Floral Isolation, Specialized Pollination, and Pollinator Behavior in Orchids

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Key Words

pollinator isolation, floral scent, floral morphology, Orchidaceae, speciation, isolation genes

Abstract

Floral isolation is a form of prepollination reproductive isolation mediated by floral morphology (morphological isolation) and pollinator behavior (ethological isolation). Here we review mechanisms and evolutionary consequences of floral isolation in various pollination systems. Furthermore, we compare key features of floral isolation, i.e., pollinator sharing and specialization in pollination, in different orchid pollination systems. In orchid pollination, pollinator sharing is generally low, indicating strong floral isolation. The pollinators' motivation to visit flowers (specifically) can be due to both foraging or reproductive behavior. In both types of behavior, innate preferences for floral signals can be quickly overruled by learning. In pollination systems in which reproductive behavior of pollinators triggers flower visits, lower pollinator sharing was evident compared with systems with foraging behavior, probably because pollinators displaying reproductive behavior show higher fidelity in their visitation patterns. Orchids pollinated through reproductive behavior also use fewer pollinators than orchids pollinated through foraging behavior. No association between specialization and pollinator sharing was found. Thus, generalized pollination does not impede floral isolation, as orchids with many pollinators may nonetheless have low pollinator sharing. Specialization in pollination was, however, linked to orchid species richness in our analysis. Flower size, spur, and column morphology are most important for morphological isolation, and floral scent is most important for ethological isolation. These traits may be based on few genes, implying that floral isolation can be brought about by few genes of large effect.

INTRODUCTION

What is Floral Isolation?

Most angiosperms, by far the most diverse plant group, use animals as vectors for gamete transfer. Because plants are often dependent on pollinator attraction for sexual reproduction, pollinator-driven selection is a likely outcome, influencing the evolution of flowers (30, 65, 83, 89, 109, 140). Pollinators not only transfer gametes among plants, they also play an important role in the reproductive isolation of plant taxa, through interplay of their behavior and morphology with floral traits (46, 65, 139, 140). Such assortative pollen delivery mediated by pollinators and floral traits is called floral isolation, a form of prepollination (prezygotic) isolation, i.e., an early-acting reproductive barrier unique to plants (106). Floral isolation can be an important process during plant speciation, by establishing reproductive isolation. It can also facilitate the accumulation of genetic differences and thus the evolution of genetic barriers (late-acting barriers) through a feedback loop (106). Floral isolation between a given set of plants is, however, not necessarily a part of their speciation process, as those plants may represent long-separated lineages that evolved without the influence of pollinators. In such cases floral isolation enhances the coexistence of species, for example by reducing the wastage of gametes and fostering the delivery of compatible pollen onto the stigmas in the presence of genetic incompatibilities. Here we review ecological (pollinator behavior, floral traits) and molecular (genes underlying floral traits) mechanisms, as well as evolutionary consequences (reproductive isolation) of floral isolation. In a meta-analysis, we compare key features of floral isolation, i.e., pollinator sharing and specialization in pollination, in different orchid pollination systems and investigate the association between these two factors and their association with orchid species richness.

Verne Grant (46), who introduced the concept of floral isolation, discriminated between (*a*) morphological isolation, in which the primary cause of isolation is flower structure, and (*b*) ethological isolation, in which pollinator behavior causes isolation (**Figure 1**). Accordingly, the term pollinator isolation is sometimes used instead of floral isolation (106). Floral isolation is most easily recognized when it is linked to a shift in pollination syndromes, e.g., from hawkmoth to hummingbird pollination (the *Salvia* type of morphological isolation sensu Grant) (47) (**Figure 1**). In classic model systems of pollinator-mediated plant evolution, such as *Mimulus* (104, 111), *Ipomopsis* (1), and *Aquilegia* (39, 58), such shifts between different pollination syndromes, resulting in efficient floral isolation, are evident. However, floral isolation can also be caused by more subtle differences in floral morphology causing different positional placement of pollen on the body of the pollinator (e.g., the *Pedicularis* type of morphological isolation) (48) (**Figure 1**). This type of isolation is commonly found among orchids, for example in *Platanthera chlorantha* and *P. bifolia* (**Figure 1**), in which differences in the structure of the column lead to the placement of pollinia on either the proboscis or the eyes of the pollinating moths (30, 89). Thus, floral isolation can also be found among plant species sharing a functional group of pollinators, or even the same pollinators (72), and a lack of major shifts in pollinator syndrome does not necessarily mean that floral isolation is

