ABSENCE OF LONG-PROBOSCID POLLINATORS FOR LONG-COROLLA-TUBED HIMALAYAN *PEDICULARIS* SPECIES: IMPLICATIONS FOR THE EVOLUTION OF COROLLA LENGTH

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The genus *Pedicularis* manifests great variation in corolla morphology, including corolla tube length. Previous observations conducted during daytime have documented bumblebees to be important pollinators for short- and long-tubed *Pedicularis* species. However, the potential role of night-flying long-proboscid insects (moths) as selective agents for the evolution of corolla length has not been determined. Three *Pedicularis* species, *Pedicularis densispica*, *Pedicularis gruinea*, and *Pedicularis siphonantha*, in montane meadows of Yunnan Province, southwestern China, have extremely divergent corolla tube lengths of 7.1, 22.0, and 50.6 mm, respectively. We find that all three species are pollinated by similar bumblebee species. Pollination treatments indicate that seed sets of inflorescences enclosed in fine-meshed nylon nets in the night are not different from open-pollinated inflorescences, but those bagged in the day have significantly decreased seed set, indicating that moth pollinators are not important for the study species. We present anatomical evidence that the loss of nectaries in long-tubed species, and hence the loss of nectar production, is a derived trait. The loss of nectaries may be a key innovation, releasing the corolla from the mechanical constraint imposed by pollinators reaching for nectar.

Keywords: bumblebee pollinator, corolla length, flower divergence, phenotypic variation, *Pedicularis*, pollinator-mediated selection.

Introduction

Pollinator-mediated selection resulting in floral divergence has been inferred through the correspondence of intraspecific floral variation to pollination ecotypes (Grant and Grant 1965; Miller 1981; Armbruster 1985; Pellmyr 1986; Galen 1989; Robertson and Wyatt 1990; Johnson and Steiner 1997; Fenster et al. 2004) and between trait evolution and pollinator shifts as revealed by phylogenies (Armbruster 1993; Hodges and Arnold 1994; Goldblatt and Manning 1996; Beardsley and Olmstead 2003; reviewed in Fenster et al. 2004). Furthermore, direct experimental evidence for pollinator-mediated selection also exists (e.g., Campbell et al. 1996; Fulton and Hodges 1999; Schemske and Bradshaw 1999). While these studies demonstrate pollinator-mediated selection to modify floral shape, it remains a major challenge to understand how flowers diverge in related plants pollinated by similar types of animals (Wilson and Thomson 1996). Similar pollinators visiting related species leads to the expectation of selection for similar floral forms.

Pollinator-mediated speciation has been invoked to explain adaptive radiation in *Pedicularis* (Orobanchaceae, formerly Scrophulariaceae), one of the largest genera of angiosperms in the north temperate zone, with more than 500 species (Macior

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1971; Hong 1983). The genus is characterized by great variation in the corolla, particularly in the galea (the hoodlike upper lip of the corolla) and the length of the corolla tube, which in some species can be greater than 110 mm. The center of *Pedicularis* diversity is in eastern Asia, where more than 200 species exhibit the greatest interspecific variation in corolla morphology (Li 1951; Tsoong 1955, 1956; Hong 1983).

The evolution of long corolla tubes driven by the mechanical fit with long-tongued (or long-proboscid) pollinators has been hypothesized since Darwin (1862) to be due to mutual gain from the plant-animal interaction. Experimental reduction of the corolla tube reduces reproductive success both in *Platanthera* (Nilsson 1988) and in *Disa* (Johnson and Steiner 1997), supporting Darwin's model of pollinator-mediated selection. Therefore, it is not surprising that several earlier workers suggested that long-tubed *Pedicularis* species that occur only in the Himalayas should be pollinated by very long-proboscid Lepidoptera (Pennell 1943; Li 1951; Sprague 1962; Macior and Tang 1997). Recent studies of long-tubed species through field observations indicate that bumblebees are major pollinators in addition to being important pollinators of short-tubed *Pedicularis* species (Macior 1990; Macior and Sood 1991; Macior and Tang 1997; Wang and Li 1998; Macior et al. 2001). Thus, we are left with the puzzling observation that both short- and long-tubed *Pedicularis* species are pollinated by bumblebees. For long-tubed species, there is a mismatch of tongue length of the bumblebee pollinators with corolla tube length because the proboscis of bumblebees is not able to penetrate very deeply into the relatively longer corolla tubes.

