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Screening game in plant–pollinator interactions: compound screening mechanism in flower morphology of Delphinium caeruleum

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Abstract

The screening mechanism is an important component of the screening game between plants and pollinators, and also as a theoretical framework for understanding the maintenance and drivers of co-evolution by animals and plants. However, the composite screening mechanism in plants from biomechanical and morphological perspectives was seldomly studied. In this study, we measured the insects and flower traits in different populations of *Delphinium caeruleum*, and compared the operative strength of staminodes with the strength that insects were capable of exerting, as well as the length of the nectar spur and proboscis in insects. The results showed that the flower traits, insect species, visitor frequency, and efficiency differed among three population. The strength and proboscis length of the insect visitors matched with the operative strength of staminodes and the nectar spur length, whereas the opposite was found for non-visitors. Our results demonstrate that *Delphinium caeruleum* can screen for efficient pollinator insects through a complex mechanism based on a combination of length screening and biomechanical screening. Pollinators contribute significantly to promoting both the convergence and divergence of flower characteristics, which may be a consequence of plants adapting to local pollinators.

1 Introduction

The traits of angiosperm flowers are strikingly diverse, where they vary in terms of color, scent, size, and the type and amount of reward they offer to pollinating animals (Conner, 1997). The evolution of floral traits has always been a focus of evolutionary biologists, but the evolutionary mechanism is still unclear. As early as 1970, Stebbins (1970) proposed the "most effective pollinator principle" where a flower's traits will be molded by those pollinators that are most effective and frequent in the local area (Mayfield et al. 2001; Huang, 2014). This view was supported by studies on species and phylogenetic levels (Whittall and Hodge, 2007; Pauw et al. 2009; Paudel et al. 2016; Johnson & Raguso, 2016). For instance, the emergence of many specialized pollination systems in plants is related to cooperation between plants and pollinators, and selection for their traits, where the different traits and foraging preferences of pollinators mediate the formation of different flower traits (Hattori et al. 2015). Classically, plant assemblages pollinated by the same pollinator are under similar selective pressures, so they should exhibit convergence in terms of their floral characteristics (Johnson and Steiner, 2000; Johnson and Raguso, 2016). By contrast, ecotypic divergence will occur when plants are pollinated by behaviorally or morphologically different insects in different populations (Fenster et al. 2004; Sun et al. 2014; Peter and Johnson, 2014). Therefore, flower traits reflect adaptations to specific pollinator groups (Fenster et al. 2004; Faegri and van der Pijl, 1979; Pyke, 2016), thereby leading to the emergence of the conceptual framework of pollination syndromes. The evolution of a structure should be consistent with the function that it performs, and thus, function is the main driving force related to structural evolution. Therefore, in order to understand the special structures and morphology of flowers, adaptive pollination (or improving reproductive fitness) has been the main focus of researchers, such as changes in flower color and shape (Irwin & Strauss, 2005; Veiga et al. 2016), heterostyly (Gilmartin, 2015; Yuan et al. 2017), the staminal lever mechanism (Claßen-Bockhoff et al. 2004), and a flexible style for pollination (Li et al. 2001), which are plant adaptations to various environmental pressures to satisfy the function of reproduction. Due to the identification of these features, researchers have increasingly focused on the diversity and sophistication of plant morphological structures under various selection pressures (biotic and abiotic), and the function of plant staminode are among these structures.

Staminodes are stamens that have lost the main function of producing pollen and they often perform important secondary flower functions (Jennifer and Harder, 2000). Studies of the phylogenetic distribution of staminodes indicate that staminodes usually appeared during evolutionary reduction of the androecium as a result of long-term natural selection in the evolutionary process. Previous studies by botanists and evolutionary biologists focused on

morphological descriptions of staminodes, and deeper research into functional staminodes has only begun to conducted in recently (Botnaru and Schenk, 2015, Hou et al. 2022a; b). Staminodes form a physical barrier structure in some genuses, such as *Penstemon, Verticordia* and *Darwinia*, and it is generally considered that the ecological function of these staminodes is to protect the internal structures of flowers from insects (Rodríguez-Riaño, 2015). However, recent studies have found that these staminodes may have functions in insect screening, where plants present biomechanical barriers in the form of staminodes to limit visitors from reaching flower rewards (or increase the cost of rewards) in order to screen for effective pollinators (Córdoba and Cocucci, 2011; Hou et al. 2022a, b). The screening mechanism is an important component of the screening game between plants and pollinators, and it serves as a useful theoretical framework for understanding the maintenance and drivers of animal and plant coevolution. In addition, studying screening mechanisms provides a theoretical basis for understanding insect-mediated plant flower morphology evolution as well as the specialized and generalized pollination mechanisms involving plants and pollinators. However, only a few studies have investigated insect screening mechanisms and that is mainly descriptive reports (Córdoba and Cocucci, 2011; Hou et al. 2022a), which are not sufficient for developing a theoretical understanding of insect screening mechanisms.

