Phylogeny and Evolution of Bracts and Bracteoles in *Tacca* (Dioscoreaceae)

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Abstract

Most species in the genus *Tacca* (Dioscoreaceae) feature green to black purple, conspicuous inflorescence involucral bracts with variable shapes, motile filiform appendages (bracteoles), and diverse types of inflorescence morphology. To infer the evolution of these inflorescence traits, we reconstructed the molecular phylogeny of the genus, using DNA sequences from one nuclear, one mitochondrial, and three plastid loci (Internal Transcribed Spacer (ITS), *atp*A, *rbc*L, *trn*L-F, and *trn*H-*psb*A). Involucres and bracteoles characters were mapped onto the phylogeny to analyze the sequence of inflorescence trait evolution. In all analyses, species with showy involucres and bracteoles formed the most derived clade, while ancestral *Tacca* had small and plain involucres and short bracteoles, namely less conspicuous inflorescence structures. Two of the species with the most elaborate inflorescence morphologies (*T. chantrieri* in southeast China and *T. integrifolia* in Tibet), are predominantly self-pollinated, indicating that these conspicuous floral displays have other functions rather than pollinator attraction. We hypothesize that the motile bracteoles and involucres may facilitate selfing; display photosynthesis in the dim understory, and protect flowers from herbivory.

Keywords: *atp*A; bracteoles; bracts; ITS; molecular phylogeny; *rbc*L; *Tacca*; *trn*H-*psb*A; *trn*L-F.

Introduction

Flowering plants possess an extraordinary diversity in reproductive traits, even among closely related species. The floral diversification that has accompanied the co-evolution of flowers and animal pollinators is particularly striking and has resulted in contrasting suites of floral characters associated with different pollinator groups (e.g., Grant and Grant 1965; Armbruster 1993; Johnson et al. 1998; Schemske and Bradshaw 1999).

In many species, animal pollinators have shaped floral evolution resulting in distinct pollination syndromes (Faegri and Van Der Pijl 1971; Proctor et al. 1996; Fenster et al. 2004). Investment in attractive structures represents an allocation cost that animal-pollinated plants pay to secure the fitness advantages that accrue from out-crossing (Charlesworth and Charlesworth 1987). In contrast, for self-pollinating species, investment in attractive structures should provide no benefit and resources should instead be redirected to alternative

structures or activities (Charnov 1982; Lloyd 1987). According to resource allocation models, species that invest in an extravagant floral display should be primarily outcrossed (Charlesworth and Charlesworth 1987). However, many plant species with extravagant floral displays also have strong capacity for nonpollinator-assisted self-pollination (Wang et al. 2004; Zhang et al. 2005; Liu et al. 2006; Fenster and Marten-Rodriguez 2007).

Tacca is comprised of approximately 15 species of acaulescent forest understory herbs (see The World Checklist of Monocotyledons (http://apps.kew.org/wcsp/home.do)) and is included in the family Dioscoreaceae (Caddick et al. 2002; but see Ding and Larsen 2000). It is primarily Paleotropical in distribution, and its current distribution center is Southeast Asia: About 13 species are restricted to Indo-Malesia (Southeast Asia to the Solomon Islands), one species occurs in tropical South America, and one species is distributed from the tropical west coast of Africa eastward to Easter Island in the eastern Pacific Ocean (Drenth 1972). These unusual plants produce a scapose, umbellate inflorescence with long, filiform bracteoles mixed in with the flowers.

Although *Tacca* is a small genus with about 15 species, its floral displays are diverse. Generally, species of *Tacca* can be sorted into three groups based on their inflorescences characters: (i) inconspicuous inflorescences with small bracts and short bracteoles, e.g., *T. leontopetaloides*, *T. plantaginea* and *T. parkeri* (**Figure 1A–C**); (ii) inconspicuous inflorescences with bracts but without bracteoles, e.g., *T. palmata* and *T. palmatifida* (**Figure 1D, E**); and (iii) very showy inflorescences with large bracts and long bracteoles, represented by *T. subflabellata*, *T. integrifolia*, *T. ampliplacenta*, *T. chantrieri* (**Figure 1F–J**). For species in the third category, the two bracts (involucres) are large and conspicuous, dark purple or white in color, positioned above the dark purple flowers and long dangling filiform bracteoles (**Figure 1F–J**).

These elaborate structures have long been assumed to function as a "deceit syndrome" in which the inflorescence resembles decaying organic material, attracting flies to facilitate cross-pollination (sapromyiophily) without providing any reward (Faegri and Van Der Pijl 1971; Drenth 1972; Saw 1993), because *Tacca* species produce no nectar and only a small amount of pollen (Zhang et al. 2005). On the other hand, *Tacca* species in the second category seem to lack any attraction to pollinators. This may be due to septal nectary loss in monocots driven by loss of pollinators (Smets et al. 2000). As such, the relationships between those diverse floral display and their pollinators in *Tacca* need more detailed exploration.