To explore the diversification of corolla morphology in *Pedicularis*, we investigate the potential role of long-proboscid pollinators as mediators of natural selection. Although studies have shown that long-tubed species are mainly pollinated by bumblebees, these studies have been limited to daytime observations. The role of night-flying lepidopteran insects as pollinators has not been determined. In this study, we conduct field observation through the day and use a bagging experiment on a long-tubed *Pedicularis* species to quantify the potential role of long-proboscid pollinators as mediators of selection for long-tubed *Pedicularis*. We also make observations of the floral nectary by scanning electron microscopy to determine the role of nectar and, consequently, selection for mechanical fit between pollinator tongue length and nectar access in the diversification of *Pedicularis*.

Material and Methods

Study Species and Site

The corolla of *Pedicularis* consists of a corolla tube, a trilobate lower lip and an upper lip covering four introrse anthers, and a style projecting from the upper lip, with the stigma protruding from the tip. The upper lip can be straight with or without teeth or shaped into a hood or helmet through the fusion of two petals (galea). We studied three sympatric species at a montane meadow (3300 m) occurring in Shangri-La County, Yunnan Province, southwestern China (lat. 27°52′N, long. 99°40′E), during mid-August to September 2005. Thousands of flowering individuals of the three *Pedicularis* species in this study occur in the meadow of ca. 2 km². Individuals of *Pedicularis densispica* Franch. produce one to three erect racemes, each with five to 30 beakless and short-tubed pink flowers. Individuals of *Pedicularis gruina* Franch. produce three to 10 erect short racemose or subcapitate inflorescences, each with three to eight beaked and short-tubed red flowers. Individuals of *Pedicularis siphonantha* Don are rosette and have five to 10 lax stems each, with ca. 20 axillary beaked and long-tubed red flowers (fig. 1).

Nectary Anatomy

To compare the nectary in the three species, we made preliminary observations using light microscopy to determine that the nectaries are located at the base of the ovary. Thereafter, fresh ovaries were collected in the field from randomly chosen flowers of each species and preserved in FAA. Fixed ovaries to be used for scanning electron microscopy were subjected to a dehydration series to 100% ethanol. The ovaries were then dried in CO_2 to the critical point, coated with gold, and observed using a Hitachi S-450 SEM at 20 kV.

Measurements of Floral Parameters

We measured corolla length, the distance from the base of the flower to the tip of the lower lobes, in two newly anther-dehiscing flowers on each of 20 individuals for each of the three *Pedicularis* species. To measure floral nectar, we first randomly checked 30 flowers in each of the three species and found that only the shortest-tubed species, *P. densispica*, produced nectar. Then, we measured nectar volume and sugar concentration of standing crop from one bagged flower from 20 *P. densispica* individuals using micropipettes and a hand-held refractometer, respectively.

Pollinator Assemblage

We took two approaches to quantify pollinators of the three *Pedicularis* species. We constructed three 1×1-m quadrats for each species on the meadow for spot watches. Three observers recorded all insect visitors to *Pedicularis* flowers within these nine quadrats from early morning to dusk, ca. 0800 to 1900 hours local time, from August 28 to September 9, overlapping with the time of peak flowering of the three species. We observed each quadrat for at least three consecutive days to quantify pollinator visitation to *Pedicularis* flowers. Another observer walked the meadow to track potential pollinators and recorded insects visiting the three *Pedicularis* species. Insects that consistently contacted both anthers and stigmas within one flowering species were considered pollinators. We recorded pollinator behaviors and photographed all types of flower visitors. We follow Harder's (1982) method to measure proboscis length of bumblebees. We dissected bumblebee specimens that were preserved in 70% alcohol and measured the distance from the basiglossal sclerite to the labellum as the proboscis length. Insect specimens were sent to the Institute of Zoology, Chinese Science Academy, Beijing, for identification.

