Did Pollination Shifts Drive Diversification in Southern African *Gladiolus*? Evaluating the Model of Pollinator-Driven Speciation

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ABSTRACT: The pollinator-driven ecological speciation model has frequently been invoked to explain plant richness in biodiversity hotspots. Here, by focusing on *Gladiolus* (260 species), a flagship example of a clade with diverse pollination biology, we test the hypothesis that high species diversity in southern Africa, one of the world's most floristically rich regions, has primarily been driven by ecological shifts in pollination systems. We use phylogenetic methods to estimate rates of transition between the seven highly specialized pollination strategies in *Gladiolus*. We find that pollination systems have evolved multiple times and that some pollination strategies arose by a variety of evolutionary pathways. Pollination shifts account for up to one-third of all lineage splitting events in the genus, providing partial support for the pollinator-driven speciation model. Transitions from the ancestral pollination mode to derived systems have also resulted in increased rates of diversification, suggesting that certain pollination systems may speed up speciation processes, independently of pollination shifts per se. This study suggests that frequent pollination shifts have played a role in driving high phenotypic and species diversity but indicates that additional factors need to be invoked to account for the spectacular diversification in southern African *Gladiolus*.

Keywords: pollination, *Gladiolus*, Iridaceae, phylogeny, radiation, speciation.

Introduction

The interaction between plants and their animal pollinators has been hypothesized to play a significant role in angiosperm diversification (Stebbins 1970; Kay et al. 2006). The study of the evolution of pollination systems can thus be expected to provide valuable insights into the

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causes of extant patterns of species richness in biodiversity hotspots, particularly in clades characterized by specialized pollination systems (Johnson 2010). The existence of a causal relationship between diversity of pollination systems and species richness, however, still remains controversial, even for regions and clades characterized by an unusual variety of floral types (Kay and Sargent 2009; Smith 2010).

One of the evolutionary settings where the role of pollination systems as drivers of angiosperm diversification has been most often invoked is the southern African subcontinent (Johnson 2010). This is a region of exceptional botanical diversity, harboring more than 20,000 plant species comprising 5%–8% of the world's total number of plant species (Goldblatt and Manning 2002). This diversity is mostly concentrated in the Cape Floristic Region, Succulent Karoo, and the Maputaland-Pondoland-Albany biodiversity hotspots (Linder 2003; Mittermeier et al. 2005). Several large plant clades in southern Africa exhibit a high diversity and specialization of pollination systems (e.g., clades in Amaryllidaceae, Geraniaceae, Iridaceae, Orchidaceae, Scrophulariaceae), leading to the hypothesis that the radiation of these florally diverse lineages has been driven by plant-pollinator interactions (Johnson 1996; Van Der Niet and Johnson 2009). This model has received much attention in the literature (Johnson 2010) but evolutionary evidence to support it remains sparse. Previous empirical studies of genera with high species richness in the Cape region of southern Africa, namely in Orchidaceae (Johnson et al. 1998; Johnson and Kurzweil 1998; Waterman et al. 2011) and Iridaceae (Goldblatt and Manning 1998), have found a link between plant diversity in the region and adaptive radiation of pollination systems. The diverse, albeit scarce, pollinator fauna and a complex geographic mosaic of pollinators in southern Africa have been

proposed as potential reasons for strong selection for floral traits that attract pollinators in this region (Johnson 1996; Barraclough 2006; Linder et al. 2010). However, a recent study of southern African clades by Schnitzler and colleagues (2011) found that other ecological factors, such as edaphic preferences, were better predictors of speciation rates than were pollination systems. More generally, just five (*Disa*, *Erica*, *Gladiolus*, *Pelargonium*, and *Moraea*) of the 20 largest genera (>80 species) in the Cape Floral Region (Goldblatt and Manning 2002) have diversified florally to any extent that is relevant to their pollination biology, with the great majority of the genera highly conserved in functional floral morphology and thus pollination system. Pollinator-related radiation is only one of several characteristics of the southern African flora, and in only a minority of plant groups can it be anticipated to be the primary driver of speciation.

An important prerequisite for understanding the role of pollinator shifts in southern Africa is the quantification of the degree of evolutionary flexibility of pollination systems within the major plant clades in the flora. Evidence from empirical studies on a variety of southern African lineages dominated by specialized pollination systems has revealed that some floral traits associated with different classes of pollinator can indeed be highly labile (e.g., Johnson et al. 1998; van der Niet and Johnson 2009; Schnitzler et al. 2011). However, little is known about the frequencies, directionality, and rates of evolutionary shifts between different pollination systems, not only in southern African clades but also in angiosperms in general (Perret et al. 2003; Pérez et al. 2006; Bastida et al. 2010; Smith 2010). If pollinator-mediated divergence is the predominant model of speciation in southern Africa, then the assumption of high evolutionary flexibility of pollination systems must be fulfilled.

Here, we test the hypothesis that high species diversity in southern Africa has been caused by frequent ecological shifts in pollination system, as proposed by Johnson (1996), by focusing on *Gladiolus*, one of the largest genera in southern Africa and the most striking example of a clade with diverse pollination biology in the region. *Gladiolus* (Iridaceae) comprises more than 260 species and presents one of the widest spectra of pollination systems known in angiosperms, with at least seven specialized systems described among the southern African species alone (fig. 1; Goldblatt and Manning 1998). The genus extends through temperate and tropical Africa, Madagascar, the Mediterranean basin, and the Middle East, but its main center of diversification is in southern Africa, where more than 65% of the species occur.

Gladiolus is an ideal system to evaluate the pollinator-driven ecological speciation model because it forms a monophyletic assemblage (Valente et al. 2011), and its

species are typically highly specialized for pollination by a single animal group (Goldblatt et al. 2001), allowing the frequency and directionality of pollinator shifts to be quantified in a rigorous fashion. In addition, in contrast to other large genera, the pollination biology of *Gladiolus* is well understood, particularly in southern Africa, where more than half of the species have been subject to intense field observations of pollination ecology (for a review, see Goldblatt et al. 2001). Detailed information on types of animal visitors and pollination mechanisms is available for more than 80 species. Analysis of the main flower types in *Gladiolus* has shown that flower type correlates closely with pollination system (Goldblatt et al. 2001). As a result, Goldblatt and Manning (2006) were able to infer, with a high degree of confidence, the pollination strategies of a further 133 species based on floral syndromes. Of the 213 species of *Gladiolus* whose pollination systems are now known or inferred, more than 50% are pollinated by long-tongued bees in the family Apidae that forage primarily for nectar. This system is the predominant mode of pollination in several species-rich southern African genera of Iridaceae and is thought to be ancestral in many (Goldblatt and Manning 2006), raising the question as to whether it may have led to elevated rates of diversification in these clades. Other specialized pollination systems recorded in the genus are, in order of importance, long-proboscid flies (Nemestrinidiae and Tabanidae), sunbirds (*Nectarinia* spp.), night-flying moths (Noctuidae and Sphingidae), butterflies (*Aeropetes tulbaghia*), and short-tongued female pollen-collecting bees (Halictidae and Andrenidae). The rarest pollination strategy in the genus is by hopliine beetles (Scarabaeidae: Hopliini), which has been confirmed in only one species.

In this study, we reconstruct the evolutionary history of pollination biology in *Gladiolus*, taking advantage of a recent species-level phylogenetic study of the genus by Valente et al. (2011), who sampled more than 80% of southern African species (132 spp.) and all seven Mediterranean basin species. We (i) estimate the degree of lability and also the directionality of pollination system transitions in *Gladiolus* in order to quantify the possible role of these ecological shifts in speciation; (ii) test whether transitions between the predominant pollination system in the genus—long-tongued bee pollination—and the rarer pollination systems might have influenced rates of diversification; and (iii) examine the evolution of pollination strategies in a rapidly diversifying subclade of the genus endemic to southern Africa in order to gain insights into the way that ecological shifts operate at lower phylogenetic levels.

