

Homoplasy, Pollination, and Emerging Complexity During the Evolution of Floral Development in the Tropical Gingers (Zingiberales)

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Abstract With their impressive array of floral diversity and a largely-understood phylogenetic relationships, the Zingiberales provide an ideal model clade to test for the roles of genetic and ecological factors driving floral diversification. Many Zingiberales have close associations with particular suites of pollinators, a species-level interaction that is reflected in their overall floral morphology. Here we first discuss the importance of understanding developmental evolution in a phylogenetic context, then use the evolution of floral morphology across the Zingiberales to test the hypothesis that shifts in rates of diversification among these tropical monocots is correlated with shifts in pollination syndrome, suggesting an important role of pollination specificity in driving speciation and floral diversification in the Zingiberales.

Keywords Floral evolution · Developmental evolution · Pollination syndrome · Zingiberales · Diversification rates · Model clade · Key innovation

“...evolutionary approaches and the exploration of developmental diversity offer a powerful probe into developmental mechanisms and the architecture of ontogeny, in a distinct and perhaps complementary manner to that provided by developmental genetics”—Rudolf A. Raff, 1996

“Reciprocal Illumination!”—Dennis Wm. Stevenson

Introduction

The most fundamental questions in developmental evolution are those that classic morphologists and developmental biologists have been asking for centuries, involving ecological (adaptation) and historical (evolution, phylogenetic) mechanisms (Darwin, 1859; Gompel et al., 2005; Rudwick, 1997; Wittkopp et al., 2003). The contribution of molecular and genetic tools (transgenics, DNA sequencing, genomic/next-generation sequencing, DNA expression analysis) to these questions is significant, and can provide new depth to studies of morphological evolution while expanding the breadth of questions that can be addressed (Carroll, 2006). This expanding research area will enable scientists to develop a better understanding of the link between genetic and morphologic diversification. To do so, one must incorporate data from morphology, ecology, population biology and developmental genetics and use tools from phylogenetic analysis, coalescent theory, and tests of molecular and morphological adaptation in addition to utilizing data from transgenic, functional genomic and gene expression analyses.

Recent increases in the number of species with whole-genome sequence data means that we now have large-scale genetic information spanning evolutionary space and time (Patel, 2004). These data provide a new empirical base for studies in comparative morphology and developmental evolution. Using model systems, gene function and expression can be studied and developmental mechanisms can be proposed. The role of these developmental mechanisms in the evolution of form and function can then be tested in non-model systems that demonstrate variation in phenotype that is correlated with fitness or adaptive significance. Data from developmental genetics and phylogenetic analysis provides the comparative framework for studying the evolution of developmental pathways, and becomes the foundation for investigating the role of homoplasy, the repeated evolution of developmental similarity, in developmental evolution.

Unfortunately, diversity is often and intentionally avoided when studying development. The search for developmental paradigms creates a bias toward seeking common patterns and mechanisms for development. Diversity is an inconvenience, or at best a novelty to be explained via essentialist models derived from understanding development in model organisms. The very characteristics that make model organisms ideal for genetic studies (short generation time, reduced phenotypic variability, robust canalized growth) make them less than ideal for evolutionary studies. As such, model organism research cannot address how selection might interact with development to create the diversity of forms we see in nature. The limited number of model organisms combined with limited diversity represented within each model system means that neither species-level diversity nor naturally occurring teratisms can be investigated. Adapting genetic models of development to explain diversity inevitably requires ad hoc hypotheses that are more about explaining away diversity than explaining the factors that drive diversification. The transition from a hunt for general mechanisms to an understanding of how those mechanisms have evolved and have contributed to diversity of form and function requires an understanding of developmental genetics in a comparative phylogenetic context.

In addition to the logistical differences, the fields of developmental and evolutionary biology have long been hindered by a conceptual separation. While advances in evolutionary theory have overturned some basic tenants of developmental biology, these concepts have not yet been incorporated into modern developmental research design (Raff, 1996). For example, the concept of *developmental constancy*, which

hypothesizes a hierarchical ontogeny of expanding complexity, predicts that features appearing early in development should be more conserved throughout evolutionary history (Wimsatt & Schank, 1988). This idea of generative entrenchment is highly influential in interpretations of developmental genetics: the stability of early development seems logical in light of seemingly precise regulation of gene cascades that show increased complexity and absolute dependency on the action of preceding genes. However, constancy in early development is not absolute, and in fact evolution of early developmental stages may be a major force driving morphological diversification (Kirchoff, 1998). Dramatic changes can occur even between closely related species, indicating that supposedly hard-wired developmental mechanisms are actually quite plastic, and that remodeling of development is a common phenomenon in evolution (Kirchoff, 1998). Changes in genome size and gene order, gene duplication events (especially of regulatory genes), horizontal gene transfer, and changes in the timing or location of gene expression are all mechanisms by which early developmental shifts may result in drastic morphological differentiation among closely related organisms.

Although body plans may be stable over long periods of time and across large evolutionary distances, basic phenotypic elements may be arrived at by different genetic pathways, indicating that the body plan can be conserved despite changes in the underlying genetic mechanisms. Alternatively, shifts in timing or location of expression of a particular gene cascade can result in drastically different morphologies without notable or significant nucleotide changes. This dissociation between organismal form and developmental genetics highlights the importance of phylogenetically-based comparative developmental studies. Without better documentation of the diversity of developmental paradigms and the order in which they occur throughout the tree of life, it is impossible to understand the genetic pathways and patterns underlying morphological diversification. Detailed studies of closely related species from developmental and genetic perspectives will provide insight to the mechanisms involved in the evolution of diversity.

The Model Clade Approach

There are two fundamental ways in which developmental genetics and evolution can be combined. The first is the comparison among model systems, which has been used extensively within the developmental genetics community often in demonstrating conservation of developmental mechanisms across great evolutionary distances (eg. Schierwater & Kuhn, 1998). Alternatively, closely related organisms that show naturally-occurring and fixed differences in developmental patterns can be used to examine the genetic basis of the characterized morphological diversity, focusing on difference rather than similarity. Once homologies in ontogeny are established, the developmental comparisons between closely related species with different morphologies is similar to developmental studies in model organisms, only both species represent viable modified ontogenies rather than a wild type and a mutant, and the genetic differences are unknown (not defined by the researcher). This second method, combined with detailed phylogenetic data, enables the identification and study of developmental pathways that are selected for (or against) during evolution, and eventually—via morphologic, ecologic and even geographic information—why such selection is occurring.