Although conspicuous inflorescences and floral structures should attract pollinators and increase the likelihood of outcrossing, we have observed previously that *T. chantrieri*, with substantial bracts and whisker-like dark purple filiform bracteoles (**Figure 1J**), and *T. subflabellata*, with large white

or pink inflorescences) (**Figure 1F**), are self pollinating and lack effective pollinators (Zhang et al. 2005; Zhang L. pers. obs., 2008). These observations about the reproductive biology of *Tacca* disagree with general assumptions about the diversity of inflorescence structures, suggesting a paradox: why have predominantly selfing species in Yunnan, China evolved such luxuriant and metabolically expensive floral displays when they serve no function in attracting pollinators? One explanation for this paradox is that conspicuous involucres and bracteoles are a relictual syndrome with no current utility (Zhang et al. 2005). To properly understand the evolutionary context of this paradox, we must first determine the phylogenetic relationship among the species to understand the sequence of character trait evolution, particularly for the three inflorescence types described above.

The taxonomy of *Tacca* remains controversial and species circumscriptions vary widely. Originally, the genus was included in the Taccaceae (Dumortier 1829). Seventy-seven specific epithets exist, but only about 15 have been accepted within *Tacca* (http://apps.kew.org/wcsp/home.do). A few other genera were assigned to Taccaceae but only *Schizocapsa* remains in current use (Ding and Larsen 2000). *Schizocapsa* was recognized by Hance (1881) because of the dehiscent capsular fruit in *S. plantanginea* Hance, compared with the indehiscent fruits of *Tacca*. The recognition of *Schizocapsa* still remains controversial. Drenth (1972) did not accept *Schizocapsa* as a separate genus, merging it with *Tacca*, arguing that dehiscent fruits were insufficient for delimiting a new genus, while Ling (1985) also described anomocytic stomata on the leaf epidermis as unique to *Schizocapsa* and also recognized a second species, *S. guangxiensis* P. P. Ling & C. T. Ting (Ling and Ding 1982). More recently, Caddick et al. (2002) moved *Tacca* into Dioscoreaceae (APG II, following their results), because it shares numerous characters with other Dioscoreaceae, such as tuberous underground parts rich in steroidal saponins, petiolate reticulate-veined leaves, and reflexed stamens, while its acaulescent habit and unilocular ovaries with parietal placentation are distinctive.

To investigate the phylogenetic relationships and the evolutionary history of variation in bracts and bracteoles in *Tacca*, we reconstructed the phylogeny of nine species in the genus using DNA sequence data from five genetic regions of three genomes: *atp*A from the mitochondrial genome, the two ITS spacer regions (including 5.8S) from the nuclear genome and *trn*L-F, *trn*H-*psb*A and *rbc*L from the chloroplast genome. We then mapped bracts and bracteoles characters onto the resulting phylogeny to reconstruct the ancestral states. Given this analysis, we can then address the following questions: (i) What are the phylogenetic relationships of *Tacca* species within the genus? (ii) What are the evolutionary trends of floral displays in this group?
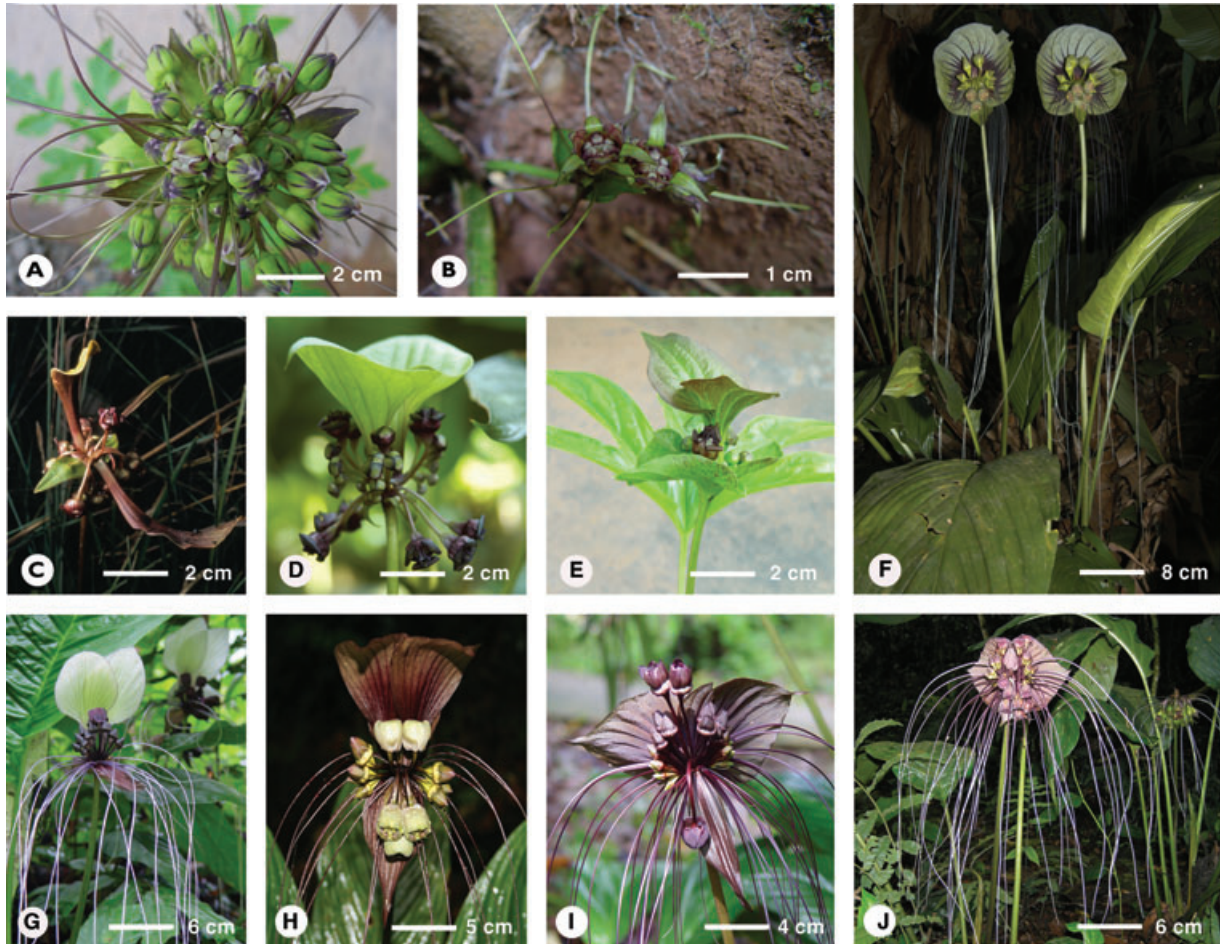
Figure 1.　The diversity of reproductive traits among species of *Tacca*.