Methods

Phylogenetic Framework

Phylogenetic analyses were conducted based on the *Gladiolus* species-level molecular data set of Valente et al. (2011). This matrix consists of five plastid regions (*matK, psbA-trnH, trnS-trnG, rpl32-trnL*, and *trnQ-rps16*) and includes 148 species of *Gladiolus*. The five-marker plastid data set was reanalyzed using both maximum likelihood and Bayesian phylogenetic methods, following the approach of Valente et al. (2011). However, unlike as in this previous study, we also added information from indels. A matrix of indel characters was produced using the "simple indel coding" method (Simmons and Ochoterena 2000) as implemented in Seqstate (Müller 2005). In the Bayesian analyses, the indel character matrix was analyzed under a binary model implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). For the maximum likelihood analyses, we used a BINCAT approximation to the indel data, as implemented in RaxML 7.2.1 (Stamatakis 2006).

Scoring Pollination Systems

Data on *Gladiolus* pollination systems was extracted from the following published accounts: Goldblatt 1996; Goldblatt and Manning 1998; and Goldblatt et al. 2001. In addition, new field observations were conducted for the Mediterranean basin species *Gladiolus communis* and *Gladiolus illyricus*. Detailed direct field observations are thus available for 87 species of *Gladiolus* (available in table A1 in Dryad: http://dx.doi.org/doi:10.5061/dryad .7pr554s8).

Pollination system was assigned based on the primary floral visitor type observed, which we consider to be the pollinator group following Goldblatt et al. (2001). For all species of *Gladiolus* (except for *Gladiolus meliusculus*; see below), flowers were visited either by a single animal species or by only one of seven ecologically homologous groups of species (functional groups) as defined by Goldblatt and Manning (2006). Given that each species of *Gladiolus* was pollinated only by one animal group, all species of *Gladiolus* can be considered to have a specialized pollination system (Fenster et al. 2004). The only exception was *G. meliusculus*, which is unusual in the genus in demonstrating a bimodal pollination system by both hopliine beetles and long-tongued bees (Goldblatt et al. 2001). For simplicity we refer to this system as "beetle pollination" from here onward.

Pollination system was inferred for 58 species for which no field data was available, as it has been shown that flower type correlates strongly with pollination system in *Gladiolus* (Goldblatt et al. 2001), and there is a strong correspondence between pollination system assigned and actual observations (Goldblatt et al. 2001). For three species (*G. decoratus* and *G. erectiflorus* from tropical Africa; and *G. horombensis* from Madagascar) assignation of pollination system based on floral syndrome was not possible, and the character was coded as unknown.

The pollination systems of each of the 148 species sampled and the method of pollination system scoring are shown in table A1 (available in Dryad: http://dx.doi.org/doi:10.5061/dryad.7pr554s8). Each species was assigned one of the following character states, based on the classification of specialized pollination systems proposed by Goldblatt et al. (2001): pollination by long-tongued apid and anthophorine bees that forage for nectar (LB); pollination by short-tongued halictid and andrenid bees that collect pollen (SB); passerine bird pollination (BI); night-flying moth pollination (MO); satyriine butterfly pollination (BU); hopliine beetle pollination (BE); long-proboscid fly pollination (LF); unknown (?).

Phylogenetic Signal of Pollination System

We evaluated the phylogenetic signal of pollination system using a parsimony approach in Mesquite, version 2.72 (Maddison and Maddison 2009). Taxa were randomly reshuffled among tips of each of 1,000 trees from the MrBayes output and the number of parsimony steps required to explain the evolution of the trait in each of the new trees was counted, thus generating a null distribution of character steps. We then calculated the average number of parsimony steps required to explain evolution of the character in the original 1,000 Bayesian trees without reshuffled terminal taxa. If this value was lower than the lower 5% percentile of the null distribution, this was considered as evidence for significant phylogenetic signal in pollination syndrome.

Ancestral Pollination Systems

We reconstructed ancestral character states for pollination systems using a maximum likelihood method implemented in Mesquite, version 2.72. To account for uncertainty in tree topology and branch lengths, character optimizations were repeated for each of 1,000 highly probable

Figure 1: Diversity of pollination systems in *Gladiolus*; species shown and their pollination system: *A, G. saundersii*, butterfly; *B, G. meliusculus*, beetle; *C, G. reginae*, long-proboscid fly; *D, G. carinatus*, long-tongued bee; *E, G. dalenii*, sunbird; *F, G. atroviolaceus*, long-tongued bee; *G, G. quadrangulus*, short-tongued bee; *H, G. tristis*, moth. All species shown are from southern Africa, except *G. atroviolaceus* from Eurasia.

trees from the MrBayes output, excluding the outgroup. We selected the single-parameter Markov *k*-state model, thus assigning equal probability to any type of pollination system shift. The best estimate of character state for each node in each of the trees was determined using the likelihood ratio test with a decision threshold of two, which was chosen in order to make a conservative assignment of character states at nodes (Maddison and Maddison 2009). Reconstructions were considered equivocal if the difference in log likelihood between alternative reconstructions was below the threshold.

Pollination System Shifts: Frequency and Rates

To estimate the frequency of shifts between each of the seven pollination systems we used the "summarize state changes over trees" application in Mesquite. The number of transitions between different character states was counted in each of the 1,000 resampled Bayesian trees using both maximum likelihood and unordered parsimony reconstruction methods.

The relative rates of transition between pollination systems were estimated using SIMMAP, version 15, build 26022010.1 (Bollback 2006). This software implements a Bayesian stochastic character mapping method (Huelsenbeck et al. 2003) that reconstructs character states and transitions based on a user-specified prior probability distribution of the rate of change. We performed two sets of SIMMAP analyses. First, in order to model the evolution of the seven pollination systems, we coded each system as a unique character state. Second, in order to explicitly investigate the rates of change from the most common pollination system—long-tongued bee—to all six remaining systems (as well as in the opposite direction), we coded pollination system as a binary character. In order to choose suitable prior parameters we followed a two-step statistical approach, as recommended for SIMMAP, version 1.5. First, we performed a Markov chain Monte Carlo analysis (1×10^5 cycles, sampling every 200 cycles) on the best tree from MrBayes to sample overall rate values (for both seven-state and binary character analyses) and bias values (binary character analysis only). We then used the posterior distribution of these parameters as the input for an R script that finds the parameter values offering the best fitting gamma and beta distributions (Bollback 2006; available from www.simmap.com). We repeated this procedure using 10 burn-in optimal trees randomly chosen from the MrBayes output and found that although optimal parameter values varied slightly, this did not strongly affect the results on relative rates of state change. We therefore report the results obtained using the parameter values estimated in the analysis of the MrBayes consensus tree. These were the following: seven-state analysis $\alpha = 6.43$, $\beta = 0.013$;

two-state analysis $\alpha = 7.46$, $\beta = 0.024$, α (beta distribution) $= 48.18$. In addition, for the seven-state analysis we assigned an empirical prior on state frequencies. In order to incorporate phylogenetic uncertainty in our data set, analyses were performed on 1,000 Bayesian trees (after pruning the outgroup). Ten realizations were sampled from the prior distributions and 10 realizations were sampled from each tree, totalling 100,000 mappings.

The analyses yielded posterior distributions for the expected number of transitions between any given state and for the expected frequency of time spent in a particular state. We calculated relative rates of state transition for each realization using the following equation: $E[i \rightarrow j]/\{E[\text{time}(i)] \times E[\text{rate}]\}$, where $E(i \rightarrow j)$ is the expected total number of transitions from state i to state j, $E[\text{time}(i)]$ is the expected dwell time in each state and $E[\text{rate}]$ is the mean gamma rate (J. Bollback, personal communication). This allowed us to obtain a posterior distribution of relative expected rates of transition for all combinations of i and j.