Figure 1

Different forms of floral isolation. (*a*) Ethological isolation mediated by floral odor differences between *Gymnadenia conopsea* (upper gas chromatographic trace) and *G. odoratissima* (lower gas chromatographic trace). Numbers refer to different scent compounds. I.S., internal standard (60). (*b*) *Pedicularis* isolation in two species of *Platanthera* mediated through different positions of the pollinia with their sticky viscidia (*yellow arrows*) and thus the placement of pollinia on different parts of the pollinators (89). (*c*) Morphological (*Salvia*-type) isolation in two species of *Gymnadenia* with different spur lengths leading to the visitation by pollinators with different tongue lengths (drawings by Silvia Pfister).
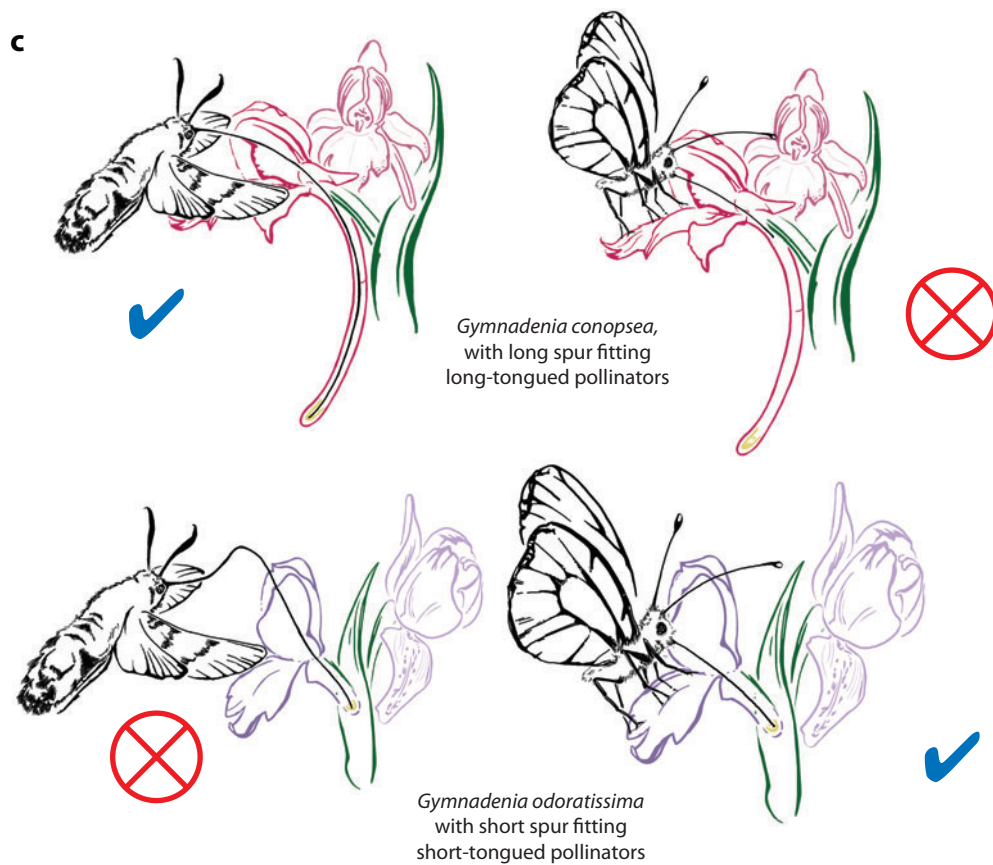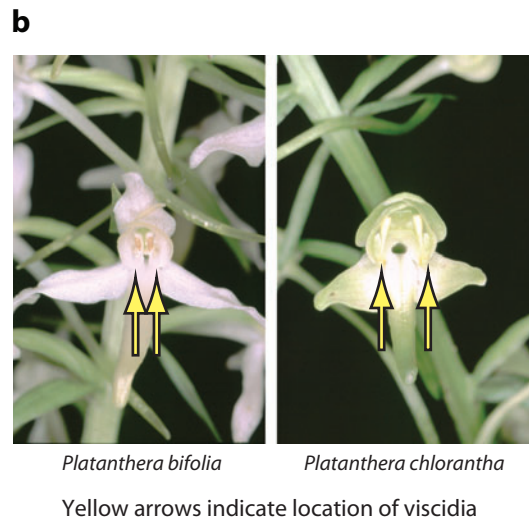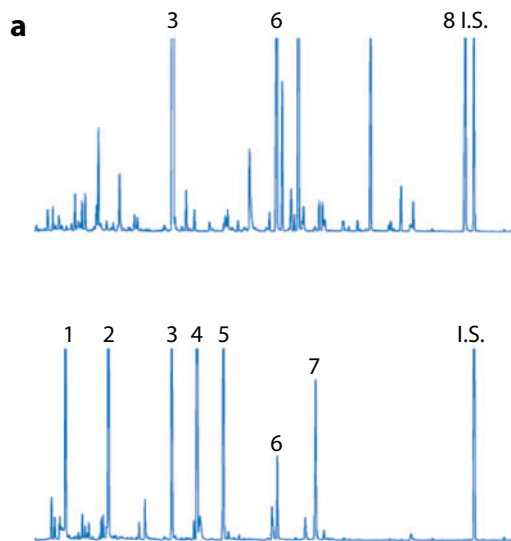
a

3 6 8 I.S.

1 2 3 4 5 I.S.

6 7

b

Platanthera bifolia   *Platanthera chlorantha*

Yellow arrows indicate location of viscidia

c

Gymnadenia conopsea,
with long spur fitting
long-tongued pollinators

Gymnadenia odoratissima
with short spur fitting
short-tongued pollinators

unimportant. Macroevolutionary studies that focus on obvious shifts in pollinator syndrome may thus often underestimate the degree of pollinator shifts and floral isolation (134). This is even more evident in ethological isolation, where differences in floral signals or morphology lead to the attraction of different pollinators or to the establishment of floral constancy to either type of flower by the same pollinators. In three species of asclepiads, a broad overlap in pollinator species was found, but mechanical and ethological factors (floral constancy) were contributing to floral isolation (74). To detect floral isolation mediated by floral constancy, quantitative approaches are necessary. In two species of *Silene*, a certain degree of assortative visitation was found by using fluorescent powder to track pollinator movements, despite a broad overlap in pollinator species (45). Although floral constancy can contribute to floral isolation, pollinators are rarely so constant as to cause strong isolation by themselves (24, 44). Floral isolation thus usually requires some form of obligatory specialization in the use of pollinators.

Floral Isolation and Specialized Pollination

Whether generalization or specialization in pollination prevails among plants is an ongoing debate (38, 68); however, orchids are among the most specialized plants with respect to their pollination. We refer to specialization in an evolutionary sense, i.e., derived plant groups use fewer pollinator species than the ancestral plant groups (38). Specialization in pollination, or, more generally, assortative visitation, is enabled by the morphology and behavior of pollinators (**Figure 1**). Accordingly, specialization can also

be found on the part of the pollinator, e.g., male euglossine bees that visit only few specific orchids or long-tongued flies that visit long-tube flowers (69). Specialized pollinators can, however, visit generalized plants, and specialized plants can be pollinated by generalist pollinators. Such nestedness in community structure has in fact been suggested to be common in mutualistic associations (91). Specialization in pollination is sometimes linked to floral isolation, but higher specialization does not necessarily lead to better pre-mating isolation, as exemplified in the genus *Cryptostylis*, in which at least five genetically incompatible species are pollinated specifically by a single but shared pollinator species (115). On the other hand, generalized pollination is believed to impede floral isolation (138, 141), but the relationship between the degree of specialization in pollination and floral isolation has not yet been explicitly tested.

Pollinator Behavior

Orchid pollination is extremely diversified, including rewarding and deceptive systems and various types of behaviors (**Table 1**) (27, 61, 112). This variation in motivation and outcome of flower visits by the pollinator is expected to affect patterns of floral isolation and pollinator specialization. Foraging behavior by pollinators is shaped by natural selection, and although innate preferences have evolved, flexibility imposed by learning is often apparent, enabling pollinators to switch between food plants. Innate preferences by naïve insects have been shown for both visual and olfactory signals (22, 32, 98, 103). Obvious shifts in color and scent linked with pollination syndromes suggest that innate preferences play an important role in floral isolation (75), and theoretical evolutionary models indicate that different preferences can lead to adaptive divergence in floral traits (40). However, preferences can differ among pollinator populations (22) and can be modified by learning (143). Hummingbirds, for example, innately prefer red to other colors but quickly change their preference to white when

Table 1 Overview of reward/deception and different types of behavior in the pollination syndromes analyzed here

Reward	Type of behavior	
	Foraging	*Reproduction*
Yes	Food reward	Scent reward
No	Food deception	Sexual deception, brood site deception

white is rewarded (85). In the honey bee, learning can erase innate preferences (42), whereas in a hawkmoth innate preferences are modified through learning of alternative food sources but can be retrieved in the presence of the innately preferred stimulus (107). An example of strict preference for a given flower type is exemplified by specialized, oligolectic bees, which collect pollen only from a limited number of food plants. Such bees, however, consume considerable amounts of pollen (88), making their role as effective pollinators questionable.