(**A**) *T. leontopetaloides*.
(**B**) *T. plantaginea*.
(**C**) *T. parkeri*.
(**D**) *T. palmatifida*.
(**E**) *T. palmata*.
(**F**) *T. subflabellata*.
(**G**) *T. integrifolia* from Motuo, Tibet.
(**H**) *T. integrifolia* from Seremban, Malaysia.
(**I**) *T. ampliplacenta*.
(**J**) *T. chantrieri*.
A, F, G, H, I photos were provided by Qing-Jun Li; **B, D, E, J** photos by Ling Zhang, and **C** photo by Dr Lisa M. Campbell from New York Botanical Garden.

Results

Phylogenetic reconstruction

The final alignment for the combined DNA sequence data for the five regions (*atp*A + ITS + *rbc*L + *trn*L-F + *trn*H-*psb*A) for the 11 samples included a total of 5 191 base pairs of which

217 (4.18%) were phylogenetically informative (**Table 1**). The mitochondria gene *atp*A was the least variable (30 out of 1 193 base pairs), of which only 11 (0.92%) were informative. The three chloroplast regions consist of 703 (*trn*H-*psb*A), 1 425 (*rbc*L), and 1 068 bp (*trn*L-F), which contribute 1%, 1.89%, and 2.52% of the informative sites, respectively. The nuclear ITS region provided the majority of the informative characters,

Table 1. DNA site variation and tree statistics for the five datasets used in the cladistic analyses presented in this study

DNA region	No. taxa	No. characters	No. variable sites	No. informative sites	Percent informative sites	No. trees	Tree length	CI	RI	RC
*atp*A	11	1 193	30	11	0.92	1	30	1.000	1.000	1.000
ITS	11	802	306	145	18.08	1	438	0.826	0.723	0.597
*rbc*L	11	1 425	107	27	1.89	4	127	0.898	0.690	0.620
trn L-F	10	1 068	65	27	2.52	10	78	0.897	0.842	0.709
*trn*H-*psb*A	11	703	43	7	1.00	5	46	0.957	0.875	0.837
combined	11	5 191	334	217	4.18	2	734	0.834	0.678	0. 566

CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

with 306 of 802 variable sites, of which 145 (18.08%) were informative. To assess the congruence between the nuclear ITS region and cytoplasmic regions (*atp*A + *rbc*L + *trn*L-F + *trn*H-*psb*A), we performed both separate and combined reconstructions of these two genomic partitions in PAUP*4.0b10 (Swofford 2001). We observed no strong topological conflicts (see supplementary materials) and all subsequent analysis was based on the combined data.

Using these five loci, we obtained a single, well-supported phylogenetic tree for the 11 samples from three (maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI)) reconstruction methods (**Figure 2**). The 10 *Tacca* samples were monophyletic and the geographically widespread species, *T. leontopetaloides,* was sister to the rest of the species. Successively, *T. parkeri* from South America and *T. plantaginea* from Southeast Asia formed a monophyletic clade, and was sister to the other *Tacca* species. The two species completely lacking bracteoles, *T. palmata* and *T. palmatifida* also formed a small distinct clade. The remaining species, with large showy bracts and whisker-like filiforms bracteoles, all found in Southeast Asia, formed the most highly derived clade. The two different morphs of *T. integrifolia*, one from Malaysia and one from Tibet, were not monophyletic with the Malaysia sample sister to *T. integrifolia*. In this clade, two sister subclades, one containing *T. chantrieri* and *T. subflabellata*, and the Tibetan *T. integrifolia* and *T. ampliplacenta*, were also supported, although with substantially shorter branch lengths.

