblance of decomposed leaves to dracontiid leaves, which putatively arise through programmed cell death, highlights the need for careful developmental studies of both types. *Amorphophallus paeoniifolius* has a long history of cultivation in Asia, but research has largely focused on corm characteristics (Hetterscheid and Ittenbach, 1996; Lebot, 2009). The only study of leaf development in *Amorphophallus* is a study on the morphology and development of the vegetative shoot of *Amorphophallus rivieri*, a publication which has thus far been inaccessible (Chao-Nien Sun, 1948).

The following study was performed to: 1) determine whether dissected and deeply lobed leaves of independent evolutionary origin in *Anthurium* utilize the same developmental mechanism; 2) determine whether the decomposed leaf in *Amorphophallus* arises through



Figure 2.1 Current phylogeny of *Anthurium* based on a combined nuclear (*CHS* first intron and partial flanking coding regions) and chloroplast (*trnG* intron, *trnH-psbA* and *trnC-ycf6* intergenic spacers) sequence alignment, adapted from Carlsen and Croat (2013). Stars denote clades in which dissected or palmately/pedately-lobed leaf morphology occurs.

blastozone fractionation; and 3) describe the development of species of *Anthurium* and *Amorphophallus* using the descriptive terminology of Ray (1987b,c).

2.2 Materials and Methods

2.2.1 Living and embedded plant material and species verification

Species of *Anthurium* and *Amorphophallus* used in this study, which were germinated from seed or propagated by cuttings in the aroid greenhouse at the Missouri Botanical Garden are listed in Table 2.1 and shown in Figure 2.2.

Table 2.1 Species included in studies of dissected/lobed leaf development.

Genus/species	MBG Collection No.	Voucher No.	Provenance
<i>Amorphophallus bulbifer</i> BLUME	T. Croat 77292	(MO) 6649284	
<i>Anthurium clavigerum</i> POEPP. & ENDL.	T. Croat 84498	(MO) 6649275	Peru: Junin
<i>Anthurium pentaphyllum</i> var. <i>bombacifolium</i> (SCHOTT) MADISON	T. Croat 84951, 2014-0003-5	(MO) 6649282	
<i>Anthurium podophyllum</i> KUNTH	2012-1643-3	(MO) 6647388	Mexico
<i>Anthurium polyschistum</i> R.E.SCHULT. & IDROBO	T. Croat 78265, 2013-2467-2	(MO) 6649283	Ecuador
<i>Anthurium sp. nov.</i>	2012-1437	(MO) 6647400	

A phylogenetic analysis using sequence data from the chloroplast region *ycf6* to *trnC* was performed to verify species identities (data not shown). DNA was extracted from *Anthurium* species in Table 2.1, following the DNA extraction protocol in Chapter One. The remaining sequences were obtained from Carlsen and Croat (2013) stored in Genbank.



Figure 2.2 Species included in studies of dissected and lobed leaf development. A. *Anthurium polyschistum*, B. *Anthurium clavigerum*, C. *Anthurium pentaphyllum* var. *bombacifolium*, D. *Anthurium* sp. nov., E. *Anthurium podophyllum*, F. *Amorphophallus bulbifer*.

2.2.2 Scanning Electron Microscopy and Histology

Dissections of developing leaves were fixed in 15 ml FAA overnight (50% EtOH, 5% glacial acetic acid, 10% 37% formaldehyde solution (formalin), 35% dH₂O), then taken through a dehydration series of 50% EtOH, 70% EtOH, 80% EtOH, 90% EtOH, 95% EtOH, 100% EtOH with 1.5 to 2 hours between each change and left in 100% EtOH overnight. The following morning 100% EtOH was changed for fresh (newly opened) 100% EtOH. Samples were then prepared for SEM imaging or histological studies.

For critical point drying the SamDri-780 Critical Point Dryer was employed following standard operating procedures and samples were left at an equilibrium pressure of ~1300 psi and temperature of ~36°C between 12 and 20 minutes. Critical point dried samples were then sputter coated using a Tousimis Samsputter-2a for two minutes under a vacuum pressure of 130-140 mTorr, with a current of 10 mA and an Argon tank reading of ~4 psi. Sputter coated samples were mounted and imaged using the S-2600H Scanning Electron Microscope at the Department of Otolaryngology's Electron Microscopy Core at Washington University in St. Louis.

For histological studies, dehydrated samples were infiltrated, embedded, sectioned, mounted and de-paraffinized as follows: All incubations were performed for two hours at room temperature with shaking. In the morning, 100% EtOH was replaced with fresh pre-chilled 100% EtOH and placed on the shaker at room temperature. 100% EtOH was replaced with histoclear:EtOH at a ratio of 1:3, then 1:1, then 3:1, then 100% histoclear, then fresh 100% histoclear and left overnight, shaking at room temperature. In the morning, histoclear was replaced with fresh 100% histoclear and left to incubate for 2 hours. Then ¼ volume of Paraplast Plus chips (McCormick #15159-464) was added to the tubes and incubated several more hours. At the end of the day more chips were added, then left overnight with shaking. In

the morning more paraplast chips were added and tubes were placed at 42°C until chips completely dissolved, then ½ the volume was poured off and replaced with 100% melted Paraplast Plus kept at 60°C, tubes were transferred to the 60°C oven for the remainder of the infiltration steps. At the end of the day, tubes were left in 100% melted paraplast. Paraplast was renewed every 8-10 hours, until it had been changed 5 times. Samples were embedded, then sectioned on a rotary microtome and mounted on ProbeOn Plus slides (Fisher #22-230-900) and placed on a slide warmer at 42°C overnight. De-paraffinized sections were re-hydrated through the reverse order of the dehydration series (above) with two minutes between each transfer. Staining of re-hydrated sections followed ‘Sass’s Safranin and Fast Green’ protocol (Ruzin, 1999), modified by staining in Safranin O (1% w/v) for 3 minutes and Fast Green (0.1% w/v) for 2 minutes.

2.3 Results

2.3.1 *Phylogenetic position of species of Anthurium*

The species of *Anthurium* studied here fall into two independent clades - the northern Central American clade (Clade 16) and a clade corresponding to section *Dactylophyllum* (Clade 3; Figure 2.1) (Carlsen and Croat, 2013). *Anthurium podophyllum*, *A. lezamai* and *A. sp. nov.* all belong in Clade 16 (data not shown). *Anthurium podophyllum* and *A. lezamai* are Mexican endemics, while *A. sp. nov.* is of unknown origin (Croat, 1983). In the phylogenetic analysis based on the *ycf6* to *trnC* chloroplast region, *A. podophyllum* and *A. sp. nov.* fall out as sister taxa. Due to differences in reproductive morphology and a pronounced difference in time to reach maturity between *A. podophyllum* and *A. sp. nov.*, *A. sp. nov.* merits species rank.

2.3.2 Leaf development in *Anthurium*

All members of Clade 16 are terrestrial plants with very short internodes (Figure 2.3A; Figure 2.4A) (Croat, 1983). Mature leaves are divided pedately into 5-7 lobes. The species of *Anthurium* from Clade 16 studied here go through a heteroblastic leaf series, in which successively older leaves gradually become more divided, with lobes becoming more accentuated (Figure 2.4B). An illustration of this type of heteroblastic series can also be found in Madison (1978). Articles in species of *Anthurium* from Clade 16 studied here are all characterized by sympodial growth following the emergence of the cotyledon. Articles consist of the activity of a single meristem from its initiation to its termination by abortion or the transition to flowering (Ray, 1987c). In sympodial growth, the shoot axis is terminated by an inflorescence and subsequent growth is continued by a bud in the axil of the penultimate leaf ($n-1$) (Ray, 1986). Sympodial growth in *Anthurium* is sylleptic since there is no resting period between axis termination and continuation. Each article of sylleptic sympodial growth in *Anthurium* is composed of a bladeless (or highly reduced bladed) prophyll ($n-2$), that is 2-keeled on its abaxial side, then a bladeless 1-keeled mesophyll ($n-1$), followed by a foliage leaf and a terminal inflorescence meristem (Figure 2.3A; 2.4C-F). A prophyll is the first leaf of a new axis (Arber, 1925; Ray, 1987b) (Figure 2.3A). Sometimes additional reduced leaves termed mesophylls follow the prophyll (Ray 1987b; Tomlinson, 1970). In *Anthurium* there is one mesophyll before the emergence of the bladed foliage leaf (Figure 2.3A). Reduced leaves are generally termed cataphylls. The prophyll, mesophyll and foliage leaf are serial homologs and their designation as ($n-2$), ($n-1$) and (n), respectively, emphasizes this relationship. All leaf types encircle the axis, as is generally the case in Araceae (Mayo et al., 1997). The previously described shoot organization has been termed sylleptic, homeophyllous, triphyllous sympodial

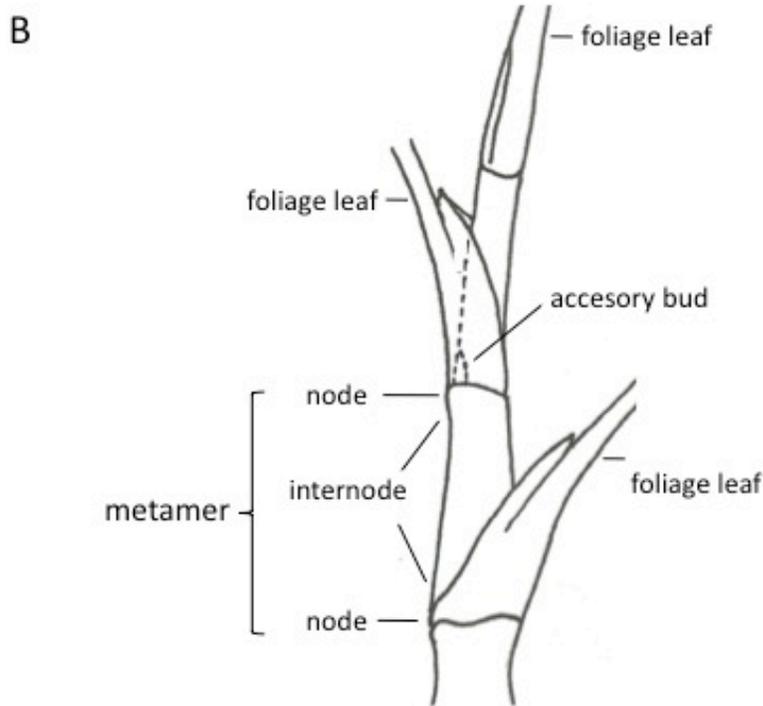
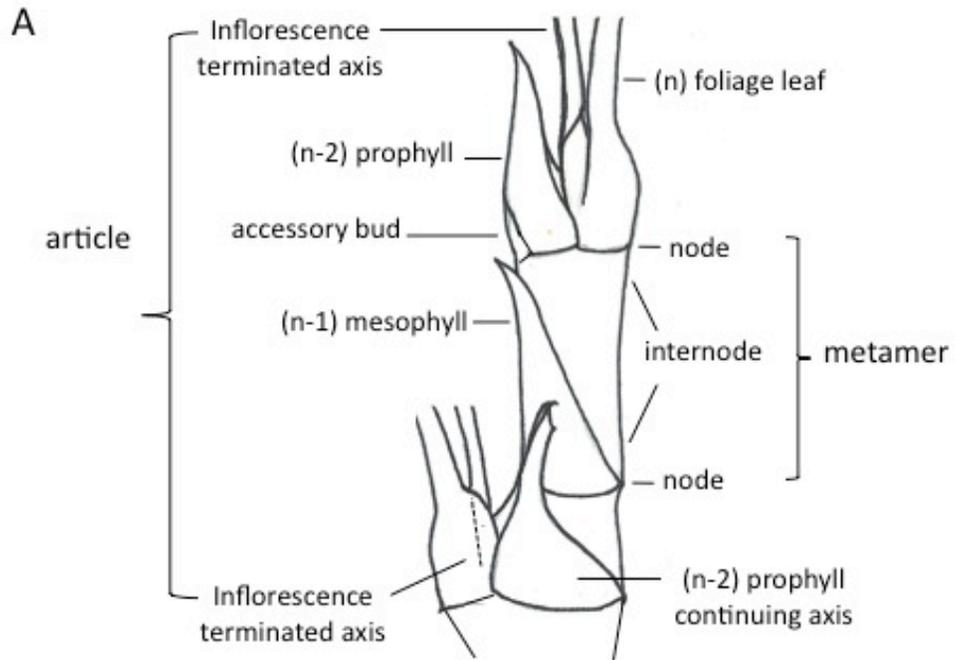


Figure 2.3 Growth modes in *Anthurium*. A. Sympodial growth. Internodes between prophyll and mesophyll have been elongated to show the metamers to which they belong. B. Monopodial growth.

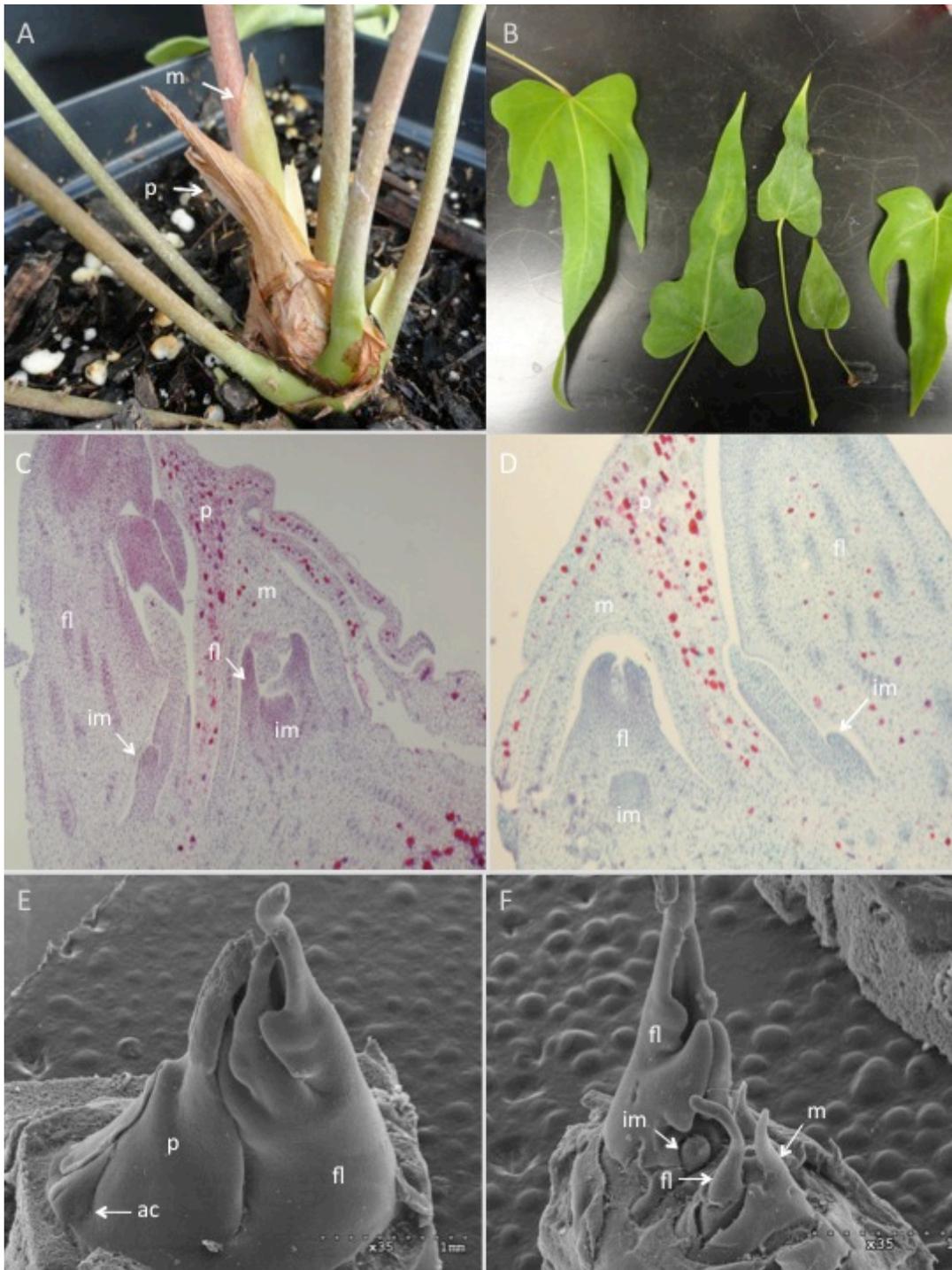


Figure 2.4 Features of sympodial growth in *Anthurium*. A. Short internodes of *A. podophyllum*, B. Heteroblastic leaf series in *A. podophyllum*, lobing increases with age, C. Longisection of the shoot apex in *A. sp. nov.*, D. Longisection of the shoot apex in *A. podophyllum*, E. SEM of the shoot apex in *A. sp. nov.* showing an accessory bud, F. SEM of the shoot apex in *A. sp. nov.* with the prophyll removed. *p* prophyll, *m* mesophyll, *fl* foliage leaf, *im* inflorescence meristem, *ac* accessory bud.

growth (Ray, 1987c) because each article has a consistent (homeophyllous) number (three) of leaf-like organs (phyllous).

Bud placement in *Anthurium* (and Araceae as a whole) is irregular in that it is not axillary per se, but rather located below the point of overlap of the margins of the prophyll (Engler et al., 1990, Ray, 1987b,c). The presence of a bud below the prophyll of the renewal shoot suggests the presence of serial, or accessory, buds in *Anthurium* (Figure 2.3A, 2.4E). It is also worth noting the complex anatomy associated with the different leaf types; cells of the prophyll contain a vast amount of secondary compounds and calcium oxalate crystals, which are a distinguishing character in Araceae (Figure 2.4C,D) (Keating, 2003, 2004a,b). Safranin stains lignified, suberized or cutinized structures, but the exact chemistry of the red cells is unknown.

The development of each leaf-like appendage and the inflorescence in this type of shoot organization is described relative to one another, as opposed to real-time measurements (Figure 2.5). Foliage leaves (n) will be referred to as p0, p1, p2, which describe the order of appearance on the shoot apical meristem (SAM). p0 is the position where the next leaf primordium will emerge, whereas p1 and p2 refer to leaf primordia that are already visible in order of appearance with p2 being older than p1. The first appendage to differentiate from the SAM of the continuing axis is the prophyll. This occurs concomitantly with the termination of the previous axis signaled by the differentiation of the inflorescence meristem (Figure 2.5A-D). While the inflorescence meristem further develops, the continuing axis becomes clearly separate as the prophyll physically tears away from the terminated axis (2.5C,D). As the margins of the prophyll finish encircling the continuing axis, the mesophyll and p1 begin to differentiate (Figure 2.5E,F). By this stage a well-developed precursor tip, or *Vorläuferspitze*, is visible on p2 of the previous axis (Figure 2.5E) (Knoll, 1948). As the mesophyll and p1 of the continuing axis further

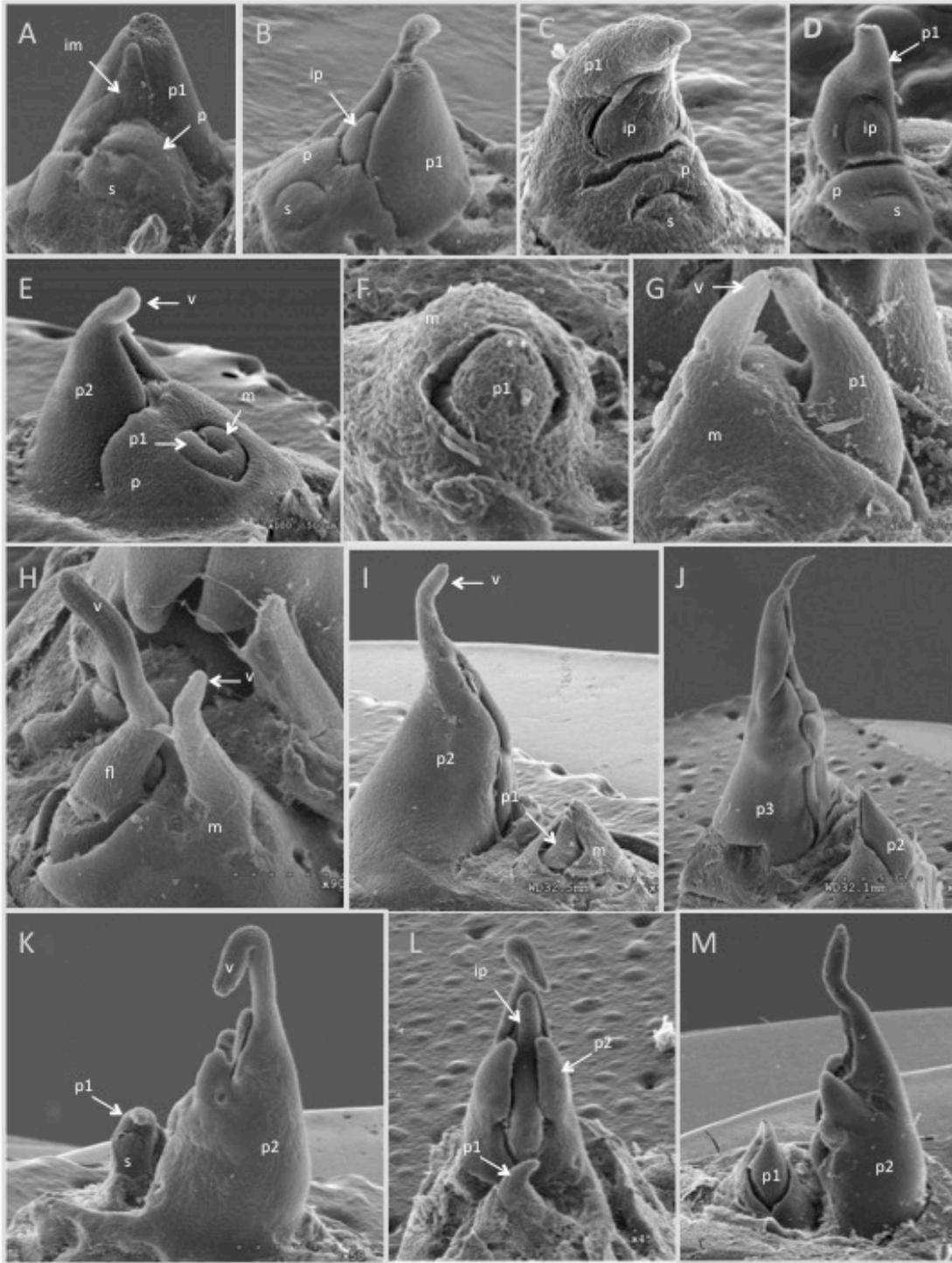


Figure 2.5 Symphyllal development in *Anthurium*. A, B. and E. *A. podophyllum*, C, D. *A. sp. nov.*, F. *A. sp. nov.*, prophyll removed G. *A. sp. nov.*, prophyll removed, H. *A. sp. nov.*, prophyll removed further in development, I. *A. podophyllum*, prophyll removed, J. *A. podophyllum* prophyll and mesophyll removed, K-L. *A. sp. nov.*, prophyll and mesophyll removed, M. *A. podophyllum*, prophyll removed. *p* prophyll, *m* mesophyll, *p1*, *p2*, *p3* foliage leaf, *im* inflorescence meristem, *ip* inflorescence primordium, *s* shoot apical meristem, *v* *Vorläuferspitze*.

differentiate, they also develop precursor tips that, depending on the species, can be moderate to massive (Figure 2.5G,H). During mesophyll and p1 differentiation on the continuing axis, p2 of the terminated axis begins to develop lobes along the marginal meristem, or blastozone. In other words, lobe formation in p2 initiates as p1 differentiates from the mesophyll, before leaf inception at p0 (Figure 2.5I,K, L). Timing of lobe formation is heterochronic between species, as seen by the further developed lobes in *A. sp. nov.* compared to *A. podophyllum* at an equivalent stage of p2 development (Figure 2.5I,K, L). Thereafter, lobes continue to develop through blastozone fractionation in a basipetal wave of maturation (Figure 2.5J,M).

The species of *Anthurium* from Clade 3, or section *Dactylophyllum*, studied here include *Anthurium clavigerum*, *A. polyschistum* and *A. pentaphyllum* (Carlsen and Croat, 2013, present study, data not shown). All are appressed-climbing or scandent plants with long internodes, and the mature leaves are dissected into 5-7 leaflets. Individual plants go through a heteroblastic leaf series, in which successively older leaves gradually increase in leaflet number. An illustration of this type of heteroblastic series can be found in Madison (1978). Growth in these species is initially monopodial, with metamers being produced from a single shoot apex (Figure 2.3B) (Ray, 1986). The base of the leaf completely encircles the stem forming a sheath (Figure 2.3B; 2.6B). An axillary bud is formed in the axils of foliage leaves (Figure 2.3B), but as mentioned above, they can be displaced upward along the internode. Ray (1988) states that vegetative buds are formed only on sympodial metamers in *Anthurium*; however, vegetative buds were found associated with monopodial metamers in this study. No inflorescence meristem is associated with this type of growth. This is the juvenile phase, or the mature phase when conditions for flowering become unsuitable. Upon flowering, however, shoot organization becomes sympodial as described above (Figure 2.6C). This growth pattern has been described as sylleptic,



Figure 2.6 Features of monopodial growth in *Anthurium*. A. *A. clavigerum*, B. *A. pentaphyllum*, C. *A. polyschistum*, D,G. Longisection of shoot apex of *A. clavigerum*, E,H. Longisection of shoot apex of *A. pentaphyllum*, F,I. Longisection of shoot apex of *A. polyschistum*. *i* internode, *n* node, *p* prophyll, *p1*, *p2*, *p3* foliage leaf, *m* mesophyll, *f* foliage leaf, *s* shoot apical meristem, *sh* sheathing leaf base, *in* inflorescence.

intermittent homeophyllous, triphyllous, sympodial because a variable number of metamers with a single foliage leaf are formed before switching to homeophyllous, triphyllous, sympodial growth during flowering (Ray, 1987c). After flowering, *Anthurium pentaphyllum* remains in sympodial growth mode, while *A. polyschistum* reverts back to monopodial growth. The individuals of *Anthurium clavigerum* used in this study were non-flowering and maintained monopodial shoot growth; however, sympodial shoot growth in this species has been described (Ray, 1987b).

Damage to the monopodial axis can also promote sympodial growth, whereby an axillary bud renews the shoot beginning with a prophyll, followed by a mesophyll, then any number of mesophylls before producing a foliage leaf. In these species, internode elongation occurs between the prophyll and mesophyll (Figure 2.6C, Ray, 1987b).

Leaf inception during monopodial growth in *Anthurium* is characterized by the emergence of a well-developed precursor tip (Figure 2.7A-C). The *Vorläuferspitze* in *A. clavigerum* (Figure 2.7C) is the longest known of any angiosperm species at an equivalent stage of leaf development. At the time the *Vorläuferspitze* emerges, the leaf base of p2 has completely encircled the shoot apex (Figure 2.7B). As the *Vorläuferspitze* of p1 elongates, leaflet formation via blastozone fractionation in p2 has already begun, and the degradation of the *Vorläuferspitze* of p2 has also commenced (Figure 2.7D-G). As leaflet formation in p2 proceeds in a basipetal maturation wave, the leaflets come to completely envelope the tip of p1 (Figure 2.7G,H). The last part of the leaf to become established is the petiole, which is the zone between the distal region of the ensheathing leaf base and proximal region of the leaf blade (marked by final leaflet primordia) (Figure 2.7H). As leaflets mature, an additional *Vorläuferspitze* is formed at the apex

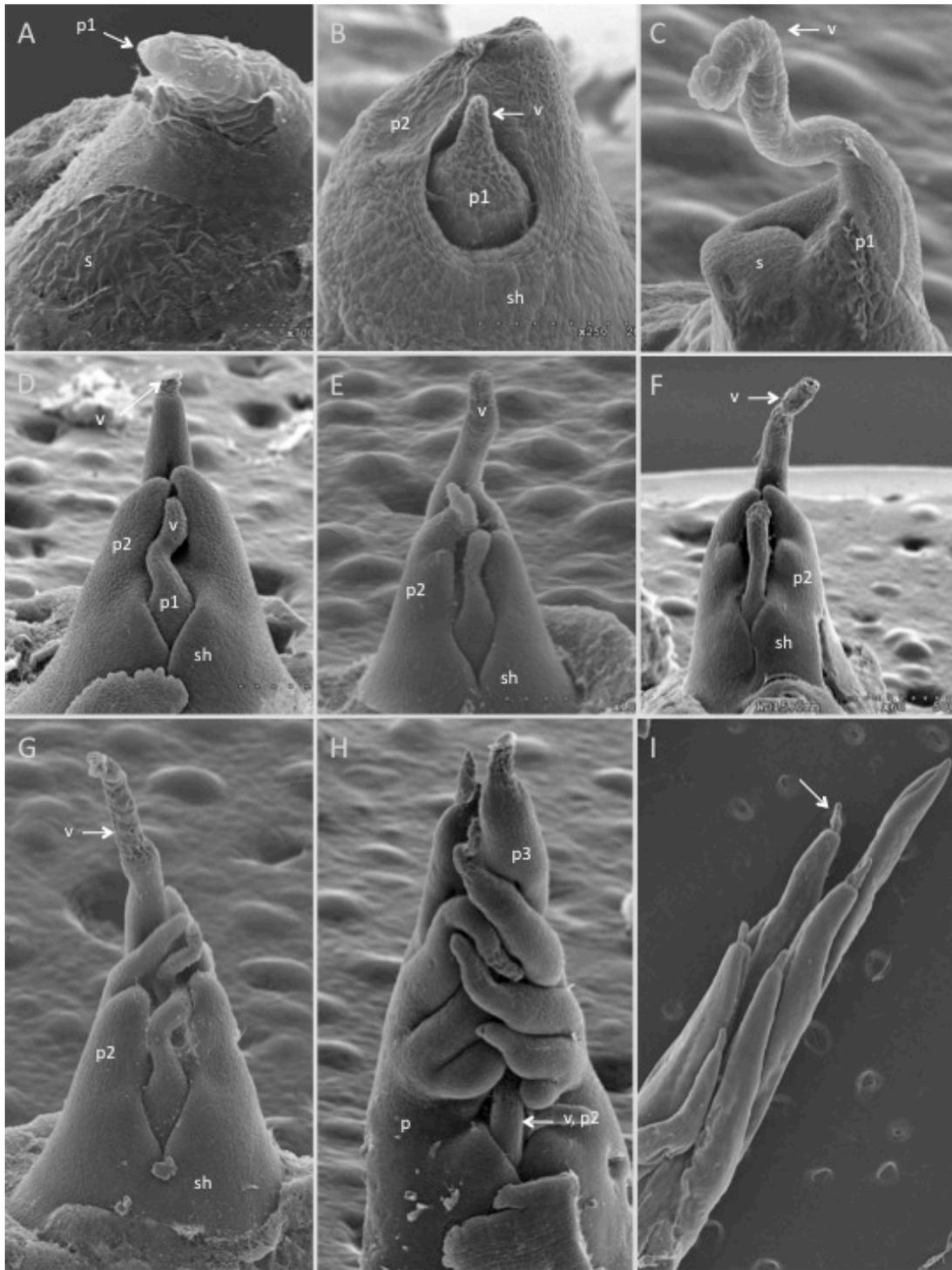


Figure 2.7 Monopodial development in *Anthurium*. A. *Anthurium polyschistum*, B. *A. polyschistum*, C. *A. clavigerum*, D. *A. pentaphyllum*, E. *A. polyschistum*, F. *A. pentaphyllum*, G. *A. polyschistum*, H. *A. polyschistum*, I. *A. polyschistum*. *s* shoot apical meristem, *v* Vorläuferspitze, *p* petiole, *p1*, *p2*, *p3* foliage leaf, *sh* sheathing leaf base, arrow indicates tip on leaflet.

of each one; these will also degrade at final maturity (Figure 2.7I). This has also been observed in leaflet development in *Dioscorea pentaphylla* (Periasamy and Muruganathan, 1985).

2.3.3 Leaf development in *Amorphophallus*

As stated above, *Amorphophallus* was separated from *Anthurium* in Engler's seventh series based on the difference in overall growth habit (Engler et al., 1990). In *Amorphophallus*, the main stem is a tuber, which in most species follows a sympodial cyclic growth pattern with alternating active and resting periods after the first flowering terminates the monopodial seedling stage (Figure 2.8) (Engler et al., 1990; Sedayu et al., 2010). Usually, the single foliage leaf (rarely two) of each shoot persists for only one vegetative period, then dies back. During the monopodial phase, a foliage leaf will emerge above ground while below ground a bud develops with a series of cataphylls with axillary buds, followed by a foliage leaf. The emerged foliage leaf will then die back and a resting/dormancy period follows. The resting leaf primordia will then resume growth the next growing season. During sympodial growth, "after-leaf" dormancy is followed by a series of inflorescence bracts surrounding an inflorescence rudiment. However, growth cycles can be more complex and vary between species; for more details see Hetterscheid and Ittenbach (1996) and Sedayu et al. (2010). Samples of *Amorphophallus bulbifer* were in the juvenile monopodial phase.

In *Amorphophallus bulbifer* three cataphylls were observed between each foliage leaf (Figure 2.8A). Early cataphyll development is similar to that of foliage leaves, but blade development in cataphylls is arrested early on, leaving a blade rudiment at the apex (Figure 2.8 C-E). Leaf development in this species is unlike any other seen in the genera of Araceae studied to date (Henriquez et al., in prep.). The regions that will give rise to each of three segments in the trisect leaf arise simultaneously, leaving a depression in the center of the primordium apex

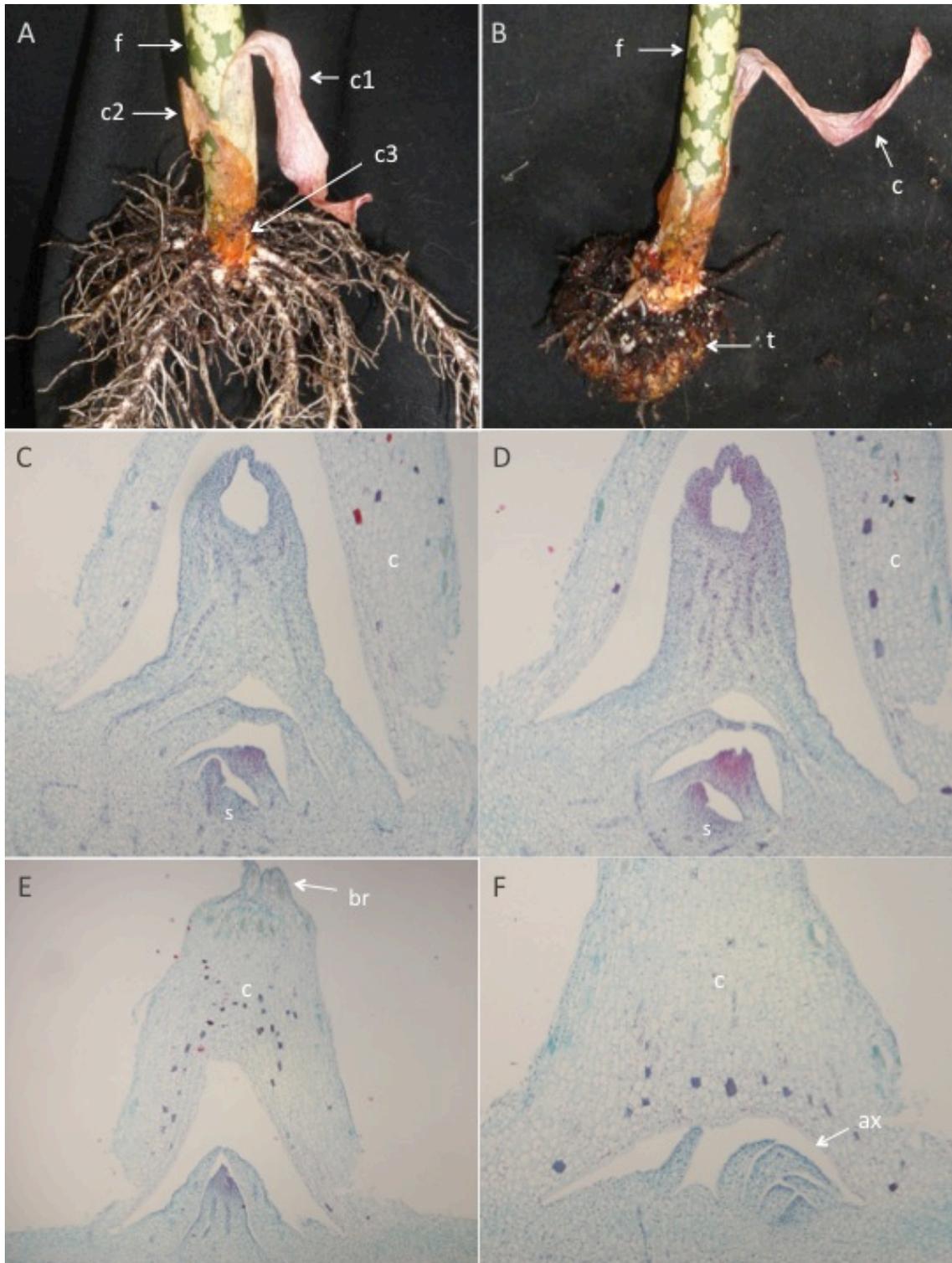


Figure 2.8 Monopodial growth in *Amorphophallus bulbifer*. A,B. tuberous stem, roots, cataphylls and petiole of foliage leaf, B. roots removed, C-F. longisections through shoot apex. *ax* axillary bud, *br* blade rudiment, *c*, *c1*, *c2*, *c3* cataphylls, *f* foliage leaf, *s* shoot apical meristem, *t* tuber.

