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Pollen size strongly correlates with stigma depth among *Pedicularis* species

Summary Darwin proposed that pollen size should be positively correlated with stigma depth rather than style length among species given that pollen tubes first enter the stigma autotrophically, then grow through the style heterotrophically. However, studies often show a positive relationship between pollen size and style length. Five floral traits were observed to be correlated among 42 bumblebee-pollinated *Pedicularis* species (Orobanchaceae) in which stigmas are distinct from styles. The phylogenetic independent contrast analysis revealed that pollen grain volume was more strongly correlated with stigma depth than with style length, consistent with Darwin's functional hypothesis between pollen size and stigma depth.

Although the evolution of pollen grain size may involve multiple physiological, morphological, ecological, and historical constraints (see Torres 2000), pollen size has often been assumed to be functionally related to style length because larger pollen grains with more energy storage may have a higher probability to reach ovaries with long styles. Based on the assumption, Delpino (1867) first proposed a positive correlation between pollen size and style length (see Cruden 2009). Indeed, a number of studies have shown a positive correlation between pollen size and style length (Torres 2000; Yang and Guo 2004; López et al. 2006; Jürgens et al. 2012; but see Bedinger et al. 2011). Darwin (1877) realized that pollen tubes should first grow autotrophically through the stigma, then heterotrophically through the style, so he rejected the interpretation by Delpino (1867) and proposed a positive correlation between pollen size and stigma depth.

Studies on pollen-pistil interactions have shown that pistil structures along the pathway of pollen tube growth perform different roles. The stigma provides an environment in which pollen grains germinate, and pollen tubes travelling along the pistils become increasingly dependent upon the specialized tissue surrounding the tract within the style for nutritive support (Labarca and Loewus 1972; Herrero and Hormaza 1996; Stephenson et al. 2003). Darwin's hypothesis seems much more reasonable than Delpino's, but examinations capable of distinguishing between these two hypotheses have been surprisingly few. To date, only Robert Cruden and colleague have tried to measure stigma depth among plant species (Cruden and Lyon 1985; Cruden 2009); however, a phylogenetically independent contrasts (PIC) analysis (Felsenstein 1985) has not been used to examine the relationship between pollen size and stigma depth.

Measurements of stigma depth are rare, probably because it is difficult to distinguish the stigma and the style in many plants. Here we use *Pedicularis* to test whether pollen size is positively correlated with stigma depth and/or style length. The suitability of *Pedicularis* species for testing Darwin's and Delpino's hypotheses is fourfold. First, corolla tube length (associated with style length and flower size) varies greatly among closely related species. A previous study has shown that pollen size is correlated with style length in 40 *Pedicularis* species (Yang and Guo 2004) but stigma depth was not measured. Second, stigma morphology of *Pedicularis* species is characterized by a bowl-shaped or spherical head and the stigmatic surface is distinctly recognizable by its generally rough with "gully-like" indentations (Figure 1). Furthermore, fresh stigmas are often distinct from the style in color. The morphological and color differences between stigmas and styles facilitate our measurement of stigma depth (Figure 1). Third, extensive studies have shown that *Pedicularis* species in the Himalayan region are pollinated by bumble bees (Liu et al. 2016). Related species share similar pollinators, reducing the confounding effect of pollination modes on pollen size (Jürgens et al. 2012). Fourth, recent molecular phylogenetic study has resulted in well-resolved evolutionary relationships among *Pedicularis* species (Yu et al. 2015), particularly the species in the Himalayan region where we worked. This permits us to conduct PIC analyses to examine the relationships of pollen size and other floral traits.

Flowers of 42 *Pedicularis* species were collected from the Hengduan Mountains in southwest China in 2012. For each species, we picked 1–3 newly opened flowers per plant from about 10 individuals from natural populations. Using digital calipers, we measured flower length, width of the lower lip, and style length. We measured the stigma depth under a stereomicroscope and pollen grain volume of hydrated pollen grains under a compound microscope with a micrometer scale. Stigma depth was measured as the vertical distance from the stigma top to the lower edge (Figure. 1). The mean values of five floral traits of each *Pedicularis* species are presented in Table S1. Pollen size and style length were positively correlated among species (Pearson coefficient $R = 0.55$, Table S2), but a stronger positive correlation was observed between pollen size and stigma depth ($R = 0.82$; Figure. S1). The strongest positive correlation was observed between flower length and style length ($R = 0.97$; Table S2), suggesting that these two correlated traits could be indicators of flower size in *Pedicularis*.

