The evolution of floral morphology in the Zingiberales: an investigation into possible roles for the *GLOBOSA*-like and *TEOSINTE BRANCHED 1*-like genes

by

Madelaine Elisabeth Bartlett

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Plant Biology

in the Graduate Division

of the

University of California, Berkeley

Committee in Charge:

Professor Chelsea D. Specht, Chair
Professor Bruce Baldwin
Professor Jennifer Fletcher
Professor Sarah Hake

Fall, 2010

The evolution of floral morphology in the Zingiberales: an investigation into possible roles for the *GLOBOSA*-like and *TEOSINTE BRANCHED 1*-like genes

by Madelaine Elisabeth Bartlett

Abstract

The evolution of floral morphology in the Zingiberales: an investigation into possible roles for the *GLOBOSA*-like and *TEOSINTE BRANCHED 1*-like genes

by

Madelaine Elisabeth Bartlett

Doctor of Philosophy in Plant Biology

University of California, Berkeley

Chair, Professor Chelsea D. Specht

The rapid rise and diversification of the angiosperms has puzzled biologists for centuries; processes leading to current angiosperm diversity remain a key question in evolutionary biology, with particular focus on the morphological diversity of flowers. The Zingiberales are an order of tropical monocots that represent an ideal group of plants to study the evolution of floral morphology. The order contains approximately 2,500 species, many of which form specialized pollination relationships with bees, birds, bats, dung beetles, moths, butterflies, and primates (lemurs) via alterations in floral form. After developing a technique for visualizing and then studying gene expression in floral apices, I investigated the role of two candidate gene families, the *GLOBOSA* (*GLO*)-like genes and the *CYCLOIDEA/ TEOSINTE BRANCHED 1* (*CYC/TB1*)-like genes, in the evolution of floral morphology in the Zingiberales.

Evolutionary developmental biology often combines methods for examining morphology (e.g. Scanning Electron Microscopy) with analyses of gene expression (e.g. RNA *in situ* hybridization). Due to differences in tissue preparation for SEM and gene expression analyses, the same specimen cannot be used for both sets of techniques. I developed a method that couples extended-depth-of-field (EDF) epi-illumination microscopy to *in situ* hybridization in a sequential format, enabling both surface microscopy and gene expression analyses to be carried out on the same specimen (Chapter 1). I first created a digital image of inflorescence apices using epi-illumination microscopy and commercially available EDF software. I then performed RNA *in situ* hybridizations on photographed apices to assess expression of two developmental genes: *Knotted1 (Kn1)* in *Zea mays* (Poaceae) and a *GLO* homolog in *Musa basjoo* (Musaceae). I demonstrate that expression signal is neither altered nor reduced in the imaged apices as compared with unphotographed controls. The demonstrated method reduces the amount of sample material necessary for developmental research and enables individual floral development to be placed in the context of the entire inflorescence. While the technique presented is particularly relevant to floral developmental biology, it is applicable to any research where observation and description of external features can be fruitfully linked with analyses of gene expression.

The MADS box transcription factor family has long been identified as an important contributor to the control of floral development. It is often hypothesized that the evolution of floral development across angiosperms and within specific lineages may occur as a result of duplication, functional diversification, and changes in regulation of MADS box genes. In Chapter 2 I examine the role of *GLO*-like genes, members of the B-

class MADS box gene lineage, in the evolution of floral development within the monocot order Zingiberales. I assessed changes in perianth and stamen whorl morphology in a phylogenetic framework. I identified *GLO* homologs from 50 Zingiberales species and investigated the evolution of this gene lineage. Expression of two *GLO* homologs was assessed in *Costus spicatus* Swartz (Costaceae) and *Musa basjoo* Siebold (Musaceae). Based on the phylogenetic data and expression results, I propose several family-specific losses and gains of *GLO* homologs that appear to be associated with key morphological changes. The *GLO*-like gene lineage has diversified concomitant with the evolution of the dimorphic perianth and the staminodial labellum. Duplications and expression divergence within the *GLO*-like gene lineage may have played a role in floral diversification in the Zingiberales.

In the Zingiberales, evolutionary shifts in symmetry occur in all floral whorls, making this an ideal group of plants in which to study the evolution of this important ecological and developmental trait. The *CYC/TB1*-like genes have been implicated in the development and evolution of floral symmetry in divergent angiosperm lineages, and I thus chose them as a candidate gene family to investigate their role in the evolution of floral symmetry within the Zingiberales (Chapter 3). I identified both Zingiberales-specific gene duplications and a duplication in the *TB1*-like (*TBL)* lineage that predates the divergence of the commelinid monocots. I examined the expression of two *TBL* genes in *Costus spicatus* (Costaceae) and *Heliconia stricta* (Heliconiaceae), two Zingiberales taxa with divergent floral symmetries. I found that *TBL* gene expression shifts concomitant with shifts in floral symmetry.

Through this body of work we have gained some insight into the mechanics of angiosperm evolution. Duplications in the *GLO*-like gene lineage in the Zingiberales may have allowed for gene sub- or neofunctionalization and the evolution of new morphologies; in particular, the evolution of differentiated sepals and petals and of the staminodial labellum. In addition, this study adds to the growing body of evidence that *CYC/TB1*-like genes have been repeatedly recruited through the course of evolution to generate bilateral floral symmetry (zygomorphy). Although this work certainly doesn't preclude the involvement of as yet uncharacterized genes and gene families, it adds to the growing body of evidence that angiosperms as a group do indeed have a genetic 'toolkit': a core set of genes that have been variously deployed through evolutionary time to generate both convergent and divergent floral morphologies.

Table of Contents

Acknowledgements

I feel like I have been writing this part of my thesis in my head for months, yet I am still unsure what to say. I have too many people to thank, and too little time to do it well. I'm happy here in Berkeley, for so very many reasons, and just as when you're sad everything makes you sad, happy is a little bit the same. I'm grateful for this happiness, and all the very many things that make it so.

I am especially grateful to Dr Tracy McLellan, for pointing me this way. I had never considered grad school before she suggested Berkeley to me. I like to say that serendipity led me here, but it was mostly her. Moving was very difficult, and I would like to thank the people who helped me more than I deserved: Cait Bray for giving me room and board in England, my mother and Mark Trebicki for packing for me, my grandmother for giving me all her stuff over here, Eugene Stockill for storing it, moving it and picking me up at the airport, Laurie Leonelli for unpacking it, and my sister for coming down to help me settle. Never has a traveler been more fortunate in the people around her.

The Specht lab has been a marvelous place to grow up as a scientist. Thank you Chelsea Specht for the lovely environment you nurtured. Thank you for your easy good nature, your integrity, your love of science, for never making me feel stupid even when I deserved it, for introducing me to the scientific community, for tirelessly supporting me and helping me. The list is too long. I have learned so much as a student in your lab, so much about biology and the profession and calling of a scientist. Thanks Heather Driscoll. You have helped me so much professionally, making my work easier and always taking interruptions in your stride, handling them with a kind of grace that I'll never have. Thank you for your razor sharp questions about what I was doing that never allowed me too much sloppiness. Thank you, above all, for your friendship and love. Thanks to Chodon Sass. We came in together and we'll be going out together (sort of). In some ways so similar and in others so different, you have taught me how to be a better person and a better scientist. I'm grateful for these years we've had together. Thank you Tanya Renner for your good nature, your kindness and your ability to always think the best of people. Thank you Kali Lader for your lovely sense of humor and your never-ceasing willingness to help everyone in your life. Thank you Ana Almeida for all your help on my project, and your excellent hospitality.

Many thanks to the rest of my thesis committee: Sarah Hake, Jennifer Fletcher, and Bruce Baldwin. All three of you have helped to make this journey mostly pleasant (none of the unpleasantness can be traced to any of you). I have learned so much from you and I am extremely grateful for the time and thought you have given to me. Thanks Sarah and Jenn especially for having me in your labs as a rotation student at the beginning. I improved. I am grateful to Barry Tomlinson and all the tropical botany students of 2009. That was an amazing class in an amazing place. Many thanks to the agencies who have funded this journey: PMB, NSF, NRF, the Botanical Society of America, NTBG, Heliconia Society International, and the MORPH research co-ordination network.

I have made so many wonderful friends here, thanks all of you for your love and support. In no particular order, especial thanks to Laurie Leonelli, Rebecca Daly, Kelly Wrighton, Bonnie Brayton, Betsy Hutchsion, Anna Simonin, Cameron Thrash, and Julia Delafield. Thanks to your well-chosen partners and friends, too. It has been a pleasure getting to know them and making them my friends. Thanks to George and all the members of the St Mark's choir. What joy and splendor I have experienced singing with you. I'm never sure until I kneel at the altar rail with all of you, my second family. Thanks to those I left behind, Barry and Megan Visser, Martin Naude, Chris Greensmith and Debbie Rogers, Bronwynn Lace, Tessa Carthy, Cait Bray, Simone Bray, Michelle Grant, Belinda Bezuidenhout, Claire Greeff, Fantine Baonville, and Claire Christison. Your friendship over the years has sustained me, even when I wasn't responding to emails. Hopefully oneday we'll all be in the same city again. A city we can all agree on.

Lastly, thanks must go to my family: my parents, Gris and Ben, Norman and Megan, Sharon, Richard and Luzette, Jeni-Joy and Carlindo. I grew up in your love, of me and of each other. I never laugh like I laugh when I'm around all of you. Thank you. And special thanks to Mark: the voice of reason and the joy of home. Where would I be without you? Eating tuna out of a can and crying, that's where.

Introduction

Estimates of the number of angiosperm species lie above 250,000, and angiosperms make up more than 90% of the terrestrial flora (Crepet & Niklas, 2009). Reasons for this incredible diversity have been postulated, including the frequency of hybrid polyploidy among angiosperms (reviewed in Rieseberg & Willis, 2007), the annual growth form (Crepet & Niklas, 2009) and the advantage afforded by many angiosperms' ability to reproduce asexually (Tiffney & Niklas, 1985). The reason most often cited, however, is plant-pollinator interactions (Raven, 1977). Floral morphology, and the evolution of floral morphology, is a key determinant and possible driver of these interactions (Endress, 1994). The Zingiberales present an ideal order in which to study this morphological diversification. The eight families in the order display a wide array of floral morphologies, shifts in which can be mapped onto the relatively well-resolved phylogeny. These evolutionary changes in floral morphology are of ecological importance, often being associated with shifts in pollinator (Figure 1, Sakai *et al.*, 1999; Kress *et al.*, 2001; Kay & Schemske, 2003).

Floral morphological diversification may occur through the evolution of novel structures, for example nectar spurs (Hodges & Arnold, 1995), the corona in aclepiads (Endress, 1994), and the gynostemium of orchids (Rudall & Bateman, 2002). Floral nectar spurs in *Aquilegia* have been proposed as a 'key innovation': the derivation of this floral trait is associated with an adaptive radiation of the genus (Hodges & Arnold, 1995). Another important process in floral evolution, and broader angiosperm evolution, is the convergent evolution of particular traits (Soltis *et al.*, 2005). Convergent floral evolution is most strikingly illustrated in

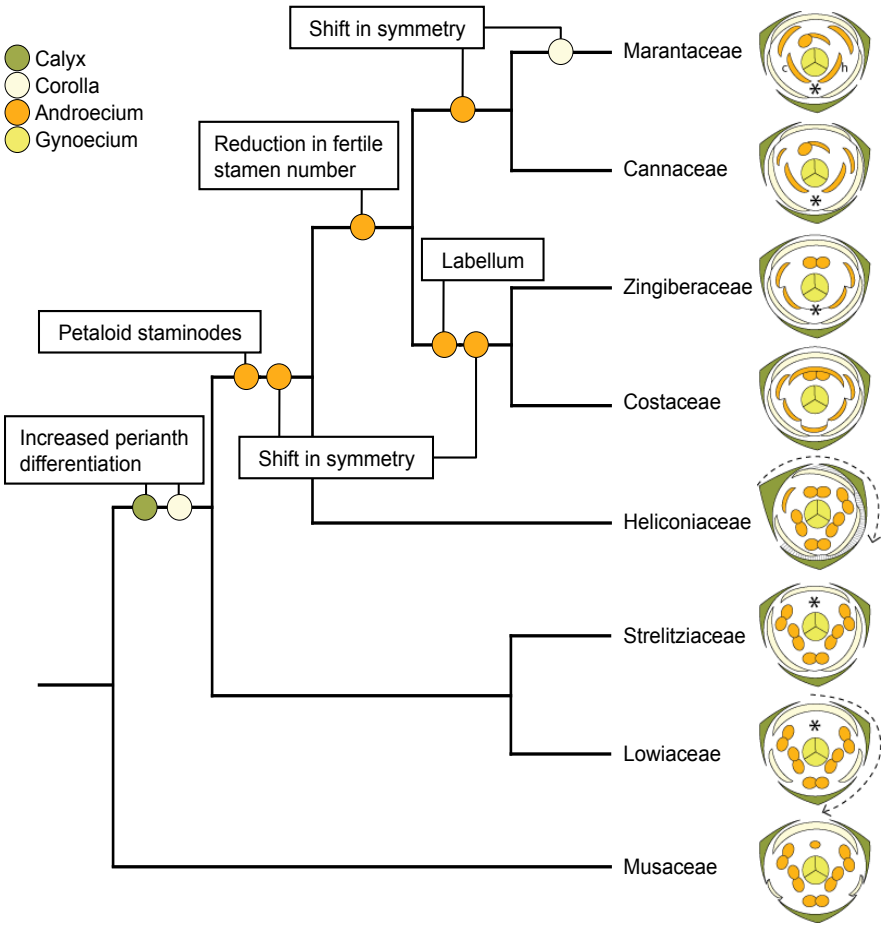

Figure 1. Floral morphology in the Zingiberales. Floral diagrams for each of the eight families are shown and shifts in morphology are mapped onto the current understanding of phylogenetic relationships in the order. Dashed arrows represent resupination (seen in some species of Heliconiaceae and Lowiaceae).

the convergence in morphology of flowers pollinated by similar pollinators (Fenster *et al.*, 2004). Through the course of my PhD research I investigated the genetics of three ecologically relevant traits, both novel and convergent: the evolution of the staminodial labellum, the evolution of a dimorphic perianth and the evolution of bilateral symmetry. The staminodial labellum is an organ found only in Zingiberaceae and Costaceae that results from the fusion of petaloid staminodes (Kirchoff, 1988a). The staminodial labellum in these families often forms the majority of the floral display, and pollinator shifts are often associated with modifications of the form and patterning of this organ (Kay *et al.*, 2005). In contrast, a dimorphic perianth, consisting of fully differentiated sepals and petals, has evolved multiple times in the angiosperms (Ronse De Craene, 2008; Endress & Doyle, 2009), as has bilateral floral symmetry, or zygomorphy (Stebbins, 1970). In the Zingiberales, the degree of perianth differentiation changes across the order, and there are multiple shifts in floral symmetry (Fig. 1). I investigated the role of two candidate gene families in the evolution of the labellum, the perianth and floral symmetry in the Zingiberales: the *GLOBOSA (GLO)*-like and the *CYCLOIDEA/TEOSINTE BRANCHED 1* (*CYC/TB1)*-like transcription factors.

In order to pursue the study of floral evolution in the order, I developed a technique that combines high resolution microscopy with detailed analysis of gene expression. I describe the development and validation of this technique in the first chapter. Studies on the evolution of development, in both plants and animals, often combine methods for examining morphology and gene expression (for example, Kim *et al.*, 2003). The most commonly utilized techniques, scanning electron microscopy and *in situ* hybridization, have very different tissue preparation schemes, rendering it impossible to image and examine gene expression in the very same material. The method I developed couples extended depth of field epi-illumination microscopy to *in situ* hybridization. While the technique presented is particularly relevant to floral developmental biology, it is applicable to any research where observation and description of external features can be fruitfully linked with analyses of gene expression.

In the second chapter I describe my work on perianth differentiation, and the evolution of the staminodial labellum in the Zingiberales. The *GLO*-like genes were chosen as candidate genes to examine the genetics of these floral traits because of their demonstrated role in the development of petals and stamens. *GLO* is a member of the MADS box transcription factor family. The ABC model for floral development proposes that three classes of MADS box genes are required for floral patterning: A-class gene expression alone specifies sepal identity, A and B genes expressed together specify petal identity, B and C genes expressed together confer stamen identity, and C gene expression alone specifies the organ identity of the carpel whorl (Coen and Meyerowitz 1991). In *Arabidopsis*, the A-class genes are *APETALA1* (*AP1*) and *APETALA2* (*AP2*) (Mandel *et al.*, 1992; Jofuku *et al.*, 1994), B-class genes are represented by *APETALA3* (*AP3*) and *PISTILLATA (PI*) (Jack *et al.*, 1992; Goto & Meyerowitz, 1994), and the C-class gene is *AGAMOUS* (*AG*) (Yanofsky *et al.*, 1990). Proper expression of *AP3*, *PI*, and *AG* are essential for stamen organ identity (Bowman *et al.*, 1991; Krizek & Meyerowitz, 1996). In addition to Arabidopsis *AP3* and *PI*, the functions of orthologs *GLOBOSA* (*GLO*) (Trobner et al. 1992) and *DEFICIENS*(*DEF*) have been examined in *Antirrhinum* (Schwarz-Sommer *et al.*, 1990), Theissen and Saedler 1995; Theissen et al. 1996). Subsequent studies in *Arabidopsis* have shown the importance of D-class genes (*AGL11*) in ovule development (Angenent *et al.*, 1995) and have identified the "E class" genes (*SEPALLATA*) as involved in specifying development of petals,

stamens and carpels (Pelaz *et al.*, 2000; Honma & Goto, 2001). (for a review, see (Kaufmann *et al.*, 2005)). It is difficult, however, to extrapolate the role of E function genes to other angiosperms, as the role of SEP genes appears to vary from taxon to taxon (Malcomber & Kellogg, 2005; Irish, 2009). We predict that this family of genes, particularly the ABC genes, is involved in the evolution of floral form and shifting organ identity in the Zingiberalean flower. Understanding the evolution and functional diversification of the ABC MADS box transcription factors in the Zingiberales may provide insight into the evolution of floral diversity in the order.

Flowers are often simply described as possessing a differentiated or an undifferentiated perianth, descriptions that often mask underlying morphological complexity. To address this issue, I developed a novel metric for assessing the degree of perianth differentiation in flowers: the perianth dimorphism score. I assessed perianth dimorphism score across the commelinid monocots and found that perianth dimorphism increased on the branch separating Musaceae from the remainder of the Zingiberales, and continued to increase gradually across the order. I sequenced *GLO*-like genes from across the order and analyzed them in a phylogenetic context. I discovered three Zingiberales-specific *GLO*-like gene duplications. Two of these duplications were associated with the increase in perianth dimorphism that occurred following the divergence of Musaceae from the ancestor of the remaining Zingiberales. The third duplication occurred prior to the divergence of Costaceae and Zingiberaceae, concomitant with the evolution of the staminodial labellum. I examined the expression of two *GLO*-like genes in *Costus spicatus* (Costaceae) and *Musa basjoo* (Musaceae) and found evidence of expression divergence associated with gene duplication and the development of a differentiated perianth in *Costus*.

In the third chapter I describe my investigation into *CYC/TB1*-like gene family evolution in the Zingiberales, and explore a possible role for these genes in evolutionary shifts in floral symmetry (zygomorphy) in the order. Floral zygomorphy has evolved at least 25 times in the angiosperms, and has been described as an adaptive trait (Endress, 1999). It is in the eudicots that the developmental genetics of zygomorphy is best understood, and there is mounting evidence that it is controlled in many lineages by *CYC/TB1*-like genes. *CYC,* from *Antirrhinum majus,* and *TB1,* from maize, are two of the founding members of the TCP transcription factor family. Double *A. majus* mutants of *CYC* and one of its paralogs, *DICHOTOMA* (*DICH*), are actinomorphic, a change from the zygomorphic symmetry apparent in the wild type flowers. *CYC* has different effects on different whorls of the *Antirrhinum* flower; it appears to promote growth of the adaxial petals and restrict growth of the adaxial stamen which aborts to becomes the staminode (Luo *et al.*, 1995). Individual organs, such as petals and stamens, also lose their adaxial-abaxial asymmetry in the mutant plants (Luo *et al.*, 1995; Corley *et al.*, 2005).

Recently it has been convincingly demonstrated that *CYC*-like genes also control symmetry in *Pisum* (Fabaceae). In *Pisum* floral symmetry is controlled by two TCP genes, *PsCYC2* and *PsCYC3,* as well as a third uncharacterized locus, *SYP1. PsCYC2* and *PsCYC3* control adaxial-abaxial symmetry at the level of the entire flower: the double mutant displays an abaxialized phenotype, but petals still display internal asymmetry. Individual organ asymmetry is controlled by the third locus, *SYP1*. The triple *pscyc2, pscyc3, syp1* mutant was radially symmetrical with all petals possessing a abaxialized, symmetrical identity (Wang *et al.*, 2008). TCP1 is the closest *Arabidopsis CYC* homolog and is expressed in the adaxial side of the early floral meristem, but doesn't appear to alter floral morphology. It is also expressed asymmetrically in vegetative

axillary meristems (Cubas *et al.*, 2001). It has been hypothesized that this asymmetric expression is plesiomorphic and has been recruited through the course of evolution to generate flowers asymmetric along the adaxial-abaxial plane (Cubas, 2004).

In Asteraceae, *CYC*-like genes rather than controlling adaxial-abaxial symmetry seem to play a role in specifying floral identity across the inflorescence. In *Gerbera* one CYC-like gene *GhCYC2* is expressed only in ray flower primordia once the disk and ray flowers being to differ morphologically. *GhCYC2* overexpresser lines have disk flowers with more ray-like characteristics. Disk flowers possess a ligular structure that resembles the bilaterally symmetric shape of ray and trans flowers, stamen development is disrupted, and in one line all the disk flower petals were fused. *GhCYC2* over-expression resulted in changed petal length in all three flower types. Petal length was increased in disk flowers and decreased in ray flowers. These results suggest that *Gerbera* CYC-like genes are contributing, along with multiple other factors, to disc vs. ray floral identity (Broholm *et al.*, 2008).

Expression patterns of *Antirrhinum* symmetry gene homologs are correlated with floral symmetry in diverse taxa. *Mohavea* is a close actinomorphic relative of *Antirrhinum. Mohavea* also differs from *Antirrhinum* in having only two, fertile abaxial stamens. The two lateral as well as the single adaxial stamens are aborted. *CYC* and *DICH* expression patterns are correlated with this morphology. Their expression is expanded into the region occupied by the lateral stamens, possibly accounting for their abortion. *DICH* expression is reduced during petal differentiation, possibly accounting for the adaxial petals greater internal symmetry as compared to the adaxial petals of *Antirrhinum* (Hileman et al., 2003). In *Chirita hetrotricha* (Gesneriaceae) *CYC*-like gene expression is also correlated with zygomorphic floral development and the abortion of stamens. Adaxial expression is correlated with reduced growth of adaxial petals, lateral expression with the abortion of lateral stamens (Gao *et al.*, 2008). In *Bournea* (Gesneriaceae) flowers are zygomorphic until fairly late in development but are almost actinomorphic at anthesis. This change in floral symmetry is also correlated with symmetry gene expression patterns. *CYC* and another floral symmetry gene, *RADIALIS* (*RAD*), are initially expressed in the adaxial stamen and petal, but expression disappears late in development, perhaps accounting for the observed early zygomorphy (Zhou *et al.*, 2008).

A relative of *Arabidopsis, Iberis amara,* has zygomorphic flowers because of larger abaxial petals. *CYC's* closest homolog in *Arabidopsis,* and presumably *Iberis*, is *TCP1*. Expression of *IaTCP1* in *Iberis amara* is correlated with reduced growth of abaxial petals in wild type, and increased equal growth of all four petals in the peloric mutant. Transgenic *Arabidopsis* plants overexpressing *IaTCP1* had reduced petal size, supporting a role for *IaTCP1* in controlling corolla zygomorphy in *Iberis* (Busch & Zachgo, 2007). In *Cadia purpurea* (Fabaceae) there has been an apparent reversal to actinomorphy. *Cadia* has actinomorphic flowers and is nested in a large clade of taxa with zygomorphic flowers. In a closely related zygomorphic species, *Lupinus, CYC* expression was localized to the adaxial side of the flower. In *Cadia*, expression was found in all petals, presumably conferring a adaxial identity upon them. This 'reversal' to actinomorphy appears to have been a homeotic transformation to adaxial identity of all petals (Citerne *et al.*, 2006).

Apart from this evidence of repeated, independent recruitment of these genes in elaborating zygomorphy in divergent plant lineages, where the evolution of zygomorphy is thought to be convergent (Stebbins, 1970), there were a number of other motivations for investigating the *CYC/TB1*-like genes in the Zingiberales. First, the TCP genes in general are thought to be controllers of cell division and growth: key processes in the elaboration of zygomorphy (Kosugi & Ohashi, 1997; Kosugi & Ohashi, 2002; Nath *et al.*, 2003; Palatnik *et al.*, 2003; Tremousaygue *et al.*, 2003; Faivre-Rampant *et al.*, 2004; Li *et al.*, 2005; Welchen & Gonzalez, 2006). Second, *CYC/TB1*-like genes seem to have a particular role in the stamen whorl (Luo *et al.*, 1995). Apart from its expression in axillary meristems and branches, *TB1* in maize is strongly expressed in the stamens of female florets - floral organs destined to abort (Hubbard *et al.*, 2002). Third, there have been numerous studies conducted showing that *CYC*-like gene copy number is dynamic. In the eudicots, the pattern that is starting to emerge is one of numerous gains and losses of *CYC*-like genes in different lineages. For example, changes in *CYC*-like copy number are correlated with changes in floral form in the Dipsacales (Citerne *et al.*, 2003; Fukuda *et al.*, 2003; Gubitz *et al.*, 2003; Hileman & Baum, 2003; Reeves & Olmstead, 2003; Howarth & Donoghue, 2005; Howarth & Donoghue, 2006; Kolsch & Gleissberg, 2006). This pattern of gene evolution suggests an ideal candidate gene family for the study of morphological evolution by gene duplication and diversification (Ohno, 1970; Lynch & Force, 2000).

I uncovered a duplication in the *TB1*-like (*TBL*) gene lineage that predates the diversification of the commelinid monocots, as well as Zingiberales-specific gene duplications. One of these gene duplications is associated with shifts in selection regime, and I found evidence of positive selection acting on one amino acid residue in one *ZinTBL* gene. I examined the expression of *TBL* genes in two taxa with divergent floral symmetries, *Costus spicatus* (Costaceae) and *Heliconia stricta* (Heliconiaceae). Expression of these genes shifts with shifting symmetry and differential stamen abortion in these two species, suggesting a role for the *TBL* genes in the establishment and evolution of zygomorphy in the Zingiberales.

Apart from further investigations into the expression patterns of these genes and their paralogs in diverse Zingiberales taxa, future studies will focus on the known interactors of these genes in other angiosperms (Krizek & Meyerowitz, 1996; Corley *et al.*, 2005), elucidating to what degree genes and gene networks have been conserved and have diversified through the course of angiosperm evolution. This work, linking gene duplications and changing expression patterns to the evolution of key floral traits, provides some insight into the mechanistics of angiosperm evolution. It supports the hypothesis that much of the floral diversity present in the angiosperms arises through the repeated recruitment of core genetic pathways, producing both convergent (differentiated perianth, zygomorphy) and divergent (staminodial labellum) morphologies.

Chapter One:

Epi-illumination microscopy coupled to *in situ* hybridization and its utility in the study of evolution and development in non-model species.

Abstract

Evolutionary developmental biology often combines methods for examining morphology (e.g. Scanning Electron Microscopy) with analyses of gene expression (e.g. RNA *in situ* hybridization). Due to differences in tissue preparation for SEM and gene expression analyses, the same specimen cannot be used for both sets of techniques. To aid in the understanding of morphological variation, it would be particularly useful to have a high-magnification image of the very same sample in which gene expression is subsequently analyzed. To address this need, we developed a method that couples extended-depth-of-field (EDF) epi-illumination microscopy to *in situ* hybridization in a sequential format, enabling both surface microscopy and gene expression analyses to be carried out on the same specimen. We first created a digital image of inflorescence apices using epi-illumination microscopy and commercially available EDF software. We then performed RNA *in situ* hybridizations on photographed apices to assess expression of two developmental genes: *Knotted1 (Kn1)* in *Zea mays* (Poaceae) and a *PISTILLATA (PI)* homolog in *Musa basjoo* (Musaceae). We demonstrate that expression signal is neither altered nor reduced in the imaged apices as compared with unphotographed controls. The demonstrated method reduces the amount of sample material necessary for developmental research and enables individual floral development to be placed in the context of the entire inflorescence. While the technique presented here is particularly relevant to floral developmental biology, it is applicable to any research where observation and description of external features can be fruitfully linked with analyses of gene expression.

Introduction

The analysis of gene expression patterns in non-model species is a major component of research on the evolution of developmental mechanisms. Spatial and temporal patterns of gene expression are best assessed using RNA *in situ* hybridization. In this technique, a labeled probe is hybridized to endogenous mRNA and detected through either autoradiography or a chromogenic reaction, depending on the label used (Jackson, 1991; Kramer, 2005). *In situ* hybridization results are often published in conjunction with scanning electron microscope (SEM) images of external morphology (Kim *et al.*, 2003). Unfortunately, once a specimen is fixed and prepared for SEM, it is no longer suitable for *in situ* hybridization. In alternative techniques to SEM, for example cryo-SEM, environmental SEM and laser scanning confocal microscopy, the tissue preparation methods themselves are not inherently damaging or incompatible with downstream gene expression analyses. The microscopy itself, however, is often destructive to tissue, precluding downstream RNA *in situ* hybridization (Lemon & Posluszny, 1998; Blancaflor & Gilroy, 2000). Because tissue fixation and preparation for epi-illumination microscopy is similar to the initial stages used for RNA *in situ* hybridization (Sattler, 1968; Kramer, 2005), it is possible to photograph a specimen using epi-illumination and continue with gene expression analysis on the same specimen. This can be advantageous in the light of the paucity of material available for developmental studies in non-model organisms.

Epi-illumination, or incident light microscopy, is a form of light microscopy in which the light source is above the object being viewed. It is used for the examination of opaque objects illuminated by reflected light (Locquin & Langeron, 1983). Epi-illumination microscopy has been used in the biomedical sciences, forensics, metallurgy, micropaleontology and botany (Nickolls, 1937; Leroy & Crane, 1964; Sattler, 1968; Locquin & Langeron, 1983; Tanaka, 2006). The technique became widely used in biology only when Leitz designed an incident light illuminator – the Ultropak - and a series of 15 objectives with magnifications ranging from 3.8x to 100x for use with the Leitz Laborlux, Ortholux, Orthoplan and Panphot microscopes. Through the use of an annular condenser and an angled annular mirror, the Ultropak illuminator and objectives allow for epi-illumination microscopy of irregular surfaces by separating the illuminating light rays from the image-forming light rays, thus reducing reflection (Leroy & Crane, 1964; Locquin & Langeron, 1983).

Epi-illumination microscopy was first employed in the investigation of floral development by Sattler (1968), and became widely used thereafter for many developmental studies in plant biology. In the twenty years following its initial publication, Sattler's paper was cited 63 times (BIOSIS). The technique has lost favor in recent years, with only 3 citations since 2000, presumably due to the reduced depth of field as compared to scanning electron microscopy and the difficulty in obtaining the now discontinued Ultropak system. With the advent of digital photography and specialized extended depth of field software, such as Nikon Imaging System (NIS) Elements, MediaCybernetics Image-Pro and Reindeer Graphics Focus Extender, increasing depth of field digitally has become relatively simple. The use of readily available metallurgical lenses in biology has also reduced the need for the Leitz system (Lacroix & Macintyre, 1995).

Many minor adjustments and additions to Sattler's (Sattler, 1968) original method have been proposed and implemented over the years. Epi-illumination microscopy has been used in combination with serial sectioning and histology (Posluszny *et al.*, 1980); depth of field has been increased by combining photographs at various focal depths in Adobe® Photoshop® (Wilson *et al.*, 2006) and staining schedules and dissecting techniques have been revised (Charlton *et al.*, 1989). This investigation, however, is the first to couple epi-illumination microscopy, newly available extended depth of field software, and *in situ* hybridization.

Our work has three goals: (1) to investigate the use of the NIS Elements Software to increase depth of field; (2) to verify that that the epi-illumination preparation techniques do not affect the *in situ* results and (3) to use epi-illumination and *in situ* hybridization to assess the expression of a floral organ identity gene in a non-model organism.

Materials and Methods

The use of the NIS Elements software was explored with inflorescence apices of *Costus cylindricus* (Costaceae), a species in which a number of flower primordia are clustered near the apex and which is thus difficult to photograph with conventional methods. The effect of epi-illumination preparation techniques on *in situ* hybridizations was investigated in maize with *Knotted1 (Kn1)*, a well-characterized gene expressed in meristems (Jackson *et al.*, 1994). The combination of these techniques was then tested in a non-model organism: the expression of *PISTILLATA (PI),* a floral organ identity gene (Coen & Meyerowitz, 1991), was assessed in inflorescences and attached flowers of *Musa basjoo* (Musaceae).

Floral material:

Maize (*Zea mays* var. *mays)* recombinant inbred line B73 seeds were grown in the greenhouses of the Department of Plant and Microbial Biology at UC Berkeley. Female inflorescences (ears) were harvested when they first became evident at approximately 6 weeks. *Costus cylindricus* (USBGH 2002-127) inflorescences were also dissected from greenhouse-grown plants. Entire inflorescences were harvested at an early developmental stage, and bracts sequentially removed to expose the early stages of floral development. In *Costus cylindricus*, a single flower is enclosed within each bract. *Musa basjoo* inflorescences were obtained from the collection of the University of California Botanical Garden at Berkeley (UCBG 89.0873). As with *Costus*, bracts were serially removed from young inflorescences to expose the youngest collateral florescences ('hands').

Fixation and Staining:

Following dissection, all floral material was immediately fixed in freshly prepared cold FAA (Jackson, 1991). Each specimen was then dehydrated from 50% to 100% ethanol using an adjusted microwave technique that decreases the time at each stage of the dehydration series, enabling the entire series to be completed in 1.5 hours (Schichnes *et al.*, 1998). Subsequent to dehydration, apices were stained for 72 hours at 4°C in a solution of 1% w/v fast green FCF in 100% ethanol (Charlton *et al.*, 1989). Apices were destained in 100% ethanol for 2 hours prior to photography.

Dissection and Photography:

One inflorescence from each species, *Costus cylindricus, Zea mays* and *Musa basjoo*, was dissected and photographed with epi-illumination microscopy. Black silicone gasket sealant that releases acetic acid (DAP, Baltimore) (Sattler, 1968) was prepared as a surface for photography by placing a small amount of the silicone in a small glass Petri dish, stirring it vigorously, letting it sit for 5-20 minutes, then flooding the dish with 100% ethanol. Samples were placed in this medium both to hold the specimen during dissection, and to provide a black background for photography. Dissection was performed in one prepared silicone dish, photography in another. The use of two dishes keeps the black background for photography free of plant debris generated during dissections. To increase depth of field in individual photographs it was important to position the objects of interest parallel to the focal plane of the camera. To keep the samples cool and to reduce streaming in the 100% ethanol, the Petri dishes were placed in a rectangular container filled with ice, which was changed approximately every 15 minutes. Photographs at various focal distances – from the top of the specimen to the bottom - were taken using the 3.8x Leitz objective on a Leitz Orthoplan microscope equipped with a Nikon Digital Sight 5M digital camera. The first photograph was taken with the flowers closest to the objective in focus; the next photograph had lower flowers, but not the lowest, in focus. This process of changing the focal depth and taking a photograph was continued until focused images of all of the flowers at all positions on the visible face of the inflorescence had been captured in a sequentially numbered series of photographs.