An important type of behavior mediated by learning of floral signals of rewarding plants is floral constancy, the tendency of pollinators to specialize on flowers of the same type during a given foraging bout (24). Floral constancy is commonly found among many pollinators (44) and can contribute to ethological isolation (48). Most studies investigating floral constancy focus on visual signals, although for example honey bees use both visual and olfactory signals to establish constancy (144). Many bees, moths, and butterflies can learn and discriminate odor bouquets that differ in only a single chemical substance (2, 6, 28, 135). Thus, even minor differences among floral odor bouquets, not necessarily detectable by the human nose, can foster constancy by pollinators. Floral constancy is expected to impose stabilizing selection on floral signals (109), and thus rewarding systems should display less variation than deceptive systems, in which pollinators learn to avoid individual plants or plant species. Such avoidance learning of pollinators, in some way the evident opposite of floral constancy, leads to switches among flower types, possibly imposing negative-frequency-dependent selection, thereby increasing signal variation (126). An interesting behavioral pattern in response to deceptive flowers, demonstrated in bumble bees, is peak shift (82); when one color is rewarded and a similar color is penalized in an experimental flight array, bumble bees shift their preference to a third color. This type of behavior may influence the evolution of floral signals in deceptive orchids, although empirical tests are not yet available.

A range of orchids are not pollinated through food-seeking animals, but by insects displaying behavior linked in some way to reproduction (**Table 1**). Such behavior can be found in rewarding systems, such as euglossine bee pollination with scent rewards, but also in deceptive pollination, such as sexual or brood site deception. Reproductive behavior is shaped by sexual selection, i.e., males are selected for optimal mating and females are selected for finding appropriate oviposition sites. Signals involved in mating behavior, such as sex pheromones, often release hard-wired behavior and are detected and processed with specific neuronal structures (4, 53). Through its often high specificity, reproductive behavior should contribute strongly to ethological isolation; however, even responses to sex pheromones are subject to learning (35). This finding was demonstrated in pollinators of sexually deceptive orchids, e.g., solitary bees, that cease responding to individual sex pheromone blends when they are associated with unreceptive females (sexually deceptive orchids) (5). Thynnine males avoid multiple visits to sexually deceptive orchids by keeping away from their flowering sites (96, 146). Such behavior may have evolved to avoid wasting time with nonreceptive females, but it is unlikely to lead to switching between species-specific pheromone signals. Consequentially, pollinator fidelity in pollination systems with reproductive behavior may be high and learning behavior may foster outcrossing, which may be two important factors influencing the evolution of pollination systems (34, 97).

Floral Isolation and Plant Speciation

Floral isolation may evolve under selection for reproductive isolation or as a pleiotropic consequence of adaptation to different pollinators (48, 65). During the adaptation process, pollinators select for floral trait divergence. Adaptation and reproductive isolation link seamlessly in the case of plants pollinated by animals, because adaptation to a new pollination niche mediated by floral traits can directly

cause isolation. Pollinators therefore have dual roles, enabling sexual reproduction and thus selecting for floral traits on the one hand and mediating prezygotic isolation on the other (63, 138, 140). The concept of adaptive speciation assumes disruptive selection driven by competition, for example, for pollen vectors in the case of pollinator-driven plant speciation (140). This is easy to envisage in deceptive pollination systems with high specificity, in which pollinators learn to avoid flowers (5), or locations with flowers (96, 146), so that individuals in large populations may generally have lower pollination success than individuals in smaller populations. Here, the recruitment of a new pollinator, through a shift in floral signals, can circumvent this habituation and thus provide a rare phenotype advantage. Fitness advantage associated with low frequency (negative-frequency-dependent selection) can lead to the establishment of an initially rare mutant, and because traits that mediate attraction of different pollinators also cause floral isolation, gene flow will be reduced. This is a scenario for sympatric speciation, because competition requires sympatry, but the possibility for sympatric divergence critically depends on the strengths of disruptive selection acting against gene flow (140). Studies quantifying such selection are thus desirable. As an alternative to sympatric divergence, plants may adapt to an allopatric or parapatric mosaic of pollinators. This is amply supported by the occurrence of allopatric sister species with different pollinators, for example, in long-tongued-fly-pollinated plants of southern Africa (65). It remains unknown whether selection for isolation per se plays an important role for the establishment of floral isolation (134). Although selection for isolation, leading to reinforcement at secondary contact after allopatric divergence, is an important component of the classical speciation scenarios (48), reinforcement in floral traits has been shown only in a few cases (79, 134). Reinforcement studies of floral traits, however, are biased toward obvious trait differences such as floral color. More subtle differences in morphology and/or scent may in fact be more important isolation factors,

and investigations on reinforcement looking at such traits hold potential for future studies.

The Genetics of Floral Isolation

Floral isolation is often not absolute in the sense that it acts in concert with other forms of isolation or allows for a certain amount of gene flow among species. As long as there is strong and consistent selection on specific traits, some level of gene flow does, however, not lead to a complete admixture of genomes (105). In fact, a moderate level of gene flow between species is fairly common among plant species (20) and can lead to the spread of adaptive alleles among species (106). Porous genomes are central to the genic view of speciation, which posits that few genes under selection may be responsible for species differences, whereas the genome can be porous with respect to gene flow at other loci (80, 147). This implies that often few genes of large phenotypic effect, rather than a multitude of genes of small effect, might be responsible for the reproductive isolation among species. Consistent with this view, quantitative trait loci (QTL) studies on the bumble bee–pollinated *Mimulus cardinalis* and hummingbird-pollinated *M. lewisii* show that in this case the evolution of reproductive isolation involves genes (or linked gene clusters) of large effect (16, 18). The two *Mimulus* species are sister species (11), and a preference of bumble bees for large flowers low in anthocyanin and carotenoid pigments has been shown, whereas hummingbirds favor nectar-rich flowers high in anthocyanins (111). By introgressively swapping the single *YUP* locus, which controls the presence of yellow carotenoids in *Mimulus* petals, into the near-isogenic lines of the other species, Bradshaw & Schemske (17) could demonstrate that the associated change in flower coloration was accompanied by a difference in pollination preference by bumble bees and hummingbirds, and that an adaptive shift in pollination may be due to a single mutation of large phenotypic effect. Similarly, a change in flower color produced by either introgressive or transgenic swapping

of a major allele of the *MYB*-type transcription factor *AN2* affected hawkmoth and bumble bee pollination rates in *Petunia axillaris* and *P. integrifolia* (56).

