

Diversification through multitrait evolution in a coevolving interaction

John N. Thompson^{a,1}, Christopher Schwind^a, Paulo R. Guimarães, Jr.^b, and Magne Friberg^{a,2}

^aDepartment of Ecology and Evolutionary Biology, University of California, Santa Cruz, CA 95064; and ^bDepartamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, 05422-970, São Paulo, Brazil

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Mutualisms between species are interactions in which reciprocal exploitation results in outcomes that are mutually beneficial. This reciprocal exploitation is evident in the more than a thousand plant species that are pollinated exclusively by insects specialized to lay their eggs in the flowers they pollinate. By pollinating each flower in which she lays eggs, an insect guarantees that her larval offspring have developing seeds on which to feed, whereas the plant gains a specialized pollinator at the cost of some seeds. These mutualisms are often reciprocally obligate, potentially driving not only ongoing coadaptation but also diversification. The lack of known intermediate stages in most of these mutualisms, however, makes it difficult to understand whether these interactions could have begun to diversify even before they became reciprocally obligate. Experimental studies of the incompletely obligate interactions between woodland star (*Lithophragma*; Saxifragaceae) plants and their pollinating floral parasites in the moth genus *Greya* (Prodoxidae) show that, as these lineages have diversified, the moths and plants have evolved in ways that maintain effective oviposition and pollination. Experimental assessment of pollination in divergent species and quantitative evaluation of time-lapse photographic sequences of pollination viewed on surgically manipulated flowers show that various combinations of traits are possible for maintaining the mutualism. The results suggest that at least some forms of mutualism can persist and even diversify when the interaction is not reciprocally obligate.

coevolution | geographic divergence | correlated traits | trait matching

Coevolving mutualisms among free-living species often favor the formation of networks of interacting species rather than reciprocally specialized pairs of species (1–3). In contrast, mutualisms between species living in intimate (i.e., symbiotic) association often favor the evolution of greater degrees of reciprocal specialization (3). This contrast is evident in pollination mutualisms: plants and pollinators generally form complex networks, but the most consistent deviations from this pattern are the interactions between plants and the insects that lay eggs in the flowers they pollinate. Pollination by these floral parasites, sometimes called nursery pollination, involves reciprocally obligate interactions between a plant species and either one insect species or a small group of insect species within a lineage, potentially driven by selection imposed on the parasitic component of the interaction. Pollination by floral parasites has arisen and diversified repeatedly among plant lineages that are common components of terrestrial communities worldwide (4–9). Examples include yuccas in North America, figs in tropical environments, globe-flowers (*Trollius*; Ranunculaceae) in boreal ecosystems, and leaf-flower (*Glochidion*; Phyllanthaceae) trees in Asia and the Pacific islands (7, 10–12). The pollinators have coradiated with the plants, but often in ways that reflect the complex historical biogeography of these interactions (13, 14).

Intermediate stages in the evolution of these obligate interactions are lacking among living species in most of these mutualisms. That makes it difficult to assess whether these interactions could have begun to diversify even before the interactions became reciprocally obligate for the partners. The interactions between

Greya moths (Prodoxidae) and their woodland star (*Lithophragma*; Saxifragaceae) host plants, however, provide clues to how these mutualisms can diversify even in the absence of, or before, the evolution of reciprocally obligate mutualism. *Greya* moths are closely related to yucca moths (15), and both of these prodoxid lineages include pollinating floral parasites (16). Yucca moths are the sole pollinators of yucca, and *Greya* moths are generally the most important pollinators of woodland stars. In some communities, however, insects other than *Greya* moths, especially solitary bees or bombyliid flies, contribute importantly to the pollination of woodland stars (17–20). Nevertheless, the interaction between woodland stars and *Greya* moths has persisted for millions of years as the plants and moths have diversified (18, 21).

We studied how the traits involved in the interaction and the potential for mutualism have changed as woodland stars and their *Greya* moth pollinators have diverged geographically in western North America. *Greya* moths pollinate at least five woodland star species within two lineages as they oviposit into the flowers (Fig. 1). The woodland star species have diverged in many floral traits, including major changes in the position of the ovary within flowers (22, 23). The species also differ in stigma structure, style length and fusion, petal size and shape, and other aspects of overall floral shape.