(Figure 2.9A). Thus, one could argue that the trisect leaf of *Amorphophallus bulbifer* is peltate very early in development, whereas peltation in other angiosperms arises much later in development (e.g. *Tropaeolum majus*) (Gleissberg, et al., 2005, Kim et al., 2003a). The early peltation of the leaf primordium, presumably through loss of adaxial identity in the cross zone (Gleissberg, et al., 2005, Kim et al., 2003a), causes a heterotopic shift in leaflet formation. Leaflets form a ring at the distal end of the proximodistal axis of the leaf primordium, instead of maintaining a lateral position (Figure 2.9C-F). As the leaf primordium continues to develop one of the segments develops more quickly and overtops the other two (Figure 2.9B,C). When each segment begins to form leaflets through blastozone fractionation, by the time the precocious segment develops three prominent leaflets, the other two segments have formed only two and the rudiment of a third (Figure 2.9F). Sedayu et al. (2010) have noted that in certain species (not *A. bulbifer*) the anterior segment is *less* divided than the posterior segments, and that this feature evolved three times from equally shaped segments; the reverse has not been observed. Clearly the anterior segment is, at least partially, developmentally independent from the posterior segments. In this respect, it is similar to what is called a “dracontiod” leaf (Cusimano et al., 2011, Hay and Mabberley, 1991; Mayo et al., 1997), in which the anterior lobe differs from the posterior lobes. Furthermore, the dracontiod leaf, found in such unrelated genera as *Anchomanes* and *Dracontium*, is thought to be elaborated from a sagittate, hastate or trisect leaf with higher order divisions resulting from cell death (Cusimano et al., 2011). Based on the results of this study, decompound leaves in *Amorphophallus* may be derived from a peltate, sagittate or hastate leaf, with the difference between a dracontiod and these decompound leaves being the presence of cell-death in the former. Detailed studies of leaf development between dracontiod- and decompound-leaved aroid species is highly desired.

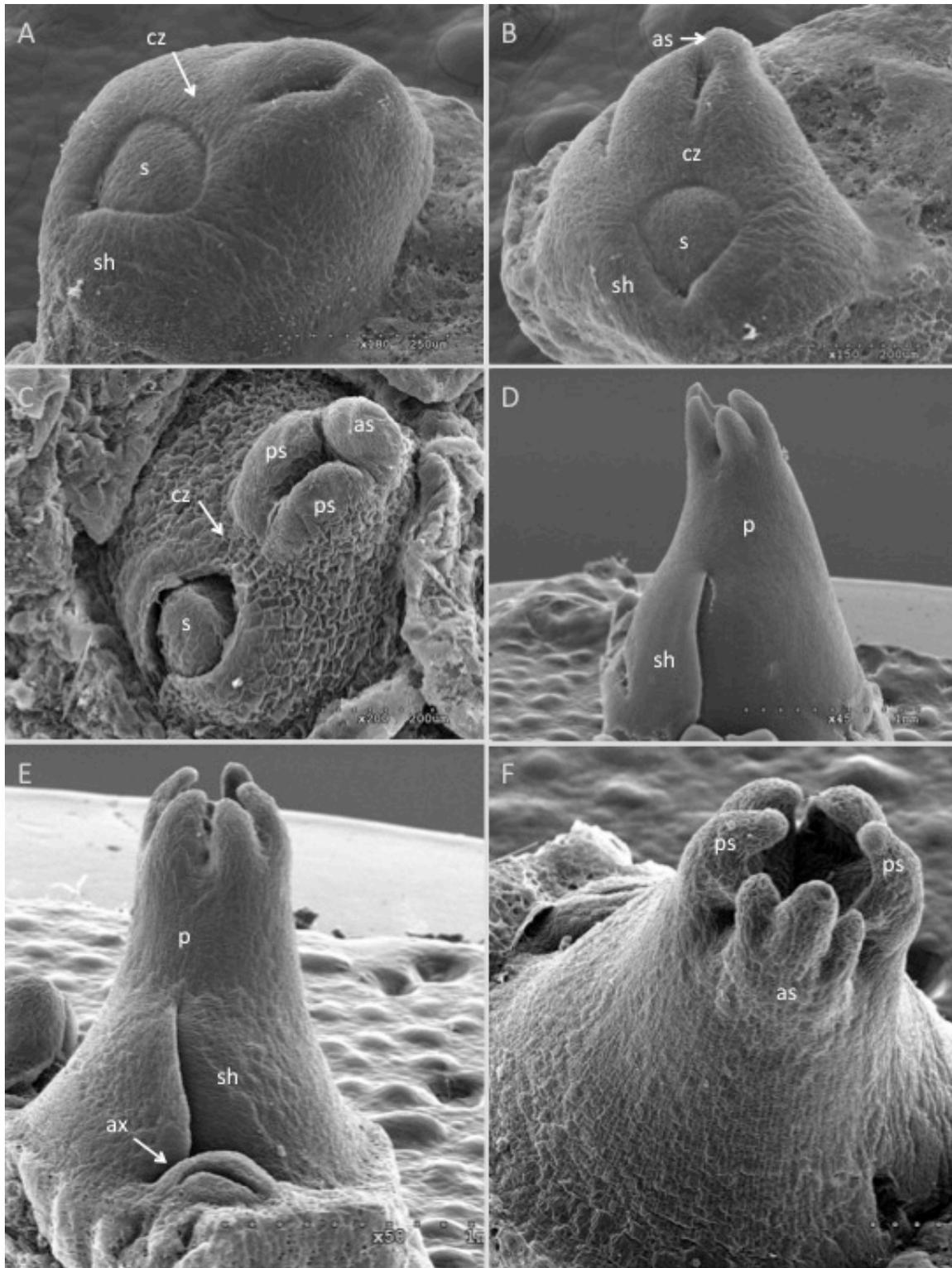


Figure 2.9 Monopodial development in *Amorphophallus bulbifer*. *as* anterior segment, *ps* posterior segment, *ax* axillary bud, *cz* cross zone, *p* petiole, *s* shoot apical meristem, *sh* sheathing leaf base.

2.4 Discussion

The present study confirms that both *Anthurium* and *Amorphophallus* employ the mechanism of blastozone fractionation to achieve dissected and lobed leaves. This is true even among species of *Anthurium* that have evolved dissected and lobed leaves independently. Having established the development of shoot organization and leaf morphology in these two genera, several salient features beg further work. These are the three leaf types in *Anthurium*, the role of adaxial-abaxial polarity genes in the peltate leaf primordium of *Amorphophallus*, the role of adaxial-abaxial polarity genes in leaflet placement on the dissected leaves of *Anthurium* and *Amorphophallus*, and the role of *KNOX1* genes in dissected leaf development in these two genera.

2.4.1 *Leaf types in Anthurium*

In his treatment of leaf types and shoot organization in Araceae, Ray (1987b,c, 1988) recognized that such complexity required an equally complex terminology to be adequately described. Although he rarely explicitly uses the term “homologous”, his observations are implicitly derived from a working concept of it, particularly with reference to two types of homology within individuals proposed by Haszprunar (1992) – iterative homology and ontogenetic homology. Iterative homology is “correspondence between different characters (or repeated characters) in the same individual at the same time”, while ontogenetic homology is “correspondence between characters of the same individual at different times” (Haszprunar, 1992). The homeophyllous, triphyllous sympodial segment, and the heteroblastic leaf series of *Anthurium* illustrate these concepts.

The terms *homeophyllous* and *intermittent homeophyllous* describe the ontogenetic homology of successive metamers on a continuing shoot, bearing monopodial or sympodial

leaves. *Triphyllous*, or three leaf types on a sympodial segment, embodies both the concepts of ontogenetic homology and iterative homology. Prophylls and mesophylls (or cataphylls to be general) and foliage leaves occupy different positions in the organism *and* are added successively through time. This example can be added to that of the heteroblastic leaf series (also found in *Anthurium*) in highlighting the lack of distinction at times between ontogenetic and iterative homology (Roth, 1994).

The triphyllous state of *Anthurium* and the nona- to dodecaphyllous state of *Amorphophallus* are also useful study cases of biological homology (Wagner, 1989). Cataphylls and foliage leaves are all phyllomes, or leaf-like structures (Arber, 1950; Sattler, 1994). The vestigial blade that can be seen at times at the apex of cataphylls supports this. With the molecular tools now available, the contribution of morphogenetic versus morphostatic mechanisms in phyllome development can be determined within a framework of leaf GRNs (Townsend and Sinha, 2012; Wagner, 1994, 2007). One can ask and answer the question “How individuated (Roth, 1991) are prophylls from mesophylls from foliage leaves”?

2.4.2 *The role of adaxial-abaxial polarity genes in the peltate leaf primordium of Amorphophallus, and leaflet placement on dissected leaves of Anthurium and Amorphophallus*

The molecular genetics of peltate leaf morphology has been studied in numerous taxa, mostly within eudicots (Gleissberg et al., 2005; Kim et al., 2003a). During peltate leaf development, the adaxial surface of the leaf between the base and apex enlarges to form a cross-zone (from *Querzone* Kaplan, 1997; Troll, 1932). In the species used in molecular genetic studies, the petiole of peltate leaves was invariably unifacial; however, Kaplan (1997) has described the formation of a peltate blade from a cross-zone that spans a bifacial (dorsiventral)

petiole in *Stephania hernandiifolia*. The petiole in *Amorphophallus* is unifacial (radialized); thus in this case the region where the bifacial blade and unifacial petiole meet is called the cross-zone (Figure 2.9A) (Gleissberg et al., 2005). Unifacial structures in angiosperms have lost one or the other adaxial-abaxial cell identities. In nature, they typically have lost the adaxial domain and are thus abaxialized. Two unrelated genes specifying adaxial-abaxial patterning, *PHANTASTICA (PHAN)* and *FILAMENTOUS FLOWER (FIL)*, are involved in the development of peltate leaves with unifacial petioles in eudicots (Gleissberg et al., 2005, Kim et al., 2003a).

The gene *PHAN*, first isolated in *Antirrhinum*, encodes an MYB domain transcription factor and is involved in the maintenance of polarity in vasculature and leaves by conferring adaxial identity (Tsiantis et al., 1999). Interestingly, the action of *PHAN* has been implicated in the great diversity of dissected leaf morphology across eudicots (Kim et al., 2003a). The extent of the adaxial domain along the proximo-distal axis of the leaf primordium determines the location of leaflet inception. In pinnately and palmately dissected leaves, expression of *PHAN* along most of the adaxial surface of the leaf primordium correlates with leaflet formation, with the palmately dissected leaf form arising through secondary extension of the basal region of the primordium where *PHAN* is down-regulated. In peltately-palmate dissected leaves, on the other hand, the expression of *PHAN*, and hence the adaxial-abaxial border, is restricted to a region at the distal tip of the leaf from which leaflets arise concentrically, while the petiole has been completely abaxialized and is radial in cross-section (Kim et al., 2003a).

FIL is a member of the *YABBY* gene family first identified in *Arabidopsis* (Sawa et al., 1999; Wang et al., 2009). *FIL* is associated with abaxial cell fates in eudicots (Sawa et al., 1999). Localized expansion of the *YABBY* gene *TmFIL* in *Tropaeolum majus* leads to

abaxialization of the petiole and formation of the cross-zone that will give rise to peltate leaf morphology (Gleissberg et al., 2005).

Genes specifying adaxial-abaxial polarity are expected to play a role in peltate leaf development in *Amorphophallus* and leaflet placement in *Anthurium* and *Amorphophallus*; however, whether *PHAN* and *FIL* orthologs in these species are expected to be involved is not so straightforward. The orthologs of *PHAN* in maize, *ROUGH SHEATH2 (RS2)* and *Arabidopsis*, *ASYMMETRIC LEAVES1 (ASI)* are not involved in adaxial-abaxial patterning (Husbands et al., 2009). In addition, members of the *YABBY* gene family have diverged in function between monocots and eudicots (Ishikawa et al., 2009; Wang et al., 2009). In rice, *YABBY* genes have a nonpolarized expression pattern and overexpression is not associated with changes in adaxial-abaxial patterning (Husbands et al., 2009). However, the action of *PHAN* in leaflet placement in dissected leaves of *Aquilegia formosa*, a member of the order Ranunculales within eudicots, makes the action of *PHAN* orthologs in specifying leaflet placement in Araceae more plausible. A transcriptomic study of peltate leaf primordium development in *Amorphophallus* could resolve which gene families in the Gene Regulatory Network (GRN) regulating leaf development are involved.

2.4.3 *The role of KNOX1 genes in dissected leaf development in Anthurium and Amorphophallus*

KNOX1 genes have been implicated in dissected leaf development in species that exhibit blastozone fractionation across the angiosperm phylogeny, except in monocots (Bharathan et al., 2002). Determining whether *KNOX1* genes are involved in dissected leaf development in *Anthurium* and *Amorphophallus* is the focus of Chapter 3.

2.5 References

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Chapter 3

The Elusive Role of *KNOX1* Genes in Dissected Leaf Development in *Anthurium* and *Amorphophallus*

3.1 Introduction

The *KNOX* gene family is one of the best-studied gene families across vascular land plants (Floyd and Bowman, 2007; Sano et al., 2005). *KNOX* genes are a class of transcription factors containing a homeobox domain that were first discovered in the maize *knotted-1* mutant (Sinha and Hake, 1990; Vollbrecht et al., 1991). *KNOX* (*knotted1*-like homeobox) genes fall into two classes based on similarity of residues within the homeodomain, intron position and expression patterns (Bharathan et al., 1999; Kerstetter et al., 1994). Class II *KNOX* genes are expressed in various locations throughout the plant. In contrast, class I *KNOX* genes (*KNOXI*) are expressed in the meristem and are absent in simple leaf primordia (Kerstetter et al., 1997; Reiser et al., 2000). The absence of *KNOXI* gene expression in leaf primordia is thought to confer determinacy to developing leaf tissue (Sinha et al., 1993). Overexpression of the *knotted1*-like gene *KNATI* in *Arabidopsis* produces ectopic meristems and induces lobe formation on simple leaves (Chuck et al., 1996). This confirms the role of *KNOXI* genes in meristem maintenance, but also implicates their action in leaf morphogenesis. Subsequent studies in tomato have shown that overexpression of *KNOXI* genes is responsible for increased complexity in leaf morphology. Specifically, the presence of *KNOXI* genes in leaf primordia prolongs the indeterminacy phase leading to an increase in leaflet number (Janssen et al., 1998; Kimura et al., 2008).

In a seminal study by Bharathan et al. (2002), the role of *KNOXI* genes in lobed and dissected leaf morphology was investigated across a broad sampling of angiosperms. This work revealed that the presence of *KNOXI* in developing leaves was associated with lobed and dissected leaf morphology. Even within simple leaves, serrated margins arise through *KNOXI* gene expression. Although the results of this study showed the function of *KNOXI* genes in

dissected leaf development to be highly conserved, two important caveats preclude the extrapolation of this mechanism to all angiosperms.

The first caveat involves the dissected pea leaf. All taxa studied in Bharathan et al. (2002) develop lobes and leaflets through blastozone fractionation, which is the alternating expansion and suppression of the blade margin (Hagemann and Gleissberg, 1996). In pea, leaflets develop through blastozone fractionation but this was shown to occur through the action of the *UNIFOLIATA (UNI)* gene (Gourlay et al., 2000; Hofer et al., 1997), not *KNOXI*. *UNI* is a homolog of the *Antirrhinum FLORICAULA/Arabidopsis LEAFY* genes, which are floral meristem-identity genes.

The second caveat involves the lack of sampling of dissected-leaved monocots in Bharathan et al. (2002). Dissected leaves are rare in monocots. Yet in addition to blastozone fractionation, monocots achieve lobed and dissected leaves by two alternative developmental mechanisms – programmed cell death, and plication followed by schizogeny – which is not seen in any other taxa outside this clade (Gunawardena and Dengler, 2006). Plication followed by schizogeny involves the folding and tearing of a simple leaf (Kaplan, 1984). This occurs in only two orders within monocots – Pandanales and Arecales (Gunawardena and Dengler, 2006). The only studies of *KNOXI* gene expression in dissected-leaved monocots were performed in members of Arecales that use the plication/schizogeny mechanism (Jouannic et al., 2007; Nowak et al., 2011). Although the two studies report different *KNOXI* gene expression patterns during plication formation, both arrive at the same conclusion of *KNOXI* transcript absence during schizogeny.

The lack of information regarding the role of *KNOXI* genes in dissected leaf development via blastozone fractionation in monocots is a major gap in our understanding of leaf

evolution in monocots and across angiosperms. The plant family Araceae is an excellent study system with which to address this issue. Leaf morphology is extremely varied throughout the group and roughly one quarter of the ca. 3,800 species have pinnately, palmately or pedately dissected leaves (Gunawardena and Dengler, 2006; Mayo et al, 1997). Dissected leaves have evolved independently many times in the family, both through blastozone fractionation and programmed cell death (Cusimano et al., 2011).

Aroids have been noted for their leaf characters that are more similar to dicots than to many other monocots (Bharathan, 1996; Kaplan, 1973). As monocots, aroids are more closely related to grasses than to eudicots; however, grasses are in a morphologically highly derived clade that has undergone multiple genome duplication events (Levy and Feldman, 2015). These genome duplications have increased the number of gene paralogs, which has led to neo- or subfunctionalization of gene family members within grasses (Preston and Kellogg, 2006). Due to the unique genomic and morphological characteristics of the grass clade, it is therefore unknown and perhaps unlikely that gene function in other monocots would mirror that in Poaceae. This is highlighted by the fact that ectopic expression of *KNOXI* genes in grasses and eudicots produce very different phenotypes (Lincoln et al., 1994; Schneeberger et al., 1995). Most *KNOXI* gene sequences available in public databases are derived from the grasses; however, sequences from banana, asparagus and palms have recently become available (D'Hont et al., 2012; Nakayama et al., 2012).

Here an attempt is made to characterize *KNOXI* gene expression patterns during lobed and dissected leaf development in two genera of Araceae – *Amorphophallus* and *Anthurium* – known to employ the blastozone fractionation mechanism (see Chapter 2). A phylogenetic analysis of *KNOXI* gene sequences obtained from *Anthurium* and a broad sampling of vascular

land plants is performed to broaden knowledge of the evolution of the gene family, particularly with respect to monocots other than grasses.

Additionally, members of Araceae include staple crops such as taro, cocoyam and elephant foot yam (Lebot, 2009). Although leaf traits have been associated with yield size in aroids, currently, there is no breeding program working on aroid leaf quality (Lebot, 2009; Simin et al., 1995). A goal of this study is to focus attention on this neglected yet agriculturally important plant family to diversify not only the field of evo-devo, but also the molecular tools available for aroid crop improvement.

3.2 Materials and Methods

3.2.1 *Living and embedded plant material*

Species of *Anthurium* and *Amorphophallus* used in this study, which were germinated from seed or propagated by cuttings in the aroid greenhouse at the Missouri Botanical Garden are listed in Table 3.1. Seeds of *Zea mays* subsp. *mays* inbred line B73 were obtained from the USDA, ARS, NCRPIS and grown in the greenhouse at Washington University in St. Louis. Embedded shoot apices of the maize Kn1-N mutant used as a positive control for immunolocalizations were supplied by the Hake lab at the USDA-ARS, Plant Gene Expression Center, Albany, CA. Histological and *KNOXI* gene expression studies in *Amorphophallus* required a composite of species due to dormancy and lack of sufficient material for a thorough analysis of any one species.

Table 3.1 Species included in studies of *KNOX1* gene expression.

Genus/species	MBG Collection No.	Voucher No.	Provenance
<i>Amorphophallus bulbifer</i> BLUME	T. Croat 77292	(MO) 6649284	
<i>Amorphophallus excentricus</i> HETT.	2013-1470	(MO) 6647394	
<i>Amorphophallus muelleri</i>	Han. 97-008-1	(MO) 6647393	
<i>Anthurium clavigerum</i> POEPP. & ENDL.	T. Croat 84498	(MO) 6649275	Peru: Junin
<i>Anthurium lezamai</i> MATUDA	83636ex		Mexico: Chiapas
<i>Anthurium pentaphyllum</i> var. <i>bombacifolium</i> (SCHOTT) MADISON	T. Croat 84951, 2014-0003-5	(MO) 6649282	
<i>Anthurium podophyllum</i> KUNTH	2012-1643-3	(MO) 6647388	Mexico
<i>Anthurium polyschistum</i> R.E.SCHULT. & IDROBO	T. Croat 78265, 2013-2467-2	(MO) 6649283	Ecuador
<i>Anthurium sp. nov.</i>	2012-1437	(MO) 6647400	

3.2.2 Western Blot Analysis

Plant nuclear protein extractions from meristems and mature leaves were performed by freezing tissue in liquid nitrogen and grinding with a mortar and pestle to a fine powder. All extraction steps were done at 4°C using the CellLytic™ Plant Nuclei Isolation/Extraction Kit (Sigma-Aldrich). Quantitation of nuclear protein extracts using the Pierce™ BCA Protein Assay Kit (Life Technologies #23227) was undertaken to normalize input protein amounts for gel electrophoresis. 50 ug of protein per sample were loaded into a NuPAGE Novex Bis-Tris 4-12% gel with 10 uL of SeeBlue Plus2 Pre-Stained Standard ladder (Life Technologies #LC5925) and run in 1X MES SDS Running Buffer at 100 volts for 2 hours in a XCell SureLock Mini-Cell. Separated proteins were electro-blotted to a nitrocellulose membrane using the Trans-Blot Turbo Midi PVDF Transfer Pack (Bio-Rad #170-4157) at a medium

setting (7 minutes, 1.3A, 25 V). The membrane was then put in a block solution of 2.5 g dry milk in 100 mL of 1X TBS-T buffer for 1 hour. After blocking, incubation with anti-full-length *KN1* polyclonal antibody provided by the Hake lab at a concentration of 1:500 was done overnight with gentle agitation at 4°C. In the morning, the membrane was washed three times for 10 minutes in 1X TBS-T, changing to new TBS-T each time, then incubated with a secondary phosphatase-labeled (AP) affinity purified antibody to rabbit IgG (KPL #05-15-06) at a concentration of 1:2000 in 1X TBS-T with gentle agitation at room temperature for 2 hours. The membrane was then washed four times for 5 minutes each, with new TBS-T each time. AP signal was detected using the CDP-Star (Roche #11685627001) chemiluminescent substrate. Film was exposed for 7, 20, 30 and 42 minutes before developing.

3.2.3 Immunolocalization

Immunolocalizations were performed according to the Hake lab protocol, as described below.

Fixation: Shoot apices were dissected and immediately put into ~15mL freshly made FAA solution (50% EtOH, 10% 37% Formaldehyde, 5% glacial acetic acid, 0.5% Triton X-100, 1% DMSO, 33.5% dH₂O) pre-chilled on ice in scintillation tubes. Tubes were put under vacuum for 10-20 min on ice, increasing to 28 psi over 6 minutes, holding for ~5 min, then releasing to normal pressure over 6 min. FAA was changed and samples were left overnight in fresh FAA with slight agitation at 4°C. Dehydration: All steps were performed at 4°C, with alcohols pre-chilled. Tubes were left for 2 hours with slight agitation between each change. The following day, half the volume of FAA was replaced with 95% EtOH three times, then all remaining solution was replaced with 95% EtOH. Saffranin was added to a final concentration of 0.1% in 95% EtOH. All 95% EtOH was replaced with 100% EtOH and left overnight.

Paraplast infiltration, embedding and sectioning: All incubations were performed for two hours at room temperature with shaking. In the morning, 100% EtOH was replaced with fresh pre-chilled 100% EtOH and placed on the shaker at room temperature. 100% EtOH was replaced with histoclear:EtOH at a ratio of 1:3, then 1:1, then 3:1, then 100% histoclear, then fresh %100 histoclear and left overnight, shaking at room temperature. In the morning, histoclear was replaced with fresh 100% histoclear and left to incubate for 2 hours. Then $\frac{1}{4}$ volume of paraplast plus chips (McCormick #15159-464) were added to the tubes and incubated several more hours. At the end of the day more chips were added, then left overnight with shaking. In the morning more paraplast chips were added and tubes were placed at 42°C until chips completely dissolved, then $\frac{1}{2}$ the volume was poured off and replaced with 100% melted paraplast plus kept at 60°C, tubes were transferred to the 60°C oven for the remainder of the infiltration steps. At the end of the day, tubes were left in 100% melted paraplast plus. Paraplast was renewed every 8-10 hours, until it had been changed 5 times. Samples were embedded, then sectioned on a rotary microtome and mounted on ProbeOn Plus slides (Fisher #22-230-900) and placed on a slide warmer at 42°C overnight.

Immunolocalization: Slides were de-paraffinized in 100% histoclear for 20 minutes, changing out for new histoclear after 10 minutes. Sectioned tissues were then re-hydrated through an EtOH series as follows, incubating with shaking for 2 minutes between each change: 100% ETOH, 100% EtOH, 95% EtOH, 85% EtOH (45ml 95% EtOH + 5 ml 8.5% NaCl), 70% EtOH (37 ml 95% EtOH + 5 ml 8.5% NaCl + 8 ml dH₂O), 50% EtOH (26 ml 95% EtOH + 5 ml 8.5% NaCl + 19 ml dH₂O), 25% EtOH (13 ml 95% EtOH + 5 ml 8.5% NaCl + 32 ml dH₂O), 0.85% NaCl, 1X PBS, 1X PBS. A proteinase K digestion was necessary for 15 minutes at room temperature in 100 ug/mL Proteinase K (Sigma-Aldrich #P6556) in 1X PBS, followed by 3

washes in 1X PBS (2 minutes each). Slides were placed in blocking solution (0.1g BSA, 0.1g dry milk, 0.5 ml Triton X-100 in 50 ml 1X PBS) for 1 hour, then incubated with either a anti-full-length *KN1* antibody, or anti-C-terminus *KN1* antibody (antibody:block solution = 1:750 and 1:500, respectively) for four hours. Slides were then washed in block solution for 5 minutes, then 1X PBS three times for five minutes, then incubated with a secondary phosphatase-labeled (AP) affinity purified antibody to rabbit IgG (KPL #05-15-06) at a concentration of 1:1000 overnight at 4°C. The following day, slides were washed in 1X PBS three times for five minutes each, then incubated in AP detection buffer for five minutes, then incubated in a solution of 20 ul NBT/BCIP (Roche #11681451001) in 1ml AP detection buffer until signal was visible using a dissecting scope (4-7 hours). At that point, slides were washed in dH2O and slide cover slips were adhered using Aqua-Poly/Mount (Polysciences #18606).

3.2.4 Class I *KNOX* gene characterization in *Anthurium*

Primers for a class I *KNOX* gene were designed based on a 654 bp mRNA sequence named *AnthuriumKnat1BTTF09-T7*, provided by the Sinha lab at the University of California, Davis. Primers were designed to capture maximum sequence length in *Anthurium* based on the sequence provided. One forward primer was designed in the MEINOX domain just upstream of the Helix-Loop-Helix region (AnthMEINF), another forward primer was designed anchored in the Helix-Loop-Helix region (AnthHLHF), a reverse primer was designed anchored in the ELK domain (AnthELKR) and another reverse primer was designed anchored in the Homeodomain (AnthHDR) (Table 3.2) (Bharathan et al., 1999). Reverse primers in the ELK and Homeodomains were designed with residues found only in class I *KNOX* genes, to avoid amplification of class II *KNOX* genes. An initial PCR reaction using all four primer pairs with cDNA from the meristem in *Anthurium clavigerum* and *A. podophyllum* (94°C for 1 minute, 35

Table 3.2 Primer sequences for *Anthurium KNOXI* and reference genes

Primer name	Primer sequence	Gene/Genic region
AnthMEINF	ATGGCTCATCCTCAGTGCTC	MEINOX domain
AnthHLHF	AGCCTACATGGACTGCCAAA	MEINOX domain, Helix-Loop-Helix (HLH)
AnthELKR	TCAGGCCACTCAAGTATCCA	ELK domain
AnthHDR	CTTCTGCCTGGCTTCTTTTG	Homeodomain
AnthEF1F	CTGCAGCGTATGGACTTGG	<i>EF-1α</i>
AnthEF1R	AGCTCCATGTCATAGCACTCA	<i>EF-1α</i>
AnthLUGF	GGAAGCGGATAAGATGCTTG	<i>LUG</i>
AnthLUGR	GGAGCATCAATCGCTACTGG	<i>LUG</i>
AnthTUBF	GATGTCGTGCGCAAGGAG	<i>TUB</i>
AnthTUBR	GGGGAACACAGAGAAGGTCA	<i>TUB</i>
AnthGAPDHF	CCGTCAATGATCCCTTCATC	<i>GAPDH</i>
AnthGAPDHR	ACGACCTTCTTGGCACCAC	<i>GAPDH</i>
AnthH3F	AGAGGCCATGGACTTCCTCA	MEINOX domain, Amphipathic Helix (H3)
AnthLNK2R	GGTCAATCTCGGGAAGCTTAG	Linker 2
AnthLNK2F	GGCTCTGCACGTTTGTCTC	Linker 2
AnthELKR2	CAGGCCACTCAAGTATCCA	ELK domain

cycles of 94°C for 30 seconds, 54°C for 1 minute, 72°C for 2 minutes, final extension at 72°C for five minutes) yielded single bands for all primer pairs (Figure 3.1A). PCR products were loaded in a 1.2% TAE gel with 1X Gel Red (Biotium #41003) and run at 90 volts for 45 minutes. The band corresponding to primer pair AnthMEINF and AnthHDR in *A. clavigerum* was gel extracted using QIAquick Gel Extraction Kit (Qiagen #28704), then ligated using the pGEM-T Easy Vector System (Promega #A1360) (Figure 3.1A). Ligated products were transformed and plated onto agar medium with ampicillin and placed at 37°C for 15 hours to select for colonies with the insert. Eight colonies that were successfully transformed (white) were selected for colony pcr using primers T7 and SP6 (92°C for 2 minute, 35 cycles of 92°C for 45 seconds, 55°C for 45 seconds, 72°C for 1 minute, and a final extension at 72°C for 5 minutes). Sequencing of colony PCR products yielded seven unique sequences of class I *KNOX* genes for *A. clavigerum*, which could be divided into two distinct groups. One group contained a single sequence that was highly divergent from all the others, while the other group contained

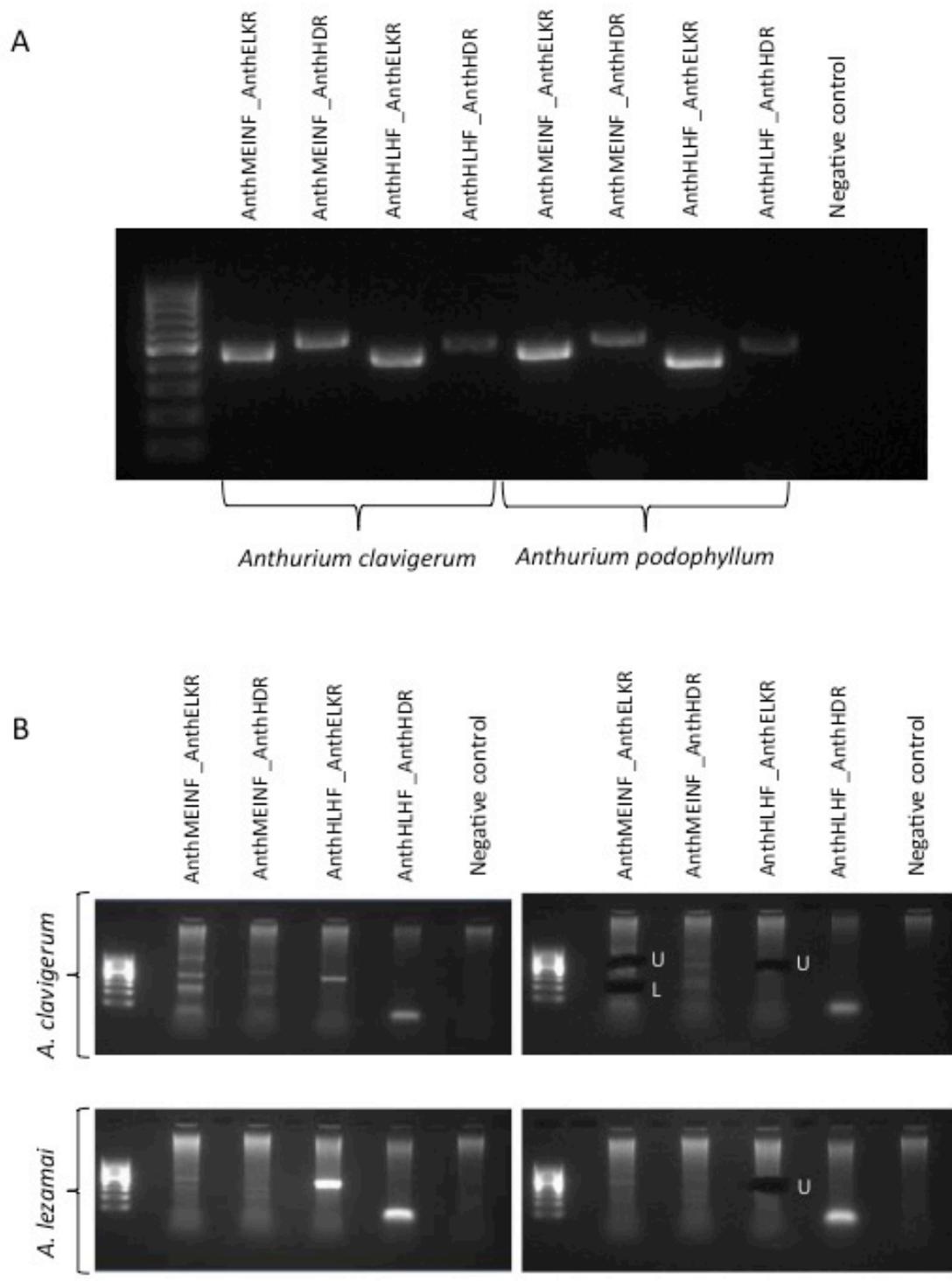


Figure 3.1 PCR products from combinations of *Anthurium KNOX1* primer pairs. A. Single band products in *A. clavigerum* and *A. podophyllum*, B. Upper and lower band products in *A. clavigerum* and *A. lezamai* that were gel extracted, cloned and sequenced. *U* upper, *L* lower.

sequences that differed by 1 to 3 SNPs and one sequence that contained a 5 bp indel in the homeodomain. Two sequences, one representing each group, were submitted to a BLAST search; top hits for both were homeobox *roughsheath1*-like protein coding sequences from banana and palms. For *Anthurium podophyllum*, PCR bands corresponding to primer pairs AnthMEINF and AnthELKR, and AnthHLHF and AnthELKR were gel extracted, cloned, with two colonies each selected for sequencing. Three unique sequences were obtained which differed by 1 to 2 SNPs.

Subsequent PCR reactions with either the AnthMEINF or AnthHLHF forward primers in combination with the AnthELKR reverse primer, using cDNA from meristems in *A. clavigerum* and *A. lezamai* yielded multiple bands (Figure 3.1B). Three bands were gel extracted, cloned, selected for eight colonies each, sequenced and submitted to BLAST searches (Figure 3.1B). Sequences from the lower band corresponding to AnthMEINF and AnthELKR in *A. clavigerum* were either bacterial or ribosomal and were discarded from further analyses. However, sequences from the upper bands yielded six additional unique sequences that differed by 1 to 3 SNPs and one sequence differed by 10 SNPs (*AcKNOX2*). For *A. lezamai*, 6 unique sequences were obtained that differed by up to 5 SNPs; however 4 were only partial sequences and were discarded from further analysis.

