Frequent shifts in pollination strategy are decoupled from diversification in the terrestrial orchids

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Abstract

Pollinator attraction strategies are central to orchid reproductive biology and have long been hypothesised to accelerate speciation rates, particularly through specialised coevolutionary interactions. However, most macroevolutionary evidence comes from studies of individual genera or tribes, leaving broad-scale patterns unresolved. Here, we reconstruct the evolution of pollination strategy in the terrestrial orchid subfamily Orchidoideae and test for associations with speciation rate. We identify multiple transitions among reward-based, deception-based, and autonomous pollination, but via an evolutionarily constrained pathway in which some transitions do not occur. We find no significant impact of any pollination strategy on speciation rates, nor degree of specialisation or pollinator identity. Despite shaping ecological interactions and reproductive isolation, pollination strategies do not strongly impact diversification in orchids. Our findings support a growing view that plant radiations are shaped by complex interactions between traits, ecological opportunity, and environmental context, rather than by any single force alone.

Introduction

Orchids are one of the most successful angiosperm families, with some ~29,500 species found in nearly every terrestrial ecosystem (1–3). They are among the most celebrated families, having held the attention of evolutionary biologists since Darwin's early work on their relationships with pollinators (4). Darwin hypothesised that the adaptation of floral traits in response to pollinator interactions could drive reproductive isolation, leading to speciation into numerous forms. This model remains influential in investigations into the origins of diversity in orchids (5–9), other flowering plant clades (10–12), and across angiosperms more broadly (13–15). A great deal of orchid variation, especially floral morphology, is linked to their extraordinary variety of pollination strategies (16–19). These range from mutualistic interactions involving nectar, oils, or sleep-site rewards, to deceptive strategies like food mimicry, brood-site deception, and sexual deception (16, 20). One iconic example of the latter is the sexually deceptive *Ophrys*, which trick male bees and wasps into pollination by having flowers that resemble female insects, and by mimicking female pheromones (21, 22). Pollinator specialisation has long been proposed as a mechanism for driving diversification, particularly in genera where small shifts in floral traits may lead to rapid reproductive isolation (23, 24). However, evidence across orchids is mixed. Some studies suggest a link between pollinator specialisation and speciation (23, 24), while others suggest other factors, such as geographical shifts and environmental change (2, 25–28), and adaptive traits such as epiphytism and CAM photosynthesis (in subfamily Epidendroideae, (5, 6)) are more powerful predictors of diversification.

One challenge in identifying the drivers of orchid diversification is data limitations. Phylogenies that are well sampled at the species level, important for analyses characterising diversification rate heterogeneity (29), have only recently become available (2, 25). As a result, most previous research into pollinator evolution has focussed on smaller lineages, such as individual genera or tribes (23, 24). Similarly, data on pollinator variation has historically had poor coverage across the diversity of orchid lineages, a problem that is also encountered in other families (30–32). However, a recent compilation of pollination traits has been published which samples the majority of genera, and ~10% of orchid species (16). While there are certainly taxonomic and spatial gaps in the data, we now have a framework for investigating the macroevolution of pollination in the orchids, if we use methods that account for incomplete and imbalanced sampling.

The subfamily Orchidoideae (~5,000 species) are an ideal study system for this hypothesis. Unlike their sister subfamily Epidendroideae, in which rapid diversification was driven by epiphytism and Crassulacean acid metabolism (CAM) photosynthesis (5, 6), Orchidoideae are predominantly terrestrial. They are extremely widespread, especially in the extratropical regions (25), and are characterised by exceptional diversity in pollination strategies, having evolved both reward-based and deceptive strategies (16). Furthermore, many genera, such as *Gymnadenia*, *Herminium*, *Ophrys* and *Orchis* were the subjects of Darwin's early observations underlying his coevolutionary hypothesis (4). Understanding the evolution of pollination strategy diversity, and impacts on diversification dynamics, is critical for fundamental evolutionary knowledge, but also for conservation. As global pollinator populations continue to decline (33, 34), the diversity of pollinator-dependent orchids is threatened (35). By understanding how past evolutionary dynamics have, or have not, changed in response to pollinator shifts, we may improve our ability to predict how orchids, many of which are threatened with extinction (3), will fare in the future.

Here, we investigate the evolutionary dynamics of pollination strategy in terrestrial orchids. Using a densely sampled phylogeny (25) and a curated dataset of pollination traits (16), we reconstruct ancestral states, estimate transition rates between strategies, and test for associations with diversification. We utilise character-free diversification methods that correct for incomplete taxonomic sampling (36, 37), in the absence of comprehensive coverage of traits, thereby avoiding the currently incalculable state-dependent sampling fractions required by classic state-dependent speciation and extinction models (38, 39). Our results reveal frequent but constrained transitions among pollination strategies, but no consistent relationship between any strategy and speciation rate. These findings suggest that while pollination strategy contributes to ecological and reproductive diversity, it plays a limited role in shaping macroevolutionary patterns of diversification across the clade.