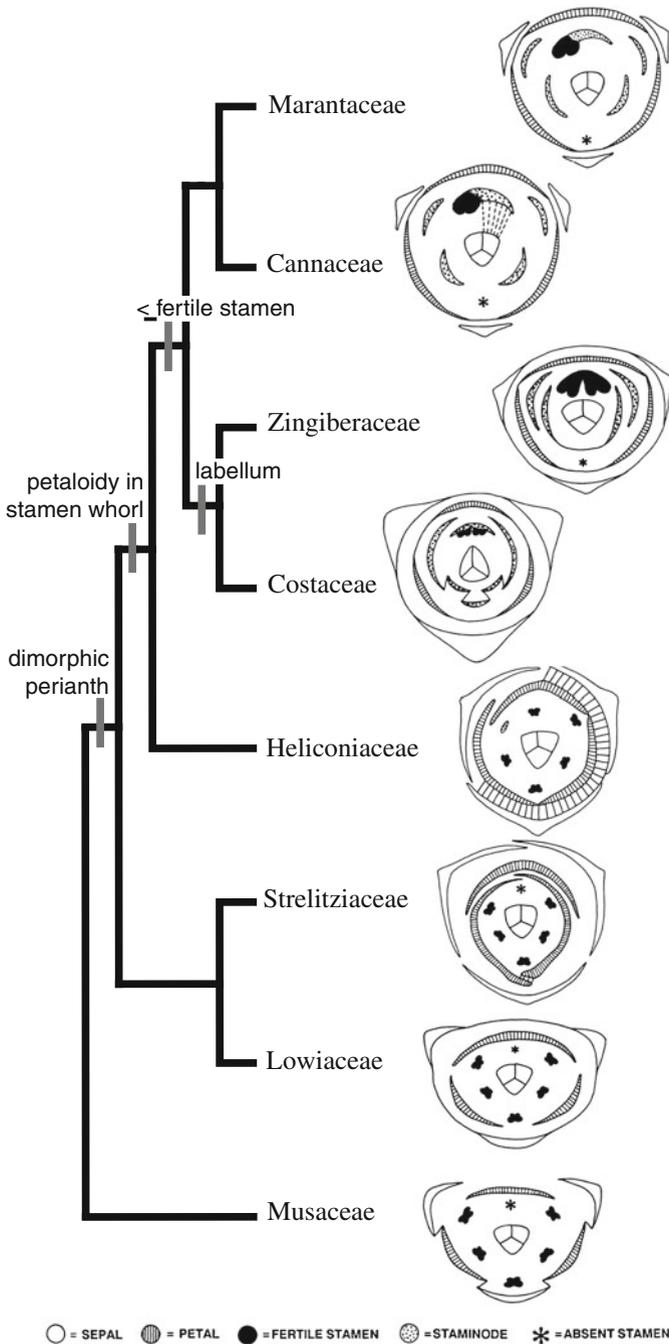


Fig. 1 Phylogeny of the Zingiberales indicating topological positions of the evolution of a dimorphic perianth, petaloid stamens, reduction in stamen number and the labellum. A representative floral diagrams of each family is indicated next to the family name. Note petal-like stamen (*stippled*) and reduction in number of fertile stamens (*black*) in ginger families. Organ fusion and positional homology among organs indicated in the placement and orientation of the floral diagrams

Floral Development of the Zingiberales

The monocot order Zingiberales (“tropical gingers”) comprises a major component of both tropical and subtropical ecosystems and includes crop plants (e.g., banana, plantain, culinary ginger), sources of traditional medicines and spices (cardamom, turmeric, galanga) and horticulturally important ornamentals (e.g., Heliconias, Bird-of-Paradise, Cannas). The order contains approximately 2,500 species that form specialized pollination relationships with bees, birds, bats, dung beetles, moths, butterflies, and primates (lemurs) via alterations in floral form. In addition to developing a phylogenetic hypothesis for the entire order (Fig. 1; Kress et al., 2001), we have estimated divergence times for the major lineages of Zingiberales (Kress & Specht, 2005, 2006; Specht, 2005) and at the species-level within several families (Specht, 2006a) using molecular phylogenetic hypotheses in combination with molecular clock or related temporal algorithms. Detailed studies of two families, Costaceae and Zingiberaceae, indicate that specialized relationships with animal pollinators have led to increased rates of diversification in bird-pollinated and bee-pollinated lineages (Kay et al., 2005; Specht, 2005, 2006a). Thus, a framework is in place for comparative studies of floral development involved in key innovations in a phylogenetic and functional context.

The Zingiberalean flower follows a typical monocot pattern in which concentric whorls of floral organs each have three members. All Zingiberales have two whorls of stamen, giving a base form of K3:C3: A 3+3:G(3), with perianth and androecial parts variously differentiated and fused throughout the order (Fig. 1).

The Zingiberales demonstrate an evolutionary trend in the ontogeny of the perianth, i.e. sepals and petals. The perianth is considered dimorphic when sepals and petals that have distinct morphologies from one another occur in the same flower. The appearance of a dimorphic perianth is variable throughout the commelinid monocots, and the transition from a monomorphic perianth to a dimorphic perianth appears to have occurred independently in the Commelinales and the Zingiberales (Bartlett & Specht, 2010). The perianth of the Musaceae is largely monomorphic with three sepals and three petals being more or less uniform in color, shape, size and texture. One petal from the inner whorl remains free while all other perianth members fuse to form a floral tube that, together with the exposed thecae (in staminate flowers), forms the floral display. Perianth dimorphism increases evolutionarily within the order (Bartlett & Specht, 2010), with the highest amount of dimorphism recognized in the Costaceae which has sepals that are significantly smaller, more coriaceous, and differently colored than the petals of the same species.

In addition to the trends observed in the evolution of perianth dimorphism (Bartlett & Specht, 2010, 2011), another important evolutionary trend that defines the evolution of the Zingiberales flower is the evolution of the androecium or stamen whorls (Fig. 1). In the banana families (Musaceae, Strelitziaceae, Lowiaceae and Heliconiaceae), which form a basal paraphyly within the order, the two consecutive stamen whorls each contain 3 fertile stamens. These stamens have elongated sterile filaments with distally located anthers comprising about half of the length of the total stamen. In many species, a single stamen aborts early in development so that there are only 5 fertile stamens at maturity (Kirchoff & Kunze, 1995; White, 1928). In *Heliconia*, a single stamen develops as an infertile, often callose, staminode (Kirchoff et al., 2009). In the derived ginger families, the number of fertile stamens

is reduced to one (Costaceae, Zingiberaceae) or 1/2 (Cannaceae, Marantaceae), the later having only a single theca (Kirchoff, 1983, 1991). The fertile stamen develops from the inner stamen whorl and is petaloid with the thecae located on the adaxial surface and the connective extending above the region of pollen production. The remaining infertile stamens (staminodes) share positional homology with stamens in the banana families but develop as petaloid structures, taking on the function (pollinator attraction) and structure (conical epidermal cells, Martin & Glover, 2007) of petals. In the Zingiberaceae and Costaceae, 2–4 (Zingiberaceae) or 5 (Costaceae) staminodes fuse together to form a novel structure, the staminodial labellum (Kirchoff, 1988). This staminodial labellum forms the main visual aspect of the floral display and is responsible for creating, via coloration patterns and floral tube morphology, the variety of pollination syndromes found in these highly diverse families. The ancestral zingiberalean flower is likely to have had 5–6 fertile stamens, with the staminode first evolving in the lineage leading to Heliconiaceae plus the ginger families (Bartlett & Specht, 2010) and ultimately occurring in 2–5 stamen positions to dominate the floral display.

