The Effects of Above- and Belowground Mutualisms on Orchid Speciation and Coexistence

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ABSTRACT: Both pollination by animals and mycorrhizal symbioses with fungi are believed to have been important for the diversification of flowering plants. However, the mechanisms by which these above- and belowground mutualisms affect plant speciation and coexistence remain obscure. We provide evidence that shifts in pollination traits are important for both speciation and coexistence in a diverse group of orchids, whereas shifts in fungal partner are important for coexistence but not for speciation. Phylogenetic analyses show that recently diverged orchid species tend either to use different pollinator species or to place pollen on different body parts of the same species, consistent with the role of pollination-mode shifts in speciation. Field experiments provide support for the hypothesis that colonization of new geographical areas requires adaptation to new pollinator species, whereas co-occurring orchid species share pollinator species by placing pollen on different body parts. In contrast to pollinators, fungal partners are conserved between closely related orchid species, and orchids recruit the same fungal species even when transplanted to different areas. However, co-occurring orchid species tend to use different fungal partners, consistent with their expected role in reducing competition for nutrients. Our results demonstrate that the two dominant mutualisms in terrestrial ecosystems can play major but contrasting roles in plant community assembly and speciation.

Keywords: coevolution, diversification, community assembly, pollination, mycorrhiza, Orchidaceae.

Introduction

Most species survive and reproduce only by interacting with other species, and the evolution of biodiversity de-

pends intimately on the evolution of these interactions (Thompson 2005). Both pollination by animals and mycorrhizal symbioses with fungi are thought to have been important factors in the success of flowering plants (Grant 1949; Remy et al. 1994). In principle, mutualisms might affect either the origin of plant species, via an effect on speciation, or the maintenance of diversity, via an effect on community assembly and species coexistence. However, which of these mechanisms operates for above- and belowground mutualisms remains obscure.

In terms of the origin of species diversity, shifts in pollinator type can cause speciation through a direct effect on patterns of gene flow as well as by exerting divergent selection pressures on populations (Grant 1992; Johnson et al. 1998; Schlüter et al. 2009; Stöckl et al. 2009; Vereecken et al. 2010). However, although it has been argued that shifts in mycorrhizal fungi might also drive plant speciation by promoting ecological divergence of plant populations (Thompson 1987; Cowling et al. 1990; Otero and Flanagan 2006), few studies have explored specificity and shifts of mycorrhizal fungal partners among related plant species (but see Barrett et al. 2010; Roche et al. 2010). If mutualisms are important drivers of speciation, then recently diverged species should tend to differ in their mutualistic partners or the nature of their interaction with their partners.

In terms of the maintenance of diversity, mutualistic interactions can play two contrasting roles in structuring plant communities (Elias et al. 2008; Sargent and Ackerly 2008). First, where species are competing for a limited resource, coexistence is thought to depend on species partitioning that resource and therefore having different niches (Diamond 1975). This will lead to communities in which species are less similar in traits or resulting interactions than expected, compared to the regional species pool; that is, they

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are phenotypically overdispersed. For example, local evolution to avoid competition through interspecific pollen transfer leads to the formation of plant communities with phenotypically diverse flowers (Armbruster et al. 1994; Muchhala and Potts 2007). Similarly, different preferences for mycorrhizal fungi between plant species can promote coexistence by reducing competition for nutrients (Vandenkoornhuyse et al. 2003; van der Heijden et al. 2003).

The second way that mutualisms can be important for structuring plant communities is by habitat filtering, in which only species with a certain trait (or interaction) are able to persist in a particular environment (Keddy 1992). This will lead to communities in which species are more similar than expected, compared to the regional species pool; that is, they are phenotypically clustered. For example, co-occurring plant species can form “pollination guilds” with shared traits that attract a local pollinator (Pauw 2006), while in other plant communities shared mycorrhizal fungi can facilitate interplant nutrient exchange (Simard and Durall 2004).

Although these mechanisms have been identified in several cases, evolutionary and ecological mechanisms have never been considered jointly for both types of mutualisms simultaneously, which means that assessment of the relative importance of both types of mutualism for each of the above mechanisms has not been possible. Here, we determined the role of pollinator and mycorrhizal interactions in a diverse group of orchids, the subtribe Coryciinae from southern Africa. Orchids are ideal for testing the relative importance of these mechanisms because of their obligate and often highly specialized interactions with both pollinators and mycorrhizal fungi (Waterman and Bidartondo 2008).

Coryciinae orchids secrete oil from a lip appendage on their flowers, and pollination occurs when female *Rediviva* bees (Melittidae) collect the oil, probably for use as a larval provision (Pauw 2006; fig. 1A). As with other orchids, pollen is placed onto precise locations on the body of the pollinator in packages called pollinaria (Pauw 2006; fig. 1B). The oil-secreting lineages of Coryciinae are largely endemic to South Africa, with two centers of diversity: a summer-rainfall area centered in the Drakensberg range and a winter-rainfall area in the Western Cape province and Namaqualand (Linder and Kurzweil 1999). This distribution mirrors that of the bee genus *Rediviva* (Whitehead and Steiner 2001; Whitehead et al. 2008). Because the diversity of orchid species is higher than that of pollinating bees, many orchid species share the same pollinator. A recent study demonstrated a pollination syndrome among the orchids pollinated by one bee species, *Rediviva peringueyi* (Pauw 2006). Fifteen orchid species were identified as members of this pollination guild (Pauw 2006), members of which share an assortment of character traits to attract a shared pollinator, in this case including yellow-

Figure 1: A, The flowers of oil-secreting orchids, such as *Pterygodium magnum*, attract specific female oil-collecting bees, here *Rediviva brunnea*. B, Pollen is attached onto precise locations in packages called pollinaria. Photographs by Anton Pauw.

green flower coloration, a distinctive pungent scent, a September peak in flowering time, and, in common with their pollinator, a strong preference for clay soil. By “guild” we mean a group of orchid species sharing the same pollinator or sets of pollinators.