Character-Dependent Speciation and Extinction Rates

In order to investigate whether given types of pollination system have promoted diversification in *Gladiolus*, we used the Binary State Speciation and Extinction (BiSSE) model (Maddison et al. 2007; FitzJohn et al. 2009) implemented in the R package diversitree (FitzJohn et al. 2009). This method does not test the pollination shift speciation hypothesis, given that it does not assess whether higher numbers of shifts lead to higher rates of speciation. It estimates binary trait-dependent speciation and extinction rates in a Bayesian framework. We use BiSSE here solely to evaluate whether there is a character-dependent effect on speciation or extinction rates. A multistate extension of BiSSE (MuSSE) is also available, but we did not use it as the character we are interested in has seven states, which would require the model to estimate 56 parameters and would limit the interpretation of results (R. FitzJohn, personal communication). We therefore restrict our analyses to the comparison of the effect on diversification rate of the predominant pollination system (long-tongued bee; see table A1, available in Dryad: http://dx.doi.org/doi:10.5061/dryad.7pr554s8) versus all other systems (rare systems). We did not run similar models for other types of pollination systems (e.g., moth pollination versus all other systems) because the number of species with each of the derived systems is small, and this would reduce the accuracy of parameter estimation (Maddison et al. 2007). Given that our species sample is random with respect to pollination system, we assume that the number of species with each system included in this study is proportional to the total number of species that exist with that given char-

Cape radiation
clade

Continued in Fig. 2B
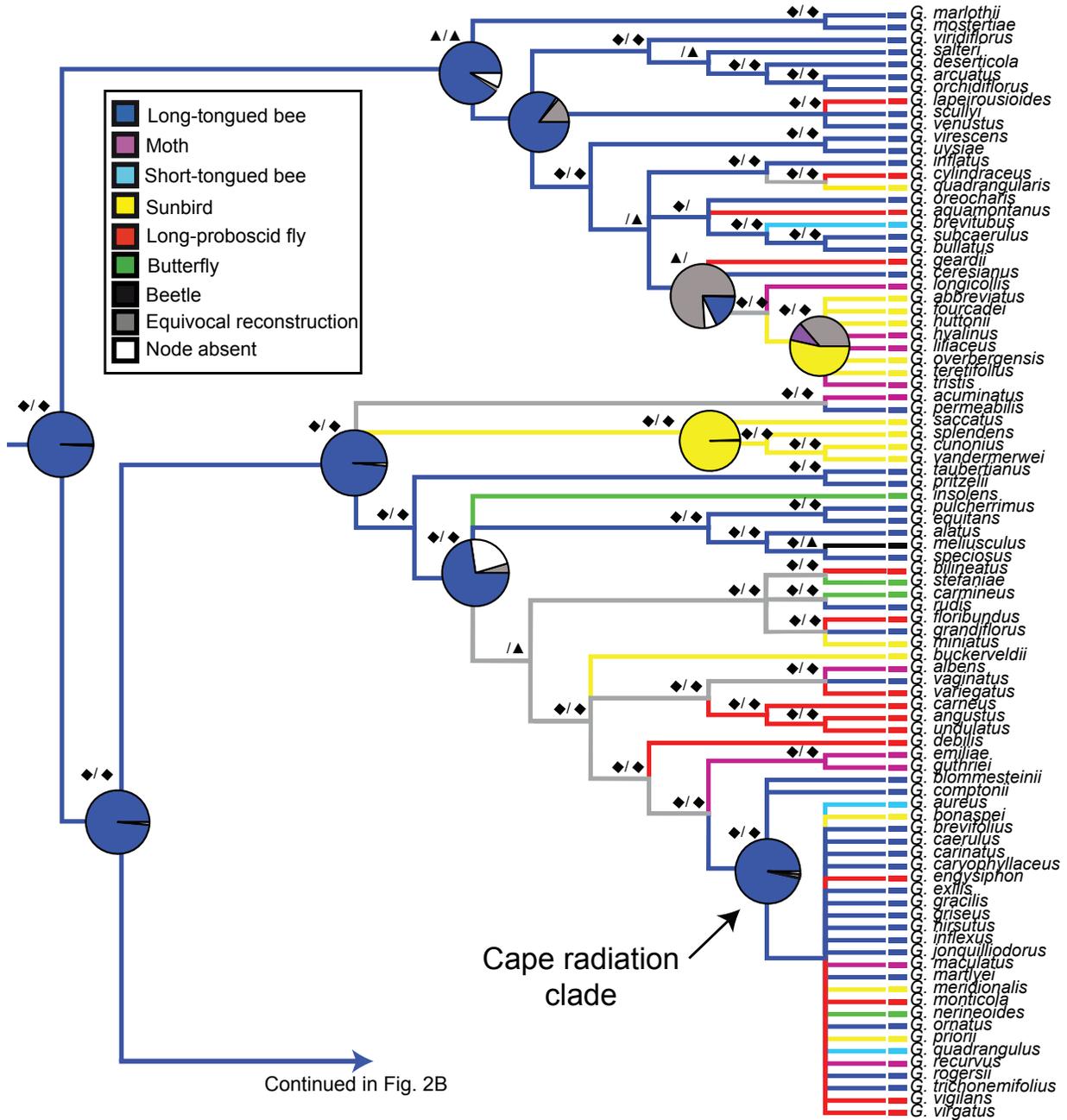
acter state. In order to obtain phylogenetic trees that are proportional to time, we used the uncorrelated lognormal divergence dating method implemented in BEAST (Drummond and Rambaut 2007), fixing the stem age of *Gladiolus* at 25.8 million years following Valente et al. (2011). Note that we are interested only in the relative rates of diversification, and therefore the absolute divergence time estimates are not relevant. In diversitree, we ran two independent MCMC chains for 10×10^4 steps for each of 10

randomly chosen trees using an exponential distribution prior for the rates. We discarded the first 25% of steps of each chain as burn-in.