Results

Evolutionary model and ancestral states

We investigated the evolutionary history of pollination strategies in a taxonomically-wide sample of Orchidoideae, sampling 485 species in 88 genera (~10% of species and ~43% of genera (1)). We reconstructed ancestral states after comparing support for six different transition models (equal rates (ER), symmetrical (SYM) and all-rates-different (ARD)), all with and without hidden rates (40). The best-supported model was ARD without hidden states (AIC weight ~ 1), indicating variation and asymmetry in transition rates among strategies. Ancestral state reconstruction under this model in deep nodes is uncertain, without majority support for any state at the root (Figure 1). The highest supported root state is lipid reward (0.47), and there is much weaker support for other strategies. We inferred origins and losses of major pollination strategies across the phylogeny. The earliest branches are predominantly reward-based, with nectar reward in branches leading to tribes Diurideae, Codonorchideae and Cranichideae and lipid reward in branches leading to tribe Orchideae. Transition counts among pollination strategies were highly asymmetric (Supp Mat). The most frequent transition was from nectar reward to autonomous selfing (median n transitions = 51), followed by shifts from food deception to nectar reward (median n = 30), and from autonomous selfing to food deception (median n = 19). Transitions into sex deception were rare, with low counts from food deception (median n = 3) and autonomous selfing (median n = 9), and there were no transitions from other strategies into lipid reward. Notably, lipid reward appears to be evolutionarily constrained, with transitions away from it (median n = 12) but none toward it, consistent with its early origin and subsequent persistence within Orchideae.

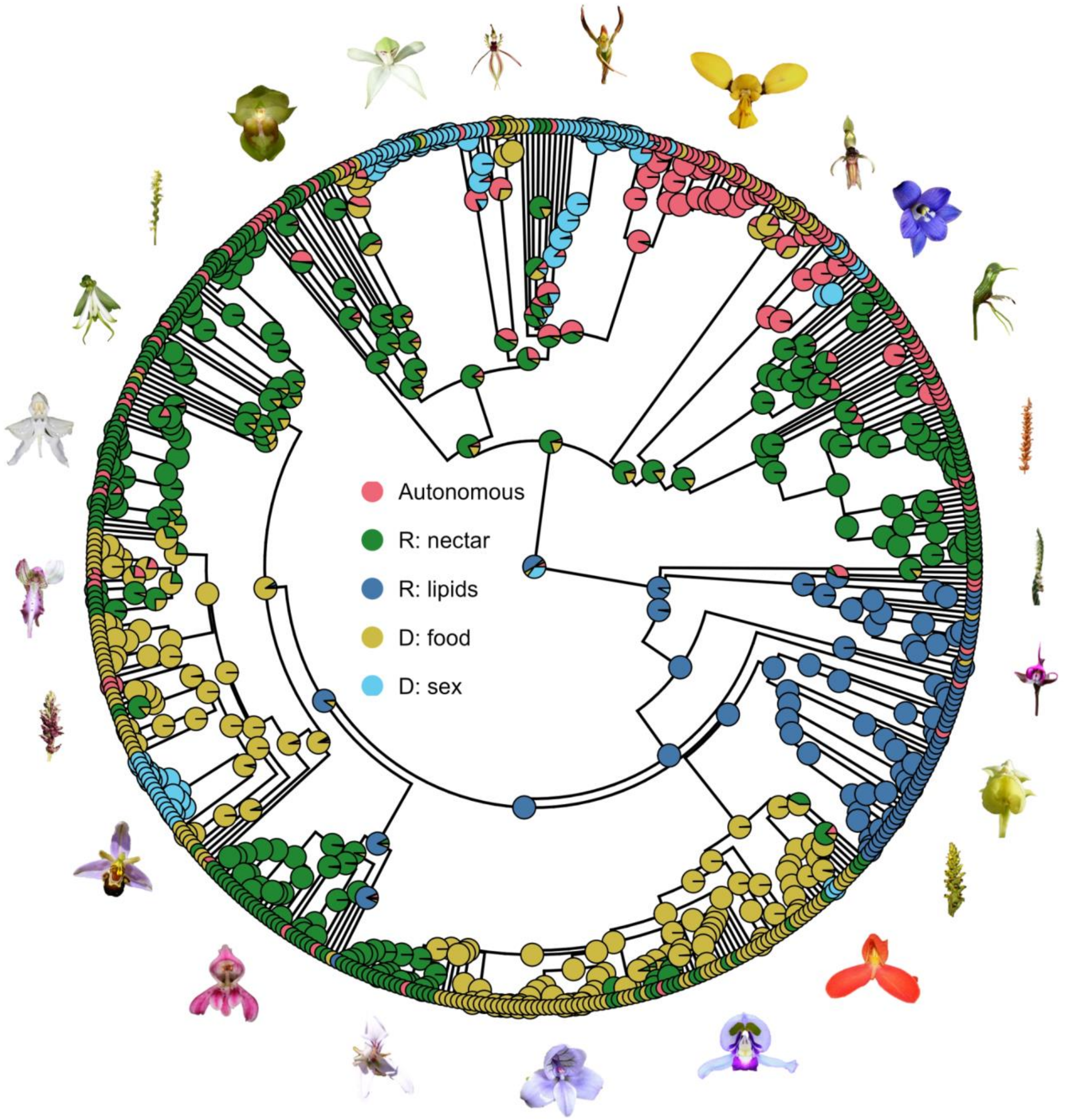

Figure 1. Phylogenetic distribution of pollinator attraction strategies in Orchidoideae. Tips and nodes are coloured by pollination strategy: autonomous selfing/agamospermy (red), nectar reward (green), lipid reward (dark blue), food deception (yellow), and sex deception (light blue). Pie charts at internal nodes represent posterior probabilities of ancestral states based on 300 stochastic character maps estimated with an all-rates-different transition model, using corHMM (40). The root state is uncertain, with highest support for a lipid reward strategy. Flower photographs are provided under Creative

Commons licences, sourced primarily from Flickr and Wikimedia Commons (details in Supp Mat), and are positioned approximately at their respective species or genera in the tree, but spaced for visual clarity.