The mycorrhizal fungi of Coryciinae species have not been investigated previously. However, in general, orchids depend entirely on mycorrhizal fungi for nutrients and energy, especially during the early part of their life cycle (Smith and Read 2008). The fungi belong to a broad range of taxa of predominantly free-living decomposers, but the extent to which orchids specialize on particular fungal species is often uncertain.

We assembled comprehensive data on pollination mode (both pollinator species and pollinarium attachment sites), mycorrhizal fungal diversity, and orchid phylogenetic relationships to test two predictions. If shifts in pollination mode or fungal partner are frequent causes of speciation, a high proportion of recently diverged orchid species should differ in pollination mode or fungal partner, more so than expected if speciation occurs independently of such shifts (Barracough et al. 1999; Whittall and Hodges 2007). In turn, a role of each mutualism in coexistence would be apparent if the diversity of interactions within communities was either significantly greater (in the case of niche partitioning) or significantly less (in the case of habitat filtering) than expected in null models of community assembly (Webb et al. 2002; Losos et al. 2003). Similarly, the number of interactions shared by co-occurring orchids is expected to be low in the case of partitioning and high in the case of habitat filtering. Given the many confounding factors that could potentially influence the distribution of traits among related species and communities, there is a surprising lack of studies combining field experiments with phylogenetic analyses (Vamosi et al. 2009). Therefore, in addition to phylogenetic analyses, we used field and laboratory experiments to test further the mechanisms by which mutualisms might influence speciation and community assembly in these plants. The following tests were conducted: (1) reciprocal transplantation of seeds and inflorescences across orchid range boundaries to test for preferences toward mutualists in different geographic areas; (2) pollinator-choice experiments aimed at elucidating the mechanism by which pollinator-driven selection might act; (3) cross-pollination experiments to evaluate the role of differences in pollinarium placement sites in speciation and coexistence; (4) stable-isotope analysis to investigate the role of mycorrhizal associations in the partitioning of resources among co-occurring orchids.

Methods

Data Collection

Orchid Molecular Phylogeny. The orchid phylogeny was reconstructed through Bayesian analysis of plastid and nuclear DNA regions, as described in detail in a previous phylogenetic analysis of the tribe Diseae (Waterman et al.

2009). Plastid regions were the plastid *trnL* intron and the *trnL-trnF* intergenic spacer region (Taberlet et al. 1991; Bellstedt et al. 2001) and part of the *matK* gene and *trnK* intron (Goldman et al. 2001). The nuclear gene was the ribosomal internal transcribed spacers and 5.8S region (White et al. 1990; Sun et al. 1994). Five additional taxa were included here: the late-flowering forms of *Pterygodium catholicum* (L.) Sw., *Pterygodium cafferum* (L.) Sw., and *Pterygodium cruciferum* Sond.; the white-flowered form of *Disperis capensis* var. *capensis* (L. f.) Sw.; and the recently described species *Pterygodium vermiferum* E.G.H. Oliv. & Liltved (table A1 in the online edition of the *American Naturalist*). Our sample includes 52 out of 60 recognized oil-secreting species; only extremely rare taxa were not sampled. The five genera within the subtribe (*Disperis*, *Pterygodium*, *Corycium*, *Ceratandra*, and *Evotella*) have traditionally been grouped together on the basis of shared floral characters (Steiner 1989). However, the results of Waterman et al. (2009), confirmed here, show that Coryciinae is diphyletic, with *Disperis* forming a distinct clade separated from the other four genera by several non-oil-secreting clades within the tribe Diseae (Waterman et al. 2009). For subsequent analyses, the consensus tree from Bayesian analysis was pruned to include only the oil-secreting clades, and the branch lengths were made ultrametric by means of penalized likelihood with optimal smoothing estimated by cross-validation in the program r8s (Sanderson 2003).

Identification of Pollinators and Attachment Sites. *Rediviva* bees were captured with insect nets on oil-secreting Scrophulariaceae and Orchidaceae or on nectar plants and were identified according to the latest revision of the genus (Whitehead and Steiner 2001; Whitehead et al. 2008). Pollinaria on captured bees were identified with a reference collection or by DNA barcoding of the *MatK* region, which has previously been found to discriminate well between Coryciinae species and was sequenced according to methods described by Waterman et al. (2009). Direct observation was lacking for 10 out of 52 species (tables A2, A3 in the online edition of the *American Naturalist*). In these cases, pollinators were predicted from geographical distribution, flowering time, and floral syndrome (Pauw 2006), and pollinarium attachment site was predicted by fitting recently killed bees onto fresh flowers with their oil-collecting front tarsi on the oil-secreting region. Predicting pollination mode without direct observations can be unreliable; however, previous studies have shown that pollination syndromes in Coryciinae can be predicted accurately because of the highly specialized relationship with oil-collecting bees and the allopatric distribution of the main pollinating bee species (Steiner 1989; Pauw 2006).