Pollination Treatments

To investigate whether night-flying long-proboscid insects are pollinators for long-tubed *Pedicularis* species, we conducted two pollination treatments on 12 randomly selected individuals of *P. siphonantha* using fine-nylon nets with openings of $1 \text{ mm} \times 1 \text{ mm}$. On each individual, we bagged one inflorescence in the day from 0800 to 1900 hours to isolate day pollinators and removed the nets during the evening to expose the inflorescence to potential night pollinators. The alternative treatment was made on another inflorescence of the same plant by bagging the inflorescence in the evening from 1900 hours to the next morning and then removing the nets during the day to expose the inflorescence to day pollinators. The timing of the placement and removal of the bags was appropriate because there was only limited seed set in the night treatment. Before manipulating these 24 inflorescences, we marked where flowers had blossomed using a cotton thread because of acropetal growth in the inflorescence. We repeated the manipulation of covering and uncovering these inflorescences for 10 d, overlapping with the peak phenology of this species. Control inflorescences were simultaneously marked. We collected these inflorescences 1 wk later and counted seed production in four flowers on each inflorescence that had experienced the treatments. Previous study has shown that ovary expansion is a reliable indicator of fertilization because of seed abortion caused by inbreeding depression in this species (Yang et al. 2005). To examine the capacity for autonomous self-pollination and therefore the importance of insect visitors to pollination, we bagged one inflorescence of eight individuals. As a control, we collected one inflorescence from each of 10 naturally pollinated individuals. Seed production of four flowers from each inflorescence was counted. We compared seed set (ratio of seeds to ovules)

Fig. 1 Floral traits and pollinator behaviors on three *Pedicularis* species. *a*, Single flower and pistil of three *Pedicularis* species, showing different lengths of corolla and style. *b*, *Bombus richardsi* removing nectar from and nototribic pollinating *Pedicularis densispica*. *c*, *Bombus richardsi* collecting pollen from and sternotribic pollinating *Pedicularis gruina*. *d*, *Bombus richardsi* collecting pollen from and sternotribic pollinating *Pedicularis siphonantha*.

among the four pollination treatments on *P. siphonantha* using ANOVA (Proc GLM) followed by Tukey tests (SAS Institute 2004).

Results

Pollinator Assemblage

Bumblebees were the sole pollinators for all three *Pedicularis* species as determined from more than 400 h of observation from four observers at quadrats or through haphazard walks through the meadow. Only *Bombus richardsi* Renig and *Bombus yunnanicola* Bischoff were observed to pollinate

the three *Pedicularis* species despite butterflies, flies, and hover flies constantly observed visiting other flowering plants in the same meadow. At nine quadrats, our observation documented that *B. richardsi* was the major pollinator, accounting for 90.4%, 84.5%, and 82.8% floral visits in *Pedicularis densispica*, *Pedicularis gruina*, and *Pedicularis siphonantha*, respectively (table 1; fig. 1). The two pollinators behaved differently when they were visiting the three *Pedicularis* species. Nectariferous short-tubed *P. densispica* was nototribic pollinated when bumblebees foraged for nectar. The nectarless, short-tubed *P. gruina* and long-tubed *P. siphonantha* were sternotribic pollinated when bumblebees collected pollen grains (fig. 1). *Bombus richardsi* is larger than *B. yunnanicola*, and

Table 1

Floral Traits (Mean ± SE), Coefficient of Variation (CV) in Tube Length, and Number of Visits by Two *Bombus* Pollinators for Three *Pedicularis* Species in a Montane Meadow in Southwestern China

Species	Tube length (mm)	CV	Nectar volume (μL)	Sugar concentration (%)	No. flowering individuals/m^2	Visits by *Bombus richardsi*	Visits by *Bombus yunnanicola*
Pedicularis densispica	7.1 ± .17	.128	3.0 ± .06	31.2 ± .83	13.6	1276	135
Pedicularis gruina	22.0 ± .56	.135	0	0	4.2	240	44
Pedicularis siphonantha	50.6 ± 1.44	.149	0	0	10.6	390	81

tongue lengths are 8.74 ± 1.14 (mean ± SE; $N = 12$) and 6.9 ± 0.86 mm ($N = 10$), respectively.