Interestingly, changes in the number of fertile stamen are inversely correlated with petaloidy throughout the evolutionary history of the Zingiberales. As stamens abort, petaloid structures develop and tend to dominate the floral display (Fig. 2). In

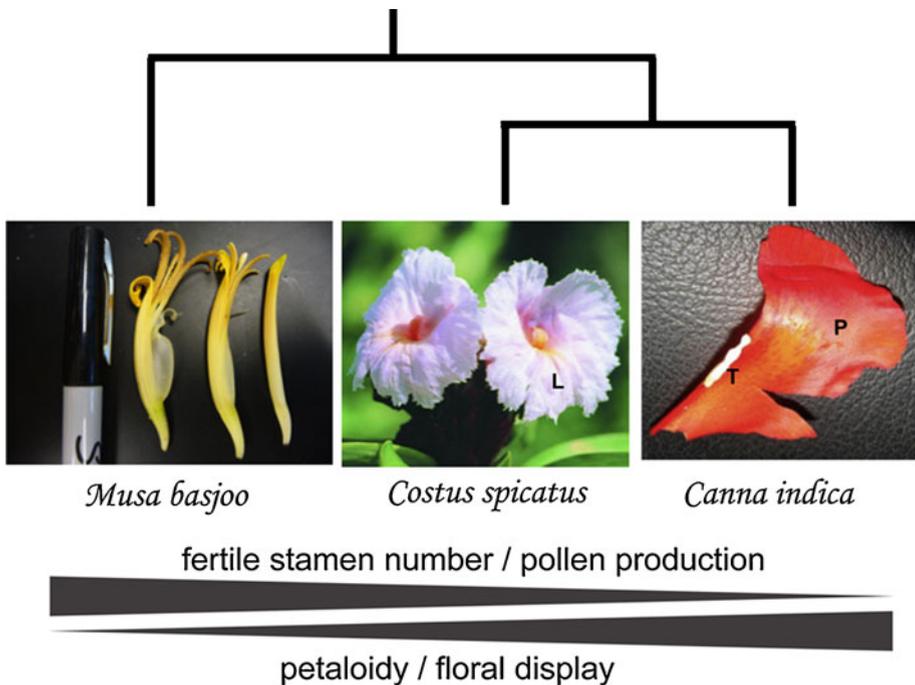


Fig. 2 Inverse correlation between the number of fertile stamens and petaloidy in the Zingiberales. In *Musa basjoo*, five fertile stamens develop, while petaloidy is inexpressive. In *Costus spicatus*, the formation of the labellum (L) is a direct result of the fusion of infertile, petaloid staminodes. In this species, only one fertile stamen develops. *Canna indica* is a striking example of extreme reduction on the number of fertile stamens, where only half a stamen (T, a single theca) is formed, while the rest of the stamen develops into a petaloid structure (P)

Musaceae, the basal-most member of the order, five of the six perianth members fuse to form a floral tube (Figs. 1 and 2) that dominates the floral display while 5–6 stamens are fertile and produce thecae containing pollen. As the number of fertile stamens is reduced in the ginger families, petaloid structures are formed in the stamen whorls instead of pollen-producing stamens. In all four ginger families, the petaloid staminode structures produce the bulk of the floral display (color, symmetry and pattern). This switch from fertile stamen (high pollen production) to petaloid, colorful structures (reduced pollen production; specialized pollination syndromes) may have important ecological implications. A reduction in production of pollen is potentially compensated for by the formation of specialized pollinator relationships that enable precise pollen placement and increase opportunity for fertilization.

Thus, major changes in the petal and stamen whorls enable the development of different pollination syndromes throughout the Zingiberales order. In order to address the evolution of developmental pathways underlying these changes in development, it is important to understand the role that differential floral development plays in driving rates of diversification. Here we use a supertree approach to investigate whether certain categories of floral forms are responsible for, or responsive to, shifts in rates of speciation and species diversification.

Adaptive Evolution and Pollination in the Zingiberales

The role of adaptive evolution in speciation and the generation of morphological diversity via adaptive or ecologically-driven phenotypic variation has been the subject of much attention in the past decade (Hodges, 1997b; Losos & Miles, 2002). One important mechanism of ecological speciation is the adaptation to different pollinators leading to pollinator partitioning and reproductive isolation (Givnish, 2010). Modifications in floral morphology and the use of specialized systems to ensure efficient and effective pollination are well known mechanisms for species diversification within many plant lineages (Castellanos et al., 2004; Hodges, 1997a). In flowering plants, the success of a population is strongly linked to the ability of the individuals to reproduce via animal-mediated pollination (Fenster et al., 2004; Pellmyr, 2002). Within the monocotyledonous plants, elaborate pollination systems involving birds, insects and even mammals have evolved multiple times throughout the entire lineage, making the monocots ideal for an analysis of the role of pollination systems on species diversification rates (Stevenson et al., 2000).

A fundamental question in the study of adaptive radiation is whether a novel morphology evolves at the same time as a shift in the rate of speciation, indicating a causal relationship between the novel morphology (i.e. the putative key innovation) and an increased rate of speciation (Berenbaum et al., 1996; Losos & Miles, 2002; McKenna & Farrell, 2006; Sanderson & Wojciechowski, 1996; Schena et al., 1995). The concept of a 'key innovation' as defined here is thus dependent upon the assumption that certain traits have enabled their corresponding lineages to proliferate (speciate, diversify) at an increased rate by opening up new adaptive zones (Burger, 1981). If this concept is correct, we would expect to find that observed differences in speciation rates between clades would be correlated with presence (or absence) of particular traits that are proposed to function as key innovations. We can thus test for

the function of a characteristic as a key innovation by testing for such a correlation in a hypothesis-testing framework.

In order to assess whether shifts in diversification rate coincide with the acquisition of a key innovation, such as a pollination syndrome, several elements are required. First, a phylogenetic hypothesis is needed to determine where and when shifts in pollination associations have occurred within an evolutionary framework and what floral forms are ancestral as well as which are derived. Phylogenetic information is then analyzed in a temporal context in order to determine the comparative rates of diversification across all studied lineages and allow estimates to be made of number of species in a lineage per unit of time. Pollination records and floral morphology are compared with shifts in diversification rate within and between lineages to determine if evolutionary changes in pollination syndromes are associated with changes in rates of diversification. Thus, we can use phylogenetic information combined with an understanding of organismal evolution to test for the role of a particular phenotype as a 'key innovation' in the evolution of a lineage.

The strength of such a correlation can be used to test the functionality of a particular ecologically relevant feature of an organism to act as a key innovation in enabling speciation, decreasing extinction, or otherwise enabling a net increase in diversification within a lineage. Historically, scenarios involving the role of a particular adaptation on increased rates of speciation were based on reported increase in number of species once the particular ecologically-relevant phenotype in question was acquired and maintained in a lineage. Such scenarios were dependent upon untestable claims of species numbers and lineage associations and were therefore unscientific (Slowinski & Guyer, 1993) in explaining diversity of individual groups or clades. Variability in species number per clade is easily consistent with simple stochastic models of phylogenetics since, under a null model of random speciation, all degrees of species diversity are equally likely (Farris, 1976; Slowinski & Guyer, 1993).