Identification of Mycorrhizal Fungi. Roots were collected from adult plants during three successive flowering seasons (2005–2007) from sites throughout South Africa. At each site, all co-occurring Coryciinae were sampled. Where several patches of the same species occurred at a site, multiple individuals of that species were sampled (see table A4 in the online edition of the *American Naturalist* for the number of plants and sites sampled). Upon collection, roots were kept cool and then stored in ethanol within 24 h. After being washed, roots were hand sectioned and viewed under a microscope to confirm colonization by mycorrhizal fungi, apparent as fungal pelotons within plant root cortical cells. DNA was extracted from colonized root sections, and fungi were identified by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region. Following methods described elsewhere (Bidartondo et al. 2004; Bidartondo and Read 2008), the fungal-specific primer pair ITS1F/ITS4 was used for polymerase chain reaction (PCR) amplification and sequencing. If no PCR products were produced with this method, the tulasnelloid-specific primer pair ITS1/ITS4-tul was used. The few PCR products that could not be sequenced directly were cloned with TOPO-TA (Invitrogen), and four cloned DNA amplicons were sequenced. During the first year of sampling, multiple root sections for PCR and sequencing were taken from different parts of the same individual plants' root systems. As the roots of these 80 plants always yielded identical fungal DNA sequences, in subsequent years only one root section was sequenced per plant (a further 151 plants). Fungal sequences were aligned in six separate alignments, corresponding to the six major taxa of fungi involved, as ITS sequences were too variable to align between these groups. The fungi within each clade were grouped further into operational taxonomic units (OTUs) defined by 95% sequence similarity, as determined by the furthest-neighbor algorithm in DOTUR (Schloss and Handelsman 2005). It is possible that the use of ITS sequence similarity cutoffs to define OTUs representing putative species may underestimate fungal diversity; however, this methodology is widely used in mycorrhizal research (Nilsson et al. 2008; McCormick et al. 2009; Lievens et al. 2010), because of the difficulties associated with obtaining multigene phylogenetic data from environmental samples. Note that for a subset of orchid taxa, we confirmed that identified fungi were mycorrhizal and not other associated fungi by field seed-germination experiments described below. For the remainder, we rely on microscopic isolation of mycorrhizal root sections to infer that obtained sequences represent mycorrhizal fungi rather than non-mycorrhizal symbionts. Fungal DNA sequences have been submitted to the GenBank database: accession numbers FJ788666–FJ788894 and FJ808567–FJ808570.

Hypothesis Testing

Effects of Mutualisms on Speciation. The relationship between mutualistic associations and orchid diversification was investigated with Jordan indices, J (Fitzpatrick and Turelli 2006). For a given pair of orchid species, $J = 1$ indicates that the species have different interactions, whereas $J = 0$ indicates that they share the same interaction. If speciation tends to involve changes in mutualistic interaction, we expect a general trend of high J across recently diverged species. In contrast, a trend of low J would indicate that mutualistic interactions are conserved among closely related species and that shifts in interaction type cannot be a frequent cause of speciation. We performed several versions of the analyses to investigate the robustness of the results. First, we calculated J for all sister-species pairs. To test for significance against random distribution of interactions (pollinator, pollinarium attachment site, fungal clade, and fungal OTU), the average J for each of the above interactions was recalculated for 999 random associations of recently diverged taxa, by shuffling the character states of interactions among orchid taxa each time (Fitzpatrick and Turelli 2006). A two-tailed test was subsequently performed to test whether changes in interactions were either significantly more different or significantly more conserved between recently diverged orchid species than expected by chance. Therefore, a mutualistic interaction was considered significantly more divergent between closely related species than expected by chance if less than 2.5% of the randomizations had a lower J —and significantly conserved if less than 2.5% of the randomizations had a higher J —than the observed value.

Because of the limited number of strict sister-species pairs in our phylogeny, we also repeated the analysis by calculating J for all pairwise comparisons of species related to each other by at least a certain threshold of divergence time. We repeated this version of the test with thresholds of 5%, 10%, and 15% of the age of the root node to check the robustness of the findings to the exact threshold used. We also tested for an interaction between pollinator species and attachment site. As differences in either of these variables would potentially lead to reproductive isolation, we tested whether recently diverged orchids were more likely than expected to differ in either pollinator or attachment site. Therefore, for this test, $J = 1$ if orchid species differ in either of these variables and $J = 0$ if they differ in neither. As an alternative, we also tested whether orchid species were more likely than expected to differ in both pollinator and attachment site.

Effects of Mutualisms on Community Assembly and Coexistence. We tested for significant niche partitioning or habitat filtering of interaction types across sites. The fungal

OTUs associating with orchid species were known for 16 sites, and we performed our most stringent analyses across those sites. However, to maximize sample size, we also repeated the analyses across our entire set of 37 sites, by inferring which of the six fungal clades each orchid species associates with for those 21 sites lacking direct fungal evidence. The reliability of inferring fungal associations is discussed further in "Results." For both versions of the analysis, the number of pollinators and fungal clades was counted at each site, and the average value across sites was recorded. A high average count is expected with niche partitioning, whereas a low average count is expected with habitat filtering. The number of times that different co-occurring orchid species associated with the same fungal OTU or used the same pollinarium attachment site was also recorded. A low incidence of sharing is expected with partitioning, and a high incidence is expected with habitat filtering.

To test for significance, test statistics were recalculated for null communities in which the species richness of each community was maintained, but the members of each community, along with their associated interaction traits, were randomly allocated from the regional pool of sampled taxa. Co-occurrence of different populations of the same species was prevented. The observed counts were compared against expected distributions generated from 999 randomizations. Observed values were subsequently compared to null distributions by means of a two-tailed test, with one tail representing significant phenotypic overdispersion (niche partitioning) and the other representing phenotypic clustering (habitat filtering).

An observation of partitioning of traits between co-occurring orchids could potentially be caused either by ecological assembly of species with different interaction traits or by evolutionary character displacement acting on different populations. For pollinators and attachment sites, no differences were observed within OTUs; therefore, character displacement at this level is unlikely. However, different populations of the same orchid do associate with different fungal OTUs. Therefore, we also tested an alternative null model in which the composition of orchid taxa within communities was kept constant and orchid-fungus groupings were shuffled only within each orchid taxon (analogous to the "evolutionary model" used by Muchhala and Potts [2007]). While the original model tests whether communities differ from expectations by chance irrespective of the process responsible, the second, "character-displacement" model tests specifically for the local evolution of species by character displacement.