To determine orthologous and paralogous relationships among the sequenced copies of *KNOXI* genes in *Anthurium* and *KNOX* genes in the literature, sequences of class I and II *KNOX* genes across Tracheophytes were downloaded from Genbank (Table 3.3) and aligned in Geneious versions 5.6-6.0.3 (created by Biomatters) using the MAFFT alignment algorithm (Kato et al., 2002). One alignment (alignment A) included only three sequences of *Anthurium* (*AnthuriumKnat1BTTF09-T7*, and one sequence each representing the two major groups found

Table 3.3 Genbank accession numbers and taxonomic rank of species in *KNOX* phylogenetic analysis

Genbank No.	Taxon/Gene	Taxonomic ranking
	Anthurium clavigerum RS1-like 1	Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Alismatales; Araceae, Pothoideae, Anthurieae
AY072736.1	Anthurium clavigerum RS1-like 2 AnthuriumKnat1BTTF09-T7 Antirrhinum majus hirzina (hirz) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Lamiales; Plantaginaceae; Antirrhineae
KC854334.1	Aquilegia coerulea KN (KN) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Ranunculales; Ranunculaceae; Thalictrioideae
U14174.1	Arabidopsis thaliana clone KNAT1 knotted-like homeobox protein gene, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; malvids; Brassicales; Brassicaceae; Camelineae
U14175.1	Arabidopsis thaliana clone KNAT2 knotted-like homeobox protein gene, partial cds	
X92392.1	Arabidopsis thaliana mRNA for KNAT3 homeobox protein	
U32344.1	Arabidopsis thaliana Shootmeristemless (STM) mRNA, complete cds	
AB673047.1	Asparagus asparagoides AaKNAT1 mRNA for class I knotted1-like homeobox protein, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Asparagales; Asparagaceae; Asparagoideae
AB673048.1	Asparagus officinalis AoKNAT1 mRNA for class I knotted1-like homeobox protein, complete cds	
XM_010232284.1	Brachypodium distachyon homeobox protein KNOX3 (LOC100843507), mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP clade; Pooideae; Brachypodieae
GQ120449.1	Brassica oleracea BREVIPEDICELLUS gene, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; malvids; Brassicales; Brassicaceae; Brassiceae
DQ630764.1	Cardamine hirsuta BREVIPEDICELLUS (BP) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; malvids; Brassicales; Brassicaceae; Cardamineae
DQ512732.1	Cardamine hirsuta shoot meristemless (STM) mRNA, complete cds	
AB043954.1	Ceratopteris richardii mRNA for CRKNOX1, complete cds	
AB043956.1	Ceratopteris richardii mRNA for CRKNOX2, complete cds	Tracheophyta; Moniliformopses; Polypodiidae; Polypodiales; Pteridaceae
AB043957.1	Ceratopteris richardii mRNA for	

DQ317421.1	CRKNOX3, complete cds Chasmanthium latifolium KNOTTED1 homeodomain protein (KN1) mRNA, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACMAD clade; Panicoideae; Chasmanthieae
AB753769.1	Chelidonium majus subsp. asiaticum mRNA for KNAT1 protein, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Ranunculales; Papaveraceae; Papaveroideae
XM_004498877.1	Cicer arietinum homeobox protein knotted-1-like 2-like (LOC101490927), mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Cicereae
GU937114.1	Cocos nucifera KNOTTED-like homeobox protein 1 (KNOX1) mRNA, complete cds	Tracheophyta;Spermatophyta; Magnoliophyta; Liliopsida; Areaceae; Arecoideae; Cocoseae; Attaleinae
EU331440.1	Corytoplectus speciosus class 1 KNOX protein mRNA, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Lamiales; Gesneriaceae; Gesnerioideae; Gesnerieae; Columneinae
JQ799053.1	Cuscuta pentagona KNOTTED-like 1-3a homeodomain protein (Knat1-3a) mRNA, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Solanales; Convolvulaceae; Cuscutae
AY608889.2	Dendrobium nobile class 1 knox mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Asparagales; Orchidaceae; Epidendroideae; Dendrobiinae
AB514533.1	Diospyros kaki Dk672 mRNA for Knotted1-like homeobox protein, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; Ericales; Ebenaceae
DQ890420.1	Elaeis guineensis class I KNOX-like 1 protein (KNOX1) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Areaceae; Arecoideae; Cocoseae; Elaeidinae
XM_010937904.1	Elaeis guineensis homeotic protein knotted-1-like (LOC105055893), mRNA	
XM_010945104.1	Elaeis guineensis homeotic protein knotted-1-like (LOC105061139), mRNA	
DQ133604.1	Eschscholzia californica class 1 Knotted 1-like protein (Knat1) mRNA, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Ranunculales; Papaveraceae; Eschscholzioidae
HQ337627.1	Eschscholzia californica subsp. californica KNAT1 protein (KNAT1) mRNA, complete cds	
XM_010061531.1	Eucalyptus grandis homeobox protein knotted-1-like 2 (LOC104447808), transcript variant X2, mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; malvids; Myrtales; Myrtaceae

EF636205.1	Euphorbia esula transcription factor STM1 mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Malpighiales; Euphorbiaceae; Euphorbioideae; Euphorbieae
EF636206.1	Euphorbia esula transcription factor STM4 mRNA, complete cds	
HM107002.1	Glycine max homeobox protein knotted-1-like 2-like (LOC100807602), mRNA >gi 302135384 gb HM107002.1 Glycine max KNOX-like DNA-binding protein mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae
547796 AY096803.1	Glycine max Sbh1 protein (H1), mRNA Helianthus annuus knotted-1-like protein 2 (kn2) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; campanulids; Asterales; Asteraceae; Asteroideae; Heliantheae alliance; Heliantheae
AF544045.1	Hordeum vulgare knotted 1 (kn1) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP clade; Pooideae; Triticeae
AF022390.1	Hordeum vulgare knotted class 1 homeodomain protein (k) mRNA, complete cds	
AB283028.1	Ipomoea batatas Ibkn2 mRNA for class-I knotted1-like homeobox protein IBKN2, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Solanales; Convolvulaceae; Ipomoeae
AB283029.1	Ipomoea batatas Ibkn3 mRNA for class-I knotted1-like homeobox protein IBKN3, complete cds	
DQ317423.1	Leersia virginica KNOTTED1-like homeodomain protein mRNA, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP clade; Ehrhartoideae; Oryzaceae
AY790247.1	Lotus corniculatus var. japonicus homeobox knotted-1-like protein KNOX2 (KN2) mRNA, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Loteae
AF000141.1	Lycopersicon esculentum class I knotted-like homeodomain protein (LeT6) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Solanales; Solanaceae; Solanoideae; Solaneae; Solanum
AF000142.1	Lycopersicon esculentum class II knotted-like homeodomain protein (LeT12) mRNA, complete cds	
U76407.1	Lycopersicon esculentum knotted 2 protein (TKn2) mRNA, complete cds	
Z71980.1	Malus domestica mRNA for knotted1-like homeobox protein	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Rosales; Rosaceae; Maloideae; Maleae

XM_008349368.1	Malus x domestica homeobox protein knotted-1-like 1 (LOC103410692), mRNA	
XM_009413056.1	Musa acuminata subsp. malaccensis homeobox protein knotted-1-like 6 (LOC103993104), mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Zingiberales; Musaceae
XM_009409420.1	Musa acuminata subsp. malaccensis homeobox protein rough sheath 1-like (LOC103990327), transcript variant X2, mRNA	
AB004797.1	Nicotiana tabacum NTH23 mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Solanales; Solanaceae; Nicotianoideae; Nicotianeae
FJ940208.1	Oryza sativa Japonica Group clone KCB937E04 homeobox protein mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP clade; Ehrhartoideae; Oryzeae
D16507.1	Oryza sativa Japonica Group OSH1 mRNA for homeobox protein, complete cds	
AB028884.1	Oryza sativa mRNA for knotted1-type homeobox protein OSH43, complete cds	
AF050180.1	Oryza sativa strain Indica IR58 KNOX class homeodomain protein (Oskn2) mRNA, complete cds	
GQ409544.1	Petunia x hybrida KNOTTED1-like protein mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Solanales; Solanaceae; Petunioideae
DQ317424.1	Pharus lappulaceus KNOTTED1-like homeodomain protein mRNA, partial cds	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; Pharoideae; Phareae
XM_007160904.1	Phaseolus vulgaris hypothetical protein (PHAVU_001G032200g) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae
XM_008800700.1	Phoenix dactylifera homeobox protein rough sheath 1-like (LOC103713687), mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Areaceae; Coryphoideae; Phoeniceae
AF483277.1	Picea abies KNOTTED1-like homeodomain protein 2 (HBK2) mRNA, partial cds	Tracheophyta; Spermatophyta; Pinidae; Pinales; Pinaceae
AF063307.1	Pisum sativum Knox class 1 protein (Hop1) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Fabeae
AF080105.1	Pisum sativum knotted I class homeodomain protein (PsKn2) mRNA, partial cds	

KJ162335.1	Prunus mume knotted1-like homeobox transcription factor (KNAT2) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Rosales; Rosaceae; Maloideae; Amygdaleae
GQ281775.1	Prunus persica class I KNOX homeobox transcription factor STM-like 2 mRNA, complete cds	
XM_009363161.1	Pyrus x bretschneideri homeobox protein knotted-1-like 2 (LOC103951709), mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Rosales; Rosaceae; Maloideae; Maleae
AB971253.1	Rorippa aquatica RaSTM mRNA for class I knotted1-like homeobox protein, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; malvids; Brassicales; Brassicaceae; Cardamineae
AY667449.1	Selaginella kraussiana KNOTTED1-like protein (KNOX1) mRNA, complete cds	Tracheophyta; Lycopodiidae; Selaginellales; Selaginellaceae
AY667450.1	Selaginella kraussiana KNOTTED1-like protein (KNOX2) mRNA, complete cds	
AY667451.1	Selaginella kraussiana KNOTTED1-like protein (KNOX3) mRNA, complete cds	
XM_011076017.1	Sesamum indicum homeotic protein knotted-1 (LOC105159076), mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Lamiales; Pedaliaceae
NM_001247012.2	Solanum lycopersicum class I knotted-like homeodomain protein (T6), mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Solanales; Solanaceae; Solanoideae; Solaneae; Solanum
U65648.1	Solanum tuberosum homeodomain protein POTH1 mRNA, complete cds Solanum tuberosum homeotic protein knotted-1-like (LOC102601860), transcript variant X1, mRNA	
XM_007018337.1	Theobroma cacao KNOTTED-like from (TCM_034627) mRNA, complete cds	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; malvids; Malvales; Malvaceae; Byttnerioideae
XM_007026122.1	Theobroma cacao KNOX/ELK homeobox transcription factor isoform 1 (TCM_030302) mRNA, complete cds	
XM_002285485.3	Vitis vinifera homeobox protein knotted-1-like 2 (LOC100255834), mRNA	Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; Vitales; Vitaceae
X61308.1	Z.mays Knotted-1 (Kn-1) gene	Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACMAD clade; Panicoideae; Andropogoneae
NM_001156179.1	Zea mays homeobox protein rough sheath 1 (gnarley1), mRNA	
AF457118.1	Zea mays knotted1-like homeodomain protein liguleless4a (lg4a) mRNA,	

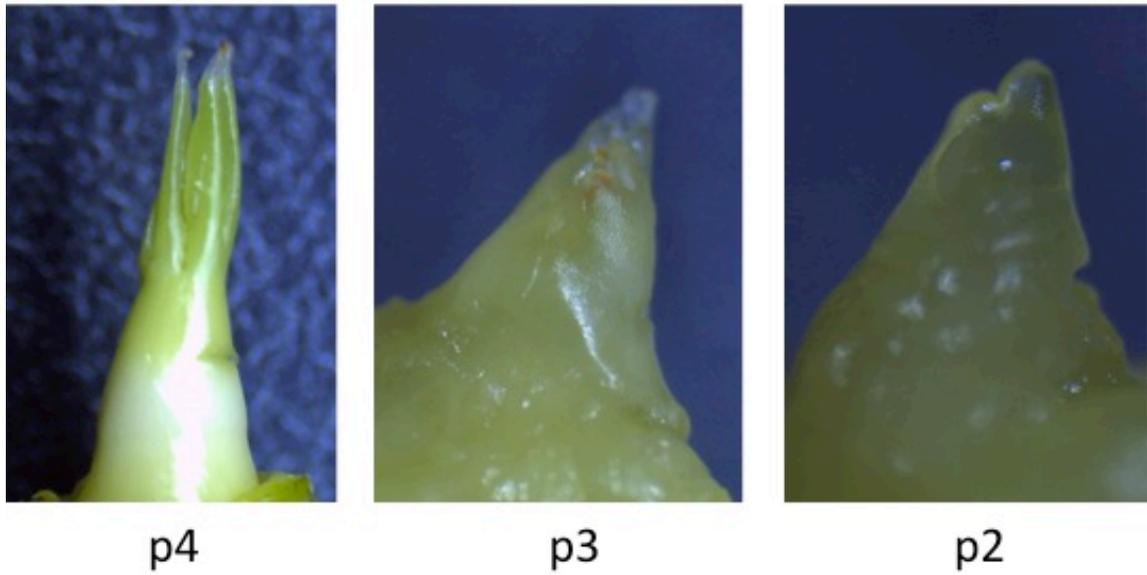
	complete cds
AF457119.1	Zea mays knotted1-like homeodomain protein liguleless4b (lg4b) mRNA, complete cds
NM_001112038.1	Zea mays liguleless3 (lg3), mRNA
L44133.1	Zea mays RS1 mRNA, complete cds

in *A. clavigerum*), which consisted of 670 bp spanning the AnthMEINF to AnthHDR region. A separate alignment (alignment B) included all sequences of *Anthurium* obtained in this study (AnthuriumKnat1BTTF09-T7, 13 from *A. clavigerum*, 3 from *A. podophyllum* and 2 from *A. lezamai*), which consisted of 579 bp spanning the AnthHLHF to AnthELKR region. A maximum likelihood analysis was performed for both alignments in RAxML according to Henriquez et al. (2014).

3.2.5 RT-PCR

Due to the conflicting results obtained from immunolocalizations (see Results section below), an RT-PCR assay was designed to determine whether class I *KNOX* genes are expressed during dissected leaf development in *Anthurium*. For this, the dissected-leaved species *Anthurium clavigerum* was chosen because it had consistently better results than *A. polychistum* for RNA extractions (data not shown). Additionally, a simple-leaved species (*Anthurium lezamai*) was included for comparison. Tissue dissections of leaves at various developmental stages (mature, p4, p3, p2 and meristem) from both *A. clavigerum* and *A. lezamai* were immediately frozen in liquid nitrogen and ground by mortar and pestle to a fine powder. Because *KNOXI* genes are expressed at the junction of the leaf base with the meristem (Goliber et al., 1999; Johnston et al., 2014), dissections were careful to avoid the leaf base (Figure 3.2). Dissections were also careful to avoid inflorescence primordia in *A. lezamai*, which was in sympodial growth mode (Figure 3.2B). Three biological replicates were collected for each

A. *Anthurium clavigerum*



B. *Anthurium lezamai*

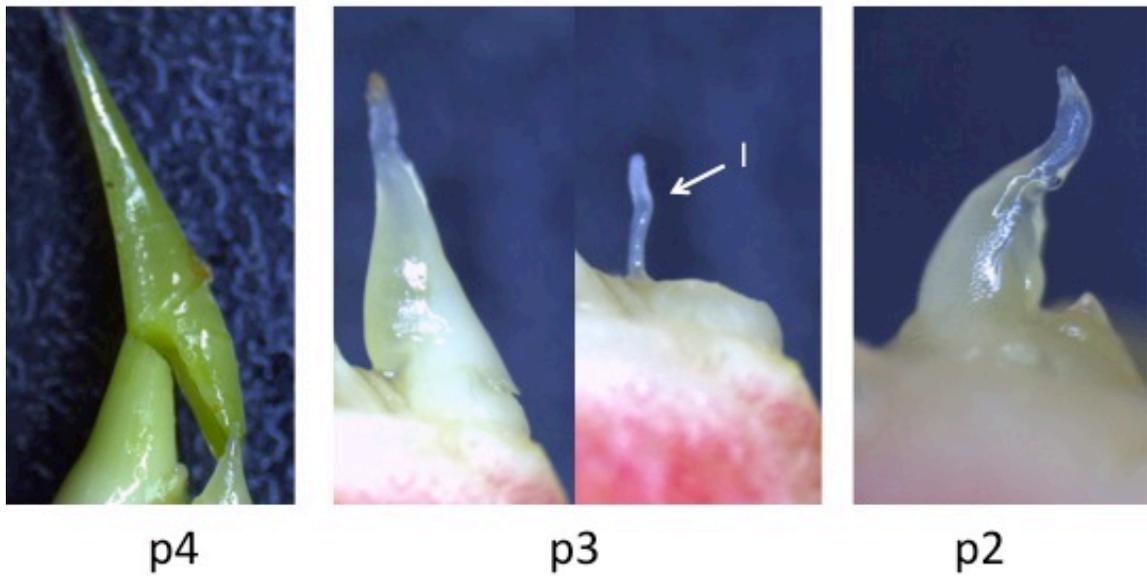


Figure 3.2 Dissections of developing leaf stages for RNA extractions for RT-PCR. A. *Anthurium clavigerum*, B. *Anthurium lezamai*. I inflorescence primordium.

species at each developmental stage. RNA extractions of mature and p4 leaves, and meristems were performed using the PureLink RNA Mini Kit (Life Technologies #12183018A), while RNA from p3 and p2 leaf stages was extracted using the Arcturus PicoPure RNA Isolation Kit (Life Technologies #KIT0204). A DNase I digestion was included in both protocols using PureLink DNase (Life Technologies #12185-010) and RNase-Free DNase (Qiagen #79254), respectively. RNA quality and concentration was assessed using Nanodrop 260/280 and 260/230 ratios and Agilent 2100 Bioanalyzer assays (Table 3.4). RNA was stored at -70°C for downstream application. cDNA synthesis was performed using SuperScript VILO MasterMix (Life Technologies #100012386). PCR conditions were as follows: 25°C for 10 minutes, 42°C for 90 minutes, 85°C for 5 minutes. cDNA quality and concentration were determined using Nanodrop 260/280 and 260/230 ratios. Input RNA quantities, cDNA quality and concentrations are listed in Table 3.4.

Candidate reference genes putatively expressed throughout all stages of leaf development were chosen from the literature to be tested as positive controls (Czechowski et al., 2005; Manoli et al., 2012; Zhang et al., 2013). These included *LUG*, *TUB*, *GAPDH* and *EF-1 α* . Primers for *Anthurium* were designed using Primer3 (Rozen and Skaletsky, 1998) based on monocot gene sequences downloaded from Genbank and aligned in Geneious using the MAFFT alignment algorithm. Primers for *EF-1 α* (AnthEF1F, AnthEF1R) and *LUG* (AnthLUGF, AnthLUGR) were designed to produce PCR products of ~250 base pairs or less, but were not designed to span an intron for initial testing. Primers for *TUB* (AnthTUBF, AnthTUBR) and *GAPDH* (AnthGAPDHF, AnthGAPDHR) were designed to produce PCR products of ~250 base pairs or less to maximize PCR efficiency (Real-time PCR handbook, Life Technologies), and targeted exons that spanned an intron to avoid amplifying possible genomic DNA contamination.

Table 3.4 RNA and cDNA quality and quantities used for RT-PCR.

<i>Anthurium</i>	Tissue	RIN	ng/uL	ng RNA cDNA	cDNA 260/280	cDNA 260/230	cDNA ng/uL	dil. <i>KNOX</i>	dil. <i>GAPDH</i>
<i>clavigerum</i>	mature leaf	8.5	63.43	317.12	1.82	2.19	1851.6	1/25X	1/25X
<i>clavigerum</i>	mature leaf	9.2	54.68	273.4	1.82	2.18	1822.8	1/25X	1/25X
<i>clavigerum</i>	mature leaf	4.1	41.78	208.9	1.81	2.11	1771.5	1/25X	1/25X
<i>clavigerum</i>	p4	5.9	64.98	324.9	1.64	1.95	1494	1/10X	1/25X
<i>clavigerum</i>	p4	5.9	52.72	263.6	1.67	2.02	1665.3	1/10X	1/25X
<i>clavigerum</i>	p4	6.9	99.07	297.21	1.81	2.2	1763.3	1/10X	1/25X
<i>clavigerum</i>	p3	7.2	4.04	32.32	1.8	2.18	2034.7	1X	1/25X
<i>clavigerum</i>	p3	7.4	0.57	5.71	1.8	2.07	2203.3	1X	1/25X
<i>clavigerum</i>	p3	7.2	0.69	6.88	1.8	2.17	2229.4	1X	1/25X
<i>clavigerum</i>	p2	6.3	0.59	5.9	1.74	2.13	2419.3	1X	1/25X
<i>clavigerum</i>	p2	7.1	0.14	1.42	1.79	2.17	2333.5	1X	1/25X
<i>clavigerum</i>	p2	7.1	0.22	2.16	1.78	2.14	2274.4	1X	1/25X
<i>clavigerum</i>	SAM	7.8	1613.8	12910.4	1.81	2.16	1833.3	1/25X	1/25X
<i>clavigerum</i>	SAM	7	313.05	626.1	1.71	2.02	1598.2	1/25X	1/25X
<i>clavigerum</i>	SAM	6.9	336.97	673.94	1.75	2.09	1709.2	1/25X	1/25X
<i>lezamai</i>	mature leaf	5.6	113.59	340.77	1.81	2.19	1893.2	1/25X	1/25X
<i>lezamai</i>	mature leaf	5.5	31	186	1.5	1.76	1312.2	1/25X	1/25X
<i>lezamai</i>	mature leaf	5.5	31	186	1.77	2.13	1614.5	1/25X	1/25X
<i>lezamai</i>	p4	6.2	224.47	448.94	1.82	2.17	1785.7	1/10X	1/25X
<i>lezamai</i>	p4	6.5	306.29	612.58	1.82	2.21	1762.9	1/10X	1/25X
<i>lezamai</i>	p4	2.8	54.49	544.9	1.58	1.88	1556	1/10X	1/25X
<i>lezamai</i>	p3	5.8	6.4	50.87	1.81	2.17	1880	1X	1/25X
<i>lezamai</i>	p3	5.9	7.05	56.39	1.81	2.2	1987.4	1X	1/25X
<i>lezamai</i>	p3	5.2	3.91	31.27	1.81	2.18	1789.7	1X	1/25X
<i>lezamai</i>	p2	7.4	0.28	2.81	1.8	2.14	2250.2	1X	1/25X
<i>lezamai</i>	p2	6.8	0.32	3.24	1.79	2.2	2451.4	1X	1/25X
<i>lezamai</i>	p2	6.5	1.03	8.25	1.8	2.18	1862.6	1X	1/25X
<i>lezamai</i>	SAM	6.4	360.91	721.82	1.61	1.88	1541.2	1/25X	1/25X
<i>lezamai</i>	SAM	6.1	81.83	327.32	1.73	2.07	1628.2	1/25X	1/25X
<i>lezamai</i>	SAM	6.6	294.57	589.14	1.66	1.99	1601.7	1/25X	1/25X

Gradient PCR reactions were used to determine optimal annealing temperatures for each primer pair (94°C for 1 minute, 35 cycles of 94°C for 30 seconds, 50-60°C for 45 seconds, 72°C for 1

minutes, and a final extension at 72°C for five minutes), with 55°C being the consensus temperature across primer pairs. Primer sequences for reference genes are listed in Table 3.2.

To detect the presence or absence of *KNOXI* gene transcripts in developing leaves of *Anthurium clavigerum* and *A. lezamai*, primers were designed by the same criteria used for reference genes *TUB* and *GAPDH* (above). Sequences of *KNOXI* genes from *A. clavigerum*, *A. pentaphyllum* and *A. podophyllum*, including one sequence from each of two highly divergent copies of *KNOXI* genes that fell into different clades from *A. clavigerum* (Results section below) were aligned with the mRNA sequence of *KN1* in maize as a positional reference. The forward primer targeting *AcKNOXI* homologs (Results section below) (AnthH3F) is anchored in the Amphipathic Helix (H3) region of the MEINOX domain, and the reverse primer (AnthLNK2R) is anchored in the Linker 2 region. The forward primer targeting *AcKNOX2,3,4* homologs (AnthLNK2F) is anchored in a region just after the Amphipathic Helix (H3) in the Linker 2 region and the reverse primer (AnthELKR2) is anchored in the ELK domain (Bharathan, et al., 1999). In *Zea mays* the regions corresponding to both primer pairs are interrupted by a 5,311 bp intron, observed in an alignment of the *KN1* mRNA transcript and a sequence of the entire gene. It is not known how the length and position of this intron in maize compares with that in *Anthurium*. All primer sequences are listed in Table 3.2.

Each RT-PCR run consisted of three separate simultaneous reactions. For all five leaf developmental stages in two species (*A. clavigerum* and *A. lezamai*) one reaction used the *AcKNOXI* primers, another reaction used the *AcKNOX2,3,4* primers, and a final reaction used the reference gene (*GAPDH*) primers as a positive control. A negative control was used for each reaction. A 20 ul PCR master mix consisted of 2 ul GoTaq DNA Polymerase Green Reaction Buffer, 1.5 ul 25mM MgCl₂, 2 ul 2.5 mM each dNTPs, 2 ul of 2 uM forward primer, 2 ul of 2

uM reverse primer, 0.25 ul high definition formamide, 0.12 ul GoTaq DNA polymerase. PCR conditions were as follows: 94°C for 1 minute, 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minutes, and a final extension at 72°C for five minutes. Four technical replicates were performed for each of three biological replicates. RT-PCR products were loaded into 1X TBE with 1% Gel Red or EtBr and run for 2 hours at 48 volts, or 30 minutes at 100 volts. After initial testing, mature leaf and meristem samples were diluted to 1/25X and p4 samples were diluted to 1/10X for both *KNOX* reactions, and all samples were diluted to 1/25X for *GAPDH* reactions to normalize signal for visualization.

3.3 Results

3.3.1 *Western Blot*

The size of the full-length *KN1* protein in maize is roughly 40 kDa (Reiser et al., 2000). In the meristem nuclear protein extraction from maize inbred line B73, a single band between 38 and 49 kDa was detected using the full-length *KN1* antibody. This band was not present in the mature leaf nuclear protein extraction from maize inbred line B73; thus, it was concluded that due to the size and expression pattern of the protein, this band corresponds to *KN1* proteins (Figure 3.3). This expression pattern was similar for *Anthurium pentaphyllum*, *Anthurium lezamai* and *Amorphophallus muelleri*, although an extra band of slightly smaller size was detected in the meristem protein extraction of *Anthurium lezamai*. In *Anthurium polyschistum* double bands were also detected in the meristem and a single band of intermediate size between the two bands in the meristem was detected in the mature leaf. In *Anthurium clavigerum* double bands were detected in the meristem and double bands of similar size were detected in the

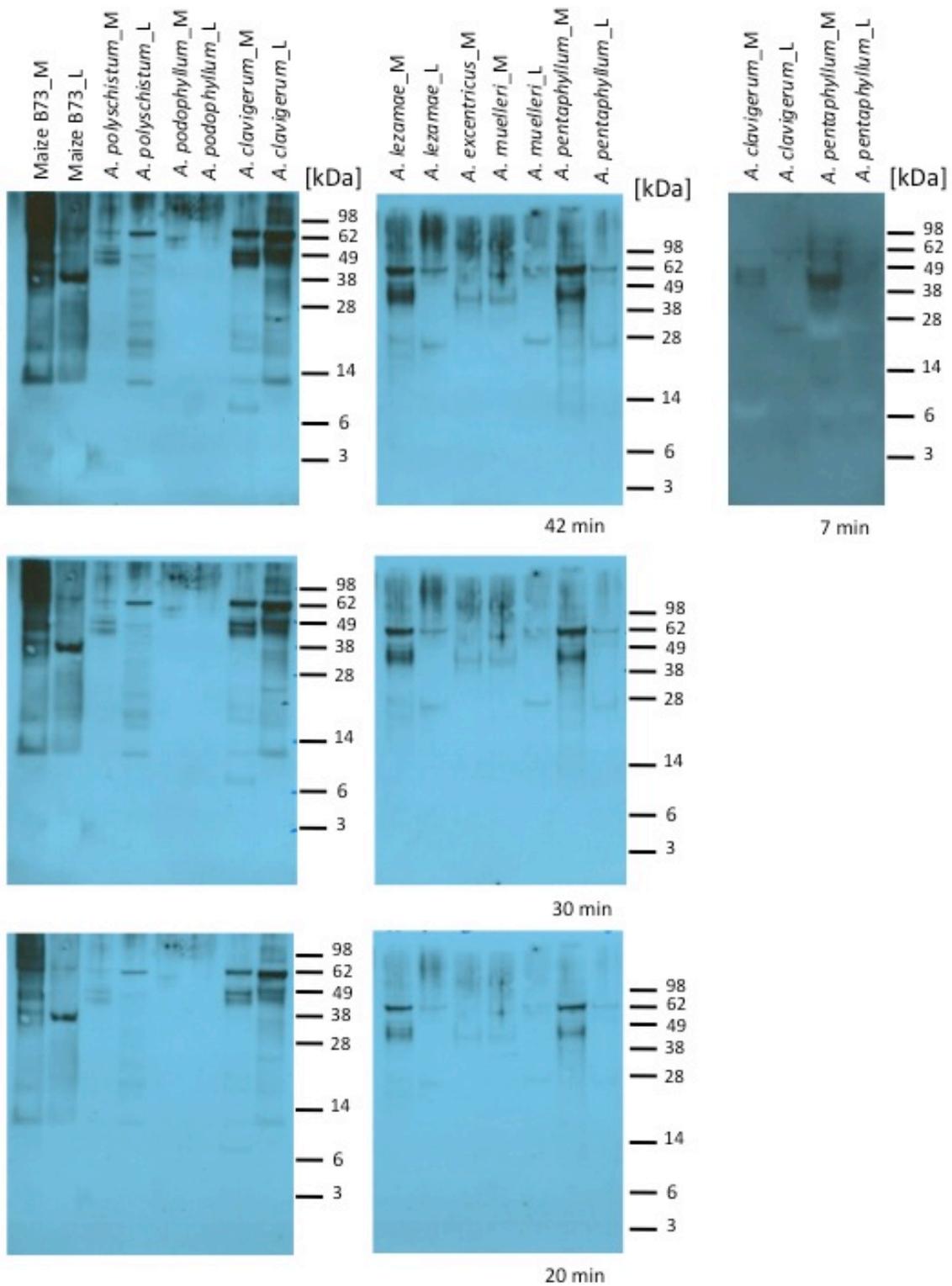


Figure 3.3 Western blot analysis using full-length anti-*KNI* antibody. *L* denotes mature leaf nuclear protein extractions, *M* denotes meristem nuclear protein extractions.

mature leaf (Figure 3.3). This pattern in *Anthurium clavigerum* is in conflict with a previous western blot analysis, in which no bands corresponding to *KNOXI* proteins were detected in the mature leaf (Figure 3.3). Bands of different sizes also appeared; the identity of the corresponding proteins is not known.

3.3.2 Immunolocalization

KNOXI protein expression was detected as expected using the anti-full-length *KNI* antibody in the maize controls (Figure 3.4). *KNI* protein expression in wild type B73 was detected in the meristem and was absent from developing leaves (Figure 3.4 D,E). Negative control immunolocalizations that were performed in B73 without the anti-full-length *KNI* antibody detected no *KNI* protein expression (Figure 3.4 F,G). The maize Kn1-N mutant used as a positive control showed *KNI* protein expression in the meristem and mature vascular tissue in the leaves (Figure 3.4 A,B,C). In contrast, *KNOXI* protein expression detected using the anti-full-length *KNI* antibody in *Anthurium* produced patterns contrary to expectation (Figure 3.5). In species of *Anthurium*, *KNOXI* protein expression was only detected in meristematic tissue of the leaf and inflorescence, while no expression was detected anywhere at any stage of leaflet or lobe development in the foliage leaf (Figure 3.5). Furthermore, the signal detected in the vegetative meristem was not distinct enough in certain cases, particularly in *Anthurium polyschistum* (Figure 3.5C), to be able to distinguish specific cell expression patterns. It is interesting to note that cells containing secondary compounds in the prophyll and *Vorläuferspitze* were darkly stained throughout; however, since staining was not nuclear it does not reflect *KNOXI* protein expression. All attempts at immunolocalization using the full-length *KNI* antibody in species of *Amorphophallus* were unsuccessful (data not shown).

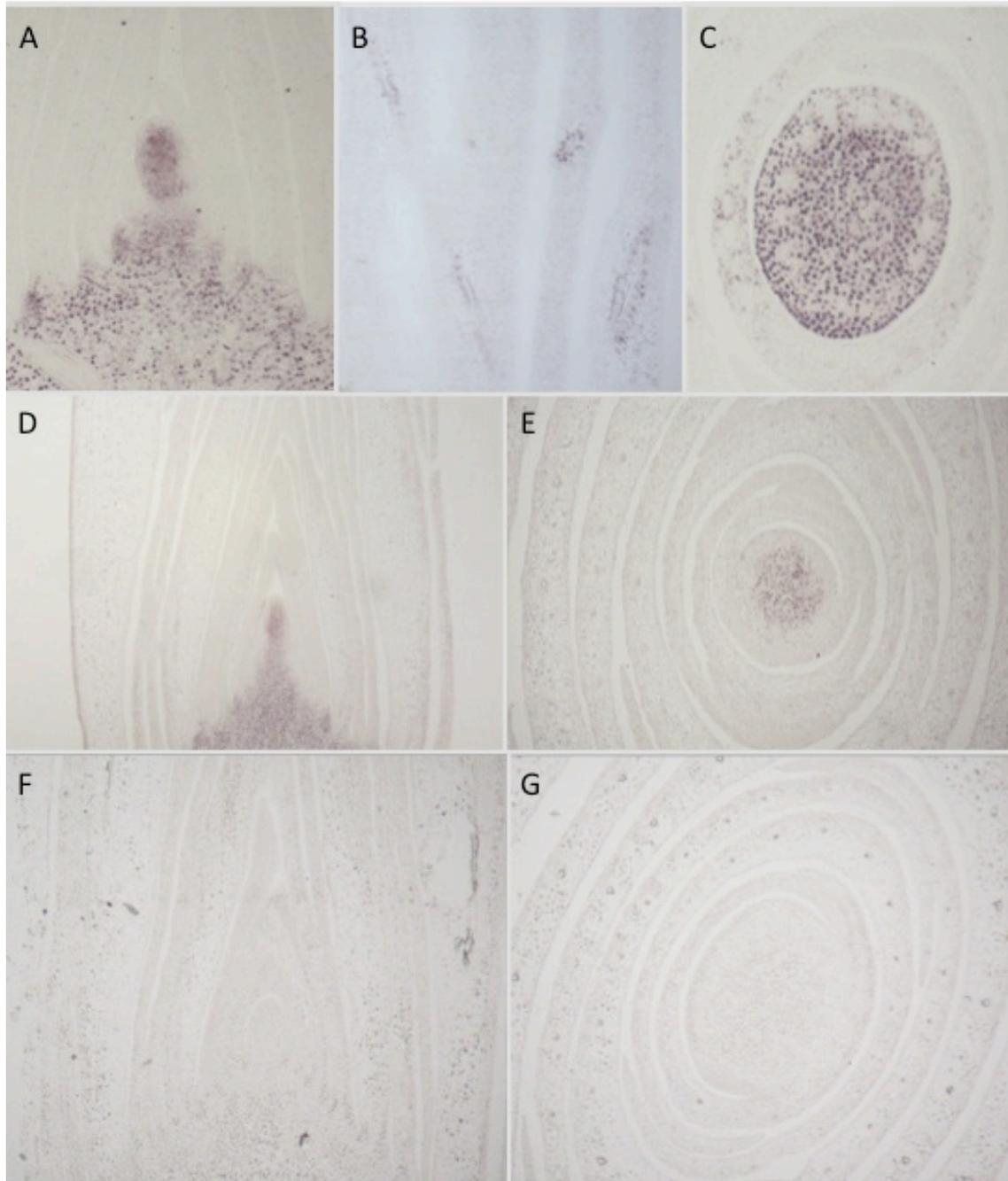


Figure 3.4 Maize positive and negative controls for immunolocalization using the full-length anti-*KNI* antibody. A-C. maize Kn1-N mutant used as a positive control. A. longisection of shoot apical meristem (SAM), B. longisection of leaves showing *KNI* protein expression in mature vascular tissue, C. cross-section showing *KNI* protein expression in mature vascular tissue, D,E. wild type maize B73, D. longisection showing *KNI* protein expression in SAM, E. cross-section showing *KNI* protein expression in SAM, F,G. wild type maize B73 negative controls, F. longisection of SAM showing no *KNI* expression, G. cross-section of SAM showing no *KNI* expression.