Ancestral reconstruction of inflorescence traits

Ancestral reconstruction of inflorescence characteristics on the phylogenetic reconstruction obtained from the DNA sequence data indicated that small and plain involucres were ancestral in the genus *Tacca*, while large, showy bracts and long well developed bracteoles were the most derived characters (**Figure 3**). Given three categories of bract size, no ambiguity existed for the reconstruction of ancestral traits at any node and evolution in bract size appeared to be strongly directional, from small (1.47–19.12 cm^2) to large (117–133.5 cm^2) (**Table 2**), and

evolution of bract color is from green (*T. leontopetaloides*, *T. plantaginea*) to other color, such as purple (*T. chantrieri*, *T. ampliplacenta*), white (*T. integrifolia*) or pink (*T. subflabellata*) etc.

For the filiform bracteoles, the reconstruction of ancestral traits in the basal clade (*T. leontopetaloides*) indicated that both the loss of these characters in non-bracteoles clade (*T. palmata*, *T. palmatifida*) and the elongation and proliferation of filiform bracteoles occurred in the most derived clade. The widespread and most basal species, *T. leontopetaloides,* appears to possess inflorescence traits most similar to the ancestral condition. The combined analysis shows that the evolution of *Tacca* was from less showy small bracts, short-filiform bracteoles types to showy large bracts and long-filiform types.

Discussion

Molecular phylogeny

Tacca has been treated as a member of Dioscoreaceae (Caddick et al. 2002), because it shares numerous characters with the family, including tuberous underground parts rich in steroidal saponins, petiolate, reticulate-veined leaves, and reflexed stamens, although it differs in its acaulescent habit and unilocular ovaries with parietal placentation. Here, the monophyly of *Tacca* was well supported in both the separate and combined analysis of five DNA sequence datasets (**Figure 2**). *Tacca leontopetaloides* is the most basal taxon while two species with smaller and less showy involucres and bracteoles, e.g. *T. parkeri* and *T. plantaginea*, are then sisters to the rest of the genus. *Tacca palmata* and *T. palmatifida*, both without filiform bracteoles, are next in the evolutionary grade leading to the species with the most conspicuous blossom structures, e.g. *T. integrifolia* and *T. chantrieri*, the most derived clade.

Based on fruit and leaf morphology, presence or absence of filiform bracteoles, and geographical distributions of each species, Drenth (1972) suggested *Tacca* may be divided
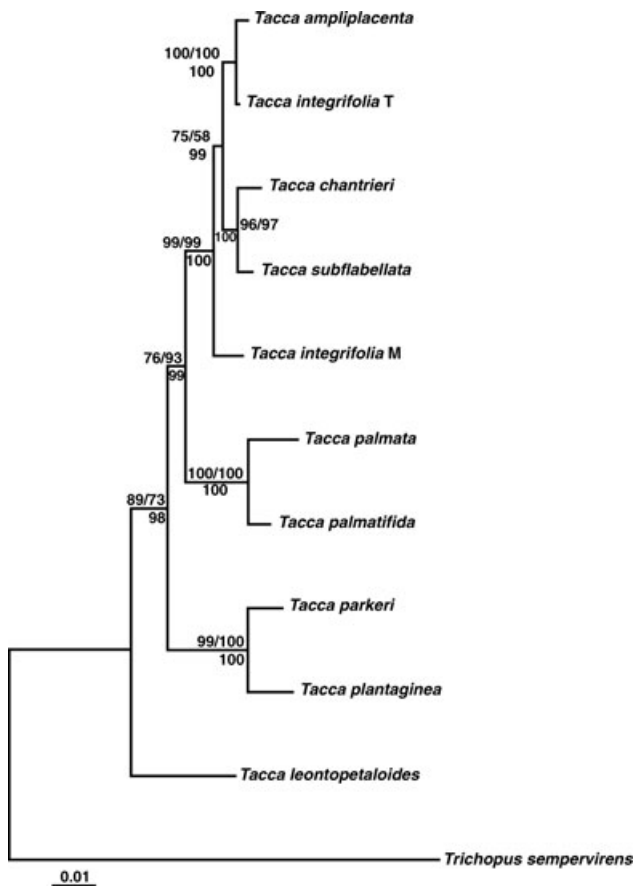
Figure 2. Phylogenetic relationships within *Tacca*.

Maximum likelihood (ML) tree for 11 taxa obtained from combined plastid, nuclear and mitochondrial sequence data. Numbers above the lines on the left indicate the ML bootstrap of each clade >50%, numbers above the lines on the right indicate the maximum parsimony (MP) bootstrap of each clade >50%, numbers below each branch are the Bayesian posterior probabilities.

into four sections. Our phylogenetic result, however, is not consistent with Drenth's system. The first section in Drenth's system includes exclusively entire-leaved Old World species with filiform bracteoles and a vertical elongate rhizome with apical growth. But in our study, the six species in this section: *T. integrifolia* (**Figure 1G,H**), *T. plantaginea* (**Figure 1B**), *T. chantrieri* (**Figure 1J**), *T. bibracteata*, *T. subflabellata* (**Figure 1F**) and *T. ampliplacenta* (**Figure 1I**) occur in different clades (**Figure 2**). All species in this section, except *T. plantaginea*, make up the most evolved clade. Within this clade, *T. integrifolia* from Malaysia is separated from those from Tibet with very high bootstrap support (**Figure 2**), and there are large morphological differences among them indicating they should be accepted as separate species. *T. subflabellata* (**Figure 1F**) from Hekou has a sister relationship with *T. chantrieri* with strong support. *T. subflabellata* is easy to differentiate from *T. chantrieri* due to its pinkish green, fan-like involucral bracts. *T. ampliplacenta*, a recent published species from west of Yunnan (Zhang and Li 2008) has a sister relationship with *T. integrifolia* from Tibet.