Many plant flowers have movable parts that must be actively handled by insects to let sexual organs be contacted (Córdoba and Cocucci, 2011). Similarly, *Delphinium caeruleum* flowers have a complex structure, where two blue staminodes form a "double door" structure which cover anthers and stigmas. The "double door" staminodes can be opened under a certain external force to expose the male and female stamen structures hidden under the staminodes. The pollinator must open the "double door" structure (staminodes) to achieve rewards, and complete pollination by contacting with the stamens and pistils below the staminodes, and thus, this structure may form an effective screen (biomechanical screening) that selects for insects with greater strength. Córdoba and Cocucci (2011) designated the mechanical strength required to open a forcible floral mechanism as operative strength. In addition, *D. caeruleum* has a spur formed by the extension of petals, with nectar at the end of the spur, which may also form a typical "length" screening structure where only insects with a long proboscis might access the nectar in the spur (Newman et al. 2014). Therefore, we hypothesized that successful pollination of *D. caeruleum* might be achieved through a complex mechanism mediated by a combination of length screening and biomechanical screening.

Based on the hypotheses described above, we examined the ecological functions of the staminodes and nectar spur in *D. caeruleum* to determine whether this composite mechanism performs the function of screening for effective pollinators. We conducted our study in three population of *D. caeruleum* in northwest China, i.e., in Hezuo, Haibei, and Tianzhu. We investigated the ecological functions of these special flower morphological traits (a compound coevolution mechanism combined with length coevolution), and then explored the evolutionary mechanism associated with the flower morphology and structure. In particular, we plan to solve following questions. (1) What are the differences in the flower traits and pollinators among different populations? (2) Do the delicate structures of the staminodes and nectar spur play roles in screening efficient pollinators for *D. caeruleum*? (3) If this is the case, will the different combinations of insects in different populations lead to differences in the operative strength of staminodes and the spur length (i.e., different local groups might differ in terms of biomechanical and length matching)?

2 Materials And Methods

2.1 Study sites and species

We investigated the following three population in China (Fig. 1). The Hezuo site is situated in Gannan, Gansu Province (alt. 2977 m; lat. 34°57'7.48"N, long. 102°52'59.79"E), where the vegetation type is alpine degraded grassland and rich in associated plants, it is mainly composed of *Compositae*, *Rosaceae* and *Gentianaceae*. The Haibei site is situated in

Qinghai province (3195 m; 37°36'45.12"N, 101°18'47.07"E), the population has relatively large human disturbance, and the associated plants are mainly composed of *gramineous* and artificially planted *Brassica campestris*. This ecological environment has been greatly affected by human activities. The Tianzhu site is situated in Wuwei, Gansu province (alt. 2906 m; lat. 37°11'42.81"N, long. 102°47'2.06"E), where the vegetation type is alpine arid grassland with few associated plants, it is mainly composed of *Compositae* and *Gramineae*.

Delphinium caeruleum Jacquem. ex Cambess is an herbaceous perennial plant in the family Ranunculaceae with a widespread distribution in the plateau area of Northwest China. The corymb inflorescence can bear 1–7 clustered flowers (Fig. 2a). The individual *D. caeruleum* flower has a complex structure, with five large and bright sepals, where one sepal extends to form a calyx spur. The ends of the two petals extend to form a nectar spur within the calyx spur. The nectary produces a large amount of nectar, which is stored in the nectar spur (Figs. 2b, 2d). Two blue staminodes form a "double door" structure and they are covered with anthers and stigmas. The "double door" staminodes can be opened under a certain external force to expose the male and female stamen structure hidden under the staminodes (Fig. 2c).