A relationship between a trait and increased diversity can only be fully tested if several groups possessing the same trait are considered in a comparative context (Mitter et al., 1988; Zeh et al., 1989), and if the groups considered are of the same age (Slowinski & Guyer, 1993) or their ages are known such that number of species per unit time can be calculated. Molecular sequence data can be used to determine relative ages of species in a phylogenetic context, and fossils reliably assigned to taxonomic groups can then be used to determine absolute ages of lineages in a known phylogeny thus enabling examination of comparative diversification rate changes over time (Eriksson & Bremer, 1992; Magallon & Sanderson, 2001; Ricklefs et al., 2007). Within the Zingiberales, such a study has been conducted for the family Costaceae (Specht, 2005) where dense species-level sampling across the family includes molecular data for each taxon so that branch lengths could be estimated and a molecular clock approach used to develop age estimates for each of the major bifurcations indicating diversification events in the form of cladogenesis or speciation. Once ages are established, diversification rates can be estimated using a method-of-moments estimator that takes into account a variable extinction rate (Magallon & Sanderson, 2001; Specht, 2005) and provides a relative rate of diversification for each clade of interest across the entire topology. These rates are compared with shifts in morphology associated with pollination syndromes such that a topological correlation is made between clades where presence or absence of a particular pollination syndrome corresponds to clades with increased or decreased rates of diversification.

To test the role of pollination in diversification across the Zingiberales, it is important to compare diversification rates in distantly related groups to determine if independently derived pollination syndromes are associated with increased rates of diversification at each appearance. However, a single phylogenetic analysis that includes a single dataset for species-level sampling within each family has not been completed for this species-rich order, and supertree approaches have resulted in low resolution due to challenging alignments.

A backbone phylogenetic hypotheses for the eight families of the order Zingiberales exists based on 20 exemplar taxa (Kress, 1995; Kress et al., 2001). In addition, more taxonomically complete phylogenies exist for each of the families within the order: Musaceae (Liu et al., 2002); Lowiaceae (Johansen, 2005); Heliconiaceae (Specht et al., 2006); Cannaceae (Prince, 2010); Costaceae (Specht et al., 2001; Specht & Stevenson, 2006); Zingiberaceae (Kress et al., 2002); Marantaceae (Prince & Kress, 2006). These species-level phylogenies are combined into a single phylogenetic hypothesis using a supertree approach that creates a single ordinal-level tree from the combined independent family-level analyses (Funk & Specht, 2007). A topological approach, which analyzes overall degrees of tree symmetry to identify nodes at which greater than expected diversification has occurred (Chan & Moore, 2002, 2005; Moore et al., 2004), is here used to determine the relative rates of diversification among lineages of Zingiberales. This allows for the identification of shifts in rates of diversification to be detected and correlated with shifts in pollination syndrome, testing for the role of pollination syndrome as a key innovation in the evolution and diversification of the Zingiberales.

Materials and Methods

Tree Building and Character-State Reconstruction

Because we do not currently have complete sampling for any single dataset across the entire Zingiberales, a supertree was constructed using the meta-tree approach (Funk & Specht, 2007). The base tree is based on the Kress et al. (2001) phylogeny of Zingiberales (Fig. 1). Taxa are grafted onto the base tree using the most recent phylogenetic hypothesis for each of the major lineages to provide the phylogenetic structure of terminal relationships. Only published phylogenies were used in order to facilitate replication of this study. Each published and supported node was coded using a basic matrix representation technique in MacClade. Overlapping sampling in data sets was coded to retain the integrity of the published phylogenetic results, intercalating taxa and lineages where necessary. The majority of nodes were not in conflict, however where conflict in nodes existed between analyses (position of *Hornstedia* taxa, position of *Alpinia oxymitra*) the topology of the analysis with the greatest taxonomic coverage was used. This is due to the fact that these taxa were on long branches in at least one of the conflicting analyses, and the use of the topology representing the most complete taxon sampling helps to ensure that the effect of any long branches in a particular data set on obscuring taxon placement is not being repeated in the meta-tree construction. The meta-tree comprises 509 terminal taxa and was produced in PAUP* based on a parsimony analysis of the matrix produced in MacClade. A heuristic search

was performed using TBR and stepwise addition with either a simple or random (10) sequence. In each case, a single tree was recovered ($L=439$).

Pollination syndrome was coded as having 4 character states [bird (includes hummingbirds, sunbirds, and honeyeaters, all conferring similar floral characters with little overlap in biogeographic distribution), insect (includes bee, beetle, fly, moth) bat and, non-flying mammal] or 8 character states [bee, beetle, bat, moth, honeyeaters, sunbirds, hummingbirds and non-flying mammal]. Various alternative codings of pollinators were attempted, varying from two states (invertebrate v. vertebrate) to 14 states with birds and bees divided taxonomically into orders; the character states presented here most closely reflect defined functional groups in their influence on floral morphology (Fenster et al., 2004). The character states were coded as the first character of the MacClade file and then removed when performing the parsimony analysis so that the pollination syndromes can be mapped as a multi-state character on to the final meta-tree topology. Ancestral character reconstructions were conducted under maximum parsimony in MacClade and with Bayesian Stochastic character mapping (Huelsenbeck et al., 2003) in SIMMAP 1.5.2 (Bollback, 2006). Because SIMMAP only accepts up to seven character states for its stochastic mapping analysis, we eliminated non-flying mammal pollination as this state is only derived in a single species. A full table of all pollination syndrome-coding for each species and the source of the coding information (published pollination record, unpublished pollination observation, or morphology-based assignment of pollination syndrome) is available from the corresponding author upon request. The supertree was converted in a radial tree using FigTree v1.2.3 (<http://tree.bio.ed.ac.uk/>) and edited using Adobe Illustrator CS4 (Adobe System Incorporated).

Analysis of Diversification Rates

In the absence of temporal information (molecular sequence data), a topological approach was used to determine if the branches of the Zingiberales supertree had likely diversified under significantly different rates, and to locate the branches along which significant shifts in rates of diversification have occurred. SymmeTREE (Chan & Moore, 2005) uses the topological distribution of species across the entire tree to investigate diversification rate shifts along branches that do not need discrete lengths associated with them. The program, which uses a likelihood framework to identify and locate shifts in diversification rates, is freely available (http://www.phylodiversity.net/bmoore/software_symmetree.html).

The constructed supertree was imported as a saved treefile. Results for the two likelihood ratio-based shift statistics, Δ_1 and Δ_2 , were calculated using SymmeTREE (Chan & Moore, 2005). Default options were selected with the exceptions that the minimum & maximum values were calculated by analysis (option c), and the number of species for the quick shift statistic calculation (for generating a likelihood ratio array for comparison with the input tree) was set to 600. Polytomies in the supertree dataset were resolved into dichotomous solutions by generating 1,000 random resolutions using the taxon-size sensitive (TSS) equal-rates Markov (ERM) random branching model as the taxon addition algorithm (TSS-ERM), providing an estimate of confidence intervals for p -values associated with each shift statistic associated with a polytomy. The taxon-size sensitive (TSS)

ERM algorithm is generally most conservative with respect to the null hypothesis (i.e., no significant diversification rate variation), thus was chosen as a conservative approach in handling ambiguities associated with polytomies in the data.