The Cape Radiation Clade

A radiation involves proliferation of species over a short period of time from an initial ancestor and may reflect the potential role of pollinator shifts in the long term. We
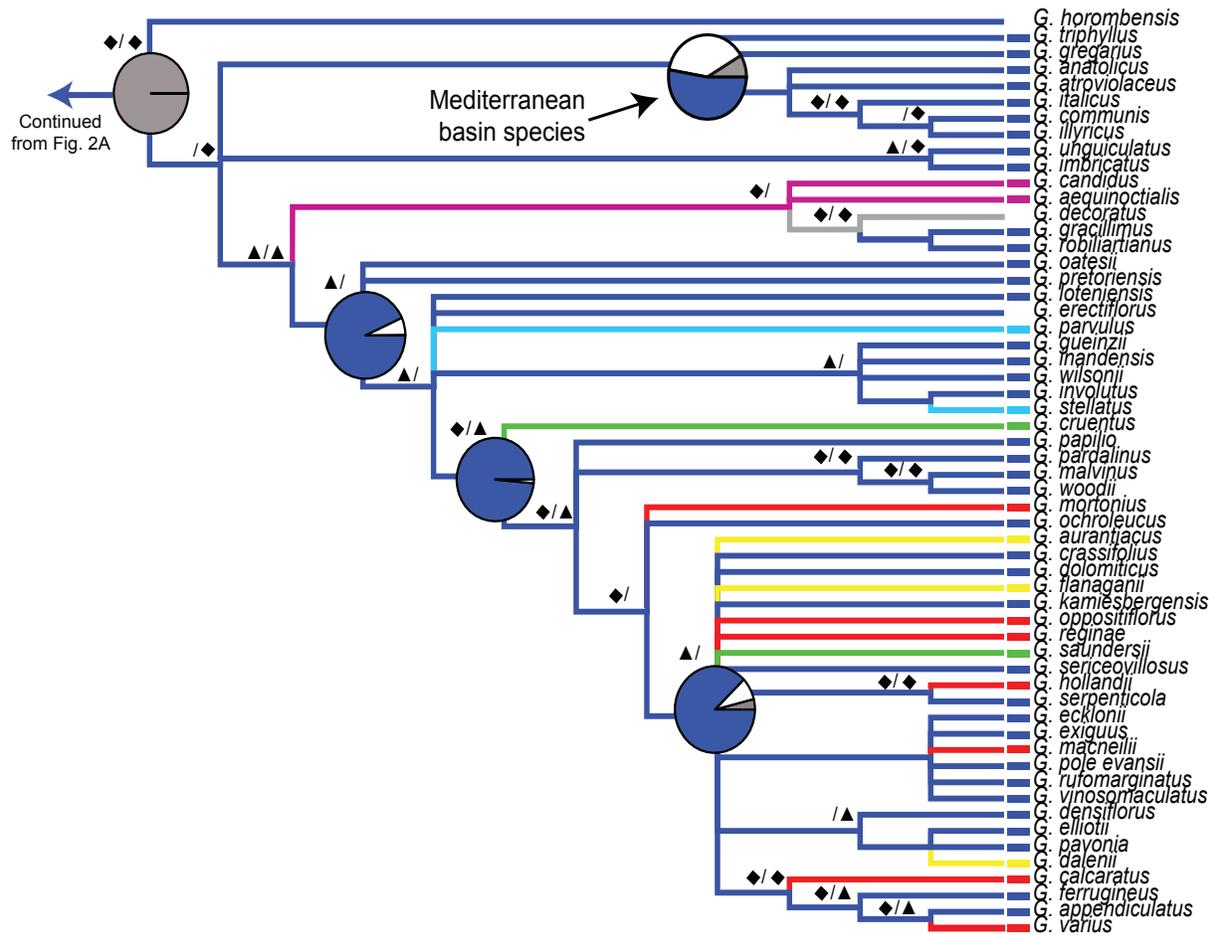
Figure 2: Evolution of pollination systems in *Gladiolus*. The tree shown is the majority rule consensus from the MrBayes (Ronquist and Huelsenbeck 2003) analysis. Symbols above the nodes represent Bayesian posterior probabilities/maximum likelihood bootstrap values. Lozenges represent posterior probabilities above 95% and bootstrap values above 80%. Triangles represent posterior probabilities above 90% and bootstrap values above 60%. Pie charts at selected nodes summarize the results of the maximum likelihood character optimization analyses in Mesquite (Maddison and Maddison 2009), conducted for 1,000 highly probable Bayesian trees. Each chart shows the percentage of trees for which a given pollination system was reconstructed as ancestral for that node. Branches are colored according to a parsimony mapping of ancestral pollination systems conducted in Mesquite onto the tree shown in the figure. The crown nodes of the Cape radiation clade and the Mediterranean basin lineage are indicated by arrows.

provide new molecular data for a lineage of 28 species of *Gladiolus* endemic to the southwesternmost region of the Cape of southern Africa. Despite an apparently recent origin (1.5–3.0 million years; Valente et al. 2011), this lineage is strikingly diverse in terms of floral morphology, with six different specialized pollination systems confirmed. Due to low sequence divergence, however, the molecular matrix of Valente et al. (2011) could not resolve relationships among the species of this clade, which they named the "Cape explosion" (Cape radiation clade, fig. 2). We therefore expanded the sampling of the genome for these taxa, producing new sequences for three additional plastid

regions (*petL-psbE*, *psbJ-petA*, and *ndhJ-trnF*) for the 28 species that comprise the radiation (Genbank accession numbers JQ796185–JQ796273). We investigated relationships among plastid haplotypes within 27 of the 28 species (we excluded *Gladiolus caryophyllaceus* as we were unable to generate sequences for two of the eight markers). The eight plastid regions were concatenated and the resulting matrix was analyzed using a coalescence-based method implemented in TCS (Clement et al. 2000). A haplotype network was constructed, with gaps treated as missing data and with a connection limit of 95%.

We used Bayes factors (BFs) to test whether different

pollination systems have evolved multiple times within the Cape radiation clade. The likelihood obtained under an unconstrained Bayesian inference analysis was compared with that obtained under analyses with the different pollination systems constrained to be monophyletic. This test was not conducted for butterfly pollination because there is only one species within the Cape radiation clade with this pollination system. BFs were calculated using Tracer, and a monophyly hypothesis was rejected if $-2 \log (BF) > 10$.

Results

Phylogenetic Analyses

The topology retrieved in the reanalysis of the *Gladiolus* matrix from Valente et al. (2011) was almost identical to that obtained in the previous study, except for differences at certain terminal nodes, reflecting low variation of sequences rather than strongly supported conflict (phylogenetic tree available in Dryad: http://dx.doi.org/doi: 10.5061/dryad.7pr554s8) The addition of the indel character matrix led to an increase in both posterior probabilities and bootstrap support values for the majority of nodes. The maximum likelihood topology recovered in the RaxML analysis was fully congruent with that obtained in the majority-rule consensus tree in MrBayes, and we therefore used the trees obtained by Bayesian inference for all subsequent analyses.

Evolutionary History of Pollination Systems

Pollination system exhibited a significant level of phylogenetic signal according to the analysis integrating over several topologies in Mesquite. The average number of parsimony steps required to explain the evolution of pollination systems in *Gladiolus* was 50.6 (mean number of steps in 1,000 trees). This value is less than the 0.05 percentile threshold (56 steps) of the distribution of character steps generated by reshuffling taxa in 1,000 randomizations. Therefore, closely related species tend to share pollination systems more often than expected by chance.

The results of the maximum-likelihood optimizations of pollination system evolution conducted more than 1,000 Bayesian trees are summarized in figure 2. Long-tongued bee pollination was unambiguously reconstructed as the ancestral state for the root of *Gladiolus* in 99.3% of trees.

The average, minimum, and maximum number of gains and losses of each pollination system in the 1,000 trees analyzed are shown in table 1. The differences found between the parsimony and maximum likelihood results stem from the fact that our choice of a conservative like-

Table 1: Number of unequivocal independent gains and losses of each of the pollination systems throughout the evolutionary history of *Gladiolus*

	Parsimony			ML		
	Average	Min	Max	Average	Min	Max
Total shifts	51.4	48	53	27.5	14	40
Independent gains:						
Long-tongued bee	3.4	1[a]	8	.4	1[a]	3
Moth	7.9	5	10	3.4	1[a]	8
Short-tongued bee	4.9	4	5	4.2	2	5
Sunbird	11.4	8	14	5.7	2	10
Beetle	1.0	1	1	.9	1[a]	1
Long-proboscid fly	17.1	12	20	9.5	4	15
Butterfly	5.7	4	6	3.5	1	6
Independent losses:						
Long-tongued bee	38.4	28	47	24.9	14	38
Moth	2.4	0	10	.4	0	4
Short-tongued bee	.2	0	5	.0	0	2
Sunbird	3.1	0	11	1.5	0	5
Beetle	.0	0	0	.0	0	1
Long-proboscid fly	6.3	0	14	.6	0	6
Butterfly	1.0	0	8	.2	0	3

Note: As inferred in the character optimization analyses conducted in Mesquite more than 1,000 highly probable trees, using parsimony and maximum likelihood (ML) methods.

[a] Minimum gains were set to 1 in the cases where Mesquite could not infer any unequivocal shifts toward a particular state.

lihood threshold resulted in more equivocal assignations of character states at nodes in the likelihood method, and thus fewer transitions in the likelihood analysis.