Directional transitions and trait lability

We further explored transition rates using an ARD reversible-jump MCMC framework, which, unlike corHMM models, incorporates rate uncertainty and infers support for whether transitions occur at non-zero rates (41). This analysis suggests that certain strategies, especially food deception and sex deception, are relatively labile and have transitioned to several other states. Autonomous selfing also shows some evolutionary lability, with transitions to food and sex deception. In contrast, reward-based strategies, particularly lipid reward, show strong evolutionary constraint. Lipid reward only significantly transitions to autonomous selfing, and no other strategy transitions to lipid reward at a significant rate. Nectar reward similarly shows low lability, with significant transitions only to autonomous selfing, though several strategies appear able to transition into nectar reward.

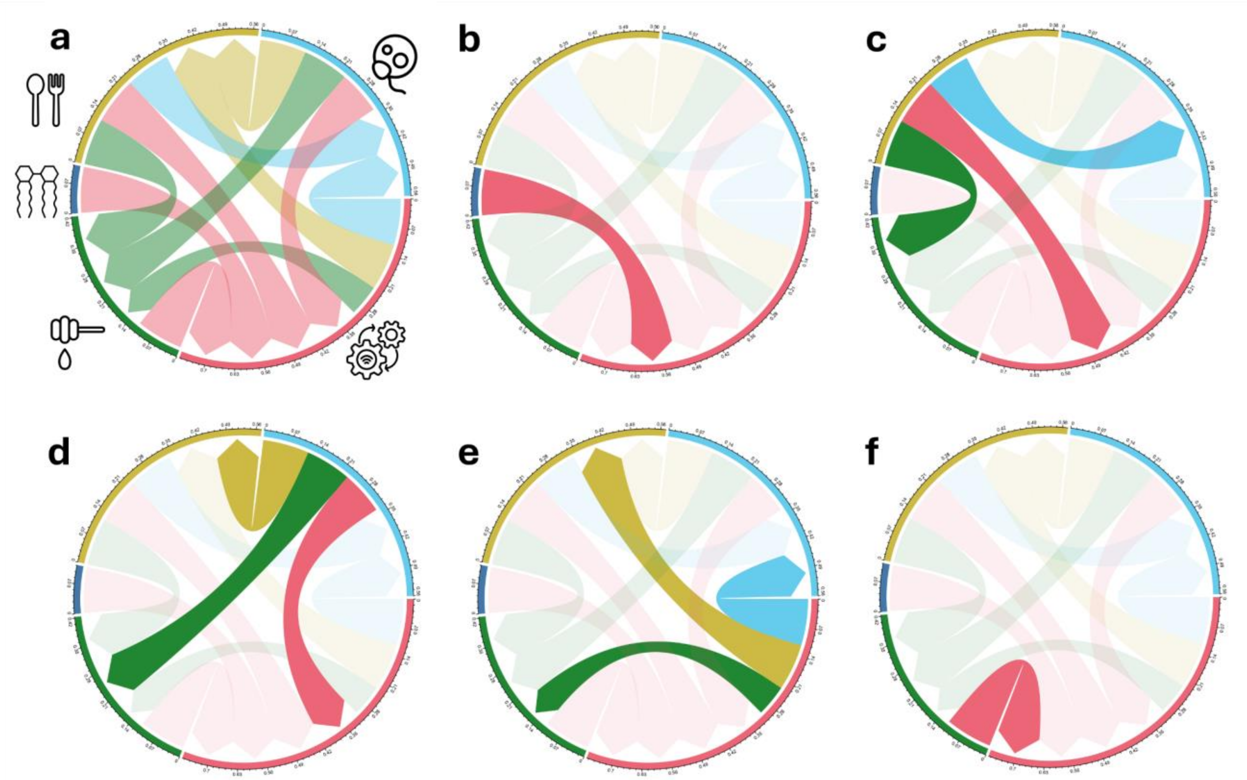

Figure 2. The complex evolution of diverse pollination strategies in Orchidoideae. Reward-based strategies are less evolutionarily labile than deception-based strategies, and autonomous selfing is a common macroevolutionary destination. (a) A chord diagram showing all significantly non-zero pairwise transition rates among pollination strategies, estimated using an all-rates-different model with reversible-jump MCMC. Chord widths are proportional to posterior median transition rates. (b–f) Directionality of transitions from each focal strategy, individually (b = lipid reward, c = food deception, d = sex deception, e = autonomous selfing, f = nectar reward). Icons are provided under Creative Commons licences, sourced from The Noun Project (details in Supp Mat).

Speciation rates are not strongly shaped by pollination strategy

We tested whether pollinator attraction strategies are associated with differences in tip-speciation rate using rates estimated with BAMM (36) by Thompson et al. (25) (Figure 3). Across strategies, we observed substantial variation in speciation rates. Species with the lipid reward strategy exhibited the lowest average tip speciation rates with narrow variance (mean = 0.25, SD = 0.07). In contrast, sex-deceptive species had the highest average speciation rates but with broad variance (mean = 2.98, SD = 1.85). Species with autonomous selfing, food deception, and nectar reward have intermediate mean speciation rates, broad variances, and heavily overlapping distributions (Figure 3). However, when assessing with a phylogenetic method that controls for background diversification rate-heterogeneity (42), we find no significant associations either when treating different polymorphic combinations as a single trait (e.g. autonomous selfing with nectar reward) (Kruskal-Wallis $p = 0.091$) or removing polymorphic species (Kruskal-Wallis $p = 0.11$). Non-significance is also found when binarising each strategy (Mann-Whitney $p > 0.05$), and when categorising species broadly, comparing autonomous selfing versus reward-based versus deception-based strategies (Kruskal-Wallis $p = 0.14$) (Supp Mat). It must be noted that lipid reward and sex deception had “nearly significant” impacts on diversification in these binary tests (Mann-Whitney $p = 0.058$ and 0.079, respectively), with lipid reward decelerating and sex deception accelerating diversification. The bimodal distribution of speciation rates among sex-deceptive species is partly driven by monotypic genera such as *Leporella* and *Spiculaea*, as well as other small genera.