Generation of focused images:

Photographs were merged to create a single focused image using the extended depth of focus (EDF) function (purchased as an add-on) of the NIS Elements D software package (Nikon). We used two of the different ways of creating focused images: smoothing and local. The smoothing function relies heavily on the quality of the first photograph and blends all lower images into the first. The local function stitches together areas that are in focus in each photograph (Nikon 2006). For maize ear primordia, the 'smoothing' function achieved better results, whereas for taller objects (*Costus* and *Musa* inflorescences) the 'stitching' function achieved better results. In the case of maize, where the inflorescence was too large to fit into a single field of view, focused images of the length of the ear were stitched together using Adobe® Photoshop® CS2 (version 9.0.2).

RNA *in situ* hybridization:

To ensure that neither the heat generated during photography nor the staining and mounting needed for dissection and photography interfere with RNA *in situ* hybridization, hybridizations were performed first on maize. Experiments with maize included the following controls: (1) eliminate staining in fast green; (2) eliminate dissection in black silicone medium; (3) eliminate photography (i.e. potential heat from illumination). Once it was ascertained that the dissection and photography did not negatively affect *in situ* results, we performed *in situ* hybridizations on a photographed *Musa basjoo* inflorescence. In both the maize and *Musa in situs,* one pair of slides was probed with sense probe as a negative control. All *in situs* were performed as described below, modified from Jackson (1991) and Kramer (2005).

Probe development:

Probe for *Kn1* corresponded to bp 364-999 (59%) of the *Zea mays Kn1* coding sequence (GenBank accession number NM_001111966). The *Musa basjoo PI* probe (GenBank accession number EU433562) corresponded to 68% of the coding sequence of *MADS4* (*PI* homolog) from *Oryza sativa* (GenBank accession number L37527) and spanned the MADS, I and K domains of the gene. For both *Kn1* and *PI*, sequences were maintained in pBluescript SK vectors. Polymerase chain reaction (PCR) was performed using M13 primers, the vector containing transcript was used as template. The amplified region included a T7 RNA polymerase start site and a T3 RNA polymerase start site. Probe was labeled through *in vitro* transcription from the PCR products using DIG-labeling mix (Roche) and T7 (antisense probe) or T3 (sense probe) RNA polymerases (Invitrogen). Probe was quantified by comparing it to dilutions of DIG-labeled control RNA (Roche). The 642 bp *Kn1* probe was hydrolyzed to 150bp stretches using sodium carbonate and sodium bicarbonate hydrolysis (Kramer, 2005). The *PI* probe was only 431bp in length and, consequently, was not hydrolyzed.

Microtechnique and Hybridization:

Following photography but before infiltration with paraffin, a final change of 100% ethanol was performed to remove any contaminating water that could have entered the solution from the ice bath. Samples were infiltrated using a tissue-processing microwave oven (Microwave Research and Applications, MRA BP111RS) following the protocol of Schichnes et al. (Schichnes *et al.*, 1998). Paraffin blocks containing the apices were trimmed and sectioned at 8 μm on a Micron retracting rotary microtome. Sections were mounted on positively charged Probe-on plus slides (Fisher Scientific) by incubation at 42°C overnight.

Wax was removed from slides with xylene, and sections were hydrated through a graded ethanol series and incubated for 20 minutes in 2 μg/ml Proteinase K solution to digest cell walls and improve probe penetration. After the destabilizing Proteinase K treatment, sections were re-fixed in 4% formaldehyde in Phosphate Buffered Saline (PBS) for 10 minutes. To reduce background, excess positive charges were acetylated using a triethanolamine-acetic anhydride treatment. Slides were dehydrated through a graded ethanol series, following which 200 μl of probe in hybridization solution (Kramer, 2005) was placed on slides. The *Kn1* probe was used at a concentration of 1.33 ng/μl/kb, the *PI* probe was used at a concentration of 2.5 ng/μl/kb. Slides were sandwiched together in pairs, with the probe inserted between the pairs. The slide sandwiches were elevated above 50% formamide-wet paper towels in a slide box, which was, in turn, placed in a sealed plastic bag. The slides were incubated overnight in an oven preheated to 53°C.

The following day, slides were separated and washed twice in 0.2X Sodium chloride – Sodium citrate buffer (SSC) for 30 minutes at 53°C and twice in 1X Sodium-Tris-EDTA buffer (NTE) for 5 minutes at 37°C. An RNase A treatment (20 μg/ml RNase A in 1X NTE, 30 minutes at 37°C) was performed to digest single-stranded RNA and reduce background. This was followed by two 5 minute washes in 1X NTE (37°C), one 60 minute wash in 0.2X SSC (53°C) and 5 minutes in PBS (4°C). Slides were blocked using 0.5% w/v Boehringer block in 1X Tris Buffered Saline (TBS) (45 minutes, room temp.) and washed in buffer A (1.0% BSA, 100 mM

Tris pH 7.5, 150 mM NaCl, 0.3% Triton X-100). Alkaline Phosphatase-conjugated anti-DIG antibody was diluted 500X in buffer A, and 200 μL of antibody solution was used to make slide sandwiches as described above. Slides were incubated with antibody above water-wet paper towels in a slide box at room temperature for 1 hour. They were then separated and washed in detection buffer (0.1 M Tris pH 9.6, 0.1 M NaCl, 0.05 M $MgCl_2$). Finally, slide sandwiches were made again, this time using 200 μL of detection buffer plus substrate (1.6 μL 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), 2.2 μL Nitro blue tetrazolium chloride (NBT) per mL of detection buffer) and incubated in a slide box as described in a drawer to prevent light contamination. Slide sandwiches were periodically assessed for color development. Once signal was evident, the reaction was stopped by dipping the slides in water. The slides were dehydrated through a graded ethanol series, washed twice in xylene to remove any residual ethanol, and coverslipped using Cytoseal-60 mounting medium (Richard Allen Scientific). Sections were photographed using a Zeiss Axiophot 381 microscope, equipped with a QImaging Color camera.

Results and Discussion

Extended depth of field (EDF) epi-illumination microscopy:

The epi-illumination technique with NIS Elements EDF software was able to deliver a single high quality image of the *Costus cylindricus* inflorescence with relative ease (Fig. 1). This result was achieved in considerably less time than the method suggested by Wilson *et al* (Wilson *et al.*, 2006). They created a focused image by taking 6-22 photographs, and manually selecting and stitching together the focused areas of each photograph in Adobe Photoshop (Wilson *et al.*, 2006).

The combined image of *Costus cylindricus* shows all of the developing flowers on the floral apex in focus. The path of floral development can be traced using this single information-rich image (Fig. 1e). The youngest flower is closest to the inflorescence apex, the next oldest flower is to the right of the previous flower, moving in a right-handed spiral around the floral apex. The basic steps in *Costus* floral development, as described for *Costus scaber* (Kirchoff, 1988b) can be discerned in this single image. Development proceeds from initiation of the first sepal, through development of the common stamen-petal ring primordium, through differentiation of the petals, stamen and labellum, and ending with gynoecium development.

Although the cost of the microscope, accessories and camera necessary to produce EDF images is high, it is trivial compared to electron microscopy and, perhaps most importantly, the technique is manageable at the laboratory rather than at the institutional level. The technique can be implemented even at smaller institutions where there is unlikely to be an institutional commitment to establishing a SEM facility. Unfortunately, it is increasingly difficult to obtain the equipment used by Sattler (1968) since Leitz, now Leica Microsystems, has ceased manufacture of the Ultropak. Recently, however, similar results have been achieved using conventional and readily available metallurgical objectives (Lacroix & Macintyre, 1995).

EDF epi-illumination microscopy coupled to *in situ* hybridization

A single, composite photograph was created for the *Zea mays* female inflorescence (Fig. 2a). RNA *in situ* hybridization on this same inflorescence using antisense *Kn1* probe (Fig. 2b) demonstrates *Kn1* expression in spikelet meristems and vasculature. Expression patterns and levels are in keeping with published expression patterns of *Kn1* in maize (Jackson *et al.*, 1994). Expression levels are also comparable to those in the control that was not subjected to any staining with fast green, dissection, photography or exposure to the silicone dissection media (compare Figs. 2c, d).

One potential disadvantage of the epi-illumination technique is that the inflorescence apex is subjected to tissue damage because of the dissection necessary for photography. Typically, protective bracts are not removed from inflorescences prior to fixation and *in situ* hybridization (Jackson *et al.*, 1994). Minor damage, however, can be used as an indicator of location in an inflorescence when interpreting sections. The damage that occurred close to the base of the *Zea* inflorescence during the initial dissection (Fig. 2a, green box) was used to orient the sections. The inflorescence was oriented in the paraffin wax so that the sections were made in the same plane as the damaged flower. The damaged flower was then located in the sections as a means of determining their orientation on the slides. Using both measurement and information about orientation, we can link florets visible on the epi-illumination image with florets showing *Kn1* expression in the *in situ* results. The two spikelet meristems highlighted in the epi-illumination image are the same pair of meristems as those highlighted in the *in situ* results (red boxes, Fig. 2a and Fig. 2b). This process of floret identification can be repeated for the entire inflorescence. This is particularly useful in the study of non-model organisms where material is often limited. In addition, this technique reduces the dependence on developmental series with defined developmental stages. Gene expression patterns can be precisely referenced back to single flowers rather than to a generalized developmental stage as defined by examining flowers of another inflorescence or individual.

In order to demonstrate the utility of these techniques in a non-model organism, we repeated epi-illumination photography and *in situ* hybridization in *Musa basjoo* with a gene for which there are no published expression patterns in this species. *PISTILLATA* (*PI*) is a B class MADS box gene expressed in petals and stamens (Coen & Meyerowitz, 1991). We analyzed the expression of a *PI* homolog in *Musa basjoo* flowers that had previously been photographed (Fig. 2f). Expression was observed in the entire androecium and in the petals (Fig. 2g).

In inflorescences such as those of *Costus, Zea* and *Musa*, many floral developmental stages can be captured through sectioning a single inflorescence, thereby providing gene expression data across a developmental series. The positions of the flowers within the inflorescence, and the relationships between flowers are retained in the sections and can be traced back to the epi-illumination micrographs. This is particularly useful when studying inflorescences with complex structure, where tying a particular flower back to its position in the inflorescence is necessary for interpretation of development. Removal, dissection and probing of individual flowers would result in the loss of positional and possibly developmental information. For example, the homologies of *Heliconia* (Heliconiaceae) floral organs can only be understood in the context of the entire inflorescence (Kirchoff, 2003). Finally, the technique provides a method whereby the variability between specimens (and in the case of floral development within an inflorescence

(Bateman & Rudall, 2006)), is no longer of as much concern as when working with individual flowers, or when using SEM micrographs of similar but not identical flowers.

Conclusion

The study of evolution and development in non-model plant species is often hampered by a paucity of material available for observation and experimentation, and the inability to investigate variability within an individual or species by using sequential high magnification visualization and gene expression analyses. The technique presented here makes it possible to perform both surface microscopy and gene expression analyses on the same specimen, thereby reducing the amount of fresh material required and enabling detailed study of within-species developmental variation. This technique has particular relevance to the investigation of floral development and evolution, but has the potential to be used in any research where observation and description of external features can be fruitfully linked with studies of gene expression.

Acknowledgements

We especially acknowledge Sankar Sridaran and Solomon Stonebloom for their help in developing these techniques. We also thank Sarah Hake and her lab (PGEC, USDA) for providing *in situ* protocols and the *Kn1* probe, and members of the Specht lab for insightful comments on earlier versions of this manuscript.

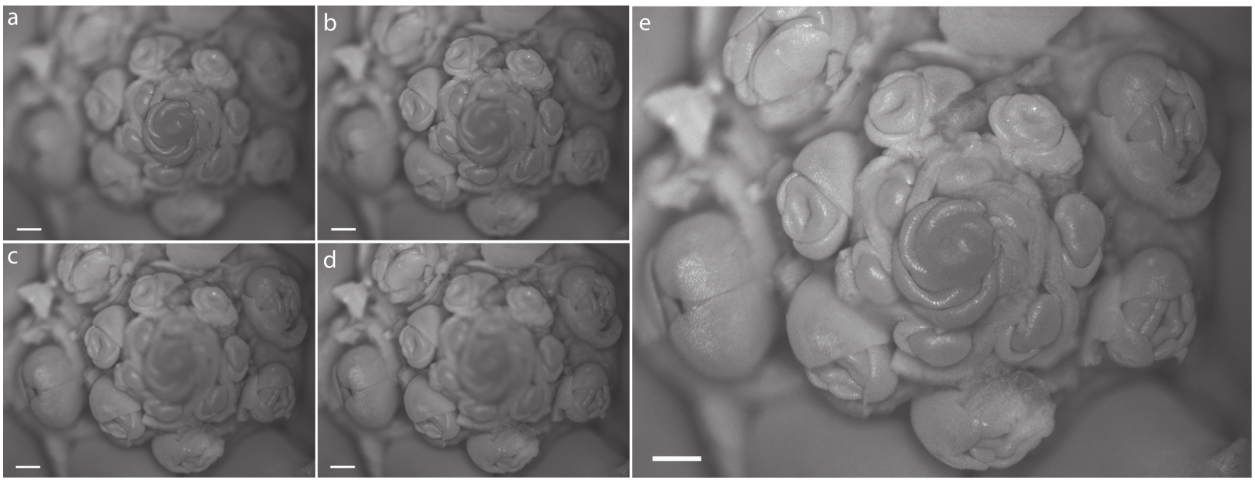

Figure 1 Extended depth of field (EDF) epi-illumination microscopy of *Costus spicatus*. The four unfocused images (**a-d**) were combined using the NIS Elements software to produce a single, focused image (**e)**. Scale bars, 200 μm.

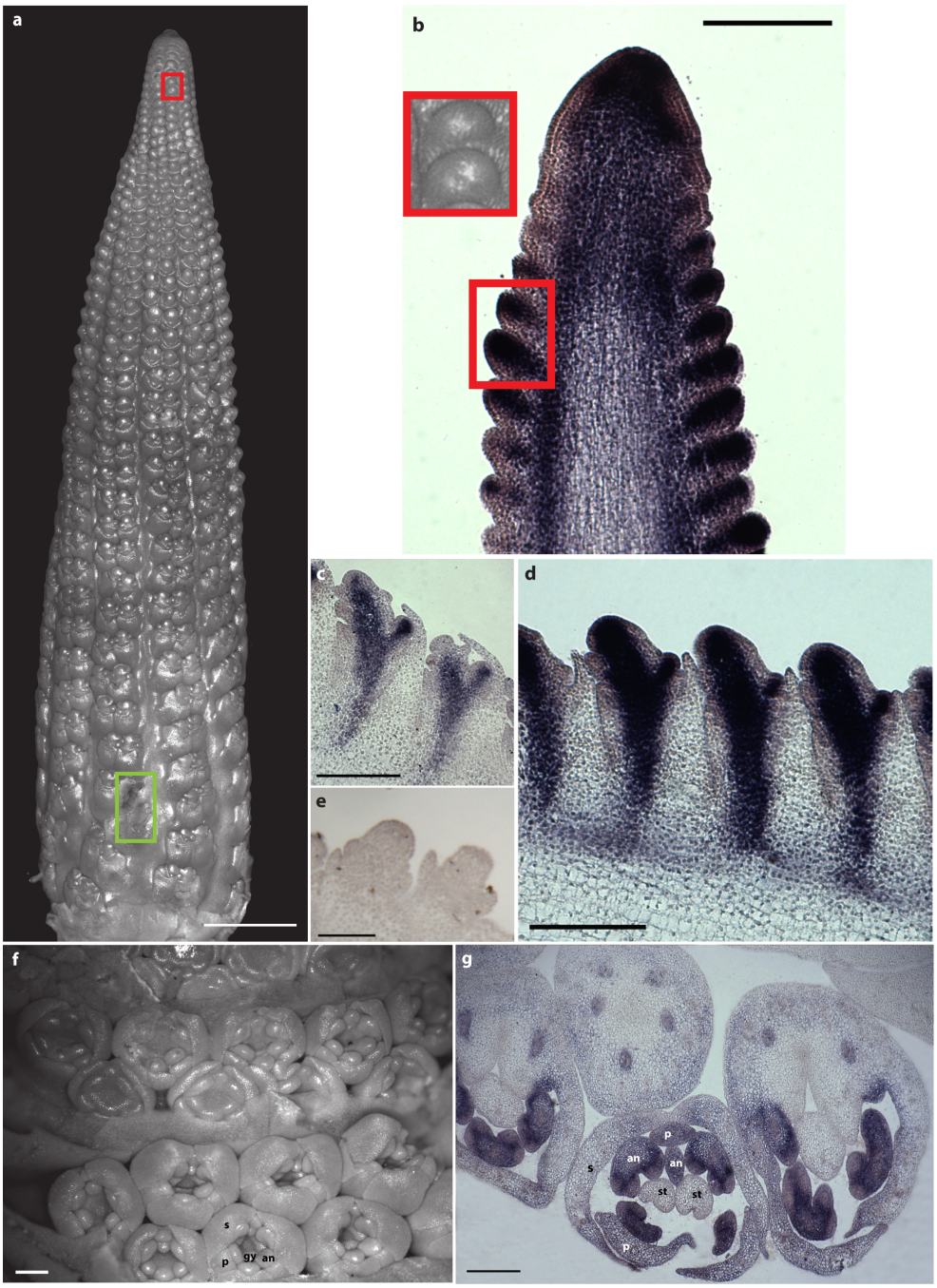

Figure 2 Epi-illumination microscopy coupled to *in situ* hybridization of *Zea mays* and *Musa basjoo*. **a-d** *Zea mays*. **a** EDF-epi-illumination image of a female inflorescence. **b** *Kn1* expression in the same inflorescence, and enlargement of two spikelet meristems from the inflorescnece. The red boxes surround identical spikelet meristems. The green box highlights the damage used to orient sections and locate individual flowers (see text). **c** *Kn1* expression in an apex that was not exposed to the silicone dissecting material, stain, or photographed with epi-illumination. The signal is comparable to that observed in the photographed inflorescence (Fig. 2a). **e** Negative *in situ* control. The sections were probed with a sense probe. **f-g** *Musa basjoo*. **f** EDF-epi-illumination image of two hands of flowers. Androecium (*an)*, petals (*p),* sepals (*s*) and developing gynoecium (*gy*) indicated. **g** *PI* expression in slightly older flowers. Expression is seen in the androecium and petals, but not in the sepals or style (*st*). All scale bars 200 µm, except **a** 1mm.

This chapter was published previously as:
Bartlett M, Kirchoff B, Specht C. 2008. Epi-illumination microscopy coupled to *in situ* hybridization and its utility in the study of evolution and development in non-model species. *Development Genes and Evolution* 218(5): 273-279.

Chapter Two:

Evidence for the involvement of *GLOBOSA*-like gene duplications and expression divergence in the evolution of floral morphology in the Zingiberales

Abstract

The MADS box transcription factor family has long been identified as an important contributor to the control of floral development. It is often hypothesized that the evolution of floral development across angiosperms and within specific lineages may occur as a result of duplication, functional diversification, and changes in regulation of MADS box genes. Here we examine the role of *GLOBOSA (GLO)*-like genes, members of the B-class MADS box gene lineage, in the evolution of floral development within the monocot order Zingiberales. We assessed changes in perianth and stamen whorl morphology in a phylogenetic framework. We identified *GLO* homologs from 50 Zingiberales species and investigated the evolution of this gene lineage. Expression of two *GLO* homologs was assessed in *Costus spicatus* Swartz (Costaceae) and *Musa basjoo* Siebold (Musaceae). Based on the phylogenetic data and expression results, we propose several family-specific losses and gains of *GLO* homologs that appear to be associated with key morphological changes. The *GLO*-like gene lineage has diversified concomitant with the evolution of the dimorphic perianth and the staminodial labellum. Duplications and expression divergence within the *GLO*-like gene lineage may have played a role in floral diversification in the Zingiberales.

Introduction

Zingiberales as an evolutionary model system:

The monocot order Zingiberales comprises a major component of both tropical and subtropical ecosystems and includes crop plants (e.g., banana, plantain, ginger), sources of traditional medicines and spices (cardamom, turmeric, galanga) and horticulturally important ornamentals (e.g., *Heliconia*, bird-of-paradise, prayer plants, *Canna*). The order contains approximately 2,500 species, many of which form specialized pollination relationships with bees, birds, bats, dung beetles, moths, butterflies, and primates (lemurs) via alterations in floral form (Frost & Frost, 1981; Itino *et al.*, 1991; Kress *et al.*, 1994; Sakai & Inoue, 1999). The order has long been recognized as a 'natural' group of plants (Bentham & Hooker, 1883) and more recent phylogenetic analyses confirm that the Zingiberales forms a monophyletic lineage (Duvall *et al.*, 1993) and is part of the commelinid monocots (Chase *et al.*; Davis *et al.*; APGIII). The eight families currently recognized within the order are often divided into two informal groups: the four 'banana families' Musaceae, Strelitziaceae, Lowiaceae and Heliconiaceae; and the four 'ginger families' Cannaceae, Marantaceae, Zingiberaceae and Costaceae (Kress, 1990a; Kress *et al.*, 2001). A summary of currently accepted phylogenetic relationships within the order is presented in Figure 1a.

Several significant changes in floral morphology have occurred through the course of Zingiberales evolution involving the perianth and androecial whorls (Kress, 1990a; Rudall & Bateman, 2004). Of particular relevance to the evolution of pollination syndromes are the derivation of a well-differentiated perianth, the development of petaloid staminodes, and the fusion of staminodes to form the staminodial labellum of Costaceae and Zingiberaceae (Fig.1). Flowers in the Zingiberales have two trimerous whorls of tepals, two trimerous androecial whorls, and a tricarpellate gynoecium. In the banana families, flowers typically have five or (rarely) six fertile stamens at maturity. In those taxa with five fertile stamens, the sixth androecial member may abort and become completely absent in the mature flower (Strelitziaceae and Lowiaceae, some Musaceae) or may develop as an infertile staminode (Heliconiaceae, some Musaceae). In the ginger families, the number of fertile stamens is reduced to one (Zingiberaceae, Costaceae) or one-half, with only a single theca (Cannaceae, Marantaceae) (Kirchoff, 2003; Rudall & Bateman, 2004). The remaining androecial members in the ginger families develop as petaloid staminodes. These infertile stamens share positional homology with stamens in the banana families and other monocots, but develop as petaloid structures, taking on the function (pollinator attraction) and structure (conical epidermal cells, Specht, unpublished) of petals (Kirchoff, 1991). In Zingiberaceae and Costaceae, these staminodes fuse in various combinations to form a novel structure, the staminodial labellum (Kirchoff, 1988b; Kirchoff, 1988a; Kirchoff, 1998; Kirchoff, 2003). The staminodial labellum provides the majority of the visual floral display and underlies the variety of pollination syndromes found in these diverse families (Kress & Specht, 2005; Specht, 2005).

MADS box candidate genes:

A group of transcription factors, many of which belong to the MADS box gene family, have been shown to be involved in controlling floral organ identity in the model plants *Antirrhinum*

majus and *Arabidopsis thaliana* (reviewed in Krizek & Fletcher, 2005; Theissen & Melzer, 2007). The ABC model for floral development proposes that three classes of MADS box homeotic genes are required for floral patterning in *Arabidopsis*: A-class gene expression specifies sepal identity, A and B genes expressed together specifies petal identity, B and C genes expressed together confers stamen identity, and C gene expression specifies carpel identity (Coen & Meyerowitz, 1991). In *Arabidopsis*, the A-class genes are *APETALA1* (*AP1*) and *APETALA2* (*AP2*) (Mandel *et al.*, 1992; Jofuku *et al.*, 1994), B-class genes are represented by *APETALA3* (*AP3*) and *PISTILLATA (PI*) (Jack *et al.*, 1992; Goto & Meyerowitz, 1994), and the C-class gene is *AGAMOUS* (*AG*) (Yanofsky *et al.*, 1990). The names of the *Antirrhinum* orthologs of *PI* and *AP3, GLOBOSA* (Troebner *et al.*, 1992) and *DEFICIENS* (Schwarz-Sommer *et al.*, 1990) respectively, have precedence; we heretofore apply these names to any Zingiberales orthologs.

It has been hypothesized that the ABC model's definition of B-class activity in the development of petals was applicable only to the core eudicots because of the unclear homology of petals across angiosperms (Kramer & Irish, 1999). However, investigations in non-core eudicots have begun to support an expanded role for the B-class genes in the development of petals in many angiosperm lineages, despite separate evolutionary derivations of petals (Endress & Doyle, 2009). In basal eudicots *Aquilegia* and *Papaver* (Ranunculales), B-class genes appear to be necessary for the development of both second whorl petals and third whorl stamens (Drea *et al.*, 2007; Kramer *et al.*, 2007). The *GLO* homologs from *Agapanthus* and *Elaeis*, monocot flowers with petaloid inner perianth organs, have been shown to rescue the *pi-1* mutant of *Arabidopsis* (Nakamura *et al.*, 2005; Adam *et al.*, 2007). Zmm16, a maize *GLO* ortholog, is also able to rescue petal development in an *Arabidopsis pi* mutant (Whipple *et al.*, 2004). Although data from heterologous expression studies are difficult to interpret, these results support the hypothesis that B-class genes are playing similar roles in both monocots and eudicots.

In addition, B-class mutants of maize and rice support the homology of petals and lodicules, the second-whorl organs of grasses. Lodicules of B-class mutants are transformed into palea-like organs (i.e. first whorl organs) and stamens are transformed into carpel-like (i.e. fourth whorl) organs (Ambrose *et al.*, 2000; Prasad & Vijayraghavan, 2003; Yadav *et al.*, 2007; Yao *et al.*, 2008). A number of morphological and gene expression studies on grasses and their closest non-grass relatives have further demonstrated the homology of lodicules and petals (Whipple *et al.*, 2004; Whipple *et al.*, 2007; Sajo *et al.*, 2008; Preston, Jill C. *et al.*, 2009). Presumably it is the downstream targets of B-class genes that have been modified throughout the course of grass evolution to produce the unique morphology of lodicules (Whipple *et al.*, 2007). It seems reasonable that the B-class genes are playing similar roles in controlling perianth and androecium identity in the Zingiberales. We hypothesize that the evolution and functional diversification of these transcription factors has contributed to the diversification of floral morphology in the Zingiberales, particularly in perianth and androecial whorls.

Changes in the Zingiberales androecium have been investigated in an evolutionary context (Rudall & Bateman, 2004), but there has been less focus on evolution of perianth morphology, perhaps due to the complexity of homology statements in petaloid monocots. Across monocots there are examples of flowers with almost indistinguishable (i.e. undifferentiated) perianth parts (tepals) or distinguishable (i.e. differentiated) perianth parts (sepals and petals). The characters

most often used to define sepals and petals are based on eudicots (Warner *et al.*, 2008), and even so there is no single set of characters, taken in isolation, that would make an organ inherently a 'sepal' or a 'petal' (Endress, 1994). In the monocots, a fully differentiated perianth has been derived multiple times separately from eudicot sepals and petals (Endress & Doyle, 2009). In Zingiberales, the perianth is considered to be differentiated in the ginger families (Givnish *et al.*, 1999; Ronse De Craene *et al.*, 2003; Heywood *et al.*, 2007), but the banana families are sometimes described as having an undifferentiated perianth (Ronse De Craene *et al.*, 2003; Heywood *et al.*, 2007) and sometimes as having a differentiated perianth (Givnish *et al.*, 1999).

In this study, we examine perianth morphology in the light of Zingiberales phylogeny, investigating the evolution of character states associated with perianth differentiation. Given the importance of homeosis in the evolution of Zingiberalean flowers, particularly the development of petaloidy in the androecial whorls (Kirchoff, 1991; Kirchoff, 1997; Kirchoff, 1998; Kirchoff *et al.*, 2009), we further focus on the evolution and function of the B-class gene lineages in Zingiberales. We present data on changes in copy number across the order and expression of *GLO* orthologs in two Zingiberales families with divergent floral morphologies. We discuss uncovered relationships between gene duplications, gene expression patterns, and changes in floral morphology.

Materials and Methods

Perianth definition and ancestral character state reconstruction:

To maintain a continuity of terminology from previous literature on floral morphology in the Zingiberales, we refer to outer whorl tepals as sepals, and to inner whorl tepals as petals in all members of the order. To assess evolutionary changes in Zingiberales perianth morphology, we scored 10 characters that may differ between sepals and petals and thus may contribute to a 'differentiated' perianth. Outgroup taxa from all commelinid monocot orders were selected based on the most recent published phylogenies (Table 1). Recognizing that ancestral character state reconstruction is highly dependent on taxon choice (Ronse De Craene, 2008), we preferentially chose early-diverging taxa and taxa with less-derived floral morphology. Original species descriptions and accompanying illustrations, as well as descriptions, illustrations and photographs from regional flora were used to determine character states. Character states were mapped onto a diagram of currently accepted commelinid monocot (Givnish *et al.*, 2006; Graham *et al.*, 2006) and Zingiberales phylogenetic relationships (Kress, 1990a; Kress, 1995; Kress *et al.*, 2001). Ancestral character states were assessed under a reversible parsimony model, as implemented in Mesquite v2.72 (Maddison & Maddison, 2009).

We defined two hypothetical perianth states: one in which the perianth is completely undifferentiated and there are no discernable differences between sepals and petals, and a second in which sepals and petals differed in every character assessed. The characters we assessed were (1) size: 0 = all tepals equal in size, 1 = sepal and petal size differs (when described as 'subequal', sepals and petals were scored as equal in size); (2) color: 0 = sepals and petals are the same color, 1 = different colors; (3) texture: 0 = sepals and petals have the same texture, 1 = different textures; (4) pubescence: 0 = sepals and petals show the same pubescence patterns, 1 = pubescence patterns differ; (5) adnation: 0 =sepals and petals adnate, 1 = no adnation between

whorls; (6) corolla connation: 0 = petals free, 1 = petals connate for some to most of their length; (7) calyx connation: 0 = sepals free, 1 = sepals connate; (8) shape: 0 = sepals and petals of approximately the same shape, 1 = shape differs; (9) zygomorphy within calyx and (10) corolla: 0 = whorl actinomorphic 1 = whorl zygomorphic. We also coded staminode characteristics (11): presence=0, absence=1, petaloid staminodes=2, staminodial labellum=3.

Perianth character states (characters 1 to 10) for each taxon were summed to yield a 'dimorphism score'. A completely undifferentiated perianth (dimorphism score of 0) would have identical sepals and petals, fused (adnate) into a single floral tube. A fully differentiated perianth (dimorphism score of 10) would have sepals and petals that differed in size, color, shape, pubescence and texture and were fused into separate calyx and corolla tubes. Calyx and corolla zygomorphy (characters 9 & 10) were included to account for perianth morphology in some Zingiberales and Commelinales. In Musaceae and many Pontederiaceae a single petal is differentiated; in Heliconiaceae, a single sepal is differentiated. The single tepal most commonly differs from the other tepals in either size or color, or both (see references included in Table 1), however these organs were not considered when scoring size, shape and color differences. Rather, their differentiation was included in the zygomorphy characters. The dimorphism score was reconstructed as both a discrete and a continuous character and the results compared.

RNA Extraction and cDNA synthesis:

Taxa were selected to represent floral diversity within each family of the order Zingiberales (Table 2). Floral material was preserved in RNAlater (Ambion). After one night of incubation at 4°C and up to two weeks at -20°C, tissue in RNAlater was archived at -80°C. RNA was extracted from floral material representing a range of developmental stages using Plant RNA Extraction Reagent (Invitrogen), following the manufacturer's protocol. The RNA pellet was re-eluted in 10-30μL of water and also stored at -80°C. RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). DNA was removed from two micrograms of RNA with RQ1 RNAse-free DNase (Promega) and reverse transcribed into cDNA using a polyT primer and M/MLV reverse transcriptase (Promega) following the maufacturer's protocols. The success of the reverse transcription reaction was assessed by amplifying β-actin from the cDNA, using the following intron-spanning primer pair: *ACT-F*, GGA CGA ACA ACT GGT ATC GTG CTG, and *ACT-R* GAT GGA TCC TCC AAT CCA GAC ACT GTA.

Amplification of *GLO* homologs:

cDNA was diluted 1 to 10, and 2μL of the dilution used in 20μL PCR reactions containing 0.5pmol of forward and reverse primer, 0.4U iProof DNA polymerase (BioRad), 4μmol dNTPs and 1μg BSA. Final $MgCl_2$ concentration was 2.5mM. We achieved limited success amplifying *GLO*-like genes using forward primers degenerate for the MADS domain and a polyT reverse. Consequently, we designed less degenerate primers after comparing *GLO*-like sequences across commelinid monocots. Multiple primer pair combinations were used for each cDNA sample: *ZinGLO-F*, CAG GTS ACC TTC TCC AAG C and *ZinGLO-R*, AGG TTD GGY TGG YTG GGT TG; *ssMADS-F*, CAR GTK ACC TTC TGC AAG and *3'GLO*, CAT ATA AGT CAG TTG CTT GTT CTC CTC CTC; *ZinGLO-R2*, GGY TGS TSR CGG AAG GCC AT, was used in combination with *ZinGLO-F* and *ssMADS-F*. The full volumes of the PCR products were run out

on 1.2% agarose gels and bands of the appropriate size were gel extracted and cloned. Between four and 20 clones were picked from each cloning reaction and sequenced using vector-specific primers. The number of colonies sequenced for each family ranged from 25 (Lowiaceae) to 140 (Costaceae). In order to saturate gene recovery for at least two species across the order, 100 *C. spicatus* colonies and 98 *M. basjoo* colonies were sequenced. In addition, *ZinGLO* clade-specific primers were designed and used on taxa where particular paralogs had not been recovered. The primer combinations used in this serial PCR approach were: *ZinGLO1*-F, GAG TAC TGC AGC CCA TCC AC; *ZinGLO1*-R CAA TTC CTT AGG GTT GAG AGA A; *ZinGLO2*-F, ATC AAG AAG GCG AGG GAG AT, *ZinGLO2*-R AAG TTC CTT GGG ATA AAG CGA G; *ZinGLO3*-F, GGG ATC ATT AAG AAA GCG AGA GAA; *ZinGLO4-F*, CAT ATT CTC AAG CTC TGG C and *ZinGLO3&4-R*, TGG AAT TAA TTC TTT TGG G. All sequences were obtained using BigDye v3.1 on an ABI 3100 Genetic Analyzer (Applied Biosystems). All sequences were deposited in GenBank (accession numbers GU594899- GU594995)

Multiple Sequence Alignment and Phylogenetic Analysis:

Zingiberales *GLO*-like sequences have been shown to form a clade (pp=0.91) within a broader monocot *GLO*-like phylogeny (Mondragon-Palomino *et al.*, 2009). For this reason, we included only commelinid monocot *GLO* homologs (retrieved from GenBank) as outgroups in our analysis. Nucleotide sequences were translated into protein using MacClade v4.08 (Maddison & Maddison, 1998) and aligned using MUSCLE (Edgar, 2004). The resulting protein alignment was transferred manually to the nucleotide data and the final alignment edited by eye. Once primer sequences had been removed, the final alignment was 597 nucleotides in length.