Overall, a minimum of 48 (parsimony) or 14 (maximum likelihood [ML]) pollination shifts was inferred. The pollination system that was reconstructed to have been gained most frequently is long-proboscid fly pollination, with a minimum of 12 (parsimony) or four (ML) independent gains. Bird pollination also has a high number of origins, having evolved a minimum of eight (parsimony) or two (ML) times. Moth, butterfly, and short-tongued bee pollination systems have all emerged more than once as revealed by both methods. Long-tongued bee pollination has been lost a minimum of 28 (parsimony) or 14 (ML) times. According to most trees, all pollination systems, with the exception of short-tongued bee and beetle pollination, have each been lost at least once (table 1). Character optimization analyses rendered similar results using parsimony, maximum likelihood, and Bayesian stochastic approaches (table 2). Of the 42 possible types of character state transitions, the one that has occurred most often has been from pollination by long-tongued bee to long-proboscid fly. Transitions from long-tongued bee pollination to moth, short-tongued bee, bird, and butterfly pollination have all occurred more than once.

Table 2: Frequency of each of the 42 types of possible pollination system transitions in *Gladiolus*

Transition	Parsimony (Mesquite)	ML (Mesquite)	Bayesian stochastic mapping (SIMMAP)
LB→MO	4.4 (1–7)	2.0 (0–5)	5.7 (0–16)
LB→SB	4.7 (3–5)	4.1 (2–5)	5.0 (0–12)
LB→BI	8.2 (5–11)	5.3 (1–8)	10.8 (1–23)
LB→BE	1.0 (1)	.9 (0–1)	1.2 (0–5)
LB→LF	15.5 (11–20)	9.2 (4–15)	18.0 (5–33)
LB→BU	4.7 (2–6)	3.4 (1–6)	4.8 (0–14)
MO→LB	.7 (0–4)	.2 (0–3)	4.1 (0–22)
MO→SB	.1 (0–1)	.02 (0–1)	.2 (0–4)
MO→BI	1.4 (0–5)	.2 (0–3)	1.7 (0–10)
MO→BE	0 (0)	0 (0)	.01 (0–3)
MO→LF	.2 (0–2)	0.1 (0–1)	1.5 (0–11)
MO→BU	.03 (0–1)	0 (0–1)	.2 (0–5)
SB→LB	.03 (0–1)	0 (0–1)	1.6 (0–16)
SB→MO	.1 (0–2)	0 (0–1)	.1 (0–5)
SB→BI	.1 (0–2)	0 (0–1)	.2 (0–6)
SB→BE	0 (0)	0 (0)	.01 (0–2)
SB→LF	.1 (0–1)	0 (0–1)	.3 (0–6)
SB→BU	.02 (0–1)	0 (0)	.09 (0–4)
BI→LB	.2 (0–2)	.1 (0–1)	6.1 (0–27)
BI→MO	1.9 (0–5)	1.3 (0–4)	2.4 (0–11)
BI→SB	.1 (0–2)	.02 (0–1)	.4 (0–5)
BI→BE	0 (0)	0 (0)	.03 (0–3)
BI→LF	.8 (0–4)	.1 (0–2)	2.0 (0–13)
BI→BU	.1 (0–2)	.02 (0–1)	.5 (0–5)
BE→LB	0 (0)	0 (0–1)	.3 (0–10)
BE→MO	0 (0)	0 (0)	.02 (0–3)
BE→SB	0 (0)	0 (0)	.01(0–2)
BE→BI	0 (0)	0 (0)	.04 (0–4)
BE→LF	0 (0)	0 (0)	.06 (0–4)
BE→BU	0 (0)	0 (0)	.01 (0–4)
LF→LB	2.2 (0–7)	.2 (0–3)	13.1 (0–36)
LF→MO	1.6 (0–4)	.1 (0–2)	2.7 (0–11)
LF→SB	.1 (0–1)	.04 (0–1)	.6 (0–7)
LF→BI	1.6 (0–5)	.3 (0–3)	2.8 (0–12)
LF→BE	0 (0)	0 (0)	.06 (0–3)
LF→BU	1.1 (0–4)	.02 (0–1)	1.2 (0–8)
BU→LB	.3 (0–3)	0 (0–1)	2.9 (0–17)
BU→MO	.01 (0–1)	0 (0–1)	.3 (0–5)
BU→SB	.02 (0–1)	0 (0–1)	.1 (0–4)
BU→BI	.1 (0–2)	.01 (0–1)	.7 (0–7)
BU→BE	0 (0)	0 (0)	.01 (0–2)
BU→LF	.6 (0–4)	.1 (0–2)	1.7 (0–10)

Note: Two columns show the average number of unequivocal transitions between each of the pollination systems as inferred in the character optimization analyses conducted in Mesquite (Maddison and Maddison 2009) more than 1,000 highly probable trees, using parsimony and maximum likelihood (ML) methods. One other column shows the expected number of transitions, obtained in the Bayesian stochastic mapping analysis of 1,000 highly probable trees in SIMMAP (Bollback 2006). Numbers in parentheses are the minimum and maximum number of transitions inferred. LB: long-tongued bee; MO: moth; SB: short-tongued bee; BI: sunbird; BE: beetle; LF: long-proboscid fly; BU: butterfly.

Rates of Pollination System Shifts

The posterior distributions of expected relative rates of transition obtained in the seven-state Bayesian analysis in SIMMAP are shown in figure 3. On average, the transitions that occurred at the highest relative rates were the following: long-proboscid fly to long-tongued bee (1.35, 95% credibility interval [CI] 0.73–2.07), butterfly to long-tongued bee (1.28, 95% CI 0–3.04), short-tongued bee to long-tongued bee (1.19, 95% CI 0–3.35), moth to long-tongued bee (1.16, 95% CI 0.24–2.38), and sunbird to long-tongued bee (0.95, 95% CI 0.25–1.76).

The analyses where pollination was coded as a binary character (long-tongued bee/non-long-tongued bee pollination) revealed that the transition from long-tongued bee to other pollination systems occurred at a similar rate (1.60, 95% CI 1.23–2.03) as the transition in the opposite direction (1.70, 95% CI 1.28–2.20).

Character-Dependent Speciation and Extinction Rates

The Bayesian BiSSE analyses revealed that both rates of speciation (λ_0 and λ_1) and rates of diversification (speciation minus extinction; r_0 and r_1) were significantly higher in *Gladiolus* lineages with rare pollination systems than in lineages with the predominant bee-pollinated system (table 3; fig. 4). There was no significant difference between the rates of transition between the two states (q_{01} and q_{10}), in agreement with the SIMMAP analyses.

The Cape Radiation Clade

The new matrix for the Cape radiation clade now includes a total of eight molecular regions with 7,060 sites. Of the 35 variable sites, 17 (48.5%) were located within the three new plastid regions sequenced in this study (*petL-psbE*, *psbJ-petA*, *ndhJ-trnF*). The resulting 19 haplotypes were connected through other 19 missing haplotypes (extinct or not found) in a TCS network with no loops (i.e., no homoplasy) and distributed in two "starlike" clusters (fig. 5). The most common, interior haplotype was shared by eight species pollinated by four different animal groups (long-tongued bee, short-tongued bee, moth, and butterfly). Pollination systems did not form independent clusters, and no haplotype was exclusive to a single pollination system. Instead, species sharing pollination strategies were mostly found scattered throughout the haplotype network.

Monophyly of long-tongued bee and moth pollination was strongly rejected in the Bayes factors analysis (table 4), suggesting that these systems have either evolved more than once within the Cape radiation clade or have evolved only once and are paraphyletic. However, the monophyly of short-tongued bee, long-tongued fly, and sunbird pol-

lination could not be rejected, revealing that they only evolved once within this clade.