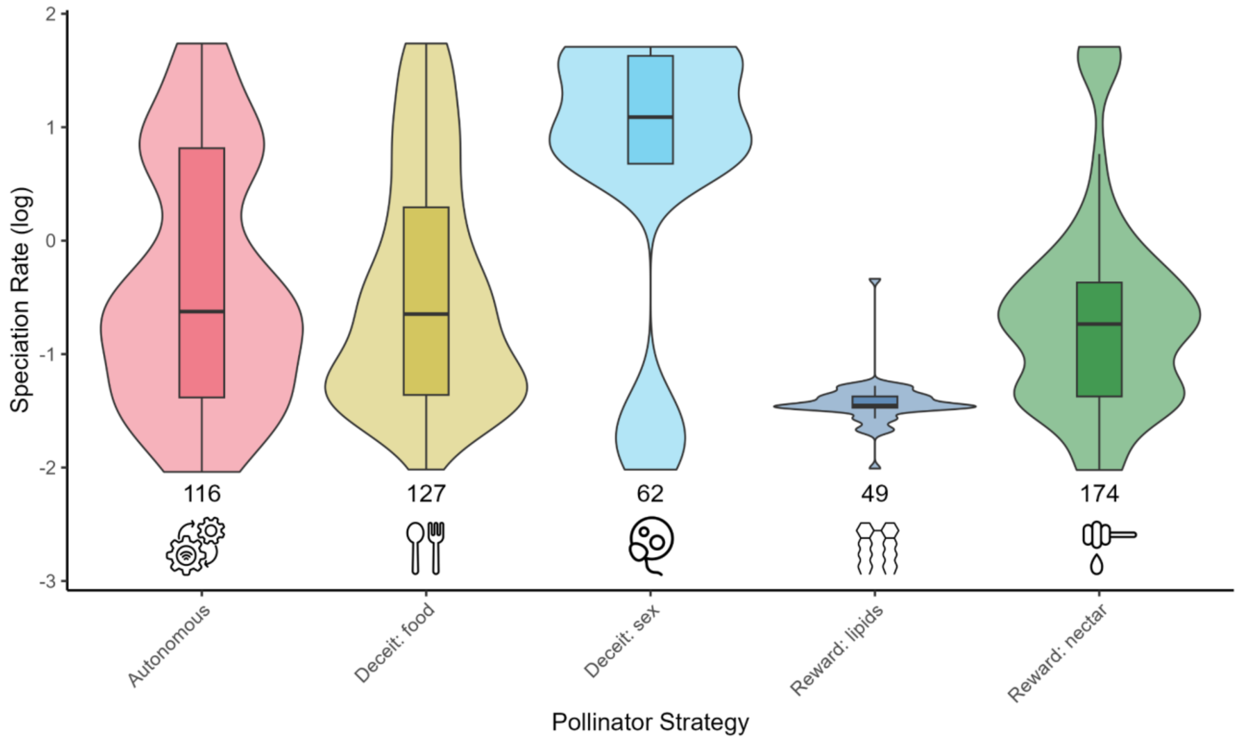

Figure 3. Speciation rates vary across pollination strategies but differences are not statistically significant. Violin plots show the distribution of log-transformed tip speciation rates estimated using BAMM, grouped by pollination strategy. Sample sizes are indicated below violins. While species with sex deception tend to have higher median rates and those with lipid rewards lower, STRAPP tests indicate that differences among strategies are not significant ($p > 0.05$), when background rate heterogeneity and effects of shared ancestry are accounted for. Icons are provided under Creative Commons licences, sourced from The Noun Project (details in Supp Mat).

Pollinator specificity and identity are not associated with diversification rates

We tested whether BAMM speciation rates in Orchidoideae are shaped by pollinator number, functional pollination specificity (FPS, defined as the number of pollinator families multiplied by the number of pollinator orders (16)), specialisation level (generalist versus specialist), and dominant pollinator order (Hymenoptera and Lepidoptera). Across all tests, we found no significant association with BAMM speciation rates (Figure 4). Pollinator number and FPS (Figure 4a) showed wide variation across species but did not correlate with tip speciation rates. STRAPP tests confirmed that neither variable was a significant predictor of diversification (pollinator number Spearman $p = 0.81$, FPS $p = 0.80$), including when excluding autonomous selfing species (number p = 0.65, FPS p = 0.76). Similarly, speciation rates did not differ significantly among autonomous selfing, generalist, and specialist species (Kruskal-Wallis, p = 0.82; Figure 4b). Nor did we find significant differences between orchids pollinated by Hymenoptera and all other orchids (Mann-Whitney, p = 0.84; Figure 4c), or between those pollinated by Lepidoptera and other orchids (Mann-Whitney, p = 0.87; Figure 4d).