Model selection was performed using MrModeltest (Nylander, 2004). The alignment was partitioned into 1st and 2nd vs. 3rd codon positions and each partition assessed. No difference in model selection was identified for the two partitions (GTR+I+G selected for all partitions and unpartitioned dataset). When the data were partitioned according to protein domain (M, I, K and C), MrModeltest selected separate models for each partition (Table 3). The resulting partitioned dataset was analyzed using Bayesian inference of phylogeny, as implemented in MrBayes v3.2.1 (Huelsenbeck & Ronquist, 2001). Gaps in the alignment were treated as missing data. Two analyses were run in parallel until both converged on similar log likelihood scores (average standard deviation of split frequencies < 0.01). The log likelihood scores reached a plateau after approximately 10% of the generations completed (assessed using Tracer, Rambaut & Drummond, 2007). Consequently, the first 10% of the trees were discarded as 'burnin' and a 50% majority rule tree was constructed from the remaining trees. Maximum likelihood searches using the unpartitioned dataset and 500 ML bootstrap replicates were performed using GARLI (Zwickl, 2006) on the CIPRES web portal (Miller *et al.*, 2009). A consensus of bootstrap trees was constructed using the sumtrees script in Dendropy (Sukumaran & Holder, 2009). Trees were edited using Mesquite (Maddison & Maddison, 2009), Dendroscope (Huson *et al.*, 2007) and Illustrator CS4 (Adobe).

RNA *in situ* Hybridization:

Expression of *ZinGLO1* was assessed in *Musa basjoo* and *Costus spicatus*. The expression of *ZinGLO2* was assessed in *C. spicatus*. *In situ* hybridizations were performed as described in

Bartlett *et al.* (2008). The RNA probes for *csGLO1* and *csGLO2* included the MADS box, I region, K region and a section of the C region of MIKC MADS box genes (Genbank GU594899 and GU594931). We are confident there was no cross-hybridization between probes because of distinct expression patterns obtained using similarly designed probes for *csGLO1*, *csGLO2* and a *C. spicatus AGAMOUS* ortholog (data not shown). The *Musa* probe (Genbank GU594929) was designed to exclude the MADS box.

Semi-Quantitative RT-PCR:

Floral organs were dissected from *C. spicatus* flowers shortly before anthesis. RNA extraction and cDNA synthesis were performed as described above. Primers were designed to flank multiple introns and to amplify different sized bands for the *C. spicatus ZinGLO1* ortholog (*csGLO1-F,* GAG TAC TGC AGC CCA TCC AC; *csGLO1-R* CAA TTC CTT AGG GTT GAG AGA A) and the *ZinGLO2* ortholog *(csGLO2 –F,* ATC AAG AAG GCG AGG GAG AT; *csGLO2-R*, AAG TTC CTT GGG ATA AAG CGA G). Reagent concentrations were as described above. Cycling conditions were as follows: initial denaturation of 98°C for 4 minutes, 28 amplification cycles (98°C for 20s, 62°C for 20s, 72°C for 30s), a final extension step of 72°C for 7min. β-actin was amplified from all tissues as a reference. All bands were sequenced to verify identity. One PCR product from each of the *csGLO1* and *csGLO2* RT-PCR experiments were cloned and multiple clones sequenced to verify that the bands represented single sequences.

Results

Character evolution in the Zingiberales and the broader commelinid monocots:

Perianth morphology in the Zingiberales is diverse (Fig. 1). In the banana families, the perianth provides the majority of the floral display (Fig. 1b-d, f, g, j). In the ginger families, it is the petaloid staminodes that are large and brightly colored, while the perianth is often inconspicuous (Fig. 1e, h, i, k-p). Ancestral character state reconstruction of major features of perianth morphology helps to assess the characters contributing to perianth differentiation (Fig. 2).

Perianth Dimorphism: Both hypothetical morphologies (a dimorphism score of 0 or 9) are rare in the commelinid monocots, although both do occur (Fig. 3). Flowers of *Musa* have a perianth dimorphism score of 1. The only distinction between the perianth whorls lies in the single free, larger adaxial petal, resulting in zygomorphy of the petal whorl (character 10, Fig. 2j). Although a completely differentiated perianth is not ancestral in the Zingiberales, this reduced perianth dimorphism appears to be a derived condition in Musaceae (Fig. 3). The adnation between the sepal and petal whorls observed in Musaceae and Heliconiaceae is derived separately in each family (Fig. 2f). The perianth dimorphism scores of Lowiaceae, Strelitziaceae, Zingiberaceae, Cannaceae and Marantaceae are all comparable, lying between 5 and 6. This similarity in scores, however, does not necessarily imply homology of the differentiated perianth in these families, as similar scores result from the presence or absence of different characters.

The reconstruction of dimorphism score as a continuous character summarized the results of individual reconstructions fairly adequately, while reconstructing dimorphism as a discrete character obscured much of the complexity revealed by the individual character state

reconstructions (data not shown). As such, the results and discussion will be restricted to the continuous character reconstruction. The rounded scores at most nodes correspond with the results obtained by assessing each character individually (Figs 2, 3). Using the continuous reconstruction, we can trace the gradual accretion and subsequent losses of morphological differences between sepals and petals in the commelinids.

The perianth dimorphism score both at the base of Commelinales plus Zingiberales and at the base of the commelinid monocots is 3. This score is in agreement with the reconstruction of individual character states (Fig. 3). The ancestral commelinid monocot perianth is reconstructed as being differentiated in shape, not a single floral tube and may or may not have been differentiated in size. This score was consistent regardless of the phylogenetic hypothesis used to reconstruct character history in the commelinid monocots (Chase *et al.*, 2006; Givnish *et al.*, 2006; Graham *et al.*, 2006). The perianth dimorphism score at the base of the Zingiberales is also 3, in accord with the results from reconstructing individual characters (Figs. 2, 3). A zygomorphic corolla, shape differences between outer and inner whorl tepals and an absence of adnation are the inferred ancestral conditions for the Zingiberales. Size difference between sepals and petals is reconstructed as equivocal.

The well differentiated perianth, a characteristic of flowers in the ginger families, Commelinaceae, Arecales and the basal Poales (i.e. dimorphism scores >5, Fig. 3), appears to be independently derived in these four groups. Zygomorphy in the corolla was reconstructed as ancestral in the Zingiberales (Fig. 2j), as has been found for the androecium in a separate investigation (Rudall & Bateman, 2004).

Stamen whorls: The presence of staminodes is not reconstructed as ancestral in the Zingiberales (Fig. 2k). Petaloid staminodes most likely evolved on the branch leading to Heliconiaceae plus the ginger families (Fig. 2k).

'Labellum' is a term used to describe non-homologous floral organs in divergent taxa (Rudall & Bateman, 2002). Many of the families of the Zingiberales have been described as possessing a labellum, all of varying homologies (Eichler, 1878; Thompson, 1933; Kirchoff, 1983; Kirchoff, 1988b; Kirchoff & Kunze, 1995; Kirchoff, 1997; Kirchoff, 1998). The labellum of Costaceae and Zingiberaceae, however, arises from the fusion of multiple petaloid staminodes (Fig. 1). It is this novel compound organ to which we refer when we use the term 'staminodial labellum'. The staminodial labellum is reconstructed to have evolved before the divergence of Costaceae and Zingiberaceae (Fig. 2k)

Gene tree: homology, duplications and losses:

Phylogenetic reconstruction of *GLO*-like gene evolution in the Zingiberales revealed a complex history of gene duplications and losses. We identified at least four *GLO* homologs in the Zingiberales (Fig. 4). These may be the result of Zingiberales-specific duplications: all *GLO* homologs form a clade to the exclusion of other monocot sequences, but the Zingiberales *GLO*-like clade received only moderate support (pp=0.81). Support levels are low throughout the Zingiberales, particularly at deeper nodes.

Bayesian phylogenetic analysis resolved three clades and one grade of *GLO* homologs (Fig. 4). Two of the three *ZinGLO* lineages, *ZinGLO3* and *ZinGLO4*, form distinct clades (pp=0.88 for both clades). The *ZinGLO1* clade is only weakly supported (pp=0.56), although internal branching patterns are consistent with broader organismal phylogeny. We have designated the grade leading to the *ZinGLO3*/*ZinGLO4* node as *ZinGLO2*. Each of the well-supported clades (pp 0.93-1.00) within the *ZinGLO2* grade shows an internal branching structure consistent with the Zingiberales organismal phylogeny. Because there is no evidence for further duplications within any of the *ZinGLO2* clades, the grade could more accurately represent a single *GLO* homolog. We tested the hypothesis that the *ZinGLO2* grade could form a monophyletic *GLO*-like gene lineage by repeating the phylogenetic analysis, constraining the *ZinGLO2* grade to be monophyletic. The evidence against the constrained model was assessed using the Bayes Factor (Kass & Raftery, 1995; Nylander *et al.*, 2004). The harmonic mean of log likelihoods in the constrained analysis (M_0), as estimated by MrBayes, equaled -21,210.14; that of the unconstrained (M_1) equaled -22,044.54. The Bayes $Factor_{10}$ thus equaled 1.04. A Bayes Factor between 1 and 3 implies very little evidence against M_0 (Kass & Raftery, 1995; Nylander *et al.*, 2004). We therefore did not reject the hypothesis that the *ZinGLO2* grade is monophyletic. In all further discussion, we refer to the genes belonging to this putative clade as orthologs of *ZinGLO2*.

Nucleotide sequence divergence between individual *ZinGLO* paralogs is low (81.2 to 95.9%), but the evidence supports our claim that the clades represent paralogs rather than alleles. Both alleles and paralogs can be discerned in the *ZinGLO* phylogeny. For example, we uncovered multiple alleles of both *ZinGLO2* and *ZinGLO1* from *Strelitzia reginae* (Strelitziaceae), *Halopegia azurea* (Marantaceae), and *C. spicatus* (Costaceae). Similarly, multiple alleles of *ZinGLO3* and *ZinGLO4* were recovered from *Globba laeta* (Zingiberaceae) (Fig. 4). Sequence divergence is particularly low between *ZinGLO3* and *ZinGLO4* (90-95.9% similarity). To confirm that these are indeed paralogs rather than persistent alleles, we sequenced intron 6 of these genes. Intron 6 of *ZinGLO3* is consistently 80bp shorter than intron 6 of *ZinGLO4* (data not shown). This adds support to the hypothesis that the *ZinGLO3* and *ZinGLO4* clades represent separate *GLO* paralogs.

To investigate the order of inferred gene duplications, we scored each of the families in the Zingiberales for the number of *GLO*-like genes retrieved (Fig. 5). Commelinaceae, the only family in the Commelinales from which *GLO* homologs have been sequenced, was used as the outgroup. Two *GLO*-like genes are recorded from *Tradescantia reflexa*, but only a single copy has been retrieved from *Commelina communis* (Ochiai *et al.*, 2004). Commelinaceae was therefore coded as polymorphic (1 or 2 *GLO*-like copies). When gene copy number was reconstructed as a discrete character using parsimony (Maddison & Maddison, 2009), the common ancestor of Zingiberales is found to have a single *GLO* homolog (Fig. 5a). The ancestor at the node separating Musaceae from the remaining Zingiberales is reconstructed to have three *GLO* homologs. Tracing the history of individual *ZinGLO* homologs yielded similar results (Fig. 5b-e). *ZinGLO2* was gained after the divergence of Musaceae from the remainder of the Zingiberales. *ZinGLO3* was either gained at the same point and subsequently lost in the Strelitziaceae, or, equally parsimoniously, was gained independently in Lowiaceae and in the ancestor prior to the divergence of Heliconiaceae from the ginger families. This second hypothesis of two separate gains seems unlikely considering the low sequence divergence

between *ZinGLO3* orthologs (92.2 to 99.8% similarity, Fig. 5d): the Lowiaceae *ZinGLO3* sequences are nested within a clade of ginger *ZinGLO3* sequences, indicating common origin. These results imply that two separate gene duplication events occurred along the branch directly after the divergence of Musaceae.

ZinGLO4 appears to be the result of a gene duplication event prior to the divergence of Costaceae and Zingiberaceae. The presence of *ZinGLO4* in only these two families, coupled with the extreme similarity of *ZinGLO4* orthologs from Costaceae and Zingiberaceae (96.3 to 99.5% similarity), adds credence to this reconstruction (Fig. 5e).

ZinGLO1 expression in *Musa basjoo* and *Costus spicatus:*

Plants in the Musaceae are monoecious, with separate female, transition, and male flowers produced on the same inflorescence but at different times during development. Transitional flowers are functionally male, but have gynoecium morphology intermediate between male and female flowers (Simmonds, 1966). Female flowers in *M. basjoo* possess five staminodes in place of fertile stamens, and male flowers have a reduced, aborted gynoecium. Early floral development appears to be similar in both male and female flowers, and differentiation between the female, transition and male flowers occurs later in development (White, 1928), Fig. 1b and c). The flowers examined in this study were all male. White (1928) observed that the floral meristems start as rounded domes and become flattened during the course of development. Sepals are initiated first, and the petals are formed in the gaps between sepals. The three outer whorl stamens are antisepalous and form internally to the perianth whorls. Inner whorl stamens are antipetalous and appear to initiate after the outer whorl stamens. The staminode in the accession of *M. basjoo* we examined appears to arise from the anterior side of the adaxial free petal (Fig. 6a). The gynoecium is inferior and initiates last.

Orthologs of *ZinGLO1* were not detected in early floral meristems of *M.basjoo* or *C. spicatus* (Fig. 6g-h). In *M. basjoo, mbGLO1* RNA was detected at high levels in the region internal to the developing sepals of young flowers (Fig 6b). It is unclear whether petals and stamens in *M. basjoo* originate from common petal-androecium primordia, which occur frequently in the Zingiberales (Kirchoff, 1983; Kirchoff, 1988b; Kirchoff & Kunze, 1995; Kirchoff, 1997; Kirchoff *et al.*, 2009), or from individual organ primordia. As such, the observed *mbGLO1* expression may have been in common petal-androecium primordia, or in individual androecial and/or petal primordia. Petals in *M. basjoo* are always formed opposite stamens, not opposite other petals (Fig. 6a), and identical expression patterns were observed in multiple sections at varying levels. Therefore, whether or not stamens and petals arise from common primordia, *mbGLO1* expression was detected in developing petals and stamens. Expression in older flowers was detected in the five fertile stamens, the adaxial staminode and the free petal (Fig 6d).

For *C. spicatus*, the development of a closely related species, *C. scaber,* has been well characterized (Kirchoff, 1988b) and was useful in determining when and where *ZinGLO* orthologs were expressed. The perianth of *C. spicatus* consists of three sepals and three petals fused proximally into a floral tube, but free distally. Five infertile staminodes are fused to form the abaxial labellum, with a single adaxial fertile stamen (Fig. 6d). Early in development, expression of *csGLO1* was detected in the common petal-androecium primordia (Fig. 6e).

Expression later became restricted to the fertile stamen, the labellum and the petal margins (Fig. 6f). Based on results from semi-quantitative RT-PCR in flowers just prior to anthesis, *CsGLO1* expression was strongest in the labellum, but was also in petals, stamens, and the gynoecium (Fig. 6p).

ZinGLO2 expression in *Costus spicatus:*

The ortholog of *ZinGLO2* was not retrieved from *Musa*. Expression of *CsGLO2,* the *C. spicatus* ortholog of *ZinGLO2,* was not detectable in early floral meristems (Fig. 6n). Expression became evident once sepals and the common petal-androecium primordia had differentiated (Fig. 6k). At this stage, *csGLO2* was expressed throughout the developing flower at a relatively higher level than that observed for *csGLO1*, a result corroborated with RT-PCR (Fig. 6p). *CsGLO2* expression was strongest in the common androecium-petal primordia and weakest in the sepals. Expression in the sepals was undetectable using *in situ* hybridization in mature flowers (Fig. 6l-m). RT-PCR indicated *CsGLO2* was expressed weakly in the sepals, with stronger expression in the petals, stamens, labellum and gynoecium of mature flowers.

Discussion

Perianth evolution in the commelinid monocots:

The ancestral perianth character state for monocotyledons has been reconstructed as 'undifferentiated' in several analyses (Ronse De Craene *et al.*, 2003; Zanis *et al.*, 2003; Endress & Doyle, 2009). A differentiated perianth is likely to have evolved multiple times in the monocots (Ronse De Craene *et al.*, 2003; Zanis *et al.*, 2003; Endress & Doyle, 2009). In Ronse De Craene's (2003) analysis, the character state at the base of the Zingiberales was reconstructed as equivocal, but *Strelitzia*, *Orchidantha* and *Heliconia* were all scored as undifferentiated and phylogenetic relationships among the Zingiberales families were not well resolved (Ronse De Craene *et al.*, 2003). Givnish et al. (1999) reconstructed the ancestral perianth state in commelinids as differentiated. Their perianth character states for Musaceae and Phylidraceae were in conflict with those of the current analysis, but even if the character states and the tree topology were adjusted to be congruent with our analysis, the ancestral state of the commelinid monocots and the Zingiberales would still be reconstructed as ancestrally differentiated. The terms 'differentiated' and 'undifferentiated' are, however, imprecise and ultimately obscure the exact changes in morphology that have occurred through the course of evolution.

In our analysis, a fully differentiated perianth was not reconstructed as ancestral in the commelinid monocots. At least three separate derivations of a well-differentiated perianth were reconstructed: at least one in the Zingiberales, at least one in the Commelinales, and either a single derivation at the base of the Arecales plus Poales, or separately in each order if they are not sister (Chase *et al.*, 2006; Graham *et al.*, 2006). The results of our individual character state and dimorphism score reconstructions suggest that the well-differentiated perianth characteristic of Lowiaceae, Strelitziaceae and the ginger familes, especially Costaceae, was not derived in a single saltatory event, but rather may have been derived through the gradual accretion of differences between outer and inner whorl tepals (Fig. 2, 3, 7). This could be interpreted as the

progressive partitioning and canalization of the perianth into outer and inner whorl organs (Flatt, 2005).

ZinGLO gene duplications and losses:

Our results indicate three duplication events giving rise to four *GLO* homologs in the Zingiberales. There are low levels of nucleotide divergence between all four *GLO* homologs, particularly between *ZinGLO3* and *ZinGLO4*. The level of divergence between *ZinGLO3* and *ZinGLO4* within a single species is similar to that observed between pairs of duplicate maize MADS box genes generated in an allotetraploidy event that occurred between 11 and 21 mya (Mena *et al.*, 1995; Theissen *et al.*, 1995; Gaut & Doebley, 1997; Cacharron *et al.*, 1999; Munster *et al.*, 2001). Ancestral character state reconstructions put the *ZinGLO3*-*ZinGLO4* duplication on the branch leading to Zingiberaceae plus Costaceae (Fig. 5 and Fig. 7). These families are estimated to have diverged from each other approximately 105 mya (Kress & Specht, 2006).

ZinGLO4 and *ZinGLO3* may be confoundingly similar for a number of reasons: they may have arisen in a more recent duplication event than our analysis suggests; these *GLO* paralogs may be under strong purifying selection; or the observed similarity may be due to gene conversion (there is evidence for extensive gene conversion in rice (Wang *et al.*, 2007)). The assessment of nucleotide sequence divergence highlights the importance of phylogenetic analysis and dense taxonomic sampling in determining gene homologies, especially in the absence of a sequenced genome. Because of the high degree of similarity between *ZinGLO* paralog sequence divergence (81.2 to 95.6 %) and within-ortholog sequence divergence (84.9 to 99.8%), a simple BLAST search would not have been able to discern orthologs, paralogs and alleles.

There is evidence for multiple losses of *GLO*-like genes in the order. These 'gene losses' may have been sampling artifacts rather than true losses, but this seems unlikely as the same sampling strategy was used on all taxa investigated. The primer combinations we used also amplified *DEF*-like and *AP1*-like genes, indicating broad amplification of closely-related MADS box gene lineages. Although nucleotide divergence between individual *ZinGLO* paralogs is low, the possibility remains that these 'lost' paralogs have diverged so extensively in their nucleotide sequences that our primers were unable to amplify them. Alternatively, they may be expressed at extremely low levels as compared with their orthologs in different taxa and thus remained undetected. Assuming a common origin of *ZinGLO3*, losses occur in three places on the tree: *ZinGLO1* was lost in Zingiberaceae (Fig. 5b), and *ZinGLO3* was lost in Strelitziaceae and Cannaceae. *ZinGLO3* also seems to have been lost in specific lineages within Costaceae. Although *ZinGLO3* was repeatedly retrieved from *Tapeinochilos* and *Dimerocostus*, attempts to amplify this gene from *Costus* were unsuccessful.

ZinGLO gene duplications are associated with increasing perianth dimorphism:

A central concept in evo-devo is the hypothesized role of the diversification of transcriptional regulation (Levine & Tjian, 2003; Wray *et al.*, 2003). The raw material for transcriptional diversification is thought to be duplicate genes. Genes may be duplicated by a whole genome duplication event (polyploidy), a tandem duplication or transposition-duplication (reviewed in

(Freeling, 2009)). These maintained duplicates may decay and become pseudogenes, they may retain their ancestral functions and expression patterns or they may subfunctionalize or neofunctionalize over time, leading to phenotypic novelty (Ohno, 1970; Lynch *et al.*, 2001; Freeling & Thomas, 2006; Freeling, 2009).

GLO-like gene duplication and diversification may have contributed to floral diversification in the Zingiberales. *ZinGLO* gene duplications are reconstructed to have occurred on the branch following the divergence of Musaceae and before the divergence of Costaceae and Zingiberaceae. Perianth dimorphism score remains constant at a level of 3 from the origin of the commelinid monocots to the base of the Zingiberales, but begins to increase following the divergence of Musaceae from the ancestor of the remaining Zingiberales. The first two hypothesized gene duplication events coincide with the beginning of this gradual increase in perianth dimorphism (Fig. 7). Gene duplication and subsequent subfunctionalization may have allowed for the partitioning and subsequent differentiation of the perianth. It has been hypothesized that rather than directly controlling petal identity, B-class genes define a particular region in the developing flower (Irish, 2009). Increasing B-class gene diversity may allow for an increased number of floral regions, resulting in increased modularization and diversification of the perianth (Mondragon-Palomino & Theissen, 2009). *CsGLO1* is expressed early in common petal-stamen primordia, and later only in the androecium and gynoecium. *CsGLO2* is expressed early in the sepals and common petal-stamen primordia, and later in the petals, androecium and gynoecium. These overlapping but not identical expression domains may be the result of sub- or neo-functionalization of the duplicated *ZinGLO* genes, allowing for the formation of new floral regions and ultimately further differentiation of sepals and petals.

If the orchid labellum is interpreted as an elaborated inner whorl tepal (see (Rudall & Bateman, 2002), for a discussion), perianth morphology in the Heliconiaceae, Musaceae, Pontederiaceae, and Orchidaceae shares some striking similarities. A perianth consisting of five tepals, with a single differentiated posterior tepal occurs in all of these families. It has been hypothesized that this within-whorl modularization in Orchidaceae may have been due to duplications and subsequent sub- and neo-functionalization in the *DEF* gene lineage (Mondragon-Palomino & Theissen, 2008; Mondragon-Palomino *et al.*, 2009; Mondragon-Palomino & Theissen, 2009). Intriguingly, *mbGLO1* was found to be expressed in the single free petal of *M.basjoo*, while it was not detected in the remaining perianth members (Fig. 6c). Modularization within individual perianth whorls, mediated by B-class MADS box gene evolution, may be a trend in these more derived monocot lineages.

<u>*ZinGLO* gene duplications and the evolution of the androecium in the Zingiberales:</u>

The staminodial labella in Costaceae and Zingiberaceae are probably homologous organs. There have been no studies of floral development in the earliest diverging Zingiberaceae lineages, the monotypic subfamilies Tamijieae and Siphonochiloideae (Kress *et al.*, 2002), but in both subfamilies the well-developed lateral staminodes are fused to the labellum as is the case in Costaceae (Kirchoff, 1988b; Sakai & Nagamasu, 2000; Kress *et al.*, 2002). It remains unclear whether the labella in *Tamijia* and *Siphonochilus* are the product of the fusion of four, or five, staminodes. The labella are, however, bilobed in both genera (Sakai & Nagamasu, 2000; Kress *et al.*, 2002), as in other Zingiberaceae where the anterior staminode initiates in a position confluent

with the rest of the labellum but ultimately aborts (Kirchoff, 1997; Kirchoff, 1998; Box & Rudall, 2006).

A single gene duplication event was reconstructed to have occurred in the common ancestor of Costaceae plus Zingiberaceae, leading to the presence of *ZinGLO4* exclusively in these two families. This hypothesized gene duplication occurred concurrently with the reconstructed derivation of the staminodial labellum. In contrast, the acquisition of petaloid staminodes is not associated with any detected *GLO*-like gene duplication event. This does not preclude the involvement of *GLO*-like genes in the evolution of petaloid staminodes: the possibility remains that *ZinGLO2* and *ZinGLO3* have been modified through the course of evolution in the lineage that led to *Heliconia* plus the ginger families, but not in *Strelitzia* or *Orchidantha*. Expression of a *ZinGLO3* ortholog has been assessed in *Alpinia oblongifolia* (Zingiberaceae) (Gao *et al.*, 2006). As there is a fairly low degree of sequence divergence between *ZinGLO3* from *A.oblongifolia* and *ZinGLO4* from *A.hainanensis* (90.8% similarity), *in situ* hybridization studies in *A. oblongifolia* may well have been detecting the expression of both *ZinGLO3* and *ZinGLO4.* Expression was detected early on in the common petal-androecium primordia, later in the petals and the androecium. This expression pattern is very similar to that observed for *CsGLO2*, except *CsGLO2* is expressed in the sepals early in development. The expression of these homologs needs to be investigated more fully across the order.

If the acquisition of *ZinGLO4* was one of the key events associated with the evolution of the staminodial labellum, it may be the case that *ZinGLO4* is the 'labellum gene' and is expressed and functions only in the labellum, or it may show a wider expression pattern. It may be the combination of multiple *GLO* homologs, rather than expression of a single ortholog, that confers labellum identity (Fig. 7). Considering the combinatorial functioning of MADS box genes (Egea-Cortines *et al.*, 1999; Honma & Goto, 2001; Theissen, 2001), and the broad expression patterns observed for *ZinGLO1, ZinGLO2, ZinGLO3* and possibly *ZinGLO4* (Gao *et al.*, 2006), the combination hypothesis seems more likely. There may be stoichiometric competition between *GLO* paralogs for binding partners in higher-order MADS complexes. A certain combination of MADS tetramers in a certain ratio is what is necessary for conferring organ identity (Fig. 7b). There is evidence for the combination hypothesis in *Petunia,* where dosage effects have been observed for B-class genes (Vandenbussche *et al.*, 2004). There is suggestive evidence for differences in expression levels between paralogs in *Costus* (this study) and Ranunculaceae (Kramer *et al.*, 2007; Rasmussen *et al.*, 2009). Petal-specific expression of *AP3-3* is seen in *Aquilegia*, but none of the *AP3* or *PI* homologs in *Aquilegia* have been found to be expressed only in the staminodia (Kramer *et al.*, 2007). Similarly, none of the Orchid *DEF* homologs are expressed exclusively in the tepaloid labellum, but it is perhaps the combination of all four that is necessary for labellum development (Mondragon-Palomino & Theissen, 2009). We plan to explore and test this hypothesis in the Zingiberales by examining the expression of all four *ZinGLO* paralogs across the order.

In conclusion, we have uncovered multiple gene duplication events within the *GLO* gene lineage in the Zingiberales. These events are separately associated with the increased modularization of the perianth and the acquisition of the staminodial labellum. In addition, the *GLO*-like gene lineage has diversified in association with the increased differentiation of the perianth. These

results provide suggestive evidence that *GLO* gene family evolution has contributed to floral morphological evolution in the Zingiberales.

Acknowledgements

This work was supported by US National Science Foundation awards to CDS (IOS 0845641) and MEB (DEB 0808298), the South African National Research Foundation, the Botanical Society of America and The Heliconia Society International. The authors would like to thank Ana Almeida, Katrina Hong, Sankar Sridaran and Solomon Stonebloom for assistance with primer design, gene recovery and RNA and DNA extractions, Bruce Kirchoff for thoughtful discussion and the Specht lab for comments on the manuscript.

Table 1. Taxa scored for perianth character state reconstructions.

Order	Family	Phylogeny reference(s)	Genus or Taxon	Authority	Character State Reference(s)
	Dasypogonaceae	(Chase *et al.*, 2006)	*Kingia*	R. Br.	(Kubitzki, 1998)
Arecales	Arecaceae	(Baker *et al.*, 2009)	*Oncocalamus*	Mann & H.Wendl.	(Uhl, 1987)
			Laccosperma	Drude	
			Eugeissona	Griff.	
Commelinales	Commelinaceae	(Evans *et al.*, 2003)	*Cartonema philydroides*	F. Muell.	(Brown, 1810; Wheeler, 2002)
			Commelina	L.	(Hardy, 2009)
			Plowmanianthus	Faden & C. R. Hardy	(Hardy *et al.*, 2004; Hardy & Faden, 2004)
	Haemodoracee	(Hopper *et al.*, 1999; Hopper *et al.*, 2009)	*Dilatris*	P. J. Bergius	(Jesson *et al.*, 2003)
			Tribonanthes	Endl.	(Macfarlane *et al.*, 1987)
	Hanguanaceae	(Saarela *et al.*, 2008)	*Hanguana*	Blume	(Rudall *et al.*, 1999)
	Philydraceae	(Saarela *et al.*, 2008)	*Philydrum*	Banks ex Gaertn.	(Jacobs, 1993; Jesson *et al.*, 2003)
	Pontederiaceae	(Kohn *et al.*, 1996; Graham *et al.*, 1998)	*Heteranthera*	Ruiz & Pav.	(Jesson *et al.*, 2003; Strange *et al.*, 2004)
			Hydrothrix	Hook f.	(Hooker, 1887; Strange *et al.*, 2004)
Poales	Bromeliaceae	(Givnish *et al.*, 2007)	*Brocchinia*	Schult. f.	(Baker, 1882; Kubitzki, 1998)
	Rapataceae	(Givnish *et al.*, 2004)	*Rapatea paludosa*	Aubl.	(Linne, 1801)
Zingiberales	Cannaceae	(Kress, 1990a; Kress *et al.*, 2001)	*Canna*	L.	(Stevenson & Stevenson, 2004a)
	Costaceae	(Specht *et al.*, 2001)	*Dimerocostus strobilaceus*	Kuntze	(Maas, 1972)
			Costus spicatus	Swartz	
	Heliconiaceae	(Specht and Kress labs, unpublished)	*Heliconia laufao*	W. J. Kress	(Kress, 1990b)
			Heliconia paka	A. C. Sm.	
	Lowiaceae	(Johansen, 2005)	*Orchidantha maxillarioides*	K. Schum.	(Ridl., 1893; Kunze, 1986)
	Marantaceae	(Prince & Kress, 2006)	*Marantochloa leucantha*	(K.Schum.) Milne-Redh.	(Milne-Redhead, 2000)
			Thaumatococcus	Benth.	(Milne-Redhead, 2000)
	Musaceae	(Kress, 1990a; Kress *et al.*, 2001)	*Musa basjoo*	Siebold	(Baker, 1891)
	Strelitziaceae	(Kress, 1990a; Kress *et al.*, 2001)	*Strelitzia reginae*	Aiton	(Aiton, 1789)
	Zingiberaceae	(Kress *et al.*, 2002)	*Siphonochilus*	J. M. Wood & Franks	(Lock, 1985)
			Zingiber officinale	Roscoe	(Sabu, 2006)

Table 2. Taxon sampling for phylogenetic analysis of *GLO* homologs in the Zingiberales.