Discussion

High Lability of Pollination Systems

The evolution of pollination systems in *Gladiolus* has been remarkably dynamic. All pollination systems, with the exception of hopliine beetle pollination, have evolved multiple times. The lability of pollination systems in *Gladiolus* was accompanied by unusual versatility in the direction of shifts: species sharing the same pollination system have, in some cases, arisen independently from ancestors with different systems. Long-tongued bee pollination was reconstructed as the ancestral state for *Gladiolus*, supporting the untested deduction by Goldblatt and Manning (1998). The loss of pollination by nectar-collecting bees accounts for more than 70% of the pollination shifts that have taken place throughout the radiation of the genus, and the rate of loss of this system was roughly equal to all combined rates of transition in the opposite direction (table 3). The derived pollination strategies were also unusually versatile: the five highest rates of transition were those that took place from "derived" pollination systems back to the ancestral long-tongued bee pollination system (fig. 3). The flexibility of pollination strategies in *Gladiolus* rivals that encountered in the genera with the most dynamic histories of pollination system evolution documented to date, including southern African *Disa* (Johnson et al. 1998) and *Babiana* (Schnitzler et al. 2011) as well as New World *Aquilegia* (Whittall and Hodges 2007), *Calochortus* (Patterson and Givnish 2004), *Costus* (Kay et al. 2005), *Iochroma* (Smith et al. 2008), and *Ruellia* (Tripp and Manos 2008).

Most studies on character evolution tend to find that although there is often a high diversity of character states, only a few of the possible types of transitions that could occur have actually taken place (Whittal and Hodges 2007). In *Gladiolus*, the percentage of realized character transitions was also unusually high, with at least 12 of the 42 possible types of pollination system transitions occurring (table 2). Certain specialized pollination strategies in *Gladiolus* have evolved from more than one ancestral system; for example, sunbird pollination has evolved independently from moth-, long-tongued bee- and long-proboscid fly-pollinated ancestors (table 2; fig. 3). Furthermore, at least five of the pollination systems have been secondarily ancestral to other systems in the genus (table 1). As far as we know, such lability of pollination systems has never been documented in any other group. The seemingly unconstrained directionality of transitions found in *Gladiolus* is rare in plants, not only between
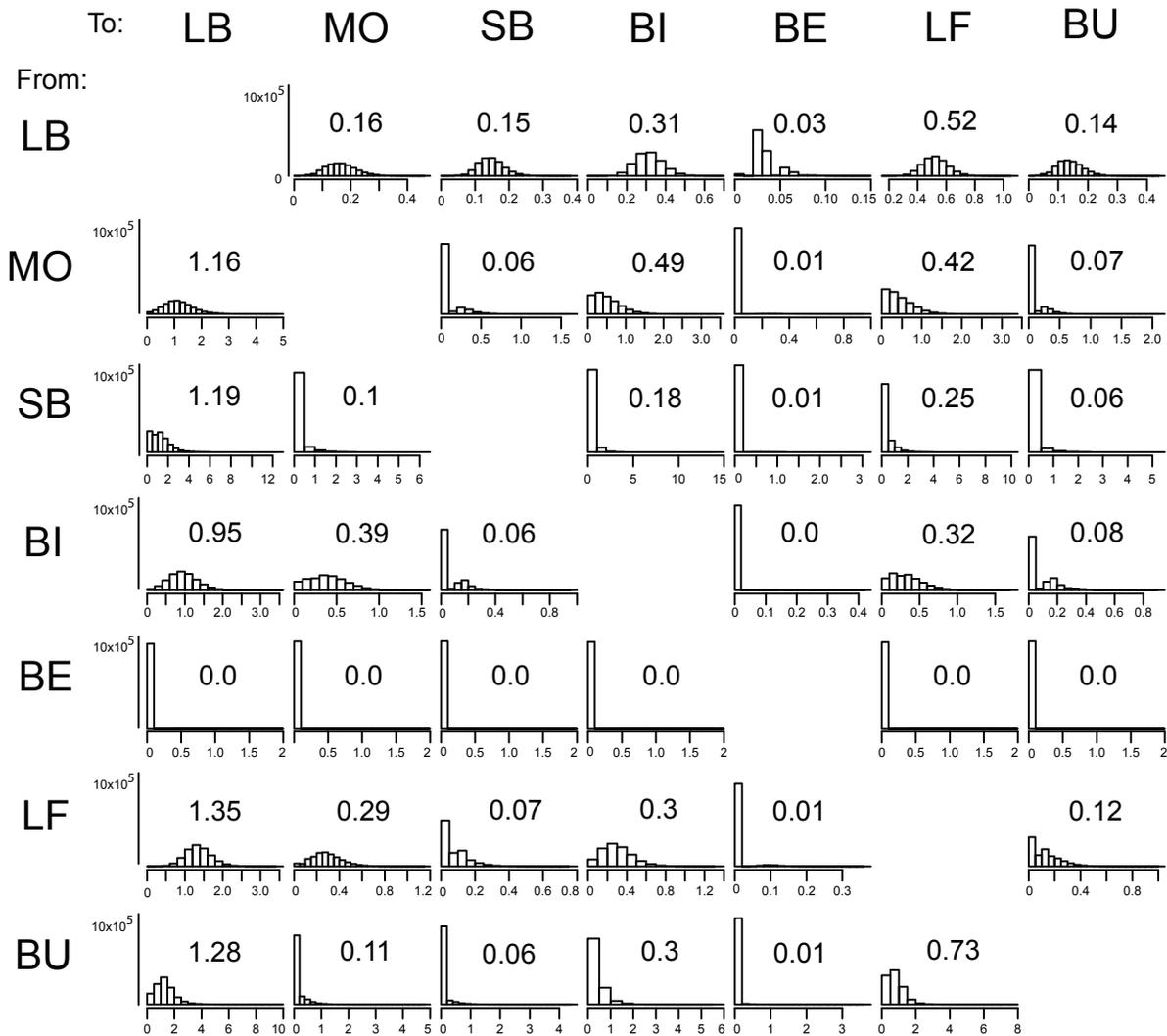
Figure 3: Posterior distributions of the expected relative rates of transition between the seven pollination systems in *Gladiolus*, obtained in the Bayesian stochastic character mapping analyses conducted more than 1,000 highly probable trees in SIMMAP (Bollback 2006). Each histogram refers to the transition from the pollination system shown at the start of the row to the pollination system shown at the top the column. The pollination systems are (in order of appearance from top to bottom in rows and from left to right in columns) LB (long-tongued bee), MO (moth), SB (short-tongued bee), BI (sunbird), BE (beetle), LF (long-proboscid fly), and BU (butterfly). The numbers above each histogram are the averages of the posterior distributions.

pollination states (Tripp and Manos 2008) but also between character states in other types of traits whose transitions also involve major morphological changes (e.g., sexual systems; Renner et al. 2009; leaf shape, Jones et al. 2009).