Topological Uncertainty

The backbone of the supertree was based on the published phylogeny of the order (Kress et al., 2001). However, the support for the relationships of the basal lineages in this phylogeny, the ‘banana families’ (Musaceae, Strelitziaceae, Lowiaceae and Heliconiaceae), is weak. To test if changes in the basal topology would affect our results, we examined the possible topologies changing the relationships of the banana families. For each topology, pollination syndromes were reconstructed and divergence rates were calculated. We did not find significant differences between the topologies. Hence, we present here only the results obtained with a topology that reflects the published phylogeny based on total evidence.

Results

Evolution of Pollination Syndromes

In order to consistently reconstruct the ancestral state of the order, we reconstructed the ancestral pollination syndrome for the sister group of the Zingiberales, the order Commelinales. Bee pollination is common in this order (Buchmann, 1980; Faden, 1992; Hardy et al., 2009; Hopper & Burbidge, 1978; Husband & Barrett, 1992; Orth & Waddington, 1997) with bird pollination reported only for the genus *Anigozanthos* (family Haemodoraceae) (Hopper & Burbidge, 1978) and bat pollination not reported at all (Fleming et al., 2009). In a tree representing the relationships among the five families of the Commelinales, insect (bee) pollination syndrome was reconstructed as the ancestral state; thus insect (bee) pollination was assigned as the state for the outgroup of the Zingiberales.

Two different analyses were run; one in which four different pollination syndromes were reconstructed on the Zingiberales supertree (Fig. 3), and a second in which the states ‘bird’ and ‘insect’ were each subdivided into three separate states: hummingbirds, sunbirds, and honeyeaters (meliphagid); and bee, beetle and moth respectively. The methods were mostly consistent, but parsimony resulted in more ambiguous nodes (25 nodes) than Bayesian stochastic character mapping (10 nodes). We therefore present only the results obtained by stochastic character mapping.

Insect pollination is the syndrome most commonly found in Zingiberales (76.6 %) in which bee pollination corresponds to 68 %. Numerous species also exhibit bird pollination (19 %) and bat pollination (3.9 %) syndromes. Using the commelinid outgroup for rooting, the ancestral pollination syndrome in the order Zingiberales was reconstructed as insect pollination syndrome ($P=0.99$), more specifically bee pollination syndrome ($P=0.82$) (Fig. 3).

During the evolution of the Zingiberales, several independent shifts to different pollination syndromes have occurred. The majority of the transitions (62 %) occurred from insect pollination syndrome to other pollination syndromes (Fig. 4). The

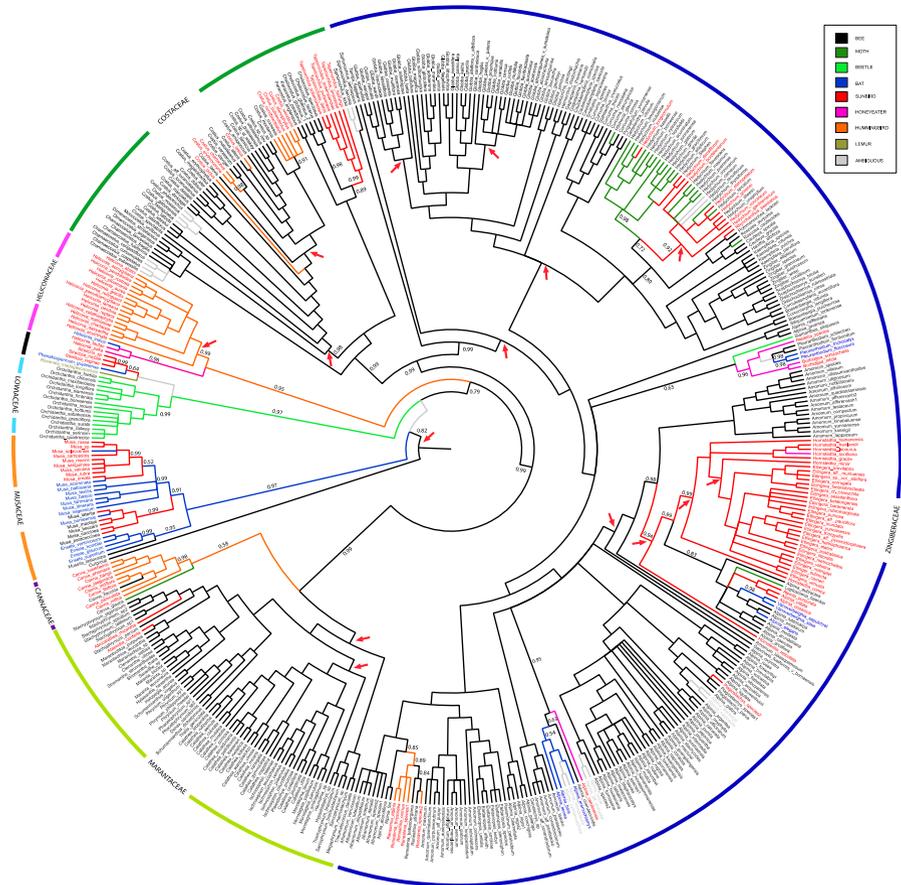


Fig. 3 Pollination syndrome evolution reconstructed under Bayesian stochastic character mapping for a metatree of the Order Zingiberales with the eight families designated. Branch color denotes eight different pollination syndromes as detailed in text and legend. Taxon name color denotes the four character state reconstruction of pollination syndromes: *black* = insect, *red* = bird, *blue* = bat, and *brown* = non-flying mammal. *Red arrows* indicate significant shifts in rate of diversification as detected by SymmeTREE. Number at a node indicates the posterior probability (pp) of the character state reconstruction given the eight character state condition. PP of the character state reconstruction is only provided for nodes that correspond with a shift in pollination syndrome

majority of transitions are from insect to bird pollination (43 %) with the opposite trend (bird to insect) occurring much less frequently (12 %). Shifts from insect to bat pollination are also frequent (12.68 %). Considering the 8 pollination states, the most frequent transitions are from bee to sunbird pollination (13.41 %) and bee to hummingbird (12.97 %) pollination. Other frequent transitions were found from bee to moth pollination (8.46 %) and bee to bat pollination (8.73 %). The opposite trends (e.g. bat to bee) are significantly less frequent (Fig. 4).