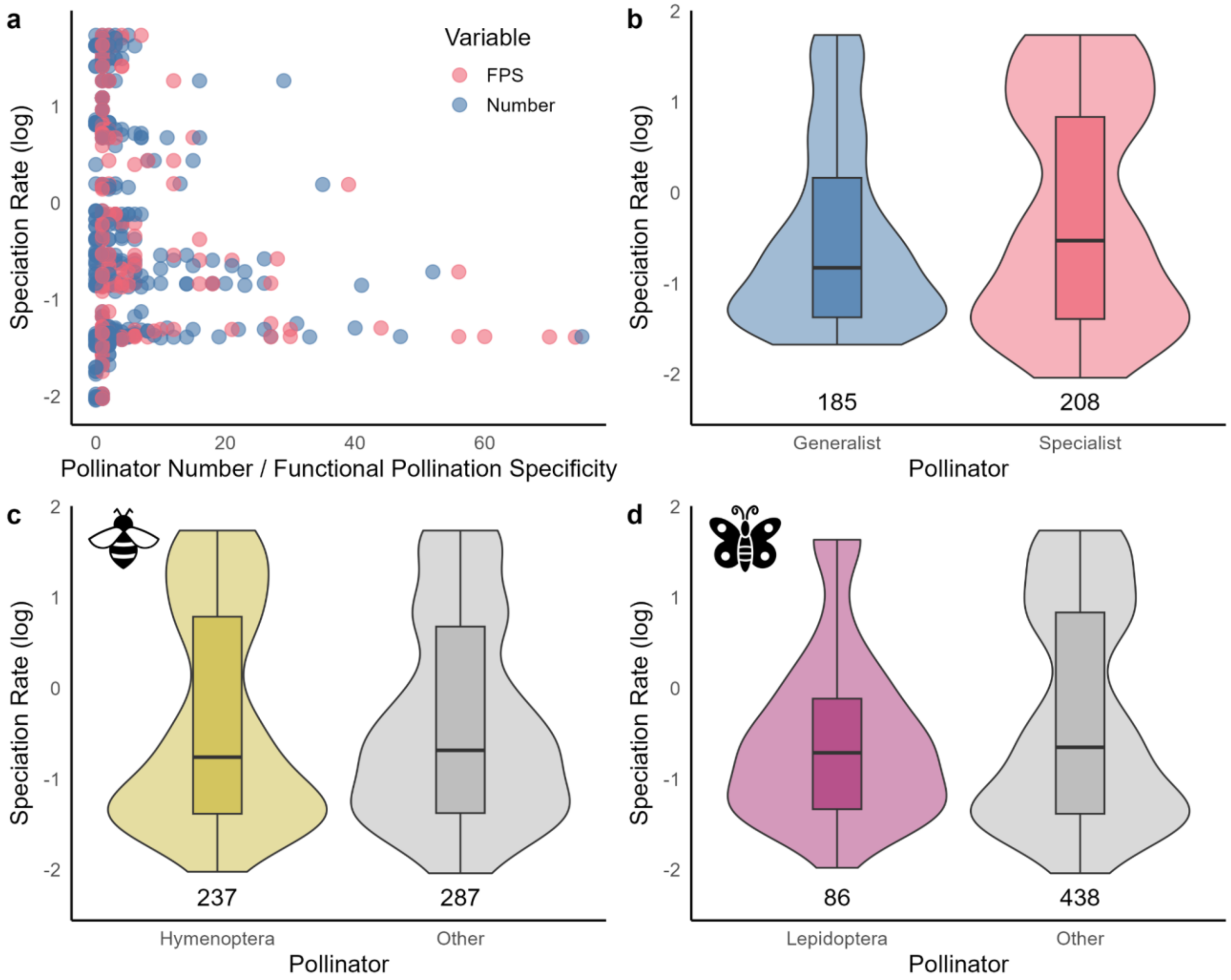

Figure 4: Speciation rates are not significantly influenced by pollination breadth or pollinator identity. (a) Scatterplot showing no correlation between speciation rate and either pollinator number or functional pollinator specificity. (b) Violin plots comparing generalist and specialist species (autonomous selfing species are not shown but were included in statistical tests). Speciation rates by Hymenoptera versus others (c), and Lepidoptera versus others (d). Statistical tests for all comparisons were non-significant ($p > 0.05$). Icons are provided under Creative Commons licences, sourced from The Noun Project (details in Supp Mat).

Robustness to alternative methods of estimating diversification rates

We re-analysed the time-calibrated phylogeny of (25) using an alternative method, Missing State Speciation and Extinction (MiSSE) (37). MiSSE and BAMM differ in assumptions and methods, notably by correcting rates to a global sampling fraction instead of genus-level fractions, which has been criticised (43). We replicated all previous analyses of state-dependent diversification and found qualitatively the same results (Supplementary Materials). Agreeing with results of analyses of BAMM rates, none of pollinator strategy, number and FPS, specialism or identity, have significant impacts on MiSSE-estimated speciation rates ($p > 0.05$, in all cases).