Species in Drenth's second section are palmate-leaved Old World species without filiform bracteoles, a distinct alliance with four species. This section was further divided into two smaller groups. The first group including *T. palmata* (**Figure 1E**) and *T. ebeltajae,* possesses a short tuberous rhizome from which leaves and inflorescences emerge from an apical cavity and in which the flowers are placed between the involucres. The second group consists of *T. palmatifida* (**Figure 1D**) and *T. celebica*, which have a horizontally growing rhizome with leaves and inflorescences that are spaced, no distinct apical growth, and flowers inserted at the base of the inner involucral bracts. Our molecular evidence indicates that this section is a well supported clade.

The third section of Drenth contains a single species, *T. leontopetaloides* (**Figure 1A**) that is widely distributed in the
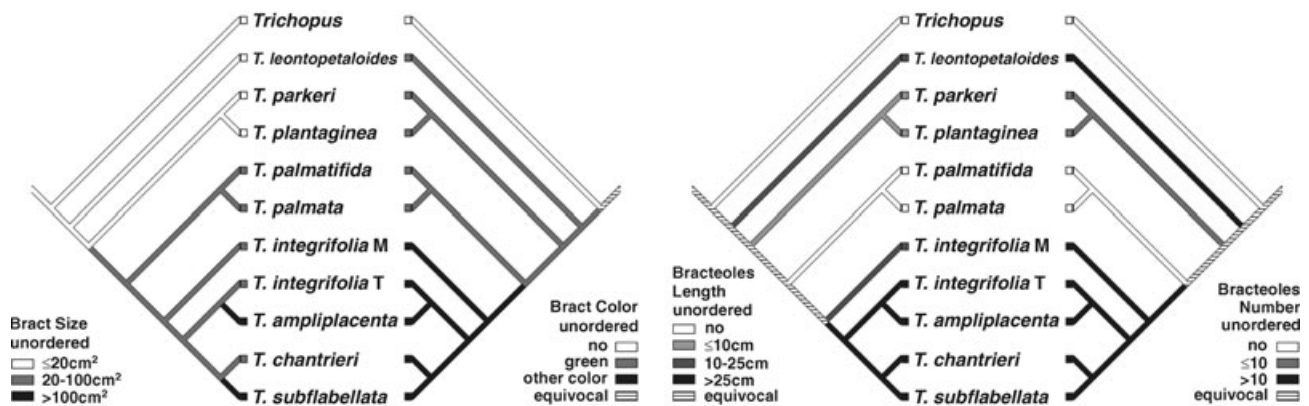

Figure 3. The evolution of bract size, color and bracteole length and number in *Tacca*.

The maximum likelihood tree was inferred and the results indicated that ancestral inflorescence traits in the genus were small, inconspicuous involucres with short filiform bracteoles.

Table 2. Inflorescence traits of *Tacca* species

Species names	Involucral bract length			Involucral bract width			Bract size (length × width)	Bract color	Bracteoles number	Bracteoles length
	Min.	Max.	Average	Min.	Max.	Average				
Trichopus	0	0	0	0	0	0	0	0	0	0
T. leontopetaloides	2.5	10	6.25	0.7	5	2.35	14.69	Green	30	25
T. parkeri	4	13	8.5	1	3.5	2.25	19.13	Green	4	5.5
T. plantaginea	1.2	1.6	1.4	0.7	1.4	1.05	1.47	Green	8	8
T. palmata	4.5	10	6.25	2.5	6	4.25	26.56	Green	0	0
T. palmatifida	6.5	12.5	9.25	4.5	7	5.75	53.19	Green	0	0
T. integrifolia M	2.5	22	12.25	1	11	6	73.5	Purple	16	25
T. ampliplacenta	10	16	13	8	10	9	117	Purple	18	40
T. integrifolia T	10	12	11	6.5	6.8	6.75	74.25	White	22	37
T. chantrieri	2.5	10	6.25	1.5	9	5.25	32.81	Green/purple	21	30
T. subflabellata	10	15	12.5	18	20	19	237.5	Pink/white	20	55

Relative bract size was measured by the average value of bract length times the average value of the bract width, data were obtained from the descriptions of published reports and the flora of China (Drenth 1972; Ding and Larsen 2000).

Note: Average length and width of involucral bract are accounted by (minimum + maximum value)/2. For bract size, we use average length times average width. Morphological characters were categorized when matching onto the phylogenetic tree. Bract size: $1 \leq 20$ cm^2, $2 = 20$–100 cm^2, $3 > 100$ cm^2; Bract color: $0 =$ no, $1 =$ green, $2 =$ other color; Bracteoles length: $0 =$ no, $1 \leq 10$ cm, $2 = 10$–25 cm, $3 > 25$ cm; Bracteoles number: $0 =$ no, $1 \leq 10$, $2 > 10$.

Old World. Drenth suggested that this section occupies an intermediate position between the first two sections, but our molecular information indicates that it is basal in the genus.