2.2 Floral traits

We randomly bagged 30 flower buds on 30 individual plants at each population to assess the flower traits. We measured the flower length, staminodes length, staminodes width, calyx spur, nectar spur, flower width, sepal length, and sepal width after anthesis using digital calipers. The operative strength of staminodes was detected with a biological tension sensor (BL-420s biological function experimental system and FT-102 biological tension sensor, 0.001 g to 5 g; Techman Soft, Chengdu, China), the determination methods described by Hou et al. (2022a, b) (Fig. 3). To measure the operative strength required to open the staminodes, 30 flower buds from 30 individual plants were randomly selected at each population, and the strength was measured after anthesis by artificially pressing the staminodes until the stamens were exposed (three measurements for each flower) (Fig. 3). We used only fresh flowers in situ without picking to exclude the influence of picking on the operative strength. The normality of the flower characteristics was tested using the one sample Komogorov–Smirnov test (1-K-S), and each flower trait was compared separately at different populations by one-way analysis of variance (ANOVA, with Tukey's multiple contrasts). The results were expressed as the mean ± standard deviation (SD). The correlations between each of the flower traits were analyzed using Origin 9.1 software (OriginLab, Northampton, MA, USA).

2.3 Observations of visitors

To quantify and identify visitors to *D. caeruleum* flowers, we conducted surveys in the field at each population in the full anthesis phase. 30 flowers were randomly selected from 30 individual plants at each population. We monitored all of the visitors to *D. caeruleum* at three population between 09:00 and 18:00 on sunny days for 5 days (45 h/each population). We recorded the visiting species, behaviors, and visit time per flower, the observation methods described by Duan et al. (2005). Insect specimens were collected in specimen boxes for later identification.

To detect the pollen removed and deposited by each visitor, we bagged 30 flowers on 30 inflorescences at each population before anthesis. All anthers and stigmas on an individual flower were carefully collected in centrifuge tubes immediately after one visit and the visitor was captured for identification. The deposited pollen was measured by microscopic examination after staining with lactophenol cotton blue. The pollen removed by visitors was recorded as the difference between the average number of pollen grains before dispersal and the number of remaining pollen grains after a visit. The normality was checked for the pollen removal and pollen deposition data using the 1-K-S test, and one-way ANOVA was conducted for variables with a normal distribution.

2.4 Biomechanical screening mechanism

To elucidate the biomechanical filtering mechanism in *D. caeruleum*, we measured the strengths of frequent insects including visitors and non-visitors (some most frequent Bees without visiting flowers in the locality), and the operative strength of the staminodes (the determination method is the same as 2.2) at all three populations. At each population, 30 insects were captured from each species to measure their strength, the determination methods described by Hou et al. (2022a, b). A biological tension sensor (BL-420s biological function experimental system and FT-102 biological tension sensor, 0.001 g to 5 g; Techman Soft, Chengdu, China) was used to measure the strength of each insect (Fig. 3). In order to measure the strength generated by weight of the insect and the muscle movement, we tie a string to the chest of the insect, and the other end of the string is tied to metal plate of the sensor. Then, put the insect to crawl forward. The maximum strength during crawling is simulated as the maximum strength of insects in the flower (measured 15 times for each insect). Insect weights, body lengths, and body widths were measured after the experiment using an analytical balance and digital calipers. We checked the normality of the insect strength and staminodes operative strength data with the 1-K-S test, and the insect strengths and staminodes operative strengths at each population were compared separately by one-way ANOVA (with Tukey's multiple contrasts).

2.5 Spur length screening mechanism

At each population, 30 insects from each common species (visitor and non-visitor) were caught with a net and killed by gassing with potassium cyanide fumes. We measured the proboscis length (maximum length that an insect could extend its proboscis) by using digital calipers (Anderson et al. 2005). To determine the spur length in *D. caeruleum*, we randomly selected 30 flowers from 30 individual plants at each population, and measured the spur length in each flower by using digital calipers. We checked the normality of the proboscis length and spur length data with the 1-K-S test, and the proboscis length and spur length at each population were compared separately by one-way ANOVA (with Tukey's multiple contrasts).