Discussion

The idea that pollinator evolution drives orchid diversification goes back to Darwin, who linked orchid variation to interactions with insect pollinators (4). Later work expanded this to a broader theory of pollinator-driven speciation across angiosperms (24, 44), which remains a powerful hypothesis. The terrestrial orchids of subfamily Orchidoideae (~5,000 species) are an ideal case study for pollinator evolution, as they have remarkable variation in both pollinators and diversification rates, but lack epiphytism, which accelerated diversification of their sister subfamily Epidendroideae (>21,000 species) (5). In our study, by analysing the most comprehensive Orchidoideae phylogeny (25) and pollinator dataset (16), we find no strong link between pollinators and diversification rates, a result that is robust to methodological differences. Our analysis includes 485 species from 88 genera (~10% of species and ~43% of genera (1)), with some unevenness in sampling, notably 99 species of *Disa*. While *Disa* dominates the tree, this is not necessarily a drawback. *Disa* is a model genus for pollination biology and captures a wide range of pollination strategies (24, 45, 46). With current data limitations (1, 2, 16, 25), this represents the best-sampled analysis possible. While transitions to different pollination strategies are common and rapid, albeit evolutionarily constrained, they do not accelerate speciation rates. We suggest that other factors, such as environmental forces and complex ecological interactions, may play a larger role in shaping speciation, a hypothesis that is gaining in strength in different taxa (5, 16, 25, 47–51).

Transition patterns reflect ecological lability and evolutionary constraint

Pollination strategies in terrestrial orchids are evolutionarily labile, showing frequent transitions among strategies and a high level of ecological flexibility. However, these transitions are not uniform and we recovered asymmetries, which suggests there are evolutionary constraints (52–54). Our ancestral state reconstructions and transition rate models indicate that some strategies, especially food deception, nectar reward, and autonomous selfing, are common evolutionary destinations. In contrast, some lipid reward and sexual deception are rarely gained. These patterns likely reflect a combination of developmental constraints, physiological trade-offs, and ecological selection pressures (55, 56). Reward-based strategies such as nectar and lipid production often involve substantial non-selfish investment and may only be advantageous in environments with reliable pollinator communities. Once lost, these strategies may be difficult to regain, especially if alternatives such as deception or selfing provide adequate reproductive success. In contrast, deceptive strategies may offer a lower-cost method of selfish pollination (55, 57), favouring transitions from other strategies without the same level of investment into nectar or lipid production. The relative rarity of sexual deception may reflect its greater ecological specificity regarding floral mimicry of female bees (21, 22), while the infrequent gains of lipid reward are consistent with its inferred ancestral status and apparent evolutionary stability within Orchideae.

Autonomous selfing appears to be a relatively common macroevolutionary destination, although the degree to which different strategies transition to autonomous selfing differs by method, with more source strategies inferred by rj-MCMC than corHMM. While up to 88% of orchids are self-compatible (16), the evolution of autonomous selfing may provide a low-cost mechanism when pollinators are scarce or unreliable. The observed asymmetry in transitions may therefore reflect both physiological costs of floral trait expression and ecological selection for reproductive assurance, making autonomous selfing an evolutionarily accessible and frequently favoured solution. However, it must be noted that this pattern could also arise from data scarcity. As Ackerman et al. (16) acknowledge, a substantial proportion of species classified as autonomously selfing were scored based on indirect evidence, such as high fruit set without observed pollinators, rather than experimental exclusion trials. In fact, only 44% of the species in their total dataset assigned to autonomous selfing or agamospermy were supported by experimental data. This introduces the possibility that the prevalence of autonomous selfing may be overestimated, especially when recorded from fieldwork or short-term studies, where pollinators may simply go undetected. Given that many orchids are visited infrequently and require extended observation to confirm pollination, caution is warranted in interpreting the evolutionary accessibility or prevalence of this strategy.

Pollination specialisation is not a direct driver of diversification

Despite long-standing hypotheses that pollination specialisation promotes orchid speciation, our results challenge this. We find no impact of pollination strategy, pollinator number, functional pollinator specificity, or pollinator order (Hymenoptera and Lepidoptera) on speciation rate. This implies that the observed variation in speciation rates is shaped by background lineage variation that may be linked to other forces, rather than being coupled with pollination (42). While important examples of rapid speciation through pollinator shifts have been documented in iconic genera such as *Ophrys (23)* and *Disa* (24), these appear to be clade-specific rather than generalisable processes. An important example of this is comparing speciation rates of the larger sex deceptive genera with those from monotypic, or species poor, sex deceptive genera, such as *Leporella* and *Spiculaea*. While some sex deceptive groups certainly do speciate faster than relatives with other strategies, rapid speciation of sex deceptive species is clearly not a universal rule. Despite disagreeing with decades of theory, our findings do agree with work previously discussed. This pattern is consistent with broader macroevolutionary observations across the orchid family. Using a phylogenomic reconstruction, Givnish et al. (5) found that while deceit pollination, commonly suggested as a trigger of diversification, did greatly increase overall species richness, this was not via accelerated diversification. They also find a strong impact by pollination via Lepidoptera and euglossine bees, that we do not recover here in a better-sampled but more taxonomically-focussed phylogeny. Givnish et al. (5) instead found strong impacts of pollinia, epiphytism, CAM photosynthesis and tropical distributions. Similarly, Gravendeel et al. (6) reported no association between pollinator specialisation and species richness across orchid subfamilies, instead finding a stronger impact of epiphytism in some genera.