We chose six sites that encompassed the latitudinal range of the interactions between woodland stars and *Greya* moths. Together the sites included all of the major *Lithophragma* species involved in the interaction (Table S1). *Lithophragma* is a well-defined genus of seven to nine species restricted to western North America (24). The species pollinated by *Greya* moths occur mostly within two clades. The *Lithophragma parviflorum* clade includes two species with indistinct boundaries, *L. parviflorum* and *Lithophragma affine* (24). *L. parviflorum* is widespread in western North America, whereas *L. affine* occurs mostly in the Coast Ranges of California and some areas of the central Sierra Nevada. The *Lithophragma heterophyllum* clade is distributed as a ring around the Central Valley of California, with *L. heterophyllum* in habitats north, northeast, and west of the valley, *Lithophragma cymbalaria* southwest of the valley in the Coast Ranges and Transverse Ranges, and *Lithophragma bolanderi* east and southeast of the valley in the southern Sierra Nevada (Table S1).

Woodland star species are pollinated by moths in the *Greya politella* species complex, which includes up to four geographically separated, and molecularly divergent, cryptic species (21). The

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¹To whom correspondence should be addressed. E-mail: jnthomp@ucsc.edu.

²Present address: Department of Plant Ecology and Evolution, Evolutionary Biology Centre, University of Uppsala, SE-75236 Uppsala, Sweden.

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Fig. 1. Divergence in morphology and the interaction between woodland star (*Lithophragma* spp.) species and their *Greya* moth pollinators. A window has been surgically cut into the side of each flower to show how pollination occurs during oviposition. As she punctures the floral ovary with her ovipositor, or slides through an opening in the side of the style to lay eggs, a moth simultaneously pollinates the flower with pollen adhering to her abdomen. Flowers vary among species from those having a completely inferior ovary, short style, and a stigma receptive only along the sides (Upper Left) to those having a completely superior ovary, a long style, and a stigma receptive over the upper stigmatic surface (Lower Right). For scale, length of a moth wing averages 6.6–7.7 mm among these populations, and floral width averages 3.0–4.0 mm. (Upper, Left to Right) Plants in the *L. parviflorum* clade: *L. parviflorum* North, *L. parviflorum* South, and *L. affine*. (Lower, Left to Right) Plants in the *L. heterophyllum* clade: *L. bolanderi*, *L. cymbalaria*, and *L. heterophyllum*. Moths are all members of the *G. politella* species complex.

study sites included all four lineages, hereafter labeled *G. politella* PNW, OR, CA, and SN for Pacific Northwest, southern Oregon, California, and southern Sierra Nevada, respectively. These divergent lineages are separated geographically and generally differ in the *Lithophragma* species they use as hosts. Most *G. politella* populations, including all of the populations in this study, visit, pollinate, and lay eggs only in *Lithophragma* (25).

As in other prodoxid moths that are the major pollinators of their host plants (i.e., yucca moths), the entire life history of *G. politella* moths is closely associated with the local host plant population. Adults mate on the host plants and take nectar only from flowers of the host plants. When a female inserts her abdomen through the corolla to oviposit, pollen adhering to one or more parts of her abdomen rubs off onto the stigma, thereby pollinating the flower (Fig. 1). Larvae complete their development on a single host individual. Movie S1 shows examples of moths ovipositing into two *Lithophragma* species.

At three of the study sites (Turnbull, Taylor, and Marble) *G. politella* was the only *Greya* species. At the other three sites an inefficient pollinator of *Lithophragma*, *Greya obscura*, cooccurred with *G. politella*. The inefficient pollinator contributes to pollination only during nectaring, because it does not oviposit directly into the flowers (18). *G. obscura* is normally found only in populations that also have *G. politella* and may depend on the

interaction between *G. politella* and woodland stars for its local persistence. The study therefore encompassed a broad range of the ecological contexts in which *Lithophragma* interacts with its major pollinator, *G. politella*.

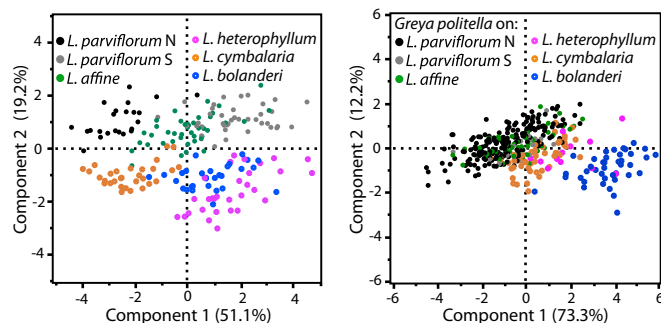


Fig. 2. Divergence in size and shape among *Lithophragma* plant populations (Left) and *G. politella* moth populations (Right) analyzed using principal component analysis. Values in parentheses after the axis labels are the percentage of variance attributable to that principal component axis. n = (plants; moths): *L. parviflorum* North (36; 200), *L. parviflorum* South (40; 20), *L. affine* (42; 48), *L. heterophyllum* (30; 12), *L. cymbalaria* (30; 48), *L. bolanderi* (30; 40).