It is noteworthy that bat pollination only appears in clades in which bird pollination syndromes (sunbird or meliphagid) are also present. Half of the transitions to bat pollination syndrome come from either sunbird or honeyeater pollination syndromes with the remaining derived from insect pollination (Fig. 4). Bat to sunbird pollination

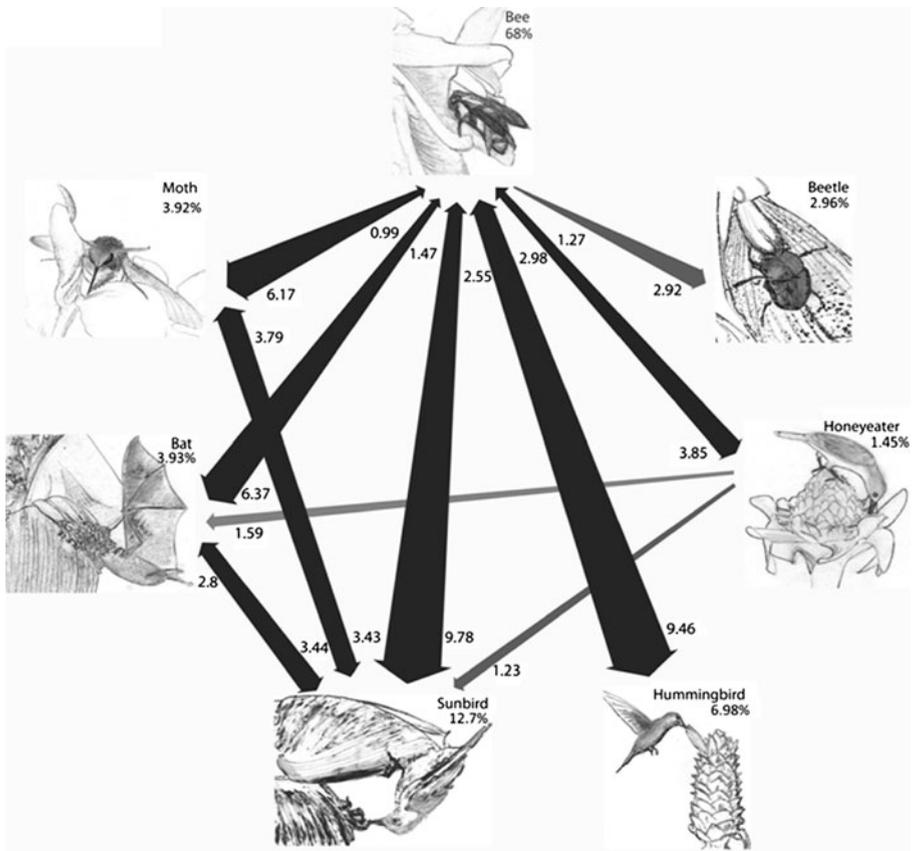


Fig. 4 Probability and directionality of transitions between pollination states. The relative size of the line and number indicated at *arrow tips* indicates the percentage of the estimated transitions between each pollination syndrome in the direction indicated. The *number* with each drawing indicates the total presence of that syndrome across the sampled members of the Zingiberales as a percentage

shifts are concentrated in the family Musaceae, where there are few reversals back to bat pollination. Otherwise, bat pollination is retained once it has evolved from either bee or honeyeater pollination syndromes with limited reversals and no shifts to a novel derived pollination syndrome. Similarly, hummingbird pollination is exclusively derived from plants that are bee pollinated, and reversals from hummingbird back to bee pollination are infrequent. As with bat pollination, hummingbird pollination does not evolve to other types of pollination syndrome, indicating potential selection for the retention of this syndrome: if hummingbird pollination is lost it is via reversal to the immediate ancestral state.

Sunbird pollinated plants are derived in their majority from plants with a bee pollination syndrome (13.41 %) but they can also be derived from moth (4.7 %) and bat pollination syndromes (4.71 %). Honeyeater pollination is significantly less frequent than other bird pollination syndromes found in the Zingiberales (1.45 %) likely due to their restricted distribution in Australia, New Guinea and a few pacific islands where Zingiberales are present but not extremely diverse or dominant in ecosystems (with the exception of *Tapeinochilos* (Costaceae) in New

Guinea). As a whole, Zingiberales are more diverse in the distribution areas occupied by sunbirds (Africa & Southeast Asia) and hummingbirds (Americas), therefore their bird pollination syndromes are frequented most by sunbirds and hummingbirds.

Pollination syndromes are differentially distributed among families. The family Musaceae is characterized by a vertebrate pollination (bird or bat). Stochastic character mapping (SM) using reconstructed bat pollination ($P=0.97$) as the ancestral pollination syndrome for this family, since species of the genus *Ensete* and several species of *Musa* are bat pollinated. Bird (sunbird) pollination has evolved independently at least twice from bat pollination in the genus *Musa*. The only species pollinated by bees in the family is the monotypic *Musella lasiocarpa* (Franch.) C.Y., although this species is reported to be pollinated by other insects (butterflies) as well.

The ancestral pollination syndrome for Lowiaceae and Strelitziaceae is insect pollination ($P=0.99$) in particular bee pollination ($P=0.97$). While the family Lowiaceae shifted to beetle pollination ($P=0.972$), the family Strelitziaceae shifted to vertebrate pollination syndromes. The three different Strelitziaceae genera have a particular vertebrate pollination syndrome: bat pollination for the monotypic *Phenakospermum guyanense*, lemur pollination for *Ravenala madagascariensis* and sunbird pollination for the five included species of the genus *Strelitzia*. The family Heliconiaceae is mostly pollinated by birds (hummingbirds and honeyeaters). Our results suggest that hummingbird pollination is the ancestral state for this family ($P=0.95$).

Reconstructions show that the ancestral pollination syndrome for the clade of ginger families (Costaceae, Zingiberaceae, Marantaceae and Cannaceae) is insect pollination ($P=0.99$), specifically bee pollination ($P=0.99$). For the families Costaceae, Zingiberaceae and Marantaceae, the ancestral pollination syndrome is insect or bee pollination ($P>0.99$). The ancestor of the family Cannaceae was most likely pollinated by insects ($P=0.97$), but the family promptly shifted to bird pollination given that all modern cannas are hummingbird pollinated ($P=0.98$).

In the family Costaceae, five independent shifts from bee to bird pollination have occurred in this supertree, four shifts from bee to hummingbird pollination in the genus *Costus* and one shift from a generalist to sunbird pollination in the Southeast Asian genus *Tapeinochilos*. Species of the large family Zingiberaceae present several pollination syndromes that appear to be derived from insect pollination syndromes. The reconstruction resulted in 15 shifts from invertebrate to vertebrate pollination. Vertebrate pollination is concentrated mostly in five independent genera. The ancestor of *Hedychium* shifted to moth pollination and within the genus at least three independent shifts to sunbird pollination have occurred. The clade comprising *Etilingera* and *Hornstedtia* shifted to sunbird pollination. *Renealmia* is mostly pollinated by hummingbirds and the clade comprising *Riedelia*, *Burbidgea* and *Pleurothodium* contains species pollinated by honeyeaters and bats.