Experimental Evidence

Reciprocal-Transplant Experiments. We used reciprocal-transplant experiments to investigate whether recently di-

verged orchids with parapatric distributions are specialized toward particular pollinators or fungi found in their home region or are able to interact with the partners used by their close relatives. In the transplant experiments, a "home-field advantage" would be consistent with the hypothesis that shifts in interaction partner can drive population divergence and speciation. Transplants were conducted between three pairs of recently diverged parapatric orchid taxa: *P. caffrum*/*Pterygodium pentherianum*, *P. catholicum* (typical form)/*P. catholicum* (late-flowering form), and *Pterygodium schelpei*/*Pterygodium volucris* (for locations of transplant sites, see table A5 in the online edition of the *American Naturalist*). Wilcoxon signed-rank tests were used to test for a significant effect of transplantation treatment for both pollination and germination studies, with species paired by site.

We tested first for differences in pollinator species between recently diverged orchids by reciprocally transplanting orchid inflorescences between regions. Inflorescences in bud were collected from the field by cutting the stem with a scalpel, leaving the tuber and leaves intact. Inflorescences were inserted in water-filled test tubes every 2 m, with species alternating, and collected into 70% alcohol 5 days later. Flowers were examined microscopically for pollen receipt (massulae of pollen adhered to stigma) and removal (missing pollinaria), either of which indicates a *Rediviva* visit (see table A6 in the online edition of the *American Naturalist*).

To test for differences in fungal partners between recently diverged orchids, we measured germination success of orchid seeds transplanted between regions. Seeds were collected from pollinated orchids and placed inside separate 3-cm² compartments constructed from 50- μ m nylon mesh. Approximately 300–500 seeds of each orchid taxon were placed inside a compartment, and the seed packets were sealed with a heat sealer. Placing seeds within separate compartments of the same seed packet ensures that the different sets of seeds are exposed to the same mycorrhizal fungi (Bidartondo and Bruns 2005). Between 40 and 80 seed packets were planted in November/December 2005 and 2006 at two of the sites that their seeds were collected from, in close proximity to populations of adult plants. Seed packets were collected the following October, stored in sealed plastic bags, and kept cool until they were examined within 3 or 4 days. The number of germinated, mycorrhizal seedlings was counted for each packet. A selection of eight germinated seedlings from each site was stored in CTAB buffer, and fungal-specific primers were later used to amplify the ITS region and identify the mycorrhizal fungi, with methods identical to those described above for identifying mycorrhizal fungi in orchid root tissue.

Pollinator-Choice Experiments. We conducted pollinator-choice experiments to investigate whether any change in pollination success in transplanted orchids could be the result of either a reduced visual or olfactory attraction to the pollinator or a mechanical mismatch between flower and pollinator. We observed pollinator choices in the field, using paired inflorescences of sister species attached to a 25-cm T-bar at the end of a long stick (pollinator: *Rediviva longimanus*; orchids: *P. schelpei* and *P. volucris*). One inflorescence from each species was used per choice experiment. *Pterygodium schelpei* is restricted to the range of *R. longimanus*, while the parapatric *P. volucris* is restricted to the range of *Rediviva peringueyi*. Bees visiting oil-secreting plants in the field were approached with the choice stick, and their first choice was recorded. New inflorescences were used after every visit to prevent depletion of oil, and their positions were alternated. The experiment was conducted at a site where *P. schelpei* occurs (experienced bees, Biedouw) and also at a site where bees had no prior experience of orchids (naive bees, Sevilla). The choice experiments were conducted by two investigators working simultaneously for two full days at Biedouw (September 5–6) and for one day at Sevilla (September 9), with all visits observed during these periods recorded. At Sevilla, *R. longimanus* obtains oil exclusively from *Diascia* “white-headii” (Scrophulariaceae). The experiment was repeated at Sevilla, where no orchids occur, to test the possibility that the bees had simply learned to visit the locally abundant orchid species. In the pollinator-choice and reciprocal-transplant experiments, pollinaria were removed where necessary to prevent genetic contamination of wild populations.

To test for the role of scent, the choice experiments were repeated with inflorescences hidden from view. Five inflorescences of either *P. schelpei* or *P. volucris* were put together in each of 10 water-filled jars and covered with opaque mesh bags. Ten control bags contained only water-filled jars. The 20 jars were laid out in a mixed array with 1 m between jars, and the number of times *R. longimanus* landed on a bag or approached it closely was recorded. The experiment was conducted at Biedouw and ran for 3 h (9:00 a.m.–noon, September 10). For this and the other pollinator experiments, the order in which species were collected from the field was alternated, so that neither species was consistently fresher.

Cross-Pollination Experiments. Cross-pollination experiments were conducted to investigate the processes that might lead to partitioning of pollinarium attachment sites among co-occurring orchids. One possibility is that co-occurring orchids sharing the same pollinator may use different pollinarium attachment sites in order to avoid hybridization and the subsequent production of unfit hy-

brids. Alternatively, a high diversity of attachment sites among co-occurring orchids may have evolved to avoid competition for stigma space, such that different parts of the bee represent a series of discrete niches.

First, we tested for genetic incompatibility between co-occurring orchids with the same pollinator and between sister species with different pollinators. Genetic compatibility among co-occurring orchids would be consistent with the hypothesis that different attachment sites have evolved to avoid hybridization. Inflorescences in bud were collected from the field and kept in water-filled test tubes until the flowers opened. Heterospecific and conspecific crosses were performed on different flowers on a single inflorescence, while the position of each of these treatments on the inflorescence was rotated. Pollinaria were dabbed onto the stigma and observed under a dissecting microscope to ensure that massulae adhered. Five to 10 massulae were deposited on stigma lobes in each treatment to simulate pollen loads observed in the field. The water was replaced regularly. When the capsules dehisced, seeds were shaken into a petri dish and examined under a dissecting microscope with backlighting. The seed coat is translucent, allowing easy distinction between filled and unfilled seeds. Crossing experiments were performed in the lab to avoid contaminating local populations with artificially produced hybrid seed. To control for lab conditions, the success of heterospecific crosses was evaluated relative to that of conspecific crosses that were performed on the same inflorescences.