Pollination treatments to examine whether night-flying insects pollinate long-tubed *P. siphonantha* indicated no significant difference in the seed set of open-pollinated (control) individuals and plants bagged only in the evening (thus subjected to day pollinators). Seed sets of bagged individuals (presumably autonomous self-pollination) and those bagged in the day (thus subjected to night pollinators) were not significantly different (fig. 2). Seed sets of individuals subjected to day pollinators and open pollinated, however, were significantly higher than those subjected to potential night pollinators or self-pollination ($F_{3, 38} = 35.96$, $P < 0.001$). The cage experiment thus indicates that day pollinators (bumblebees) are the only pollinators of long-tubed *P. siphonantha*.

Corolla Length and Nectary Morphology

Corolla length varied greatly among the three species. Compared with *P. densispica*, *P. siphonantha* and *P. gruina* have corolla tubes that are approximately threefold and sevenfold longer, respectively (table 1). Nectar was observed in the short-tubed *P. densispica* only, with corolla length of ca. 7 mm (table 1).
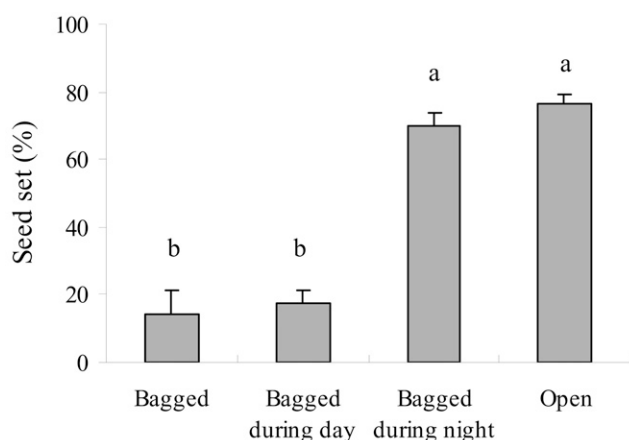

Fig. 2 Comparison of seed sets (mean ± SE) of four pollination treatments in long-tubed *Pedicularis siphonantha*. Seed sets of open-pollinated (control) individuals and those bagged in the evening and opened in the day were not significantly different, but both were higher than those of individuals bagged in the day or bagged both in the evening and in the day (self-pollination). The same letters above bars indicate that differences between treatments are not significant ($P > 0.05$) using a Tukey post hoc comparison.

We observed a conspicuous, discoidal floral nectary at the ovary base in short-tubed *P. densispica* (fig. 3a, 3d). In *P. gruina*, one or two protuberant nectaries were small (fig. 3b, 3e), and in *P. siphonantha*, the nectary was inconspicuous (fig. 3c, 3f).

Discussion

Absence of Long-Proboscid Pollinators

Our pollination experiments exclude the possibility that night-flying insects are pollinators of the long-tubed study species. The *Pedicularis* species we studied are diurnally pollinated. It is consistent with fact that these species are not light colored and do not emit fragrance or begin anthesis in the evening, traits that are strongly associated with pollination by moths (Fenster et al. 2004). Proboscis lengths of bumblebee pollinators match corolla tube length in nectariferous *Pedicularis densispica*, concordant with the requirement of access to nectar by pollinators. In contrast, we did not observe a similar fit of pollinator proboscis lengths with corolla tube lengths in *Pedicularis gruina* and *Pedicularis siphonantha*. Field observations (including those reported here) from this area and from Europe, North America, and Japan have shown that bumblebees are major pollinators for almost all *Pedicularis* species (Macior 1982, 1988, 1990; Macior and Sood 1991; Philipp et al. 1996; Macior and Tang 1997; Wang and Li 1998, 2005; Macior et al. 2001).

A recent phylogenetic study demonstrates a significant association between short-tubed flowers and nectar production in *Pedicularis* (Ree 2005) and confirmed earlier hypotheses of the direction of floral character evolution (Li 1951; Tsoong 1955, 1956; Yang et al. 2003), as the gain of long corolla tubes was much more likely than their loss. Our comparative observations on floral ontogeny among *Pedicularis* species with different corolla tube lengths, though the species were not each other's closest relatives, reveal remnant nectar secretory tissues in long-tubed species, consistent with the notion that loss of nectar is derived in *Pedicularis* species providing only pollen reward. Therefore, the mapping of traits on phylogeny (Ree 2005) and anatomical evidence that loss of nectaries is derived is consistent with nectar-producing species of *Pedicularis* being constrained by bumblebee pollinators to have short tubes.