In contrast, the large family Marantaceae does not at least superficially demonstrate a diversity of pollination syndromes; bee pollination syndrome is common throughout the family, although differences in species of bee may play a large role in floral specialization and diversification. Only two species of Marantaceae represented in the supertree are reported to be sunbird pollinated (Ley & Classen-Bockhoff, 2009).

Analysis of Diversification Rates

The Δ shift statistics were used to locate significant shifts in diversification rates for the Zingiberales supertree (Fig. 3), demonstrating a total of 15 significant rate shifts ($p \leq 0.05$) across the entire topology. Significant rate shifts were detected within four families of the order (Heliconiaceae, Costaceae, Zingiberaceae and Marantaceae). Eight of the significant shifts are associated with nodes in which transitions from insect to bird pollination syndromes have taken place: five to sunbird and three to hummingbird pollination. In one node, transitions from moth to sunbird pollination also occur.

The other six significant rates are not associated with pollination shifts and are located (1) at the base of the order, (2) within the family Marantaceae and (3) within the family Zingiberaceae (two in *Globba* and one at the base of the family).

To explore if the plant species richness is related to the evolution of the pollination syndromes, we calculated Spearman rank correlations between species richness per family and number of pollination shifts or pollination syndromes. A weak correlation exists between the number of species and the number of pollinator shifts ($\rho = 0.67$, $P = 0.066$). However, there is no association between the number of pollination syndromes and the number of species ($\rho = 0.24$, $P = 0.557$).

The correlation between the lineage age and number of species in families, using the spearman rank correlation coefficient, was also calculated in order to determine if species diversity is simply a function of age (birth only model). Lineage ages were taken from Specht and Kress (2006). The association between these two parameters is not significant ($\rho = 0.547$, $P = 0.171$).

Discussion

Sampling

One of the major important issues to testing for key innovations is the need to have full taxonomic sampling or to accommodate incomplete taxonomic sampling if one is to claim correlated cladogenesis. A complete phylogeny coupled with trait databases can be used to investigate ‘correlates of cladogenesis’ or key innovations (Gittleman et al., 2004). Metatrees (grafted supertrees) offer the opportunity to examine the nature of evolutionary processes over large tree space using resolution gained from small lineage phylogenies and avoiding alignment and analytical problems associated with the generation of single gene trees for large scale phylogenies.

Testing Key Innovations

The supertree of the Zingiberales was constructed using a matrix representation with parsimony (MRP) and considering the cladistic principles evaluated by Bryant (2004) such that the supertree in this analysis is considered to act as a heuristic synthesis of hierarchical information rather than a rigorous phylogenetic analysis of the included taxa. MRP was used only as a mechanism to produce a single phylogenetic tree from the available topological data and to code terminals and reconstruct ancestral character states for pollination syndrome.

A total of 508 species of the Zingiberales were included in the meta-tree (around 25 % of the whole order) representing the eight families and the majority of the genera (84 from 92 recognized genera). The families in the order differ in species richness ranging from 1,200 species (Zingiberaceae) to 7 species (Streliziaceae). But lineage ages do not explain the variation in species richness among families as shown by the spearman correlation ranks ($\rho=0.547$, $P=0.171$). Other studies have shown that this pattern rarely exists (Vamosi & Vamosi, 2010). Gentry (1982) sustained that high diversity of tropical flora cannot be explained by a gradual diversification but by an explosive speciation and adaptive radiation. Key innovations such as pollination shifts can alter the evolutionary success of lineages (Vamosi & Vamosi, 2010). A new floral adaptation that leads to pollination specialization could increase the effectiveness of intraspecific flow, affecting the reproductive success, population viability and ability to colonize new areas. That would be translated into lower extinction rates and higher species richness (Armbruster & Muchhala, 2009). A positive but weak relationship between species richness and pollination shifts was found using the spearman correlation ranks ($\rho=0.67$, $P=0.066$), suggesting that pollination shifts could be considered an important trait for the diversification of this order.

Pollination Syndromes Evolution

Pollination syndromes provide great utility for understanding mechanisms of floral diversification (Fenster et al., 2004). While pollinator specificity is not absolute, pollinators can be organized into functional groups based on types of selection pressures they exert on floral evolution. Certain functional groups have been shown to exert different selection pressures on floral traits (Fenster et al., 2004). Major gaps exist in our knowledge of specific pollinators for many species of Zingiberales as a whole; however similar convergent floral morphologies involving entire suites of correlated characters indicate strong selection for pollinator preference and provide an indication of the selection mechanisms underlying the evolution of pollination syndromes. The relative importance of specific traits, the selective factors that favor shifts between groups, and whether selection acts on different traits independently or in combination in response to specific pollinators cannot be addressed at this time. However, using the concept of functional groups from the perspective of floral morphology, an accurate phylogenetic reconstruction allows us to address the role of history in the evolution of the Zingiberales flower and its association with pollinator preferences, and to investigate the role that particular combined morphological character evolution may have played in the history of diversification across this group.

The diverse morphology of the Zingiberales flowers attracts diverse animal pollinators. The most abundant pollination syndrome in the order is bee pollination, but Zingiberales flowers are also pollinated by moth, beetle, bird and bat species. The bird pollinators belong to three families: the Neotropical hummingbirds (Trochilidae), the Old World sunbirds (Nectariniidae) and the Asiatic honeyeaters (Meliphagidae). From the ancestral floral state, which is reconstructed as light-colored and open (non-tubular) flowers pollinated by insects, at least 21 independent transitions to bird pollination and bat pollination have occurred (Fig. 3). As compared with the insect pollinated ancestral state, bird pollinated flowers are prominently tubular in form and are brightly colored (red, orange, yellow) while bat pollinated flowers are larger and

pale in color, typically opening (and presumably peaking in nectar production) at nightfall.

In Costaceae, species pollinated generally by insects have open light-colored flowers with a large petaloid labellum, forming the ancestral floral form of this family. Bee pollination floral morphology evolved from a generalist insect pollination floral form once in African species of the genus *Costus* (Specht, 2006a, b). In bee-pollinated species, the labellum is used as landing platform and nectar guide, with ultraviolet markings that are oriented visually toward the floral center. Bird pollinated flowers in the family Costaceae are reported to have evolved multiple times from a bee pollinated ancestral floral form in the New World genus *Costus* (Kay et al., 2005; Specht, 2006b) and from a generalist insect ancestral morphology to form the Melanesian genus *Tapeinochilos*. The bird pollinated flowers in *Costus* are red, yellow or orange in color and the labellum is contained within the petals maintaining a rigid tubular structure (Specht, 2006b).

Bat pollinated plants always occur in clades containing bird pollinated plants, in particularly sunbird and honeyeaters pollination syndromes, as shown in other plant families (Fleming et al., 2009). Bat pollinated plants evolve less frequently than bird pollinated plants. This result is not surprising because to assure visitation, bat pollinated flowers should be bigger and produce more nectar, thus more energetically expensive (Fleming et al., 2009). In Zingiberales, bat pollinated flowers are large, accessible and produce copious amounts of nectar and pollen (Fleming et al., 2009). Bat pollination is a syndrome essentially from Old World Zingiberales, with the exception of the South American *Phenakospermum guyannense* pollinated by phyllostomid bats. This pattern is opposite to that found in other bat pollinated plant lineages, where bat pollination appears in twice as many genera and species in the New World than in the Old World (Fleming et al., 2009).