Family	Species	Authority	Location[a]	Accession[b]
Cannaceae	*Canna sp.*	L.	Lyon	MB0854
Costaceae	*Costus erythrophyllus*	Loes.	NMNH	1994-680
	Costus osae	Maas & H. Maas	NMNH	L-92.0409
	Costus spicatus	Swartz	NMNH	2002-127
	Dimerocostus strobilaceus	Kuntze	Lyon	L-68.0278
	Tapeinochilos solomonensis	Gideon	Lyon	2003.0170
Heliconiaceae	*Heliconia griggsiana*	L. B. Sm.	McBryde	930123-001
	Heliconia lennartiana	W. J. Kress	McBryde	0611775-005
	Heliconia lingulata	Ruiz & Pav.	McBryde	061178-006
	Heliconia metallica	Planch. & Linden ex. Hook	McBryde	266002
	Heliconia rostrata	Ruiz & Pav.	UCBG	90.1606
	Heliconia pendula	Wawra	McBryde	711003-003
Lowiaceae	*Orchidantha maxillarioides*	(Ridl.) K. Schum.	McBryde	970091
Marantaceae	*Afrocalathea rhizantha*	K. Schum.	Lyon	2003.0237
	Ataenidia conferta	(Benth.) Milne-Redh.	Lyon	L-74.0401
	Calathea burle-marxii	H. A. Kenn.	McBryde	770488-001
	Ctenanthe compressa	(A. Dietr.) Eichler	Lyon	L-79.0210
	Halopegia azurea	K. Schum.	Lyon	2003.0185
	Marantochloa leucantha	Milne-Redh.	Lyon	L-80.0376
	Monotagma guianense	K. Schum.	Lyon	L-78.1340
	Phrynium oliganthum	Merr.	Lyon	L-96.0226
	Stachyphrynium jagorianum	(K. Koch) K. Schum.	Lyon	2003.0192
	Schumannianthus virgatus	Rolfe	Lyon	L-83.0899
	Stromanthe jacquinii	(Roem. & Schult.) H. Kenn. & Nicolson	Lyon	L-68.0354
Musaceae	*Musa basjoo*	Siebold	UCBG	89.0873
	Musa velutina	H. Wendl. & Drude	Lyon	L-67.0284
Strelitziaceae	*Phenakospermum guyannense*	(Rich.) Endl.	PTBG	047865
	Strelitzia reginae	Aiton	UC	MB0607
Zingiberaceae	*Aframomum angustifolium*	(Sonn.) K. Schum.	Lyon	L-80.0617
	Alpinia luteocarpa	Elmer	McBryde	990315-004-CL
	Burbidgea nitida	Hook. F.	NMNH	1996-282
	Burbidgea schizocheila	Hackett	Lyon	L-93.0039
	Curcuma sp	L.	Lyon	MB0825
	Elettaria cardamomum	Maton	Lyon	L-67.1100
	Elettariopsis smithiae	Y. K. Kam	Lyon	L-93.0137
	Etlingera corneri	Mood & Ibrahim	Lyon	L-91.0443
	Globba laeta	K. Larsen	Lyon	L-92.0182
	Hedychium greenii	*W.W. Sm.*	NMNH	1994-776
	Hornstedtia gracilis	R. M. Sm.	Lyon	L-99.0505
	Kaempferia sp. "Grande"	J. Banta	NMNH	2001-115
	Kaempferia rubromarginata	(S. Q. Tong) R. J. Searle	Lyon	2003.0153
	Mantisia saltatoria	Sims	Lyon	L-2001.0365
	Pleuranthodium hellwigii	(K. Schum.) R. M. Sm.	Lyon	L-99.0492
	Scaphochlamys kunstleri	(Baker) Holttum	NMNH	1994-749
	Zingiber officinale	Roscoe	UC	MB0876

[a] Location of live accessions or herbarium sheets. Lyon Arboretum, Oahu, Hawaii USA; McBryde Botanical Garden, Kauai, Hawaii USA; University of California Botanical Garden (UCBG); University of California Berkeley Herbarium (UC), Smithsonian Greenhouses (NMNH)

Table 3. Models of nucleotide evolution selected for *GLO* dataset partitioned according to protein domain

Data Partition	**Model**
M-domain	GTR + G
I-domain	SYM + I + G
K-domain	GTR + I + G
C-domain	HKY + I + G

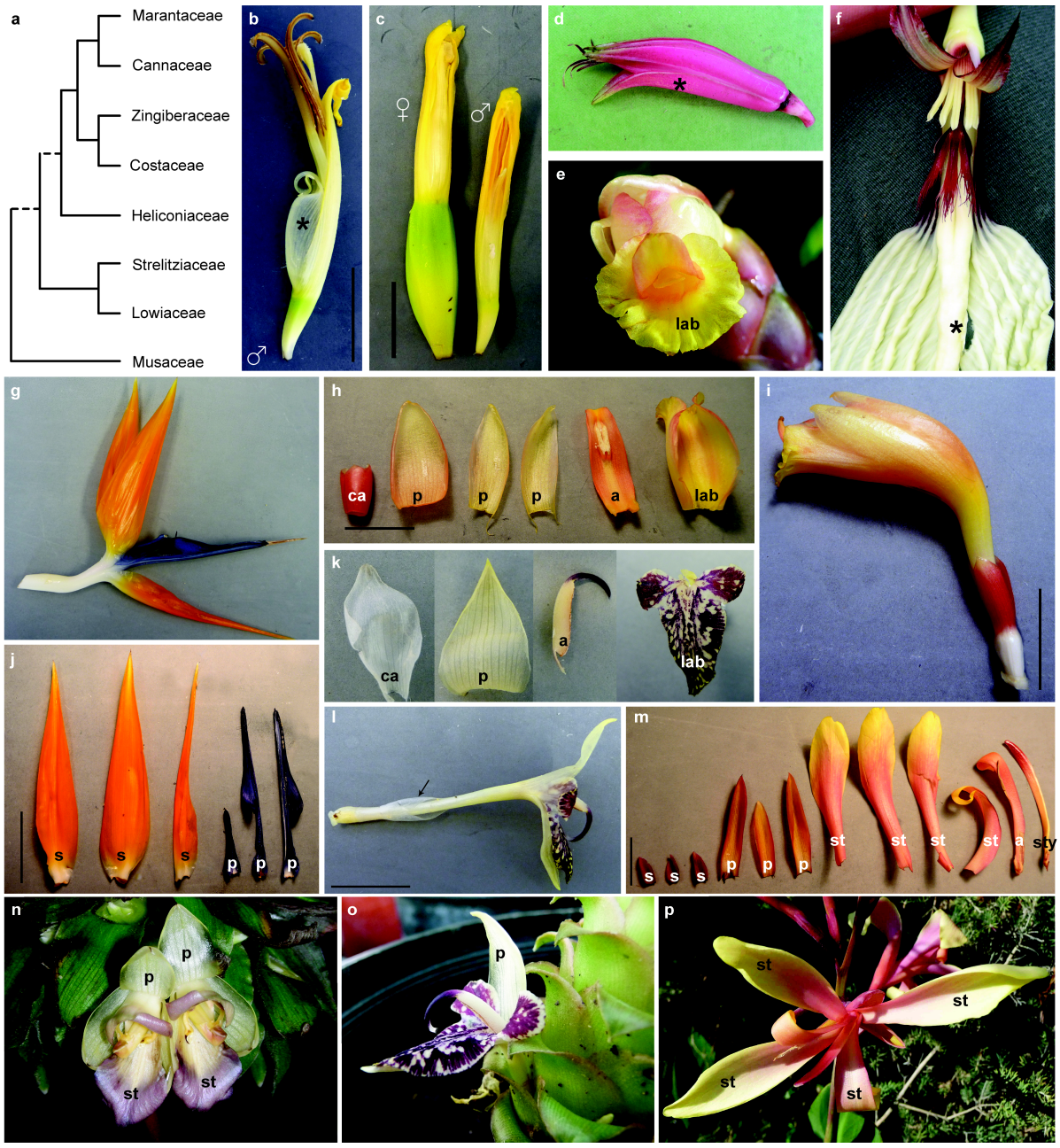

Figure 1. Floral morphology in the Zingiberales. (a) Currently accepted phylogenetic relationships between families in the Zingiberales (Kress, 1990a,1995; Kress et al., 2001). Dashed lines indicate branches with weak support. (b) A single Musa basjoo flower. *, free adaxial petal. (c) Single male and female M. basjoo (Musaceae) flowers shortly before anthesis. (d) Heliconia metallica (Heliconiaceae) flower. *, free adaxial sepal. (e, h, i) Costus spicatus (Costaceae) (e) single flower (h) perianth and androecium of a single flower (i) single flower removed from inflorescence. (f) Partially dissected Orchidantha maxillarioides (Lowiaceae) flower, with sepals having been removed. *, adaxial petal (labellum). (g, j) Strelitzia reginae (Strelitziaceae) (g) single flower (j) dissected perianth of a single Strelitzia flower. (k, l, o) Zingiber wrayi (Zingiberaceae). (k) Dissected calyx tube, single petal lobe, fertile stamen and labellum. (l) Flower removed from an inflorescence (o) single flower. The arrow indicates a membranous calyx tube. (m, p) Canna sp. (Cannaceae) (m) Dissected perianth, androecium and petaloid style of one Canna flower (p) single flower. (n) Calathea princeps (Marantaceae) flower pair. a, fertile stamen; ca, calyx tube; s, sepal; p, petal; st, petaloid staminode; lab, staminodial labellum; sty, style. Bars, 2 cm.

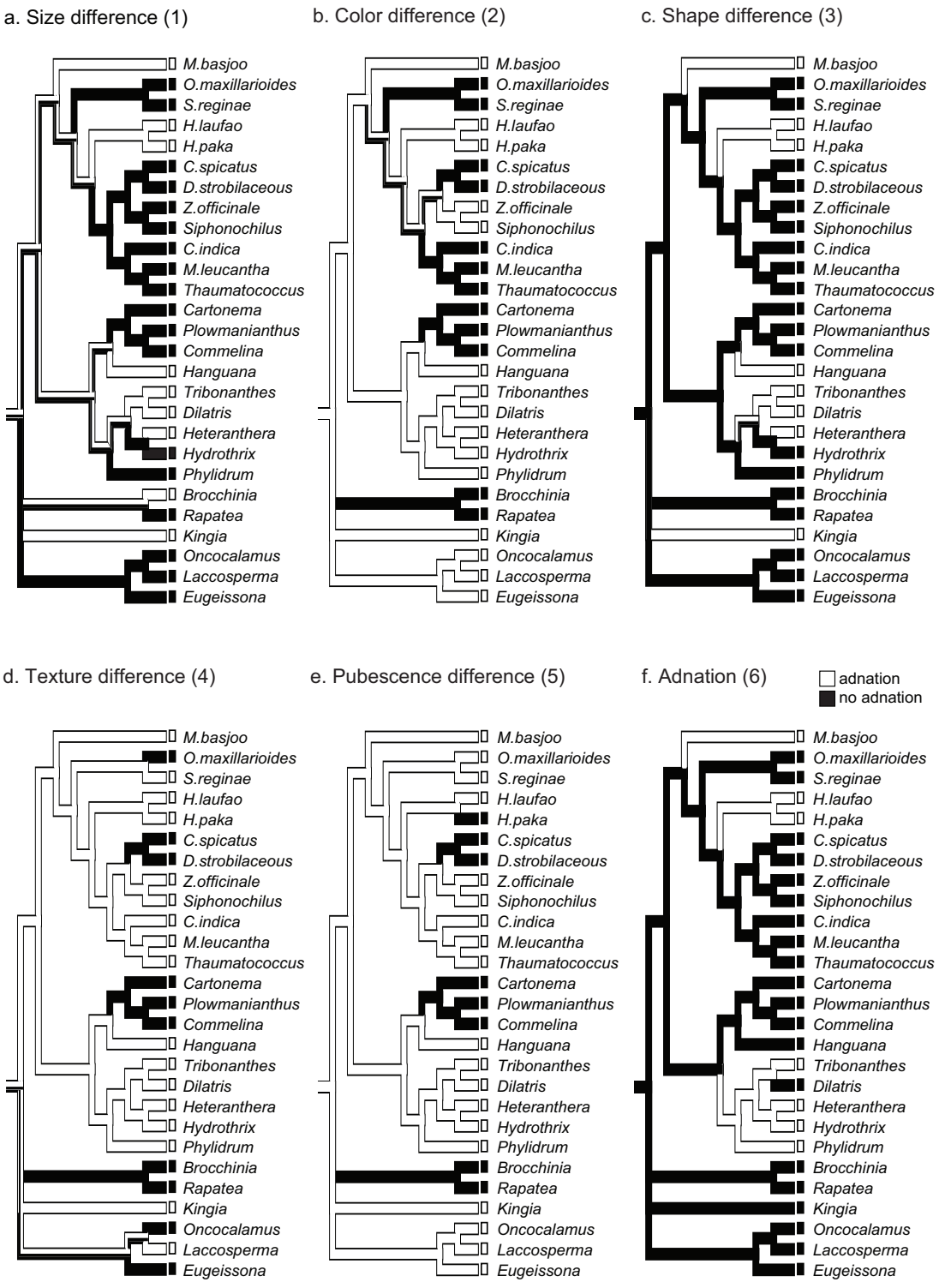

Figure 2. Ancestral character state reconstructions in the commelinid monocots. (a–e) Differences between sepals and petals have been coded. White, no difference between the whorls; black, difference between the whorls. (f) White, adnation between sepals and petals: black, no adnation; (g, h) Black, connation within the calyx or the corolla; white, an absence of connation within each whorl. (i, j) Black, the presence of zygomorphy within a perianth whorl; white, no zygomorphy within a whorl. (k) Presence or absence and nature of staminodes when present.

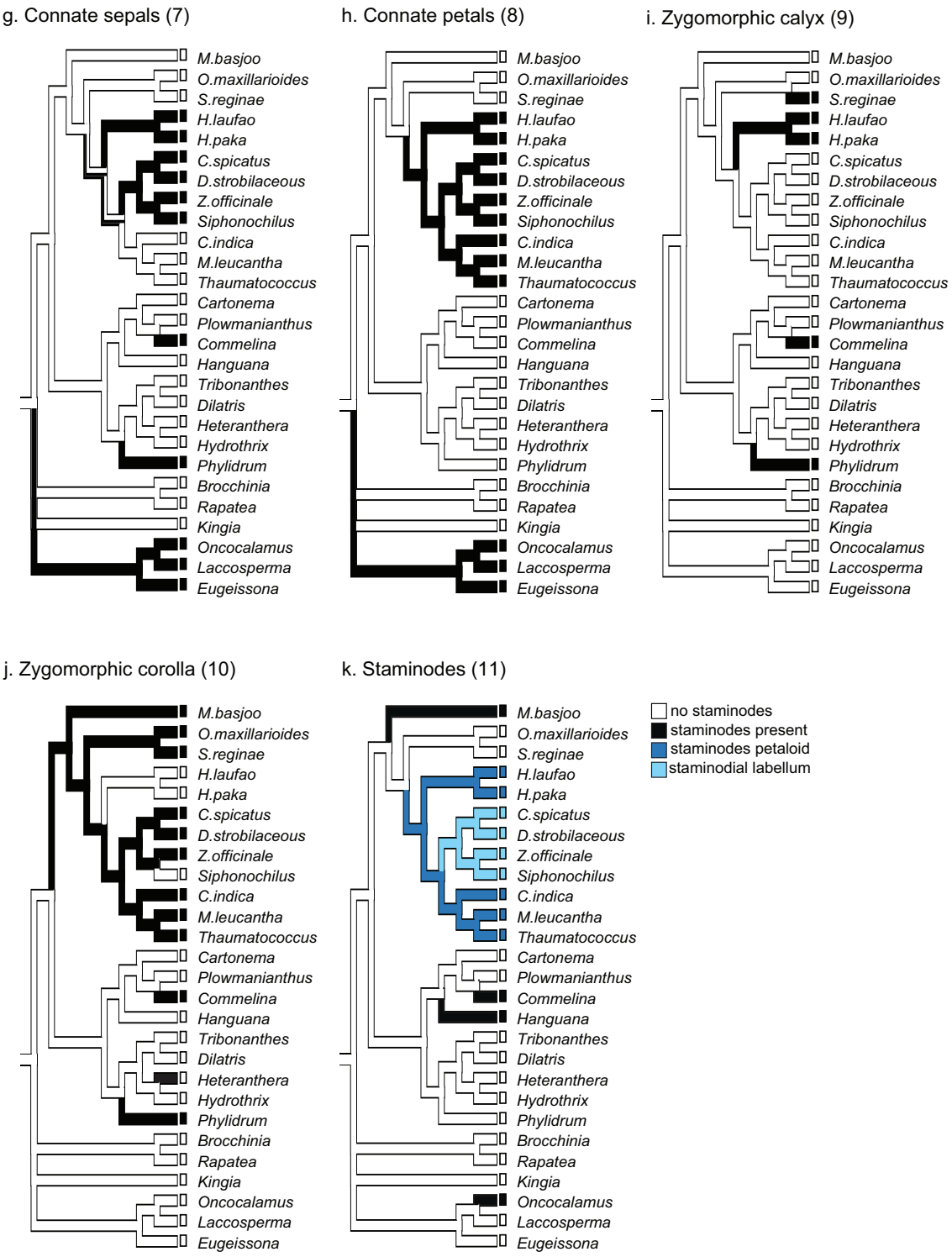

Figure 2 *(Continued)*.

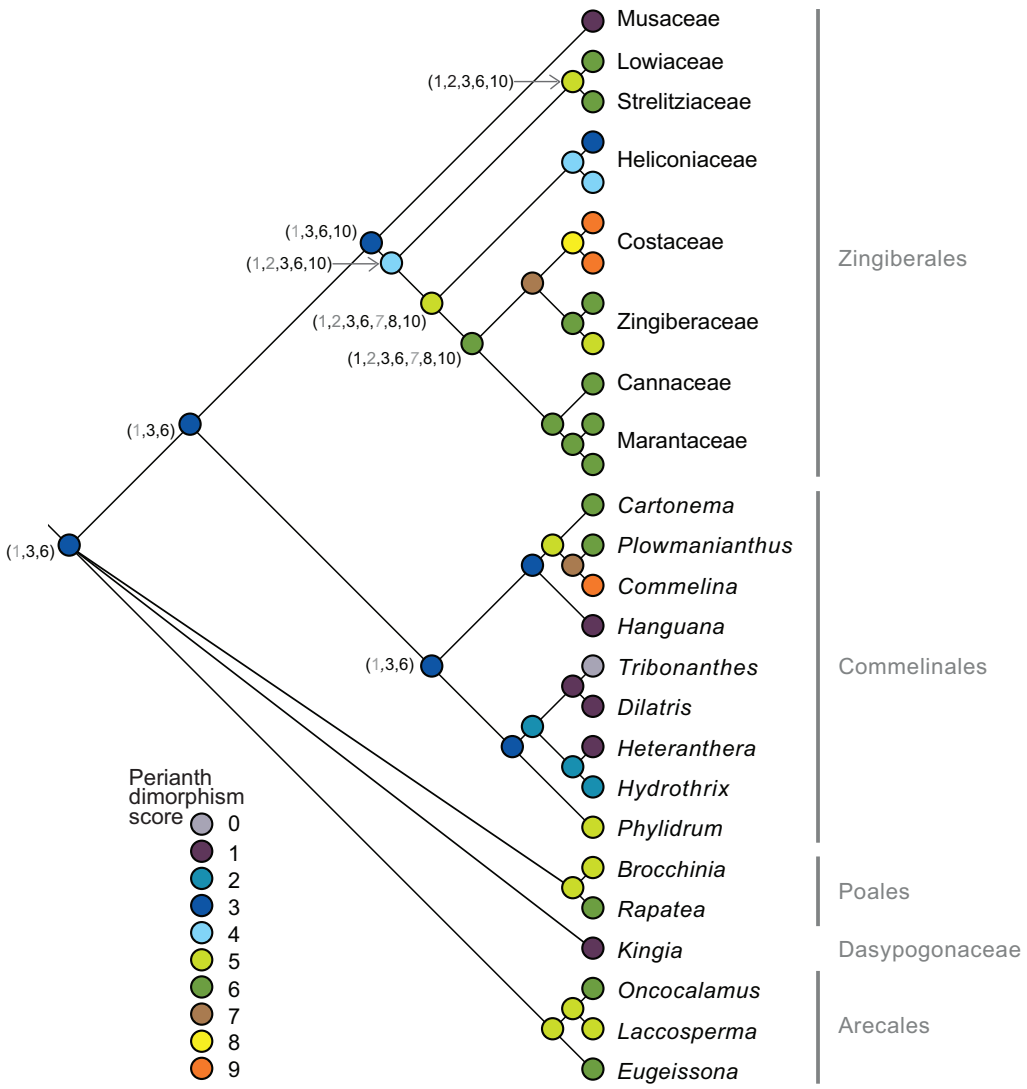

Figure 3. Individual character states tallied and summarized as a dimorphism score and mapped onto the phylogeny as a continuous character. Individual character state reconstructions at key nodes are shown in parentheses. Characters reconstructed as equivocal are shown in grey, unequivocal reconstructions in black.

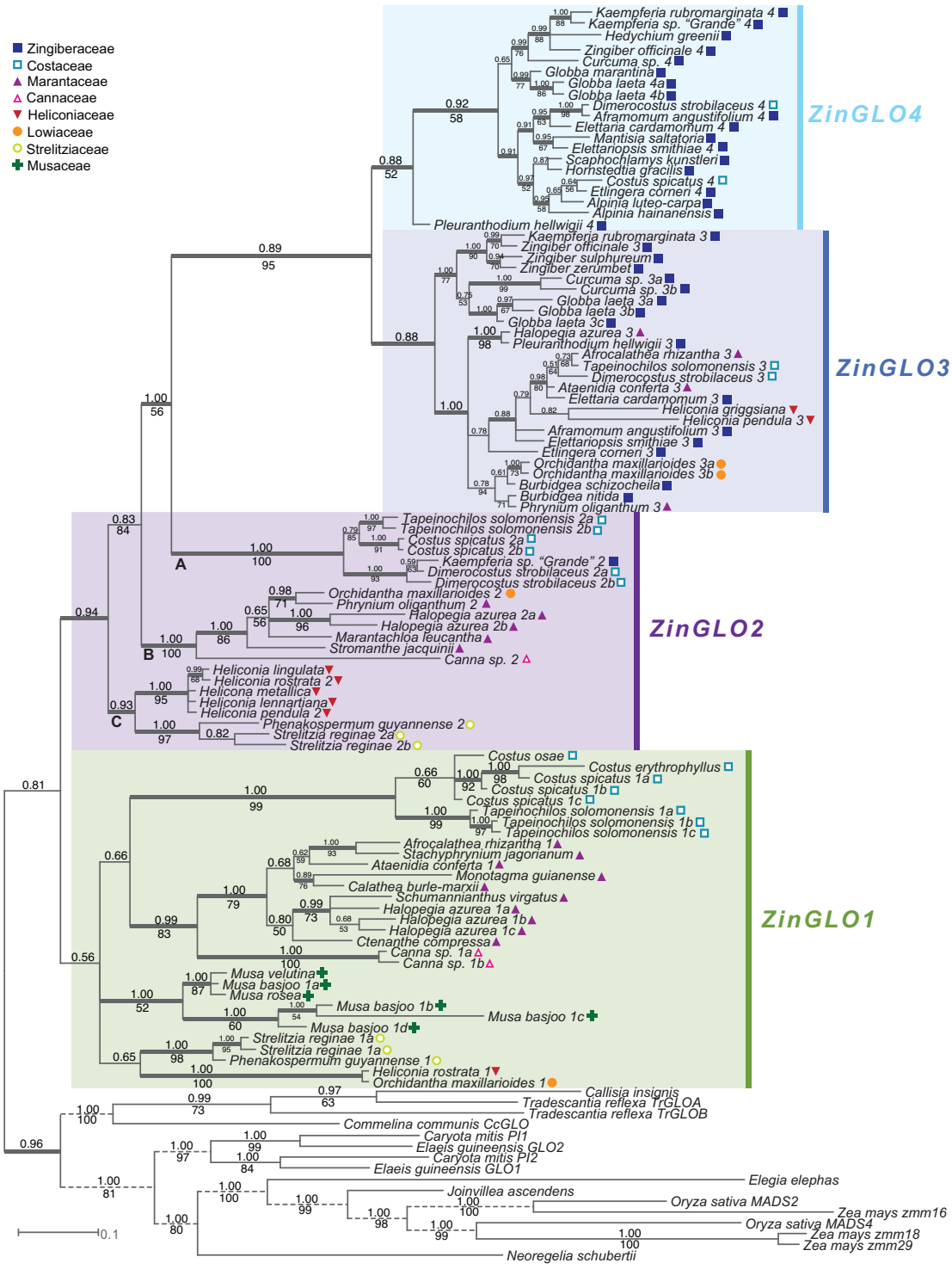

Figure 4. Bayesian phylogenetic hypothesis of *GLO* homolog relationships in the Zingiberales. When *ZinGLO2* is constrained to be monophyletic, relationships among the A, B and C lineages are unresolved. Clade posterior probabilities are shown above branches, ML bootstrap support >50% below branches. Thick branches have a posterior probability ≥ 0.85. Accession numbers for commelinid monocot *GLO* sequences retrieved from Genbank: EF521817, AY621154, DQ005582, AB177807, AB177805, AB177804, DQ005602, DQ005585, AF227195, AF411848, DQ005601, DQ005600, DQ662246, DQ662245, NM_001111667, NM_001111666, AJ292960, L37527, L37526.

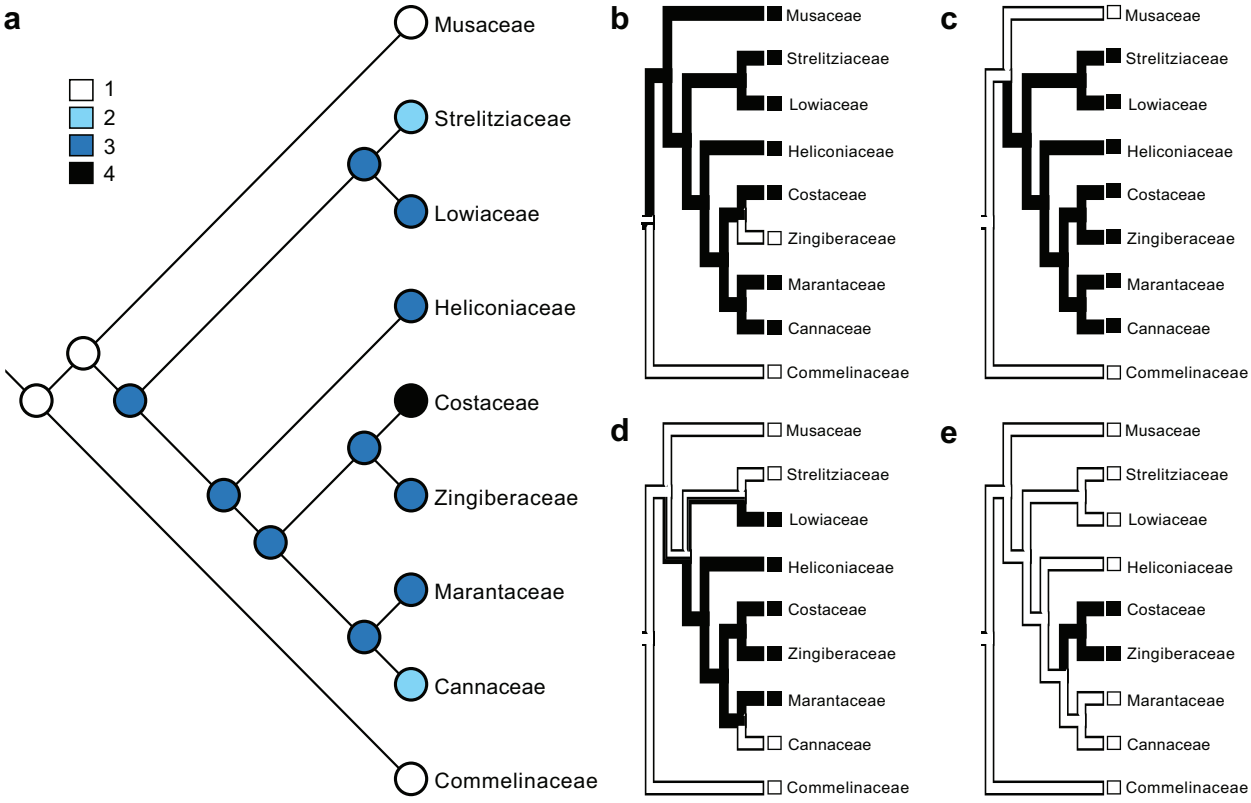

Figure 5. Character state reconstructions of *GLO* copy number in the Zingiberales. (a) *GLO* copy number reconstructed as a discrete multistate character. (b-e) Reconstructions of individual *GLO* homologs (*ZinGLO1* to *ZinGLO4*).

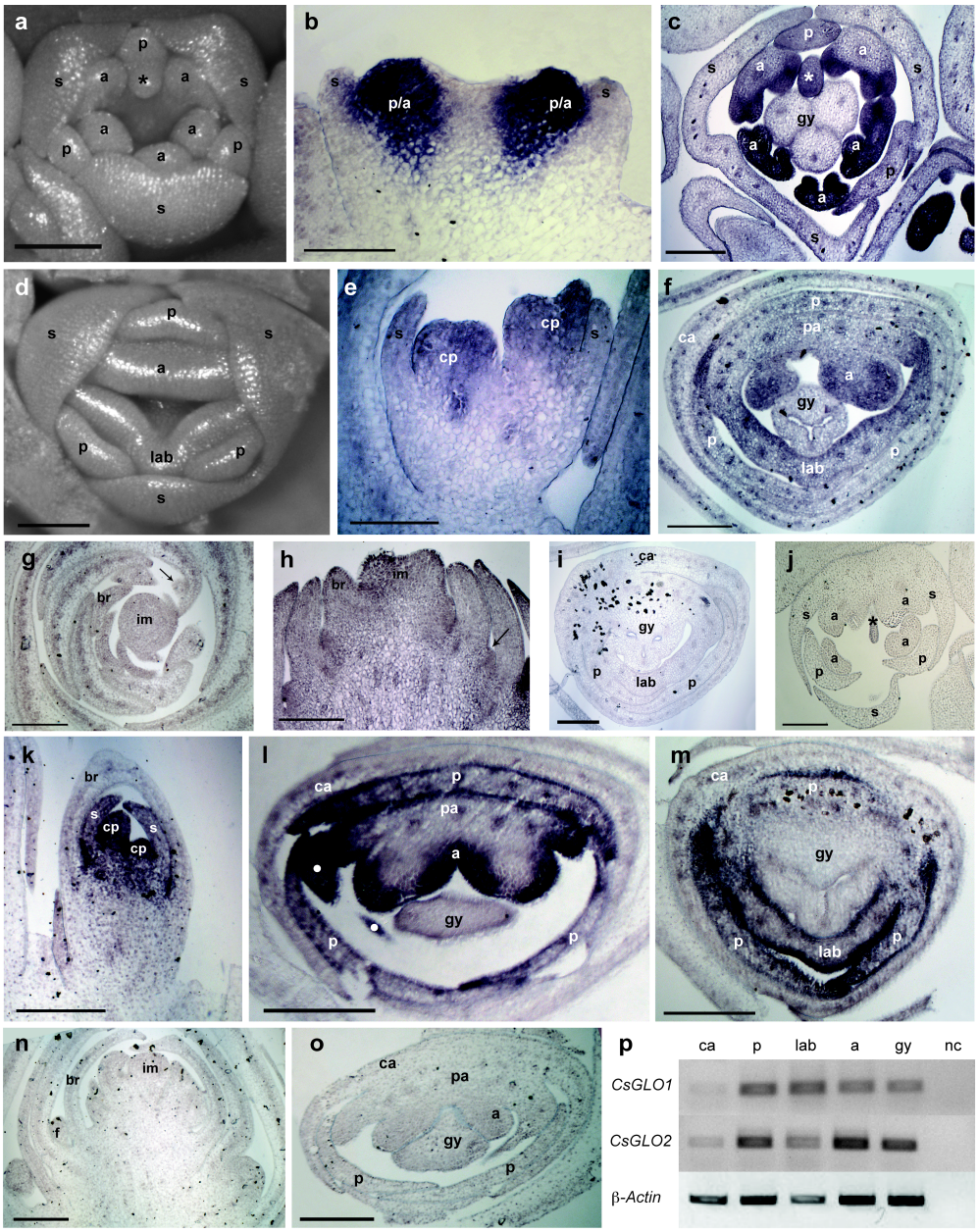

Figure 6. *ZinGLO1* expression in *Musa basjoo* (Musaceae), *ZinGLO1* and *ZinGLO2* expression in *Costus spicatus* (Costaceae). All images presented with the adaxial side of the flower uppermost in transverse sections, towards the left in longitudinal sections. (a) EDF epi-illumination image of single *M. basjoo* flower (Bartlett *et. al,* 2008). *=staminode. (b-c) *in situ* hybridization of *MbGLO1* in early (b, longitudinal section) and late (c, transverse section) *M. basjoo* floral meristems. (d) EDF epi-illumination image of single *C. spicatus* flower. (e-f) *in situ* hybridization of *CsGLO1* in early (e, longitudinal section) and late (f, transverse section) *C. spicatus* floral meristems. (g-h) *ZinGLO1* is not expressed in the inflorescence meristem or early floral meristems of *C. spicatus* (g) or *M. basjoo* (h). Sense *csGLO1* controls in individual *C. spicatus* (i) and *M. basjoo* (j) flowers. (k-o) *in situ* hybridization of *CsGLO2* in early (k, longitudinal section) and late (l-m, cross sections) *C. spicatus* floral meristems. Staminodial labellum members marked with white circles in (m). (n) *CsGLO2* is not expressed in the inflorescence meristem or early floral meristems of *C. spicatus*. (o) Sense *csGLO2* control. (p) results of semi-quantitative RT-PCR of *csGLO1* and *csGLO2* in *C. spicatus*. *a,* fertile stamen; *br,* bract; *ca,* calyx tube; *cp,* common petal-androecium primordium; *gy,* gynoecium; *im,* inflorescence meristem; *lab,* staminodial labellum; *p,* petal; *pa* petaloid appendage of fertile stamen; *s,* sepal;. All scale bars represent 200μm.

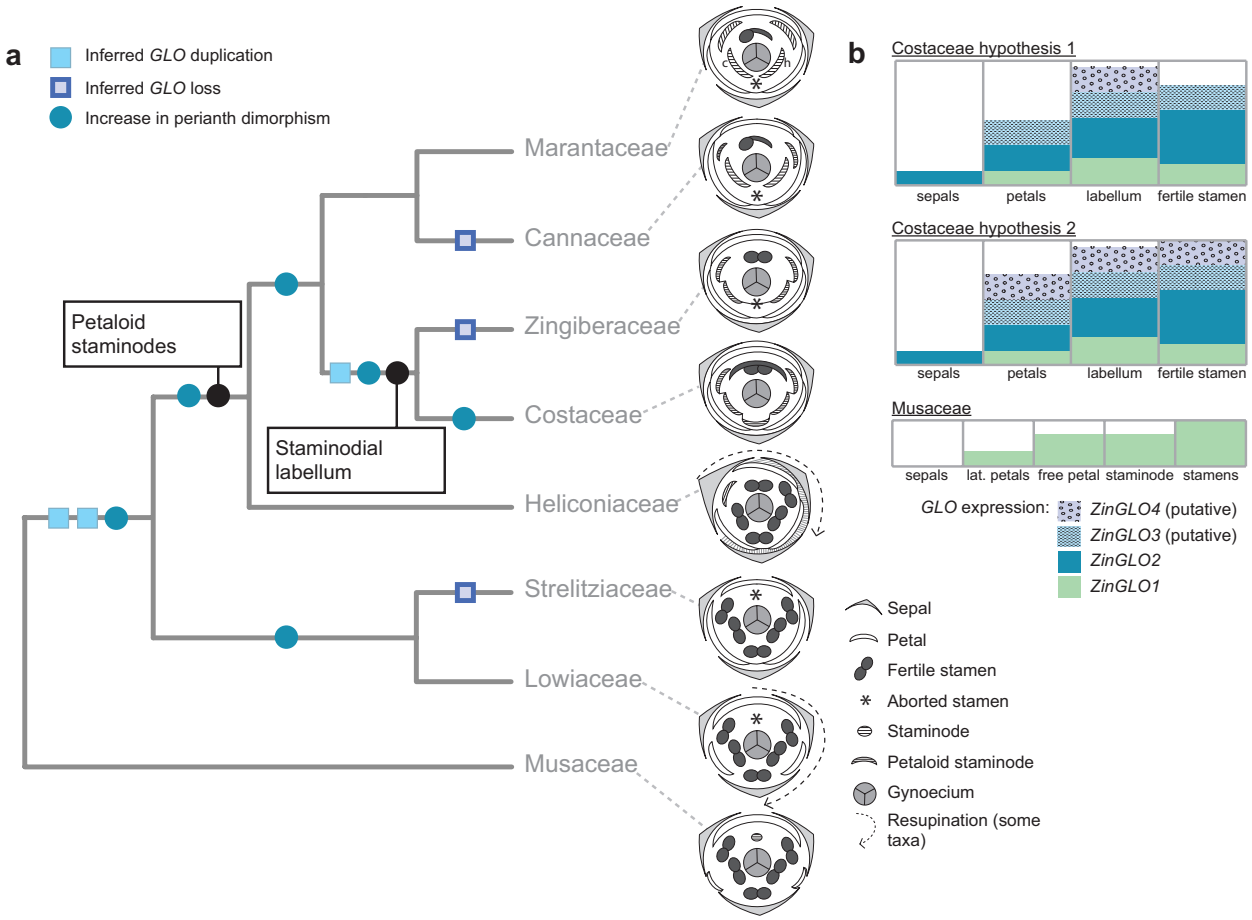

Figure 7. *GLO* gene family history and floral morphological evolution in the Zingiberales. (a) Morphological character state changes and Zin*GLO* gene duplications and losses shown on Zingiberales phylogeny. There appears to be a relationship between increasing perianth dimorphism and *GLO* gene duplications. In addition, one *GLO* duplication occurred concurrently with the derivation of the staminodial labellum. Floral diagrams were based on (Eichler, 1878; Kirchoff, 1983; Kunze, 1984; Kirchoff, 1988a; Kress, 1990a; Kirchoff, 1991; Kirchoff *et al.*, 2009) as well as the authors' observations. *c,* callose staminode; *h,* hooded staminode.(b) *GLO* gene expression in *Costus* and *Musa* and two hypotheses for *GLO* gene expression in the development of the staminodial labellum. *GLO* expression in individual floral organs has been summarized over time and space, and approximate expression levels are shown as shaded boxes. Costaceae hypothesis one represents *ZinGLO4* as the 'labellum gene'. Costaceae hypothesis two is the alternative (the combination hypothesis), where all four *ZinGLO* genes are broadly expressed and organ identity is based on the correct *ZinGLO* gene expression ratio.

This chapter was published previously as:
Bartlett ME, Specht CD. 2010. Evidence for the involvement of GLOBOSA-like gene duplications and expression divergence in the evolution of floral morphology in the Zingiberales. *New Phytologist*. 187:521-541

Chapter Three:

The evolution of *TEOSINTE BRANCHED* like genes in the Zingiberales and a possible role for these genes in the evolution of floral symmetry across the order.