A single species, *T. parkeri* (**Figure 1C**), from the New World, has a rather isolated position in Drenth's system. This species shares generally entire leaves with the first section, possesses no or a few filiform bracteoles, the bracts are erect but small, and it shares the palmate-leaved and horizontal rhizome. Our molecular evidence shows that *T. parkeri* has a sister relationship with *T. plantaginea* in the first section.

Elaborate floral displays are derived in *Tacca*

Based on the evolutionary trends inferred from the molecular phylogenetic results, bracts and bracteoles in *Tacca* have become larger and more conspicuous, plus a proliferation of bracteole number, from an ancestor with small and inconspicuous features. Our previous investigations into the pollination and mating biology of *Tacca* species show that these plants might be largely self-fertilized. For instance, *T. chantrieri* populations from Yunnan Province in southwestern China exhibited high levels of self-fertilization in different years as well as in multiple populations (Zhang et al. 2005, 2006a). Research on the population genetic structure of *T. integrifolia* in Tibet and Malaysia also implied individuals were primarily self-fertilized (Zhang et al. 2006b). Several species, including *T. palmata*, *T. leontopetaloides*, *T. plantaginea*, and *T. subflabellata* set fruit either when isolated in the greenhouse or when flowers were bagged to exclude pollinators in natural populations (Zhang

L. pers. obs., 2005–2008), indicating clear self-compatibility. Although we don't have strong evidence of self mating systems in all *Tacca* species, self-pollination appears to be a constant trait in some species of the genus; with no indication of a correlated evolutionary shift towards outcrossing from selfing along with the development of showy inflorescences. These findings contradict the resource allocation model, which states that species with extravagant floral displays should be primarily out-crossed (Charlesworth and Charlesworth 1987).

The evolution of self-fertilization from primarily outcrossing ancestors is one of the most common evolutionary transitions in plants (e.g., Stebbins 1974; Grant 1981; Barrett et al. 1989, 1996; Shimizu et al. 2004), but in those cases, reduction of floral attractants is usually observed (Charlesworth and Charlesworth 1981, 1987; Charnov 1982; Campbell 2000; Tang and Huang 2007). Paradoxically, the evolution of inflorescences in *Tacca* has proceeded from simple and reduced to conspicuous and elaborate floral displays, without a corresponding transition to increased out-crossing. Previously, we proposed that showy bracts and bracteoles in *Tacca* are "relictual" ancestral traits (Zhang et al. 2005) and have no current utility. However, results here disprove this hypothesis, suggesting instead that these displays do perform some unknown function. So, the question remains, why do self-pollinated plants invest a significant amount of resources in these structures?

In order to explore possible functions, a detailed review of the reproductive and pollination biology of these plants should provide insights. Despite the conspicuous involucres

and whisker-like filiform bracteoles, the single flower structure of *Tacca* species is appropriate for automatic self pollination. First, the anthers dehisce several hours before flowers have opened while the anthers and stigmas are in close proximity. Self pollen grains are easily deposited on the stigmatic surface. No *Tacca* flowers secrete nectar and very few pollen grains are produced, and thus almost no nutritional rewards are available for pollinators. As anthesis proceeds and flowers become pendent, additional pollen grains accumulate on the stigma through autonomous intrafloral self-pollination (Zhang et al. 2005, 2006a; Zhang and Li 2008). As such, the enlarged bracts and motile filiform appendages could increase the movement of their elaborate floral display in the moist and still understory habitats in tropical forests, providing an increased chance for self pollen grain deposit on the stigma under the lack of pollinators. Though the previous manipulation experiments didn't support this selfing-promotion hypothesis on maternal fitness (fruit and seed set) (Zhang et al. 2005), we still need to test for effects on paternal fitness (pollen deposition and competition).

Flower or inflorescence structures with multiple functions may reflect the net effect of conflicting or additive selective pressures (Anderson 1976; Fenster et al. 2004; Armbruster et al. 2005; Waser and Ollerton 2006). Bracts can serve many functions and can perform more than one function at a time, resulting in a morphology that is a compromise among these functions. For example, in the dove tree (*Davidia involucrata*), the bracts are white to attract pollinators, while one bract is substantially larger than the other to protect pollen from rain. The bracts of the dove tree dramatically illustrate the many important reproductive roles that vegetative organs, like bracts, can play (Sun et al. 2008). In *Dalechampia* vines, bracts indicate to the pollinator whether floral reward is available (Armbruster et al. 2005) and protect flowers from florivores and pollen thieves (Armbruster 1997). Bracts can function both before and after pollination, as in *Talia*, where they not only attract nocturnal pollinators but persist in the fruit, promoting wind dispersal of the seed (Anderson 1976). Moreover, plant coloration of bracts has been hypothesized to be a means of defense since in the mycoheterotrophic plant *Monotropsis odorata*, its dried vegetative bracts facilitate herbivore avoidance, possibly promoting plant fitness (Klooster et al. 2009). Consequently, we hypothesized that the large bracts in some *Tacca* species may perform several functions, including protection against herbivores or photosynthetic surfaces. Plants may invest resources in the large involucres to attract potential herbivores away from their flowers. Support for this idea comes from observations of heavily grazed involucres on some individuals of *T. chantrieri* in natural habitats (Zhang L. pers. obs., 2005). Finally, most species of *Tacca* inhabit moist and shaded understory habitats in Southeast Asian tropical forests. Given their leaflike structure and size, the large involucral bracts in *Tacca* could also be a

significant source of photosynthetic carbon for developing fruits after the ovule has been fertilized.