Pollination Syndromes as Key Innovations

It has long been argued that specialized biotic pollination is a key factor in diversification and success of angiosperms. Here, we show that the increase of the species richness in the order Zingiberales is associated to biotic pollination shifts.

Fourteen nodes have significant shifts in rates of evolution in the supertree of the order. Eight of these nodes also have simultaneously experienced a shift in pollination syndrome, in particular shifts from insect to bird pollination (Fig. 3). This result indicates that bird pollination specialization in Zingiberales may increase the speciation rates via reproductive specialization. Due to the nature of our analysis, we cannot differentiate this from the potential that a shift to bird pollination may also (or instead) decrease extinction rate with the net result being a significant increase in diversity in bird pollinated lineages.

One clade that has a significant shift in diversification rate is the Neotropical members of the family Heliconiaceae. This family, mostly bird pollinated, underwent a rapid radiation in the Neotropics that occurred approximately 18 Ma ago (Specht, Kress and Driscoll, unpublished data) resulting in close to 215 neotropical species in comparison with only 6 old world species. The neotropical *Heliconia* are exclusively pollinated by hummingbirds, indicating that both colonization of the neotropics combined with exploiting a novel pollinator relationship promoted speciation in this

lineage. Ongoing work is investigating the possibility for co-diversification between new world *Heliconia* and their hummingbird pollinators.

In the family Costaceae, a lineage with a generalist insect ancestral pollination state, diversification rates are increased in two nodes both of which occur within the genus *Costus*. The genus *Costus* originated in Africa where it is either generalist insect or bee pollinated (here coded as bee). The lineage underwent a rapid radiation following dispersal to the Neotropics associated with at least four and perhaps more independent shifts from bee-pollinated flowers to hummingbird-pollinated flowers (Kay et al., 2005; Specht, 2006b). While other genera of Costaceae (*Monocostus*, *Dimerocostus*, and *Chamaecostus*) have an older neotropical presence without experiencing species radiations (1–8 extant species per genus), the *Costus* neotropical radiation (~120 species) is more recent and is alone associated with multiple shifts from bee pollination syndrome to hummingbird pollination (Specht, 2006b). To date, no shifts from hummingbird pollination syndrome back to bee pollination have been recorded. The shift to bird pollination in *Tapeinochilos* is not associated with a shift in diversification rate in the supertree presented; however, if the full number of *Tapeinochilos* species (18) are included as a polytomy, a significant shift in diversification is correlated with this node.

Bee pollination is reconstructed as ancestral within the Zingiberaceae. Major shifts in pollination include shifts to moth and sunbird pollination in *Hedychium*, to sunbird pollination in *Burbridgea* plus *Riedelia*, *Etilingera* and several groups of *Alpinia*, to bat pollination in *Pleuranthodium* and *Vanoverberghia* and other *Alpinia*, and to hummingbird pollination in the new world *Renealmia*. Significant shifts in rates of diversification in the family Zingiberaceae are consistently associated with shifts in pollination syndrome. The *Hedychium* lineage shifted from ancestral bee pollination to moth pollination, and within this clade at least three shifts from moth pollination to sunbird pollination have occurred. At least one shift from moth to sunbird pollination is correlated with a significant shift in diversification rate, and the larger clade containing *Hedychium* also appears to have diversified more rapidly than the sister clade containing bee-pollinated *Globba*. Another significant shift is found in the clade that unites species of *Etilingera* and *Hornstedtia* which have shifted from bee pollination to sunbird pollination.

Other significant shifts in divergence rate not associated with shifts in pollination syndrome are located in the family Zingiberaceae and Marantaceae, the two richest families in the order. Unfortunately, information about pollination syndromes is not always available; however, some of the identified shifts in divergence rates could be indirectly explained by shifts in pollination syndromes. For example, two significant shifts are located within the asiatic genus *Globba* for which pollinators are not known. However, this genus, containing around 100 species, presents small tubular flowers with nectaries at the base of the tube suggesting an adaptation to moth pollination (Box & Rudall, 2006). Williams et al. (2004) hypothesize that innovations of colorful bracts and variable inflorescence morphologies in the richest sections of *Globba* are consistent with a hypothesis of pollinator driven selection.

In the case of the family Marantaceae, species are mostly pollinated by bees (Kennedy, 2000; Ley & Classen-Bockhoff, 2009). This family presents a particular adaptation in its pollen transfer mechanism, called explosive pollination, that has been hypothesized to enhanced rate of speciation (Kennedy, 2000). The style is placed under

tension by differential growth of the style and hooded staminode, and the tension released by the pollinator moving the trigger appendage of the hooded staminode. As a result, the style springs rapidly and irreversibly forward, mediating pollen transfer (Classen-Bockhoff, 1991; Pischtschan & Classen-Bockhoff, 2008). Precise pollen placement, protection of the pollen by the cucullate staminode and possible adaptation to pollination by distinctive morphological changes are innovations that may explain the richness of Marantaceae compared to its sister group, the Cannaceae (Kennedy, 2000).

In our analyses, we considered bees as a functional group because we do not have enough data to consider different types of bee morphology (long-tongued vs. short tongued bees) or behavior and its resulting influence on floral morphology. By doing that we are likely missing several pollination shifts that play an important role in the evolution of certain groups. For example, the New World Marantaceae flowers shifted to larger tube length as adaptation to pollination by long tongued, traplining, euglossine bees (Kennedy, 2000). By contrast, Old World Marantaceae species are primarily adapted to pollination by short-medium tongued bees with few exceptions (Ley & Classen-Bockhoff, 2009). This pollination shift may be responsible for the radiation of American species, especially of the genus *Calathea* that contains around 300 species (Kennedy, 2000). In fact, in our tree one of the nodes experiencing significant shifts in diversification corresponds to the node that separates *Calathea* species and some other American species from the African *Haumania*. This would suggest that shifts in specific pollinator could play a role in the diversification of the family Marantaceae.

Conclusions

Floral isolation and specialization in pollination have been assumed to affect plant speciation particularly as it relates to the diversification of floral forms. This association seems likely within the Zingiberales, a monocot lineage with flowers having diverse floral morphologies that reflect specialized pollination syndromes. Increases in rates of diversification are found in clades that have undergone shifts in pollination syndromes, indicating a role of pollination morphology in species divergence and diversification. Additional studies focused on pollination ecology, species level phylogenetics, and ongoing studies in the genetics and evolution of developmental morphology will further aid in our understanding of how changes in floral traits lead to species isolation and floral diversification in this order.

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research is actively driving the fields of plant morphology, plant systematics, plant ontology and developmental evolution—all from a phylogenetic perspective. The entire field benefits from his expansive, library-like knowledge of plant morphology and diversity of plant form and function and his ability to see a lesson to be taught or a research question to be answered in every shoot apical meristem or axillary bud.

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