We conclude that selection for access to nectar does not act on corolla length in contemporary populations of long-tubed *Pedicularis* species. These results suggest two phenomena: (1) the occurrence of selection on tube length in nectariferous *Pedicularis* species is via mechanical fit of the pollinator and (2) very long-tongued lepidopteran pollinators have not been
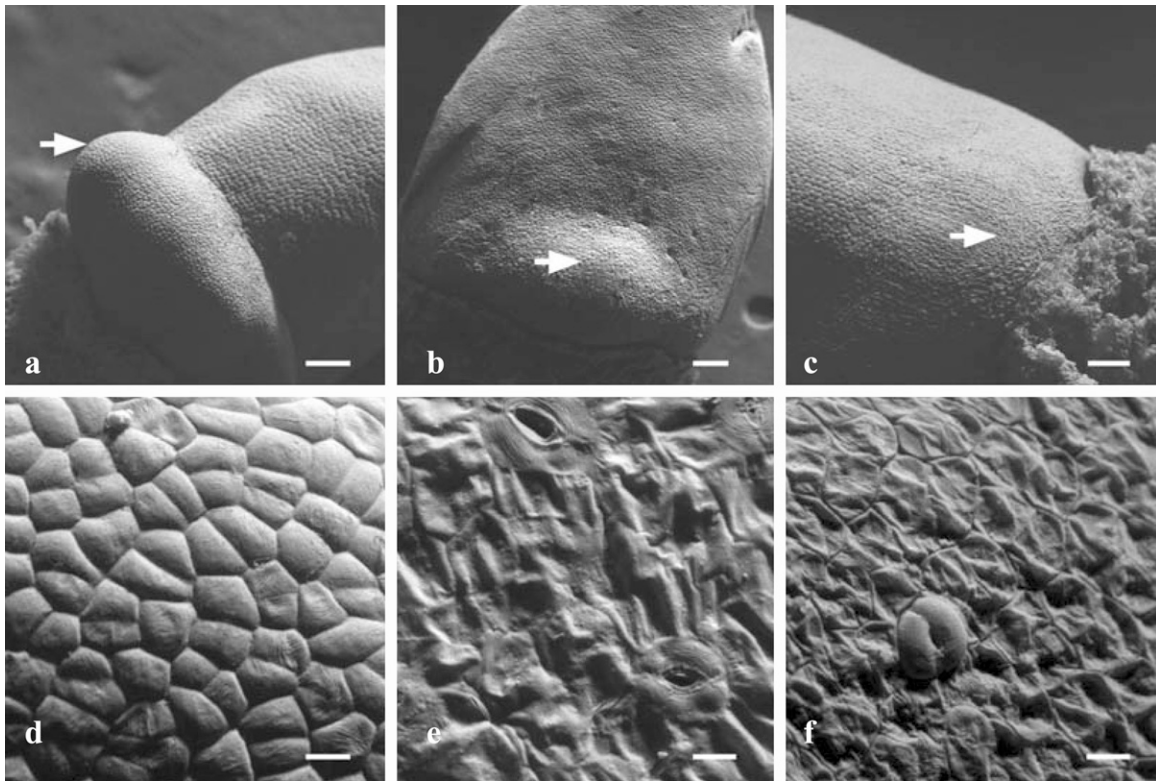
Fig. 3 Nectary SEM micrographs of three *Pedicularis* species. *a*, *d*, *Pedicularis densispica*. *b*, *c*, *Pedicularis gruina*. *c*, *f*, *Pedicularis siphonantha*. *a–c*, Views of ovary with the nectary (arrow) at its base, showing different sizes of nectary. Scale bars = 100 μm. *d–f*, Detail of the nectary epidermis, showing different fullness of epidermis cells among three species. Scale bars = 10 μm.