To test whether co-occurring orchids would compete if they shared the same attachment site, we pollinated flowers of one orchid with pollen from three other co-occurring orchids on day 1 and with conspecific pollen on day 2. If pollination were reduced by the prior application of heterospecific pollen, it would suggest that there would be selection to avoid such contamination. From the *R. peringueyi* pollination guild, we used *P. catholicum* (donor/recipient), *P. volucris* (donor/recipient), *Pterygodium alatum* (donor/recipient), *Corycium orobanchoides* (recipient), and *Disperis villosa* (donor). The success of the mixed-pollen-load treatment was assessed relative to that of conspecific crosses performed on a flower on the same inflorescence. The position of the two treatments on the inflorescence was rotated.

Stable-Isotope Analysis. Potential mechanisms by which the use of different fungal partners could promote orchid coexistence were investigated via stable-isotope analysis. If different fungi allow partitioning of the nutrient pool by providing access to different nutrient sources, orchids associating with different fungi should have unique isotope signatures (Gebauer and Meyer 2003). For example, orchids associating with fungi deriving more nutrients from

other autotrophic plants should have higher $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values, whereas those associating with fungi deriving nutrients from decomposition should have lower values. Isotopes of carbon and nitrogen were analyzed for *P. catholicum* and *P. volucris*, which were shown to associate with different fungi (see "Results"). Samples were collected from three sites in the Western Cape province of South Africa: Gydo Pass, Romansrivier, and Tygerberg. At each site, between five and seven 1-m² plots were selected for sampling. From each plot, leaf material was collected from orchid species and from three autotrophic reference plants. A soil sample was also collected. Reference plants always belonged to the genera *Athrixia*, *Oxalis*, and *Pelargonium*, but species sometimes differed between sites. In total, five samples of each orchid species were collected from each site, with a total of 54 reference-plant samples.

Leaf material was oven dried and ground to a fine powder in a Retsch MM301 mixer mill. Relative C and N isotope abundances of leaf material and soil were measured with an elemental analyzer coupled with a gas-isotope ratio mass spectrometer, using methods described by Bidartondo et al. (2004). Relative abundances, denoted as δ values, were calculated according to the equation $\delta^{15}\text{N}$ or $\delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000\text{‰}$, where R_{sample} and R_{standard} are the ratios of the heavy isotope to the light isotope in the samples and the standard, respectively. Standard gases were calibrated with respect to international standards by means of reference substances provided by the International Atomic Energy Agency (Vienna). For each site, differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values between plant species were tested for significance with Kruskal-Wallis nonparametric tests, with subsequent Mann-Whitney *U*-tests for post hoc comparisons. To compare the ^{15}N or ^{13}C abundance of the orchids across all three sites, enrichment factors ($\epsilon^{15}\text{N}$ and $\epsilon^{13}\text{C}$) were calculated, using the following formula (where $X = ^{15}\text{N}$ or ^{13}C):

$$\epsilon X_{\text{sample}} = \delta X_{\text{sample}} - \text{mean } \delta X_{\text{reference plants}}$$

Using enrichment factors eliminates site-dependent differences in δ values. The enrichment factors of the three reference plants will, by definition, cluster around 0. Enrichment factors of the reference plants and each orchid species were pooled across all three sites and compared with Kruskal-Wallis nonparametric tests, with subsequent Mann-Whitney *U*-tests for post hoc comparisons.

Results

Phylogenetic Analyses

All phylogenetic tree nodes in common with the previous analysis of Waterman et al. (2009) were found again here, and we refer to that paper for detailed discussion of re-

lationships. The five additional taxa added here fell into regions of the tree expected from their taxonomy and previously resolved groupings.

Aboveground Interactions

Pollination modes were identified from observations of *Rediviva* bees collecting oil from orchid flowers, from DNA barcoding and morphological analysis of 254 pollinaria attached to captured bees, and from records in the literature (tables A2, A3). Orchid species were found to belong to six parapatric pollination guilds analogous to the *Rediviva peringueyi* pollination guild (Pauw 2006), within which they share the same pollinator. One pollination guild in the Drakensberg region differed by using three species of *Rediviva*; orchids within this guild are pollinated by any of the three bee species, while in each of the other guilds orchids are pollinated by a single species of *Rediviva* (figs. 2D, 3). The different *Rediviva* species have different soil preferences for nesting sites and therefore rarely co-occur, leading to a geographic mosaic of pollinator species (fig. 2D). Therefore, the orchid pollination guilds are all parapatric, with very limited overlap at range margins (fig. 2D).

Reconstructing pollinator traits onto a phylogenetic tree shows that a high proportion of recently diverged taxa differ in mode of pollination (fig. 3; 61%–79% differ in pollinator species, and 50%–67% differ in attachment site, the ranges being the range of values depending on whether sister species or all species that diverged within 5%, 10%, or 15% of the age of the root node were compared). The Jordan index calculations were largely insensitive to the cutoff criteria used to define recently diverged orchids (table A8 in the online edition of the *American Naturalist*); hereafter, we report results for a cutoff of 10%. Recently diverged taxa do not differ in pollinator species significantly more than expected by chance (Jordan index $J = 0.65$; two-tailed $P = .936$; table A8), but they are significantly more likely to differ in either pollinator type or pollinarium attachment site ($J = 0.62$, $P < .001$; table A8). This pattern is a consequence of most allopatric sister species differing in pollinator species but not pollinarium attachment site, while sympatric orchids differ in attachment site but share the same pollinator. Recently diverged orchids are significantly less likely than expected to differ in both pollinator type and attachment site ($J = 0.28$, $P < .001$; table A8).