The analysis of the rapidly diversifying Cape radiation clade illustrates how dynamic pollination system evolution has been in the genus. The haplotype network revealed that radiation of pollination systems has proceeded remarkably rapidly in this clade (fig. 5). The six specialized

pollination syndromes that occur within this lineage have probably all evolved in the last 1.5–3.0 million years (Valente et al. 2011), but little genetic variation has accumulated in the molecular regions sampled. The six pollination strategies did not cluster in distinct lineages within the haplotype network, indicating that rapid parallel shifts have occurred. A contrasting scenario was encountered in the lineage comprising all seven species of *Gladiolus* from the Mediterranean basin (fig. 2): speciation in this region has clearly not been associated with diversification of pol-

Table 3: Mean and 95% credibility intervals (CIs) of the parameters estimated in the Bayesian binary state speciation and extinction analyses

Parameter	Mean	95% CI
λ_0	.160	.024–.360
λ_1	.794	.487–1.178
μ_0	.262	.017–.633
μ_1	.218	.007–.684
r_0	−.102	−.455–.151
r_1	.576	.269–.899
q_{01}	.543	.097–1.265
q_{10}	1.034	.398–2.047

Note: Summary statistics are shown for all Markov chain Monte Carlo runs combined (each run was conducted independently for each of 20 Bayesian trees). λ_0 = speciation rate in state 0; λ_1 = speciation rate in state 1; μ_0 = extinction rate in state 0; μ_1 = extinction rate in state 1; r_0 = diversification rate in state 0; r_1 = diversification rate in state 1; q_{01} = transition rate from state 0 to 1; q_{10} = transition rate from state 1 to 0; where state 0 is long-tongued bee pollination and state 1 is all other pollination systems combined.

lination systems, as we found that no pollinator shifts have taken place within this clade (all species are melittophilous), even though the Mediterranean lineage is older (>4 million years old; Valente et al. 2011) than the Cape radiation clade. A lower pollinator richness of Europe has previously been invoked to explain the narrow array of pollination strategies in this region in *Aquilegia* (Bastida et al. 2010), but whether this hypothesis holds for *Gladiolus* remains to be tested.

Our estimates of evolutionary flexibility of pollination systems rely on the topology and branch lengths of the phylogenetic trees presented in this study. The uncertainty associated with the lack of support of some branches is a common feature of phylogenetic species-level studies of recently diverged groups (Bakker et al. 2005). In all post-tree-reconstruction phylogenetic analyses we have explicitly taken this limitation into account by integrating parameter estimations over a large set of Bayesian trees that capture the uncertainty in the relationships amongst species of *Gladiolus*. The fact that the *Gladiolus* phylogenetic trees used in this study are based solely on plastid data could mask additional sources of diversity, particularly hybridization, and associated plastid DNA capture, masking deeper relationships that could be revealed if nuclear data were available. However, the topology presented here receives external support from the fact that most of the clades retrieved in our analysis correspond broadly to the morphology-based series or species groups proposed by Goldblatt and Manning (1998) in their monograph of the genus, rather than to geography (Valente et al. 2011), as

would be expected if plastid DNA capture had not been prevalent.

Pollination Shifts and Speciation in Southern Africa

The complexity of the evolutionary history of pollination systems in *Gladiolus* is also illustrated by the high number of lineage-splitting events that have been associated with a pollination shift, accounting for between 18.7% and 34.9% of all branching events (average of 27.5 shifts with maximum likelihood and 51.4 shifts with parsimony). The fact that an important percentage of branching events in the genus are linked to pollinator divergence suggests that pollinator-driven speciation may have been frequent. Nevertheless, our study revealed that the majority of speciation events in *Gladiolus* were not associated with a transition between major pollinator functional groups, therefore providing only partial support for the pollinator-driven speciation hypothesis (Johnson 1996). Indeed, our analysis of phylogenetic signal of pollination system confirms that, overall, pollination systems tend to be conserved in *Gladiolus* and that closely related species tend to share pollination strategy (Pagel 1999). In addition, the Bayes factor analysis (table 4) revealed that single origin of at least four (butterfly, long-proboscid fly, short-tongued bee, sunbird) of the six pollination systems found within the rapidly evolving Cape radiation cannot be rejected within this lineage. This suggests that although shifts between pollinator functional groups may have partly driven speciation in this endemic southern African clade, an additional explanation is required to account for its unusually rapid rates of diversification (Valente et al 2011), given that most speciation events were not accompanied by a change in pollinator category.

Overall, our results show that pollinator shifts have played a significant role in driving phenotypic diversity and possibly speciation in one of the most florally diverse genera. However, it seems that diversification in *Gladiolus* follows the pollinator-driven speciation model only to some extent (Johnson 1996; van der Niet and Johnson 2009), as more than half of the branching events in the phylogenetic tree appear to be associated with factors other than pollination biology. This result is particularly relevant given that it was obtained from the prime example of a southern African clade exhibiting high diversity of floral types and pollination strategies (Goldblatt and Manning 2002). This work joins other studies in demonstrating only a moderate link between speciation and pollinator divergence in the southern Africa flora. Previous analyses of Cape clades *Lapeirousia* (Goldblatt and Manning 1996), *Babiana*, *Moraea*, Podalyrieae, and *Protea* (Schnitzler et al. 2011), have found that pollinator systems show an unexpectedly high degree of conservatism and have alter-
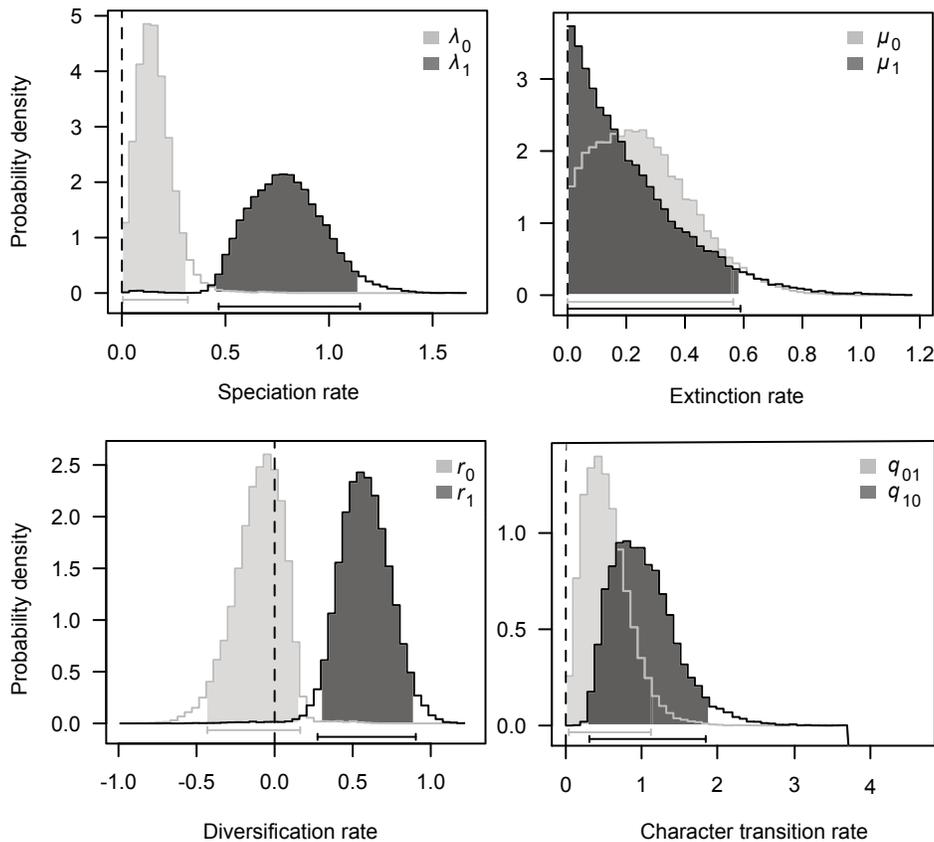
Figure 4: Posterior distributions of parameters estimated in the Bayesian binary state speciation and extinction analyses. Horizontal bars indicate the 95% credibility interval for each parameter. Abbreviations according to table 3.

natively proposed a primary role of edaphic factors in driving divergence (see van der Niet et al 2006). We are currently unable to test the edaphic shifts hypothesis in *Gladiolus* due to the lack of available relevant data for such a large group, which has been little studied in an important part of its range outside of southern Africa. Johnson (2010) has recently proposed that a link between pollinator distribution and habitat type would facilitate parallel shifts in pollination system and edaphic specialization. Operation of this model requires that pollination systems are composed of single pollinator species, in order that shifts in habitat types become shifts out of an existing pollination ambit. This has not been demonstrated to apply in *Gladiolus*, nor in other southern African Iridaceae, in which the majority of pollinators are functional groups rather than single species, or have foraging ranges that extend well beyond individual habitat boundaries.