One possible explanation for this decoupling between pollinator variation and diversification rates lies in the demographic context of orchid reproduction. As Tremblay et al. (7) describe, many orchids are pollen-limited and have skewed reproductive success, with a few individuals achieving pollination while the majority do not. In this context, pollinator specialisation may constrain reproductive assurance, thereby reducing the potential for long-term speciation. Furthermore, even flexible or rapidly evolving pollination strategies may not increase the probability of speciation, especially if gene flow remains limited or inconsistent. Another potential explanation lies in abiotic forces, which can shape macroevolutionary dynamics beyond the effect of adaptive traits (47). Recently, Thompson et al. (25) demonstrated that speciation in Orchidoideae is associated with global cooling throughout the Cenozoic, and Guo et al. (26) found an impact of sea level on *Paphiopedilum* diversification. An impact of abiotic drivers was also found by Pérez-Escobar et al. (28), who found that Neotropical epiphytic orchid diversification was associated with Andean uplift and geographic shifts, rather than different pollinators. Subsequent work by Pérez-Escobar et al. (58) strengthened this by showing that environmental sex determination (ESD) is associated with increased diversification in Neotropical epiphytic orchids, but ESD itself is triggered to evolve by habitat fragmentation and climatic instability. Therefore, the impact of reproductive traits on diversification is likely to be context-dependent, shaped by interactions with abiotic forces and lineage-specific constraints, rather than being a general macroevolutionary driver.

The complexity of triggering rapid diversification

Our results highlight that rapid diversification in orchids likely arises from complex interactions among ecological, geographic, and evolutionary factors, rather than from single traits like pollination strategy. This contributes to the growing appreciation showing that triggers of diversification are rarely explained by simplistic models (39, 47, 48, 59). While pollination strategy in terrestrial orchids is certainly a powerful driver of ecological differentiation (7), it does not consistently trigger increased diversification. Recent work in another diverse family also found this pattern. It was previously thought that pollinator divergence was a driver of cactus diversification (11), but by sampling biotic and abiotic variables widely, Thompson et al. (47) found that it is not among the strongest predictors, when accounting for complex interactions. Instead, diversification was shaped primarily by abiotic variables, and the interplay between these and biotic traits. Such results challenge the traditional narrative that key traits can act in isolation as direct drivers of speciation, and instead highlight the multifactorial nature of evolutionary radiations. Bouchenak-Khelladi et al. (49) offer a useful framework for interpreting these patterns. They argue that adaptive radiations require not just adaptive traits, but the optimal ecological conditions, which they delimit as “backgrounds”, “triggers”, and “modulators”. In orchids, pollination strategy may serve as a modulator or context-dependent trigger that facilitates divergence when coupled with ecological opportunity or environmental change, but cannot drive radiation in isolation. Bouchenak-Khelladi et al. (49) distinguish “polymorphic” traits from “simple” traits that are conserved and necessary for survival. Polymorphic traits are those which are labile and variable, and can promote diversification by allowing species to partition ecological niches or undergo reproductive isolation. They are not necessarily the cause of radiation, but can facilitate or maintain it once it begins. The recovered lability of pollination strategies, and the different ways in which they mediate interactions with the environment and other organisms, may fit this description. Unless matched with extrinsic triggers or ecological shifts, pollinator strategy alone may not lead to accelerated diversification.

Conclusion

Orchids have long captured the attention of evolutionary biologists and horticulturalists with their remarkable floral adaptations (1, 2, 4, 5, 7, 16). Darwin posited that pollinator specialisation, and specific deceptive strategies, were the result of coevolution and could lead to reproductive isolation. However, our results suggest that while pollinator strategy is very labile, it is not consistently associated with increased diversification. Together with recent work in other groups (47, 48), our findings underscore the complexity of macroevolution. They support a growing consensus that evolutionary radiations emerge from the complex interplay of traits, ecological context, and historical effects (49). Further work should begin to integrate all forces hypothesised to be important simultaneously within an analytical framework, to understand the conditions in which pollinator transitions do or do not accelerate orchid diversification.

Materials and methods

Pollinator strategy data

Data on pollinator attraction strategy were sourced from a recently-published and comprehensive database of orchid reproductive biology (16). These data were assembled from an exhaustive search of literature published since Darwin (4), and scored attraction strategies as deceit-based (sex and food deceit) or reward-based (fragrance, nectar, oil, and sleeping site). Taxonomy was corrected against the World Checklist of Vascular Plants (60) using the R package rWCVP (61). The majority of species names were exactly matched to species in WCVP and the few “fuzzy matches” were checked manually. Species names with multiple matches were resolved automatically, by keeping the accepted name where exactly one was accepted, and keeping a synonym (as opposed to invalid or illegitimate names) when exactly one was a synonym. Finally, synonyms were corrected to their accepted name and duplicates that resulted from the correction steps were removed.

Species were categorised based on autonomous selfing/agagamospermy (119 species), nectar reward (174 sps), lipid reward (50 sps), sleep site reward (four sps), brood site deception (two sps), food deception (131 sps) and sex deception (65 sps). A small number of species (50) were scored for more than one category, which were either the result of more than one category being operational, or conflicting source reports (Ackerman, personal communication).

Phylogenetic framework

The cleaned dataset was matched with species from a recently published molecular phylogeny (25), and species not sampled in the dataset were pruned for analysis, leaving 485 species. This phylogeny was chosen because it is currently the most taxonomically comprehensive phylogeny for subfamily Orchidoideae, with 1,475 of ~5,000 species sampled. It was reconstructed using the supermatrix approach, sampling up to nine commonly-sequenced nucleotide loci publicly available in GenBank, and calibrated against geological time with RelTime (62), using robust phylogenomic estimates implemented as secondary calibrations (5, 62), given the lack of an extensive fossil record for orchids (2).