3 Results

3.1 Floral traits

The flower length was longest at Haibei, but not significantly different between Hezuo and Tianzhu. The staminodes length and staminodes width did not differ significantly between Hezuo and Haibei, but they were significantly smaller at Tianzhu. The operative strength of staminodes was strongest at Haibei, followed by Hezuo and weakest at Tianzhu. No significant differences were found in the calyx spur, nectar spur, flower width, sepal length, and sepal width among the three populations (Table 1). In addition, except for the calyx spur and nectar spur, the other floral traits were significantly positively correlated with each other. The calyx spur was only correlated with the nectar spur, and the nectar spur had significant positive correlations with the staminodes length, staminodes width, and operative strength of staminodes (Fig. 4).

 Table 1

 Comparison of flower traits in *Delphinium caeruleum* at different populations. The results represent the mean ± standard deviation. Different letters in columns indicate significant differences at *P*<0.05.</td>

	Calyx	Nectar	Flower	Flower	Sepal	Sepal	Staminodes length (mm)	Staminodes width (mm)	Operative strength of staminodes (mN)
	Spur (mm)	Spur (mm)	Length (mm)	Width (mm)	Length (mm)	Width (mm)			
Hezuo	24.12 ± 2.23ª	15.12 ± 2.23ª	36.03 ± 4.61 ^b	31.25 ± 2.01ª	16.61 ± 1.63ª	8.97 ± 0.89 ^a	8.67 ± 1.36ª	7.94±1.37ª	29.02 ± 2.86 ^b
Haibei	24.29 ± 2.03 ^a	15.82 ± 1.82ª	39.83 ± 2.60ª	30.72 ± 4.22ª	17.32 ± 2.56ª	9.24 ± 1.07 ^a	9.16±1.20ª	7.63 ± 1.41ª	34.74 ± 5.32ª
Tianzhu	24.17 ± 2.54 ^a	15.16 ± 1.44ª	34.37 ± 4.39 ^b	31.35 ± 5.32ª	16.34 ± 0.93ª	9.41 ± 1.36 ^a	5.84 ± 0.63 ^b	5.72 ± 0.87 ^b	23.05± 4.32°

3.2 Observations of visitors

In 135 h of observations, we recorded a total of six species and 199 visits (70 visits at Hezuo, 81 visits at Haibei, and 48 visits at Tianzhu) on *D. caeruleum*. The visitor assemblages varied among the populations, where *Bombus impetuosus*, *B. lepidus*, and *B. pyrosoma* were observed at Hezuo, *B. filchnerae* and *A. mellifera* were observed at Haibei, and only *B. rufofasciatus* was observed at Tianzhu (Fig. 5; Fig. 6). The six visiting species exhibited the same behavior when they visited flowers, where the forelegs clasped onto the staminodes, and the hind legs were placed on the sepals and vertically pressed downward on the staminodes. The proboscis then penetrated between the two petals to enter the spur and make contact with the sex organs (Fig. 6). All visitors to *D. caeruleum* were efficient at pollen deposition and removal, but the efficiency varied among individuals (Fig. 7).

In addition, we observed three non-visitor (most frequent) insect species at Hezuo comprising *B. sichelii*, *B. kashmirensis*, and *A. mellifera*, as well as three species at Haibei comprising *A. cerana*, *B. personatus*, and *B. sushkini* and two species at Tianzhu comprising *B. supremus* and *B. waltoni*.

3.3 Biomechanical screening mechanism

Significant differences (P < 0.05) were found in the operative strength of staminodes among the three populations, with the strongest at Haibei (mean ± SD = 34.74 ± 5.32 mN), followed by Hezuo (29.02 ± 2.86 mN) and the weakest at Tianzhu (23.05 ± 4.32 mN). No significant differences were found between the strengths of local visitors and the operative strength of staminodes (Fig. 8). However, except for *B. sushkini* (P>0.05), significant differences (P < 0.05) were found between the strengths of non-visitor insects and the operative strength of staminodes in local flowers (Fig. 8).

3.4 Spur length screening mechanism

The spur length in *D. caeruleum* did not differ significantly (P > 0.05) among the three population. No significant differences (P > 0.05) were found between the nectar spur length and proboscis length in visitors at Hezuo and Tianzhu, but the nectar spur length was significantly longer than the proboscis length in non-visitors. However, different results compared with the other two population were found for two insects at Haibei, where the proboscis length of the visitor *A. mellifera* was significantly(P < 0.05) shorter than the spur length, and that of the non-visitors *B. sushkini* was also significantly longer(P < 0.05) than the spur length (Fig. 9).