Comparisons to null models show that co-occurring orchids interact with fewer pollinator species than would be expected if orchid communities were assembled at random (37 sites: two-tailed $P < .001$; fig. 2A), consistent with pollinator type acting as a habitat filter. However, orchid communities show a high diversity of pollinarium attach-

Figure 2: Relationship between mutualistic interactions and co-occurrence. Orchid distributions are reduced to one dimension by means of nonmetric multidimensional scaling (NMDS); orchid species with similar Y-axis values share similar distributions. Each point in the three plots represents a different orchid taxon. This is plotted against pollinator type (A), pollinarium attachment site (B), and fungal clade (C). Points represent orchid taxa, with a different symbol representing each pollinator type. D, Different orchid-visiting *Rediviva* bee species have nonoverlapping distributions, leading to a geographic mosaic of pollinators. This, in turn, leads to the formation of regional pollination guilds. Within guilds, co-occurring orchid species share pollinators but possess a wide range of attachment sites and mycorrhizal fungi. Points on the map represent bee distribution records obtained from the latest revisions and the collections of the South African museum. See table A7 in the online edition of the *American Naturalist* for pollinarium attachment site abbreviations.

ment sites, and co-occurring orchids are less likely to share the same attachment site than would be expected if community assembly were random (37 sites: $P < .001$; fig. 2B), consistent with the partitioning of the bee's body into a series of discrete niches.

Reciprocal transplants of orchid inflorescences between the ranges of parapatric sister species demonstrate significantly lower pollination success among orchids transplanted across range boundaries (Wilcoxon signed-rank test: $n = 8$, $W = 36$, $P = .005$; fig. 4). Pollinator-choice

Figure 3: Above- and belowground mutualisms show contrasting patterns of evolution. While pollination mode differs more than expected between closely related orchid species, fungal preferences are highly conserved. Note that all cases with a shift in pollinator type are allopatric and all cases without a shift in pollinator type are sympatric. Coryciinae associate with fungi from the six fungal clades listed at the top of the table; numbers correspond to the number of individuals found to associate with each clade. Symbols correspond to pollinator (*Rediviva*) species. See table A7 in the online edition of the *American Naturalist* for pollinarium attachment site abbreviations. The orchid phylogeny was constructed via Bayesian analysis of plastid and nuclear DNA regions. All nodes are supported by >95% posterior probability except where indicated.

experiments demonstrate that bees visit orchids found within their range significantly more than closely related orchids normally found within the range of a different bee species ($\chi^2 = 12.5$, $df = 1$, $P < .001$; fig. A1 in the online

edition of the *American Naturalist*). These results are consistent even where individual bees have no prior experience of visiting either orchid species ($\chi^2 = 7.1$, $df = 1$, $P = .008$; fig. A1); that is, one orchid species is not naturally

Figure 4: Reciprocal-transplant experiments confirm the observed phylogenetic pattern of interaction preferences. Inflorescences and seeds were reciprocally transplanted for three pairs of recently diverged orchid taxa: typical and late-flowering forms of *Pterygodium catholicum* (A); *Pterygodium volucris* and *Pterygodium schelpei* (B); and *Pterygodium cafferum* and *Pterygodium pantherianum* (C). Inflorescences were transplanted, and the number of pollinated flowers was recorded after 5 days. Seeds were transplanted in mesh packets, and the number of seed packets containing germinated seeds was measured after 1 year in the field. Orchid pollination rates were significantly higher at their home site (Wilcoxon signed-rank test:

found in the area where the experiment was conducted but does come from a region where the same bee species is also found, whereas the other species comes from a region with a different bee species. The preference is also found where flowers are concealed so that only olfactory cues are available ($\chi^2 = 25.1$, $df = 1$, $P < .001$; fig. A1). Empty control bags received no visits.

Cross-pollination experiments demonstrate that parapatric orchid sister species are genetically compatible but that co-occurring species tend to be genetically incompatible, even when mechanical barriers are artificially overcome (fig. 5; also fig. A2 in the online edition of the *American Naturalist*). In addition, seed set from conspecific crosses is found to be significantly reduced by prior contamination of the stigma with heterospecific pollen (Mann-Whitney U -test: $N = 18$, $P < .01$ for all species; figs. 5, A2).

Belowground Interactions

DNA sequencing of the ITS region from the roots of 231 individual plants shows that orchids associate with six distinct fungal clades (fig. 3). No major differences were apparent in the fungal preferences of orchids sampled over multiple sampling years. The six main orchid lineages each show a strong preference for one of the six clades of fungi, with individuals rarely associating with fungi outside their preferred clade (fig. 3). The three earliest-diverging orchid clades each have a preference for one of three fungal clades—Ceratobasidiaceae, *Tulasnella*, and Sebaciniales-B—that are all rhizoctonia-forming fungi typically utilized by photosynthetic orchids worldwide (Smith and Read 2008). The three more derived orchid clades have shifted to three fungal clades—Sebaciniales-A, *Tricharina*, and *Peziza*—that are not commonly reported from photosynthetic orchids; the Sebaciniales-A clade, in particular, has been reported previously only as mycorrhizal associates of nonphotosynthetic orchids (Weiß et al. 2004). No sister species of orchids associate with different fungal clades ($J = 0$, $P < .001$; table A8). The 231 identified fungi were grouped into 55 unique fungal OTUs. Orchid sister species were found to associate with the same fungal OTU more often than expected by chance ($J = 0.25$, $P < .001$; table A8).