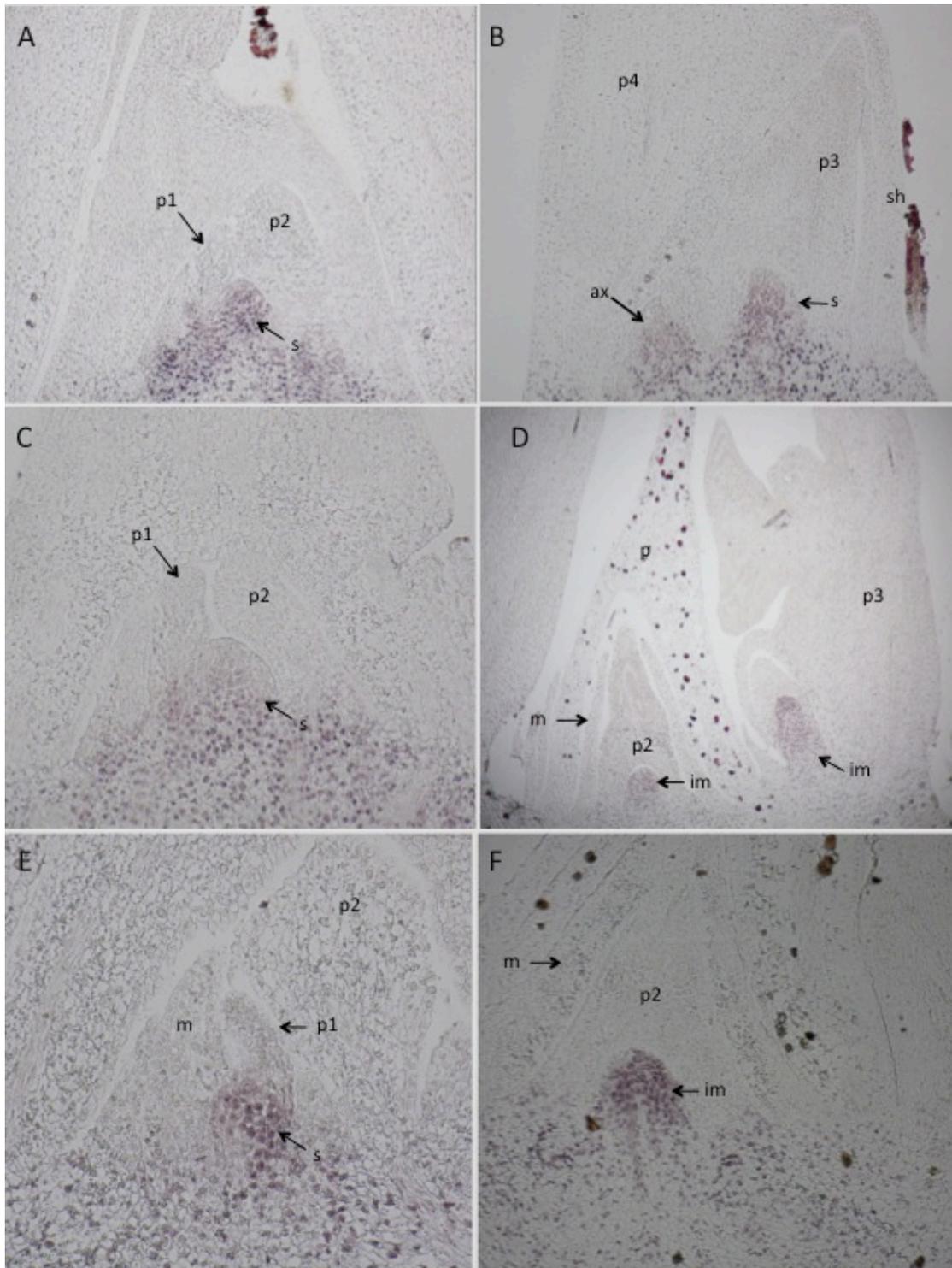


Figure 3.5 Immunolocalization using full-length anti-KNI antibody in *Anthurium*. A. *Anthurium clavigerum*, B. *A. polyschistum*, C. *A. polyschistum*, D. *A. sp. nov.*, E. *A. sp. nov.*, F. *A. podophyllum*. p1, p2, p3 foliage leaf, s shoot apical meristem, ax axillary meristem, sh sheathing leaf base, m sylleptic mesophyll, p sylleptic prophyll, im inflorescence meristem.

In contrast, *KNOXI* protein expression detected using the anti-C-terminus *KNI* antibody in *Anthurium* and *Amorphophallus* produced patterns similar to those detected in dissected leaf development across angiosperms (Figure 3.6) (Bharathan et al., 2002). Moreover, signal quality and intensity were similar to those seen in Bharathan et al. (2002). In *Anthurium polychistum*, *KNOXI* protein expression is detected at the location of incipient leaflet formation in p1, and is excluded from p0 on the flank of the meristem (Figure 3.6A). Serial sections deeper into the meristem, beyond p0, reveal *KNOXI* protein expression as expected (Figure 3.6B). Developing vasculature in p1 and p2 also express *KNOXI* proteins (Figure 3.6C, D). The region of p2 corresponding to sheath and petiole surrounding p1 also shows *KNOXI* protein expression (Figure 3.6A-D). In p3, the region corresponding to sheath and petiole surrounding p2 also shows *KNOXI* protein expression except for a band that is full of secondary compounds in closest proximity to p2. In *Amorphophallus bulbifer*, *KNOXI* protein expression is found in the shoot apical meristem, in developing lobes at the apex of the leaf, in the developing leaf base, in ground tissue of the tuber, and unexpectedly in developing cataphylls (a general term for prophylls and mesophylls) (Figure 3.6E-H). *KNOXI* protein expression is absent from cataphylls (proleptic mesophylls) later in development, in a band corresponding to the developing petiole between the leaf base and apex, and from a band in between the ground tissue of the tuber and the leaf base (Figure 3.6E).

3.3.3 Characterization of class I KNOX genes in *Anthurium*

According to the results of the phylogenetic analyses performed on both alignments, at least three copies of *KNOXI* genes were present in the common ancestor of monocots and eudicots. These include the *STM* clade (green), the *KNAT2/liguleless* clade (purple) and a *KNAT1/(KNI/RSI)* clade (blue, gold, magenta) (Figure 3.7). In both phylogenies the

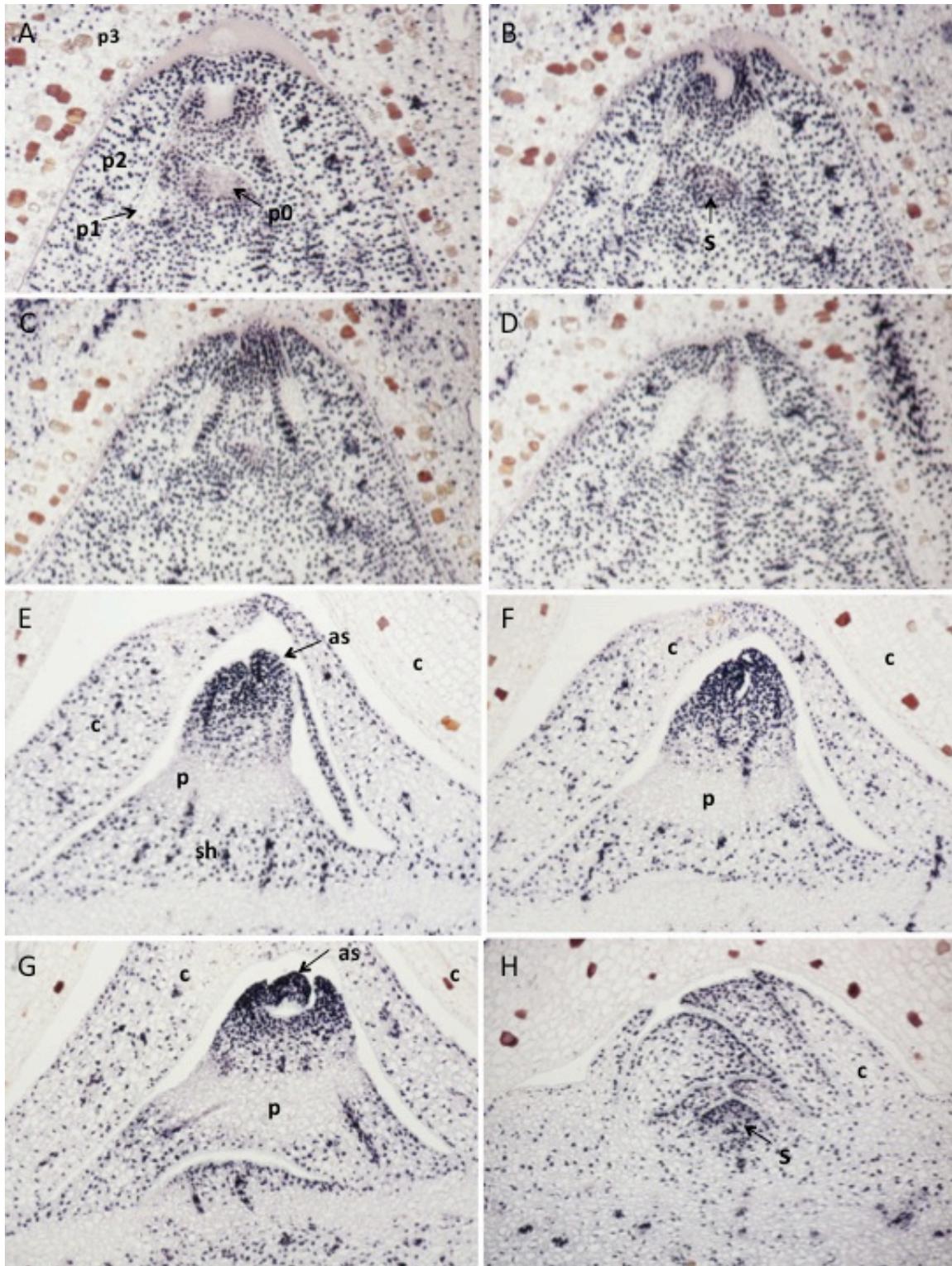


Figure 3.6. Immunolocalization with C-terminus anti-KNI antibody. A-D. *Anthurium polyschistum*, E-H. *Amorphophallus bulbifer*. *as* anterior segment, *p* petiole, *p0* site of next leaf inception, *p1*, *p2*, *p3* foliage leaf, *c* cataphyll, *s* shoot apical meristem, *sh* sheathing leaf base.

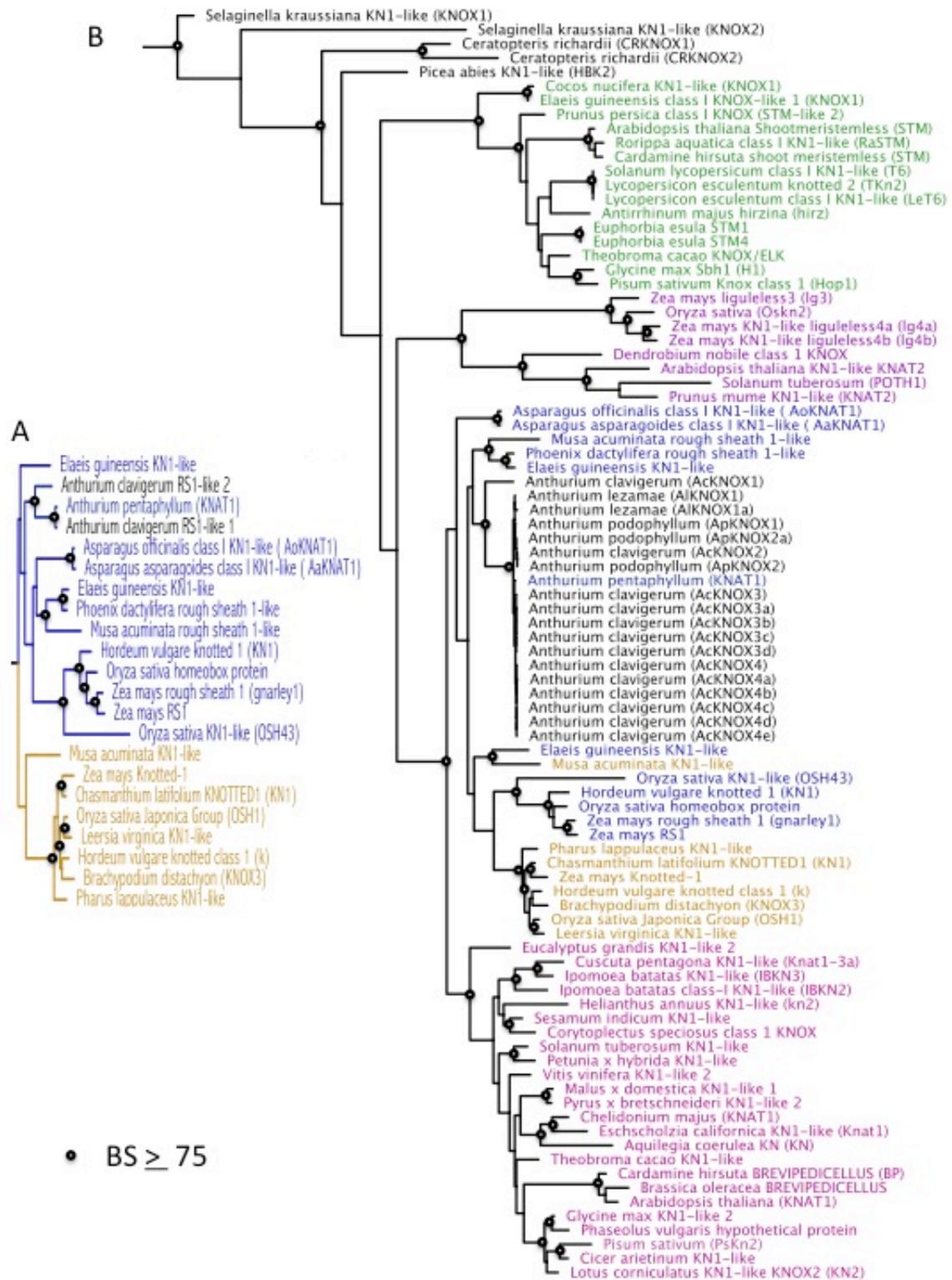


Figure 3.7 Maximum likelihood phylogeny of *KNOX1* sequences from taxa across tracheophytes. A. phylogeny based on an alignment of 670 bp, B. phylogeny based on an alignment of 579 bps. Circles denote nodes with bootstrap support of 75% and above.

relationships among the *STM*, *KNAT2/liguleless* and *KNATI/(KNI/RSI)* clades were similar, and thus not shown in phylogeny A. The separation of eudicot *KNATI* homologs from monocot *KNI/RSI* homologs is also similar and well supported in both phylogenies (A: BS=84%, B: BS=86%), and thus not shown in phylogeny A. Within the *KNI/RSI* clade, however, relationships are not stable, nor well supported. Within grasses *KNI*-like and *RSI*-like copies consistently fall into two separate clades, which are strongly supported (A: *KNI*-like BS=90%, *RSI*-like BS=98%; B: *KNI*-like BS=96%, *RSI*-like BS=89%). In phylogeny A (alignment of 670 bp), *KNI*-like copies diverged from *RSI*-like copies in the common ancestor of the (Poales (Zingiberales, Commelinales)) clade, while all copies in earlier diverging lineages were *RSI*-like (Stevens, 2001). In phylogeny B (alignment of 579 bp), the split between *KNI*-like and *RSI*-like copies occurred within Poaceae, while earlier diverging monocots contained copies of *KNOXI* genes that are neither more closely related to *KNI* or *RSI*. Support values for both topologies are low; however, and lack of sampling is an obvious shortcoming.

Anthurium pentaphyllum, *A. clavigerum* and *A. lezamai* are diploid species (Bliss and Susuki, 2012). Within *Anthurium*, all copies of *KNOXI* genes form a clade that is well supported in both phylogenies (A: BS=88%, B=87%). The placement of *KNOXI* copies of *A. clavigerum* into three separate clades suggests that there are at least three different copies within the species. The highly divergent copy of *A. clavigerum* (*AcKNOXI*) is outside a clade containing all other copies found in four species of *Anthurium* (A: BS=100%, B=100%). Another copy of *A. clavigerum* (*AcKNOX2*) falls into a clade containing the Mexican endemics *A. podophyllum* and *A. lezamai* (B: BS=91%), and is more closely related to *A. podophyllum* (B: BS=94%). Contamination is highly unlikely since sequences of *A. podophyllum* and *A. clavigerum* that form

a clade were cloned on separate occasions. All eleven other *KNOXI* sequences of *A. clavigerum* fall into a clade (B: BS=90%) that is the sister group to *A. pentaphyllum* as originally expected, as both species are members of section *Dactylophyllum* (B: BS=93%). The eleven sequences of *A. clavigerum* further fall into two clades: *AcKNOX3a-d* (B: BS=71%) and *AcKNOX4a-e* (B: BS=88%). Sequences from these two clades differ by only several SNPs and thus it is not clear whether they are actually separate copies, or simply allelic variants.

3.3.4 RT-PCR

The reference gene *GAPDH* was chosen as a positive control because it is expressed at all stages of leaf development (Figure 3.8). However, expression levels of all reference genes were variable across loci and tissues. Initial tests were performed on meristem and mature leaf cDNA of *Anthurium clavigerum* and *A. lezamai*. *EF-1 α* was expressed in the meristem of both species and in the mature leaf of *A. lezamai*, but not in the mature leaf of *A. clavigerum*. *LUG* was expressed in both tissues in both species, but to a minimal degree in the mature leaf of *A. clavigerum*. *TUB* and *GAPDH*, on the other hand, were expressed stably in both tissues from both species. During initial testing, *TUB* primers amplified a region using the genomic DNA of *A. lezamai*, whereas the primers for *GAPDH* did not (data not shown). Therefore, *GAPDH* was used as a positive control in the RT-PCR reactions testing for *KNOXI* gene expression. However, in subsequent reactions primers for *GAPDH* (AnthGAPDHF, AnthGAPDHR) amplified regions in genomic DNA as well, but the products were much larger than those in cDNA samples confirming the absence of genomic DNA in cDNA samples (Figure 3.8).

The results of RT-PCR for all biological and technical replicates demonstrate that class I *KNOX* genes are expressed in developing leaves in *Anthurium*, making the expression patterns seen in immunolocalizations using the anti-C-terminus *KNI* antibody more accurate than those

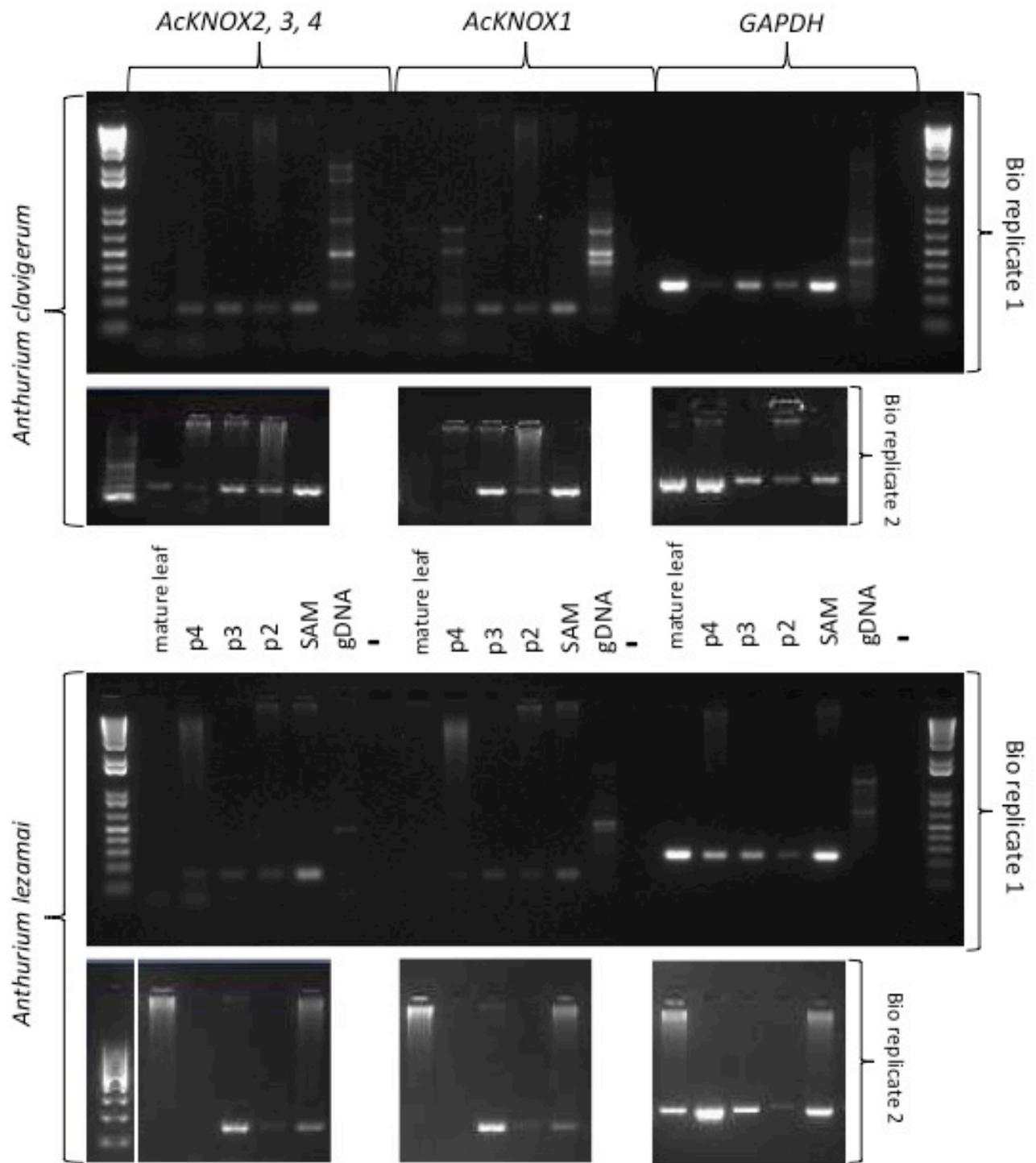


Figure 3.8 RT-PCR of *KNOX1* gene transcripts in successive stages of leaf development in *Anthurium clavigerum* and *A. lezamai*.

using the full-length *KNI* antibody (Figure 3.8). Additionally, upon cloning and sequencing RT-PCR products from the meristem and developing leaves in *A. clavigerum* revealed identical copies of *KNOXI* genes expressed in the meristem and developing leaves. This result suggests that the immunolocalization reaction using the full-length *KNI* antibody was inhibited in developing leaf tissue. Both targeted copies of *KNOXI* genes (*AcKNOXI* and *AcKNOX2,3,4*) were expressed in the meristem, p2 and p3 in both simple-leaved *A. lezamai* and dissected-leaved *A. clavigerum*. This result is unsurprising as *KNOXI* genes are expressed in developing vascular tissue (Taylor, 1997).

Stage p4 *KNOXI* gene expression for both copies was variable in both species, depending on the sample. In biological replicate 1, both copies of *KNOXI* genes were expressed in p4 in *A. lezamai*, while in biological replicate 2 neither is expressed in p4. A similar pattern can be seen between p4 biological replicates in *A. clavigerum* for *AcKNOXI* (Figure 3.8). The expression of *AcKNOXI* was invariably lower than *AcKNOX2,3,4* at this stage (Figure 3.8). Two extra bands at stage p4 in biological replicate 1 of *Anthurium clavigerum* that are of similar size as the bands in the genomic DNA sample are odd in that they did not appear in the other two reactions using the same cDNA nor were they present in earlier technical replicates of the same sample. The bands in the genomic DNA samples were gel extracted, cloned, sequenced and submitted to BLAST searches - no *KNOXI* sequences were found.

The most striking result was the expression of *AcKNOX2,3,4* genes in two biological replicates of the mature dissected leaf in *A. clavigerum*, whereas no *KNOXI* genes were expressed in the mature simple leaf of *A. lezamai*. Dissections of mature leaves for RNA extraction were taken from the most recent fully expanded leaf in both species. This suggests that *KNOXI* genes are expressed much later in dissected leaf development in *Anthurium* than

previously expected. This result is confirmed in the western blot analysis, where *KNOXI* protein expression appears in the “mature” leaves of *A. clavigerum* and *A. polyschistum* (Figure 3.3). Interestingly *AcKNOXI* was not expressed in the same samples of the mature leaf in *A. clavigerum*. Additionally, upon cloning and sequencing PCR products of the various stages from both copies in both species, a unique sequence of *AcKNOX2,3,4* containing a 5 amino acid deletion appeared in the mature leaf of *A. clavigerum* and nowhere else.

3.4 Discussion

A comparison of the development of deeply lobed and dissected leaves in *Anthurium* and *Amorphophallus* confirms that they arise through blastozone fractionation (see Chapter 2). The results of immunolocalization using the antibody against the C-terminus of the maize *KNI* protein suggest that *KNOXI* genes are expressed during lobe and leaflet formation in both genera. The presence of *KNOXI* sequences in developing leaves in the RT-PCR assay supports the C-terminus *KNI* immunolocalization result, but it provides no insight into the precise location or function of *KNI*-like genes in the developing leaf in *Anthurium*. However, the expression of *KNOXI* genes much later in development in the dissected leaf of *A. clavigerum* compared with the simple leaf of *A. lezamai* is further evidence for a role of *KNOXI* genes in dissected leaf development in *Anthurium*.

All *KNOXI* sequences from *Anthurium* in this study form a clade with those of other monocots. As more *KNOXI* sequences from Araceae become available, it will be interesting to see whether dicot-like copies are found as they were in palms, which have at least one copy that is more closely related to dicots than to *KNOXI* sequences of other monocots (Figure 3.7). This may represent a lack of sampling in monocots and/or a loss of copies in the grass clade.

Although the relationship between *KNOXI* genes in *Anthurium* and *KN1* and *RS1* in maize are not clear, the fact that *Anthurium KNOXI* genes fall into a larger clade with *KNATI*, which has been shown to induce lobe formation in *Arabidopsis*, further supports the role of *Anthurium KNOXI* genes in lobed and dissected leaf development.

The conflicting results between immunolocalizations performed with two antibodies that should be detecting the same proteins are odd to say the least, especially considering that the same *KNOXI* sequences were found in both the meristem and the developing leaf. It has been noted elsewhere that anti-*KN1* antibodies could be detecting the antigenic activity of more than one *KNOXI* protein (Bharathan et al., 2002). This indicates that the protein expression patterns seen using the full-length and C-terminus anti-*KN1* antibodies could be a composite of the activity of multiple *KNOXI* genes. Given that there are *at least* three copies of *KNOXI* genes in *Anthurium clavigerum*, the expression domain of each copy and the roles of different copies in leaf development need to be determined before a clearer picture of *KNOXI* gene function in *Anthurium*, let alone *Amorphophallus* or Araceae, emerges. *In situ* hybridization using probes designed specifically to the *AcKNOXI* and *AcKNOX2,3,4* copies in *Anthurium clavigerum* is the requisite next step.

That being said, the present study is a step in the right direction, and will hopefully serve as a foundation from which to proceed. Studies of *KNOXI* genes in Araceae will: 1) help bridge the gap in our understanding of dissected leaf development between monocots and dicots; 2) expand knowledge of the evolution and functional divergence of members of the *KNOX* gene family; and 3) broaden the molecular genetic tools available for aroid crop improvement, which are staples for food security in many developing countries.

3.5 References

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Chapter 4

Leaf Development in Araceae: A New Look at the “Monocot Leaf vs. Dicot Leaf” Paradigm

4.1 Introduction

The idea that there is a broad distinction between monocot and dicot leaves permeates botanical literature. Here, dicot refers to a paraphyletic clade containing all angiosperms other than monocots. Many monocot leaves have closed, parallel venation, sheathing leaf bases and a linear blade, while dicots have petiolate leaves with an expanded lamina and reticulate venation (Kaplan, 1973, 1975; Rudall and Buzgo, 2002). In addition to these general differences, two unique conditions exist in monocot leaf morphology. These are the terete (radially symmetric) leaf and the ensiform leaf, which can be seen as an elaboration of the terete leaf (Yamaguchi et al., 2010). Several developmental modes are also found only in monocots including plication, plication followed by schizogeny, and programmed cell death (Gunawardena and Dengler, 2006; Periasamy and Muruganathan, 1986). Leaf morphology has been used along with roots, anatomy, and seed structure/cotyledonary condition as support for the monophyly of monocots (Arber, 1918; Chase, 2004; Kaplan, 1973). Botanists have proposed various hypotheses as to the evolutionary origin of monocots and their particular leaf morphology. Most notable are the ‘Phyllode theory’ (Arber, 1918; de Candolle, 1827; Henslow, 1911) and the ‘Leaf-base theory’ (Hagemann, 1970; Kaplan, 1973, 1975; Knoll, 1948; Troll, 1955).

4.1.1 *The Phyllode theory*

de Candolle (1827) considered the entire monocot leaf to represent only the leaf base and petiole of a dicot leaf. According to this theory, linear dorsiventral monocot leaves with parallel venation are derived from petioles with an open arc of bundles, while the terete monocot leaf is derived from petioles with a ring of bundles. In monocots, the expanded blade of the dicot leaf is completely absent (Arber, 1925). The ensiform leaf is considered a terete leaf that has been flattened in the median plane so that opposing vascular bundles maintain their orientation and

appear inverted on one surface with respect to dorsiventral leaves (Arber, 1925). The phyllode theory was supported by the presence of inverted vascular bundles in both ensiform leaves in monocots and the phyllodes of *Acacia* and *Oxalis*, from whence the theory's name derived. Henslow (1911) elaborated this theory, suggesting that the expanded blade in monocots is derived from an expansion of the apical region of the petiole and is thus, implicitly, again not homologous with the blade of dicotyledons. Arber (1918, 1925) supported and bolstered the phyllode theory by a vast anatomical and morphological survey of angiosperm leaves. Influenced by her mentor (Sargent, 1904), Arber considered the ensiform leaf of phyllodic origin the ancestral character state of monocots (Arber, 1918, 1925). Henslow (1911), Sargent (1904) and Arber (1918) all suggested that monocots were a monophyletic group; however Sargent and Arber considered the common ancestor to be geophilous, while Henslow proposed that the common ancestor was of aquatic origin. Although the exact conditions under which monocots arose have not been settled, there is a general consensus that high levels of moisture were involved (Carlquist, 2012).

The phyllode theory did not go uncontested. Goebel (1891) argued that the submerged linear leaves of *Sagittaria* were homologous with the entire dicotyledonous leaf, but they represented a rudimentary, or arrested stage, of the mature leaf (Arber, 1918). Troll (1939) argued against the phyllode theory by noting that monocot leaves never exhibit an apical blade rudiment as seen in *Acacia* phyllodes and that radial vasculature occurs in blades and leaf bases, as well as in petioles; thus it did not aid in identifying homologies (Kaplan, 1973).

4.1.2 *The Leaf-base theory*

The leaf-base theory has at its foundation the work of Eichler (1861). Eichler noted that leaf primordia develop into two distinct regions soon after inception from the shoot apex. These

consist of a proximal, more or less axis-encircling region called the “Unterblatt”, or lower leaf zone, and a distal freely projecting region called the “Oberblatt”, or upper leaf zone (Kaplan 1973, 2001). Knoll (1948) concluded that the monocot leaf could be divided into morphological zones where the expanded, dorsiventral blade was derived from the lower leaf zone, while in dicots it was derived from the upper leaf zone. Knoll based this proposal on his observation of forms in monocots intermediate between terete leaves with a long radial axis and short dorsiventral sheathing leaf base to those with the radial portion reduced to tiny precursor tip or, *Vorläuferspitze*, with the dorsiventral sheathing leaf base elaborated into the photosynthetic surface. Troll (1955), Kaplan (1973) and Hagemann (1970 although with modification) supported this view, which is represented diagrammatically in Kaplan (1973). Thus, the leaf-base theory regards the monocot leaf blade as non-homologous with the dicot leaf blade, and that different regions of the leaf primordium can produce structurally similar analogous components (Kaplan, 1973).

4.1.3 *Araceae, Alismatales and the Leaf-base theory*

A major discrepancy in both theories is that the more typical dicot leaf condition, with reticulate venation and an expanded blade with no *Vorläuferspitze*, is also found among basal monocot lineages (Arber, 1918; Chase, 2004; Kaplan, 1973; Rudall and Buzgo, 2002). Recent studies have shown these theories incapable of explaining many of the diverse leaf forms found within monocots, particularly in the order Alismatales and in the family Araceae. In testing the applicability of the leaf-base theory to four monocot species that represent the morphological transitional series of Knoll (1948), Kaplan (1973) noted that members of Araceae displayed developmental characteristics similar to those of dicots. Monocot leaves are typically described as having basipetal maturation that proceeds from the distal apex toward the proximal base, in

contrast, the species of Araceae studied by Kaplan displayed acropetal differentiation characteristic of dicots. Likewise, he states that genera belonging to other families in the same order as Araceae, the Alismatales, lack a *Vorläuferspitze*, thus making determination of blade morphological relationships difficult or impossible. On this note, Kaplan (1973) concedes that the leaf-base model may not be universal among monocots and that a broad survey is necessary for its validation.

Taking this challenge, Bharathan (1996) who characterized leaf primordia of four dicots and fourteen monocots, including three species of Araceae. As did Kaplan (1973), she reported aroid genera had highly variable leaf developmental modes. She concluded that among monocots there are eleven leaf primordial types, of which only one represents the “monocot type.” This “monocot type” was restricted to the commelinoids and asparagoids, and thus could not be a synapomorphy for the entire monocot clade.

4.1.4 *The Transition-zone theory*

A recent study of the evolutionary history of monocot leaves proposed a new framework with which to study leaf development (Rudall and Buzgo, 2002). The transition-zone theory proposes that leaf structures can be explained through the action of an adaxial meristem within a highly plastic transition zone between the leaf apex and leaf base. It gains support through the observation that foliar structures such as the petiole, terete and ensiform blades, the peltate blade, ligules and stipules arise from morphogenetic activity within the transition zone, often on the adaxial surface (Gleissberg et al., 2005; Hagemann, 1970; Kaplan, 1970; Kim et al., 2003a; Roth, 1949). The transition-zone theory is valuable in incorporating the importance of the elaboration of the cross-zone (an adaxial region at the distal end of the leaf base) in understanding leaf morphology; however, it has two drawbacks. First, it persists in defining

discrete zones within the leaf, making inferences of homology without explaining how corresponding zones with highly different morphologies arise; and secondly, description of the cross-zone as an adaxial meristem is somewhat misleading in that studies have shown that the molecular basis of cross-zone formation involves *loss* of adaxial cell identities in many cases (Gliessberg et al., 2005, Kim et al., 2003).

Geeta (2003) incorporated aspects of the transition zone theory into a subsequent analysis of leaf development in 25 monocots and dicots that drew from Bharathan (1996). The study concludes that monocot leaf primordia may follow two modes of development. In proposing two developmental modes for monocots, however, several salient features of monocot leaf development were ignored. These include leaf plication in Araceae, Iridaceae (Rudall, 1990) and palms, schizogeny following plication to produce leaflets in palms (Gunawardena and Dengler, 2006; Kaplan, 1984), programmed cell death in Araceae and Aponogetonaceae, and ensiformity arising independently in distantly related orders (Arber, 1925; Rudall, 1990; Rudall and Buzgo, 2002).