In this study we have restricted our focus to shifts between the seven pollinator functional categories in *Gladiolus*. As a result, transitions that may have taken place within these groups—for example, between different types of long-proboscid flies or moths—and that may have been related to speciation could not be assessed. Previous studies have shown that these types of shifts have occurred between sister species in other southern African clades, namely *Diseae* orchids (Johnson and Steiner 1997; Johnson et al. 1998; Waterman et al 2011). In *Gladiolus*, a study of selection by Anderson et al (2009) found evidence for the buildup of isolating barriers between two floral morphs of the southern African species *G. longicollis* that differ in corolla tube length as a result of adaptation to two hawk-moth guilds. However, a follow-up genetic modeling analysis of this system found that divergence toward speciation was unlikely (Rymer et al 2011). Although there is currently no evidence to suggest that the *Gladiolus* pollinator functional groups considered in this study may conceal transitions that were relevant to the speciation process, we cannot exclude this possibility.

In addition to evaluating the pollinator shift speciation hypothesis, we have assessed whether different types of
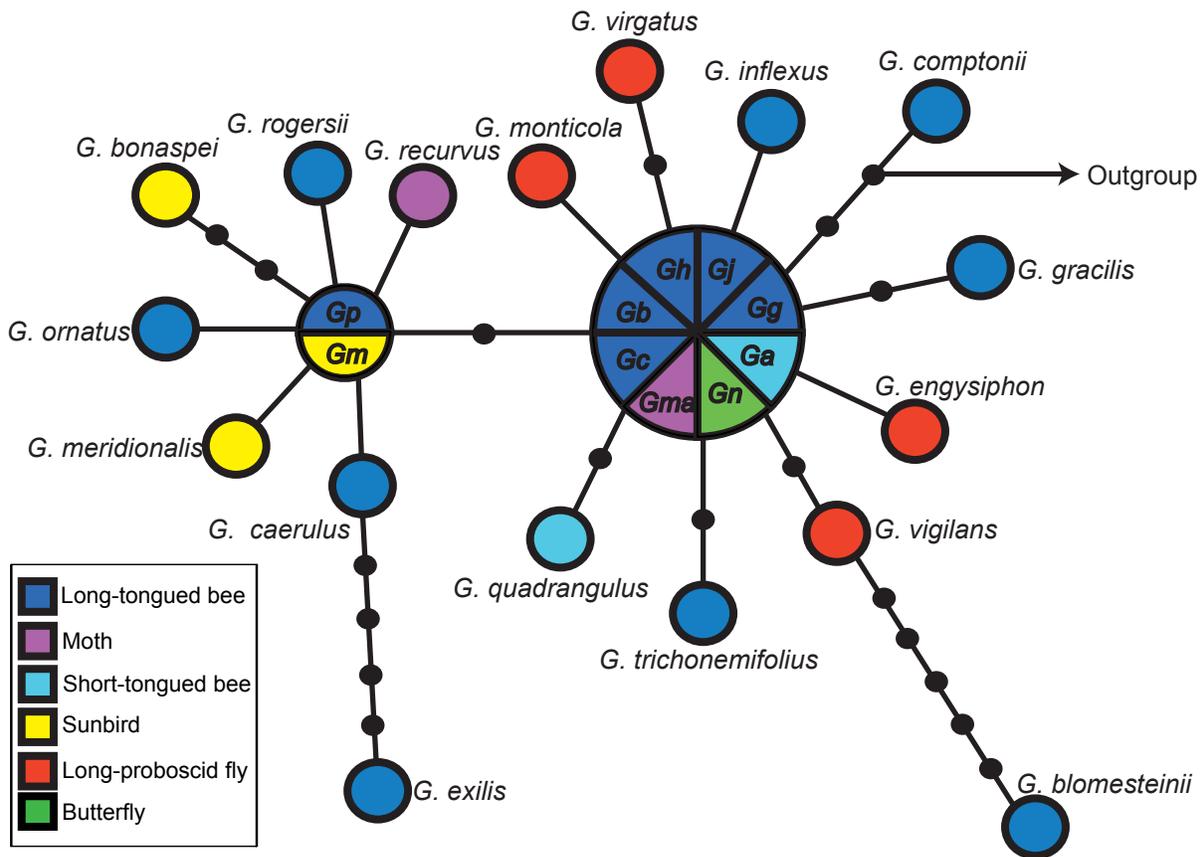
Figure 5: Parsimony haplotype network based on a concatenated eight-plastid region matrix (*matK, psbA-trnH, trnS-trnG, rpl32-trnL, trnQ-rps16, petL-psbE, psbJ-petA, ndhJ-trnF*) from a sample of 27 species of the Cape radiation clade, plus 3 outgroup species (not shown). Black circles represent mutational steps; circle size is proportional to frequency; colors indicate pollination system. *Gladiolus* species: *Ga* = *G. aureus, Gb* = *G. brevifolius, Gc* = *G. carinatus, Gg* = *G. griseus, Gh* = *G. hirsutus, Gj* = *G. jonquilliodorus, Gm* = *G. martleyi, Gma* = *G. maculatus, Gn* = *G. nerineoides, Gp* = *G. priorii.*

pollination system may have influenced diversification processes in *Gladiolus* by directly affecting rates of speciation and/or extinction. Our BiSSE analyses revealed an important character-dependent effect of pollination system on rates of evolution: species with rarer derived pollination strategies (short-tongued bee, bird, moth, long-tongued fly, butterfly, and beetle) had significantly higher combined rates of speciation and diversification than species with the ancestral long-tongued bee system. To our knowledge, this is the first group where such an effect is shown for pollination systems. Recent applications of character-dependent diversification models on southern African and tropical clades found no significant effect of the type of pollination system or of particular floral traits on diversification rate (Armbruster et al. 2009; Schnitzler et al. 2011), whereas a study of *Ipomoea* did find a strong character-dependent effect of flower traits on diversifica-

tion but did not specifically test the effect of pollination systems (Smith et al. 2010). Our finding reveals that certain types of pollination systems may accelerate species production, suggesting that specific classes of pollination system may have promoted diversification in southern Africa. In other words, in the case of *Gladiolus*, species with rarer pollination systems have overall given rise to new species more rapidly than species pollinated by the predominant long-tongued bee pollination system. Our results pose the new question as to why these rarer pollination systems are associated with rapid diversification. The answer may lie in the fact that competition to attract "derived" animal pollinators is relaxed, since such pollination systems are less commonly exploited than melittophilous strategies (Goldblatt and Manning 2006). These rarer, derived systems could be considered key innovations that have effectively opened up new, less crowded pollination niches

Table 4: Results of the Bayes factor (BF) test for different pollination system monophyly hypotheses within the Cape radiation clade

Monophyly constraint	Marginal likelihood ± SE	−2log(BF)
No constraint	−10,436.9 ± .23	
Long-tongued bee pollination	−10,463.3 ± .23	52.8
Long-proboscid fly pollination	−10,437.8 ± .18	1.9
Moth pollination	−10,452.5 ± .27	31.2
Short-tongued bee pollination	−10,436.3 ± .24	1.1
Sunbird pollination	−10,436.4 ± .19	.9

Note: Hypotheses with −2(log BF) > 10 are rejected. Monophyly of butterfly pollination was not tested given that only one species with that syndrome is found within the Cape radiation clade.

that may have facilitated divergence processes (Hunter 1998).

We suggest that the key for high plant species diversity in southern African *Gladiolus* may be the existence of a diverse array of rare pollinator groups, independently and in addition to pollination shifts per se. Additional factors such as variety of soils, ancestral geographic distributions and climatic conditions should be further explored to account for high rates of species differentiation in one of the most spectacularly diverse genera of the angiosperms.

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