Orchid communities show a higher number of fungal clades (across 37 sites including inferred fungal associations: two-tailed $P = .011$; fig. 2C), and co-occurring or-

$n = 8$, $W = 36$, $P = .005$), with their preferred pollinator (shown on X-axis). In contrast, there is no significant difference in seed germination between treatments ($n = 8$, $W = 14$, $P > .05$). Values are Wilson's point estimates, and error bars show the 95% binomial distribution.

Figure 5: Hand-pollination experiments show that crosses among sister species with different pollinators yield as much seed as conspecific crosses ($N = 5$ pairs), while crosses between co-occurring members of the same pollination guild yield relatively few seeds ($N = 13$ pairs), as do crosses using conspecific pollen mixed with that of other community members to simulate competition for the same pollination niche ($N = 4$ recipient species). Values (means + SD) are standardized per species relative to seed set following conspecific pollination. Further detail is provided in fig. A2 in the online edition of the *American Naturalist*.

chids are less likely to share fungi than would be expected if community assembly were random (across 16 sites with direct fungal associations: two tailed $P = .024$). Note that the conservatism of fungal association within orchid species, and indeed within higher clades of orchids, means that our use of inferred fungal clade across the 37 sites is likely robust to uncertainties, as indicated by the correspondence of results with the more stringent analysis of 16 sites. The number of shared fungal OTUs in communities did not differ significantly, however, from that in the character-displacement null model of community assembly (Muchhala and Potts 2007) designed to test for local evolution of fungal preferences (16 sites: $P = .107$).

Reciprocal transplantation of seeds across parapatric range boundaries shows that orchid seeds germinate just as efficiently in new environments as in their sites of origin (Wilcoxon signed-rank test: $n = 8$, $W = 14$, $P > .05$; fig. 4). In addition, germinated seeds associate with the same fungal partners in both their original and their transplanted environments (table A9 in the online edition of the *American Naturalist*). Finally, two co-occurring orchids with different fungal partners were found to have similar carbon signatures. However, *Pterygodium volucris* was significantly more enriched in ^{15}N than *Pterygodium catholicum* (Mann-Whitney U -test: $P = .0024$; fig. A3 in the online edition of the *American Naturalist*) and the reference plants ($P < .0001$). *Pterygodium catholicum* is also more enriched in ^{15}N than the reference plants ($P = .0012$).

Discussion

This study provides the first simultaneous evidence of the importance of above- and belowground mutualisms for speciation and coexistence in a lineage of plants. Interactions related to reproductive isolation (pollinators) and resource use (mycorrhizas) show different patterns of evolution, which combine to influence patterns of coexistence and speciation.

As expected because of their direct role in reproduction and, potentially, reproductive isolation, pollinator interactions differ frequently between recently diverged species in this group, consistent with the hypothesis that shifts in pollinator interactions drive speciation. The observed patterns suggest that speciation is associated either with colonization of areas with different pollinator guilds or with shifts in pollen attachment site within guilds. Of these two modes, shifts between guilds are twice as common as shifts within guilds (fig. A4 in the online edition of the *American Naturalist*). Very few recently diverged orchids differ in both pollinator and attachment site, suggesting that shifts in one of these interactions may be sufficient for reproductive isolation.

Field experiments provide support for the putative mechanisms inferred from the phylogenetic analyses. Reciprocal transplants of orchid inflorescences between the ranges of parapatric sister species show low but not negligible pollination success when orchids are transplanted across range boundaries (fig. 4). Pollinator-choice experiments provide evidence that orchids are adapted to the innate preferences of local *Rediviva* bees (fig. A1). The low pollination success of transplanted orchids is due to their reduced visual and olfactory attractiveness to pollinators outside their native range, at least in the species tested. Experiments with naive bees suggest that these preferences are not simply learned through prior exposure to the locally abundant orchid species. Therefore, orchids colonizing new areas are likely to both experience selection to improve their attractiveness to the local pollination guild and diverge from their ancestral populations. In other groups of orchids, it has been hypothesized that small changes in floral scent compounds can lead to the attraction of different pollinators and reproductive isolation (Mant et al. 2002; Huber et al. 2005; Stökl et al. 2009), and it may be likely that a similar mechanism operates here. In contrast, the same fungal partners are found in different areas, and clades of orchids display conserved preferences for a particular partner. Therefore, orchids are unlikely to shift their fungal partners when they diverge in different regions, because the same fungal partners can be recruited in each region. It remains possible that subtle differences in fungal partners within OTUs might exist between different regions but were not manifest in terms

of the percentage germination of seeds. In addition, fungal species diversity may have been underestimated by the use of a DNA sequence similarity cutoff to define OTUs. However, irrespective of the taxonomic resolution of fungi versus pollinators, our experimental results indicate very different patterns in how well orchids can recruit effective partners in different geographical regions. Effective pollination does not occur outside native regions, whereas effective fungi—indistinguishable at the level of ITS OTU—can be recruited. Overall, therefore, it seems that fungal partners cannot be important drivers of speciation in this system.

Our results strongly suggest that pollinators play a direct role in speciation, whereas mycorrhizas do not. However, we cannot conclusively demonstrate that pollination shifts have a causal role in orchid species from the phylogenetic patterns or from the experiments providing evidence for plausible mechanisms for those patterns. It remains possible that shifts in pollinator type are an incidental consequence of occupying a new area and that other factors, such as geographical isolation per se or other environmental differences between regions, play a more direct role in driving divergence and reproductive isolation. Conservatively, therefore, we conclude that there is a significant difference in patterns between pollination and mycorrhizal interactions, which matches predictions based on their roles in reproduction and resource use, respectively. Our findings are consistent with those of other studies of orchid diversification that have shown a potential role for pollinator shifts in plant diversification (Johnson et al. 1998; Schlüter et al. 2009; Stökl et al. 2009; Vereecken et al. 2010) and have found shifts in fungi to be an unlikely driver of speciation (Barrett et al. 2010; Roche et al. 2010; but see Taylor et al. 2004). Our results confirm these findings but for the first time consider the role of both mutualistic partners in the same orchid taxon.