4.1.5 *Gene Regulatory Networks of leaf development*

Recently, much progress has been made in elucidating the molecular genetics of leaf development (Husbands et al., 2009; Nakayama et al., 2013; Townsley and Sinha, 2012). The modular nature of the gene interactions regulating development has given rise to Gene Regulatory Network (GRN) theory (Erwin and Davidson, 2009; Ichihashi et al., 2014). GRN modules form a hierarchy with modules regulating highly conserved functions at the core of the network and separate modules regulating more labile functions at the periphery (Erwin and Davidson, 2009). GRN architecture is a highly plastic system, in which modules can be re-wired to affect gene expression changes spatially and temporally either between species or over the

course of ontogeny of a single individual (True and Haag, 2001). Re-wiring of GRNs can lead to major phenotypic changes on which natural selection can act, or conversely, remain neutral or nearly neutral when changes in the underlying genetics do not alter the phenotype. The latter case, known as Developmental Systems Drift (DSD), is surprisingly common (True and Haag, 2001). Both processes can act together when a change in the genetic background or environmental conditions of a GRN allow Developmental Systems Drift to access new peaks in the adaptive landscape that were once inaccessible (Townsend and Sinha, 2012). This conceptual framework is extremely useful for exploring the roles of natural selection and drift in phenotypic change over evolutionary time.

In this study, a survey of leaf primordia across angiosperms, with a focus on Araceae, is undertaken to 1) broaden sampling to determine how variable leaf development is in Araceae; and 2) ascertain whether there is a fundamental difference in leaf development between monocots and dicots. Methods include construction of a matrix of developmental, morphological and anatomical leaf characters with which to perform ancestral character state reconstruction and multivariate analyses. The results of these analyses are discussed in terms of current knowledge of the Gene Regulatory Networks (GRNs) involved in leaf development.

4.2 Materials and Methods

4.2.1 *Living plant material and literature search*

Living material was collected from the Missouri Botanical Garden and voucher specimens were deposited in the Missouri Botanical Garden herbarium. Information on other taxa was obtained from a search of leaf development literature. Taxa included in this study are

listed in Table 4.1 with accession and voucher number and/or literature sources where appropriate.

4.2.2 Preparation of living material

For SEM, dissections of developing leaves were fixed in 15 ml FAA overnight (50% EtOH, 5% glacial acetic acid, 10% 37% formaldehyde solution (formalin), 35% dH₂O), then taken through a dehydration series of 50% EtOH, 70% EtOH, 80% EtOH, 90% EtOH, 95% EtOH, 100% EtOH with 1.5 to 2 hours between each change and left in 100% EtOH overnight. The following morning 100% EtOH was changed for fresh (newly opened) 100% EtOH. For critical point drying the Tousimis SamDri-780 Critical Point Dryer was used using standard operating procedures and samples were left at an equilibrium pressure of ~1300 psi and temperature of ~36°C between 12 and 20 minutes. Critical point dried samples were mounted and then sputter coated using the Tousimis Samsputter-2a for two minutes under a vacuum pressure of 130-140 mTorr, with a current of 10 mA and an Argon tank reading of ~4 psi. Sputter-coated samples were imaged using the Hitachi S-2600H Scanning Electron Microscope at the Department of Otolaryngology's Electron Microscopy Core at Washington University in St. Louis.

4.2.3 Ancestral character state reconstruction

Leaf primordia were scored for developmental, morphological and anatomical characters chosen to describe the maximal amount of diversity seen in developing and mature leaves from 66 taxa across the angiosperm phylogeny. Data were coded as 14 unordered multistate characters. Characters and character states are listed in Table 4.2. The character matrix can be viewed in Table 4.3.

Table 4.1 Taxa included in the matrix. Taxonomic ranking within Magnoliophyta is based on APG (Stevens, 2001), Cusimano et al. (2011), Genbank and Henriquez et al. (2014).

Taxa	MBG Collection/ Voucher No.	Literature source	Taxonomic Ranking within Magnoliophyta
<i>Trithuria submersa</i>		Hamann, 1998; Rudall et al., 2007	basal Magnoliophyta; Nymphaeales; Hydatellaceae
<i>Hydatella australis</i>		Hamann, 1998, Rudall et al., 2007	basal Magnoliophyta; Nymphaeales; Hydatellaceae
<i>Nymphaea hybrid</i> <i>Pink Platter</i>	2014-0309- 1/6647391		basal Magnoliophyta; Nymphaeales; Nymphaeaceae
<i>Lactoris</i> <i>fernandeziana</i>		González and Rudall, 2001	Magnoliidae; Piperales; Aristolochiaceae
<i>Saruma henryi</i>		González and Rudall, 2001	Magnoliidae; Piperales; Aristolochiaceae; Asaroideae
<i>Asarum europaeum</i>		Kaplan, 1997	Magnoliidae; Piperales; Aristolochiaceae; Asaroideae
<i>Acorus calamus</i>		Kaplan, 1970	Liliopsida; Acorales; Acoraceae
<i>Aglaonema modestum</i> Schott ex Engl.	T. Croat 79477/6649292	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Aglaonemateae
<i>Alocasia</i> <i>macrorrhizos</i>	2013-2525-2/ 6647397	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae
<i>Amorphophallus</i> <i>henryii</i>		Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Thomsonieae
<i>Amydrium</i> <i>zippelianum</i>	T. Croat 52817/6649286	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Monsteroideae
<i>Anchomanes</i> <i>welwitschii</i>	T. Croat 69773	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Nephtytideae
<i>Anthurium clavigerum</i>	T. Croat 84498/6649281	Mayo et al., 1997	Magnoliophyta; Liliopsida; Alismatales; Araceae; Pothoideae; Anthurieae
<i>Anubias heterophylla</i>	T. Croat 95582/6421699	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae
<i>Arisaema</i> <i>leschenaultii</i>		Mayo et al., 1997, Periasamy and Muruganathan, 1986	Liliopsida; Alismatales; Araceae; Aroideae
<i>Calla palustris</i>		Lehmann and Sattler, 1992; Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae
<i>Dieffenbachia</i> <i>parvifolia</i>	T. Croat 103328/6649294	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Spathicarpeae
<i>Epipremnum</i> <i>pinnatum</i>	T. Croat 73986/6649279	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Monsteroideae
<i>Gonatopus boivinii</i>	T. Croat 56901/6649287	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Zamiodulcadoideae

<i>Hapaline brownii</i>	T. Croat 90010/6649296	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Caladieae
<i>Lasia spinosa</i>	T. Croat 71753/6441222	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Lasioideae
<i>Monstera deliciosa</i>		Gunawardena and Dengler, 2006; Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Monsteroideae
<i>Nephtytis swainei</i>	T. Croat 67403/6649289	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Nephtytideae
<i>Orontium aquaticum</i>	T. Croat 103050/6421703	Keating, 2002; Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Orontioideae
<i>Philodendron acutatum</i>	T. Croat 101775/6649278	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae
<i>Pinellia pedatisecta</i>	T. Croat 81511/5489873	Mayo et al., 1997; Murata, 1990; Keating, 2002	Liliopsida; Alismatales; Araceae; Aroideae
<i>Scindapsus siamensis</i>	T. Croat 57126/6649291	Bharathan, 1996; Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Monsteroideae
<i>Spathiphyllum cochlearispathum</i>	T. Croat 65001/6649276	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Monsteroideae; Spathiphyllaeae
<i>Stenospermatum marantifolium</i>	T. Croat 74938/6649277	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Monsteroideae
<i>Stuednera colocasiifolia</i>	T. Croat 77954/6649297	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Monsteroideae
<i>Stylochaeton bogneri</i>	T. Croat 87579/6421694	Mayo et al., 1997	Liliopsida; Alismatales; Araceae
<i>Synandropadix vermitoxicus</i>	T. Croat 101512/6649288	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Spathicarpeae
<i>Syngonium podophyllum</i>	T. Croat 49759/6649290	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Caladieae
<i>Typhonium trilobatum</i>	2013- 1471/6647399	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Areae
<i>Typhonodorum sp.</i>	6647398	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Peltandreae
<i>Xanthosoma atrovirens</i>	MBG 850652/6647395	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae; Caladieae
<i>Zamioculcas zamiifolia</i>	T. Croat 97755/6422614	Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Zamioculcadoideae
<i>Zantedeschia aethiopica</i>		Kaplan, 1973; Mayo et al., 1997	Liliopsida; Alismatales; Araceae; Aroideae
<i>Sagittaria latifolia</i>		Bloedel and Hirsch, 1979	Liliopsida; Alismatales; Alismataceae

<i>Carludovica palmata</i>		Wilder, 1976	Liliopsida; Pandanales; Cyclanthaceae; Carludovicioideae
<i>Dioscorea pentaphylla</i>		Periasamy and Muruganathan, 1985	Liliopsida; Dioscoreales; Dioscoreaceae
<i>Smilax bona-nox</i>		Martin and Tucker, 1985	Liliopsida; Liliales; Smilacaceae
	1994-2143		Liliopsida; Liliales; Liliaceae
<i>Tricyrtis formosana</i>			
<i>Narcissus pseudonarcissus</i>	2007-0041-1	Denne, 1959	Liliopsida; Asparagales, Amaryllidaceae, Amaryllidoideae, Narcisseae
<i>Crocasmia masonorum</i>		Rudall, 1990	Liliopsida; Asparagales
<i>Sansevieria suffruticosa</i>		Kaplan, 1997	Liliopsida; Asparagales, Asparagaceae; Nolinoideae
<i>Sansevieria trifasciata</i>		Kaplan, 1997, Stevenson, 1973	Liliopsida; Asparagales, Asparagaceae; Nolinoideae
<i>Calamus australis</i>		Merklinger et al., 2014	Commelinids, Arecales, Arecaceae, Calamoideae; Calameae; Calaminae
<i>Chamaedorea elegans</i>		Nowak et al., 2011	Liliopsida; Arecaceae; Arecoideae; Chamaedoreae
<i>Elaeis guineensis</i>		Jouannic et al., 2007	Liliopsida; Arecaceae; Arecoideae; Cocoseae; Elaeidinae
<i>Tradescantia zebrina</i>	1989-5211		Liliopsida; Commelinales; Commelinaceae
Bosse			
<i>Calathea mirabilis</i>	1996-3142	Croxdale, 1998	Liliopsida; Zingiberales; Marantaceae;
<i>Xyris longiscapa</i>		Sajo and Rudall, 1999	Liliopsida; Poales; Xyridaceae
<i>Zea mays</i>			Liliopsida; Poales; Poaceae; PACMAD clade; Panicoideae; Andropogoneae
<i>Eschscholzia californica</i>		Scanlon et al. (1996) Becker et al., 2005	eudicotyledons; Ranunculales, Papaveraceae, Eschscholzioideae
<i>Stephania hernandiifolia</i>		Hagemann, 1970; Kaplan, 1997	eudicotyledons; Ranunculales, Menispermaceae, Menispermoideae
<i>Caltha palustris</i>		Hagemann, 1970; Kaplan, 1997	eudicotyledons; Ranunculales; Ranunculaceae, Ranunculoideae; Caltheae
<i>Trifolium repens</i>		Denne, 1966	eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Trifolieae
<i>Astragalus cicer</i>		Kaplan, 1997	eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae;

Papilionoideae; Galegeae

<i>Prunus sibirica</i>	Kaplan, 1997; Slade, 1956	eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Rosales; Rosaceae; Maloideae; Amygdaleae
<i>Pelargonium betulinum</i>	Kaplan, 1997	eudicotyledons; Gunneridae; Pentapetalae; rosids; malvids; Geraniales; Geraniaceae;
<i>Tropaeolum majus</i>	Gleissberg et al., 2005	eudicotyledons; Gunneridae; Pentapetalae; rosids; malvids; Brassicales; Tropaeolaceae
<i>Muehlenbeckia platyclada</i>	Kaplan, 1997	eudicotyledons; Gunneridae; Pentapetalae; Caryophyllales; Polygonaceae; Polygonoideae; Polygoneae
<i>Polemonium caeruleum</i>	Kaplan, 1997	eudicotyledons; Gunneridae; Pentapetalae; asterids; Ericales; Polemoniaceae; Polemonioideae
<i>Kohleria bogotensis</i>	Barth et al., 2009	eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Lamiales; Gesneriaceae; Gesnerioideae; Gesnerieae; Gloxiniinae
<i>Senecio serpens</i>	Timonin et al., 2006	eudicotyledons; Gunneridae; Pentapetalae; asterids; campanulids; Asterales; Asteraceae;Asteroideae; Senecioneae; Senecioninae

Ancestral character state reconstruction was performed in Mesquite version 3.02 (Maddison and Maddison, 2015). The character matrix was imported into Mesquite and used to create an unordered cladogram. The cladogram was then rearranged into two different topologies, one following the conservative evolutionary relationships proposed by APG III (2009) and the other following the relationships proposed by Zeng et al. (2014). The major difference between the two phylogenies is the placement of magnoliids, which in the former are the sister taxon to a clade containing monocots and eudicots, whereas in the latter they are the sister taxon to eudicots. Taxa included in the morphological matrix that are missing from the Zeng et al. (2014) tree topology, such as Caryophyllales and Ericales, were inserted according to

Table 4.2 Developmental, morphological and anatomical characters with character states used in matrix construction. For further discussion of the characters and states see section 4.3.1.

Character	Character-states
<i>Vorläuferspitze</i>	0=absent, 1=present
Cross-meristem	0=absent, 1=present
Differentiation direction	0=acropetal, 1=basipetal
Sheathing leaf base	0=absent, 1=present
Plication	0=absent, 1=present
Dorsiventrality	0=unifacial terete, 1=unifacial ensiform, 2=bifacial
Petiole	0=absent, 1=present
Blade peltation	0=absent, 1=present
Blade margins	0=entire to lobed, 1=entire w/ schizogeny, 2=entire w/cell death, 3=dissected
Blade division	0=absent, 1=pinnate, 2=pedate, 3=trisect w/ further elaboration, 4=palmate, 5=trifid, 6=trisect
Midrib	0=absent, 1=present
Primary venation of ultimate units	0=parallel, 1=pinnate, 2=palmate, 3=undifferentiated, 4=campylodromous, 5=palmate-parallel
Higher order venation	0=reticulate, 1=transverse reticulate, 2=parallel pinnate, 3=parallel, 4=secondary laterals parallel-pinnate, connected by transverse tertiary veins, 5=combination of 0 and 2, 6=combination of 1 and 2
Stipules/ligule/ochrea	0=absent, 1=present

the relationships in APG III. Evolutionary relationships in Araceae followed the topologies in Henriquez et al. (2014). Araceae included in the morphological matrix that were not included by Henriquez et al. (2014) were placed according to the relationships seen in Cusimano et al. (2011). One difference between the tree topologies for Araceae used in the Zeng et al. (2014) and APG III tree is the placement of *Calla*. In the Zeng et al. (2014) topology, *Calla* is the sister taxon to (*Anubias*, *Zantedeschia* clade), whereas in the APG tree, *Calla* is the sister taxon to the *Dracunculus* clade. This reflects the two different placements of *Calla* in Henriquez et al. (2014), which were included to explore how this difference would affect ancestral character-state reconstruction.

Ancestral character-state reconstructions were performed using maximum parsimony in Mesquite. Maximum likelihood reconstructions were not done because branch lengths for the tree are unknown.

Table 4.3 Character matrix

	Vorläuferspitze	Cross-meristem	Differentiation direction	Sheathing leaf base	Plication	Dorsiventrality	Petiole	Blade peltate	Blade margins	Blade division	Midrib	Primary venation	Higher order venation	Stipules/Ligule/Ochrea
<i>Trithuria submersa</i>	0	0	0	0	0	0	0	0	0	0	1	?	?	0
<i>Hydatella australis</i>	?	0	0	0	0	0	0	0	0	0	1	?	?	1
<i>Nymphaea hybrid Pink Platter</i>	0	?	0	0	0	2	1	1	0	0	1	2	0	1
<i>Lactoris fernandeziana</i>	1	1	?	1	0	2	1	0	0	0	1	1	0	1
<i>Saruma henryi</i>	0	0	0	0	0	2	1	0	0	0	1	2	0	0
<i>Asarum europaeum</i>	0	0	1	0	0	2	1	0	0	0	1	2	0	0
<i>Acorus calamus</i>	1	1	0	1	0	1	0	0	0	0	1	0	3	0
<i>Aglaonema modestum</i>	0	1	0	1	0	2	1	0	0	0	1	1	2	0
<i>Alocasia macrorrhizos</i>	1	1	0	1	0	2	1	1	0	0	1	1	0	0
<i>Amorphophallus henryi</i>	0	1	?	1	0	2	1	1	3	3	1	1	0	0
<i>Amydrium zippelianum</i>	0	0	1	1	0	2	1	0	0	1	1	1	1	0
<i>Anchomanes welwitschii</i>	0	1	?	1	0	2	1	0	2	3	1	1	0	0
<i>Anthurium clavigerum</i>	1	0	1	1	0	2	1	0	3	4	1	1	0	0
<i>Anubias heterophylla</i>	1	0	0	1	0	2	1	0	0	0	1	1	4	0
<i>Arisaema leschenaultii</i>	0	1	?	1	1	2	1	0	3	4	1	1	0	0
<i>Calla palustris</i>	0	0	0	1	0	2	1	0	0	0	1	3	2	1
<i>Dieffenbachia parvifolia</i>	1	0	0	1	0	2	1	0	0	0	1	1	4	1
<i>Epipremnum pinnatum</i>	0	0	0	1	0	2	1	0	2	1	1	1	5	0
<i>Gonatopus boivinii</i>	0	0	0	1	0	2	1	0	3	3	1	1	0	0
<i>Hapaline brownii</i>	1	1	0	1	0	2	1	1	0	0	1	1	0	0
<i>Lasia spinosa</i>	0	1	0	1	0	2	1	0	0	1	1	1	0	0
<i>Monstera deliciosa</i>	0	?	1	1	0	2	1	0	2	1	1	1	1	0
<i>Nephtytis swainei</i>	0	1	?	1	0	2	1	0	0	0	1	2	0	0
<i>Orontium aquaticum</i>	0	1	?	1	0	2	1	0	0	0	1	4	1	0
<i>Philodendron acutatum</i>	1	0	0	1	0	2	1	0	0	0	1	1	6	1
<i>Pinellia pedatisecta</i>	0	1	?	1	1	2	1	0	3	2	1	1	0	0
<i>Scindapsus siamensis</i>	1	0	0	1	0	2	1	0	0	0	1	3	6	0
<i>Spathiphyllum cochlearispathum</i>	1	0	0	1	0	2	1	0	0	0	1	1	6	0
<i>Stenospermaton</i>	0	0	?	1	0	2	1	0	0	0	1	3	2	0
<i>Stuednera colocasiifolia</i>	1	1	0	1	0	2	1	1	0	0	1	1	0	0
<i>Stylochaeton bogneri</i>	1	0	?	1	0	2	1	0	0	0	1	1	0	0
<i>Synandropadix vermitoxicus</i>	1	0	0	1	0	2	1	0	0	0	1	1	0	0

	Vorläuferspitze	Cross-meristem	Differentiation direction	Sheathing leaf base	Plication	Dorsiventrality	Petiole	Blade peltate	Blade margins	Blade division	Midrib	Primary venation	Higher order venation	Stipules/Ligule/Ochrea
<i>Syngonium podophyllum</i>	1	0	0	1	0	2	1	0	3	2	1	1	0	0
<i>Typhonium trilobatum</i>	0	1	0	1	0	2	1	0	0	5	1	1	0	0
<i>Typhonodorum sp.</i>	1	1	?	1	0	2	1	0	0	0	1	1	2	0
<i>Xanthosoma atrovirens</i>	1	?	0	1	0	2	1	0	0	0	1	1	0	0
<i>Zamioculcas zamiifolia</i>	0	0	1	1	0	2	1	0	3	1	1	1	0	0
<i>Zantedeschia aethiopica</i>	1	?	0	1	0	2	1	0	0	0	1	1	6	0
<i>Sagittaria latifolia</i>	0	0	?	1	0	2	1	0	0	0	1	4	0	0
<i>Carludovica palmata</i>	1	1	1	1	1	2	1	0	1	4	1	5	?	0
<i>Dioscorea pentaphylla</i>	1	?	1	0	0	2	1	0	3	4	1	1	0	0
<i>Smilax bona-nox</i>	1	0	0	0	0	2	1	0	0	0	1	4	0	1
<i>Tricyrtis formosana</i>	1	0	1	1	0	2	0	0	0	0	1	0	?	0
<i>Narcissus pseudonarcissus</i>	0	0	?	1	0	2	0	0	0	0	0	0	3	0
<i>Crocasmia masonorum</i>	1	1	1	1	1	1	0	0	0	0	0	0	3	0
<i>Sansevieria suffruticosa</i>	1	1	1	1	0	0	0	0	0	0	0	0	3	0
<i>Sansevieria trifasciata</i>	1	1	1	1	0	2	0	0	0	0	0	0	3	0
<i>Calamus australis</i>	1	1	1	1	1	2	1	0	1	1	1	0	3	1
<i>Chamaedorea elegans</i>	0	1	1	1	1	2	1	0	1	1	1	0	3	0
<i>Elaeis guineensis</i>	1	0	1	1	1	2	1	0	1	1	1	0	3	?
<i>Tradescantia zebrina</i>	0	0	1	1	0	2	0	0	0	0	1	0	3	0
<i>Calathea mirabilis</i>	?	0	?	1	0	2	1	0	0	0	1	1	?	1
<i>Xyris longiscapa</i>	1	1	1	1	0	1	0	0	0	0	0	0	3	?
<i>Zea mays</i>	1	0	1	1	0	2	0	0	0	0	1	0	3	1
<i>Eschscholzia californica</i>	0	0	0	0	0	2	1	0	3	1	1	1	0	0
<i>Stephania hernandiifolia</i>	?	0	0	0	0	2	1	1	0	0	1	2	0	0
<i>Caltha palustris</i>	0	1	?	1	0	2	1	0	0	0	1	2	0	1
<i>Trifolium repens</i>	0	1	1	1	0	2	1	0	3	6	1	1	0	0
<i>Astragalus cicer</i>	0	0	0	0	0	2	1	0	3	1	1	1	0	1
<i>Prunus sibirica</i>	0	0	0	0	0	2	1	0	0	0	1	1	0	1
<i>Pelargonium betulinum</i>	?	0	0	0	0	2	1	0	0	4	1	2	0	1
<i>Tropaeolum majus</i>	1	0	1	0	0	2	1	1	0	4	1	2	0	1
<i>Muehlenbeckia platyclada</i>	?	0	0	1	0	2	1	0	0	0	1	1	0	1
<i>Polemonium caeruleum</i>	0	0	1	0	0	2	1	0	3	1	1	1	0	0
<i>Kohleria bogotensis</i>	1	0	1	0	0	2	1	0	0	0	1	1	0	0
<i>Senecio serpens</i>	1	1	?	0	0	0	0	0	0	0	?	?	?	0

4.2.4 Multivariate analysis of leaf characters

A distance matrix was constructed using Gower's Generalized Distance Measure with the *daisy* function in package **cluster** in R version 3.1.2 (Maechler et al., 2015). Because all characters were treated as nominal, the distance measure is the same as a simple matching distance, in which each character either contributes a value of 1 if the two taxa being compared have the same state or 0 if they do not. The distance matrix was then used as the basis for a principal-coordinates analysis using the *pcoa* function in package **ape** (Paradis et al., 2004). A Cailliez correction (Legendre & Legendre 1998) was applied to remove negative eigenvalues, and the first three principal coordinate axes – corresponding to about 34% of the variation – were retained for subsequent analysis. Taxa were plotted in the rotated morphospace and colored according to taxonomic order. The first three axes and taxa were plotted in 3D morphospace using the R package **scatterplot3d** (Ligges & Mächler, 2003).

4.3 Results

A vast diversity of leaf primordia exists across angiosperms at the developmental and morphological level. Even within a single family, Araceae, diversity can be extreme. Figure 4.1 shows some of the leaf primordium forms in Araceae, with two examples from outside Araceae. The evolution of specific components of leaf development, morphology and anatomy is addressed in the results of ancestral character state reconstruction. Then, results of the morphospace analysis will examine leaf development in a more global context.

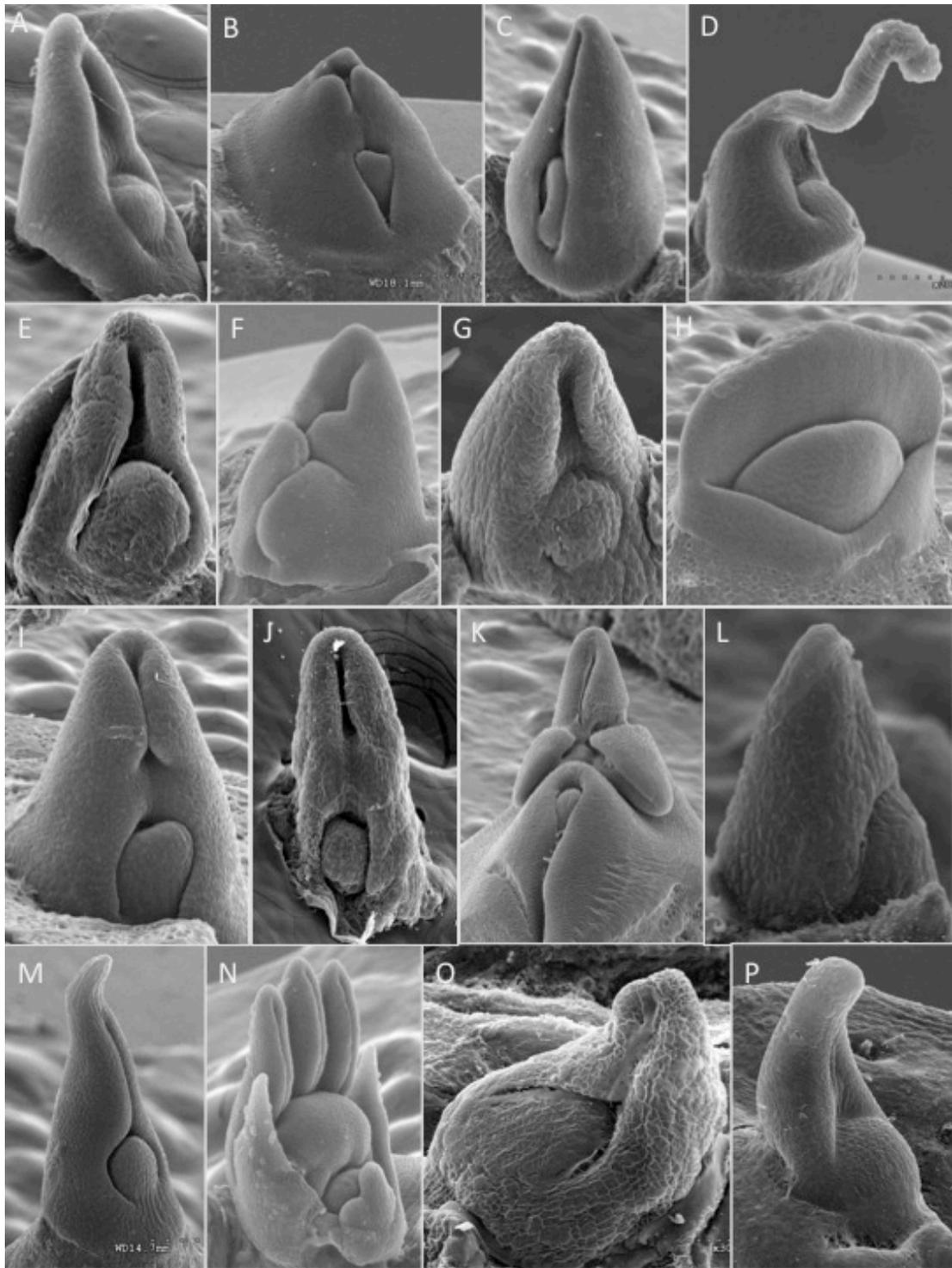


Figure 4.1 Leaf primordium diversity. A. *Alocasia macrorrhiza*, B. *Amorphophallus henryi*, C. *Amydrium zippelianum*, D. *Anthurium clavigerum*, E. *Epipremnum pinnatum*, F. *Gonatopus boivinii*, G. *Lasia spinosa*, H. *Narcissus pseudonarcissus*, I. *Nephtytis swainei*, J. *Orontium aquaticum*, K. *Pinellia pedatisecta*, L. *Stuednera colocasiifolia*, M. *Syngonium podophyllum*, N. *Trifolium repens*, O. *Typhonium trilobatum*, P. *Xanthosoma atrovirens*.

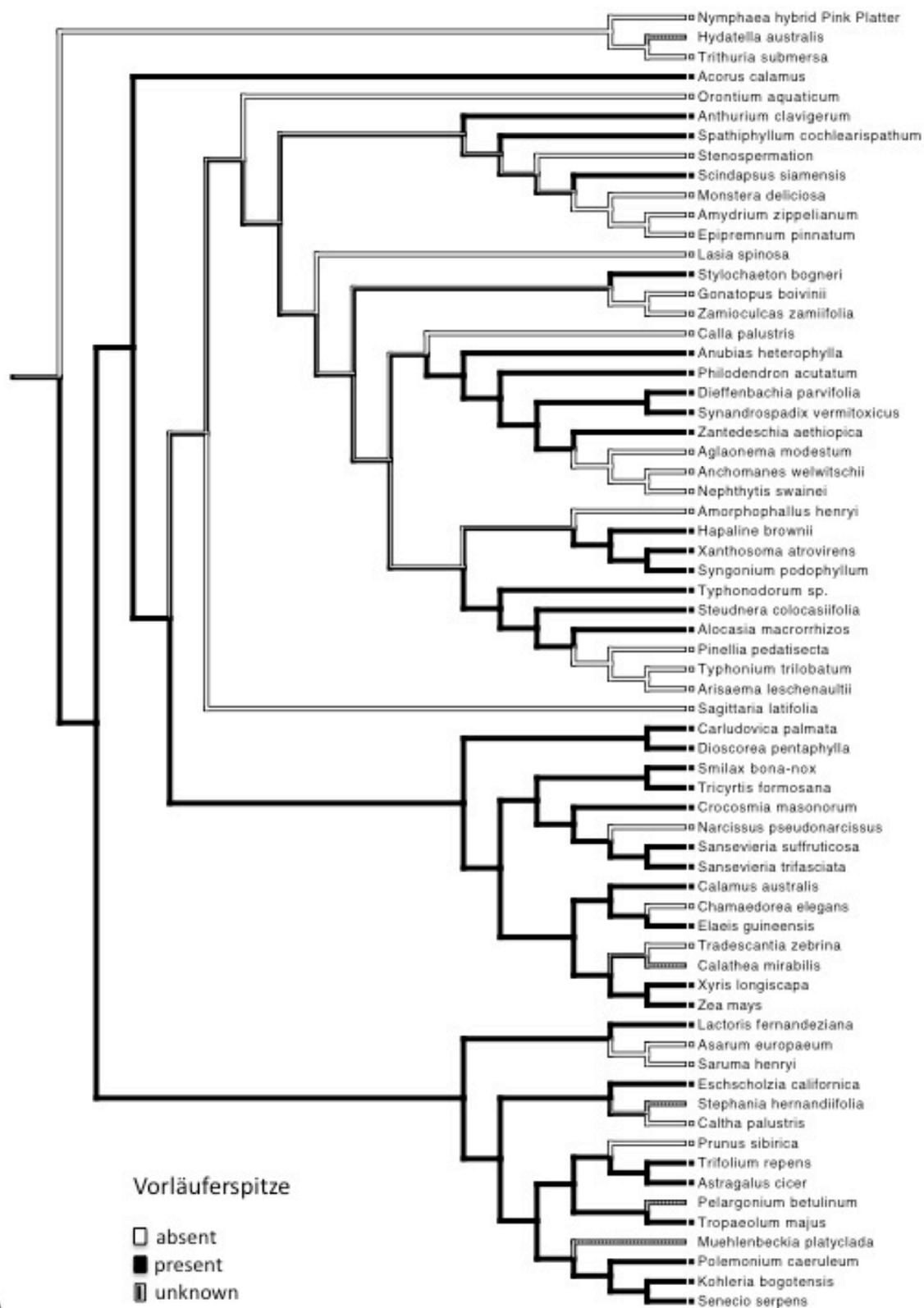
4.3.1 *Ancestral character state reconstruction*

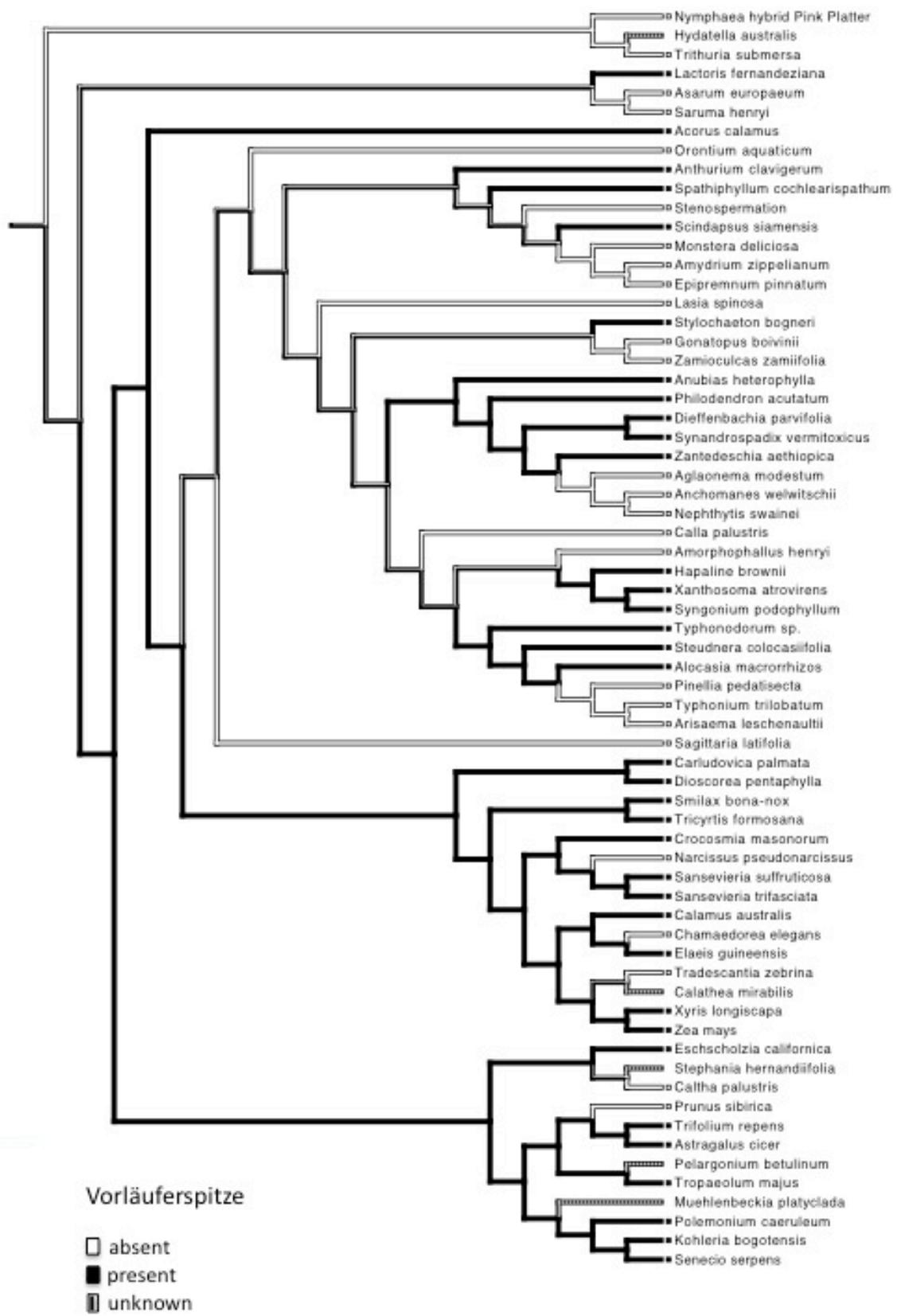
Ancestral character states are discussed in terms of the results of the maximum parsimony analysis. The evolutionary history of characters was not greatly affected by the differing tree topologies, except in the case of the *Vorläuferspitze*, shown in Figure 4.2. Differences in the evolutionary history of other characters between the two topologies are mentioned where they occur

Character 1 - *Vorläuferspitze*

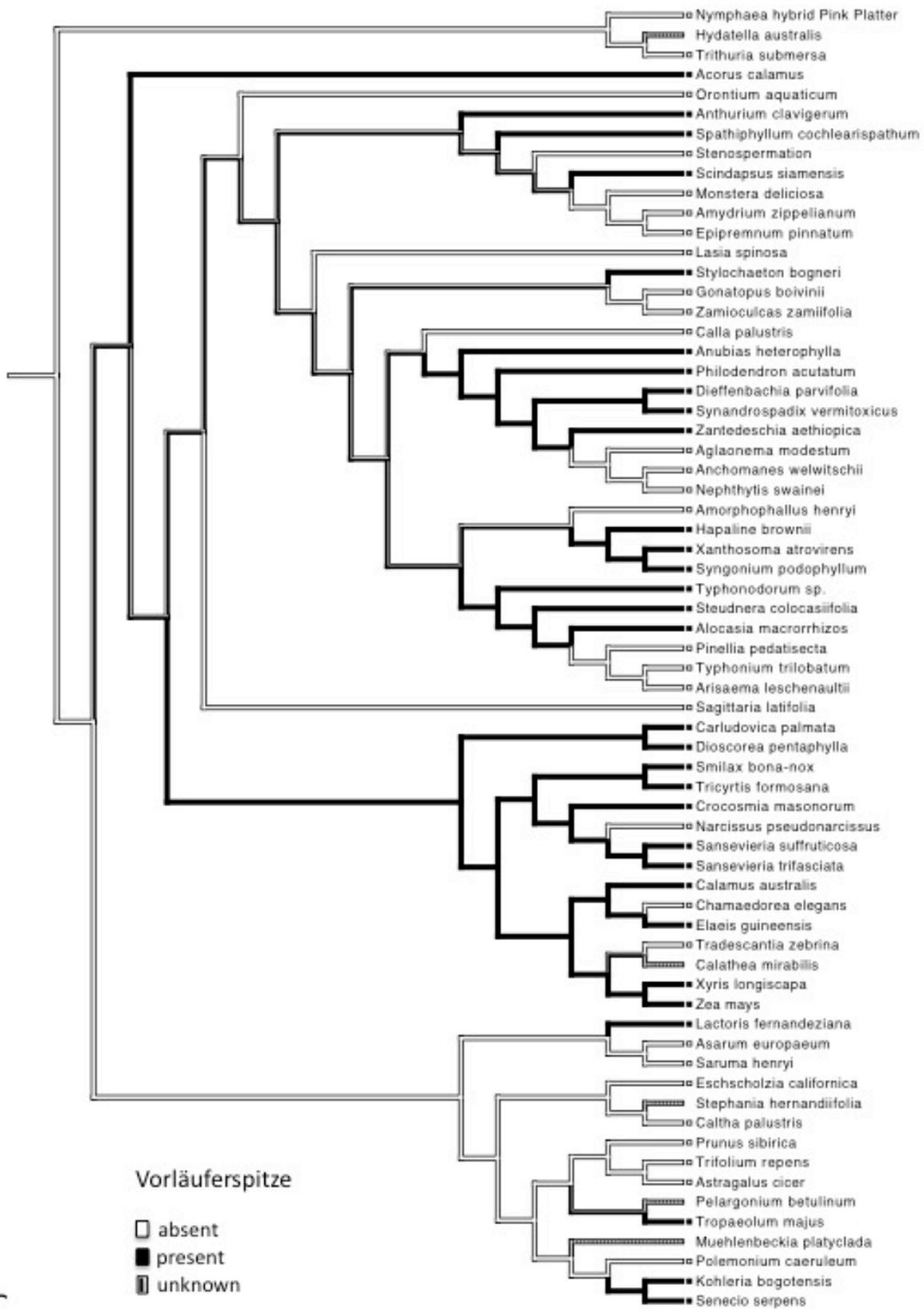
The distinction between the leaf apex and a *Vorläuferspitze* has been considered both a crucial concept for the determination of homology among bladed leaves (Kaplan, 1973) and as simply arbitrary (Rudall and Buzo, 2002). The observations of the present study show that there is a continuum between the two, with a very pronounced and unambiguous *Vorläuferspitze* at one end, and a complete lack of any radial structure at the apex on the other (Figure 4.1D,H). The two extremes occur across angiosperms. Continuous characters are difficult to code in a binary manner and some level of arbitrariness in assigning one state or the other is unavoidable; however, the *Vorläuferspitze* is a salient feature of some leaf primordia and thus was deemed worthy of analysis. In cases where the presence or absence of a *Vorläuferspitze* was unclear the character state was scored as ambiguous.