In *Silene*, ecological experiments showed that phenylacetaldehyde, which is dominant in the floral scent of *S. dioica*, contributes to species differentiation among *S. dioica* and *S. latifolia*, because application of phenylacetaldehyde to flowers of both species (making their scents more similar) resulted in increased interspecies pollen transfer (137). Because the synthesis of phenylacetaldehyde is catalyzed by phenylacetaldehyde synthase (71), this observation again raises the possibility of a single gene with a major phenotypic effect being responsible for trait differentiation among these *Silene* species. Taken together, these investigations strengthen the view that few genes of major phenotypic effect may often be responsible for plant traits involved in floral isolation, and although there are as yet no similarly detailed studies on orchids, we may expect the genetic bases for floral isolation to be comparable.

The Questions Addressed in our Meta-Analysis

Orchids are an ideal group for comparative studies on the effect of pollination variables on reproductive parameters. Orchids are often intercompatible (119), and prepollination barriers are generally thought to be of key importance for species isolation. Apart from their huge variation in pollination systems, orchids also live in diverse habitats, from epiphytic to terrestrial, and in diverse climates. In our meta-analysis we used studies investigating pollination among at least two species of closely related orchids. Using data obtained from these studies, we comparatively analyzed pollinator sharing and specialization in pollination in different pollination systems. We also surveyed the literature for available data on molecular bases of traits involved in floral isolation. Specifically, we tested the following hypothesis: (*a*) Different types of pollinator behavior, found in different pollination systems, affect pollinator sharing and (*b*) specialization in pollination. (*c*) Higher specificity in pollination leads to lower pollinator sharing (better floral isolation), and (*d*) is linked to higher species richness. (*e*) Floral isolation can have an oligogenetic basis.

METHODS

For our metastudy, we used only papers that investigate pollination of at least two orchid species of one genus (because phylogenetic analyses are often lacking, we took membership in one genus as a proxy for close relatedness). In this way, the data used for each group of species/genus were recorded by the same researcher(s), and among-species comparisons of data from different researchers, with the unavoidable differences in scrutiny, are prevented. We did not include spatial isolation in our analysis, because there is often limited information of the species' geographic distribution available. We found 31 studies that met our criteria, comprising 27 genera, eight monophyletic tribes (19), and three subfamilies of orchids (**Table 2**). Pollinators included representatives of three orders of insects (Hymenoptera, Diptera, and Lepidoptera) and birds. This data set contained a considerable number of different pollination systems, with both reward and deception, and different behavior types represented (**Table 1**). For each orchid species investigated in a given study, we recorded the pollinators in a spreadsheet. We then calculated a sharing index (S.I.) for each pollinator species using the formula S.I. = (orchid spp. sharing the pollinator - 1)/ (total number of orchid spp. in the study - 1). This S.I. varies from 0 (0% sharing) to 1 (100% sharing). For example, if five orchid species were investigated, and one given pollinator was shared by two of them, the S.I. for this pollinator would be 0.25. From the S.I. values for each pollinator, the mean pollinator sharing index was calculated for each study/orchid genus. In addition, the mean number of pollinators per genus (pollinator specialization) was calculated for the species investigated in each study. For each study, the pollination syndromes of

Table 2 Systematic placement, pollination, and floral isolation of the orchid genera included in the analysis

Subfamily, tribe, genus (species in study; total in genus)	Pollinators	Poll. syndrome[a]	Floral isolation type[b]	Key trait for floral isolation	Poll. sharing index (S.I.)	Mean no. of pollinators	Reference(s)
Cypripedioideae							
Cypripedium (2; 47)	Bees	FD	M	Flower size	0	1.5	(8)
Paphiopedilum (3; 77)	Syrphid flies	FD	n.m.	Color, scent?	0	2.67	(7)
Orchideae							
Satyrium (3, 5; 88)	Bees, moths, butterflies sunbirds, carrion flies	FR (BSD)	M, E	Flower size, column structure; scent, color; rostellum structure, spur length	0.17	2.37	(36, 62, 64)
Disa (2; 162)	Long-tongued flies	FD	M	Spur length	0	1	(67)
Disa (2; 162)	Birds	FR	n.m.	n.m.	0.33	2	(66)
Disa (2, 2; 162)	Male bees, male sphecid and pompilid wasps	SD	E	Scent?	0.1	2	(70, 128)
Brownleea (3; 7)	Bees, flies	FR	M	Spur length	0	2	(77)
Disperis (14; 84)	Bees	FR	M(P)	n.m.	0.18	1.43	(127)
Platanthera (2, 2; 85)	Moths, butterflies	FR	M(P), E	Column morphology, spur length, scent	0.46	6.75	(89, 125)
Ophrys (45; 126)	Male bees	SD	E, M(P)	Scent, trichomes on labellum	0.01	1.2	(93, 94, 117)
Anacamptis (2; 12)	Bees	FD	M, E	Spur length	0.13	9	(26)
Anacamptis (2; 12)	Bees	FD/FR	n.m.	n.m.	0.17	3.5	(29)
Orchis (4; 33)	Bees	FD	M, E	Spur length	0.09	4.5	(26)
Gymnadenia (2; 16)	Moths, butterflies	FR	M, E	Spur length, scent	0.01	5.75	(60, 136)
Habenaria (3; 600)	Moths	FR	M	Spur length	0	1	(122)
Diurideae							
Caladenia (7; 84)	Male thynnine wasps	SD	E	Scent	0.02	1.14	(129)
Chiloglottis (6; 27)	Male thynnine wasps	SD	E	Scent	0	1	(15)
Cranichideae							
Spiranthes (6; 30)	Bees	FR	n.m.	n.m.	0.06	3.5	(21)
Prescottia (3; 35)	Bees, moths	FR	n.m.	n.m.	0	2	(123)

(Continued)

Table 2 (*Continued*)

Subfamily, tribe, genus (species in study; total in genus)	Pollinators	Poll. syndrome[a]	Floral isolation type[b]	Key trait for floral isolation	Poll. sharing index (S.I.)	Mean no. of pollinators	Reference(s)
Dendrobieae							
Bulbophyllum (3; 1000)	Flies	BSD	E, M	Scent, flower size	0.33	1.66	(13)
Epidendreae							
Pleurothallis (5; 1200)	Flies	BSD	E	Scent	0.04	1.6	(14)
Earina (3; 10)	Flies, wasps	FR	n.m.	n.m.	0	2	(78)
Cattleya (2; 42)	Bees	FD	None	Habitat	0.5	1.5	(124)
Sophronitis (2; 65)	Bees	FD	M(P)	Column length, flower size	1	1	(121)
Vandeae							
Aerangis (4; 50)	Hawkmoths	FR	M, E	Spur lengths, scent?	0.13	1.75	(84)
Angraecum (4; 220)	Hawkmoths	FR	M(P), E	Resupination, spur length, scent?	0.5	1	(90, 142)
Cymbidieae							
Stanhopea (17; 58)	Male euglossine bees	SR	E, M	Scent, flower size	0.02	1.12	(33)
Catasetum (20; 166)	Male euglossine bees	SR	E, M(P)	Scent, column structure	0.05	2	(55)
Cirrhaea (3; 7)	Male euglossine bees	SR	E	Scent	0	2.5	(92)