Abstract

The *CYCLOIDEA/TEOSINTE BRANCHED1(CYC/TB1)*-like transcription factors have been implicated in the development and evolution of floral symmetry in divergent eudicot lineages. We thus chose to investigate a possible role for these genes in the evolution of floral symmetry within petaloid monocots, using the order Zingiberales as a model system. The Zingiberales are tropical monocots with a diversity of floral morphology that arises from changes in organ number and identity, evolution of novel structures, and shifts in symmetry. Evolutionary shifts in symmetry have occurred in all floral whorls, making the order ideal for studying the evolution of this important ecological trait. We analyzed *TB1*-like (*TBL*) genes from taxa spanning the order in a phylogenetic context and identified Zingiberales-specific gene duplications as well as a duplication in the *TBL* gene lineage that predates the diversification of commelinid monocots. Using RNA in situ hybridization we examined the expression of two *TBL* genes in *Costus spicatus* (Costaceae) and *Heliconia stricta* (Heliconiaceae), two Zingiberales taxa with divergent floral symmetry patterns. We found that shifts in *TBL* gene expression were concomitant with evolutionary shifts in floral symmetry and stamen abortion. *ZinTBL1a* expression was found in the posterior (adaxial) staminode of *H. stricta*, but in the abaxial staminodial labellum of *C. spicatus. ZinTBL2* expression was observed in the anterior (abaxial) sepals of *H. stricta*, but in the adaxial fertile stamen of *C. spicatus*. This study adds to the growing body of evidence that *CYC/TB1*-like genes have been repeatedly recruited throughout the course of evolution to generate bilateral floral symmetry (zygomorphy).

Introduction

Flowers are most often described as having many planes of symmetry (actinomorphic) or one plane of symmetry (zygomorphic). Rarely, they may have no observable plane of symmetry (asymmetric) (Endress, 1999). Symmetry is an important aspect of floral form, from both an ecological and developmental standpoint (Endress, 1999; Giurfa *et al.*, 1999). While the earliest flowers were likely to have been actinomorphic, zygomorphy has evolved repeatedly in the angiosperms and is a characteristic of many large and ecologically diverse clades such as Orchidaceae and Lamiales (Stebbins, 1970; Endress, 1994). Shifts to zygomorphy are thought to contribute to floral diversification and speciation through promoting pollinator specificity and subsequently increasing fitness (reviewed in Giurfa *et al.*, 1999). Some experimental evidence for this hypothesis has been found in *Erysimum mediohispanicum* (Brassicaceae) (Gomez *et al.*, 2006), and sister group comparisons confirm that zygomorphic lineages tend to be more species-rich than closely related actinomorphic lineages (Sargent, 2004).

Symmetry in the Zingiberales:

Floral symmetry in the Zingiberales is complex from both a developmental and an evolutionary standpoint. Although zygomorphy is dominant, all of the floral whorls exhibit evolutionary shifts in symmetry across the order, making this an ideal group for examining the evolution of this trait. Shifts in calyx, corolla, and androecium symmetry have been independently mapped onto the phylogeny in previous studies (Rudall & Bateman, 2004; Bartlett & Specht, 2010) and are discussed below (Fig. 1).

The calyces of representatives from six of the eight families (excluding Cannaceae and Marantaceae) in the Zingiberales are zygomorphic to varying degrees during development (Fig. 1A). In all of these taxa, the abaxial sepal (or posterior sepals in *Heliconia;* Kirchoff et al. 2009) is initiated last and delayed in development (Endress, 1999). This initial delay in abaxial development is not uncommon in flowers, particularly in taxa with bracteate inflorescences (Endress, 1999). In Heliconiaceae (Fig. 1D) and Strelitziaceae (Fig. 1B), the developmental zygomorphy of the calyx persists and is evident in the mature flower (Frost & Frost, 1981; Kress & Stone, 1993; Kress *et al.*, 1994; Kirchoff *et al.*, 2009).

Corolla zygomorphy is reconstructed as ancestral in the Zingiberales (Bartlett & Specht, 2010) (Fig. 1A). In Cannaceae, Costaceae, Lowiaceae, Musaceae, and Strelitziaceae, corolla zygomorphy is the result of differentiation of the adaxial petal. This is also true in some Marantaceae, but in many taxa of this family the corolla is actinomorphic (Fig. 1G) (Stevenson & Stevenson, 2004b; Ley & Classen-Bockhoff, 2009). The elaborated adaxial petal of Lowiaceae is often described as a labellum and is reported to be of importance in effective dung-beetle pollination (Kirchoff & Kunze, 1995; Sakai & Inoue, 1999). Perianth zygomorphy is expressed to greater and lesser degrees within the "ginger families" (the clade containing Costaceae, Zingiberaceae, Cannaceae and Marantaceae), although in these families the perianth is often less important in floral display than the staminode-containing androecial whorls (Smith, 1972; Kay & Schemske, 2003).

The androecium was also reconstructed as ancestrally zygomorphic in the Zingiberales (Rudall & Bateman, 2004) (Fig. 1A). In Lowiaceae, Musaceae, and Strelitziaceae there are five fertile stamens and the inner-whorl adaxial stamen is either completely absent or replaced with a staminode (in some *Musa* spp.) (Kress, 1990a). *Ravenala madagascariensis* (Strelitzaceae) represents an exception, possessing an actinomorphic androecium with six fertile stamens (Kress *et al.*, 1994); Rarely, six fertile stamens may be present in *Musa* flowers (Simmonds, 1966). Petaloid staminodes are reconstructed to have evolved on the branch leading to *Heliconia* and the ginger families (Fig. 1C). The androecium in *Heliconia* is zygomorphic due to the presence of a posterior, outer-whorl, petaloid staminode (Rudall & Bateman, 2004; Kirchoff *et al.*, 2009; Bartlett & Specht, 2010). Fertile stamen number is reduced to one adaxial outer-whorl petaloid stamen in Costaceae and Zingiberaceae (Fig. 1E). The remaining five androecial members develop as petaloid staminodes that fuse in various combinations to form the petaloid labellum (Kirchoff, 1988a; Kirchoff, 1988b; Kress, 1990a). In Cannaceae and Marantaceae the androecium is strongly asymmetric (Fig. 1F). There is a single half-stamen, while the remaining androecial members develop as petaloid staminodes (Kirchoff, 1983; Rudall & Bateman, 2004).

The ovary is actinomorphic for much of development in the "banana families" (the grade containing Heliconiaceae, Lowiaceae, Musaceae, and Strelitziaceae), Costaceae, and Zingiberaceae (Fahn & Benouaiche, 1979; Kunze, 1986; Kirchoff, 1988b; Kirchoff, 1992; Newman & Kirchoff, 1992; Kirchoff & Kunze, 1995). In Costaceae, Lowiaceae, and Zingiberaceae the stigma takes on a particular, not strictly actinomorphic, form (Pedersen & Johansen, 2004; Box & Rudall, 2006; Specht, 2006). In Cannaceae and Marantaceae, the gynoecium, like the androecium, is asymmetric at maturity (Kirchoff, 1983; Rudall & Bateman, 2004) (Fig. 1F).

<u>*CYC/TB1*-like candidate genes:</u>

There is evidence of repeated, independent recruitment of the *CYCLOIDEA/TEOSINTE BRANCHED1 (CYC/TB1)*-like class II TCP transcription factors in elaborating zygomorphy in divergent plant lineages where the evolution of zygomorphy is thought to be convergent (Stebbins, 1970; Luo *et al.*, 1995; Citerne *et al.*, 2003; Feng *et al.*, 2006; Busch & Zachgo, 2007). The most complete functional data comes from the asterid *Antirrhinum majus* and the rosid *Pisum sativum* (Fabaceae). In *Antirrhinum,* the class II TCP genes *CYC* and *DICHOTOMA (DICH)* are the products of a gene duplication event that predates the diversification of the tribe Antirrhineae (Hileman & Baum, 2003). In concert with the MYB domain transcription factor *RADIALIS (RAD)*, *CYC* and *DICH* specify adaxial floral identity (Luo *et al.*, 1995; Corley *et al.*, 2005). A second MYB domain transcription factor, *DIVARICATA* (*DIV*), confers abaxial floral identity (Almeida *et al.*, 1997). This distinction between adaxial and abaxial floral identity enables the development of zygomorphy (Corley *et al.*, 2005).

CYC and *DICH* have different effects on different whorls of the zygomorphic *Antirrhinum* flower. They have been shown to promote growth of the adaxial petals while restricting growth of the adaxial sepal and the adaxial stamen, which in wild type flowers aborts to become a staminode. The double *cyc/dich* mutant displays a radial, abaxialized phenotype, with six, rather than five, sepals, petals, and six fully formed stamens (Luo *et al.*, 1995). Thus, these genes play a role in controlling organ number as well as size, two key aspects of floral zygomorphy. In *Pisum*

floral symmetry is controlled by two *CYC*-like genes (*PsCYC2* and *PsCYC3)* and another uncharacterized single locus, *SYP1*. *PsCYC2* and *PsCYC3* control adaxial-abaxial symmetry at the level of the entire flower: the double mutant displays an abaxialized corolla, but petals still display internal asymmetry. Internal organ asymmetry is controlled by the third locus, *SYP1*. The triple mutant is radially symmetric with all petals possessing a abaxialized identity (Wang *et al.*, 2008).

Because of their demonstrated role driving the development of zygomorphy in model systems, recent studies have focused on the *CYC/TB1*-like genes as potential candidates for evolutionary changes in zygomorphy in a number of lineages, using expression patterns and copy number of the *CYC*-like genes to understand lineage specific evolution. *CYC*-like gene expression has been found to be correlated with floral symmetry in Gesneriaceae, Malpighiaceae, *Mohavea* (Plantaginaceae), *Cadia purpurea* (Fabaceae)*, Iberis amara* (Brassicaceae), *Veronica montana*, and *Gratiola officinalis* (Veronicaceae) (Hileman *et al.*, 2003; Citerne *et al.*, 2006; Busch & Zachgo, 2007; Gao *et al.*, 2008; Preston, J. C. *et al.*, 2009; Reardon *et al.*, 2009; Song *et al.*, 2009; Zhang *et al.*, 2010). In the eudicots, there is an emerging pattern of numerous gains and losses of *CYC*-like genes in different lineages (Citerne *et al.*, 2003; Fukuda *et al.*, 2003; Gubitz *et al.*, 2003; Hileman & Baum, 2003; Reeves & Olmstead, 2003; Howarth & Donoghue, 2005; Howarth & Donoghue, 2006; Kolsch & Gleissberg, 2006). In the Dipsacales, these changes in *CYC*-like copy number are correlated with changes in floral form (Howarth & Donoghue, 2005). This pattern of gene evolution suggests an ideal candidate gene family for the study of morphological evolution by gene duplication and diversification (Ohno, 1970; Lynch & Force, 2000).

Furthermore, the *CYC/TB1*-like genes seem to have a particular role in stamen abortion associated with zygomorphy (Hileman & Cubas, 2009; Preston & Hileman, 2009). Because this is a process of key importance when considering zygomorphy of the androecial whorl in the Zingiberales, we hypothesize that the *CYC/TB1*-like genes have been recruited in the Zingiberales to generate zygomorphy. Based on the described patterns of symmetry, we expect *CYC/TB1* expression early in development in the anterior sepals of *Costus* and *Heliconia* (Fig. 1b). We expect this expression to persist in the zygomorphic calyx of *Heliconia*, but not in *Costus* where the calyx is actinomorphic at maturity. We also expect *CYC/TB1* expression in the petaloid staminode of *Heliconia* and in the petaloid labellum of *Costus*.

In this study, we investigate the evolution of the *CYC/TB1*-like gene family in the context of floral symmetry within the Zingiberales. We retrieved *CYC*/*TB1*-like genes from 29 taxa spanning the Zingiberales. The nucleotide sequences were analyzed in a phylogenetic context, revealing evidence of a *CYC/TB1*-like (henceforth referred to as *TB1*-like or *TBL*) gene duplication that occurred before the diversification of the commelinid monocots, and at least one Zingiberales-specific *TBL* gene duplication. We assessed the expression of two of the identified *TBL* genes in *Costus spicatus* (Costaceae) and *Heliconia stricta* (Heliconiaceae) and found that the expression of these genes is correlated with evolutionary shifts in floral symmetry.

Materials and Methods

Amplification of *CYC/TB1* homologs:

TB1-like *(TBL)* genes were amplified from Zingiberales taxa (Table 1) using primers situated in the conserved SP, TCP and R regions (Lukens & Doebley, 2001; Howarth & Donoghue, 2005). We achieved the most success amplifying *TBL* genes with non-degenerate forward primers situated in the TCP domain. Once TCP genes had been recovered, clade-specific primers were designed. Multiple primer pair combinations were used on all taxa investigated and are listed in Table 2. PCR reactions had a final volume of 20μL and contained 0.5pmol each of forward and reverse primer, 0.4U iProof DNA polymerase (BioRad, Hercules, California, USA), 4μmol dNTPs, and 2μg BSA. Final $MgCl_2$ concentration was 2.5mM. The full 20μL PCR reactions were run out on 1.2% agarose gels and bands of the appropriate size were gel extracted and cloned using the CloneJet cloning kit (Fermentas, Ontario, Canada). Inserts were sequenced using vector-specific primers and BigDye v3.1 on an ABI 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). The number of colonies sequenced for each taxon ranged from 20 to 100. All sequences were deposited in GenBank (accession numbers GU123456- GU123456).

Multiple sequence alignment and phylogenetic analyses:

As of January 2010, all but one of the monocot *CYC/TB1*-like genes in GenBank were from taxa in the Poaceae. In order to explore the full complement of *TB1*-like genes present in grass genomes, *TB1*-like sequences were retrieved from the genome sequences of *Brachypodium distachyon, Sorghum bicolor, Oryza sativa,* and *Zea mays* (all Poaceae) using the COGE genome browser (Lyons & Freeling, 2008). The genomes were searched for significant BLAST hits using *TB1*, Os08g33530, and Os09g24480 (*REP1)* as query sequences in three separate searches. A similar procedure was used to search the *Vitis vinifera* (Vitaceae)*, Arabidopsis thaliana* (Brassicaceae)*, Carica papaya* (Caricaceae)*,* and *Populus trichocarpa* (Salicaceae) genomes using *CYC1, CYC2* and *CYC3* from *Antirrhinum majus.*

These genes were included with the retrieved Zingiberales sequences to generate an alignment of *CYC/TB1*-like genes. A second alignment of a broader swathe of class II TCP transcription factors included the described *CYC/TB1* sequences, *CINCINNATA* and its closest homologs from rice and *A.thaliana,* as well as those class II TCP genes from rice that were unlike *TB1*. The primer sequences were removed from all sequences, resulting in the loss of the R region for phylogenetic analysis. Due to high levels of nucleotide divergence between class II TCP transcription factors, it was possible to align only the TCP domain unambiguously. The TCP and ECE domains (when present) were aligned using MUSCLE and the final alignment edited by hand. The final alignments are included in the online supplementary materials.

Both alignments consisted of 169 nucleotide characters, with 128 variable characters in the *CYC/TB1* alignment and 131 variable characters in the Class II TCP transcription factor alignment. The HKY+I+G model of nucleotide evolution was selected for the phylogenetic analysis of both datasets under the Aikaike Information Criterion, as implemented in MrModeltest v2.3 (Nylander, 2004). Bayesian inference was used to generate a phylogeny for

both datasets, as implemented in MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003). For each dataset, two analyses were run in parallel until convergence (standard deviation of split frequencies ≤ 0.01). Likelihood scores were examined using Tracer (Rambaut & Drummond, 2007) and the first 10% of trees were discarded as burnin. Maximum likelihood searches and ML bootstrap analyses (1000 reps class II TCP, 531 reps *CYC/TB1*) were performed on both datasets using GARLI v0.96 (Zwickl, 2006) with the same model parameters.

Analysis of protein sequences:

Sequences were translated into protein using MacClade (Maddison & Maddison, 1989). MEME (Bailey *et al.*, 2009) was used to search for conserved motifs in the *CYC/TB1* like genes. All identified motifs were individually assessed and verified. The Protein Data Bank (Berman *et al.*, 2000) was searched with verified motifs using FIMO and MAST (Bailey *et al.*, 2009). The TCP domains of the Zingiberales *TBL* genes were analyzed for non-conservative amino acid replacements using Grantham's amino acid distances (Grantham, 1974) and Yang *et al's* (Yang *et al.*, 2000) categories. If an amino acid replacement was considered non-conservative using both methods, it was scored as such. Protein structure prediction for the *ZinTBL* genes was performed using the Phyre server (Kelley & Sternberg, 2009). In order to facilitate comparison between *TBL* genes in different clades, alignments of ECE regions were converted into sequence logos using WebLogo (Crooks *et al.*, 2004).

Tests for selection acting on the TCP domain of the *TBL* genes:

Tests for variation in selection regime across the *TBL* phylogeny were performed using PAML v4.2a (Yang, 2007). We employed the likelihood ratio test (d.f. =1) to compare two pairs of site models, which allow the ω ratio to vary among sites in the alignment: M2a (positive selection) vs M1a (nearly neutral); and M7 (beta) vs M8 (beta and ω>1) (Anisimova *et al.*, 2001; Wong *et al.*, 2004; Yang *et al.*, 2005). We estimated ω at individual codons using Single Likelihood Ancestor Counting (SLAC) and Fixed Effects Likelihood (FEL) analyses (Kosakovsky Pond & Frost, 2005b). These analyses were all conducted using the Datamonkey web server (Kosakovsky Pond & Frost, 2005a).

To detect selection acting on particular gene lineages, we tested 8 hypotheses of varying selection acting on particular branches of the *TBL* gene phylogeny using branch models of selection (Yang, 1998; Yang & Nielsen, 1998). Those branches that were identified as having significantly higher ω values in comparison to the remaining branches in the phylogeny were further analyzed using the branch site models. These models allow ω to vary across branches and sites and can be used to detect selection acting on particular lineages and amino acid sites (Yang *et al.*, 2005; Zhang *et al.*, 2005).

RNA *in situ* hybridization:

The expression of *ZinTBL1a* and *ZinTBL2* was assessed in *C. spicatus* and *H. stricta.* RNA in situ hybridizations were performed as described prevsiously (Bartlett *et al* (2008). Considering the extremely low levels of nucleotide divergence within the *ZinTBL1a* and *ZinTBL2* clades, a

single probe specific to each homolog was used in both *C. spicatus* and *H. stricta.* The probes were designed to exclude the conserved TCP domain.

Results

Phylogenetic analyses:

We conducted phylogenetic analyses in order to determine the evolutionary history of *TBL* genes in the Zingiberales. Maximum likelihood and Bayesian phylogenetic analysis of both the complete class II TCP transcription factor dataset and the CYC/TB1-like dataset resolved trees with very similar topologies. Analysis of the full TCP class II dataset (Fig. 2) resolved two main gene lineages: a weakly supported *CYC/TB1* clade (posterior probability (pp) = 0.55, ML bootstrap support (BS) = 55%), and a well-supported *PCF/CIN* clade (pp=1.00, BS=99%). Tree topology was in agreement with previous analyses, and confirms that the gene duplication events that led to the *CYC1, CYC2* and *CYC3* gene lineages were eudicot-specific (Howarth & Donoghue, 2006). With the exception of two PCF-like genes from *Plagiostachys albiflora* and *P. mucida* (Zingiberaceae), all genes that we recovered from the Zingiberales fell squarely within the well-supported monocot *TBL1*-like *(TBL*) clade (pp=0.81, BS=100%).

Analysis of the reduced *CYC/TB1* dataset resolved two clades of *TBL* genes from monocots, named *TBL1* and *TBL2* (Fig. 3). These clades were also present in the full class II TCP analysis, but had lower levels of support in the larger analysis. *TBL1* and *TBL2* appear to have been generated by a gene duplication event that occurred after the divergence of *Acorus* from the remaining monocots, but predated the divergence of the commelinid monocots. We have named these gene lineages *TBL1* and *TBL2*. The *TBL1* clade includes *Zea mays TB1* and its orthologs from other grasses, while the *TBL2* clade includes *REP1* (Yuan *et al.*, 2009), Os08g24480 and their orthologs from other grasses. Our analyses reconstructed a Zingiberales-specific gene duplication to have occurred in the *TBL1* gene lineage, leading to two clades of genes: *ZinTBL1a* and *ZinTBL1b.* Homologs in both of these gene lineages were retrieved from all eight families in the Zingiberales with the exception of Cannaceae (only *ZinTBL1b* was retrieved from *Canna*). *ZinTBL1b* and *ZinTBL1a* are in a moderately well-supported sister relationship with the *TBL1* clade from Poaceae, which includes *TB1* from *Zea mays*.

The well-supported *TBL2* clade (pp=0.93, BS=63%) comprises two further well-supported sister lineages containing genes from either Zingiberales (*ZinTBL2* clade) or Poaceae (*PoaTBL2* clade). There is some evidence for *TBL2* duplications in both the Poaceae and the Zingiberales lineages. Two *ZinTBL2* copies have been recovered from Zingiberaceae and Marantaceae (Fig. 3). One *ZinTBL2* homolog shows extremely low sequence divergence, similar to what is seen in *ZinTBL1a.* The second putative copy shows a higher level of sequence divergence. Each of the grasses included in the analysis are represented by two genes in the *TBL2* clade, except for *Z. mays,* which is represented by five *TBL2* genes. Thus, we have found evidence for several Zingiberales-specific *TBL* gene duplication events and for an independent gene duplication event that predated the diversification of the commelinid monocots.

ZinTBL protein evolution:

We screened the Zingiberales *TBL* genes for both previously defined and as yet unrecognized protein domains. As expected, the TCP domains of *ZinTBL1a, ZinTBL1b* and *ZinTBL2* were predicted to form a basic helix-loop-helix, distinct from the canonical domain of the bHLH transcription factors (Cubas *et al.*, 1999). No structural homologs were found and no further predictions of *ZinTBL* structure could be made. As is common in TCP transcription factors (Cubas *et al.*, 1999), there have been many non-conservative amino acid substitutions in both the basic and the helix-turn-helix domains of the protein (Fig. 4).

The glutamate-cysteine-glutamate (ECE) domain is a domain of unknown function that has been repeatedly found in *CYC/TB1*-like genes, and has been used to circumscribe the *CYC/TB1* subfamily within the class II TCP transcription factors (Howarth & Donoghue, 2005; Howarth & Donoghue, 2006). A putative ECE domain was found by visual inspection in all the Zingiberales *TBL* genes as well as in *Acorus calamus*. MEME also identified the ECE region of *ZinTBL1a, ZinTBL1b* and *ZinTBL2* as a domain, despite the fact that the *ZinTBL1a* ECE domain appears to have diverged considerably (Fig. 4c). The *PoaTBL2* genes from *Oryza sativa* have previously been described as lacking both ECE and R domains (Howarth & Donoghue, 2006; Yuan *et al.*, 2009). However, upon close inspection of *PoaTBL2* genes, what could be highly divergent ECE domains could be identified. ECE domain architecture differed between *PoaTBL2* genes, and two subdivisions could be identified, *PoaTBL2a* and *PoaTBL2b* (Fig. 4c).

Apart from the previously described SP (Lukens & Doebley, 2001), TCP (Cubas *et al.*, 1999), ECE (Howarth & Donoghue, 2005), and R domains (Cubas *et al.*, 1999), MEME identified five further motifs (Fig.4). Motif 2 was found in all Zingiberales *TBL* genes and in Poaceae *TBL1*. MEME did not identify motif 2 in either of the *Acorus calamus TBL* genes, although examining the sequences enabled us to identify a potential (highly diverged) motif 2 in *A. calamus TBLa* and *TBLb*. The *PoaTBL2* genes shared motif 4, and motif 5 was found in *PoaTBL1* and in *PoaTBL2a*. Motif 1 was found in *ZinTBL1b* and *ZinTBL1a*, while the two *ZinTBL2* clades of genes shared motif 3. None of these additional domains were found in the eudicot CYC proteins. Neither FIMO nor MAST (Bailey *et al.*, 2009) found any significant similarity between any of these domains and the protein structures stored in the Protein Data Bank (Berman *et al.*, 2000). All the identified motifs are included in the supplementary online data. In summary, apart from the previously described ECE and SP domains, we identified five new motifs in monocot *CYC/TB1*-like transcription factors.

ZinTBL genes evolve under differing selection regimes:

The ratio of non-synonymous nucleotide substitutions to synonymous substitutions (ω=dN/dS) is often used as a measure of selection acting on protein coding sequences. A value of ω less than one implies that the protein under investigation is under negative (purifying) selection, ω equal to one implies neutral evolution and ω greater than one implies positive selection acting on a protein (Kimura, 1977; Miyata & Yasunaga, 1980; Yang, 2002). Using Datamonkey (Kosakovsky Pond & Frost, 2005a) and PAML v4.2 (Yang, 2007), we implemented various tests to identify selection acting on the *TBL* genes. The models of selection acting on codon sites (M2a and M8), when ω is allowed to vary among sites in the alignment, were just as likely as

those models where positive selection is absent (Table 3). SLAC and FEL estimates of ω supported these results. The SLAC analysis identified 22 out of 52 (42%) of the amino acids in the TCP domain as being under negative selection; FEL identified 30 sites under negative selection (58%, $p<0.01$ in both analyses). The remaining sites were identified as evolving neutrally.

To detect shifts in selection following gene duplication and speciation, we employed the branch-based models of selection, where ω is different for different branches in the phylogeny (branches labeled in Fig. 3). We tested eight nested hypotheses of differing ω values across the *TBL* phylogeny: H1, the branch leading to *ZinTBL1a* (Z1a) has a distinct ω value; H2, where ω for the branch leading to *ZinTBL1b* (Z1b) is distinct; H3, the branch leading to *ZinTBL2* (Z2) has a distinct ω value; H4, Z1a and Z1b have different ω values to the remaining branches in the phylogeny; H5, Z2 and the branch leading to *PoaTBL2* (P2) have differing ω values; H6, the three main branches in the *TBL1* clade, Z1a, Z1b, and P1, have differing ω values; H7, ω differs between the branches leading to *TBL1* and *TBL2*, and lastly H8, a specific ω is estimated for the branches leading to *Acorus*, *ZinTBL1a*, *ZinTBl1b*, *PoaTBL1*, *ZinTBL2,* and *PoaTBL2*. The null model (H0) was one in which there was a single ω for all branches of the tree (Table 4). H1 ($p=0.0003$), H5 ($p=0.025$), and H8 ($p=0.0002$) were significantly more likely than their respective null hypotheses. All ω values were estimated to be less than one, similar to what was found in the SLAC, FEL, and site model analyses.

The branch leading to *ZinTBL1a* was estimated to have a significantly different ω value compared to ω values on the remaining branches (H1). In this and a number of the other branch-based models, estimates of ω on the branch leading to *ZinTBL1a,* although less than one, were 2 to 5 times greater than ω estimates for the remaining branches. For these reasons, we made *ZinTBL1a* the foreground lineage and tested branch-sites models of selection (Table 3). Positive selection acting on the Z1a branch was detected ($p=0.0192$), and the Bayes Empirical Bayes analysis found positive selection acting on amino acid site 22N ($pp=0.993$), marked with a red box in Fig. 4b. In summary, although the majority of the amino acid residues in the *TBL* genes were under strong purifying selection, we have found evidence for shifts in selection following gene duplication events. In addition, we have found evidence for positive selection acting on *ZinTBL1a*.

TBL gene expression in *Costus spicatus* and *Heliconia stricta:*

In order to investigate the *TBL* genes' roles in floral development, we examined the expression of the *ZinTBL* genes in two taxa with divergent floral symmetries: *Costus spicatus* and *Heliconia stricta*. RT-PCR experiments showed expression of *ZinTBL1a* and *ZinTBL2* in *C. spicatus* flowers, but not *ZinTBL1b* (data not shown). We therefore investigated the expression patterns of the *C. spicatus* and *H. stricta* orthologs of *ZinTBL1a* and *ZinTBL2* in developing flowers using RNA in situ hybridization.

The inflorescence of *C. spicatus* is bracteate (Fig. 5a). As in *C. scaber* (Kirchoff, 1988b), a reduced cincinnus of a single flower occurs in the axil of each primary bract. The flowers of *C. spicatus* are strongly zygomorphic and consist of three fused sepals, a floral tube formed by the

proximal fusion of the androecium and the corolla, and a trilocular inferior ovary. The petals are free distally, and the adaxial petal is larger than the lateral petals. There are two trimerous androecial whorls. A single adaxial, interior-whorl stamen is fertile. The remaining androecial members develop as petaloid staminodes and fuse to form the staminodial labellum. *CsTBL1a* expression was detected in the primary bract and in the early floral meristem of *C. spicatus* (Fig. 5b). Later in development, once floral organs were clearly discernable, *CsTBL1a* expression became restricted to the abaxial side of the flower and bract (Fig. 5c-d). Expression of *CsTBL1a* in older flowers was detected in the abaxial side of the floral tube, the labellum, the gynoecium, and the anther thecae. The *C. spicatus* ortholog of *ZinTBL2, CsTBL2,* was expressed in the fertile thecae of the single adaxial, fertile stamen (Fig. 5e). No expression of *CsTBL2* was observed in the sepals of *C. spicatus*. No signal was observed in either of the *CsTBL* sense controls (Fig. 5f-g).

The *Heliconia* inflorescence consists of showy bracts that enclose cincinni of multiple flowers. The plane of floral symmetry in *Heliconia* is not congruent with the median plane, causing oblique zygomorphy (Eichler, 1878; Kirchoff *et al.*, 2009). The trimerous calyx and trimerous corolla are similar, and fuse postgenitally to form the floral tube. The posterior sepal is larger than the other perianth members and separates from the floral tube at anthesis (Kress, 1990b; Kirchoff *et al.*, 2009). As in all Zingiberales, there are two trimerous androecial whorls. There are five fertile stamens, while the exterior-whorl posterior androecial member develops as a petaloid staminode. The ovary is trilocular and inferior. In *H. stricta*, *HsTBL1a* expression was detected in the gynoecium and the posterior staminode. Weak expression was also detected in the petals (Fig. 5h). Expression of the *H. stricta* ortholog of *ZinTBL2, HsTBL2,* was found in the anterior sepals (Fig. 5i). No signal was observed in either of the *HsTBL* sense controls (Fig. 5 j-k).

In summary, *ZinTBL1a* expression in both *C. spicatus* and *H. stricta* is found primarily in the staminodes and the gynoecium, while *ZinTBL2* expression is expressed in the adaxial fertile stamen of *C. spicatus* and the anterior sepals of *H. stricta.*

Discussion

TB1-like genes have diversified in the commelinid monocots and in the Zingiberales:

Our data show evidence of *TBL* gene duplications that predate the divergence of Zingiberales and Poales (Poaceae), and are thus possibly shared by all commelinid monocots. The duplications that led to the three clades of *CYC* genes found in core eudicots, *CYC1, CYC2* and *CYC3,* were hypothesized to have occurred prior to the origin and diversification of the core eudicots (Howarth & Donoghue, 2006). The duplications in the *TBL* gene lineage that we have uncovered in monocots mirror these *CYC* duplications in the core eudicot lineage. We still do not know, however, when these duplications occurred. They may have occurred just prior to the diversification of the commelinid monocots, or earlier in monocot history but after the divergence of the lineage leading to *Acorus*.

Based on our results, there appears to be evidence for similar gene duplication histories between *CYC*-like and *TB1*-like lineages: early duplications generated core gene lineages (i.e. *TBL1* and

TBL2; CYC1, CYC2 and *CYC3*) and were followed by lineage-specific gene duplications. There is evidence for gene diversification in both the *ZinTBL1* and the *ZinTBL2* lineages, similar to the reported *CYC2* diversification in many eudicot lineages (Fukuda *et al.*, 2003; Howarth & Donoghue, 2005; Kolsch & Gleissberg, 2006; Damerval *et al.*, 2007). Unlike what has been discovered in the Dipsacales (Howarth & Donoghue, 2005) and Veronicaceae (Reardon *et al.*, 2009), however, no correlations can be made between *ZinTBL* copy number and floral symmetry pattern.

We show evidence that at least one Zingiberales-specific gene duplication occurred in the *TBL1* gene lineage, leading to the clades *ZinTBL1a* and *ZinTBL1b.* The *ZinTBL1a* clade was well supported (pp=1.00, BS=98%), the *ZinTBL1b* clade less so (pp=1.00, BS<50). Homologs from both of these gene lineages were recovered from members of all eight families in the order Zingiberales with the exception *ZinTBL1a* from Cannaceae, suggesting that the gene duplication that led to these two clades occurred prior to the diversification of the order approximately 100-120 mya (Kress & Specht, 2006). Sequence divergence in the *ZinTBL1a* clade was extremely low. Except for the sequence from *Orchidantha maxillarioides* (Lowiaceae)*,* the amplified region spanning the TCP, ECE and R regions of the protein was identical across the order. This is in stark contrast to *ZinTBL1b* where sequence divergence is quite high. The nucleotide and protein sequences of the genes in the *ZinTBL1b* clade cannot be unambiguously aligned over much of their length, and internal resolution in the *ZinTB11b* clade is weakly supported for the most part. Taken together, this suggests that the *ZinTBL1b* clade may represent more than one *TB1* homolog. Alternatively, this clade may represent a single gene that has undergone significant diversification in the Zingiberales, indicating a potentially important role in developmental diversification. Internal clade topology is broadly congruent with taxonomic phylogeny, consistent with this hypothesis. In addition, there is evidence for rapid molecular evolution of *CYC*-like genes in many eudicot lineages (Fukuda *et al.*, 2003; Gubitz *et al.*, 2003; Citerne, 2005). Molecular evolution may be occurring at a similarly rapid rate in this clade of *TBL* genes in the Zingiberales.

There is little evidence for extensive gene duplication of either the *TBL1* or the *TBL2* gene lineages in grasses. *TBL1* is single copy in *Orzya, Sorghum,* and *Brachypodium.* In maize, there are two *TBL1* loci, *TB1* and *TB2,* which probably originated from a segmental allotetraploidy event that generated the maize genome ~12mya (Gaut & Doebley, 1997; Swigonová *et al.*, 2004). Apart from maize, *TBL1* has been found to be single copy in a large number of grasses (Lukens & Doebley, 2001). Our phylogenetic analyses confirm this finding. This is in contrast to the inferred homologs of *TB1* in the Zingiberales, *ZintBL1a* and *ZinTBL1b*, which have both been maintained in Zingiberales genomes following a duplication event in the *TBL1* gene lineage. It is also in stark contrast to *CYC*-like genes in the eudicots, which appear to have undergone several rounds of duplication and gene retention in numerous lineages (Citerne *et al.*, 2003; Gubitz *et al.*, 2003; Reeves & Olmstead, 2003; Ree *et al.*, 2004; Howarth & Donoghue, 2005; Howarth & Donoghue, 2006; Kolsch & Gleissberg, 2006; Damerval *et al.*, 2007).

TBL protein evolution:

Protein structure in the *TBL* gene family suggests an interesting history of gain and loss of particular motifs. The ECE region was found in *ZinTBL2,* as well as in the *Acorus TBL* genes. A

short stretch of the R domain beyond the primers was also present in all of the Zingiberales and *Acorus* TBL sequences. These results imply that *PoaTBL2* lost the R domain, and that the ECE domain has either diverged considerably or has been lost from these genes. A novel motif not found in *TBL2*, domain 4, was uncovered in *PoaTBL2*. Motif 5 was found only in *PoaTBL1* and *PoaTBL2a*. This motif was outside the region amplified by our primers, and may be present in the *ZinTBL* sequences. The presence and absence of domains within specific gene lineages lends support to our phylogenetic hypothesis of gene family evolution: *ZinTBL1a* and *ZinTBL1b* are in a weakly supported sister relationship, but the presence of motif 1 in both of these gene lineages allows us to be more confident in their close relationship.