selective agents for the evolution of long corolla tubes in *Pedicularis*. Given the evidence of the simultaneous gain of corolla tube length with the loss of nectaries, it seems unlikely that long corollas in *Pedicularis* are relict, representing an adaptation to long-tongued insects that have disappeared (e.g., *Schiedea lychnoides*, presumably pollinated by extinct honeyeaters; Wagner et al. 2005). Because our results are concordant with previous observations (Macior and Tang 1997; Wang and Li 1998, 2005; Macior et al. 2001), it is unlikely that an extinct long-proboscid lepidopteran pollinator is the important pollinator for the long-tubed *P. siphonantha* studied here because many long-proboscid butterflies were observed at the study site on other flowers. However, if the flowers were adapted for long-proboscid nectar-feeding insects, then it would be one of the very few examples of floral mimicry or reward deception outside the Orchidaceae (S. Johnson, personal communication).

In contrast to previous observations that long-corolla species are associated with low variation in tube length and thus supportive evidence of pollinator-mediated selection on floral shape (Fenster 1991; Cresswell 1998; Alexandersson and Johnson 2002), here (table 1) and elsewhere (S.-Q. Huang and C. B. Fenster, unpublished data), we document relatively greater variation in corolla length in long-tubed *Pedicularis* species. These results support the hypothesis that large variation in corolla length occurs where the strength of directional selection is reduced or selective pressures are no longer exerted on corolla tube length, corroborating the prediction that access to nectar by pollinators constrains the evolution of corolla length in short-tubed *Pedicularis* species (Ree 2005).

Possible Selective Agents of Corolla Length in Pedicularis

Pollinator-mediated selection has been considered one of the underlying mechanisms for floral diversification (e.g., bumblebee- and hummingbird-pollinated *Mimulus lewisii* and *Mimulus cardinalis*, respectively; Schemske and Bradshaw 1999; reviewed in Fenster et al. 2004). Given the tremendous diversity of floral form found within *Pedicularis*, it may at first be surprising (see Ollerton 1998) that we document divergent *Pedicularis* species as having overlapping *Bombus* pollinators. One expectation might be that different floral forms of *Pedicularis* reflect divergent selection imposed by different pollinators or, equivalently, that otherwise similar pollinators visiting related species results in selection for similar floral form. However, our results are mirrored in other groups. For example, *Calochortus* lilies have radiated within California while most species are pollinated by pollen-collecting beetles (Dilley et al. 2000; Patterson and Givnish 2003). Therefore, divergent selection pressures imposed by different types of pollinators are not prerequisite for floral diversification.

Floral diversity is associated with different mechanical fit of the pollinator with the flower or placement of pollen on different parts of the pollinator's body. For example, the *Platanthera* orchids of northern Europe have diverged by placing their pollinia on either the proboscis or the eye of

their moth pollinators (Nilsson 1983; Maad and Nilsson 2004). Indeed, Grant (1994*b*) named the phenomenon of reproductive isolation via placement of pollen on different parts of the pollinator's body the "*Pedicularis* type" in recognition of Sprague's (1962) and Macior's (1977) observations that related species of *Pedicularis* place their pollen either sternotrobic or nototrobic (Grant 1994*a*; Robart 2005), which is also mirrored in our observations.

We conjecture that the evolution of nectarless flowers may have released *Pedicularis* from selective constraint associated with mechanical fit of pollinator proboscis with access to nectar, thus allowing novel selection pressures to arise, including the role of different pollinator behaviors to dictate the evolution of floral form. Among the adaptive explanations, the function of the long corolla has been proposed to increase visual attractiveness through projecting a greater distance from the leaves (Macior and Sood 1991; Ree 2005). In this case, the longer flower may be functioning as a pedicel. However, this adaptive explanation does not adequately explain why long-corolla *Pedicularis* species are concentrated in the Himalayas. Alternatively, corolla-tube-length variation may reflect selection for reproductive isolation among sympatric *Pedicularis* species in the Himalayas, the diversification center of this large genus. While the adaptive explanation(s) for the evolution of corolla tube length in *Pedicularis* is as yet unknown, it is clear that *Pedicularis* represents an ideal plant group in which to investigate how flowers sharing similar pollinators diverge.

Acknowledgments

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