[a]FR, food reward; FD, food deception; SD, sexual deception; SR, scent reward; BSD, brood site deception.
[b]M, mechanical; (P) *Pedicularis* type; E, ethological; n.m., not mentioned.

the investigated species were recorded [food reward, food deception, scent reward, and sexual deception (including brood site deception)]. The syndromes were grouped according to the pollinators' behavior, namely foraging behavior and reproductive behavior (**Table 1**). If multiple studies were available for species of one genus with the same pollination syndrome, the mean values for the genus was calculated. For genera containing species with different pollination syndromes (e.g., *Disa*) the mean values for the species of each pollination syndrome were calculated. For each orchid genus,

the presence/absence of *Pedicularis*-type isolation was recorded. Differences in spur lengths were not classified as *Pedicularis*-type isolation, although they can lead to differential placement of pollinia but usually lead to the attraction of different pollinators. The genus *Ophrys* was split into section *Ophrys* (head pollination) and section *Pseudophrys* (abdomen pollination), with no *Pedicularis* isolation occurring within each group. The total number of species per genus was gathered from References 34, 99, 100, 101, 102, and for the tribes Vandeae and Cymbidieae information was gathered from

Food deception: pollination syndrome with nonrewarding plants producing signals that are associated with floral reward by pollinators

Sexual deception: pollination syndrome in which plants mimic mating signals of pollinators, leading to pseudocopulation of pollinators on flowers

Wikipedia (**http://www.wikipedia.org/**). Furthermore, we surveyed the literature for studies that identified molecular bases of traits important for floral isolation in orchids and model plant species.

Statistical Analysis

Species showing total genetic incompatibility (*Cryptostylis*, *Disa venosa*, and *D. racemosa*) were excluded from the statistical analyses. All dependent variables ("sharing index," "number of pollinators," "number of orchid species") were ln (1+x) transformed prior to analysis to approach normality and reduce heterogeneity of variances. Levene's tests of equality of error variances showed that the variances of the transformed variables were homogeneous among the groups. An univariate GLM ANOVA was performed for the dependent variable "sharing index" with "pollination syndrome" and "*Pedicularis* isolation" as fixed factors. A *t*-test was calculated for "number of pollinators" with "pollinator behavior" as an independent factor. Pearson correlations and regressions were calculated for the variables "sharing index" and "number of pollinators" as well as "number of pollinators" and "number of orchid species"; for this last analysis, the genus *Disa* was excluded because it contains several different pollination syndromes with different numbers of pollinators. All analyses were performed using the program SPSS 14.0.

Effects of Phylogeny

In a preanalysis investigating the effects of phylogeny on our results, neither tribe nor subfamily had a significant effect on the variables investigated (pollinator sharing, number of pollinators, number of orchid species; one-way ANOVA, with subfamily: $F_{2,28}$ = 2.25, 1.16, 0.53; tribe: $F_{2,28}$ = 1.52, 0.72, 0.63; all $P >$ 0.1). All correlations of variables were also calculated using phylogenetically independent contrasts; however, the (non)-significance of results did not change for any analysis. To calculate phylogenetically independent contrasts,

sequences for the chloroplast intronic *maturase K* (*matK*) regions were downloaded from GenBank and aligned using Clustal_X (132). A phylogenetic tree was calculated with PAUP* (ver. 4.0b10) (131) using the P-distance and the neighbor-joining method. Although this tree cannot be taken as an accurate phylogeny, the *matK* phylogeny was in overall agreement with previous phylogenetic studies (19) and also with an ITS phylogeny for the same set of genera (data not shown). The tree was used to calculate independent contrasts using the Contrast program from the PHYLIP package (v. 3.67) (37). The following species were used for phylogenetic reconstructions, with GenBank accessions for *matK* indicated in parentheses: *Aerangis confusa* (DQ091332), *Angraecum sesquipedale* (AF263621), *Brownleea parviflora* (DQ414994), *Bulbophyllum longiflorum* (EF079343), *Caladenia barbarossa* (AJ310026), *Cattleya skinneri* (AF263813), *Chiloglottis trapeziformis* (AJ310003), *Cirrhaea dependens* (AF239477), *Cypripedium calceolus* (AY557208), *Disa spathulata* (AY368384), *Disperis capensis* (AJ310022), *Earina autumnalis* (EF079336), *Gymnadenia conopsea* (EF612530), *Habenaria repens* (AJ310036), *Ophrys apifera* (AJ543953), *Orchis quadripunctata* (AY368385), *Paphiopedilum glaucophyllum* (AY557205), *Platanthera chlorantha* (EF612531), *Pleurothallis ochreata* (AY008458), *Prescottia plantaginea* (AJ543939), *Satyrium stenopetalum* (EF612594), *Sophronitis cernua* (EF079310), *Spiranthes cernua* (AJ543917), and *Stanhopea tigrina* (AY368430). Because no *matK* sequence data were available from *Anacamptis*, a sequence from *Himantoglossum robertianum* (AY368382) was used as a substitute, based on phylogenetic relatedness and availability of sequences in GenBank.

Pollinator Sharing

We found an overall low degree of pollinator sharing (mean sharing index $\pm$ SD = 0.14 $\pm$ 0.23), suggesting strong floral isolation among orchid species. Although pollinator records for any species are unlikely to be complete, our measurement of floral isolation

is conservative because it does not include frequency of visitation and floral constancy, both of which are components of ethological isolation but have not been investigated in most studies. Thus, more rigorous investigations will probably identify even stronger floral isolation for most pollination systems. Pollinator sharing was significantly influenced by pollination syndrome and *Pedicularis*-isolation, i.e., the different placement of pollinia on the pollinators (**Table 3**) (**Figure 1**). Estimated marginal means were higher for species with *Pedicularis* isolation, as expected because such morphological isolation enables the sharing of pollinators without reproductive interference or hybridization. Orchids pollinated by foraging behavior (food deception and food reward) had higher pollinator sharing than systems based on reproductive behavior (sexual deception, brood site deception, and scent reward) (**Figure 2**). We also found a significant interaction between *Pedicularis* isolation and pollination syndrome, indicating that this type of isolation is not found in equal frequency in all pollination syndromes, but more commonly in food reward systems. Despite the aforementioned limitation of our meta-analysis, this comparison of pollinator sharing among pollination syndromes is robust, because no systematic bias is expected for either of the groups compared.