Both mutualisms appear to play a role in coexistence. Six allopatric pollination guilds are identified among Coryciinae, and within each region successful pollen transfer is possible only for orchids that conform to a specific syndrome of traits that attract the local *Rediviva* bee. This phenotypic similarity among co-occurring orchid species is consistent with a role for pollinators as a habitat filter. In contrast, the high diversity of pollinarium attachment sites among co-occurring orchid species is consistent with niche partitioning of the pollinating bee's body. In theory, this partitioning of attachment sites among co-occurring orchid species may be caused by either reinforcement against hybridization or competition for efficient pollen transfer (Armbruster et al. 1994). Reinforcement appears to be a viable explanation in some species—hand-pollination experiments show that crosses between parapatric sister species produce filled seed embryos (figs. 5, A2), and

one pair of sympatric sister species is known to hybridize (Steiner and Cruz 2009)—however, competition for efficient pollen transfer seems likely to be the stronger force in current communities. Hand pollination showed that co-occurring species tend to be genetically incompatible (figs. 5, A2), even when mechanical barriers are artificially overcome, which would limit the strength of selection for reinforcement among those species. In contrast, seed set is significantly reduced when the stigma is clogged with heterospecific pollen before the application of conspecific pollen (figs. 5, A2), which might cause negative interactions between orchid species without partitioning of pollinarium attachment sites.

Despite conserved preferences among closely related orchid species, a high diversity of fungal partners is found within orchid communities. This is consistent with the hypothesis that different fungal partners are needed for orchid species co-occurrence, through their roles in establishment and, potentially, resource partitioning. The number of shared fungi in communities did not differ significantly from that in a character-displacement null model of community assembly designed to test specifically for local evolution of fungal preferences among different populations of each orchid species. This indicates that the observed pattern is due to the community-assembly processes rather than to character displacement or population-level adaptation.

The mechanism behind partitioning of fungal partners within orchid communities is unknown, but it is likely to reflect access to different resources by different fungal taxa. This is supported by stable-isotope abundances, which suggest that orchid species with different fungal preferences do indeed have access to different sources of nitrogen (fig. A3). However, this should be viewed as preliminary evidence until there is better general understanding of the physiological mechanisms underpinning nutrient acquisition in orchids (Leake and Cameron 2010). A thorough investigation into how orchids compete for nutrients via mycorrhizal fungi was beyond the scope of this study, but it would be a productive avenue for future research.

Our findings provide an interesting contrast to previous studies of phylogenetic community assembly, which have argued that traits relating to resource partitioning are expected to be evolutionarily labile, because it is essential that differences in these traits evolve if species are to coexist (Silvertown et al. 2006). Habitat-determining traits, in contrast, were argued to evolve more slowly. We find the opposite pattern in Coryciinae. The most rapidly evolving trait, pollinator type, acts as a habitat filter, while traits relating to pollinarium attachment site and mycorrhizal fungi, which have to differ for orchid species to co-occur, are more evolutionarily conserved. One explanation is that orchid communities in South Africa have resulted from

in situ radiation, whereas earlier studies considered plant communities assembled from relatively unrelated species. Because speciation mostly involves geographical isolation, the most rapidly evolving traits, namely, those associated with speciation, will tend to differ between species in different areas.

After geographic speciation, the buildup of alpha diversity within communities requires the origin of traits that permit coexistence. Although we have no direct evidence for competition for nutrients in these orchids, our data indicate that shifts in fungal partner have occurred that allow different orchid clades access to different resources. The three basal orchid clades each have a preference for conventional fungi used by photosynthetic orchids worldwide (Smith and Read 2008). The more derived orchid clades have shifted to three different fungal clades normally not associated with photosynthetic orchids. Mycorrhizal fungi show a high diversity of enzymatic capabilities (Bruns 1995), and different fungal lineages are likely to have access to different nutrient resources. Therefore, hypothetically, if an ancestral orchid became adapted to a novel fungal lineage, it could enter into the preexisting pollination guilds and adapt to new pollinator species without competing with sympatric orchid species for nutrients. Shifts in fungal preference may therefore represent key innovations (Hodges and Arnold 1995; Wheat et al. 2007; Futuyma and Agrawal 2009) that allowed successive clades of orchids to spread through the region. It should be noted that although seed germination experiments confirm the mycorrhizal status of some of the identified fungi, it is difficult to determine whether all the identified fungi are mycorrhizal, as opposed to being other fungal symbionts. However, the use of microscopically isolated root sections and the repeatability of ITS sequencing give us confidence that the fungi we identified are mycorrhizal partners beyond reasonable doubt.

Our results strongly suggest that mutualistic interactions have had dramatic effects on the evolution and coexistence of species within a plant radiation. With the potential for different organisms to respond differently to climate change (Parmesan 2006; Tylianakis et al. 2008) and concern over worldwide declines in pollinator abundance and soil quality (Biesmeijer et al. 2006; Pauw 2007), it is becoming increasingly vital to understand the effect of biotic interactions on plant species and communities. However, most previous studies have focused on how abiotic factors affect community assembly (Cavender-Bares et al. 2004; Slingsby and Verboom 2006), while ignoring the greater community context in which each plant species interacts with multiple other species (Strauss and Irwin 2004). In our study, the diversity of orchid communities is intimately linked to the diversity of relatively little known organisms such as oil-collecting bees and mycorrhizal fungi. This

implies that effective conservation of plant species such as these orchids requires a full understanding of the interactions that drive their divergence and coexistence.

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