Four markers (ITS, *matK*, *rbcL* and *trnL-F*) were used to reconstruct the phylogeny of the 39 *Pedicularis* species (DNA sequences of three species unavailable). PIC analyses were implemented in R program using the *ape* package. We calculated Felsenstein's contrasts for five traits based on the tree, and then performed Pearson correlations between the contrasts.

The maximum likelihood (ML) tree annotated morphological characters are shown in Figure 2. Four major clades were numbered following the study of Yu et al. (2015). Generally, species in clade 3 and 4 had relatively larger

Figure 1. Stigma and style morphology of eight *Pedicularis* species under a stereomicroscope
Images were taken at the similar magnification to show the stigma and style distinct in color and head shape in each species. Stigma depth was measured as the vertical distance from the stigma top to the lower edge (arrows marked).

flowers, whereas species in clade 5 had thicker stigmas and larger pollen grains. Flowers in clade 1 were smaller, but three species (*P. dolichoglossa*, *P. lyrata*, and *P. pectinatiformis*) had thicker stigmas and larger pollen. Felsenstein's contrast analyses showed that five floral traits were generally correlated, among them style length and flower length were highly correlated (Table 1), but flower lower lip was not correlated with stigma depth and pollen size. Pollen size was positively correlated with style length ($R = 0.47$), but more strongly with stigma depth ($R = 0.87$) (Table 1; Figure S2). We observed five obvious transitions of pollen size between two closely related species in which four transitions were consistent with Darwin's hypothesis that changes in pollen size associate with stigma depth rather than style length, but one transition between *P. cyathophylla* and *P. superba* was not (Figure 2). These results indicate that evolutionary shifts of pollen size were usually associated with corresponding changes in stigma depth but not so much in style length.

Our phylogenetic comparative analysis confirmed that pollen size was more strongly correlated with stigma depth than with style length among *Pedicularis* species (Table 1; Figure S2), consistent with the prediction of Darwin on pollen size although it does not falsify Delpino's hypothesis (Cruden 2009). Pollen size is also correlated with style length in *Pedicularis* species, probably because style length is generally correlated with stigma depth.

Delpino's hypothesis proposed that species with long styles evolved large pollen grains to support pollen tube growth. *Pedicularis armata* had the longest style (over 100 mm) among the studied species, but its pollen volume was relatively small (7,422 μm^3). Inconsistent with Delpino's hypothesis, several species had relatively large pollen grains but relatively short styles. For example, the style lengths of *P. dolichoglossa*, *P. lyrata* and *P. polyodonta* were relatively short ranging from 24 to 27 mm, but their pollen volumes were quite large (over 13,000 μm^3, Table S1). However, the three species had relatively large stigma depths, more than 390 μm, in support of a positive correlation between pollen size and stigma depth. Darwin (1877) noted obvious differences in pollen size between two morphs of distylous *Suteria* species (Rubiaceae), but their style lengths being equivalent. Likewise, in some distylous species (*Linum* and *Limnanthemum*), the style lengths were apparently different but pollen size of two morphs was equivalent (see Cruden 2009).

An analysis of nine species within the tomato clade did not find a positive correlation between style length and pollen size (Bedinger et al. 2011). Among 14 Nyctaginaceae species, pollen size – pistil length correlation was positive among seven species with starchy pollen but not among the other seven species with lipids as pollen reserves (López et al. 2006), implying that pollen reserves may affect the evolution of pollen size. Pollen tube growth in the style is probably controlled by the recipient, since donor pollen size had minimal effects on pollen fertilization ability (Cruzan 1990). Surprisingly, pollen size has rarely been related to stigma traits but has often been related to style length. The correlation of pollen size and style length may be an inevitable concomitant of the widespread correlation