Our findings support an earlier hypothesis by Dressler (34) suggesting that pseudocopulation and pollination by male euglossine bees provide better isolation among species than does pollination through foraging behavior. A possible reason may be the higher fidelity of pollinators toward their mates, sources for scent collection, and brood substrate, which evolved under sexual selection (4). Such specific responses will lead to a low propensity of switching among orchid species, enabling the effective usage of such behavior for pollen transfer in the plants (34). Pollination through reproductive behavior is present in both large subfamilies of orchids, and in five of eight tribes included in our analysis, suggesting independent evolutionary origins within the orchids. Such systems are also not limited to orchids, as pollina-

Table 3 General linear model of pollinator sharing using pollination syndrome and *Pedicularis* isolation as independent factors

Source	d.f.	SS[a]	MS	F	P
Dependent variable: pollinator sharing index					
Corrected model	6	0.52[b]	0.09	6.25	0.001
Intercept	1	0.55	0.55	39.86	<0.001
Poll. syndrome	3	0.21	0.07	5.09	0.008
Pedicularis isolation	1	0.24	0.25	17.87	<0.001
Syndrome × *Pedicularis*	2	0.12	0.06	4.48	0.023

[a]Type III.
[b]Adjusted R squared = 0.410.

tion by male euglossine bees is found in several other plant families, such as Araceae, Euphorbiaceae, Solanaceae, and Gesneriaceae (41), and brood site deception and nursery pollination are also widespread among different plant families (108). The driving force for such repeated independent evolution may be the reduced sharing of pollinators, leading to a more efficient import and export of pollen on the one hand and a reduction of hybridization on the other.

Foraging behavior is generally more flexible and subject to learning than reproductive behavior, with the likely outcome of less fidelity in pollination. In food-rewarding systems, pollinator fidelity is dependent on floral constancy, but pollinators will switch to another species when rewards are low or when plants are scarce (44, 139). In food-deceptive systems, pollinators avoided individual orchid species and switched to other species in subsequent trials (109). Indeed, our data analysis showed the highest values for pollinator sharing in food-deceptive systems, but with very high variation, indicating differences among different mimicry systems. Avoidance learning and switching to another plant species are expected to be more pronounced in generalized mimicry than in Batesian mimicry, because the latter usually involves the imitation of specific model plants and more specialized pollinators (112). In Batesian food-deceptive systems with specialized pollinators, such as long-tongued fly pollination in the South African *Disa draconis* complex (67), low pollinator sharing was evident (**Table 2**).

Batesian mimicry:
mimic imitates signals of a specific model for its own benefit
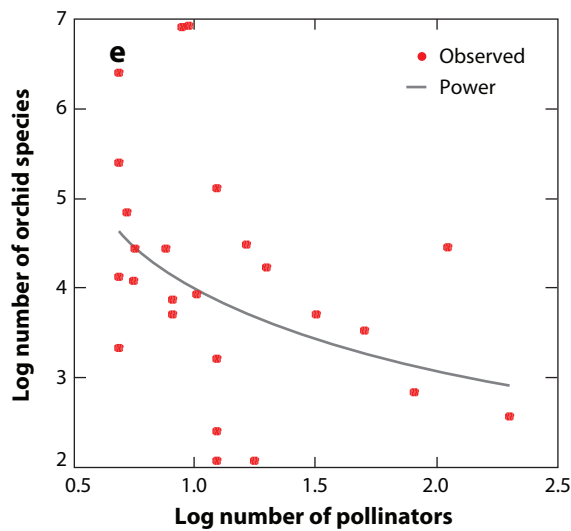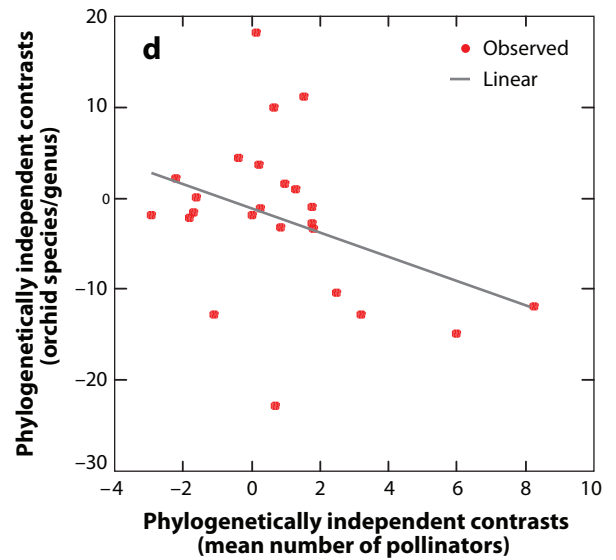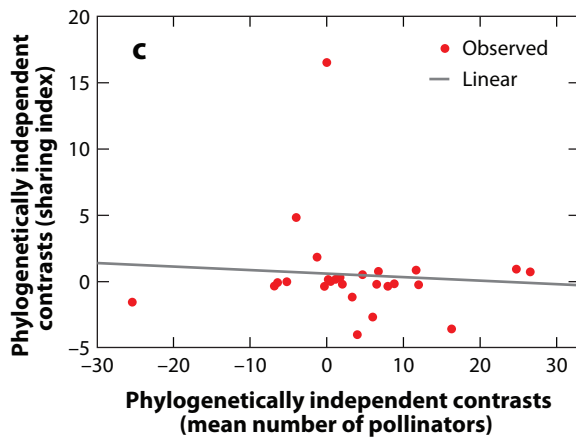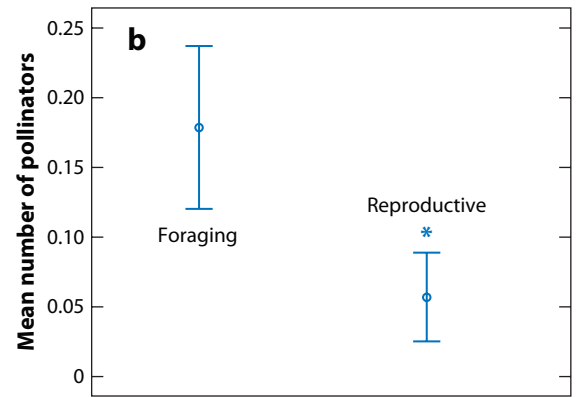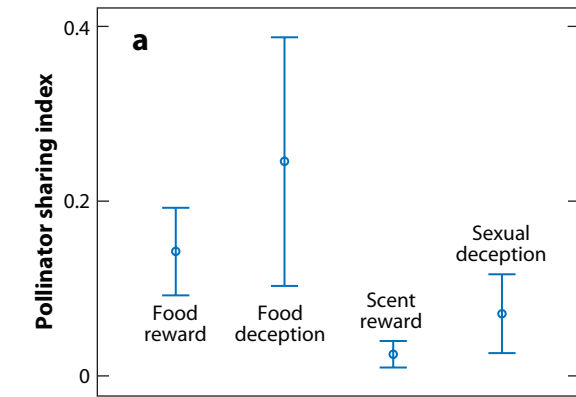
Pollinator sharing should affect the genetic divergence among species, because more pollinator sharing should lead to more hybridization, in the absence of postmating barriers, and thus homogenization of neutral loci (105). However, the high values for pollinator sharing in food-deceptive orchids do not fit their high levels of neutral genetic divergence compared with sexually deceptive orchids (27). This apparent paradox is explained by the evolution of karyotype differences leading to postzygotic isolation in food-deceptive orchids (25).