Populations differed in the moth body parts that touched an anther or the stigma during a bout (Table 1). For example, moths visiting *L. bolanderi* always touched either the anther or the stigma with the abdomen, whereas moths visiting *L. parviflorum* N did so only 27% of the time. The probability of a moth part touching the stigma or an anther did not depend on bout duration (i.e., number of photos) (length effect: $P = 0.414$, $\chi^2 = 0.67$, $df = 1$, logistic regression including effects of population and duration). Despite the differences among populations in plant morphology and in moth morphology and behavior, moths visiting plants from their local population touched either an anther or the stigma during 82–100% of bouts in all populations. The percentages did not differ significantly among populations (Table S3). Hence, different combinations of plant and moth traits all resulted in a high probability that a moth would contact the floral reproductive parts.

Pollination Efficacy. Even with these different oviposition behaviors and interactions between plant and moth body parts, every moth population was an effective pollinator of its local *Lithophragma* population. The mean number of developing seeds resulting from a single bout, however, differed significantly among populations, ranging from 85 seeds for *L. affine* to 523 for *L. bolanderi* (Fig. 4). Moreover, a pollination bout resulted in significantly more developing seeds in plants from the *L. heterophyllum* clade than in plants from the *L. parviflorum* clade (Fig. 4; $P < 0.001$ for clade effect, generalized linear model; Table S4).

The distribution of the number of seeds resulting from a bout also differed among populations and clades (Fig. 4). These distributions are, in effect, the distributions of ecological outcome of the interaction from the perspective of plant fitness. The distributions were strongly skewed toward a smaller number of seeds in the *L. parviflorum/affine* lineage, but not in the other lineage. To the extent that the number of seeds is an index of fitness, then, the potential strength of the mutualism differs between these lineages, but the moths are effective pollinators of both lineages.

Analysis of generalized linear models suggested that the differences among populations in the number of developing seeds were due primarily to the interaction of plant and moth traits (i.e., significant clade and site effects) rather than to other factors (Table S4). The total number of ovules available for pollination also contributed a significant, but smaller, effect. In all popula-

tions, however, a single bout resulted in pollination of less than two-thirds of ovules in all populations. Hence, in no population was seed set in the moth pollination trials limited by the number of available ovules. The duration of a bout, pollen load on a moth, and the number of eggs deposited during oviposition did not significantly affect the number of developing seeds. There were no significant interaction effects among the factors (Table S4).

In separate experiments, hand pollination of flowers reinforced the conclusion that the number of seeds produced per flower in the moth pollination trials was determined primarily by pollination efficacy rather than by factors extrinsic to the plant–moth interaction (Table S5). Selfed flowers produced on average five or fewer seeds in all populations ($n = 165$ crosses). Flowers outcrossed by hand to other individuals within the same population produced on average more than 100 more seeds than moth-pollinated flowers ($n = 166$ crosses). Hence, flowers of all populations were capable of developing more seeds on average than the number produced during a single moth visit. The ratio of the number of seeds resulting from a moth pollination bout compared with hand pollination of outcrossed plants was 38% in *L. parviflorum* North, 56% in *L. parviflorum* South, 27% in *L. affine*, 76% in *L. bolanderi*, 48% in *L. cymbalaria*, and 37% in *L. heterophyllum* (Table S5).

We further analyzed how moth and plant traits affect pollination efficacy by allowing moths from the *L. cymbalaria*-feeding population and the *L. bolanderi*-feeding population to oviposit into flowers from the other population. Although these two plant species are phylogenetically close relatives, the functional morphology trials had shown that moths associated with these two species differ in where they insert the ovipositor within a flower, and the pollination efficacy experiments had shown that these two species differ in pollination efficacy. *G. politella* SN moths from the *L. bolanderi* population pollinated *L. cymbalaria* plants as effectively as moths from the *L. cymbalaria* population, but moths from the *L. cymbalaria* population were poorer pollinators of *L. bolanderi* than *L. bolanderi* moths (Fig. 5). Hence, the evolution of an unusual oviposition behavior in the *G. politella* SN population has not resulted in specialization so strong that it would prevent it from being an effective pollinator of a *Lithophragma* population with a different combination of floral traits.

Discussion

The combined analyses of morphology, time-lapse photographic sequences, and pollination efficacy experiments suggest that the interactions between woodland star plants and *Greya* moths have retained the potential for mutualism even as populations have diverged in morphology, the moths have diverged in behavior, and the taxa have undergone speciation. This complex pollination mutualism has persisted and diversified over millions of years even without the interaction becoming reciprocally obligate in these two genera.