Model selection, ancestral state reconstruction and transition rates

To identify the evolutionary mode of pollinator strategy, model selection was performed using hidden Markov transition models with the R package corHMM (40). Six models were estimated: all rates different (ARD), equal rates (ER) and symmetrical (SYM), all with and without hidden rates, and were compared with Akaike information criterion weights (AICw). After initial investigation, the two states with very few species were removed (sleep site reward, n = 4; brood site deception = 2). Rare states are known to introduce artifacts to estimated transition rates (53, 63, 64) and increase uncertainty around ancestral states (Meade, personal communication), which they did here. After identifying the best model based on AIC weight, 300 SIMMAPs were estimated to explore uncertainty of ancestral states (65). To explore transition rates further, an ARD model was estimated in reversible-jump Markov chain Monte Carlo (MCMC) analyses using the MultiState module in BayesTraitsV3 (www.evolution.reading.ac.uk/). This rj-MCMC approach was chosen because it automatically allows rates to be zero if there is not enough evidence for their existence (41). A hyperexponential prior was applied to each transition rate, with the mean of the exponential drawn from a uniform 0-100. 51 million generations were sampled every

5,000 after discarding the first 1 million as burn-in. Convergence was checked with the R package coda (66), ensuring effective sample sizes of >1,000. In both corHMM and rj-MCMC analyses, polymorphic species were included, allowing transitions to reflect the full range, and uncertainty, of pollination strategies observed.

Assessing drivers of diversification

To assess evidence of drivers of diversification, Structured Rate Permutation on Phylogenies (STRAPP) tests were performed (42), implemented by the R package BAMMtools (67). These used the BAMM analysis by Thompson et al. (25) which found strong rate heterogeneity, with a mean of ~37 rate shifts across the posterior sample and ~44-fold variation in tip speciation rates. This character-independent estimation method was selected over other methods such as state-dependent speciation and extinction (SSE) methods (38), and hidden state implementations of SSE models (39), due to methodological issues. The relatively low availability of pollinator strategy data available for orchids (16) made it impossible to account for incomplete sampling of states, a crucial step in SSE analyses to ensure accurate estimation of diversification rates. BAMM accounts for incomplete lineage sampling in a character-independent framework, and Thompson et al. (25) used genus-level sampling fractions. STRAPP tests therefore represent the best-possible method to accurately estimate state-dependent diversification in this scenario. Furthermore, unlike binary state dependent speciation and extinction (BiSSE) (38), but like the hidden states implementation of BiSSE (39), STRAPP tests relax the assumption that all rate variation is associated with the focal trait (42), providing a more accurate null model given the complexity of macroevolution. Different tests were performed: (1) Binary analyses, which tested each pollination strategy individually while retaining polymorphic species, and included comparisons of generalists (pollinated by >1 species) versus specialists, Hymenoptera-pollinated versus others and Lepidoptera-pollinated versus others; (2) Multistate analyses, which tested all attraction strategies simultaneously after removing polymorphic species, and included a three-state comparison grouping species broadly into autonomous, reward-based, and deception-based strategies; (3) Continuous trait analyses, which tested the effects of pollinator number and functional pollinator specificity (FPS, defined as the multiplying counts of pollinator families by orders). STRAPP tests were performed by permuting rates across the tree, while maintaining the position of rate shifts (42). Statistical differences were assessed with a Kruskal-Wallis test for the multi-state tests, a Mann-Whitney U test for the binary tests, and a Spearman's rank correlation coefficient for the pollinator count and FPS tests. Only variation in speciation rate was considered, since extinction rates by BAMM are thought to be unreliable (68), while speciation rates are more accurate (69).

Sensitivity testing of diversification rate estimates

To account for potential concerns regarding diversification rate estimates using BAMM (68, 70) (although, see (69, 71)), especially the implementation of clade-specific sampling fractions (43), we also inferred rates with the Missing State Speciation and Extinction (MiSSE) model (37), which is implemented in the R package hisse (39). MiSSE uses a global sampling fraction, and is useful for conducting post hoc statistical tests for relationships between diversification rates and character data, including in animals (72), plants (73), and fungi (74). Similar to STRAPP (42), character data can be discrete or continuous, single variables or combinations of variables. We used MiSSE to analyze 30 possible model structures, which were generated using the function generateMiSSEGreedyCombinations from combinations of between one and ten turnover parameters and between one and three extinction fraction parameters. Prior to running MiSSE, we noted that the Orchidoideae phylogeny possesses many tips with extremely short branch lengths, possibly due to their recent quite rapid radiation (2, 25). Because the presence of taxa with very short branch lengths can lead to inaccurate diversification estimates from formal SSE models (39), we pruned the phylogeny of its 200 shortest tips. Since the original tree contained 1,475 tips (25), our input tree for analyses contained 1,275 tips in total. To calculate our global sampling fraction of 25.5%, we divided this number of tips by the approximate total number of species estimated to exist in the clade (5,000) (1). We ran MiSSE on our set of 30 models using the function MiSSEGreedy, which performs an automated function to fit MiSSE models. To estimate the likely ancestral states along our phylogeny, we used the MarginReconMiSSE function, and then we estimated diversification rates at the tips of the tree by running the function GetModelAveRates on our marginal reconstruction results. We replicated all previously-described statistical tests between trait data and MiSSE-estimated tip rates. In each case, we tested for correlation using boxplots or phylogenetic linear regression with the package phylolm (75).

Supplementary materials

Code and data used to generate these results will be made publicly available at https://github.com/jamie-thompson/orchid_pollinators.

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