Specificity in Pollination

Using specific pollinators, i.e., those that visit flowers specifically, does not necessarily imply high specialization in pollination by the plant. Sexual mimicry could, for example, incorporate the mating signals of multiple species, thus leading to the attraction of many pollinators, although each pollinator visits only one orchid. In our analysis, overall specificity in pollination was high (mean no. pollinators $\pm$ SD = 2.3 $\pm$ 1.87). Earlier studies found similar levels of specialization (49, 133), supporting the hypothesis that orchids are generally highly specialized in their pollination. Pollination systems with reproductive behavior were significantly more specific than pollination systems with foraging behavior ($t_{21.24} = 2.31, P = 0.03$) (**Figure 2**), suggesting that orchids using specialized pollinators are also more specialized in pollination. Our findings thus do not support the suggested nestedness in mutualistic associations (91); however, only the rewarding orchid-pollinator associations are mutualistic, whereas deceptive pollination can be seen as a parasitic relationship (146).

Specific pollinator attraction in reproductive pollination is often achieved by volatile signaling, e.g., through fragrance bouquets in euglossine pollination (55) and sex pheromone mimicry in sexually deceptive orchids (83, 113, 116). In both scent reward and sexual deception systems, preadaptations enable the plants to produce attractive chemical signals (3, 114). Besides the stunning specificity of reproductive pollination systems, some of the South African and tropical food reward and food-deceptive systems, such as *Angraecum*, *Habenaria*, and *Disa*, are also highly specific in their pollination (63, 69). Specific pollination is likely to be an efficient, but also risky, strategy. A recently published evolutionary model predicts the evolution of specialization when local plant abundance is low compared with other species in the community (110). This may often be the case for orchids, especially in epiphytic species with often low population densities (49). Additionally, the presence of pollinia in orchids enables pollination with low visitation rates, because more pollen is transported by a single visitor. On the other hand, pollinia can also lead to higher losses if pollinators show low fidelity, selecting for better isolation.

Do Fewer Pollinators Lead to Better Isolation?

The exclusive use of pollinators, i.e., low pollinator sharing, differs in principle from specialization in pollination, i.e., the use of few pollinator species. It is interesting to ask, however, if there is a correlation between these two factors, so that high specialization in pollination leads to low sharing. In our analysis, however, no such correlation was evident ($r_{28} = -0.02\ P = 0.92$;

Figure 2

(*a*) Mean ($\pm$ standard error of mean) pollinator sharing index in different pollination syndromes. (*b*) Specificity in pollination in orchids pollinated through foraging and reproductive behavior (*$P = 0.03$). (*c*, *d*) Phylogenetically independent contrasts in the relation between (*c*) pollinator sharing and mean number of pollinators, showing the lack of an association, and (*d*, *e*) specialization in pollination and number of species in orchid genera, indicating that more specialized orchids are richer in species (panel *e* shows data without phylogenetically independent contrasts).

phylogenetically independent contrasts: $r_{25} = -0.07$ (**Figure 2**). Although this seems counterintuitive, there are clear examples of more generalized orchid species that show strong floral isolation. *Gymnadenia* and *Spiranthes* have a mean of 5.75 and 3.5 pollinator species but a low sharing index of 0.01 and 0.06, respectively. Both genera also show a diversity in pollinator functional groups, such as moths and butterflies in *Gymnadenia* (60, 136) and long- and short-tongued bees in *Spiranthes* (21). The genus *Earina* is even more generalized, with flies and wasps as pollinators, but no sharing of pollinator species between these orchids was detected (78). In *Gymnadenia odoratissima* and *G. conopsea*, which are widely sympatric in their distribution, a combination of spur lengths and floral scent differences leads to the attraction of different pollinators (60) (**Figure 1**). Thus, these are seemingly powerful mechanisms for floral isolation in these food-rewarding orchids. At the other extreme are the specifically pollinated *Cattleya* species, with 1.5 mean pollinators but a high sharing index of 0.5 (124). These findings suggest that high specificity in pollination does not necessarily involve better isolation, nor does lower specificity make strong floral isolation unlikely (138), at least for orchids and other plant families with flowers that permit morphological isolation. Our findings call for a reassessment of the association of pollinator generalization and floral isolation on a broader scale.

Is Specialization in Pollination Linked to Species Richness?

Floral isolation and specialization in pollination have been assumed to affect plant speciation, and this association seems likely in the orchids, one of the most species-rich plant families and very specialized in pollination (34). Specific pollination may allow for more adaptive peaks in a fitness landscape or, in other words, more pollinator niches among the available pollinator community. In our data set, neither pollinator sharing nor pollinator behavior nor pollination syndrome was linked to orchid species number ($r_{24} = 0.22$, $P = 0.29$; one-way ANOVA $F_{1,24} = 2.42$; $F_{3,21} = 1.82$; $P = 0.13$; 0.17). We found, however, a significant association between number of pollinators and number of orchid species, suggesting that specialized orchid genera are richer in species than less specialized groups. In a regression analysis, a power function best explained the relation between these two factors ($y = 4.01 \cdot x^{-0.38}$; $F_{1,23} = 4.83$, $P = 0.04$); phylogenetically independent contrasts of these data showed a negative correlation ($r_{24} = -0.42$) (**Figure 2**). Earlier analyses in angiosperms indicated that animal pollination and floral spurs can foster speciation (31, 57, 73), assuming that these traits increase the specificity of pollination and reproductive isolation (57, 73). Evidence from earlier studies for a link between pollinator specialization and species richness is equivocal. A trend toward higher specialization in more recently derived subfamilies was previously suggested (133), but no such association and no significant link between orchid species richness and pollinator specificity were found in a recent study (49). Pollinator specialization alone may not drive divergence but could be linked to factors promoting speciation, such as small disjunct populations with little gene flow among them (65, 110). Relying on few pollinators should also increase the risk of extinction; however, it remains unknown how quickly specialized plants can adapt to new pollinator species and thereby escape extinction when their pollinators decline or disappear.