The view that these interactions are robust is further reinforced by ecological studies showing that *Greya* moths are the major, and sometimes almost the sole, pollinator in most populations that have been studied in detail, even though the interaction is swamped in some localities by unrelated copollinators (18, 20, 26). The results therefore provide insight into how the early stages of mutualisms between plants and pollinating floral parasites can evolve and persist. These mutualisms can remain robust even as populations diverge in suites of correlated traits and as some plant populations continue to be pollinated at least sometimes by other taxa.

Prodoxid moths have evolved relationships with plants that range in ecological outcome from parasitism to mutualism (25). Most species are parasitic as larvae on floral, leaf, or stem tissues. The two lineages that have evolved pollination mutualisms with plants, *Greya* moths and yucca moths, include a combination of parasitic and mutualistic species. Mutualistic species have evolved

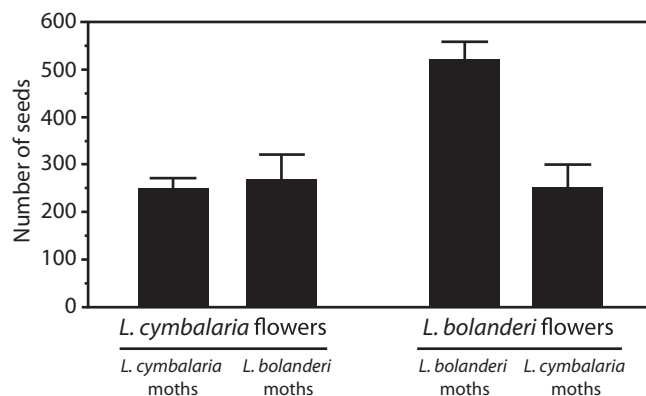


Fig. 5. Pollination efficacy of *G. politella* moths pollinating their local woodland star species compared with a woodland star species from a different locality. Values show a significant interaction effect of moth plant \times plant population (Likelihood ratio $\chi^2_{1,99} = 6.01$, $P = 0.014$, generalized linear model). Values are means \pm 1 SE. Number of trials = 45 *L. cymbalaria* moths on *L. cymbalaria* plants; 11 *L. cymbalaria* moths on *L. bolanderi* plants; 28 *L. bolanderi* moths on *L. bolanderi* plants; 19 *L. bolanderi* moths on *L. cymbalaria* plants.

from parasitic species, and parasitic species have evolved from mutualistic species. Locally parasitic or weakly mutualistic species, however, do not necessarily compromise these mutualisms. Within *Greya*, relatively inefficient *G. obscura* moths cooccur and contribute to pollination at some sites along with the more efficient *G. politella* moths (18). Within the yucca moths, some nonpollinating cheater species have evolved from mutualistic species (27, 28), but the cheater moths persist only where the mutualistic moth species are present to pollinate the flowers. Hence, although some local interactions between these plant and moth lineages may include species that are poor mutualists or even parasites, the pollination mutualism has persisted as the lineages have continued to diversify.

There is, however, nothing inevitable about the evolution of mutualism once plants have been colonized by floral parasites. Parasitism of flowers or seeds is common among insects, but many plant lineages have flowers poorly suited to pollination by ovipositing insects (25). Interactions between some plant and insect lineages, such as *Silene* plants and *Hadena* moths, seem to remain at the interface between parasitism and mutualism; however, like *Lithophragma* and *Greya*, they may possibly vary in ecological outcome among populations (4, 29). It is likely, though, that more plants are pollinated by floral parasites than is currently known. For example, only within the past decade has pollination by insects during oviposition been discovered in the Phyllanthaceae in the tropical Pacific, involving possibly hundreds of plant species, and discovery of similar pollination mutualisms within this and other plant families continues (6, 11, 30, 31).

The matching of floral and pollinator traits has long been considered evidence for coevolution and illustrative of the intricate, and potentially fragile, mutualistic relationships between species (32–36). Plant species often have pollinators whose body parts closely complement the flowers they visit, resulting in effective pollination (37–40). Evolution of the presence, size, or shape of some characters can alter pollination efficacy by creating mismatches in traits (41–44). The results for woodland stars and *Greya* moths, however, indicate that there is a wide range of trait combinations that can sustain mutualisms in codiverging lineages. No simple pattern of matching of plant and moth traits is required to maintain the mutualism, even though some local interactions result in greater pollination efficiency than others. In addition to the morphological traits assessed here, woodland stars also produce a complex array of floral volatiles that vary among species and populations (45). Hence, the number of potentially coevolving and geographically varying traits is even greater than those assessed here.