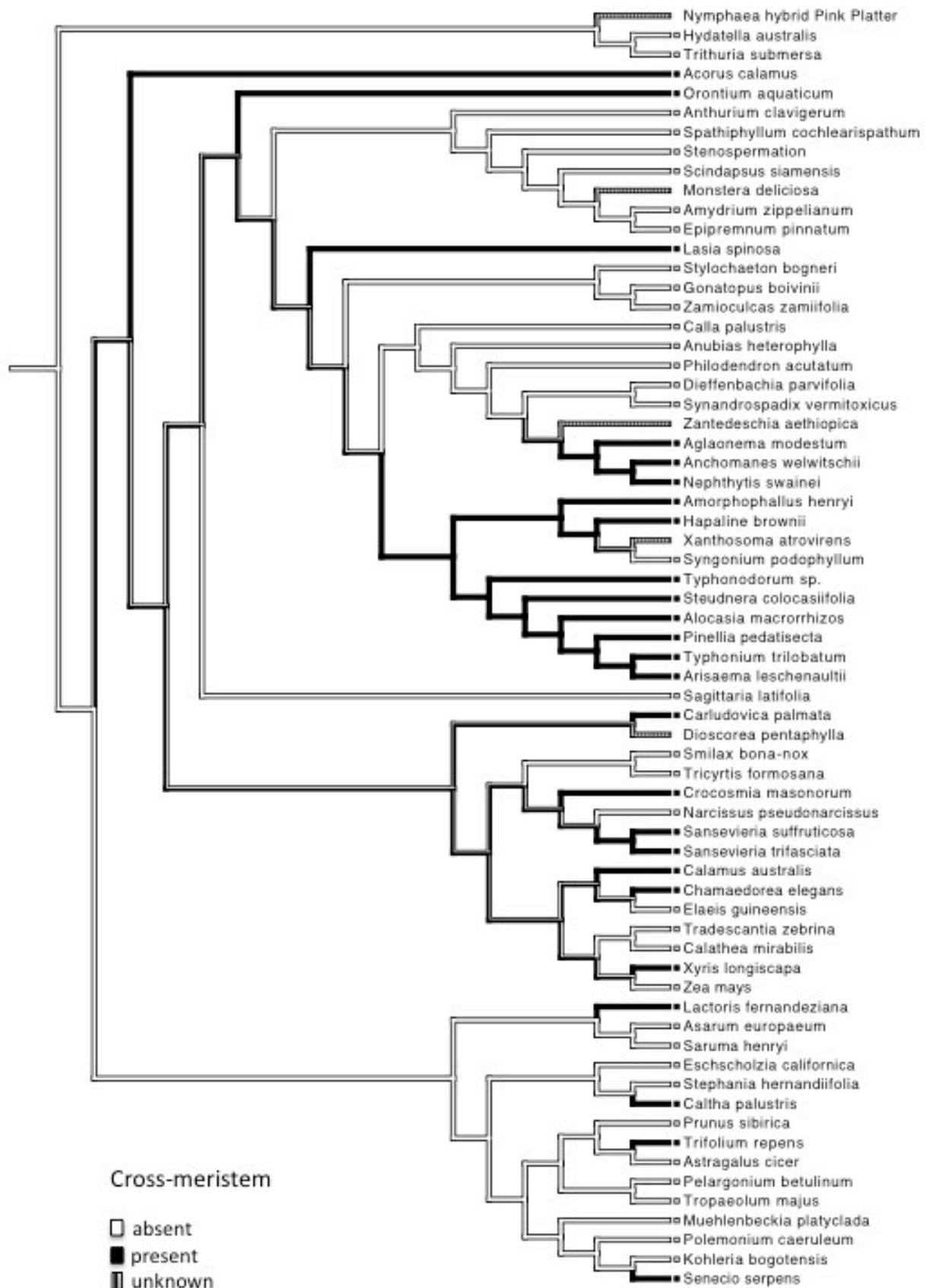
According to the Zeng et al. (2014) tree topology, the presence of a *Vorläuferspitze* is the ancestral character state for the (monocots (magnoliids, eudicots)) clade (Figure 4.2A). In the APG tree, the presence of a *Vorläuferspitze* is the ancestral character state for a clade containing monocots and eudicots, but its presence is ambiguous in the common ancestor of magnoliids (Figure 4.2B).