Based on the results of our molecular phylogenetic analyses and the reconstruction of the evolution of inflorescence traits, *Tacca* is a monophyletic group, and ancestral *Tacca* species probably had short or less showy involucres and shorter (or no) bracteoles, whereas derived species have elaborate floral displays. Because these derived species remain self-compatible without any obvious transition to increased out-crossing or pollinator service, the showy involucres and bracteoles may possibly have several other functions rather than pollinator attraction, to facilitate self-fertilization via the motile and dangling filiform bracteoles, to perform photosynthesis in the dim understory, to protect flowers from herbivory. If so, these showy inflorescence structures may be under strong selection from their biotic and abiotic environment. In this case, further detailed studies on the inflorescence structure and their functions in *Tacca* species are needed.

Materials and Methods

Taxon sampling and DNA extraction, amplification and sequencing

Taxonomically, we followed Caddick's system for the family level and Drenth's treatment for species level in this study. We examined 9 out of 15 species of *Tacca* recognized by Drenth (1972) and our previous study (Zhang 2006; also see The World Checklist of Monocotyledons (http://apps.kew.org/wcsp/home.do)). In this study, we selected 10 samples as the ingroup, including two forms of *T. integrifolia* from separate geographic regions that differ substantially in their floral morphology. These species represent almost all morphological types, although there are six species missing according to the World Checklist of Monocotyledons of Kew: *T. bibracteata* (Sarawak), *T. borneensis* (Malaysian Borneo), *T. celebica* (Sulawesi), *T. ebeltajae* (New Guinea/Solomons), *T. maculata* (Australia) and *T. ankaranensis* (Madagascar). However, from Drenth (1972) results, *T. borneensis* (Malaysian Borneo) was a synonym of *T. integrifolia* and *T. maculata* (Australia) was a synonym of *T. leontopetaloides*. We could not find *T. maculata* when we did field surveys in the wet tropics of north-east Australia, the only species of *Tacca* we could find from the published reports in Australia is *T. leontopetaloides*. The rest of the other four species, *T. bibracteata* (Sarawak), *T. celebica* (Sulawesi), *T. ebeltajae* (New Guinea/Solomons) and *T. ankaranensis* (Madagascar) are very narrow-ranged species and they are very difficult to obtain materials. We used the taxonomic system defined by Drenth (1972), and voucher specimens are preserved in the herbarium of Xishuangbanna Tropical Botanical Garden (HITBC) and living collections were planted in the nursery at XTBG (**Table 3**). We used *Trichopus*

Table 3. List of sampled species, their locations and voucher specimens used in molecular phylogenetic study

Taxon	Geographic origin	Voucher or accession number	GenBank accession #				
			atpA	rbcL	trnL-F	trnH-psbA	ITS
Tacca leontopetaloides	Singapore BG	ZL-001 (HITBC), 38 2002 1061 (XTBG)	JN850559	AJ286561	JN850599	JN850588	JN850570
T. palmata	Bogor BG	ZL-004 (HITBC), 11 2004 006 (XTBG)	JN850560	AF307479	JN850600	JN850589	JN850571
T. palmatifida	Bogor BG	ZL-003 (HITBC), 11 2004 008 (XTBG)	JN850561	JN850581	JN850601	JN850590	JN850572
T. plantaginea	Jiangcheng, Yunnan	ZL-002 (HITBC), 00 2003 2121 (XTBG)	JN850563	AF307483	JN850603	JN850592	JN850574
T. subflabellata	Hekou, Yunnan	ZL-005 (HITBC), 00 2004 0978 (XTBG)	JN850564	JN850583	JN850604	JN850593	JN850575
T. integrifolia M	Malaysia	ZL-008 (HITBC)	JN850557	JN850579	JN850597	JN850586	JN850568
T. integrifolia T	Medog, Tibet	ZL-006 (HITBC), 00 2003 2122 (XTBG)	JN850558	JN850580	JN850598	JN850587	JN850569
T. parkeri	NMNH, USA†	Wilmer diaz 7505 (NMNH)	JN850562	JN850582	JN850602	JN850591	JN850573
T. chantrieri	Xishuangbanna, Yunnan	ZL-007 (HITBC)	JN850556	JN850578	JN850596	JN850585	JN850567
T. ampliplacenta	Cangyuan, Yunnan	ZL-009 (HITBC, KUN), 00 2005 0047 (XTBG)	JN850555	JN850577	JN850595	JN850584	JN850566
Trichopus sempervirens	NMNH, USA†	Bogner 208 (NMNH)	JN850565	AY298818	–	JN850594	JN850576

†Specimen; – sequences are obtained from this study.

sempervirens as the outgroup because of the close relationship between *Tacca* and *Trichopus* (Caddick et al. 2002) (see Appendix in Supplementary Data accompanying the online version of this article).