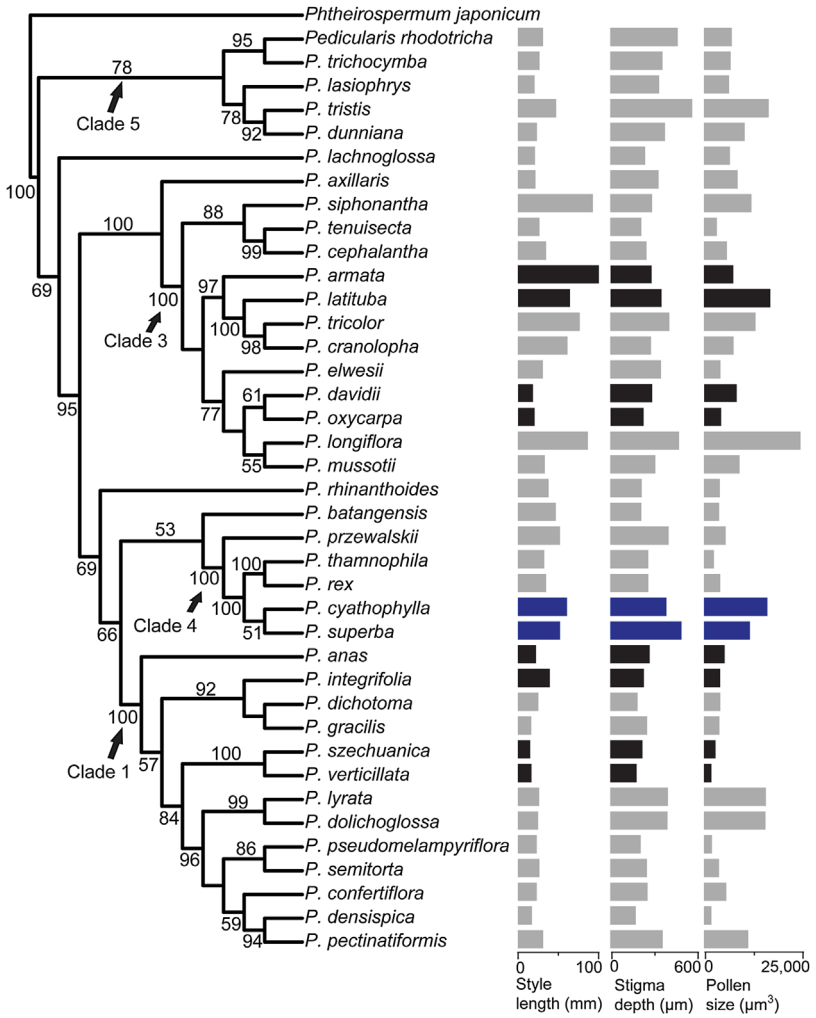

Figure 2. Phylogenetic relationship of 39 *Pedicularis* species, with *Phtheirospermum japonicum* as outgroup
Numbers beside branches indicate Maximum Likelihood bootstrap support. Bar length indicates length of floral traits or pollen grain volume with scale bars at the bottom. Four pairs of closely related species (dark-colored bars) which changes in pollen size associated with stigma depth rather than style length are consistent with Darwin's hypothesis but one transition between a pair species (blue bars) is not.

Table 1. The Pearson's correlation (upper right) and *P*-values (lower left) between Felsenstein's contrasts of two floral traits on the phylogenetic tree involving 39 *Pedicularis* species

	Flower length	Lower lip width	Style length	Stigma depth	Pollen volume
Flower length		0.704	0.980	0.418	0.477
Lower lip width	8.11E-07		0.776	0.269	0.167
Style length	2.20E-16	1.04E-08		0.419	0.472
Stigma depth	0.009	0.102	0.009		0.871
Pollen volume	0.002	0.315	0.003	1.17E-12	

between pollen size and stigma depth in numerous plant groups (including within many heterostylous species), as shown in *Polygonum* (Cruden and Lyon 1985), *Pultenaea* (Cruden 2009) and *Pedicularis* (this study). The functional basis of the correlation between style length and stigma depth remains to be explained.

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AUTHOR CONTRIBUTIONS

S.-G.S. and S.-Q.H. collected materials from the field, X.-P.W. and S.-Q.H. measured floral traits and collected data, X.-P.W. and W.-B.Y. analyzed the data. X.-P.W. and S.-Q.H. wrote the manuscript. All authors contributed in experimental design, data analysis and commented the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.
Doc 1. Materials and Methods
Figure S1. The relationship between pollen volume and style length (a) and stigma depth (b) among 42 *Pedicularis* species
Figure S2. Felsenstein's contrasts of two floral traits between pollen grain volume and style length (open circles) and stigma depth (closed blue circles) based on the PIC analysis among 39 *Pedicularis* species
Table S1. Five measured floral traits (Mean ± *SE*) of 42 *Pedicularis* species
Except where we measured 20 flowers per species, the flower number is given in parentheses.
Table S2. The Pearson's correlation (upper right) and *P*-values (lower left) between five floral traits among 42 *Pedicularis* species
Table S3. GenBank accessions of DNA sequences of four markers for 39 studied *Pedicularis* species