More generally, these results suggest that mutualisms between species may have become so pervasive worldwide because they often show considerable evolutionary flexibility rather than fragility. Divergence of all coevolving species is undoubtedly driven by multiple selection pressures and also by other evolutionary processes, such as genetic drift acting from time to time on traits in some small populations. The interplay of many evolutionary processes during population divergence and speciation, however, does not necessarily undermine the ability of coevolutionary selection to maintain mutualisms between pairs of species even when those interactions are not reciprocally obligate. Once formed, these mutualisms may often be malleable in the trait combinations that allow the maintenance of the mutualism. Any fitness advantage gained through interacting with another species can be favored by selection, even if the traits do not match optimally. In that sense, it is no wonder that all eukaryotic species have evolved mutualisms with other taxa.

Materials and Methods

Multitrait Divergence. Floral measurements included petal length, petal width, overall flower length, floral width, internal floral depth, floral flair, stigma diameter, and pistil height above the nectary disk (Fig. S1). Moth measure-

ments included wingspan, proboscis length, sixth abdominal segment length, seventh abdominal segment length, and ovipositor length. On the basis of preliminary studies, these were the traits that, directly or indirectly, were likely to affect pollination. The abdominal segments were included because, in *G. politella* females, the seventh segment is two to three times longer than other abdominal segments. This unusual abdominal elongation is not found in *G. politella* males or in other *Greya* species. Floral and moth measurements were made on individuals collected at the study sites. Floral measurements were on the second flower produced on each plant. Morphology was analyzed using principal component analysis on the correlations among traits, with principal component 1 capturing primarily variation in size and overall shape and principal component 2 capturing primarily variation in components of shape orthogonal to principal component 1.

Codivergence of Traits. We assessed codivergence of traits initially by comparing plant and moth principal component values. We then grouped plant and moth individuals hierarchically according to different assumptions to assess the combined contributions of local trait matching and larger phylogenetic effects on the observed patterns. We began by combining all individuals into one plant population and one moth population. We then hierarchically grouped individuals according to the known patterns of genetic divergence among the sampled populations: three plant species in each of the two lineages, and four cryptic moth species within the *G. politella* species complex. Using randomization and model selection procedures based on likelihood approximations, we assessed whether a grouping of plant and/or moth populations based on patterns of genetic divergence explained the data better than a completely randomized distribution of traits among populations. The full randomization sampled all individuals without replacement and randomly assigned them among populations. The structured randomization constrained assignments based on genetic relatedness among populations. The models evaluated variation in the values of the first principal component of the moth traits relative to either the first or second principal component of the plant traits. Collectively, the models analyzed how much genetic structuring of populations beyond a random model was needed to approach the observed pattern of plant and moth trait combinations in the data (further details in *SI Materials and Methods*).

Functional Morphology. Assessment of how pollination occurs during oviposition was made using time-lapse photography on flowers into which a window had been cut into the side of the calyx (Fig. 1). Females, which are unusually docile for moths, were gently placed on a flower and allowed to nectar and oviposit. Nectaring almost invariably precedes oviposition, and a single sequence of nectaring and oviposition (hereafter bout) by a moth takes up to an hour. Each digital image was analyzed to determine which moth part (proboscis, head, side of the abdomen, terminal abdominal membrane) touched which plant part (stigma, anthers) during the entire bout. Terminal abdominal membrane refers to the membrane by which the ovipositor is attached to the terminal abdominal segment. This membrane expands beyond the terminal abdominal segment when a moth extends its ovipositor. The final analysis included 7,591 scorable frames distributed over 65 bouts. Scorable frames were those in which it was possible to determine with certainty whether a female touched an anther or the stigma.

Pollination Efficacy. Pollination efficacy was assessed in the laboratory by placing a female moth directly on a previously unvisited flower in a mesh cage (1 m in height and width and 0.5 m in depth). After she contacted the petals, the moth would either rest for some time or immediately lower her head to begin nectaring. Each female was observed throughout the nectaring and oviposition bout (hereafter bout) to make certain that the trial assessed the effect on seed set of a single time that a female inserted her abdomen into the flower. As soon as she withdrew her abdomen from the flower, the trial ended. The entire procedure was then repeated using the same female but on a new flower of a plant from a different maternal lineage of the same plant population. Only virgin flowers receiving newly collected pollen were used in the assessment of pollination efficacy. Separate experiments used hand-pollination of flowers to test for self-compatibility and maximum potential seed set. Emasculated flowers were pollinated either with anthers from a different flower on the same plant or anthers from three different plants of the same population. More details in *SI Materials and Methods*.

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