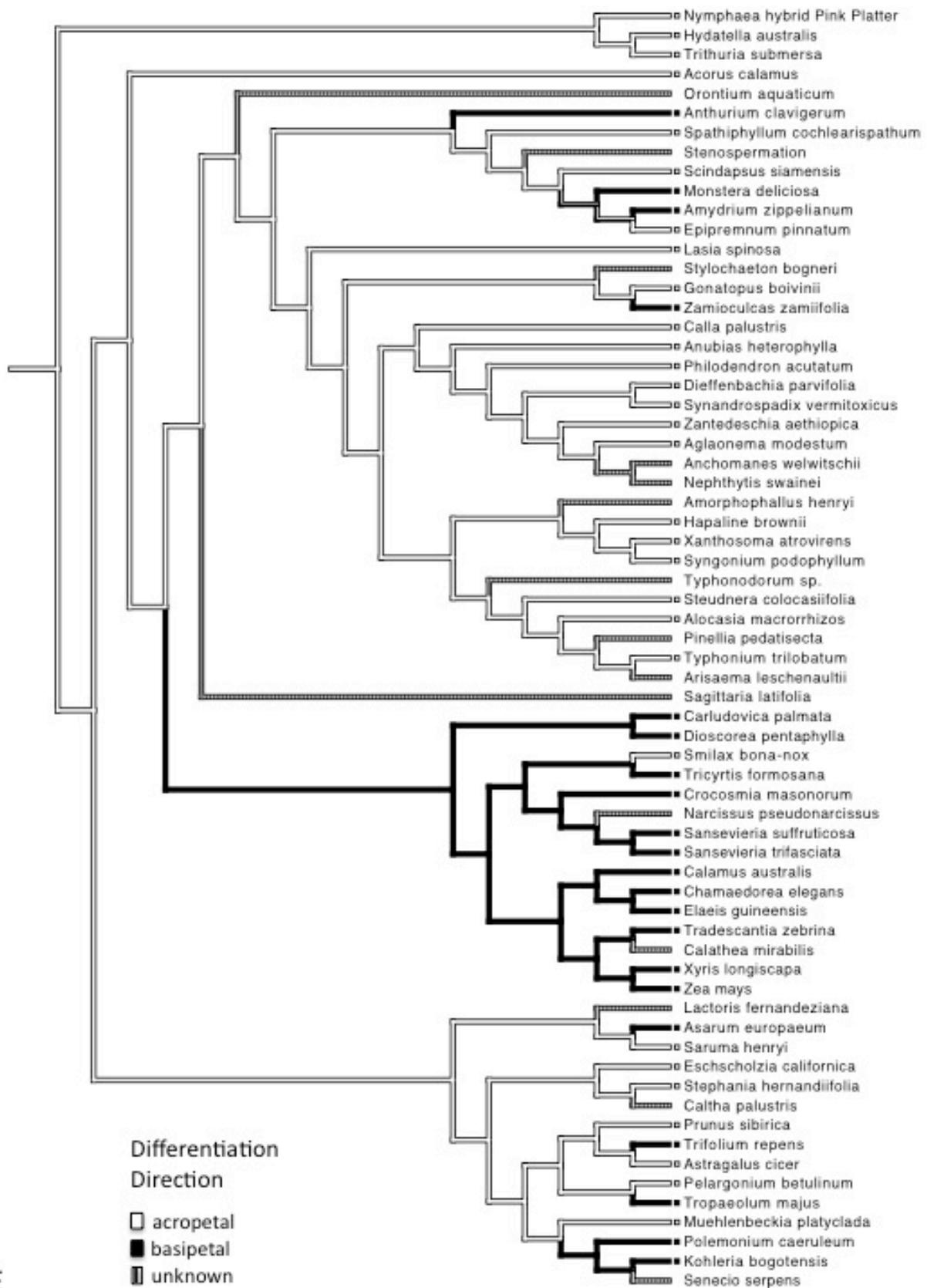




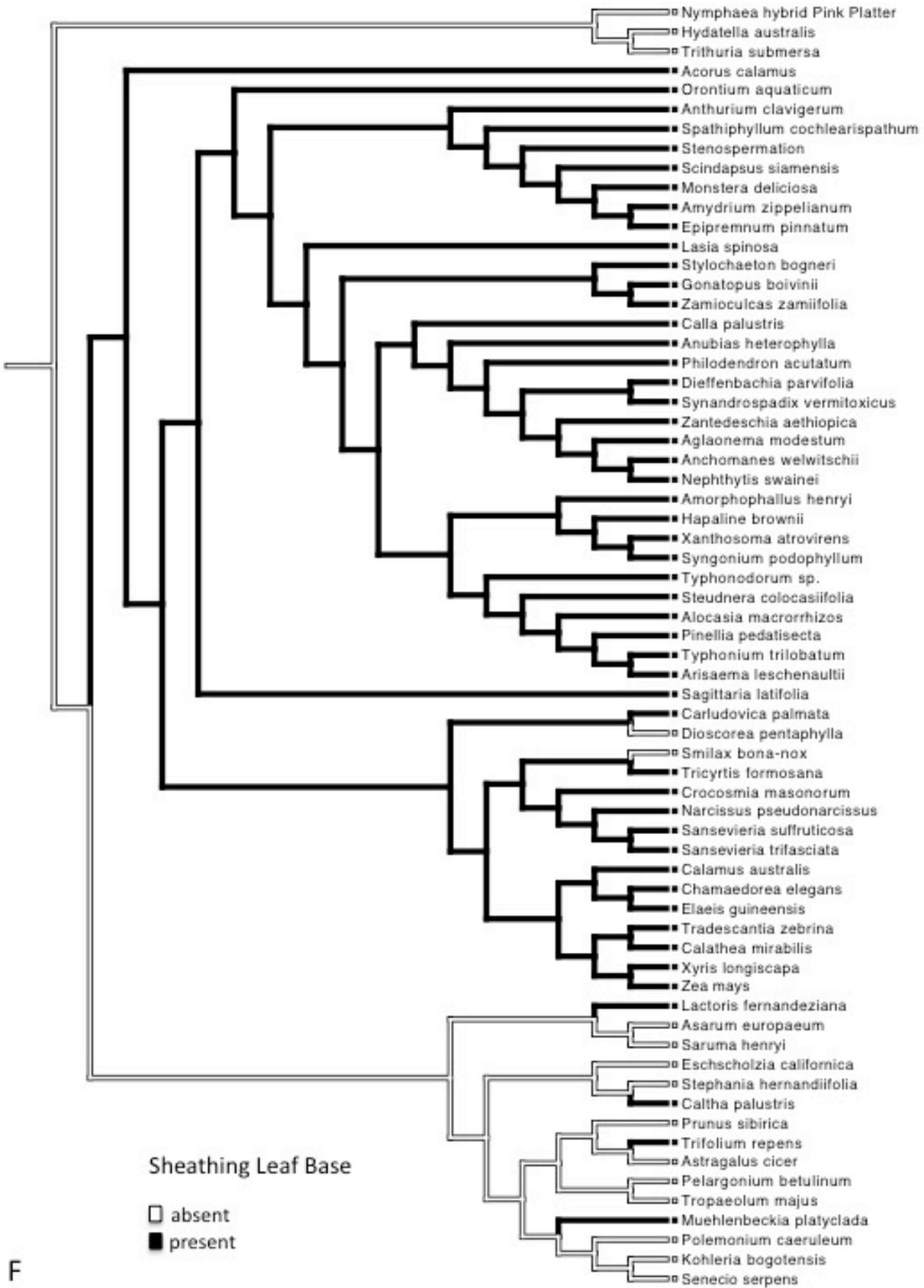
B

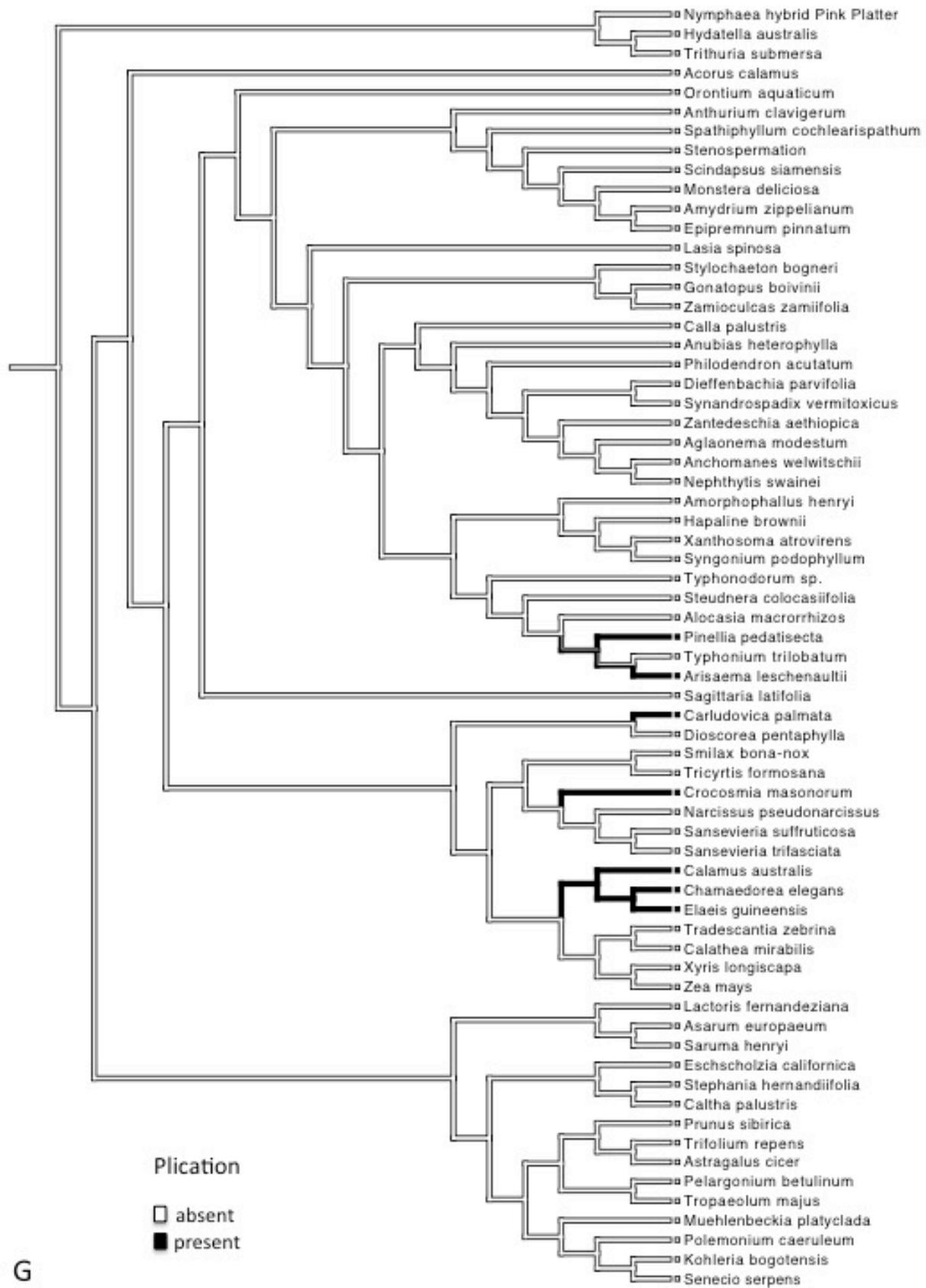


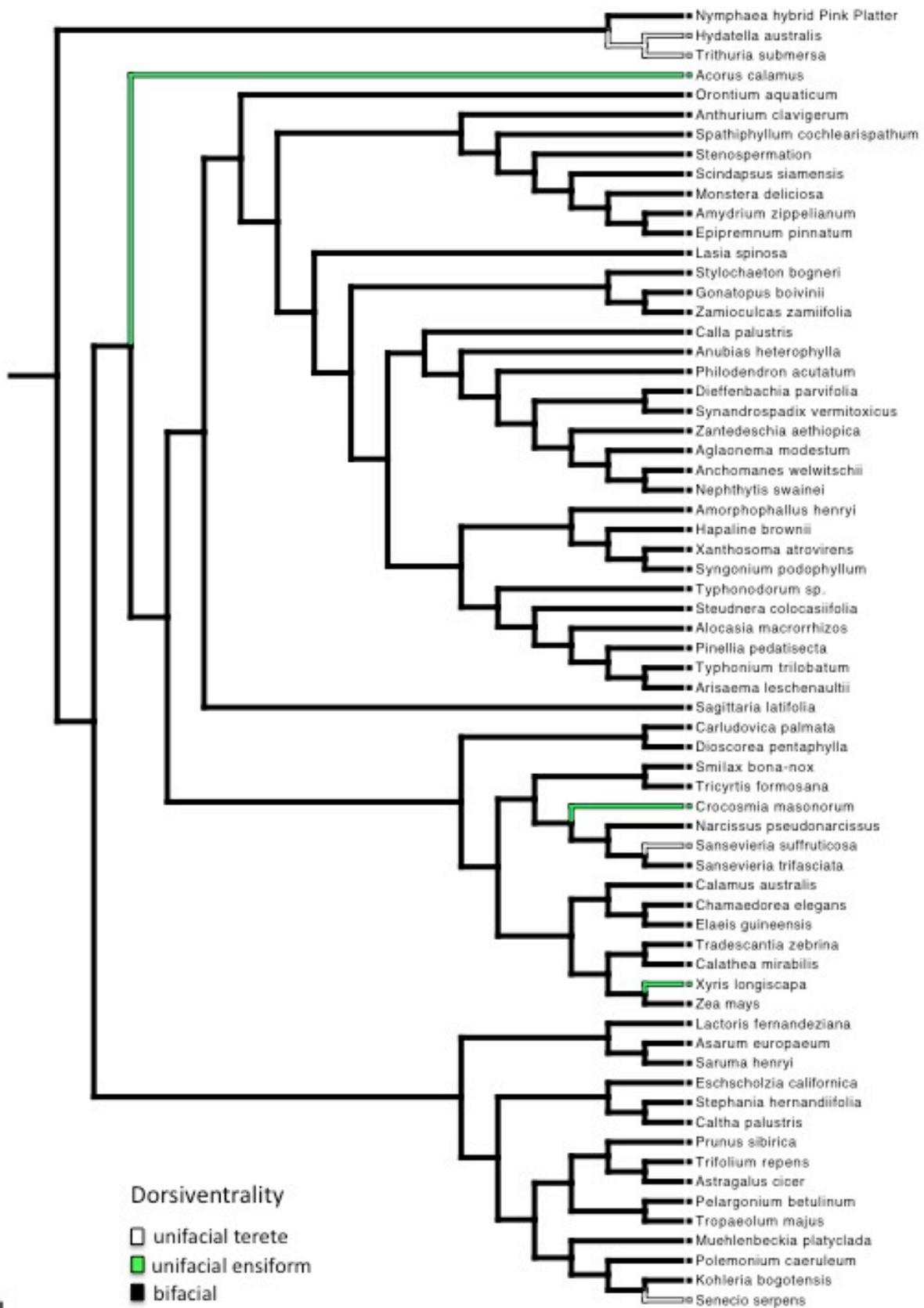




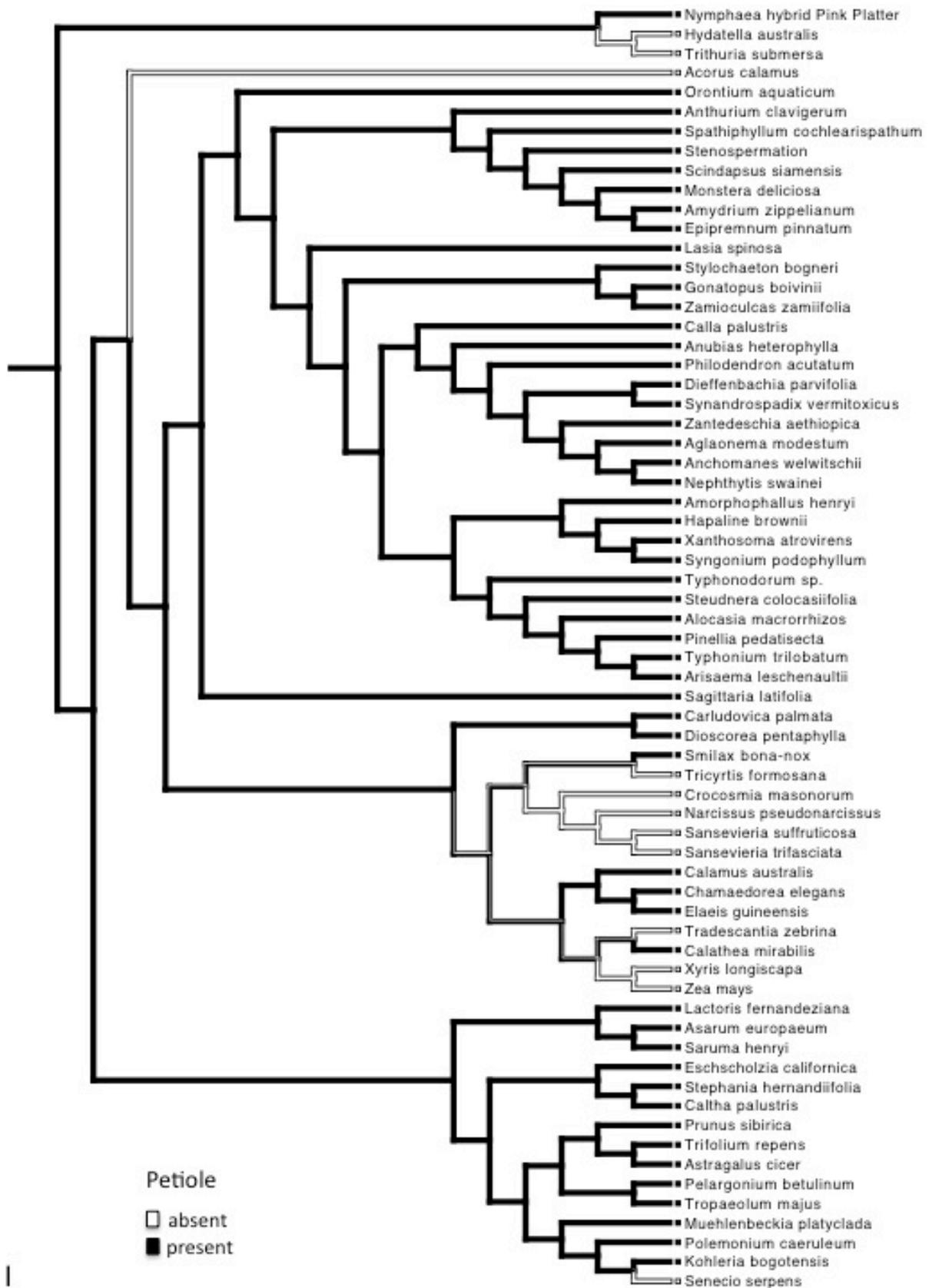
E

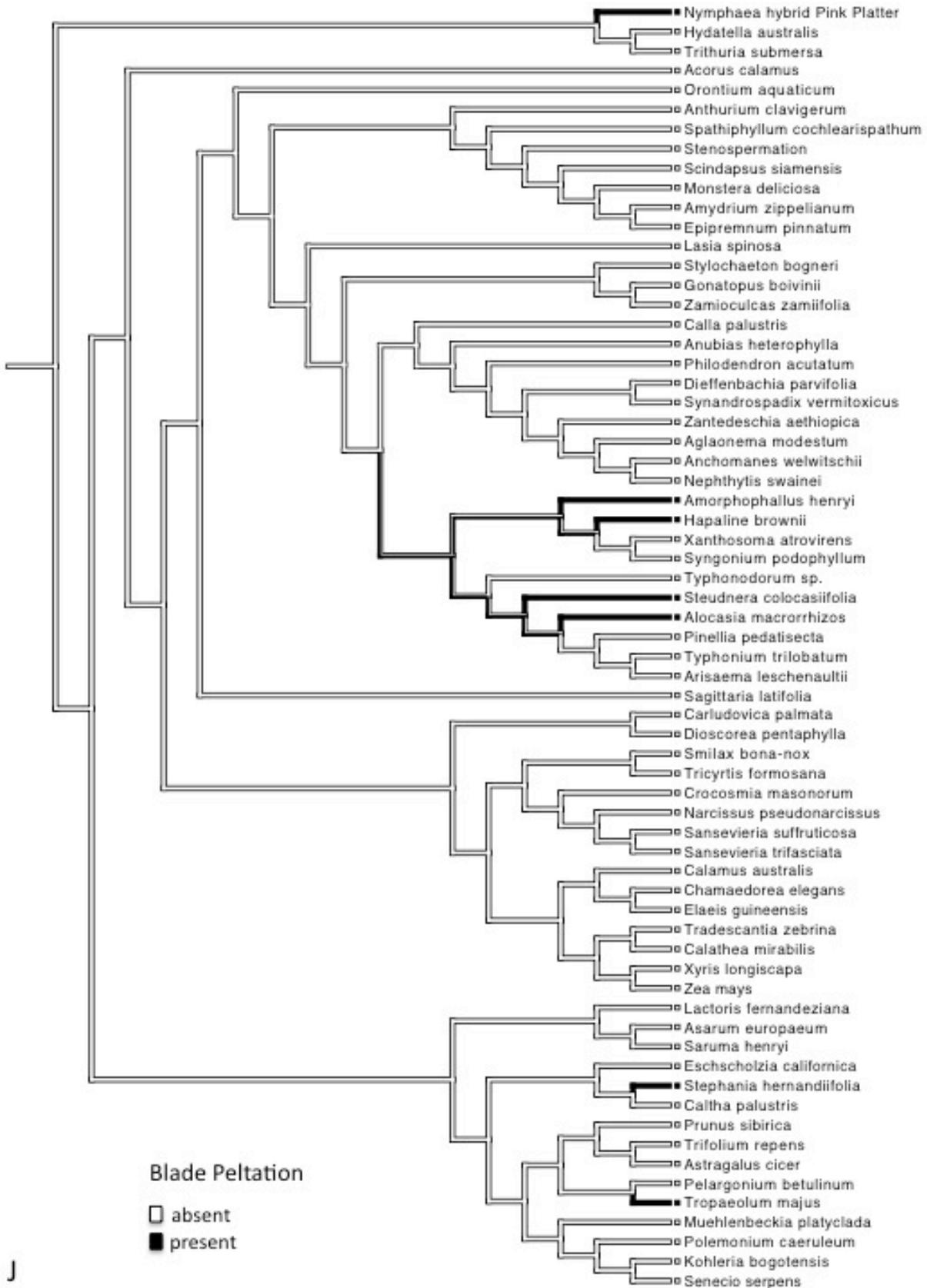




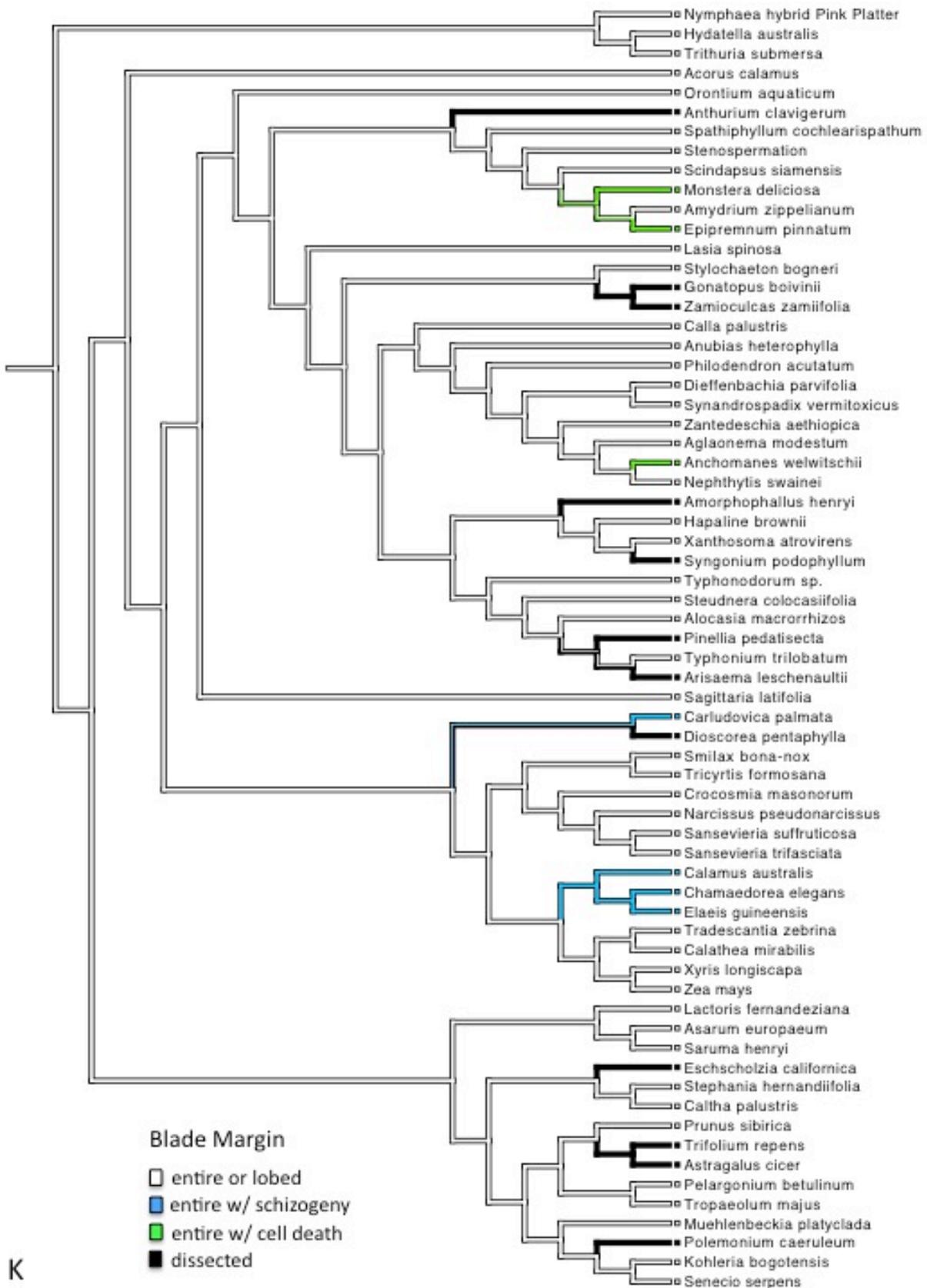


H

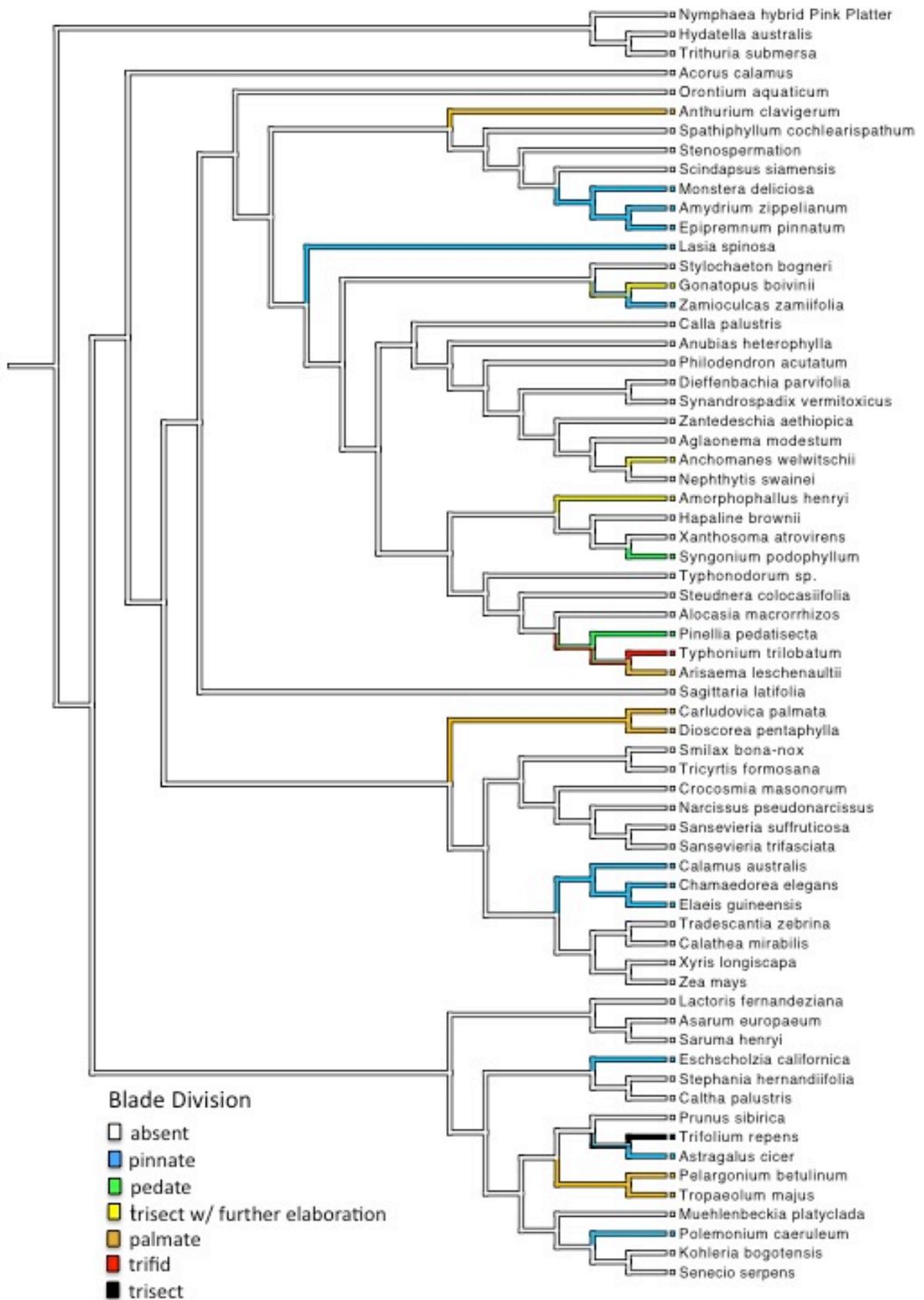




J

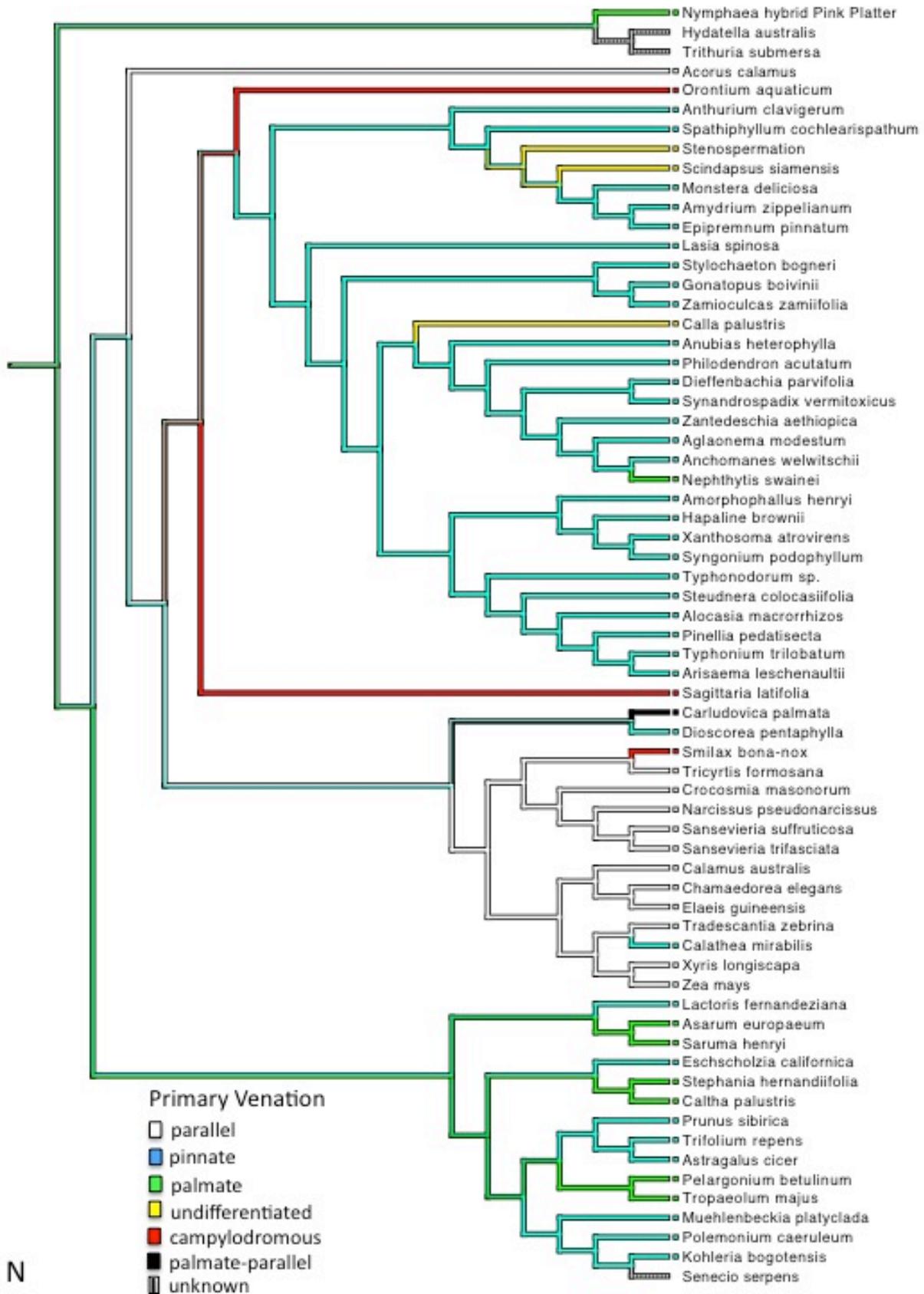


K



L

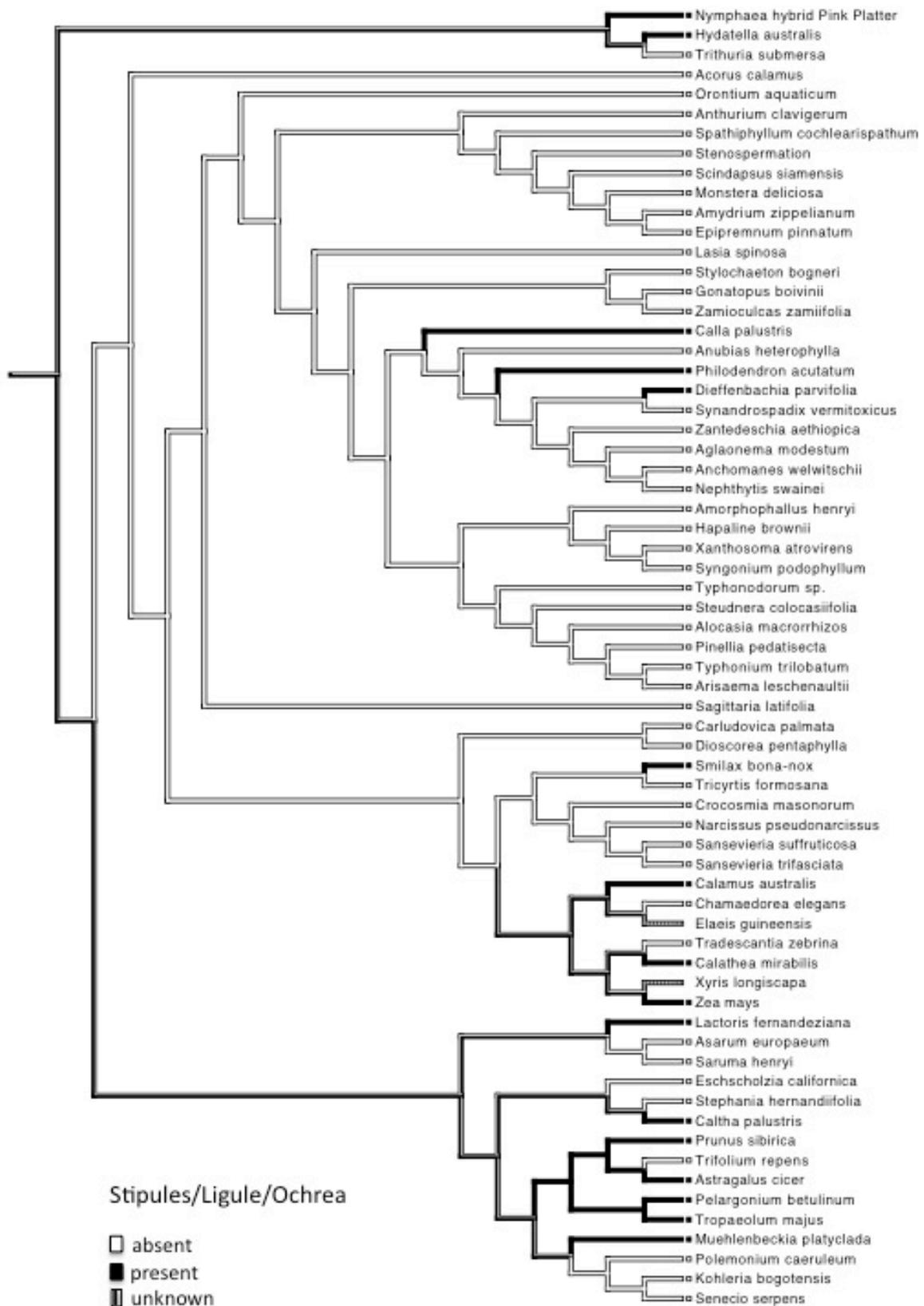




N



O



P

Figure 4.2 Maximum parsimony ancestral character state reconstruction of 14 unordered multistate leaf developmental, morphological and anatomical characters in two different phylogenies of angiosperms (APG III, 2009; Zeng et al., 2014). A. *Vorläuferspitze* in Zeng et al. (2014). B. *Vorläuferspitze* in APG. C. Modified *Vorläuferspitze* coding in Zeng et al. (2014). D-P Zeng et al. (2014) phylogeny. D. Cross-meristem, E. Differentiation direction, F. Sheathing leaf base, G. Plication, H. Dorsiventrality, I. Petiole, J. Blade peltation, K. Blade Margin, L. Blade division, M. Midrib, N. Primary venation, O. Higher order venation, P. Stipules, ligule, ochrea.

In a separate analysis, dissected leaves achieved through blastozone fractionation that possess a *Vorläuferspitze* that will ultimately become a terminal leaflet were scored as lacking a *Vorläuferspitze*. This coding scheme is in the matrix shown in Table 4.3, and was used for the multivariate analysis (below). Species that were affected by this recoding were *Eschscholzia californica*, *Astragalus cicer*, *Trifolium repens* and *Polemonium caeruleum*. This changed the evolutionary history of the *Vorläuferspitze* dramatically as shown in the Zeng et al. (2014) topology (Figure 4.2C). In this case, the absence of a *Vorläuferspitze* is the ancestral character state for angiosperms, although *Vorläuferspitzen* evolved independently numerous times outside the monocot clade. The ancestral state for monocots and Alismatales is ambiguous; however, the presence of a *Vorläuferspitze* is a synapomorphy for a clade in monocots containing Pandanales and all subsequently diverging orders. This evolutionary scenario for the *Vorläuferspitze* is much closer to what Knoll (1948) and Kaplan (1973) predicted, but there are nontrivial issues associated with this analysis besides the issue of the *Vorläuferspitze* being a continuous character.

The *Vorläuferspitze* was not defined in terms of function or the leaf morphology of the mature leaf, although there have been discussions about its functional significance (see discussion) (Kaplan, 1973). In the pinnately dissected leaf of *Eschscholzia californica* and the simple peltate leaf of *Tropaeolum majus*, the apex of the leaf primordium is radial, but later in

development becomes flattened (Becker et al., 2005; Gleissberg et al., 2005). In *Narcissus pseudonarcissus* there is no radial structure on the leaf primordium, yet later in development the tip of the leaf is clearly distinct and transparent, which is not a *Vorläuferspitze* in the strict sense, but is definitely a separate zone. Furthermore, the development of a *Vorläuferspitze* is not linked with a specific developmental stage. In certain species of *Anthurium*, the *Vorläuferspitze* is the first structure to differentiate on the primordium, while in *Crocasmia masonorum* the *Vorläuferspitze* differentiates after plications have formed (Henriquez et al., in prep; Rudall, 1990). Finally, the presence of *Vorläuferspitzen* on the tips of leaflets of dissected leaves in *Dioscorea pentaphylla* (Periasamy and Muruganathan, 1985) and *Anthurium polyschistum* (Henriquez et al., in prep) extends the question of similarity and ultimately homology beyond monocots and dicots to leaves and leaflets. Thus, the difference between a leaf apex, a leaflet apex, and a *Vorläuferspitze* is not at all clear from a strictly morphological perspective at early stages of development (see further discussion below).

Character 2 – Cross-meristem

The cross-zone (from *Querzone*: Kaplan, 1997; Troll, 1932) is a zone that appears to connect separate margins across the adaxial surface, whereas the adaxial meristem, or *Ventralmeristem* (Roth, 1949; Troll, 1939) is the outward growth of the adaxial surface of the leaf primordium. Both structures are associated with a shift in symmetry and are here considered together under the term cross-meristem (Rudall and Buzgo, 2002). The formation of a cross-meristem gives rise to a multitude of foliar structures including petioles, unifacial blades, ochreas, ligules, peltate blades, stipules, etc. (Gleissberg et al., 2005; Ichihashi et al., 2011). Geeta (2003) included this character by stating the presence or absence of the foliar structures that result from it. However, leaf primordia of various taxa possessing one or another of these

structures exhibit different levels of development of the cross-meristem at equivalent stages. Thus, the presence or absence of a cross-meristem is an issue of heterochrony across the taxa surveyed. The character describes whether a cross-meristem is present before the emergence of the next leaf primordium at p0. The cross-meristem has been shown to develop late in eudicot species with a peltate blade (Kaplan, 1997; Kim et al., 2003a). However, in Araceae the peltate blade of *Amorphophallus* develops a cross-meristem very early in development, and other species that do not have peltate leaf morphology similarly develop cross-meristems very early on.

The early establishment of a cross-meristem during leaf ontogeny was not present at the base of the tree and is unlikely for *Amborella* and so was absent from the common ancestor of angiosperms; it is ambiguous for monocots. However, an early cross-meristem characterizes the *Anchomanes* and *Dracunculus* clades in Araceae (Figure 4.2D).

Character 3 - Differentiation direction

The direction of tissue differentiation can be described in multiple ways: 1) histogenesis, whereby densely cytoplasmic, eumeristematic cells become vacuolated and progressively larger; 2) trichome formation, which signifies the end of the meristematic phase of the cell; 3) vein differentiation; and 4) morphogenesis such as the formation of leaflets (Kaplan, 1997).

Unfortunately, the various indicators of tissue differentiation are not mutually exclusive. In *Astragalus cicer*, for example, leaflets arise in an acropetal differentiation direction while trichomes form in basipetal direction (Kaplan, 1997). Here, morphogenesis was used as the primary determinant of the direction of differentiation and other indicators were used only when morphogenetic differentiation directions were unclear. Because of the dynamic nature of tissue differentiation and the conflict between the indicators, a clear phylogenetic signal for this

character was not expected. Nonetheless, a basipetal differentiation direction is a synapomorphy for a clade within monocots containing Pandanales and all subsequently diverging orders, with a reversal in *Smilax* (Figure 4.2E). Monocots have typically been described as differentiating basipetally, while aroids have been noted for their acropetal differentiation direction (Kaplan, 1973). Basipetal differentiation also occurs in several genera of Araceae and eudicots.

Character 4 – Sheathing leaf base

The presence of a sheathing leaf base is the ancestral character state for monocots. Independent evolution of a non-sheathing leaf base occurred in *Dioscorea* and *Smilax* (Figure 4.2F).

Character 5 – Plication

Plication, or folding of tissue, of the leaf primordium occurs only in monocots and only sporadically therein. Plication is a synapomorphy for Araceae and arose independently in *Carludovica*, *Crocasmia*, *Pinellia* and *Arisaema* (Figure 4.2G).

Character 6 – Dorsiventrality

A bifacial blade is the ancestral character state of angiosperms, including monocots, in this study (Figure 4.2H). However, the lack of sampling of terete- and ensiform-leaved taxa in monocots precludes coming to a firm conclusion as to the dorsiventrality (or lack thereof) of the ancestral monocot blade. Of particular interest are the extremely varied forms of leaves in Alismatales. The presence of inverted bundles in leaves of some species of *Sagittaria*, *Cymodocea*, *Potamogeton*, *Stratiotes*, *Enhalus*, *Butomus* and *Eichhornia* (Arber, 1921) must be taken into account in future studies. Tofieldiaceae appears to be the sister taxon to all other families within Alismatales (Ross et al., 2015) and they have bifacial, ensiform, and rarely unifacial leaves, and their inclusion may change the results seen here. A detailed look at leaf

developmental morphology in Alismatales with recent molecular phylogenies in mind (Ross et al., 2015) is highly desired.

Character 7 – Petiole

The presence of a petiole is the ancestral character state for angiosperms, including monocots. Within monocots, the presence of a petiole is ambiguous in the common ancestor of the sister taxon to Pandanales (Figure 4.2I).

Character 8 – Blade peltation

Within monocots, blade peltation is equivocally a synapomorphy for the *Dracunculus* clade in Araceae (Figure 4.2J). Interestingly, the early development of a cross-meristem is a synapomorphy for the *Dracunculus* clade, which may have predisposed this group to peltate blade formation (Figure 4.2D). Peltate blades have arisen independently several times outside of monocots.

Character 9 – Blade margin

This character was coded to account for the various mechanisms by which ultimate leaf form is achieved (blastozone fractionation, schizogeny and cell death). A entire or lobed blade margin is the ancestral state for angiosperms (Figure 4.2K). Previous studies have arrived at a similar conclusion, albeit without the lobed condition (Bharathan et al., 2002). Simple leaves giving rise to dissected forms through schizogeny occurs only in monocots. This mode is a synapomorphy for Arecaceae, and arose independently in *Carludovica*. Plication, which is a precursor to schizogeny, expectedly follows the same evolutionary history in these two taxa (Figure 4.2G). Plication is known from other monocots not included here, such as Hypoxidaceae and Orchidaceae, etc.

Likewise, simple leaves giving rise to dissected forms through cell-death occurs only in monocots, and in this study, only in Araceae (Aponogetonaceae was not included). Cell death arose at least twice independently in Araceae, but this number is expected to increase as other taxa unrelated to the examples included here are added. Confirmation that cell death is the developmental mechanism is important for such studies. This issue is highlighted by lobe formation in *Amydrium zippelianum*. Lobe formation in the genus *Amydrium* was expected to be through cell death (as in the closely related genus *Monstera*) because in many of the genera in subfamily Monsteroideae fenestration occurs via cell death (Gunawardena and Dengler, 2006; Mayo et al., 1997). However, I found that *Amydrium zippelianum* forms lobes through blastozone fractionation. Blastozone fractionation has evolved independently many times throughout monocots and eudicots.

Character 10 – Blade division

Blade division was included to study the evolution of ultimate leaf morphology without consideration of the underlying mechanism. A blade with no division is present at the base of the tree and is the case for *Amborella* and so is the ancestral character state for angiosperms (Figure 4.2L). More taxa need to be added to those clades for which pinnately divided leaves are a synapomorphy - i.e. (*Monstera* (*Amydrium*, *Epipremnum*)), and Arecaceae - to confirm this result. The same is true for palmate blade division in Pandanales and malvids as there is an obvious lack of sampling, but we can estimate that palmately divided leaves are not the ancestral character state for Pandanales.

Character 11 – Midrib

The midribs of dicots and many monocots are constructed differently, i.e., the monocot midrib is multistranded, whereas in dicots it is not (Inamdar et al., 1983). The molecular basis of

midrib formation may also differ between monocots and dicots (discussed below).

Furthermore, ensiform leaves produce secondary or “pseudo” midribs (Arber, 1925); such leaves are scored following whether or not they appear to have a midrib, a strictly structural view being taken. For feasibility, a strictly structural view is taken in which a midrib is either present or absent. The presence of a midrib is a synapomorphy for angiosperms (Figure 4.2M). The loss of a midrib is a synapomorphy for Asparagales and was independently lost in *Xyris*.

Character 12 – Primary venation

Venation patterns are extremely complex across angiosperms, as seen in the Manual of Leaf Architecture (Leaf Architecture Working Group, 1999). In the current study, venation patterns were greatly simplified, as detailed description for each taxon would be unfeasible. That being said, pinnate venation is unequivocally the ancestral character state of a clade within Araceae containing a majority of species in the family, while parallel primary venation is a synapomorphy for the clade in monocots that forms the sister taxon to Pandanales. Parallel primary venation evolved independently in *Acorus*. The ancestral primary venation state for all other clades is ambiguous (Figure 4.2N).

Character 13 – Higher-order venation

The cautionary statement regarding primary venation patterns applies equally for higher-order venation patterns (Leaf Architecture Working Group, 1999). Inclusion of this character was debated but given the importance of higher order venation in considering Araceae ‘dicot-like’ (Kaplan, 1973; Keating, 2002), the character was included. Character states other than ‘reticulate’ and ‘parallel’ were coded in Araceae.

Reticulate higher-order venation is a synapomorphy for angiosperms (Figure 4.2O). Parallel higher-order venation occurs only within monocots and is a synapomorphy for

Asparagales and the commelids. It may be a synapomorphy for the larger clade including Liliales, but this is ambiguous. Parallel higher-order venation evolved independently in *Acorus*.

Character 14 – Stipules, ligule, ochrea

Stipules, ligules and ochreae are here considered secondary elaborations of the leaf. The petiole may also be considered a secondary elaboration (Hagemann, 1970), and formation of these foliar structures arise through the action of a cross-meristem. However, petioles are often present without stipules, a ligule or ochrea, which is why petioles were considered separately. The presence of secondary elaborations of the leaf as the ancestral character state for angiosperms is ambiguous. Their absence is a synapomorphy for monocots (Figure 4.2P). These secondary structures have arisen multiple times independently in Araceae, once in Liliales, and are possibly a synapomorphy for the commelinids.

4.3.2 *Multivariate analysis of leaf characters*

The matrix in Table 4.3 was used in the multivariate analysis. The more traditional coding scheme for the *Vorläuferspitze* was used to emphasize the difference between monocot and dicot leaves giving the best chance for this difference to be detected in the multivariate analysis. As mentioned above, the first three principal coordinate axes corresponded to roughly 34% of the variation in leaf characters across angiosperms (Figure 4.3). The first three axes and taxa color-coded by monocot versus dicot were plotted in 3D morphospace (Figure 4.4). Axes were then compared in 2D for further analysis (Figure 4.5).

Results of the multivariate analysis indicate that there is much overlap in leaf developmental morphological morphospace between monocot and dicot taxa (Figure 4.4; Figure 4.5). Clusters in the distribution of taxa in morphospace are correlated with combinations of characters. Outliers include monocot and dicot species that possess a combination of characters

Scree Plot

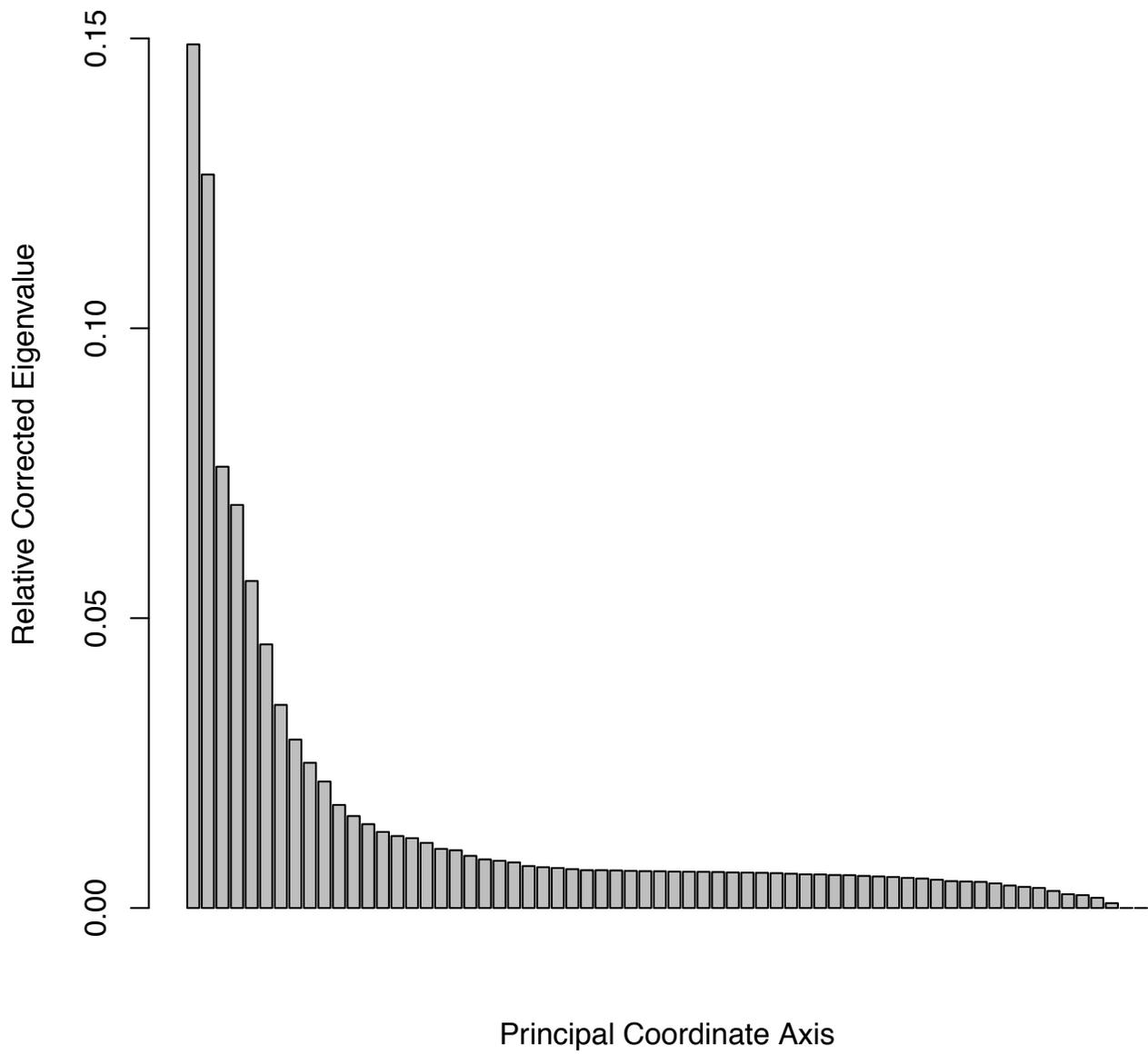


Figure 4.3 Scree plot of PCoA relative corrected eigenvalues.

Principal Coordinate Analysis of Developmental Morphology

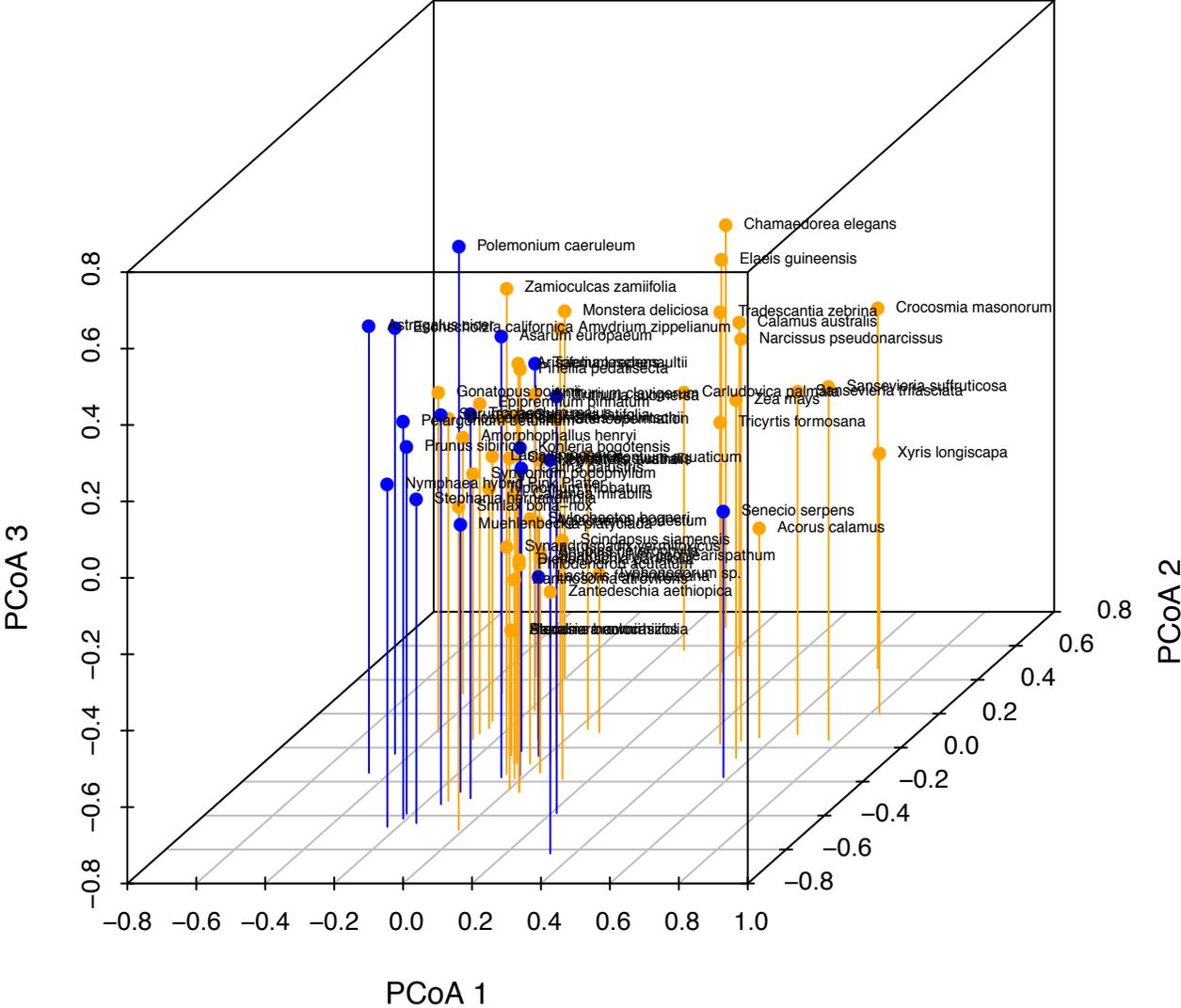
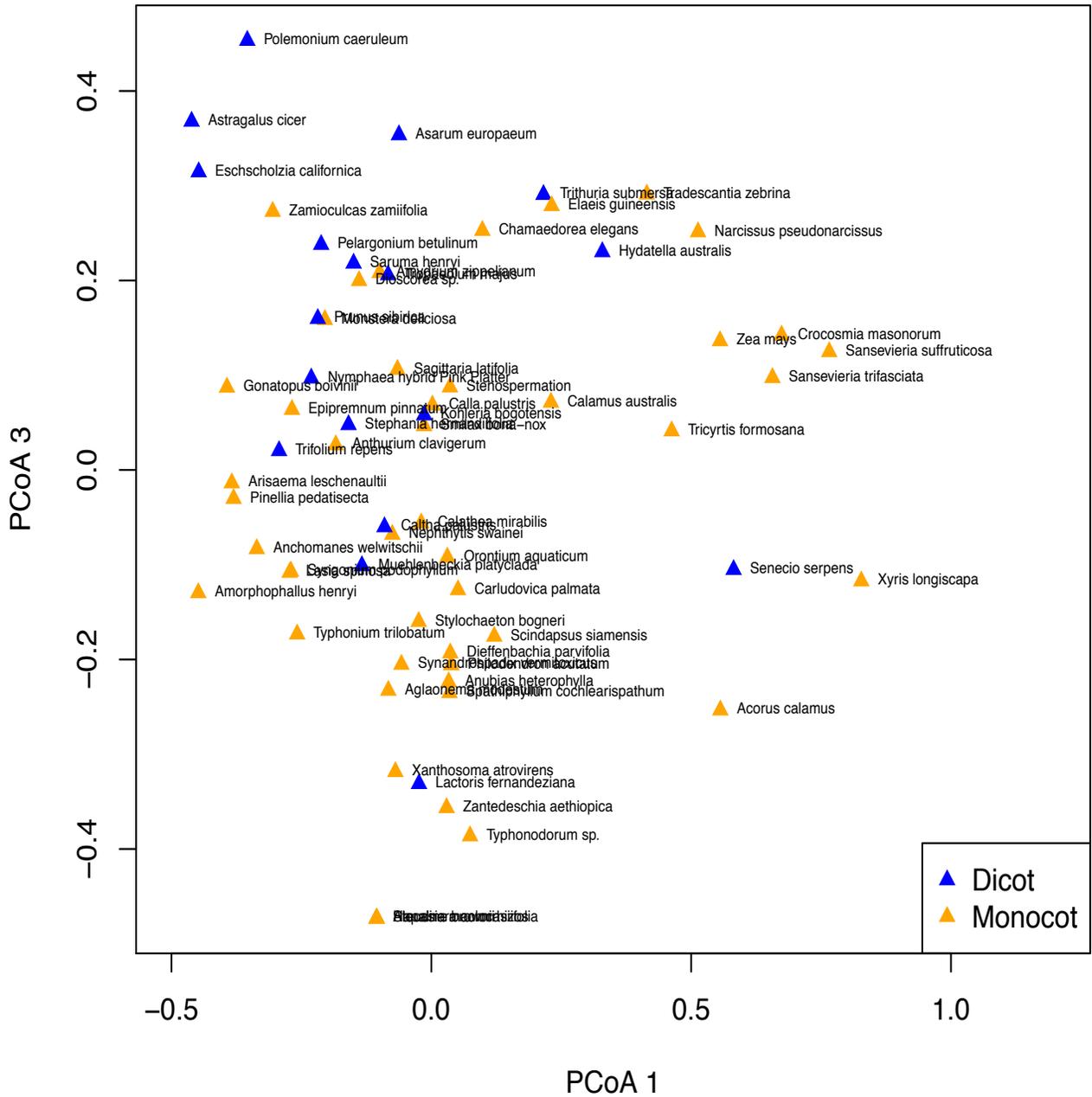


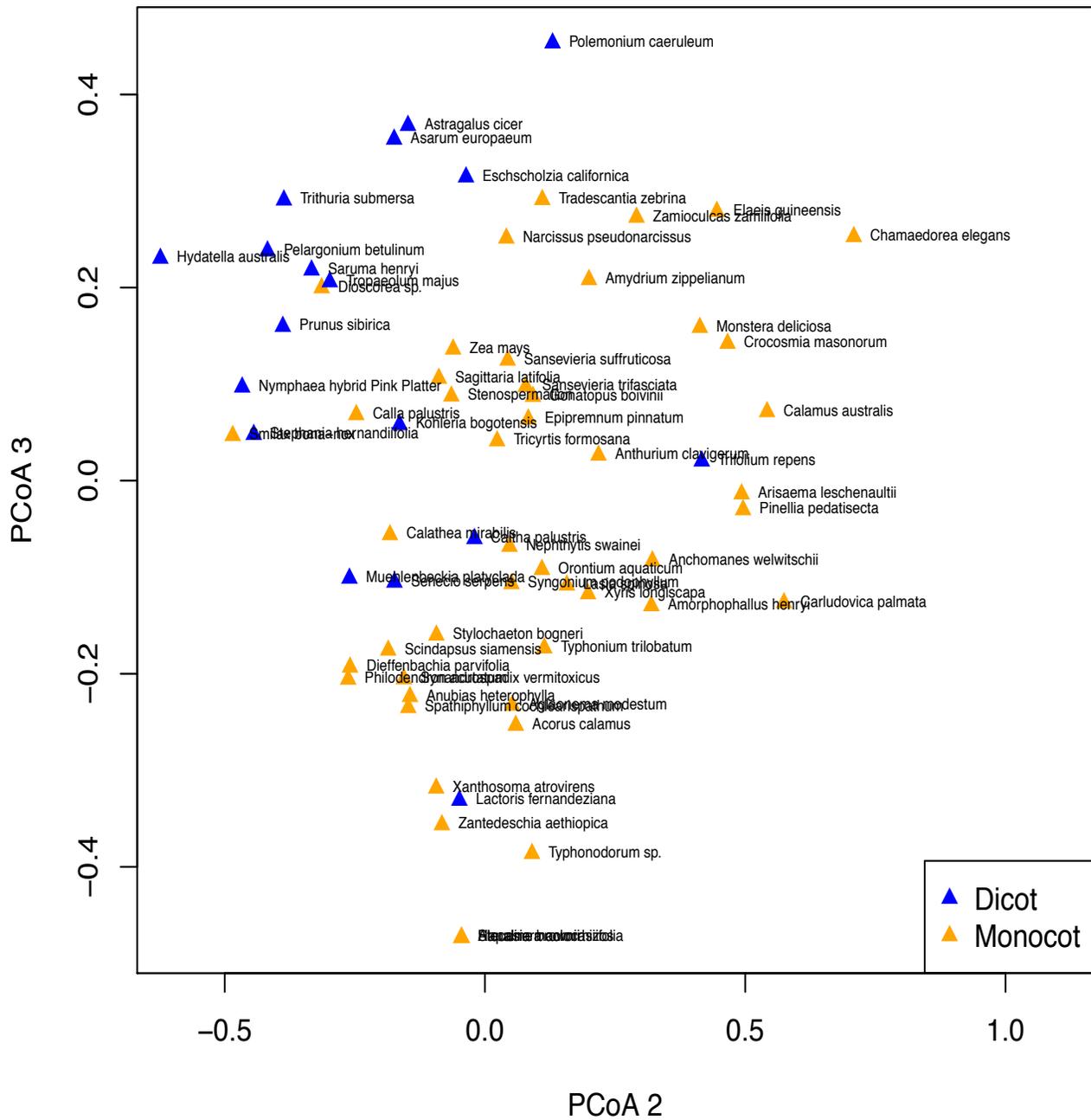
Figure 4.4 Principal coordinate analysis of leaf developmental morphology in 3D morphospace.