Total DNA was extracted from leaves using a modified cetyl trimethyl ammoniumbromide (CTAB) procedure (Doyle and Doyle 1987). We used silica gel-dried or fresh leaves for all accessions, sterilizing the surface of the leaves prior to DNA isolation. Double-stranded DNA was directly amplified by symmetric polymerase chain reaction (PCR) amplification using the ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers of White et al. (1990). For *atp*A, we use forward primer 5′-AAG TGG GAT GAG ATC GGT CGA G-3′ and reverse primer 5′-GGC ATT CGA TCA CAG A-3′ (Davis et al. 1998). For *trn*L-F, including *trn*L intron, *trn*L 3′exon and *trn*L-F spacer region, PCR amplification we used c (5′-CGA AAT CGG TAG ACG CTA CG-3′) and f (5′-ATT TTC AGT CCT CTG CTC TAC C-3′) primers of Taberlet et al. (1991). For *rbc*L, we used reverse primer 5′-TCC TTT TAG TAA AAG ATT GGG CCG AG-3′ and forward primer 5′-ATG TCA CCA CAA ACA GAA AC-3′ (Lledo et al. 1998). For *trn*H-*psb*A, we used *psb*AR primers 5′-GTTATGCATGAACGTAATGCTC (Sang et al. 1997), *trn*HF CGCGCATGGTGGATTCACAATCC (Tate and Simpson 2003).

Reaction volumes were 20 μL and each reaction contained 2.0 μL 10×buffer, 20–60 ng DNA, 0.5–0.75 μM primer, 0.4 mM dNTP, 1.5 mM MgCl$_2$, 50 mM Tris-HCl (pH = 8.3), 0.75U *Taq* polymerase, 5% dimethylsulfoxide (DMSO), and double-distilled water was added to 20 μL. PCR was performed in a T3-Thermocycler (Biometra) and 3 min at 94 °C at first, followed by 30 cycles of 1.5 min at 94 °C for template denaturation, 1 min at 50 °C for primer annealing, 1.5 min at 72 °C for primer extension, followed by a final extension of 10 min at 72 °C. PCR products were purified using Watson's purification kit prior to sequencing. Molecular datasets were produced for *atp*A, *trn*H-*psb*A, *trn*L-F, *rbc*L and ITS DNA sequences, although not all of these sequences were available for all species examined due to failure of amplification in certain species. Accession numbers for new sequences are included in **Table 3**.

Sequence alignment and phylogenetic analysis

DNA sequences were assembled using Lasergene analysis software (DNASTAR Inc., Madison, WI, USA; Burland 2000) and Muscle 3.6 (Edgar 2004) was used to generate the sequence alignment.

Phylogenetic reconstructions were generated using both MP and ML optimality criteria as implemented in PAUP*4.0b10 (Swofford 2001). Additionally, a BI was conducted using Mr-Bayes 3.12 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). All analyses presented are based on the combined datasets (ITS, *atp*A, *rbc*L, *trn*H-*psb*A, and *trn*L-F region) because no significant incongruence was detected

among data partitions. Characters were treated as unordered and unweighted. The GTR+I+G model and parameter settings were chosen by using the Akaike information criterion (AIC) as suggested by Modeltest V3.7 (Posada and Crandall 1998) for the ML and BI analyses.

For the ML analyses, optimal gene trees were found via heuristic searches of 100 replicates of random sequence addition with TBR branch-swapping, MULTREES ON. The relative clade support for the ML analyses was estimated by bootstrap support (BS) analysis of 1 000 replicates of heuristic searches using the same model and parameters.

Heuristic searches were conducted in MP analyses with Tree-Bisection Reconnection (TBR) branch swapping, MulTrees ON, and 10 000 random taxon addition replicates holding 20 trees at each step. Bootstrap support (BS) values for individual clades were calculated by running 1 000 bootstrap replicates of the data, with starting trees acquired by a single replicate of random stepwise addition of taxa, under TBR branch swapping, and MulTrees ON. The consistency index (CI), retention index (RI), and rescaled consistency index (RC) were obtained through PAUP 4.0b10.

Bayesian inference was conducted according to GTR+I+G model and parameter settings using MrBayes 3.12. Four independent Markov Chain Monte Carlo chains were run simultaneously and sampled every 100 generations for a total of 1 000 000 generations. To establish the "burn-in" phase, i.e. log probability values stationarity, a plot of generations against log likelihood scores was performed using Excel 2003 (Redmond, WA, USA); these burn-in trees were discarded from the analysis.

Phylogenetic inferences were based on comparisons of the ML tree, MP strict consensus tree and Bayesian tree.

Inflorescence characters mapping

To infer ancestral states and analyze inflorescence traits evolution, character evolution was studied with MacClade 4.08 (Maddison and Maddison 2005), which optimizes character changes on a tree based on the principle of parsimony. These inflorescence characters included bract size, bract color, bracteoles number and their length, etc. (Table 2). For bract sizes, we measured by length and width of the bract. Among these characters, the bract sizes as the key trait were used in the ancestral reconstruction of inflorescence traits of *Tacca*. Information on inflorescence characters came from the descriptions of published reports and the flora of China (Drenth 1972; Ding and Larsen 2000).

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The maximum parsimony trees from the nuclear ITS and cytoplasmic regions (*atp*A + *rbc*L + *trn*L-F + *trn*H-*psb*A).

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