The majority of the amino acid residues in the TCP domain of the *TBL* genes are under negative selection. This is unsurprising given that the TCP domain is the most conserved domain of the protein, involved in DNA binding. No positive selection was found to be acting on the full coding sequence of grass *TB1* orthologs (Lukens & Doebley, 2001). In contrast, positive selection was found to be acting on three residues in the TCP domain and one residue of the R domain of *CYC*-like genes of *Senecio vulgaris* and *Helianthus anuus* (Asteraceae) (Chapman *et al.*, 2008). In Asteraceae, *CYC*-like genes have been shown to play a role in specifying floral identity across the inflorescence, where ray florets are zygomorphic and disc florets actinomorphic (Broholm *et al.*, 2008; Chapman *et al.*, 2008; Kim *et al.*, 2008). Although negative selection is dominant, selection pressure differs significantly across branches in the *TBL* phylogeny. Hypothesis 8, where separate ω values were estimated for each of the labeled branches, was significantly more likely than a scenario in which there were three different values of ω for the branches leading to *TBL1*, *TBL2*, and *Acorus TBL*. This implies that there are distinct forces acting on each of the *TBL* gene lineages, suggesting functional diversification of the *TBL* genes in the Zingiberales.

There have been shifts in selection regime following gene duplication in the *ZinTBL1* gene lineage. In the more sensitive branch-sites test for selection, positive selection was found to be acting on the branch leading to *ZinTBL1a*, on the asparagine residue at position 22 of the amplified TCP domain. This residue is predicted to be the first residue in the first helix of the HLH domain. In bHLH transcription factors of the MyoD type, the HLH domain is thought to be involved in mediating protein dimerization (Murre *et al.*, 1989). This non-conservative amino acid replacement on the branch leading to *ZinTBL1a* may have resulted in new protein-protein interactions and new *TBL* gene function. Sequence divergence within the *ZinTBL1a* clade, however, is extremely low. These results suggest a scenario where there was positive selection acting on *ZinTBL1a* after the *ZinTBL1a/1b* gene duplication, resulting in a new protein function that was maintained through extreme purifying selection acting on the *ZinTBL1a* gene.

<u>*ZinTBL1a* expression changes correlate with changes in androecium zygomorphy:</u>

ZinTBL1a expression was found to be correlated with stamen abortion in *C. spicatus* and in *H. stricta*. *CsTBL1a* expression was observed in the abaxially placed (anterior) staminodial labellum of *Costus spicatus* while *HsTBL1a* expression was observed in the posterior staminode of *H. stricta*. The observed switch in expression domain may indicate that *ZinTBL1a* expression results in abaxial stamen abortion in *Costus* and posterior stamen abortion in *Heliconia*, thus causing the shift in androecium zygomorphy. The *CYC*-like genes have been implicated in causing stamen

abortion in many taxa, most notably in *Antirrhinum majus*, where *CYC* and *DICH,* one of *CYC*'s paralogs*,* restrict growth of the abaxial stamen*,* which aborts to become a staminode (Luo *et al.*, 1995). Apart from its expression in axillary meristems and branches, *TB1* in *Z. mays* is strongly expressed in the stamens of female florets: floral organs destined to abort. Much weaker *TB1* expression was also observed in the fertile stamens of male florets (Hubbard *et al.*, 2002). Overexpression of a *CYC2* homolog in *Gerbera hybrida* (Asteraceae) disrupts stamen development in both disc and ray florets. Stamens in the transgenic 35S::*GhCYC2* lines were discolored and unable to release pollen (Broholm *et al.*, 2008). In *Opithandra* (Gesneriaceae) expression of two *CYC2*-like genes was found to be correlated with adaxial and abaxial stamen abortion, and negatively correlated with expression of *OpdcyclinD3,* a positive regulator of cell division (Gaudin *et al.*, 2000; Song *et al.*, 2009)*. OpdCYC1* was initially found to be broadly expressed, in petals, fertile stamens, and staminodes, and later became localized to the staminodes (Song *et al.*, 2009). In *Veronica montana* and *Gratiola officinalis* (Veronicaceae), *CYC*-like gene expression is correlated with adaxial, but not abaxial or lateral, stamen abortion, suggesting a second mechanism for controlling abaxial stamen abortion in this family (Preston, J. C. *et al.*, 2009).

Expression of *CsTBL1a* was also observed in the early floral meristem, the abaxial side of the bract, and later in the abaxial side of both the bract and floral tube. This suggests that *CsTBL1a*, similar to *CYC* in *Antirrhinum*, has a broader role than causing stamen abortion and may be defining abaxial floral identity. Both *CsTBL1a* and *HsTBL1a* were expressed in the gynoecia of *Costus* and *Heliconia.* This expression domain has not yet been described for *TBL* genes, although *GoCYC2* and *GoCYC1* were both expressed at a low level in the gynoecium of *Gratiola officinalis* (Veronicaceae) (Preston, J. C. *et al.*, 2009). The *Heliconia* and *Costus ZinTBL1a* homologs are expressed throughout the gynoecium, which notably is the only actinomorphic floral whorl in both *Heliconia* and *Costus*. Similarly, symmetrical *CYC2*-like gene expression was observed in the actinomorphic flowers of *Bhesa paniculata* (Centroplacaceae), a close relative of the Malphigiaceae where asymmetric *CYC*-like gene expression is associated with zygomorphy in the perianth (Zhang *et al.*, 2010). In *Cadia purpurea* (Fabaceae) a symmetric *CYC*-like gene expression domain in the perianth is associated with a shift to floral actinomorphy (Citerne *et al.*, 2006). This actinomorphic expression of *ZinTBL1a* in the gynoecia of both *C. spicatus* and *H. stricta* may be instrumental in developing actinomorphy in this floral whorl, or it may be indicative of an as yet undescribed role of *TBL* genes in gynoecium development.

The complex *ZinTBL1a* expression patterns we have observed are not unprecedented. *CYC* is expressed in the sepals, petals and stamens of *A. majus*, and has different effects on different whorls of the *Antirrhinum* flower; it appears to promote growth of the adaxial petals and restrict growth of the adaxial sepal and the adaxial stamen, which aborts to become the staminode (Luo *et al.*, 1995). In Fabaceae, much of the investigation into zygomorphy and *CYC*-like genes has focused on the corolla, although all four floral whorls are zygomorphic to varying degrees. There is some evidence that *CYC*-like genes are controlling zygomorphy of legume flowers outside of the corolla. In *Lotus japonicus CYC*-like expression is evident in the adaxial side of the developing calyx (Feng *et al.*, 2006). When two *CYC*-like genes, *K-1* and *LST1-1*, are mutated in *Pisum sativum*, the calyx appears to have a more actinomorphic form with reduced abaxial lobes (Fig. 1d in Wang *et al.*, 2008). As our expression patterns indicate, *TBL* gene action is likely just as complex in the flowers of the Zingiberales.

ZinTBL2 is expressed in the anterior sepals of *Heliconia* and the posterior stamen of *Costus*:

Expression of the *ZinTBL2* homolog *HsTBL2* was detected in the anterior sepals of *Heliconia*. At maturity, *Heliconia* flowers look noticeably zygomorphic because the anterior sepals are considerably smaller than the posterior sepal from inception, and the posterior sepal separates from the other sepals at anthesis (Kirchoff *et al.*, 2009). *HsTBL2* may restrict the growth of the anterior sepals, causing zygomorphy of the sepal whorl throughout development. There is evidence that a putative *ZinTBL2* homolog from rice, *RETARDED PALEA 1 (REP1),* contributes to the specification of zygomorphy in the rice flower. *REP1* is expressed in the adaxially placed palea early in development, later it is expressed in the stamen thecae and the vascular bundles of the palea and lemma. The *rep1* mutant shows a partial loss of palea identity: the palea is delayed in development and has an expanded marginal tissue domain. This suggests that *REP1* is contributing to the specification of adaxial identity in the first whorl of the developing rice flower (Yuan *et al.*, 2009). Similarly, *HsTBL2* may also specify anterior sepal identity in *Heliconia* flowers.

CsTBL2 is expressed in the adaxial anther thecae of the *Costus spicatus* flower. Apart from weak *TB1* expression in the stamens of maize male florets, similar expression of *IaTCP1* was observed in the fertile stamens of *Iberis amara* (Busch & Zachgo, 2007). *REP1* is also expressed in the fertile stamens of *Oryza*, but no stamen defects were observed in the *rep1* mutant (Yuan *et al.*, 2009). This may be due to redundancy with Os08g24480 (sister to *REP1* in our analysis), both genes being the product of a potentially grass-specific *TBL1b* gene duplication event. What role *CYC/TB1* homologs might play in fertile stamens has yet to be determined. What is intriguing is the expression of *ZinTBL2* abaxially in *H. stricta* and adaxially in *C. spicatus*: *ZinTBL2* is expressed in the anterior (abaxial) sepals of developing *Heliconia* flowers, but in the adaxial fertile stamen of developing *Costus* flowers (Fig. 6). It is possible that *ZinTBL2* contributes to positional identity in both *Heliconia* and *Costus*, but that it specifies abaxial floral identity in *Heliconia* and adaxial floral identity in *Costus.* Understanding this pattern across Zingiberales will be important to assessing the possible evolution of abaxial vs adaxial identity specification within the order.

In addition to sequence changes, the regulation of *TBL* genes has likely diversified throughout the course of plant diversification. The coding sequence of the amplified region of both *ZinTBL1a* and *ZinTBL2* is identical in *Heliconia* and *Costus*, yet they have very different expression patterns during flower development in these taxa (Fig. 5 and 6). This provides some evidence for evolution of development through *cis*-regulatory evolution (Stern, 2000; but see Hoekstra & Coyne, 2007; Carroll, 2008). *Zea mays TB1* is a major maize domestication gene (Doebley *et al.*, 1997). The differences between the phenotypic effects of maize *TB1* and teosinte *TB1* are thought not to be due to differences in coding sequence, but rather due to differences in the regulation of expression (Hubbard *et al.*, 2002; Clark *et al.*, 2006). *TB1* expression was observed in axillary meristems of maize where it represses tiller outgrowth. *TB1* is not expressed in axillary meristems of the wild progenitor of maize, teosinte (Hubbard *et al.*, 2002) which has extensive axillary branching (tillering). The *TB1* gene has pleiotropic effects on plant morphology, including differences in the degree of axillary meristem outgrowth, and aspects of inflorescence architecture (Doebley *et al.*, 1995; Clark *et al.*, 2006). Introgression experiments

have demonstrated that these pleiotropic effects are under the control of distant *cis*-elements, more than 41kb upstream of the *TB1* coding region, that act to alter *TB1* transcription (Clark *et al.*, 2006). Similarly, *ZinTBL1a* may be under divergent transcriptional control in *Heliconia* and *Costus.*

CYC/TB1-like genes control both structural and presentational zygomorphy:

Floral morphology may be viewed in light of Endress' three interconnected levels of floral organization: Bauplan, construction, and mode (Endress, 1994). Bauplan refers to the basic organization of the flower, the floral diagram, and is the most deeply rooted in phylogeny. Bauplanne are often characteristic of families (e.g. the families of the Zingiberales), and sometimes orders (e.g. Orchidales). Construction refers to the architecture of flowers: flowers with different Bauplanne may look superficially similar because of similar architectural and functional constraints (e.g. the lip flowers of orchids and Lowiaceae). Mode refers to the most plastic floral traits, such as organ color and size, and may vary within a population or species (Endress, 1994). Individual flowers may be understood in terms of all three levels of organization. A striking example is found in the Marantaceae: Kunze (1984) described the secondary zygomorphy of the asymmetric flowers of *Calathea* (Marantaceae). Because of re-orientation of the floral organs at anthesis, the flowers look superficially like the lip flowers typical of Costaceae and Zingiberaceae. The orientation of this staminode as a lip is common in the Marantaceae and has been shown to be of importance in pollination in numerous African members of the family (Ley & Classen-Bockhoff, 2009). The flowers are structurally asymmetric (Bauplan), but changes in orientation (mode) render them superficially zygomorphic in order for successful pollination to occur (construction).

Rudall and Bateman in their 2004 analysis of zygomorphy in monocots made a distinction between structural zygomorphy and 'more subtle causes of bilateral symmetry' (Rudall & Bateman, 2004), what we term 'presentation zygomorphy'. Structural zygomorphy occurs as a result of organ loss, suppression, or elaboration and is thought to be more deeply rooted in phylogeny (Rudall & Bateman, 2004). Presentation zygomorphy involves smaller changes in floral form, such as differential organ coloration or differential organ expansion late in development, and can vary within a species (Endress, 2001; Rudall & Bateman, 2004). Structural zygomorphy might be thought of as changes in floral Bauplan, whilst presentation zygomorphy results from changes in floral mode (Endress, 1994).

What is intriguing is that there is a growing body of evidence that the *CYC/TB1*-like genes control floral zygomorphy at the level of both structural (Bauplan) and presentational (mode) zygomorphy. Changes in Bauplan may be considered macroevolutionary in nature, whilst changes in mode are most often considered microevolutionary (Endress, 1994): thus homologous genes may be controlling what are often considered vastly different evolutionary processes.

The calyx, corolla and androecium of *Antirrhinum* flowers are all zygomorphic. The calyx is zygomorphic because of reduced growth of the adaxial sepal (presentational zygomorphy) whereas the corolla and androecium are structurally zygomorphic because of organ elaboration and organ suppression, respectively (Vincent & Coen, 2004). Mutant analyses have revealed that *CYC* and *DICH* control both the structural zygomorphy of the corolla and androecium of

Antirrhinum, and the less pronounced presentation zygomorphy of the calyx (Luo *et al.*, 1995). *CYC* is expressed in the adaxial sepal, and both the single (*cyc*) and double (*cyc, dich*) mutants have six, rather than five sepals, indicating that *CYC*, possibly in concert with *DICH*, represses adaxial sepal initiation and growth (Luo *et al.*, 1995).

Apart from controlling the development of structural zygomorphy in the corolla of *Lotus* and *Pisum* (Fabaceae) (Feng *et al.*, 2006; Wang *et al.*, 2008), *CYC*-like genes appear to have a role in controlling the positional zygomorphy of the *Lotus* and *Pisum* calyces. In *Lotus*, expression of *CYC2* homologs was found in the adaxial sepals, which are smaller than abaxial sepals at maturity (Tucker, 2003; Feng *et al.*, 2006). Although the authors did not mention it, the zygomorphy of the calyx in *Pisum k-1, lst1-1* mutants is less pronounced than in the wildtype flowers (Fig. 1d in Wang *et al.*, 2008).

Iberis amara has flowers with a presentationally zygomorphic perianth in which the abaxial petals expand more than the adaxial petals. Higher expression of the *CYC* homolog *IaTCP1* in *Iberis amara* is correlated with decreased petal growth (Busch & Zachgo, 2007). In a peloric mutant, *IaTCP1* is expressed at a much lower level and this low expression level is correlated with increased equal growth of adaxial and abaxial petals. Transgenic *Arabidopsis* plants overexpressing *IaTCP1* had reduced petal size, supporting a role for *IaTCP1* in controlling presentational corolla zygomorphy in *Iberis* (Busch & Zachgo, 2007).

In the Zingiberales, the zygomorphy of the calyx of *Heliconia* might be considered presentation zygomorphy, whilst the androecium of both *Heliconia* and *Costus* is rendered structurally zygomorphic through differential stamen abortion (Rudall & Bateman, 2004). Our expression data supports the hypothesis that *TBL* genes are contributing to zygomorphy in both of these cases. If this is the case, homologous genes are controlling the development of non-homologous morphological features. Similarly, the decreased growth of adaxial petals observed in *Iberis* produces floral zygomorphy analogous to the zygomorphy seen in *Antirrhinum*, but both are controlled by homologous genes. Thus, *CYC/TB1*-like genes are deployed in different whorls of the developing flower and during different phases of development to control both structural and presentational zygomorphy, mostly likely via control over underlying processes such as cell proliferation, cell identity, and meristematic activity (Kosugi & Ohashi, 1997; Kosugi & Ohashi, 2002; Tremousaygue *et al.*, 2003; Li *et al.*, 2005; Wang *et al.*, 2008).

In conclusion, we have found evidence of an ancient duplication in the *TBL* gene lineage that predates the divergence of the commelinid monocots. In addition, there have been *TBL* gene duplications in the Zingiberales, one of which is associated with significant shifts in selection regime. Expression patterns of two *ZinTBL* genes are associated with differences in floral symmetry, adding to the growing body of evidence of continued recruitment of *CYC/TB1*-like genes in the evolution of floral symmetry. Further exploration of *TBL* gene evolution, expression, and function in the Zingiberales will shed more light on these intriguing phenomena.

Table 1. Taxon sampling for phylogenetic analysis of *TB1*-like genes in the Zingiberales

Family	Species	Author	Location[a]	Accession
Acoraceae	*Acorus calamus*	L.	UCBG	94.1392
Cannaceae	*Canna sp.*	L.	UC	mb0602
Costaceae	*Costus amazonicus*	(Loes.) J.F. Macbr.	NMNH	M9036
	Costus dubius	(Afzel.) K. Schum.	UCBG	89.0918
	Costus guanaiensis	Rusby	NMNH	L80.0707
	Costus spicatus	Swartz	NMNH	2002-127
	Monocostus uniflorus	(Poepp. ex Petersen) Maas	NMNH	1994-725
Heliconiaceae	*Heliconia chartacea*	Lane ex Barreiros	HLA	L96-5689
	Heliconia pendula	Wawra	McBryde	711003-003
	Heliconia stricta	Huber	NMNH	1994-637
Lowiaceae	*Orchidantha maxillarioides*	(Ridl.) K. Schum.	McBryde	970091
Marantaceae	*Calathea insignis*	Petersen	UCBG	90.1612
	Calathea ornata	*(Linden) Korn.*	UCBG	90.1624
	Maranta leuconeura	*E. Morren*	UCBG	51.0711
Musaceae	*Musa basjoo*	Siebold	UCBG	89.0873
	Musa sp.	L.	UC	mb0602
Strelitziaceae	*Strelitzia nicolai*	Regel & Körn.	UC	mb0601
	Strelitzia reginae	Aiton	UC	mb0607
Zingiberaceae	*Alpinia vittata*	W. Bull	RBGE	19691132
	Burbidgea nitida	Hook. F.	NMNH	1996-282
	Curcuma longiflora	Salisb.	NMNH	2000-056b
	Curcuma rubrobracteata	Körn., M. Sabu & Prasanthk.	NMNH	1998-172
	Elettariopsis unifolia	(Gagnep.) M.F. Newman	RBGE	19901449
	Elettaria cardamomum	Maton	HLA	L67-1100
	Globba laeta	K. Larsen	HLA	L92-0182
	Plagiostachys albiflora	Ridl.	NMNH	KSH745
	Plagiostachys mucida	Holttum	NMNH	KSH661
	Pleuranthodium hellwigii	(K. Schum.) R. M. Sm.	HLA	L-99.0492
	Riedelia lanata	(Scheff.) K. Schum. ex Valeton	NMNH	16327
	Zingiber officinale	Roscoe	UC	MB0876
	Zingiber ottensii	Valeton	NMNH	94-770

[a] Location of live accessions or herbarium sheets: Lyon Arboretum, Oahu, Hawaii, USA (HLA); McBryde Botanical Garden, Kauai, Hawaii, USA; University of California Botanical Garden (UCBG); University of California Berkeley Herbarium (UC), Smithsonian Greenhouses (NMNH).

Table 2. Primers used to amplify *TBL* genes from the Zingiberales

Primer		Sequence: 5' to 3'
Forward	CYC-F1[a]	AAA GAY CGV CAC AGC AA
	CYC-F1 (T/G)	AAA GAT CGG CAC AGC AA
	ZinTBL-F1	AAA GAT CGG CAC AGC AAG AT
	TB1_SP-F1[b]	TCC CAT CAG TAA AGC ACA TGT TTC CTT TC
	TB1-F2	AAR GAY CGG CAC AGC AA
Reverse	TB1-R1	CTC MCR CTC GCC TTS GCC CTC GWC TC
	CYCR-LH [a]	CTC GCY CTC GCY TTC GCC CTC GAC TC
	CYCR-LH (T/C)	CTC GCT CTC GCC TTC GCC CTC GAC TC
	ZinTBL1a-R1	GGC TTG ATC ACT TGC CTC TC
	ZinTBL1a-R2	CAR TTT GGT CGT CGA CTT GCC
	ZinTBL1b-R1	CAC TGT GGA TGT CGA GTG CT
	ZinTBL1b-R2	AGT ACT CGG CCT TCC TCG TC
	ZinTBL1b-R3	CTC GCA RGC GCC GAG TG
	ZinTBL2-R1	CTC GGC TTC ATC TTG TGT GGT G
	ZinTBL2-R2	TCC CTY STG AAT CTW CCR TC
	ZinTBL2-R3	ACT CGG AAG CGG ATG ACT C

[a] From Howarth and Donoghue (2005)
[b] Modified from Lukens and Doebley (2001)

Table 3. Log likelihood values and parameter estimates under models of variable ω among sites and along branches and sites

Model		L[a]	Parameter estimates
Sites	M0 (one ratio)	-2077.98	ω= 0.075
	M1a (nearly neutral, ω_1=1)	-2057.37	p_0= 0.827, p_1= 0.173, ω_0= 0.056
	M2a (positive selection)	-2057.37	p_0= 0.827, p_1=0.155, p_2=0.018 ω_0= 0.056, ω_1=1, ω_2= 1
	M7 (beta)	-1999.67	p = 0.3481 q=3.88713
	M8 (beta & ω>1)	-1999.67	p_0 = 0.999, p = 0.348, q=3.887, ω=2.672
Branch-sites	***ZinTBL1a* foreground A (ω_{fore}>1)**	**-2053.24**	p_0= 0.831, p_1= 0.148, p_{2a}= 0.182, p_{2b}= 0.003 ω_0= 0.058, ω_1= 1, $\omega_{2a,b\ fore}$= 28.128 $\omega_{2a\ back}$= 0.058, $\omega_{2b\ back}$ = 1
	A1 (ω_{fore}=1)	-2055.98	p_0= 0.780, p_1= 0.14, p_{2a}= 0.068, p_{2b}= 0.14, ω_0= 0.056, $\omega_{2a\ back}$= 0.056, $\omega_{2b\ back}$ = 1

[a] models significantly more likely than the corresponding null model are shown in bold
[b] 2δ=5.4826, d.f.=1, p=0.0192, not significant after Bonferroni correction, $\alpha_{0.05}$=0.0045

Table 4. Tests of hypotheses of ω variation amongst branches in the *TBL* gene lineage

Model[a]	ω_{Z1a}	ω_{Z1b}	ω_{P1}	ω_{Z2}	ω_{P2}	ω_{A}	LnL	d.f.	p
H0: $\omega_{Z1a}=\omega_{Z1b}=\omega_{P1}=\omega_{Z2}=\omega_{P2}=\omega_{A}$	0.075	$=\omega_{Z1a}$	$=\omega_{Z1a}$	$=\omega_{Z1a}$	$=\omega_{Z1a}$	$=\omega_{Z1a}$	-2077.98		
H1: $\omega_{Z1a}\neq\omega_{Z1b}=\omega_{P1}=\omega_{Z2}=\omega_{P2}=\omega_{A}$	0.299	0.065	$=\omega_{Z1b}$	$=\omega_{Z1b}$	$=\omega_{Z1b}$	$=\omega_{Z1b}$	-2071.30	1	**0.0003**[b]
H2: $\omega_{Z1b}\neq\omega_{Z1a}=\omega_{P1}=\omega_{Z2}=\omega_{P2}=\omega_{A}$	0.086	0.057	$=\omega_{Z1a}$	$=\omega_{Z1a}$	$=\omega_{Z1a}$	$=\omega_{Z1a}$	-2076.57	1	0.0926
H3: $\omega_{Z2}\neq\omega_{P2}=\omega_{Z1a}=\omega_{Z1b}=\omega_{P1}=\omega_{A}$	0.007	$=\omega_{Z1a}$	$=\omega_{Z1a}$	0.081	$=\omega_{Z1a}$	$=\omega_{Z1a}$	-2077.92	1	0.7328
H4: $\omega_{Z1a}\neq\omega_{Z1b}\neq\omega_{P1}=\omega_{Z2}=\omega_{P2}=\omega_{A}$	0.302	0.056	0.071	$=\omega_{P1}$	$=\omega_{P1}$	$=\omega_{P1}$	-2070.86	1	0.3458[c]
H5: $\omega_{Z2}\neq\omega_{P2}\neq\omega_{Z1a}=\omega_{Z1b}=\omega_{P1}=\omega_{A}$	$=\omega_{A}$	$=\omega_{A}$	$=\omega_{A}$	0.082	0.042	0.086	-2075.41	1	**0.025**[d]
H6: $\omega_{Z1a}\neq\omega_{Z1b}\neq\omega_{P1}\neq\omega_{Z2}=\omega_{P2}=\omega_{A}$	0.301	0.056	0.079	0.069	$=\omega_{Z2}$	$=\omega_{Z2}$	-2070.81	2	0.6089[e]
H7: $\omega_{Z1a}=\omega_{Z1b}=\omega_{P1}\neq\omega_{Z2}=\omega_{P2}\neq\omega_{A}$	0.084	$=\omega_{Z1a}$	$=\omega_{Z1a}$	0.063	$=\omega_{Z2}$	0.087	-2077.24	1	0.4757
H8:$\omega_{Z1a}\neq\omega_{Z1b}\neq\omega_{P1}\neq\omega_{Z2}\neq\omega_{P2}\neq\omega_{A}$	0.299	0.056	0.075	0.081	0.041	0.156	-2067.35	3	**0.0002**[b,f]

[a] Z1a= *ZinTBL1a,* Z1b=*ZinTBL1b*, P1=*PoaTBL1*, Z2=*ZinTBL2,* P2=*PoaTBL2,* A=*Acorus TBL*
[b] Significant after Bonferroni correction, $\alpha_{0.05}=0.0045$
[c] H4 vs H1, [d] H5 vs H3, [e] H6 vs H1, [f] H8 vs H7

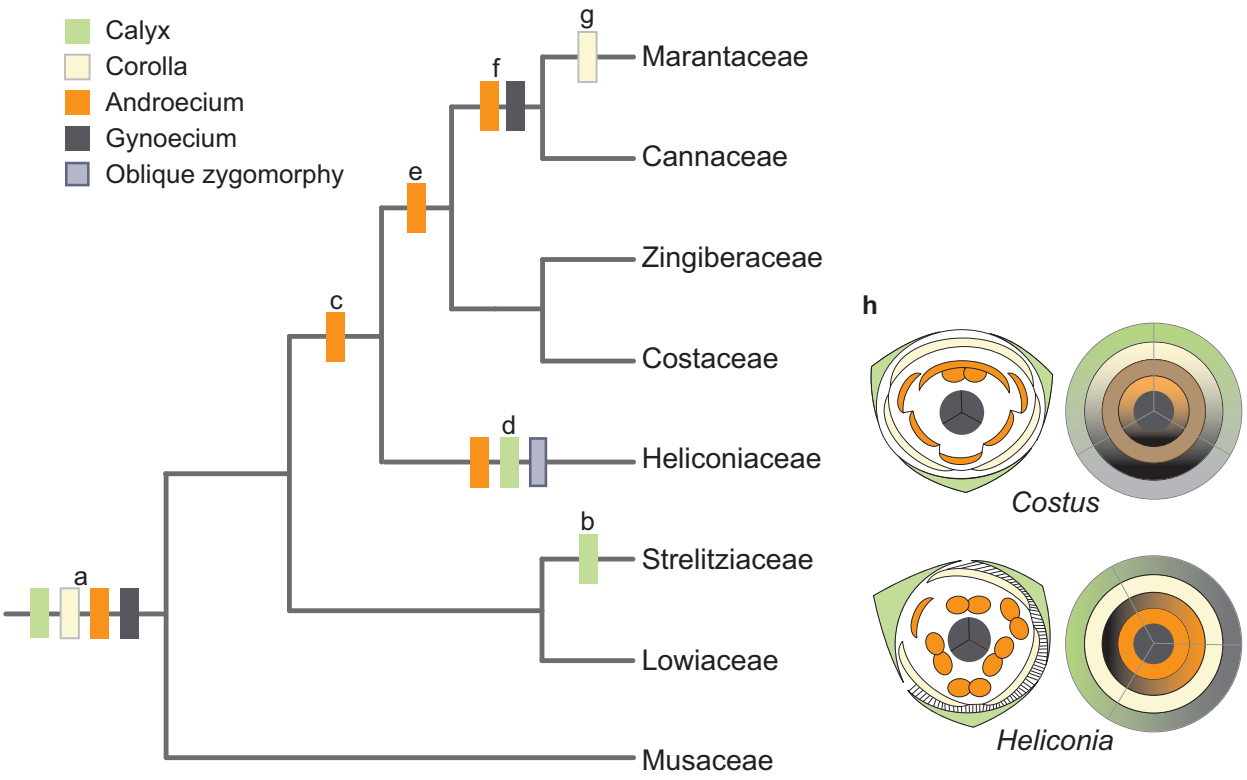

Figure 1. Changes in floral symmetry in the Zingiberales. Changes in floral symmetry in the Zingiberales. Character states and evolutionary shifts are color coded and numbered according to floral whorl and symmetry plane: (a) Abaxial sepal delayed in development, calyx actinomorphic; zygomorphic perianth forms bulk of floral display; androecium zygomorphic due to the suppression of an inner whorl, adaxial stamen; gynoecium actinomorphic. (b) Calyx zygomorphic at maturity. (c) Petaloid staminodes. (d) Calyx zygomorphic at maturity; androecium zygomorphic due to the suppression of an outer whorl, posterior stamen; flower obliquely zygomorphic. (e) Androecium forms majority of the floral display. All stamens but adaxial inner whorl stamen develop as petaloid staminodes. (f) Single anther theca develops rendering the androecium asymmetric; gynoecium asymmetric. (g) Corolla actinomorphic in many taxa. (h) Floral diagrams and predicted *TBL* gene expression in *Costus* and *Heliconia*. Darker regions of floral whorls indicate regions where we expect *TBL* expression. In *Costus*, we expect *TBL* expression early in development, but we expect this expression not to persist to floral maturity.

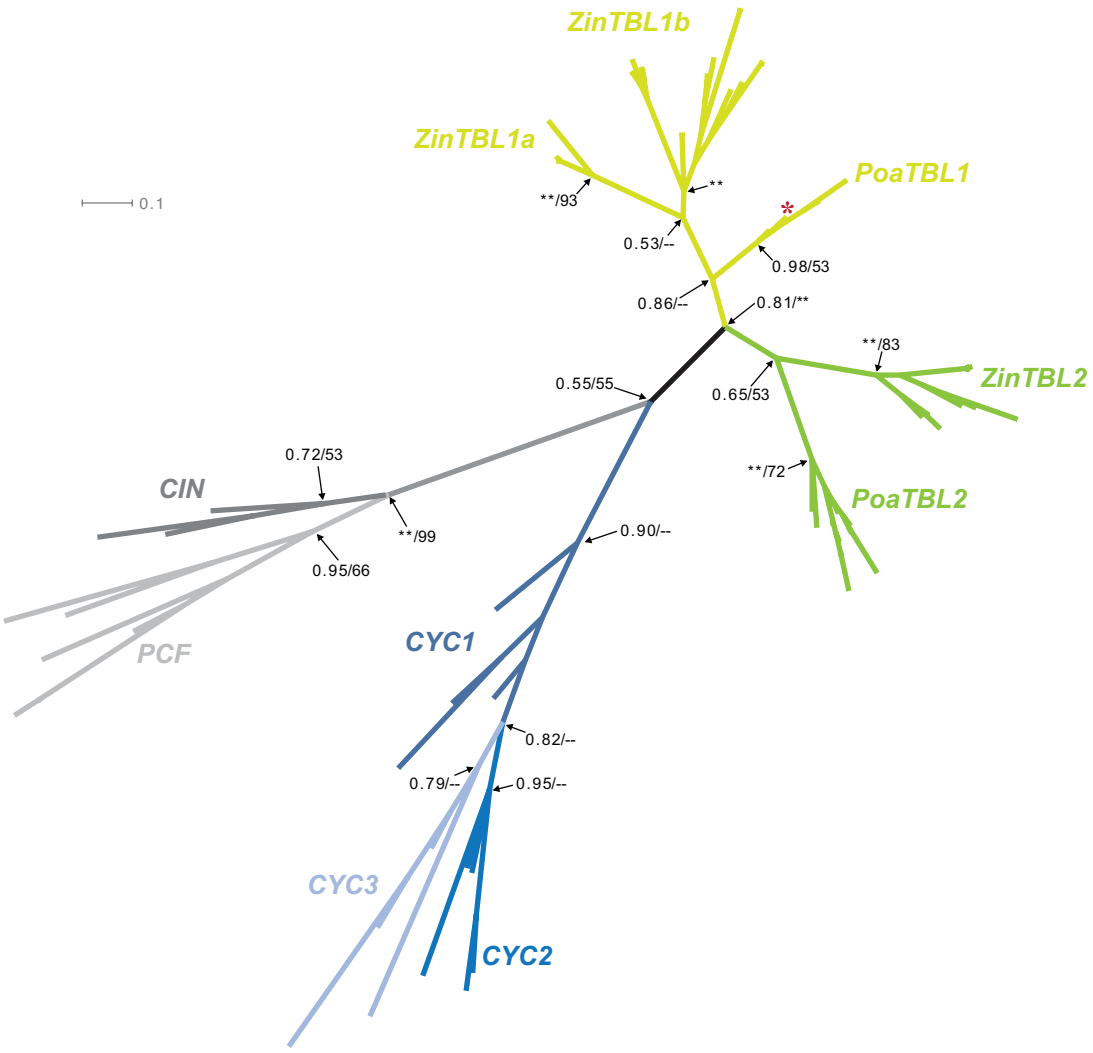

Figure 2. Bayesian phylogenetic hypothesis of class II TCP transcription factors. Bayesian posterior probabilities (first) and ML bootstrap support values >50% (second) are indicated. Where a particular relationship was not present in the maximum likelihood analysis, only the posterior probability is shown. *TB1* from *Zea mays* is marked with an asterisk.