Principal Coordinate Analysis of Developmental Data



B

Principal Coordinate Analysis of Developmental Data



C

Figure 4.5 Principal coordinate analysis of leaf developmental data in 2D morphospace. A. PCoA axes 1 and 2, B. PCoA axes 1 and 3, C. PCoA axes 2 and 3.

that are rare across angiosperms (i.e., Hydatellaceae), or single salient features such as plication and/or schizogeny (Figure 4.4; Figure 4.5).

Axes 1 and 2

In the graph of axes 1 and 2 (Figure 4.5A), the uppermost region includes *Arisaema*, *Pinellia*, *Chamaedorea*, *Carludovica*, *Calamus*, *Elaeis* and *Crocoshmia*. The species are all characterized by plication, a sheathing leaf base, and lack of a peltate blade. The only unique character found in these species is plication, indicating that it contributes heavily to axis 2. The difference separating *Pinellia* and *Arisaema* from *Chamaedorea*, *Carludovica* and *Calamus* is that they achieve dissected leaves through blastozone fractionation instead of schizogeny, indicating that margin-defining mechanisms (character 9) contribute to axis 1. Indeed, all species occupying the leftmost region along axis 1, including *Arisaema*, *Pinellia*, *Amorphophallus*, *Polemonium*, *Gonatopus*, *Eschscholzia*, *Anchomanes* and *Astragalus* all possess dissected leaves achieved through blastozone fractionation. They also possess a petiole and lack a *Vorläuferspitze*.

The right part of the graph includes *Crocoshmia*, *Sansevieria*, *Xyris*, *Acorus*, *Narcissus*, *Tricyrtis*, *Zea*, *Tradescantia* and *Senecio*, which share the absence of a petiole, entire blades, absence of blade peltation and parallel primary and higher-order venation. The only character distinguishing *Crocoshmia* is plication (hence its higher position along axis 2), while the only character distinguishing *Senecio* is lack of a sheathing leaf base (venation is unknown for this species).

Hydatella, which has the unique combination of unifacial blades that lack a sheathing leaf base (as in *Senecio*) but also possesses a midrib and stipules is at the base of the graph.

Axes 1 and 3

The distribution of taxa along axis 1 remains with petioleless, entire-bladed species to the right and blastozone-fractionated dissected-leaved species with a petiole to the left (Figure 4.5B). Taxa at the opposite ends of axis 3 are *Polemonium caeruleum* and three species of Araceae which are identical in all character-states, including *Alocasia macrorrhizos*, *Stuednera colocasiifolia* and *Hapaline brownii*. *Polemonium* and the three species of Araceae differ in that the leaf primordium of *Polemonium* lacks a *Vorläuferspitze*, a cross-meristem, a sheathing leaf base and blade peltation, and possesses a pinnately-dissected leaf whose leaflets differentiate basipetally. The three species of Araceae all have a *Vorläuferspitze*, a cross-meristem, a sheathing leaf base and entire, peltate blades.

Axes 2 and 3

Taxa at the opposite ends of Axis 2 are *Chamaedorea* and *Hydatella*, while taxa at the opposite ends of axis 3 are *Polemonium* and the three species of Araceae as mentioned above (Figure 4.5C). Together, the combinations of characters of these four taxa represent the maximum amount of leaf developmental morphological diversity across the monocots and dicots studied here.

4.4 Discussion

The results of this study show that monocots and dicots largely overlap in leaf developmental, morphological and anatomical morphospace. Moreover, outliers are due to rare combinations of characters, which can occur across the angiosperm phylogeny from Nymphaeales to Asterales. These results suggest that it is too simplistic to think about monocot and dicot leaf blades as being non-homologous. I argue that at the developmental morphological level monocot and dicot leaves are homologous. At molecular genetic level, however, the

homology versus non-homology of monocot and dicot leaves should be studied within a hierarchical framework of gene regulatory networks (GRNs) composed of conserved hubs and peripheral genes that can be rewired to produce novel morphologies. I also argue that a loss of developmental constraint occurred in the common ancestor of monocots and that over evolutionary time, canalization of several features of leaf development occurred in the sister taxon to Pandanales.

Conserved hubs of leaf development are not only shared among leaves, they are shared at a deeper level of morphogenesis, that between the shoot and leaf. The deep homology of shoots and leaves is not a new concept.

4.4.1 *The Partial-shoot theory*

Arber (1950), influenced by the work of de Candolle (1868) and Zimmerman (1965), conceived of any leaf-like appendage, or *phyllome*, as a partial-shoot (Claßen-Bockhoff, 2001). According to this theory, the plant body can be viewed as a branching system propelled by an ‘urge to self-continuance’ (Arber, 1950, p.78) that is realized in repetition. The leaf as an incomplete, or partial, shoot strives ‘towards the development of whole-shoot characters’ (Arber, 1950, p.78). The incompleteness of the shoot in leaves is expressed as dorsiventrality, which is constantly being challenged by ‘an innate pulse toward radiality’ (Arber, 1950, p. 78). She is not concerned with the origin of leaves in a phylogenetic sense, but rather with what the leaf *is*.

Although Arber’s theory is largely metaphysical in its explanations, there is ample evidence from molecular genetic studies that lend support to a holistic view of the shoot and leaf in angiosperms. These are reviewed here, by no means exhaustively, to strengthen the argument of homology between monocot and dicot leaves. However, there is also evidence that monocot

leaves and dicot leaves have diverged in modules downstream of ancestral GRN hubs. These are reviewed in the following section.

4.4.2 *The shared GRNs of shoots and leaves*

Polarity

The shoot apical meristem (SAM) in angiosperms is divided into three cyto-histological zones (central, peripheral and rib) that are characterized by distinct gene expression patterns (Floyd and Bowman, 2010). The central zone is characterized by apical initials with low rates of cell division that replenish stem cells in the peripheral and rib zones (Taiz and Zeiger, 2006). The peripheral and rib zones are characterized by higher rates of cell division, with the peripheral zone giving rise to leaf primordia at auxin maxima, and the epidermis, cortex and vasculature of the stem (Floyd and Bowman, 2010). Polarization of both stems and leaves involve the class III *Homeodomain Leucine-Zipper (HD-ZIPIII)* and *KANADI (KAN)* gene families (Floyd and Bowman, 2010; Husbands et al., 2009). The polarization of leaves in the dorsiventral plane by juxtaposition of upper and lower zones is considered necessary for laminar outgrowth, although an alternative mechanism has been identified in ensiform leaves (discussed below). Members of the *HD-ZIPIII* gene family are expressed in the central zone of the meristem and in rays that correlate with auxin flow out of the meristem toward predicted sites of organ initiation and provasculature (Husbands et al., 2009). In the leaf primordium *HD-ZIPIII* genes are restricted to the adaxial surface by the miRNA miR166 and *LITTLE ZIPPER (ZPR)* proteins, while *KANADI* gene expression is restricted to the lower or abaxial surface where it acts with *AUXIN RESPONSE FACTORS (ARFs)* to maintain abaxial cell fates. The close association between the meristem and adaxial cell fates is demonstrated by loss-of-function mutations in *HD-ZIPIII* alleles that result in a loss of central shoot identity, loss of polarized vascular bundles in the

stem, and loss of SAMs in mutant seedlings (Husbands et al., 2009). Additionally, mutants with gain-of-function *HD-ZIPIII* alleles produce larger SAMs and leaf primordia become radialized through loss of abaxial identity. In *Arabidopsis*, double and triple mutants of *KAN* family members cause ectopic outgrowths on the abaxial surface at a site of auxin maxima, as in the SAM. Thus, *HD-ZIPIII* genes promote apical/central identities, while *KANADI* genes promote basal/peripheral identity (Floyd and Bowman, 2010, Husbands et al., 2009). *HD-ZIPIII* genes were present in the common ancestor of land plants (Floyd and Bowman, 2006). It is hypothesized that the co-option of the *HD-ZIPIII/KANADI* module from maintenance of radial patterning in the stem made possible the evolution of complex steles from protosteles and also the planation of ancestral branching systems to produce leaves (Floyd and Bowman, 2010; Kenrick, 2002). Expression patterns of *HD-ZIPIII* genes in the lycophyte *Selaginella kraussiana* and in gymnosperms support the hypothesis of a conserved role for these genes throughout vascular-plant evolution (Floyd and Bowman, 2010).

Boundaries, Totipotency and Determinacy

The site of lateral organ initiation from the peripheral zone at auxin maxima is associated with the demarcation of boundaries between totipotent stem cells in the SAM and cells that will form a determinate structure. Class I *KNOTTED1*-like homeobox (*KNOXI*) genes maintain the meristematic identity of cells in the SAM (Kerstetter et al., 1997; Vollbrecht et al., 1991) and are down-regulated at the site of leaf initiation by MYB type transcription factors called the *ARP* genes for *ASYMMETRIC LEAVES1 (AS1)*/*ROUGH SHEATH2 (RS2)*/*PHANTASTICA (PHAN)* in *Arabidopsis*, maize and *Antirrhinum*, respectively. Absence of *KNOXI* gene expression in leaf primordia is thought to confer determinacy and may be a synapomorphy for seed plants since *KNOXI* genes are expressed in leaf initials in ferns (but also in tomato, Reiser et al., 2000)

(Bharathan et al., 2002; Floyd and Bowman, 2010). In *Arabidopsis*, tomato and pea, *KNOX1* genes form a positive feedback loop with a family of transcription factors, the *NO APICAL MERISTEM (NAM)/ CUP-SHAPED COTYLEDON3 (CUC3)* genes (Blein et al., 2008). *NAM/CUC3* genes are expressed in the SAM at the boundary of organ primordia in a pattern following the phyllotaxy of leaf placement, and repress growth so allowing organ separation. Polar auxin transport from shoot to root in *Arabidopsis thaliana* is mediated by *PIN-FORMED1 (PIN1)* (Leyser and Day, 2003). Regions where the *KNOX1/CUC3* feedback loop is expressed are associated with auxin minima and the down-regulation of *PIN1*. There is interaction between *PIN1/KNOX1/CUC3* during leaflet development in dissected leaves in members of Ranunculales, Solanales, Fabales and Brassicales (Blein et al., 2008)

4.4.3 *Transitions from homology to non-homology in monocot and dicot leaves*

The *WOX* gene family

The *WUSCHEL* gene in *Arabidopsis* was the founding member of the *WOX (WUSCHEL-like homeobox)* gene family (Mayer et al., 1998). *WUSCHEL* and its orthologs *TERMINATOR* from petunia and *ROSULATA* from *Antirrhinum* are required to maintain stem cells in the shoot apical meristem (Mayer et al., 1998; Vandenbussche et al., 2009). Fifteen *WOX* family members have been identified in *Arabidopsis* and seven have been analyzed genetically (Zhang et al., 2007). Loss-of-function of the *MAEWEST (MAW)* gene in petunia, an ortholog of *WOX1* in *Arabidopsis*, is associated with severely reduced lateral outgrowth of leaf blade margins (Vandenbussche et al., 2009). In maize, loss-of-function of the *NARROW SHEATH1 (NS1)* and *NARROW SHEATH2 (NS2)* genes causes reduced lateral outgrowth of the blade margin (Scanlon et al., 1996). *NS1/NS2* are members of the *WOX3/PRESSED FLOWER (PRS)* subfamily in *Arabidopsis*. Interestingly, *PRS* is expressed in developing leaf margins of *Arabidopsis*, but *prs*

null mutants lack only stipules. The reduced leaf margin phenotype is expressed only in *wox1 prs* double mutants (Vandenbussche et al., 2009). According to the Leaf-base theory, the maize leaf is derived from lower leaf zone tissue corresponding to the leaf base and stipules in dicots. Gene expression patterns of *PRS* in the blade margins of maize and *Arabidopsis* suggest that these structures are homologous and that the role of *PRS* in stipule development in *Arabidopsis* is a derived feature.

4.4.4 *Novelties in monocot leaf morphology*

The maize ligule – a novel structure with a conserved molecular basis

The maize leaf is composed of a proximal sheath and a distal blade, which are sharply demarcated at their junction by an outgrowth called the ligule. It has been stated that the ligule has no clear homologous structure in dicot species (Townsend and Sinha, 2012); however, a transcriptomic study of ligule development (Johnston et al., 2014) suggests a more nuanced answer to this issue of homology. The GRNs involved in lateral organ initiation from the SAM, including the *HD-ZIPIII*, *CUC2*-like, *ARF3a* and *NSI* genes, are also co-expressed during ligule formation. Furthermore, *PINI* accumulation in the pre-ligule and preblade region resembles *PINI* accumulation at the site of leaf initiation, while *KNOX1* expression in the preligule and presheath region resembles *KNOX1* expression at the base of leaf primordia (Goliber et al., 1999). Additionally, a *BOP*-like gene is expressed in the developing ligule and in the presheath (proximal) region at the base of the leaf primordium.

BLADE ON PETIOLE (BOP) genes in *Arabidopsis* inhibit laminar outgrowth on the petiole, with *BOP2* expressed in the proximal region of the leaf primordium (Townsend and Sinha, 2012). Hence this is an additional module in the leaf GRN that has similar function in maize and *Arabidopsis*. The maize ligule may have no morphological homologs in dicots, but at

the molecular level it shares deep homology with the leaf primordium irrespective of monocot vs. dicot distinctions.

The ensiform leaf – a novel structure with a novel molecular basis

Recently, ensiform leaf development has been analyzed at the molecular genetic level (Nakayama et al., 2013; Yamaguchi et al., 2010a,b). Based on anatomical studies, it was historically proposed that ensiform leaves are derived by flattening in the median plane of cylindrical, unifacial leaves (de Candolle 1827, Arber, 1925). Modern studies have shown that unifacial leaves lack dorsiventrality through loss of the adaxial domain, and are thus abaxialized (Yamaguchi et al., 2010a). Studies of ensiform leaf development in *Juncus prismatocarpus* (Yamaguchi et al., 2010a) show that the flattened blade is also abaxialized by expression of an ortholog of *ARF3a*. This indicates that flattened leaves have evolved by independent mechanisms in bifacial and ensiform leaves, since juxtaposition of adaxial/abaxial regions are missing in the ensiform leaf. It was found that expression of *DROOPING LEAF (DL)*, a member of the *YABBY* gene family, correlates with early extension of leaf primordia in the median plane (toward the shoot apex), while *PRESSED FLOWER (PRS)*, a *WOX* gene family member, correlates with later marginal growth of the flattened leaf blade (Yamaguchi et al., 2010a). In the bifacial-leaved monocots, grasses and lily, *DL* regulates both midrib formation and carpel specification (Ishikawa et al., 2009; Wang et al., 2005). However, in *Arabidopsis* and *Amborella*, *DL/CRC* genes regulate flower gynoecia and nectaries; thus, *DL/CRC* genes are known to be involved in leaf development only in monocots (Preston et al., 2011; Wang et al., 2009). It will be interesting to see if the functional role of *DL/CRC* genes in the earliest diverging monocots *Acorus* and Alismatales retain the dicot pattern or if *DL/CRC* mediated leaf development is a synapomorphy for the monocot clade. Additionally, the unique function of *DL/CRC* in midrib

formation in monocots is interesting when considering that at the anatomical level, most monocot and dicot midribs are very different anatomically (Inamdar et al., 1983).

YABBY genes were recently found in *Micromonas* thus dating the gene family to the origin of the green-plant lineage between 700-1500 Mya (Leliaert et al., 2011; Worden et al., 2009). Yet, despite their ancient origin and status as “ancestral toolkit” genes (Worden et al., 2009), members of the *YABBY* family appear to occupy a peripheral position in the GRN regulating leaf development. This is supported by findings that *YABBY* genes are expressed later in leaf primordium development than the *HD-ZIPIII/KANADI* module (Toriba et al., 2007), and by the fact that *YABBY* genes have diversified in function both within monocots and between monocots and dicots. Besides *DL/CRC* diversification in leaf and floral traits, *YABBY* genes specify abaxial identity in a number of eudicot species, while in monocots they have been shown to have both non-polar and adaxial expression domains (Husbands et al., 2009)

4.4.5 *Loss of developmental constraint and canalization in monocots*

The presence of unique morphologies and developmental mechanisms in monocots may represent an environment-induced release of phenotypic variation from plesiomorphic developmental constraints. The earliest diverging orders in monocots, Acorales and Alismatales, display many of the novel features of monocot developmental morphology. Both are associated with aquatic habitats, particularly Alismatales, which have evolved numerous adaptations to facilitate an existence in water and include the only submerged marine angiosperms (Les and Tippery, 2013). Araceae are one of the most ecologically versatile plant families, ranging from free-floating aquatics to epiphytes to seasonally dormant terrestrials (Mayo et al., 1997).

In West-Eberhard’s theory of phenotypic accommodation (2005) developmental variation is the expression of reorganized ancestral developmental pathways induced by a mutational or

environmental change. A change in the environment is proposed as a more powerful initiator in producing morphological novelties since it affects many individuals simultaneously, while mutation initially affects only single individuals. The reorganization of ancestral developmental pathways has been shown to be a largely neutral process called Developmental System Drift (DSD) (True and Haag, 2001). In the case of monocots, increased moisture levels in the environment may have been the requisite epigenetic cue that enabled the exploration of morphospace, through underlying DSD, on a changed adaptive landscape (Newman and Müller, 2000; Wright, 1982). An alternative hypothesis would involve genetic assimilation (Waddington, 1953). In this scenario, environmental changes would induce new morphologies in conserved ancestral GRNs through developmental plasticity. If the environmental change persists long enough, the character may become incorporated into the genetic makeup of the organism. This scenario seems less likely, however, given the labile arrangement of GRNs.

Despite the variability in monocots as whole, certain characters have become fixed during the evolution of the clade. These include a sheathing leaf base in the common ancestor of monocots, the combination of a *Vorläuferspitze sensu stricto*, parallel venation and a basipetal differentiation direction in Liliales and all subsequently diverging orders, ensiformity in Iridaceae, and plication/schizogeny in Arecales. The final step in phenotypic accommodation is genetic accommodation (West-Eberhard, 2005). Genetic accommodation requires that morphological novelty be associated with reproductive success for a change in gene frequency to occur. A prolonged selective pressure can lead to fixation or canalization of the trait (Waddington, 1942). On the other hand, canalization may have occurred through stochastic processes. A recent study has shown that, as with DSD, canalization is a property inherent to the

network configuration of interacting transcriptional regulators and does not require natural selection (Siegal and Bergman, 2002).

4.5 Conclusion

Based on the results of this study and a review of the literature, it is evident that all-or-nothing statements of homology are inadequate to describe leaf primordium development across angiosperms. I propose that at the developmental morphological level monocot and dicot leaves are homologous, and that issues of homology between monocot and dicot leaf development at the molecular genetic level be analyzed within a hierarchical framework of Gene Regulatory Networks. Vascular land plants share deep homology in the regulatory hubs specifying polarity (*HDZIPIII/KANADI*) and determinacy (*KNOX/ARP*). These modules have been recycled during stem and leaf development over the course of land-plant evolution so that at this level in the molecular genetic hierarchy all leaves can be viewed as partial-shoots. In angiosperms, the next level involves GRN modules that have a similar function in monocots and dicots, but may also be in the process of diversifying. Members of the *WOX* gene family are an example. At the most peripheral level, highly plastic GRN modules become rewired both between dicots and monocots, and within monocots. The *YABBY* gene family has been shown to operate on this level of the GRN hierarchy, and is implicated in the production of many diverse leaf structures. From this perspective, zonal patterning in the leaf also becomes hierarchical and negates the rigid boundaries set forth by the Leaf-base theory and to a lesser extent by the Transition-zone theory. Kaplan recognized that upper and lower leaf zones are useful terms "...which refer to the positional topography of the leaf primordium *without having fixed developmental or functional fates* [italics added] (Kaplan, 1997). Molecular genetic studies have much to tell us

yet about the evolution of monocot leaves. Araceae and Alismatales, with their incredible diversity spanning monocot and dicot leaf forms, should be the focus of future studies in order to understand the evolutionary diversification of leaf developmental GRNs between monocots and dicots.

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Conclusion of the Dissertation

In studying the plant family Araceae, one theme emerges time and again – diversity. From an ecological, developmental, morphological, chemical and genomic perspective, species of Araceae are an endless source of scientific inquiry. The studies included herein are focused on one particularly intriguing characteristic of the family, and that is the rich diversity of leaf forms. At a first glance, studying the generative processes of leaf form in Araceae seems an aesthetically pleasing endeavor (which admittedly it is). However, a closer look at the long-standing questions regarding leaf evolution in angiosperms, and at agricultural practices in developing countries makes clear that studies of leaf form in the family are not just botanically attractive, they are crucial.

Morphological and developmental studies of leaf-form variation have emphasized that among angiosperms two main groups can be distinguished - monocots and dicots (Arber, 1918, 1925; de Candolle, 1827; Henslow, 1911; Kaplan, 1973; Troll, 1939). Numerous theories have been proposed to account for this such as the Phyllode (Arber 1918; de Candolle, 1827; Henslow, 1911) and Leaf-base (Kaplan, 1973; Knoll, 1948; Troll, 1955) theories, which place great emphasis on the non-homology of the two leaf forms. However, careful inspection of developing leaves from certain lineages of monocots has revealed that monocot leaf development is highly variable and underrepresented by such typological models (Bharathan, 1996; Kaplan, 1973). Aroids, in particular, have leaf morphological and developmental characteristics that are representative of, and transitional between, the two extremes. Yet, the study of aroid leaf form seems to have been set aside as an oddity, as studies highlighting the ‘monocot leaf vs. dicot leaf’ dichotomy have proliferated (Ishikawa et al., 2009; Tsiantis et al., 1999; Wang et al., 2009). Previous disregard for the potential of Araceae to answer questions about the early evolution of leaves in angiosperms may be understood in light of the lack of knowledge of evolutionary

relationships among angiosperms in the pre-molecular era, and a lack of laboratory techniques. Currently however, state-of-the art technology and the knowledge now emerging regarding the Gene Regulatory Networks (GRNs) governing leaf development across a range of angiosperm taxa, make studies in non-model taxa feasible. Thus, continuing ignorance of a group of plants that may provide insight into key genetic and ontogenetic events in the evolution of monocot leaf morphology *and* which contains species that are staple crops, is no longer acceptable. This dissertation is an attempt to redress said grievance.

Three independent lines of research were pursued to form a broad foundation that will facilitate ongoing study of leaf evolution in Araceae. These include: 1) providing a strongly-supported hypothesis of the evolutionary relationships among species in the family to be able to describe the sequence of morphological and developmental modifications to leaf ontogeny over time; 2) a developmental and molecular analysis of dissected leaf development in two genera that can specifically address the gap in our understanding of blastozone fractionation versus schizogeny and cell death in monocots; and 3) analyzing the variation in aroid leaf development in the broader context of GRN-mediated angiosperm leaf development to test the hypothesis that monocot and dicot leaf structures arise from non-homologous developmental modes.

The evolutionary history of the family is characterized by several adaptive shifts in and out of aquatic habitats and shifts in reproductive structures (Cusimano et al., 2011). Subfamilies Gymnostachydoideae, Orontioideae, Pothoideae, Monsteroideae and Lasioideae possess bisexual, perigynous flowers evenly distributed along the spadix, aperturate pollen containing sporopollenin in the ectexine, and lack laticifers. The most notable evolution of reproductive and anatomical characters involves a shift to unisexual flowers that have lost the perigon with female and male zonation of the spadix, inaperturate pollen that lacks sporopollenin in the ectexine and

the presence of articulated laticifers (Cusimano et al., 2011). The phylogenomic analysis in Chapter One is the first to provide strong statistical support for the Unisexual flowers clade (containing subfamilies Zamioculcadoideae and Aroideae) in which this major adaptive shift occurred. The evolution of vegetative characters, however, is much more complex.

Vegetative characters have been studied in a phylogenetic context in *Anthurium* (Carlsen and Croat, 2013). It was shown that leaf characters are highly homoplastic and therefore cannot be used to determine species relationships. At the family-wide level, however, certain components of leaf developmental morphology may characterize individual clades. Specifically, the early formation of a cross-meristem, coupled with as yet unknown modifications to the leaf GRN, may have predisposed the *Dracunculus* clade in subfamily Aroideae to peltate blade formation. Likewise, plication during leaflet formation occurs only in a subclade within the *Dracunculus* clade, again suggesting that significant changes in leaf GRN architecture occurred during the evolution of this clade.

Dissected leaf development is a vegetative character that has evolved many times independently in the family. Two genera of Araceae, *Anthurium* and *Amorphophallus*, were confirmed to produce highly variable dissected leaf morphologies via blastozone fractionation, which is the plesiomorphic developmental mechanism for achieving dissected leaves in angiosperms. Aroids are known to use the programmed cell-death mechanism to produce fenestration and leaflets, but the phylogenetic distribution of this character is uncertain. This is highlighted by the finding that *Amydrium zippelianum*, a species suspected of using the programmed cell-death mechanism, was shown to use the blastozone fractionation mechanism to produce leaflets. Detailed analysis of leaf development in subfamilies that are noted for the prevalence of cell-death, such as Monsteroideae and Lasioideae, are required before a clearer

history of the evolution of this unique mechanism emerges. Considering that programmed cell-death as a leaf developmental mechanism has been found thus far in only one order of angiosperms, the Alismatales, and in only one other species outside Araceae, it is surprising that studies of the molecular genetics of this mechanism in leaves are completely absent. Could the p53 apoptosis pathway have been co-opted during leaf evolution in Araceae (Speidel, 2010)?

In contrast to programmed cell-death, blastozone fractionation is an extremely well studied phenomenon both at the morphological and molecular level in numerous angiosperms clades outside monocots. Blastozone fractionation of the leaf margin produces lobe and leaflet formation, which are theorized to affect internal leaf temperatures. Dissected leaves have been shown to photosynthetically outperform simple leaves at higher temperatures (Nicotra et al., 2008). Increasing global temperatures thus make studies of leaf dissection extremely important, especially in crops and closely related species. A major evolutionary question is whether leaf dissection in monocots uses the *KNOXI* GRN module, which has been shown to be the major driver of leaf dissection in almost every other angiosperm studied to date (Bharathan et al., 2002). The molecular genetic tool with which a vast majority of these studies were performed was immunolocalization using an anti-*KNI* antibody designed in maize. Results of immunolocalization using the very same antibody that was shown to perform normally in maize suggest that developing dissected leaves of *Anthurium* do not express *KNI* proteins. Strangely, immunolocalization using an antibody designed against only the C-terminus of the *KNI* protein suggest that *KNI* proteins are expressed in developing dissected leaves of *Anthurium* and *Amorphophallus* in the expected pattern. This was further confirmed by RT-PCR experiments. The finding that *KNOXI* gene transcripts are found in late stages of development in the dissected leaf of *Anthurium clavigerum* while they are absent in the simple leaf of *A. lezamai* at an

equivalent stage, argues for the involvement of *KNOXI* genes in dissected leaf development in *Anthurium*. Despite the lack of resolution of precise *KNOXI* gene expression patterns, several aspects of the experiments were fruitful nonetheless. *KNOXI* sequences of several species of *Anthurium* were produced, as well as *Anthurium*-specific primer sequences for potential reference genes for qRT-PCR assays. Future studies using different techniques such as in situ hybridization or laser capture microdissection are required to resolve the conflicting *KNOXI* data in *Anthurium* and *Amorphophallus*.

In addition to the repeated evolution of dissected leaves, other aspects of leaf morphology occur in Araceae that are rarely found in monocots but are common in dicots such as reticulate venation, and the lack of a *Vorläuferspitze* (Keating, 2002; Kaplan, 1973). These characters have been hypothesized to have arisen independently in Araceae (Kaplan, 1973; Keating, 2002). However, a review of the literature on leaf development across angiosperms reveals that hallmark characters of monocot leaf development, such as the presence of a *vorläuferspitze*, also occur in dicots (González and Rudall, 2001). Moreover, increasingly detailed description of the GRNs governing evolutionary morphology has revealed that interactions among GRN modules are highly plastic and shaped in large part by neutral processes called Developmental Systems Drift (DSD) (True and Haag, 2001). This calls into question previous statements of homology that were once widely accepted. For example, in peas floral meristem genes have been co-opted to produce dissected leaf morphology, making them an exception to the *KNOXI* story (Hofer et al., 1997). One could argue that molecular developmental mechanisms of leaflets in peas and other angiosperms are not homologous.

These findings prompted a renewed and innovative look at the relationship between monocot and dicot leaves, this time emphasizing the transitional morphology of Araceae. In

addition, development modes and morphologies previously excluded from other studies (Geeta, 2003) were included to test whether the “monocot leaf vs. dicot leaf” dichotomy would persist and to see if current knowledge of leaf GRNs could shed light on the debate.

The analysis of leaf development was based on deconstructing complex morphology and development modes into individual components that together describe the great diversity in angiosperm leaf morphology. Ancestral character-state reconstruction was performed to visualize the evolutionary history of those components to determine which of them, if any, are unique to the monocot clade. Characters found exclusively in the monocot clade are parallel primary and higher-order venation, the ensiform leaf and the developmental mechanisms of cell death, plication and schizogeny.

Results of the multivariate analysis corroborate this conclusion. Monocots and dicots largely overlap in leaf morphospace. Outliers include members of monocots and dicots that are distinguished by rare character combinations. For example, members of the family Hydatellaceae (*Trithuria submersa* and *Hydatella australis*) have a long and sordid taxonomic history due to their odd morphology (Rudall et al., 2007) and are among the outliers in morphospace.

Two major conclusions can be drawn from these analyses. The first is that there is no “monocot type” of leaf primordium. Rejection of the Leaf-base theory is a by-product of this conclusion. Similar variation in the elaboration of upper and lower leaf zones occurs in both monocots and dicots, and characters once thought to be unique to monocots, such as the terete leaf and presence of a *Vorläuferspitze*, are likewise promiscuous in their distribution. A review of leaf GRN literature provides evidence against the Leaf-base theory as well. Genes regulating proximodistal and blade marginal cell identities show similar expression patterns in *Zea mays*

and *Arabidopsis* (Johnston et al., 2014; Vandenbussche et al., 2009). This result contradicts a tenet of the Leaf-base theory, which states that the monocot blade is derived from the lower leaf zone, while in dicots it is derived from the upper leaf zone. Yet, studies of leaf GRNs also undeniably support a divergence between monocots and dicots. This leads to the second major conclusion.

All-or-nothing statements of homology are inadequate to describe evolutionary morphological differences between monocots and dicots. At the developmental morphological level monocot and dicot leaves are homologous; at the molecular genetic level issues of homology between monocot and dicot leaf development must be analyzed within a hierarchical framework of Gene Regulatory Networks. The evolutionary community has recognized the need for a hierarchical basis of comparative homology (Hall, 1994), and studies of the evolution of GRNs and of the nature of their activity in modulating morphological evolution confirm this need. This study is the first to integrate the hierarchical GRN framework in interpreting results of the “monocot leaf vs. dicot leaf” analysis.

The evolution of leaves in land plants can be viewed on the most basic level as a deep homology between leaves and shoots, which share the most conserved GRN hubs regulating polarity (*HDZIPIII/KANADI*) and determinacy (*KNOX/ARP*) (Floyd and Bowman, 2010). In angiosperms, the next level involves GRN modules that have a similar function in monocots and dicots, but may also be in the process of diversifying. Members of the *WOX* gene family are an example, since they share a similar role in margin expansion in dicots and monocots, but have also been recruited for the novel function of stipule production in *Arabidopsis* (Vandenbussche et al., 2009). At the most peripheral level, highly plastic GRN modules become rewired both between dicots and monocots, and within monocots. The *YABBY* gene family has been shown to

operate on this level of the GRN hierarchy, and is implicated in the production of such diverse leaf structures as the peltate blade in dicots, and the ensiform leaf and midrib in monocots (Wang et al., 2009).

The appearance and canalization (Waddington, 1942) of unique characters in monocots is a separate but related issue that should be addressed in future studies of the evolution of monocot leaves. Two different scenarios could account for the appearance of novel traits. One possibility is that pre-existing phenotypic plasticity of certain leaf developmental characters conferred a selective advantage, which was followed by genetic assimilation (Waddington, 1953). Or conversely, phenotypic accommodation occurred (West-Eberhard, 2005) whereby the underlying leaf GRNs in monocots experienced significant DSD as they diverged from dicots, which was not revealed until the epigenetic environment changed. These alternative hypotheses can be tested in the Acorales and Alismatales. Araceae and Tofieldiaceae are particularly well-suited for this task because of their incredible morphological, ecological and developmental diversity.

Canalization of several characters has occurred within monocots. These include the sheathing leaf base, the parallel leaf venation and basipetal differentiation direction in a subclade of monocots, plication in palms, and ensiformity in Iridaceae. Studies of the opposing roles of selection and drift during fixation of these characters should also prove to be a fruitful line of research.

Beyond the intriguing major evolutionary questions in angiosperms that studies of Araceae can address, there is the even greater issue of feeding people. Recently a report by the World Bank and the International Monetary Fund states that progress on Millennium Developmental Goals relating to food and nutrition is lagging (Global Monitoring Report 2012). As climate change and unprecedented population growth exacerbate the demands on the world

ecosystem, understanding the genetic connections between efficient photosynthesis, leaf development and productivity and swiftly applying new findings from this field is necessary for meeting our goal of sustainable ecosystem and food management. Aroid crops are of extreme importance to the poor, often cultivated by smallholder farmers in developing countries that severely lack molecular agro-technological tools. Developing an aroid research program focusing on aspects of crop improvement that are currently underrepresented, specifically aroid leaf traits, will have far-reaching effects for those populations who are at greatest risk of food instability. Ironically, the “poor man’s crop” has a wealth of potential for scientific discovery and for feeding countries where population growth is highest. For this reason it is important to educate society on the nutritional value, morphological uniqueness, cultural legacy and physical beauty of these plants until they are no longer associated with poverty. It is time to bring the orphan crop home.

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2010 – 2015 Doctoral research, *Evolutionary developmental leaf morphology of the plant family Araceae*, Washington University in St. Louis/ Missouri Botanical Garden, Principal Investigators Dr. Barbara Schaal, Dr. Thomas B. Croat

- *Phylogenomics of the plant family Araceae* in collaboration with Dr. Chris J. Pires, University of Missouri, Columbia. Methods: library prep for next-gen sequencing, quality-trimming/filtering data, reference-based genome assembly, phylogenetic analysis. Software: YASRA, Bowtie2, Geneious, DynamicTrim, PrinSeq, RAxML, Mr. Bayes,
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2007 – 2008 Undergraduate research, University of California, Berkeley, Principal Investigator Dr. Chelsea D. Specht

- *Molecular and morphological analysis of the Mexican Milla complex* in collaboration with El Instituto de Ecologia, A.C. Methods: plant collection, DNA extraction, PCR amplification, DNA sequencing with ABI 3100 Sequencer, sequence alignment, phylogenetic analysis

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ADDITIONAL TRAINING

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