Which Floral Traits Are Most Important in Floral Isolation?

Most orchid species included in our analysis rely on floral morphology (spur lengths, column structure, trichomes on labellum, flower size), floral scent, or a combination thereof for assortative pollinator attraction. Floral color was generally less important for floral isolation (**Table 2**). Floral color has probably been overestimated as a trait for filtering pollinators (68), possibly because shifts in pollinator syndrome from insect to hummingbird pollination often involve an obvious shift in flower color

(85). Such shifts have occurred in some of the model plant groups for pollinator-driven evolution, such as *Mimulus*, *Aquilegia*, and *Ipomopsis*, possibly biasing the general focus on color. More studies on the relative effect of different floral traits on floral isolation are desirable and may help to disentangle the apparent complexity of floral traits and their functions.

Candidate Genes Underlying Floral Isolation

Our current knowledge on the molecular bases of floral traits is still relatively poor. Genes for traits potentially underlying floral isolation, such as spur or trichome development, have not yet been identified or studied in detail; however, for flower color and odor, numerous candidate genes are known. Nonetheless, in light of our present state of knowledge from both Orchidaceae and nonorchid plant genetic model organisms, it seems likely that changes in many floral traits (e.g., color, odor, presence of spurs) may indeed be the result of genetic changes in a small number of genes.

Considerable information on candidate genes for floral traits is available from genetic model plants such as *Arabidopsis thaliana* and *Antirrhinum majus*, and a number of homologs of these have also been identified in the orchids (118). Overall flower morphology is determined by the floral genetic program that includes the determination of organ identities and the establishment of directionality, whereas specific floral traits such as coloration, scent, and epidermal cell structure are downstream of the overall floral program. Organ identity is determined by the combinatorial action of transcription factors (mostly MADS-box proteins), and a number of studies have investigated these genes in orchids (86, 118, and references therein). Furthermore, other transcription factors such as *KNOX* genes may be involved in the formation of specific floral structures such as spurs (43) that have been linked to pollination syndromes [e.g., in *Aquilegia* (145)]. Little is known about the genes responsible for determining directionality and zygomorphy in orchid flowers or about the transcriptional targets of MADS-box proteins in orchids. However, studies in *Arabidopsis* and *Antirrhinum* indicate that the transcriptional targets of the MADS-box proteins responsible for perianth identity in these plants include genes involved in scent, color, and wax biosynthesis and only a few other transcription factors (12, 148). Nonetheless, other transcription factors, such as MYB and basic loop-helix-loop (bHLH)-type proteins, are involved in the regulation of downstream floral traits such as floral coloration and epidermal cell shape and overall petal morphology (10, 50, 76, 120).

Ultimately, it is the activity of biosynthetic enzymes in floral color and odor synthesis pathways that is responsible for the formation of these traits. Several of these biosynthetic genes in orchids have been identified. Genes involved in the synthesis of anthocyanidin and carotenoid pigments were cloned from the orchids *Bromheadia* (81), *Dendrobium* (87), *Phalaenopsis* (51, 52, 130), and *Oncidium* (54). Scent genes in the monoterpene synthesis pathway have been identified from expressed sequence tag (EST) libraries in the orchid *Phalaenopsis* (59). Monoterpenes play important roles in fragrance bouquets attracting euglossine bees (55); such scent genes are probably involved in specific pollinator attraction in euglossine-pollinated orchids. Furthermore, in sexually deceptive orchids such as *Ophrys*, which use wax layer components such as alkanes and alkenes for specific pollinator interaction, genes influencing fatty acid and wax biosynthesis may be involved in regulating traits important for pollinator-mediated isolation (118). These biosynthetic genes and their regulators are candidate isolation genes, as they may underlie the strong floral isolation found in orchids pollinated through reproductive behavior.

OUTLOOK

Quantitative studies on floral isolation are rare, but floral isolation is often only detectable by

MADS: MCM1 (yeast MIMICHROMO-SOME MAINTENANCE 1), AG (*Arabidopsis* AGAMOUS), DEF (*Antirrhinum* DEFICIENS), SRF (human SERUM RESPONSE FACTOR)

taking into account visitation frequencies and floral constancy (20, 39). Stains for pollinia (95) and fluorescent powder as pollen analogs (45) are useful tools for such studies. Reciprocal transplant experiments and experimental sympatric arrays are important in this context to control for the effects of different pollinator communities in different habitats in naturally allopatric plant species. As a further complication, pollinator assemblages can vary as a function of geography, so studies on floral isolation should be conducted on a larger geographic scale (14). Further investigation of the evolutionary forces driving floral isolation is needed. Quantifying selection against hybridization would be highly desirable, but this is difficult to achieve in orchids because of complicated germination and long generation times. To link floral isolation to reproductive isolation in general, we need more studies examining the hierarchy and relative importance of different components of reproductive isolation in plants (72, 104).

Our knowledge of the molecular basis of floral isolation is still in its infancy. Orchids, through their long generation times, are not ideal for quantitative genetic approaches, but genomic approaches (e.g., EST libraries) and molecular tools for gene silencing are promising for future studies of molecular gene function (118). Increasing numbers of studies support the view that key adaptations that also bring about isolation can be based on few genes of large effect. Such genes are expected to be key players in plant evolution, and knowledge of their phylogenetic patterns would be highly informative with regard to the evolution of reproductive isolation. Variation in such genes on a population level can provide important information on the adaptability of plants to new pollinators. Whether selection on preexisting genetic variation or on new mutation brings about such novel adaptation is an important and timely question (9), given ongoing climatic changes that will likely change pollinator communities.

SUMMARY POINTS

1. We review the literature on floral isolation, focusing on the occurrence of floral isolation, pollinator behavior that is important in floral isolation, and mechanisms and genetics of floral isolation.

2. In a meta-analysis we show that pollination syndrome has a significant impact on pollinator sharing, i.e., reproductive behavior of pollinators leads to lower pollinator sharing as compared with foraging behavior of pollinators.

3. Reproductive behavior is linked to higher specialization in pollination by the plants.

4. Specialization in the use of pollinators is not related to pollinator sharing.

5. Specialization in the use of pollinators is related to orchid species number.

6. Floral morphology and scent are key traits for floral isolation.

7. Floral isolation may be mediated by few genes of large effect.

ACKNOWLEDGMENTS

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