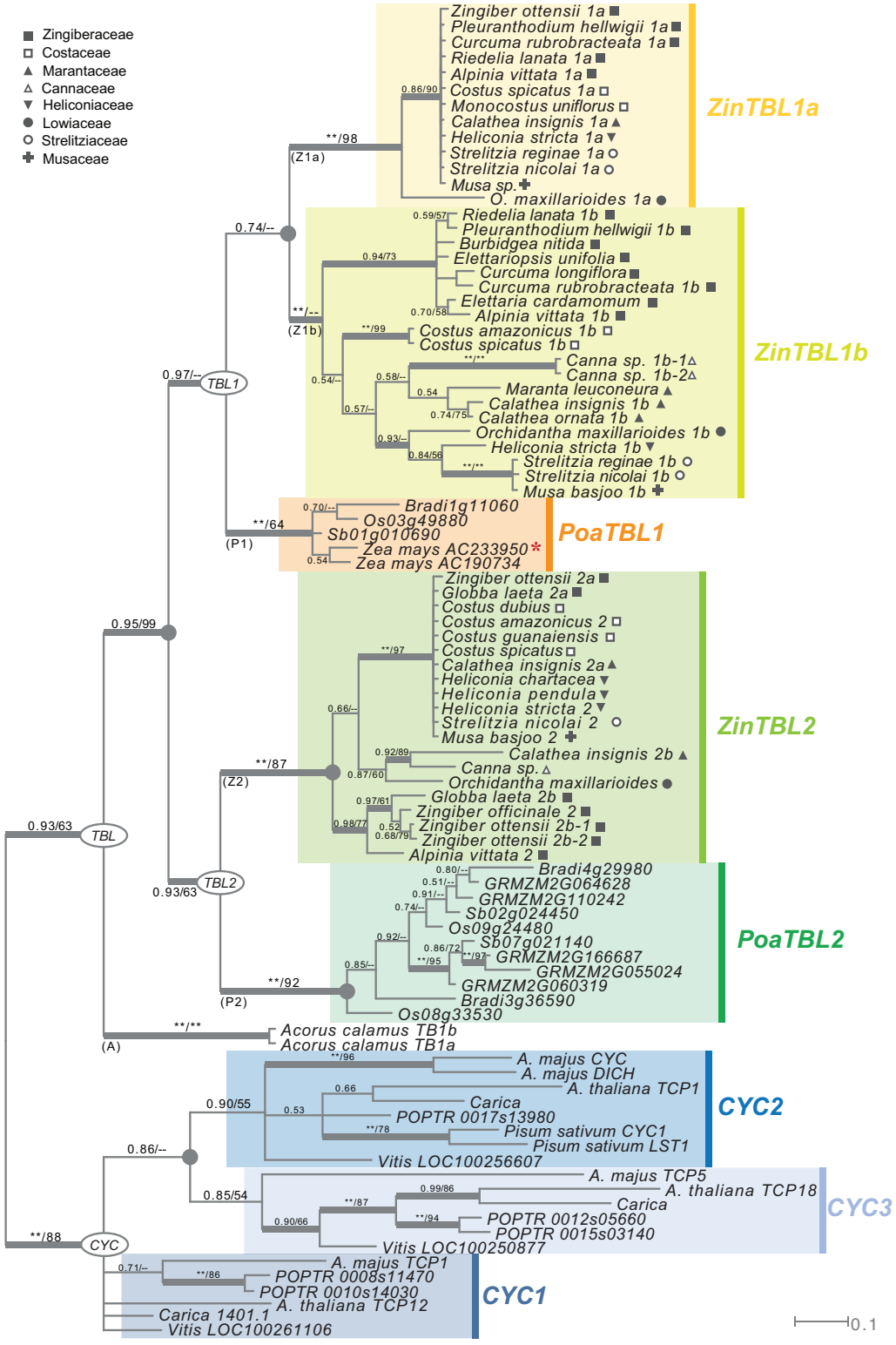

Figure 3. Bayesian phylogenetic hypothesis of *CYC/TB1*-like genes. Posterior probabilities (pp, first) and ML bootstrap support values >50% (second) are indicated. Where a particular relationship was not present in the maximum likelihood analysis, only the posterior probability is shown. Thicker branches indicate strong support (pp > 0.9 and ML bootstrap > 60%, or pp >0.95, or ML bootstrap >80%) *TB1* from *Zea mays* is marked with an asterisk. Putative gene duplication events are marked with grey circles.

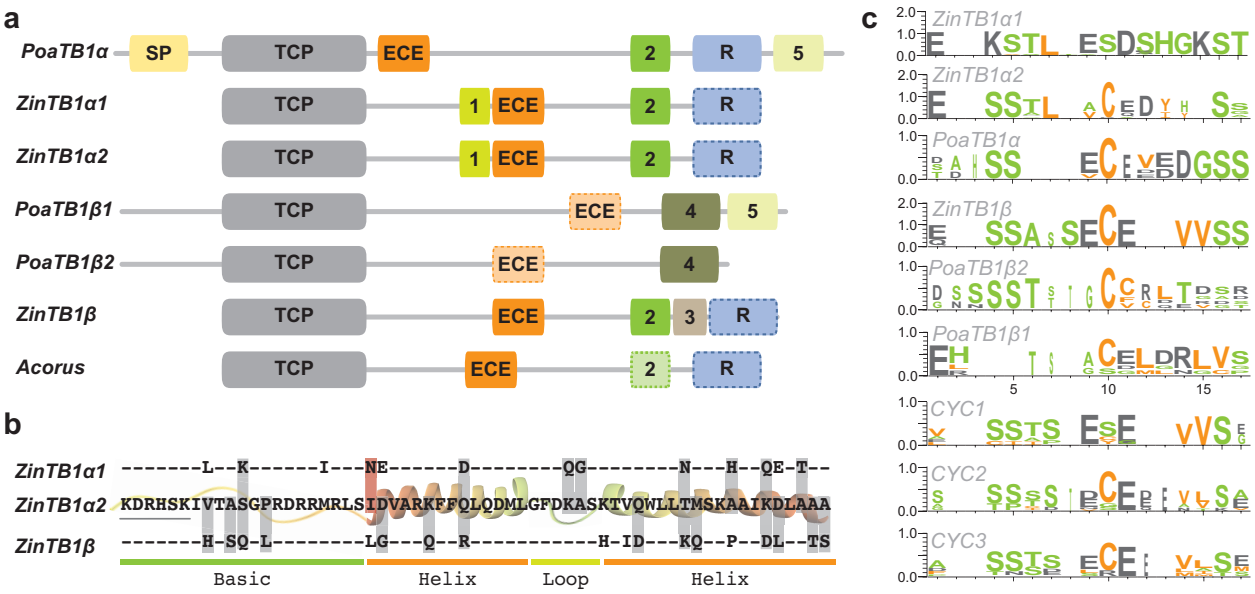

Figure 4. TBL protein evolution in the monocots. (a) Protein motifs detected by MEME are shown in their approximate positions in TBL proteins from *Acorus*, Zingiberales and Poaceae. Domains that we could identify, but were not detected by MEME, are shown with dashed outlines. (b) TCP domain structure prediction. Non-conservative amino acid replacements are boxed in grey. The red box indicates the *ZinTBL1a* residue identified as under positive selection in the PAML BEB analysis. (c) Aligned sequence logos of the ECE domain. Amino acids are colored according to hydrophobicity.

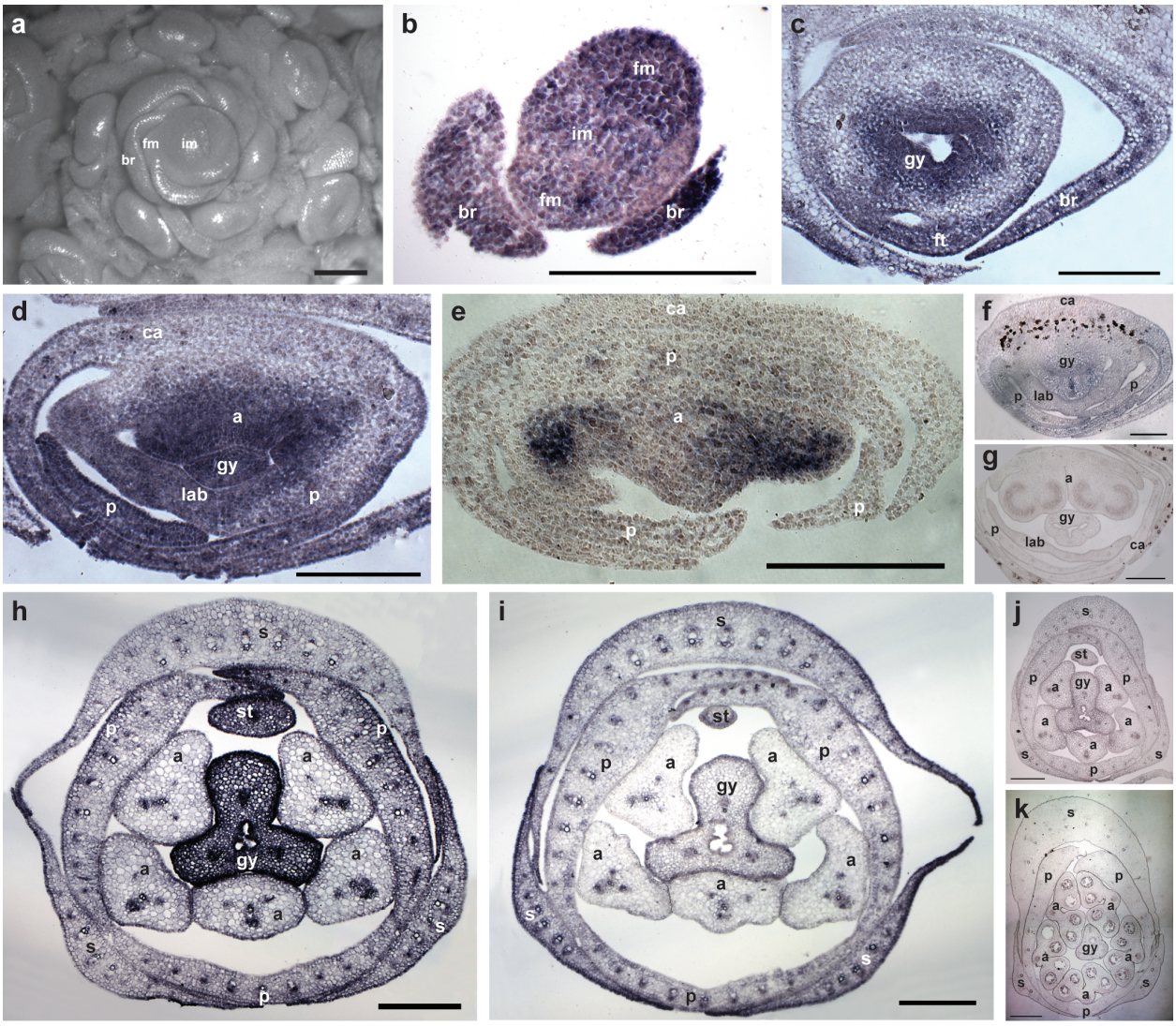

Figure 5. *ZinTBL1a* and *ZinTBL2* expression in *Heliconia stricta* and *Costus spicatus.* The *C. spicatus* inflorescence shown in (a) was photographed, sectioned, and probed for *CsTBL1a* and *CsTBL2* expression. *CsTBL1a* expression was detected in the incipient floral meristem and the abaxial side of the primary bract (b). Later in development (c-d) *CsTBL1* expression was detected in the gynoecium, the labellum and the abaxial side of the floral tube and bract. (e) *CsTBL2* expression was detected in the thecae the fertile stamen. There was no significant signal development with sense probe of *CsTBL1a* (f) or *CsTBL2* (g). In *H. stricta, HsTBL1a* expression was detected in the petaloid staminode and gynoecium (h) and *HsTBL2* expression was detected in the posterior sepals (i). No significant signal development was observed with sense probe of *HsTBL1a* (j) or *HsTBL2* (k). *a,* fertile stamen; *br,* bract; *ca,* calyx tube; *cp,* common petal-androecium primordium; *fm,* floral meristem; *ft,* floral tube; *gy,* gynoecium; *im,* inflorescence meristem; *lab,* staminodial labellum; *p,* petal; *s,* sepal; *st,* petaloid staminode. The adaxial side of *Costus* flowers and the posterior side of *Heliconia* flowers is uppermost in all micrographs. All scale bars represent 200μm.

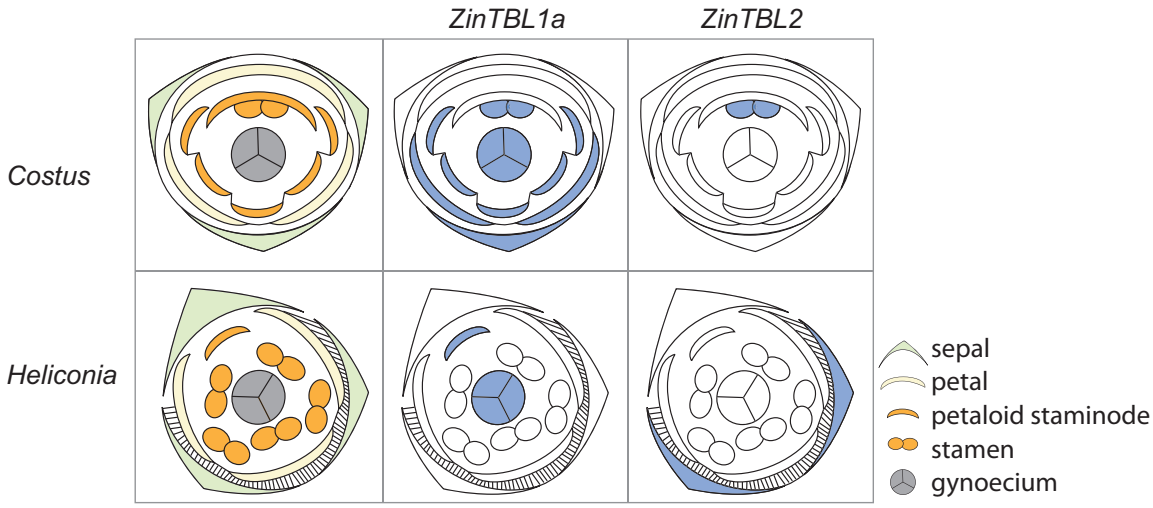

Figure 6. *ZinTBL* genes are expressed in different floral domains in *C. spicatus* and *H. stricta*. *ZinTBL* expression is shaded in blue. The adaxial side of the flowers is uppermost in all diagrams.

Future Directions

MADS box genes in the Zingiberales:

The conclusions and hypotheses arising from this research should be confirmed and expanded by further investigation of the *GLO*-like genes in the order. Expression of all four *ZinGLO* homologs should be assessed in more Zingiberales taxa. Are the divergent *ZinGLO1* and *ZinGLO2* expression patterns we observed maintained across the order? Is there any tie between *ZinGLO3* and *ZinGLO4* expression and the development of the staminodial labellum? Much remains to be discovered about MADS box gene family evolution in the Zingiberales. Beyond the *GLO*-like genes, it would be of value to explore the evolutionary history of other MADS box genes in the order, particularly the *DEF*-like genes. Are the duplications we have uncovered in the *GLO*-like gene lineage mirrored by duplications in the *DEF*-like gene lineage? Or do the two interacting partners have disparate evolutionary histories?

GLO-like and DEF-like proteins in *Arabidopsis* and *Zea mays* act as obligate heterodimers (Ambrose *et al.*, 2000; Yang *et al.*, 2003). There is some evidence that GLO-like proteins in *Lilium* and *Tulipa* can act as homodimers. Obligate DEF-GLO heterodimerization may have evolved separately in the monocots and the dicots (Winter *et al.*, 2002). It would be of some interest to uncover the interacting B class protein networks in the Zingiberales in order to assess how many times in the course of angiosperm evolution this protein-protein interaction arose.

TBL genes in the monocots:

The *CYC/TB1*-like genes represent an intriguing transcription factor family that appears to have played a major role in the evolution of plant form. However, the *TBL* genes remain underexplored in the monocots. Wider sampling of *TBL* genes across a broad swathe of monocots will aid in more accurately dating the gene duplication event that led to the *TBL1* and *TBL2* gene lineages in the commelinids. Deeper sampling of *TBL* genes in the Zingiberales will also help pinpoint the gene duplications we found evidence for in the *ZinTBL2* and *ZinTBL1b* gene lineages. It would also be of some interest to explore expression of genes from all three clades in more Zingiberales taxa. *TB1* and its orthologs have demonstrated roles in controlling shoot branching in a number of grasses (Doebley *et al.*, 1995; Takeda *et al.*, 2003; Kebrom *et al.*, 2006). There is also considerable vegetative morphological diversity in the Zingiberales (Tomlinson, 1962), the *ZinTBL* genes may have played a part in the evolution of this diversity.

Final flower orientation, particularly of zygomorphic flowers, has been shown to be of importance in pollination biology (Ushimaru & Hyodo, 2005; Fenster *et al.*, 2009; Ushimaru *et al.*, 2009). Along with shifts in symmetry in all floral whorls, the plane of zygomorphy and floral orientation also varies across the Zingiberales. In *Scaphochlamys* and *Hedychium*, transverse zygomorphy occurs in every second flower of the cincinnus (Kirchoff, 1997; Kirchoff, 1998). There has been some debate surrounding the inflorescence in Musaceae; it has variously been interpreted as a spike (Payer, 1857; Eichler, 1875), a raceme (Bentham & Hooker, 1883) a panicle (Baker, 1891) and a cincinnus (Fahn, 1953). If the inflorescence in *Musa acuminata* and *Musa balbisiana* is a cincinnus (Fahn, 1953), transverse zygomorphy occurs in every second flower of the cincinnus, as is found in *Hedychium* and *Scaphochlamys* (Gao *et al.*, 2004). How

widely these characters of oblique and transverse zygomorphy occur in the gingers and what role the *TBL* genes might play in the development of these traits is yet to be determined. We intend to conduct a broad survey of inflorescence architecture in the order to assess the distribution and evolution of oblique and transverse zygomorphy.

Resupination has evolved multiple times in the Zingiberales, occurring in Heliconiaceae, Lowiaceae and Zingiberaceae (Kirchoff & Kunze, 1995; Pedersen, 2001). Floral position changes throughout anthesis in all three genera in the Strelitziaceae. This precise floral re-orientation, similar to resupination, presumably occurs as a result of bending of the prolongation of the ovary (Frost & Frost, 1981; Kress & Stone, 1993; Kress *et al.*, 1994; Kirchoff & Kunze, 1995). Within-flower reorientations may be common in the Zingiberaceae. Flexistyly has been reported in 24 species in the subfamily Alpineae (Li *et al.*, 2001; Kress *et al.*, 2005). In *Curcumorpha longiflora* resupination of the anthers occurs after the pollen has been released (Gao *et al.*, 2004). The *CYC* homolog, *GhCYC2*, influences inflorescence orientation in *Gerbera hybrida.* When *GhCYC2* is overexpressed, the scape does not bend during any phase of development (Broholm *et al.*, 2008). A single large effect QTL has been shown to be of importance in determining flower orientation in *Aquilegia* (Hodges *et al.*, 2002), this may be a TCP transcription factor. We intend to examine resupination in a phylogenetic context in the Zingiberales, and explore the association of *TBL* genes with this trait.

References

Adam H, Jouannic S, Orieux Y, Morcillo F, Richaud F, Duval Y, Tregear JW. 2007. Functional characterization of MADS box genes involved in the determination of oil palm flower structure. *Journal of Experimental Botany* **58**(6): 1245-1259.

Aiton W. 1789. *Hortus Kewensis*. London.

Almeida J, Rocheta M, Galego L. 1997. Genetic control of flower shape in *Antirrhinum majus*. *Development* **124**(7): 1387-1392.

Ambrose BA, Lerner DR, Ciceri P, Padilla CM, Yanofsky MF, Schmidt RJ. 2000. Molecular and genetic analyses of the *silky1* gene reveal conservation in floral organ specification between eudicots and monocots. *Molecular Cell* **5**(3): 569-579.

Angenent GC, Franken J, Busscher M, van Dijken A, van Went JL, Dons HJ, van Tunen AJ. 1995. A novel class of MADS box genes is involved in ovule development in *Petunia*. *The Plant Cell* **7**: 1569-1582.

Anisimova M, Bielawski JP, Yang Z. 2001. Accuracy and power of the likelihood ratio test in detecting adaptive molecular evolution. *Molecular Biology and Evolution* **18**(8): 1585-1592.

APGIII. 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. *Botanical Journal of the Linnean Society* **161**(2): 105-121.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Research* **37**(S2): W202-208.

Baker. 1882. Brocchinia reducta. *Journal of Botany, British and Foreign* **20**: 331.

Baker. 1891. Musa basjoo. *Curtis's Botanical Magazine* **117**.

Baker WJ, Savolainen V, Asmussen-Lange CB, Chase MW, Dransfield J, Forest F, Harley MM, Uhl NW, Wilkinson M. 2009. Complete Generic-Level Phylogenetic Analyses of Palms (Arecaceae) with Comparisons of Supertree and Supermatrix Approaches. *Systematic Biology* **58**(2): 240-256.

Bartlett ME, Kirchoff BK, Specht CD. 2008. Epi-illumination microscopy coupled to in situ hybridization and its utility in the study of evolution and development in non-model species. *Development Genes and Evolution* **218**(5): 273-279.

Bartlett ME, Specht CD. 2010. Evidence for the involvement of GLOBOSA-like gene duplications and expression divergence in the evolution of floral morphology in the Zingiberales. *New Phytologist* **187**: 521-541.

Bateman RM, Rudall PJ. 2006. Evolutionary and morphometric implications of morphological variation among flowers within an inflorescence: A case-study using European orchids. *Annals of Botany (London)* **98**(5): 975-993.

Bentham G, Hooker JD. 1883. *Genera Plantarum*. London.

Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. 2000. The Protein Data Bank. *Nucl. Acids Res.* **28**(1): 235-242.

Blancaflor EB, Gilroy S. 2000. Plant cell biology in the new millennium: New tools and new insights. *American Journal of Botany* **87**(11): 1547-1560.

Bowman JL, Smyth DR, Meyerowitz EM. 1991. Genetic Interactions among Floral Homeotic Genes of Arabidopsis. *Development (Cambridge)* **112**(1): 1-20.

Box MS, Rudall PJ. 2006. Floral structure and ontogeny in *Globba* (Zingiberaceae). *Plant Systematics and Evolution* **258**(1-2): 107-122.

Broholm SK, Tahtiharju S, Laitinen RAE, Albert VA, Teeri TH, Elomaa P. 2008. A TCP domain transcription factor controls flower type specification along the radial axis of the *Gerbera* (Asteraceae) inflorescence. *Proceedings of the National Academy of Sciences of the United States of America* **105**(26): 9117-9122.

Brown R. 1810. *Prodromus Florae Novae Hollandiae*.

Busch A, Zachgo S. 2007. Control of corolla monosymmetry in the Brassicaceae *Iberis amara*. *Proc Natl Acad Sci U S A* **104**(42): 16714-16719.

Cacharron J, Saedler H, Thessien G. 1999. Expression of MADS box genes *ZMM8* and *ZMM14* during inflorescence development of *Zea mays* discriminates between the upper and the lower floret of each spikelet. *Development Genes and Evolution* **209**(7): 411-420.

Carroll SB. 2008. Evo-devo and an expanding evolutionary synthesis: A genetic theory of morphological evolution. *Cell* **134**(1): 25-36.

Chapman MA, Leebens-Mack JH, Burke JM. 2008. Positive Selection and Expression Divergence Following Gene Duplication in the Sunflower *CYCLOIDEA* Gene Family. *Mol Biol Evol* **25**(7): 1260-1273.

Charlton WA, Macdonald AD, Posluszny U, Wilkins CP. 1989. Additions to the Technique of Epi-Illumination Light-Microscopy for the Study of Floral and Vegetative Apices. *Canadian Journal of Botany-Revue Canadienne De Botanique* **67**(6): 1739-1743.

Chase MW, Fay MF, Devey DS, Maurin O, Ronsted N, Davies TJ, Pillon Y, Petersen G, Seberg O, Tamura MN, Asmussen CB, Hilu K, Borsch T, Davis JI, Stevenson DW, Pires JC, Givnish TJ, Sytsma KJ, McPherson MA, Graham SW, Rai HS. 2006. Multigene analyses of monocot relationships: A summary. *Aliso* **22**: 63-75.

Citerne HL. 2005. A primer set for specific amplification of two *CYCLOIDEA*-like genes in the Genistoid clade of Leguminosae subfam. Papilionoideae. *Edinburgh Journal of Botany* **62**(03): 119-126.

Citerne HL, Luo D, Pennington RT, Coen E, Cronk QC. 2003. A phylogenomic investigation of *CYCLOIDEA*-like TCP genes in the Leguminosae. *Plant Physiology* **131**(3): 1042-1053.

Citerne HL, Pennington RT, Cronk QC. 2006. An apparent reversal in floral symmetry in the legume *Cadia* is a homeotic transformation. *Proceedings of the National Academy of Sciences* **103**(32): 12017-12020.

Clark RM, Wagler TN, Quijada P, Doebley J. 2006. A distant upstream enhancer at the maize domestication gene *tb1* has pleiotropic effects on plant and inflorescence architecture. *Nature Genetics* **38**(5): 594-597.

Coen ES, Meyerowitz EM. 1991. The War of the Whorls: Genetic Interactions Controlling Flower Development. *Nature* **353**(6339): 31-37.

Corley SB, Carpenter R, Copsey L, Coen E. 2005. Floral asymmetry involves an interplay between TCP and MYB transcription factors in *Antirrhinum*. *Proceedings of the National Academy of Sciences of the United States of America* **102**(14): 5068-5073.

Crepet WL, Niklas KJ. 2009. Darwin's second 'abominable mystery': Why are there so many angiosperm species? *Am. J. Bot.* **96**(1): 366-381.

Crooks G, Hon G, Chandonia J, Brenner S. 2004. WebLogo: A sequence logo generator. *Genome Research* **14**(1): 188-1190.

Cubas P. 2004. Floral zygomorphy, the recurring evolution of a successful trait. *BioEssays : news and reviews in molecular, cellular and developmental biology; BioEssays : news and reviews in molecular, cellular and developmental biology* **26**(11): 1175-1184.
Cubas P, Coen E, Zapater JM. 2001. Ancient asymmetries in the evolution of flowers. *Current biology : CB* **11**(13): 1050-1052.
Cubas P, Lauter N, Doebley J, Coen E. 1999. The TCP domain: a motif found in proteins regulating plant growth and development. *The Plant Journal* **18**(2): 215-222.
Damerval C, Le Guilloux M, Jager M, Charon C. 2007. Diversity and evolution of *CYCLOIDEA*-like TCP genes in relation to flower development in Papaveraceae. *Plant Physiology* **143**(2): 759-772.
Davis JI, Petersen G, Seberg O, Stevenson DW, Hardy CR, Simmons MP, Michelangeli FA, Goldman DH, Campbell LM, Specht CD, Cohen JI. 2006. Are mitochondrial genes useful for the analysis of monocot relationships? *Taxon* **55**(4): 857-870.
Doebley J, Stec A, Gustus C. 1995. *teosinte branched1* and the origin of maize: evidence for epistasis and the evolution of dominance. *Genetics* **141**(1): 333-346.
Doebley J, Stec A, Hubbard L. 1997. The evolution of apical dominance in maize. *Nature* **386**(6624): 485-488.
Drea S, Hileman LC, de Martino G, Irish VF. 2007. Functional analyses of genetic pathways controlling petal specification in poppy. *Development* **134**(23): 4157-4166.
Duvall MR, Clegg MT, Chase MW, Clark WD, Kress WJ, Hills HG, Eguiarte LE, Smith JF, Gaut BS, Zimmer EA, Learn GH. 1993. Phylogenetic Hypotheses for the Monocotyledons Constructed from Rbcl Sequence Data. *Annals of the Missouri Botanical Garden* **80**(3): 607-619.
Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**(5): 1792-1797.
Egea-Cortines M, Saedler H, Sommer H. 1999. Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in *Antirrhinum majus*. *EMBO Journal* **18**(19): 5370-5379.
Eichler A. 1878. *Blüthendiagramme: Construirt und Erläutert*. Leipzig, Germany: Engelmann.
Eichler AW 1875. Scitaminae. *Blüthendiagramme Constructert und Erläutert*. Leipzig: Wilhelm Engelmann, 172-178.
Endress PK. 1994. *Diversity and evolutionary biology of tropical flowers*. Cambridge: Cambridge University Press.
Endress PK. 1999. Symmetry in Flowers: Diversity and Evolution. *International Journal of Plant Sciences* **160**(S6): S3-S23.
Endress PK. 2001. Evolution of floral symmetry. *Current Opinion in Plant Biology* **4**(1): 86-91.
Endress PK, Doyle JA. 2009. Reconstructing the Ancestral Angiosperm Flower and Its Initial Specializations. *American Journal of Botany* **96**(1): 22-66.
Evans TM, Sytsma KJ, Faden RB, Givnish TJ. 2003. Phylogenetic relationships in the Commelinaceae: II. A cladistic analysis of rbcL sequences and morphology. *Systematic Botany* **28**(2): 270-292.
Fahn A. 1953. The Origin of the Banana Inflorescence. *Kew Bulletin* **8**(3): 299-306.
Fahn A, Benouaiche P. 1979. Ultrastructure, Development and Secretion in the Nectary of Banana Flowers. *Ann Bot* **44**(1): 85-93.
Faivre-Rampant O, Bryan GJ, Roberts AG, Milbourne D, Viola R, Taylor MA. 2004. Regulated expression of a novel TCP domain transcription factor indicates an

involvement in the control of meristem activation processes in Solanum tuberosum. *Journal of Experimental Botany* **55**(398): 951-953.

Feng X, Zhao Z, Tian Z, Xu S, Luo Y, Cai Z, Wang Y, Yang J, Wang Z, Weng L, Chen J, Zheng L, Guo X, Luo J, Sato S, Tabata S, Ma W, Cao X, Hu X, Sun C, Luo D. 2006. Control of petal shape and floral zygomorphy in *Lotus japonicus*. *Proceedings of the National Academy of Sciences of the United States of America* **103**(13): 4970-4975.

Fenster CB, Armbruster WS, Dudash MR. 2009. Specialization of flowers: is floral orientation an overlooked first step? *New Phytologist* **183**(3): 502-506.

Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics* **35**(1): 375-403.

Flatt T. 2005. The evolutionary genetics of canalization. *Quarterly Review of Biology* **80**(3): 287-316.

Freeling M. 2009. Bias in Plant Gene Content Following Different Sorts of Duplication: Tandem, Whole-Genome, Segmental, or by Transposition. *Annual Review of Plant Biology* **60**: 433-453.

Freeling M, Thomas BC. 2006. Gene-balanced duplications, like tetraploidy, provide predictable drive to increase morphological complexity. *Genome Research* **16**(7): 805-814.

Frost SK, Frost PGH. 1981. Sunbird pollination of *Strelitzia nicolai*. *Oecologia* **49**: 379-384.

Fukuda T, Yokoyama J, Maki M. 2003. Molecular evolution of *CYCLOIDEA*-like genes in Fabaceae. *Journal of Molecular Evolution* **57**(5): 588-597.

Gao J-Y, Zhang L, Deng X-B, Ren P-Y, Kong J-J, Li Q-J. 2004. The floral biology of *Curcumorpha longiflora* (Zingiberaceae): a ginger with two-day flowers. *American Journal of Botany* **91**(2): 289-293.

Gao Q, Tao JH, Yan D, Wang YZ, Li ZY. 2008. Expression differentiation of *CYC*-like floral symmetry genes correlated with their protein sequence divergence in *Chirita heterotricha* (Gesneriaceae). *Development Genes and Evolution* **218**(7): 341-351.

Gao XM, Xia YM, Li QJ. 2006. Isolation of two putative homologues of *PISTILLATA* and *AGAMOUS* from *Alpinia oblongifolia* (Zingiberaceae) and characterization of their expression. *Plant Science* **170**(3): 674-684.

Gaudin V, Lunness PA, Fobert PR, Towers M, Riou-Khamlichi C, Murray JA, Coen E, Doonan JH. 2000. The expression of D-cyclin genes defines distinct developmental zones in snapdragon apical meristems and is locally regulated by the Cycloidea gene. *Plant Physiology* **122**(4): 1137-1148.

Gaut BS, Doebley JF. 1997. DNA sequence evidence for the segmental allotetraploid origin of maize. *Proceedings of the National Academy of Sciences of the United States of America* **94**(13): 6809-6814.

Giurfa M, Dafni A, Neal PR. 1999. Floral Symmetry and Its Role in Plant-Pollinator Systems. *International Journal of Plant Sciences* **160**(S6): S41-S50.

Givnish TJ, Evans TM, Pires JC, Sytsma KJ. 1999. Polyphyly and convergent morphological evolution in Commelinales and Commelinidae: Evidence from rbcL sequence data. *Molecular Phylogenetics and Evolution* **12**(3): 360-385.

Givnish TJ, Millam KC, Berry PE, Sytsma KJ. 2007. Phylogeny, adaptive radiation, and historical biogeography of Bromeliaceae inferred from ndhF sequence data. *Aliso* **23**: 3-26.

Givnish TJ, Millam KC, Evans TM, Hall JC, Pires JC, Berry PE, Sytsma KJ. 2004. Ancient vicariance or recent long-distance dispersal? Inferences about phylogeny and South American-African disjunctions in Rapateaceae and Bromeliaceae based on ndhF sequence data. *International Journal of Plant Sciences* **165**(4, Suppl. S): S35-S54.

Givnish TJ, Pires JC, Graham SW, McPherson MA, Prince LM, Patterson TB, Rai HS, Roalson EH, Evans TM, Hahn WJ, Millam KC, Meerow AW, Molvray M, Kores PJ, O'Brien HE, Hall JC, Kress WJ, Sytsma KJ. 2006. Phylogenetic relationships of monocots based on the highly informative plastid gene ndhF: Evidence for widespread concerted convergence. *Aliso* **22**: 28-51.

Gomez JM, Perfectti F, Camacho JP. 2006. Natural Selection on *Erysimum mediohispanicum* Flower Shape: Insights into the Evolution of Zygomorphy. *The American Naturalist* **168**(4): 531-545.

Goto K, Meyerowitz EM. 1994. Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. *Genes and Development* **8**(13): 1548-1560.

Graham SW, Kohn JR, Morton BR, Eckenwalder JE, Barrett SCH. 1998. Phylogenetic congruence and discordance among one morphological and three molecular data sets from Pontederiaceae. *Systematic Biology* **47**(4): 545-567.

Graham SW, Zgurski JM, McPherson MA, Cherniawsky DM, Saarela JM, Horne EFC, Smith SY, Wong WA, O'Brien HE, Biron VL, Pires JC, Olmstead RG, Chase MW, Rai HS. 2006. Robust inference of monocot deep phylogeny using an expanded multigene plastid data set. *Aliso* **22**: 3-21.

Grantham R. 1974. Amino Acid Difference Formula to Help Explain Protein Evolution. *Science* **185**(4154): 862-864.

Gubitz T, Caldwell A, Hudson A. 2003. Rapid molecular evolution of *CYCLOIDEA*-like genes in *Antirrhinum* and its relatives. *Molecular Biology and Evolution* **20**(9): 1537-1544.

Hardy CR, Davis JI, Stevenson DW. 2004. Floral organogenesis in Plowmanianthus (Commelinaceae). *International Journal of Plant Sciences* **165**(4): 511-519.

Hardy CR, Faden RB. 2004. Plowmanianthus, a new genus of Commelinaceae with five new species from tropical America. *Systematic Botany* **29**(2): 316-333.

Hardy CRS, Lindsey L. Faden, Robert B. 2009. Floral organogenesis and the developmental basis for pollinator deception in the Asiatic dayflower, *Commelina communis* (Commelinaceae) *American Journal of Botany* **96**(7): 1236-1244.

Heywood VH, Brummit RK, Culham A, Seberg O. 2007. *Flowering Plant Families of the World*. Buffalo: Firefly Books.

Hileman LC, Baum DA. 2003. Why do paralogs persist? Molecular evolution of *CYCLOIDEA* and related floral symmetry genes in Antirrhineae (Veronicaceae). *Molecular Biology and Evolution* **20**(4): 591-600.

Hileman LC, Cubas P. 2009. An expanded evolutionary role for flower symmetry genes. *J Biol* **8**(10): 90.

Hileman LC, Kramer EM, Baum DA. 2003. Differential regulation of symmetry genes and the evolution of floral morphologies. *Proceedings of the National Academy of Sciences* **100**(22): 12814-12819.

Hodges SA, Arnold ML. 1995. Spurring plant diversification: Are floral nectar spurs a key innovation? *Proceedings of the Royal Society of London Series B Biological Sciences* **262**(1365): 343-348.

Hodges SA, Whittall JB, Fulton M, Yang JY. 2002. Genetics of Floral Traits Influencing Reproductive Isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *The American Naturalist* **159**(3): S51-S60.

Hoekstra HE, Coyne JA. 2007. The locus of evolution: evo devo and the genetics of adaptation. *Evolution* **61**.

Honma T, Goto K. 2001. Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* **409**(6819): 525-529.

Hooker JD. 1887. On *Hydrothrix*, a new genus of Pontederiaceae. *Ann Bot* **os-1**(2): 89-94.

Hopper SD, Fay MF, Rossetto M, Chase MW. 1999. A molecular phylogenetic analysis of the bloodroot and kangaroo paw family, Haemodoraceae: Taxonomic, biogeographic and conservation implications. *Botanical Journal of the Linnean Society* **131**(3): 285-299.

Hopper SD, Smith RJ, Fay MF, Manning JC, Chase MW. 2009. Molecular phylogenetics of Haemodoraceae in the Greater Cape and Southwest Australian Floristic Regions. *Molecular Phylogenetics and Evolution* **51**(1): 19-30.

Howarth DG, Donoghue MJ. 2005. Duplications in *CYC*-like genes from Dipsacales correlate with floral form. *International Journal of Plant Sciences* **166**(3): 357-370.

Howarth DG, Donoghue MJ. 2006. Phylogenetic analysis of the *"ECE" (CYC/TB1)* clade reveals duplications predating the core eudicots. *Proceedings of the National Academy of Sciences* **103**(24): 9101-9106.

Hubbard L, McSteen P, Doebley J, Hake S. 2002. Expression patterns and mutant phenotype of *teosinte branched1* correlate with growth suppression in maize and teosinte. *Genetics* **162**(4): 1927-1935.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754-755.

Huson DH, Richter DC, Rausch C, Dezulian T, Franz M, Rupp R. 2007. Dendroscope: An interactive viewer for large phylogenetic trees. *Bmc Bioinformatics* **8**: Article No.: 460.

Irish VF. 2009. Evolution of petal identity. *Journal of Experimental Botany* **60**(9): 2517-2527.

Itino T, Kato M, Hotta M. 1991. Pollination ecology of two wild bananas, *Musa acuminata* subsp. *Halabanensis* and *M. salaccensis*: chiropterophily and ornithophily. *Biotropica* **23**: 151-158.

Jack T, Brockman LL, Meyerowitz EM. 1992. The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens *Cell* **68**(4): 683-697.

Jackson D 1991. In-Situ Hybridization in Plants. In: Gurr SJ, M. J. Mcpherson and D. J. Bowles ed. *Molecular Plant Pathology: A Practical Approach*. Oxford: Oxford University Press, 163-174.

Jackson D, Veit B, Hake S. 1994. Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. *Development (Cambridge)* **120**(2): 405-413.

Jacobs SWL, ed. 1993. *Philydraceae*. Flora of New South Wales. Kensington: New South Wales University Press.

Jesson LK, Kang J, Wagner SL, Barrett SCH, Dengler NG. 2003. The development of enantiostyly. *American Journal of Botany* **90**(2): 183-195.

Jofuku KD, Den Boer BGW, Van Montagu M, Okamura JK. 1994. Control of *Arabidopsis* Flower and Seed Development by the Homeotic Gene *APETALA2*. *Plant Cell* **6**(9): 1211-1225.

Johansen LB. 2005. Phylogeny of *Orchidantha* (Lowiaceae) and the Zingiberales based on six DNA regions. *Systematic Botany* **30**(1): 106-117.

Kass RE, Raftery AE. 1995. Bayes Factors. *Journal of the American Statistical Association* **90**(430): 773-795.

Kaufmann K, Melzer R, Theissen G. 2005. MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. *Gene* **347**(2): 183-198.

Kay KM, Reeves PA, Olmstead RG, Schemske DW. 2005. Rapid speciation and the evolution of hummingbird pollination in neotropical Costus subgenus Costus (Costaceae): evidence from nrDNA ITS and ETS sequences. *American Journal of Botany* **92**(11): 1899-1910.

Kay KM, Schemske DW. 2003. Pollinator assemblages and visitation rates for 11 species of neotropical *Costus* (Costaceae). *Biotropica* **35**(2): 198-207.

Kebrom TH, Burson BL, Finlayson SA. 2006. Phytochrome B represses Teosinte Branched1 expression and induces sorghum axillary bud outgrowth in response to light signals. *Plant Physiology* **140**(3): 1109-1117.

Kelley L, Sternberg M. 2009. Protein structure prediction on the web: a case study using the Phyre server *Nature Protocols* **4**: 363 - 371.

Kim M, Cui M-L, Cubas P, Gillies A, Lee K, Chapman MA, Abbott RJ, Coen E. 2008. Regulatory Genes Control a Key Morphological and Ecological Trait Transferred Between Species. *Science* **322**(5904): 1116-1119.

Kim M, McCormick S, Timmermans M, Sinha N. 2003. The expression domain of PHANTASTICA determines leaflet placement in compound leaves. *Nature* **424**(6947): 438-443.

Kimura M. 1977. Prepondence of synonymous changes as evidence for the neutral theory of molecular evolution. *Nature* **267**: 275-276.

Kirchoff BK. 1983. Floral Organogenesis in 5 Genera of the Marantaceae and in *Canna* (Cannaceae). *American Journal of Botany* **70**(4): 508-523.

Kirchoff BK 1988a. Floral ontogeny and evolution in the ginger group of the Zingiberales. In: Leins P, Tucker SC, Endress PK eds. *Aspects of floral development*. Berlin: J. Cramer, 45-56.

Kirchoff BK. 1988b. Inflorescence and flower development in *Costus scaber* (Costaceae). *Canadian Journal of Botany* **66**: 339-345.

Kirchoff BK. 1991. Homeosis in the flowers of the Zingiberales. *American Journal of Botany* **78**(6): 833-837.

Kirchoff BK. 1992. Ovary structure and anatomy in the Heliconiaceae and Musaceae (Zingiberales). *Canadian Journal of Botany* **70**(12): 2490-2508.

Kirchoff BK. 1997. Inflorescence and flower development in the Hedychieae (Zingiberaceae): Hedychium. *Canadian Journal of Botany* **75**: 581-594.

Kirchoff BK. 1998. Inflorescence and flower development in the Hedychieae (Zingiberaceae): *Scaphochlamys kunstleri* (Baker) Holttum. *International Journal of Plant Sciences* **159**(2): 261-274.

Kirchoff BK. 2003. Shape matters: Hofmeister's rule, primordium shape, and flower orientation. *International Journal of Plant Sciences* **164**(4): 505-517.

Kirchoff BK, Kunze H. 1995. Inflorescence and floral development in *Orchidantha maxillarioides* (Lowiaceae). *International Journal of Plant Sciences* **156**(2): 159-171.

Kirchoff BK, Lagomarsino LP, Newman WH, Bartlett ME, Specht CD. 2009. Early floral development of *Heliconia latispatha* (Heliconiaceae), a key taxon for understanding the evolution of flower development in the Zingiberales. *Am. J. Bot.* **96**(3): 580-593.

Kohn JR, Graham SW, Morton B, Doyle JJ, Barrett SCH. 1996. Reconstruction of the evolution of reproductive characters in Pontederiaceae using phylogenetic evidence from chloroplast DNA restriction-site variation. *Evolution* **50**(4): 1454-1469.

Kolsch A, Gleissberg S. 2006. Diversification of *CYCLOIDEA*-like TCP genes in the basal eudicot families Fumariaceae and Papaveraceae s.str. *Plant Biology* **8**(5): 680-687.

Kosakovsky Pond SL, Frost SDW. 2005a. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics* **21**(10): 2531-2533.

Kosakovsky Pond SL, Frost SDW. 2005b. Not So Different After All: A Comparison of Methods for Detecting Amino Acid Sites Under Selection. *Molecular Biology and Evolution* **22**(5): 1208-1222.

Kosugi S, Ohashi Y. 1997. PCF1 and PCF2 specifically bind to cis elements in the rice proliferating cell nuclear antigen gene. *The Plant Cell* **9**(9): 1607-1619.

Kosugi S, Ohashi Y. 2002. DNA binding and dimerization specificity and potential targets for the TCP protein family. *The Plant Journal : for cell and molecular biology* **30**(3): 337-348.

Kramer EM 2005. Methods for studying the evolution of plant reproductive structures: Comparative gene expression techniques. *Methods in Enzymology*, 617-636.

Kramer EM, Holappa L, Gould B, Jaramillo MA, Setnikov D, Santiago PM. 2007. Elaboration of B gene function to include the identity of novel floral organs in the lower eudicot *Aquilegia*. *Plant Cell* **19**(3): 750-766.

Kramer EM, Irish VF. 1999. Evolution of genetic mechanisms controlling petal development. *Nature* **399**(6732): 144-148.

Kress WJ. 1990a. The Phylogeny and Classification of the Zingiberales. *Annals of the Missouri Botanical Garden* **77**(4): 698-721.

Kress WJ. 1990b. The taxonomy of old world *Heliconia* (Heliconiaceae). *Allertonia* **6**(1): 1-58.

Kress WJ 1995. Phylogeny of the Zingiberanae: morphology and molecules. In: Rudall P, Cribb PJ, Cutler DF, Humphries CJ eds. *Monocotyledons: systematics and evolution*: Royal Botanic Gardens, Kew, 443-460.

Kress WJ, Liu A-Z, Newman M, Li Q-J. 2005. The molecular phylogeny of *Alpinia* (Zingiberaceae): a complex and polyphyletic genus of gingers. *Am. J. Bot.* **92**(1): 167-178.

Kress WJ, Prince LM, Hahn WJ, Zimmer EA. 2001. Unraveling the evolutionary radiation of the families of the Zingiberales using morphological and molecular evidence. *Systematic Biology* **50**(6): 926-944.

Kress WJ, Prince LM, Williams KJ. 2002. The phylogeny and a new classification of the gingers (Zingiberaceae): Evidence from molecular data. *American Journal of Botany* **89**(10): 1682-1696.

Kress WJ, Schatz GE, Andrianifahanana M, Morland HS. 1994. Pollination of *Ravenala madagascariensis* (Strelitziaceae) by lemurs: evidence for an archaic coevolutionary system? *American Journal of Botany* **81**: 542-551.

Kress WJ, Specht CD 2005. Between Cancer and Capricorn: phylogeny, evolution, and ecology of the tropical Zingiberales. In: Friis I, Balslev H eds. *Proceedings of a symposium on plant diversity and complexity patterns - local, regional and global*

dimensions. Copenhagen: Biologiske Skrifter, The Royal Danish Academy of Sciences and Letters.
Kress WJ, Specht CD. 2006. The evolutionary and biogeographic origin and diversification of the tropical monocot order Zingiberales. *Aliso* **22**: 621-632.
Kress WJ, Stone DE. 1993. Morphology and floral biology of *Phenakospermum* (Strelitziaceae), an arborescent herb of the Neotropics. *Biotropica* **25**: 290-300.
Krizek BA, Fletcher JC. 2005. Molecular mechanisms of flower development: an armchair guide. *Nat Rev Genet* **6**(9): 688-698.
Krizek BA, Meyerowitz EM. 1996. The Arabidopsis homeotic gene *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function. *Development* **122**(1): 11-22.
Kubitzki K, ed. 1998. *Flowering Plants: Monocotyledons. Alismatanae and Commelinanae (except Graminae)*. Families and Genera of Vascular Plants. Heidelberg: Springer-Verlag.
Kunze H. 1984. Comparative Studies of the Flower in Cannaceae and Marantaceae. *Flora* **175**(5): 301-318.
Kunze H. 1986. Inflorescence and Flower Morphology of *Orchidantha maxillarioides* Ridl. K. Shum. Lowiaceae. *Beitraege zur Biologie der Pflanzen* **61**(2): 221-234.
Lacroix CR, Macintyre J. 1995. New Techniques and Applications for Epi-Illumination Light-Microscopy. *Canadian Journal of Botany-Revue Canadienne De Botanique* **73**(11): 1842-1847.
Lemon GD, Posluszny U. 1998. A new approach to the study of apical meristem development using laser scanning confocal microscopy. *Canadian Journal of Botany-Revue Canadienne De Botanique* **76**(5): 899-904.
Leroy DO, Crane MJ. 1964. The Ultropak, an Aid in Photomicrography. *Micropaleontology* **10**(1): 105-110.
Levine M, Tjian R. 2003. Transcription regulation and animal diversity. *Nature* **424**(6945): 147-151.
Ley AC, Classen-Bockhoff R. 2009. Pollination syndromes in African Marantaceae. *Annals of Botany* **104**(1): 41-56.
Li C, Potuschak T, Colon-Carmona A, Gutierrez RA, Doerner P. 2005. Arabidopsis TCP20 links regulation of growth and cell division control pathways. *Proceedings of the National Academy of Sciences of the United States of America* **102**(36): 12978-12983.
Li Q-J, Xu Z-F, Kress WJ, Xia Y-M, Zhang L, Deng X-B, Gao J-Y. 2001. Study on the flexistyly pollination mechanism in *Alpinia* plants (Zingiberaceae). *Acta Bot. Sin.* **43**(364-369).
Linne Cv. 1801. *Species Plantarum*: Berolini : Impensis G.C. Nauk, .
Lock JM 1985. Zingiberaceae. In: Polhill RM ed. *Flora of Tropical East Africa*. Rotterdam: A. A. Balkema.
Locquin M, Langeron M. 1983. *Hand Book of Microscopy*. London, England; Boston, Mass., USA.: Butterworths.
Lukens L, Doebley J. 2001. Molecular evolution of the *teosinte branched* gene among maize and related grasses. *Molecular Biology and Evolution* **18**(4): 627-638.
Luo D, Carpenter R, Vincent C, Copsey L, Coen E. 1995. Origin of floral asymmetry in *Antirrhinum*. *Nature* **383**(6603): 794-799.

Lynch M, Force A. 2000. The probability of duplicate gene preservation by subfunctionalization. *Genetics* **154**(1): 459-473.
Lynch M, O'Hely M, Walsh B, Force A. 2001. The probability of preservation of a newly arisen gene duplicate. *Genetics* **159**(4): 1789-1804.
Lyons E, Freeling M. 2008. How to usefully compare homologous plant genes and chromosomes as DNA sequences. *The Plant Journal* **53**(4): 661-673.
Maas PJM 1972. Costoideae (Zingiberaceae). *Flora Neotropica*. New York: Haner Publishing Co.
Macfarlane TD, Hopper SD, Purdie RW, George AS, Patrick SJ, eds. 1987. *Haemodoraceae*. Flora of Australia. Canberra: Australian Government Publishing Services.
Maddison W, Maddison D 1998. MacClade: analysis of phylogeny and character evolution.In: Sinauer, Sunderland, Mass.
Maddison W, Maddison D 2009. Mesquite: a modular system for evolutionary analysis.In: http://mesquiteproject.org.
Maddison WP, Maddison DR. 1989. Interactive Analysis of Phylogeny and Character Evolution Using the Computer Program Macclade. *Folia Primatologica* **53**(1-4): 190-202.
Malcomber ST, Kellogg EA. 2005. SEPALLATA gene diversification: brave new whorls. *Trends in Plant Science* **10**(9): 427-435.
Mandel MA, Gustafson-Brown C, Savidge B, Yanofsky MF. 1992. Molecular characterization of the Arabidopsis floral homeotic gene *APETALA1*. *Nature* **360**(6401): 273-277.
Mena M, Mandel MA, Lerner DR, Yanofsky MF, Schmidt RJ. 1995. A characterization of the MADS-box gene family in maize. *Plant Journal* **8**(6): 845-854.
Miller M, Holder M, Vos R, Midford P, Liebowitz T, Chan L, Hoover P, Warnow T 2009. The CIPRES Portals.In: http://www.phylo.org/.
Milne-Redhead E 2000. Marantaceae. In: Polhill RM ed. *Flora of Tropical East Africa*. Rotterdam: A. A. Balkema.
Miyata T, Yasunaga T. 1980. Molecular evolution of mRNA: a method for estimating evolutionary rates of synonymous and amino acid substitutions from homologous nucleotide sequences and its applications. *Journal of Molecular Evolution* **16**: 23-36.
Mondragon-Palomino M, Hiese L, Haerter A, Koch MA, Theissen G. 2009. Positive selection and ancient duplications in the evolution of class B floral homeotic genes of orchids and grasses. *BMC Evolutionary Biology* **9**: Article No.: 81.
Mondragon-Palomino M, Theissen G. 2008. MADS about the evolution of orchid flowers. *Trends in Plant Science* **13**(2): 51-59.
Mondragon-Palomino M, Theissen G. 2009. Why are orchid flowers so diverse? Reduction of evolutionary constraints by paralogues of class B floral homeotic genes. *Annals of Botany* **104**(3): 583-594.
Munster T, Wingen LU, Faigl W, Werth S, Saedler H, Theissen G. 2001. Characterization of three *GLOBOSA*-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. *Gene* **262**(1-2): 1-13.
Murre C, McCaw PS, Baltimore D. 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding daughterless, MyoD and MYC proteins. *Cell* **56**(5): 777-784.

Nakamura T, Fukuda T, Nakano M, Hasebe M, Kameya T, Kanno A. 2005. The modified ABC model explains the development of the petaloid perianth of *Agapanthus praecox* ssp *orientalis* (Agapanthaceae) flowers. *Plant Molecular Biology* **58**(3): 435-445.

Nath U, Crawford BC, Carpenter R, Coen E. 2003. Genetic control of surface curvature. *Science* **299**(5611): 1404-1407.

Newman SWH, Kirchoff BK. 1992. Ovary Structure in the Costaceae (Zingiberales). *International Journal of Plant Sciences* **153**(3): 471-487.

Nickolls L. 1937. Physics and the detection of crime. *Journal of Scientific Instruments* **XIV**(1): 1-8.

Nylander JAA 2004. MrModeltest v2. .In. Evolutionary Biology Centre, Uppsala University: Program distributed by the author.

Nylander JAA, Ronquist F, Huelsenbeck JP, Nieves-Aldrey JL. 2004. Bayesian phylogenetic analysis of combined data. *Systematic Biology* **53**(1): 47-67.

Ochiai T, Nakamura T, Mashiko Y, Fukuda T, Yokoyama J, Kanno A, Kameya T. 2004. The differentiation of sepal and petal morphologies in Commelinaceae. *Gene* **343**(2): 253-262.

Ohno S. 1970. *Evolution by Gene Duplication*. Berlin: Springer-Verlag.

Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D. 2003. Control of leaf morphogenesis by microRNAs. *Nature* **425**(6955): 257-263.

Payer JB. 1857. Ordre des Zingiberacees. *Traite d'Organogenie Comparee de la Fleur*: 674-676.

Pedersen LB. 2001. Four new species of *Orchidantha* (Lowiaceae) from Sabah. *Nordic Journal of Botany* **21**(2): 121-128.

Pedersen LB, Johansen B. 2004. Anatomy of the unusual stigma in Orchidantha (Lowiaceae). *American Journal of Botany* **91**(3): 299-305.

Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF. 2000. B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature* **405**(6783): 200-203.

Posluszny U, Scott MG, Sattler R. 1980. Revisions in the Technique of Epi-Illumination Light-Microscopy for the Study of Floral and Vegetative Apices. *Canadian Journal of Botany-Revue Canadienne De Botanique* **58**(23): 2491-2495.

Prasad K, Vijayraghavan U. 2003. Double-stranded RNA interference of a rice *PI/GLO* paralog, *OsMADS2*, uncovers its second-whorl-specific function in floral organ patterning. *Genetics* **165**(4): 2301-2305.

Preston JC, Christensen A, Malcomber ST, Kellogg EA. 2009. Mads-box gene expression and implications for developmental origins of the grass spikelet. *American Journal of Botany* **96**(8): 1419-1429.

Preston JC, Hileman LC. 2009. Developmental genetics of floral symmetry evolution. *Trends Plant Sci* **14**(3): 147-154.

Preston JC, Kost MA, Hileman LC. 2009. Conservation and diversification of the symmetry developmental program among close relatives of snapdragon with divergent floral morphologies. *New Phytol* **182**(3): 751-762.

Prince LM, Kress JW. 2006. Phylogenetic relationships and classification in Marantaceae: insights from plastic DNA sequence data. *Taxon* **55**(2): 281-296.

Rambaut A, Drummond A 2007 Tracer.In: Available from http://beast.bio.ed.ac.uk/Tracer

Rasmussen DA, Kramer EM, Zimmer EA. 2009. One size fits all? Molecular evidence for a commonly inherited petal identity program in Ranunculales. *Am. J. Bot.* **96**(1): 96-109.

Raven PH. 1977. A suggestion concerning the Cretaceous rise to dominance of the angiosperms. *Evolution* **31**: 451-452.
Reardon W, Fitzpatrick D, Fares M, Nugent J. 2009. Evolution of flower shape in *Plantago lanceolata*. *Plant Molecular Biology* **71**(3): 241-250.
Ree RH, Citerne HL, Lavin M, Cronk QC. 2004. Heterogeneous selection on LEGCYC paralogs in relation to flower morphology and the phylogeny of Lupinus (Leguminosae). *Molecular Biology and Evolution* **21**(2): 321-331.
Reeves PA, Olmstead RG. 2003. Evolution of the TCP gene family in Asteridae: cladistic and network approaches to understanding regulatory gene family diversification and its impact on morphological evolution. *Molecular Biology and Evolution* **20**(12): 1997-2009.
Ridl. 1893. Protamomum maxillarioides Ridl. *Transactions of the Linnean Society of London* **3**: 383.
Rieseberg LH, Willis JH. 2007. Plant Speciation. *Science* **317**(5840): 910-914.
Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics (Oxford)* **19**(12): 1572-1574.
Ronse De Craene LP. 2008. Homology and evolution of petals in the core eudicots. *Systematic Botany* **33**(2): 301-325.
Ronse De Craene LP, Soltis PS, Soltis DE. 2003. Evolution of floral structures in basal angiosperms. *International Journal of Plant Sciences* **164**(5 Supplement): S329-S363.
Rudall PJ, Bateman RM. 2002. Roles of synorganisation, zygomorphy and heterotopy in floral evolution: The gynostemium and labellum of orchids and other lilioid monocots. *Biological Reviews* **77**(3): 403-441.
Rudall PJ, Bateman RM. 2004. Evolution of zygomorphy in monocot flowers: iterative patterns and developmental constraints. *New Phytologist* **162**(1): 25-44.
Rudall PJ, Stevenson DW, Linder HP. 1999. Structure and Systematics of *Hanguana*, a monocotyledon of uncertain affinity. *Australian Systematic Botany* **12**: 311-330.
Saarela JM, Prentis PJ, Rai HS, Graham SW. 2008. Phylogenetic relationships in the monocot order Commelinales, with a focus on Philydraceae. *Botany* **86**(7): 719-731.
Sabu M. 2006. *Zingiberaceae and Costaceae of South India*. Kerala: Indian Association for Angiosperm Taxonomy.
Sajo MG, Longhi-Wagner HM, Rudall PJ. 2008. Reproductive morphology of the early-divergent grass *Streptochaeta* and its bearing on the homologies of the grass spikelet. *Plant Systematics and Evolution* **275**(3-4): 245-255.
Sakai S, Inoue T. 1999. A new pollination system: dung-beetle pollination discovered in *Orchidantha inouei* (Lowiaceae, Zingiberales) in Sarawak, Malaysia. *American Journal of Botany* **86**: 56-61.
Sakai S, Kato M, Inoue T. 1999. Three pollination guilds and variation in floral characteristics of Bornean gingers (Zingiberaceae and Costaceae). *American Journal of Botany* **86**(5): 646.
Sakai S, Nagamasu H. 2000. Systematic studies of Bornean Zingiberaceae: III. *Tamijia*: A new genus. *Edinburgh Journal of Botany* **57**(2): 245-255.
Sargent RD. 2004. Floral symmetry affects speciation rates in angiosperms. *Proceedings of the Royal Society Biological Sciences Series B* **271**(1539): 603-608.
Sattler R. 1968. A technique for the study of floral development. *Can J Bot* **46**((5)): 720-722.

Schichnes D, Nemson J, Sohlberg L, Ruzin SE. 1998. Microwave protocols for paraffin microtechnique and in situ localization in plants. *Microscopy and Microanalysis* **4**(5): 491-496.

Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H, Sommer H. 1990. Genetic Control of Flower Development by Homeotic Genes in *Antirrhinum majus*. *Science* **250**(4983): 931-936.

Simmonds NW. 1966. *Bananas*. London: Longmans.

Smith RM. 1972. The genus *Burbidgea*. *Notes from the Royal Botanic Garden Edinburgh* **31**: 297-306.

Soltis DE, Soltis PS, Endress PK, Chase MW. 2005. *Phylogeny and Evolution of Angiosperms*. Sunderland, Massachussets: Sinauer Associates.

Song CF, Lin QB, Liang RH, Wang YZ. 2009. Expressions of *ECE-CYC2* clade genes relating to abortion of both dorsal and ventral stamens in *Opithandra* (Gesneriaceae). *BMC Evolutionary Biology* **9**.

Specht CD 2005. Phylogenetics, floral evolution and rapid radiation in the tropical monocot family Costaceae (Zingiberales). In: Sharma AK, Sharma A eds. *Plant genome: biodiversity and evolution*. Calcutta: Scientific Publications, Inc., 29-60.

Specht CD. 2006. Systematics and Evolution of the tropical monocot family Costaceae (Zingiberales): a multiple data set approach. *Systematic Botany* **31**(1): 88-105.

Specht CD, Kress WJ, Stevenson DW, DeSalle R. 2001. A molecular phylogeny of Costaceae (Zingiberales). *Molecular Phylogenetics and Evolution* **21**(3): 333-345.

Stebbins GL. 1970. *Adaptive Radiation of Reproductive Characteristics in Angiosperms Part 1: Pollination Mechanisms*.

Stern DL. 2000. Perspective: evolutionary developmental biology and the problem of variation. *Evolution* **54**(4): 1079-1091.

Stevenson DW, Stevenson JW 2004a. Cannaceae. In: Smith N, Mori SA, Henderson A, Stevenson DW, Heald SV eds. *Flowering plants of the Neotropics*. Princeton, N. J.: Princeton University Press.

Stevenson DW, Stevenson JW 2004b. Marantaceae. In: Smith N, Mori SA, Henderson A, Stevenson DW, Heald SV eds. *Flowering plants of the Neotropics*. Princeton, N.J. : Princeton University Press.

Strange A, Rudall PJ, Prychid CJ. 2004. Comparative floral anatomy of Pontederiaceae. *Botanical Journal of the Linnean Society* **144**(4): 395-408.

Sukumaran J, Holder M 2009. DendroPy Phylogenetic Computing Library.In: http://packages.python.org/DendroPy/.

Swigonová Z, Lai J, Ma J, Ramakrishna W, Llaca V, Bennetzen JL, Messing J. 2004. Close Split of Sorghum and Maize Genome Progenitors. *Genome Research* **14**(10a): 1916-1923.

Takeda T, Suwa Y, Suzuki M, Kitano H, Ueguchi-Tanaka M, Ashikari M, Matsuoka M, Ueguchi C. 2003. The OsTB1 gene negatively regulates lateral branching in rice. *The Plant Journal : for cell and molecular biology* **33**(3): 513-520.

Tanaka M. 2006. Dermoscopy. *J Dermatol* **33**(8): 513-517.

Theissen G. 2001. Development of floral organ identity: Stories from the MADS house. *Current Opinion in Plant Biology* **4**(1): 75-85.

Theissen G, Melzer R. 2007. Molecular mechanisms underlying origin and diversification of the angiosperm flower. *Annals of Botany* **100**(3): 603-619.

Theissen G, Strater T, Fischer A, Saedler H. 1995. Structural characterization, chromosomal localization and phylogenetic evaluation of two pairs of *AGAMOUS*-like MADS-box genes from maize. *Gene* **156**(2): 155-166.

Thompson J. 1933. Studies in advancing sterility. Part VI. The theory of Scitaminean flowering. *Publications of the Hartley Botanical Laboratories* **11**: 1-111.

Tiffney BH, Niklas KJ 1985. Clonal growth in land plants: A paleobotanical perspective. In: Jackson JBC, Buss LW, Cook RE eds. *Population biology and evolution of clonal organisms*. New Haven, Connecticut, USA: Yale University Press.

Tomlinson PB. 1962. Phylogeny of the Scitamineae - morphological and anatomical considerations. *Evolution* **16**: 192-213.

Tremousaygue D, Garnier L, Bardet C, Dabos P, Herve C, Lescure B. 2003. Internal telomeric repeats and 'TCP domain' protein-binding sites co-operate to regulate gene expression in *Arabidopsis thaliana* cycling cells. *The Plant Journal* **33**(6): 957-966.

Troebner W, Ramirez L, Motte P, Hue I, Huijser P, Loennig W-E, Saedler H, Sommer H, Schwarz-Sommer Z. 1992. *GLOBOSA*: A homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. *EMBO Journal* **11**(13): 4693-4704.

Tucker SC. 2003. Floral development in legumes. *Plant Physiology (Rockville)* **131**(3): 911-926.

Uhl NW. 1987. *Genera Palmarum*. Lawrence, Kan.: Allen Press.

Ushimaru A, Dohzono I, Takami Y, Hyodo F. 2009. Flower orientation enhances pollen transfer in bilaterally symmetrical flowers. *Oecologia* **160**(4): 667-674.

Ushimaru A, Hyodo F. 2005. Why do bilaterally symmetrical flowers orient vertically? Flower orientation influences pollinator landing behaviour. *Evolutionary Ecology Research* **7**(1): 151-160.

Vandenbussche M, Zethof J, Royaert S, Weterings K, Gerats T. 2004. The duplicated B-class heterodimer model: Whorl-specific effects and complex genetic interactions in *Petunia hybrida* flower development. *Plant Cell* **16**(3): 741-754.

Vincent CA, Coen ES. 2004. A temporal and morphological framework for flower development in Antirrhinum majus. *Canadian Journal of Botany* **82**(5): 681-690.

Wang XY, Tang HB, Bowers JE, Feltus FA, Paterson AH. 2007. Extensive concerted evolution of rice paralogs and the road to regaining independence. *Genetics* **177**(3): 1753-1763.

Wang Z, Luo YH, Li X, Wang LP, Xu SL, Yang J, Weng L, Sato SS, Tabata S, Ambrose M, Rameau C, Feng XZ, Hu XH, Luo D. 2008. Genetic control of floral zygomorphy in pea (*Pisum sativum* L.). *Proceedings of the National Academy of Sciences* **105**(30): 10414-10419.

Warner KA, Rudall PJ, Frohlich MW. 2008. Differentiation of perianth organs in Nymphaeales. *Taxon* **57**(4): 1096-1109.

Welchen E, Gonzalez DH. 2006. Overrepresentation of elements recognized by TCP-domain transcription factors in the upstream regions of nuclear genes encoding components of the mitochondrial oxidative phosphorylation machinery. *Plant Physiology (Rockville)* **141**(2): 540-545.

Wheeler JR. 2002. *Flora of the South West : Bunbury, Augusta, Denmark* Canberra: A.C.T. : Australian Biological Resources Study

Whipple CJ, Ciceri P, Padilla CM, Ambrose BA, Bandong SL, Schmidt RJ. 2004. Conservation of B-class floral homeotic gene function between maize and *Arabidopsis*. *Development* **131**(24): 6083-6091.

Whipple CJ, Zanis MJ, Kellogg EA, Schmidt RJ. 2007. Conservation of B class gene expression in the second whorl of a basal grass and outgroups links the origin of lodicules and petals. *Proceedings of the National Academy of Sciences* **104**(3): 1081-1086.

White PR. 1928. Studies on the banana; an investigation of the floral morphology and cytology of certain types of the genus *Musa* L. *Zeitschr Zell Forsch U Mikrosk Anat* **7**(5): 673-733.

Wilson TC, Charlton WA, Posluszny U. 2006. Increased image depth for epi-illumination microscopy. *Canadian Journal of Botany-Revue Canadienne De Botanique* **84**(3): 508-513.

Winter K-U, Weiser C, Kaufmann K, Bohne A, Kirchner C, Kanno A, Saedler H, Theissen G. 2002. Evolution of class B floral homeotic proteins: Obligate heterodimerization originated from homodimerization. *Molecular Biology and Evolution* **19**(5): 587-596.

Wong WSW, Yang Z, Goldman N, Nielsen R. 2004. Accuracy and power of statistical methods for detecting adaptive evolution in protein coding sequences and for identifying positively selected sites. *Genetics* **168**: 1041-1051.

Wray GA, Hahn MW, Abouheif E, Balhoff JP, Pizer M, Rockman MV, Romano LA. 2003. The evolution of transcriptional regulation in eukaryotes. *Molecular Biology and Evolution* **20**(9): 1377-1419.

Yadav SR, Prasad K, Vijayraghavan U. 2007. Divergent regulatory *OsMADS2* functions control size, shape and differentiation of the highly derived rice floret second-whorl organ. *Genetics* **176**(1): 283-294.

Yang Y, Fanning L, Jack T. 2003. The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. *Plant Journal* **33**(1): 47-59.

Yang Z. 1998. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Molecular Biology and Evolution* **15**: 568-573.

Yang Z. 2002. Inference of selection from multiple species alignments. *Current Opinion in Genetics & Development* **12**: 688-694.

Yang Z. 2007. PAML 4: Phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution* **24**: 1586-1591.

Yang Z, Nielsen R. 1998. Synonymous and nonsynonymous rate variation in nuclear genes of mammals. *Journal of Molecular Evolution* **46**: 409-418.

Yang Z, Nielsen R, Goldman N, Pedersen A-MK. 2000. Codon-Substitution Models for Heterogeneous Selection Pressure at Amino Acid Sites. *Genetics* **155**(1): 431-449.

Yang Z, Wong W, Nielsen R. 2005. Bayes empirical Bayes inference of amino acid sites under positive selection. *Mol Biol Evol* **22**(4): 1107-1118.

Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM. 1990. The protein encoded by the *Arabidopsis* homeotic gene *AGAMOUS* resembles transcription factors. *Nature* **346**(6279): 35-40.

Yao S-G, Ohmori S, Kimizu M, Yoshida H. 2008. Unequal genetic redundancy of rice *PISTILLATA* orthologs, *OsMADS2* and *OsMADS4*, in lodicule and stamen development. *Plant and Cell Physiology* **49**(5): 853-857.

Yuan Z, Gao S, Xue DW, Luo D, Li LT, Ding SY, Yao X, Wilson ZA, Qian Q, Zhang DB. 2009. *RETARDED PALEA1* controls palea development and floral zygomorphy in rice. *Plant Physiol* **149**(1): 235-244.

Zanis MJ, Soltis PS, Qiu YL, Zimmer E, Soltis DE. 2003. Phylogenetic analyses and perianth evolution in basal angiosperms. *Annals of the Missouri Botanical Garden* **90**(2): 129-150.

Zhang J, Nielsen R, Yang Z. 2005. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. *Molecular Biology and Evolution* **22**: 2472-2479.

Zhang W, Kramer EM, Davis CC. 2010. Floral symmetry genes and the origin and maintenance of zygomorphy in a plant-pollinator mutualism. *Proceedings of the National Academy of Sciences* **107**(14): 6388-6393.

Zhou X-R, Wang Y-Z, Smith JF, Chen R. 2008. Altered expression patterns of TCP and MYB genes relating to the floral developmental transition from initial zygomorphy to actinomorphy in Bournea (Gesneriaceae). *New Phytologist* **178**(3): 532-543.

Zwickl DJ. 2006. *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation.* The University of Texas at Austin.