4 Discussion

Pollination is a plant–insect interaction and a significant selective force that drives the evolution of flower traits. The pollinator landscapes determined by the behavior/morphology of visitors lead to geographically divergent floral ecotypes that impose divergent selective pressures (Fenster et al. 2004). Similarly, we found that the floral traits of *D. caeruleum* varied significantly among the different populations, especially the flower size, staminodes size, and operative strength of staminodes. In addition, the strong correlations between the flower morphological characteristics and operative strength of staminodes indicated that the operative strength may be determined by a combination of the floral characteristics comprising part of the functional module in the flower, thereby leading to differences in the operative strength of staminodes among populations. Therefore, we consider that the flower morphological traits affected the mechanical functions of the flowers, thereby supporting the view that flowers contain modules that integrate flower characteristics into different functions.

Pollination is an important factor that affects the success of plant reproduction and many experimental studies have demonstrated that it can be influenced by different environmental conditions (Genung et al. 2017). The richness and assemblages of insect populations may be influenced by the geographical location and environmental differences. Unsurprisingly, differences were found in the insect species among the three populations investigated in this study, and the pollinator species and visiting numbers on *D. caeruleum* also differed among the three populations. According to the morphological structure of the flower and visiting behavior of the pollinator, the pollinator was required to possess sufficient strength to open the staminodes and a sufficiently long proboscis to obtain nectar from the long nectar spur. The two traits in *D. caeruleum* may prevent potential visitors from reaching the flower reward or they could force visitors to invest more energy and time in accessing the flower reward because plants prefer to interact with high-quality visitors by providing a demanding environment. Visitors struggle to overcome the demanding environment to obtain a flower reward and a cost is incurred in this process (Archetti, 2011). However, the visitor can also decide whether to visit a plant after assessing the potential costs and rewards of the environment. When the rewards and costs are set correctly, high-quality visitors will decide to enter a flower in a demanding environment. Thus, visitors will be screened according to their own quality even though the plants cannot assess the quality of the visitors (Archetti et al. 2011; Archetti 2011). Therefore, D. caeruleum may separate high-quality pollinators from other visitors with this screening mechanism, which was confirmed by the high pollen deposition and removal efficiency of the pollinators (Fig. 7), although it was difficult to assess the pollen deposition and removal efficiency of non-visitors. The complex structure of *D. caeruleum* flowers can be explained by interactions between the plant and pollinators.

Geographic differences in visitor behavior or morphology can affect the intensity and direction of selection for plant traits, and drive divergent patterns in the evolution of plant traits (Grant and Grant, 1965; Stebbins, 1970). We also found that differences in pollinators resulted in geographically divergent plant traits across the range of *D. caeruleum*. At different populations, the operative strength of staminodes and nectar spur size matched with the strength and proboscis length of pollinators, but not those of non-visitors. These findings confirmed our prediction of a biomechanical and length screening mechanism in *D. caeruleum*. However, two insect species at Haibei were exceptions, where *A. mellifera* (pollinator) and *B. sushkini* (non-visitor) had sufficient strength to open the staminodes but their proboscis lengths were significantly shorter than the spur length. Interestingly, these two species had different pollination behaviors, where ones visited flowers by opening the two staminodes whereas the other did not. We suggest that insect rewards determined these differences. Pollen is the main protein source for larval nutrition in honey bees (Brodschneider and Crailsheim, 2010), and although the proboscis of *A. mellifera* was too short to obtain nectar, its strength allowed it to open the staminodes to collect pollen. However, the main reward is nectar for bumblebees, and the short proboscis length in *B. sushkini* was clearly not conducive to maintaining the mutual

relationship between the plant and bumblebee pollinator. Thus, the length screening function of the nectar spur imposes a stricter limit on the proboscis length in bumblebees. He and Liu (2004) previously showed that *B. sushkini* is a dependable and effective pollinator of *Gentiana straminea* because its proboscis length matches well with the depth of the corolla tubes of *G. straminea* at Haibei Station. In summary, our results suggest that the staminodes and nectar spur have functions in insect screening, where the staminodes and nectar spur form biomechanical barriers that limit visitors from accessing flower rewards (or increase the cost of rewards) to screen effective pollinators (Castellanos *et al.* 2004; Córdoba and Cocucci, 2011). In addition, we consider that the variations in flower traits usually reflect adaptation to local visitors, and thus phenotypic plasticity may be related to mutual adaptation between plants and their visitors (Anderson and Johnson, 2008; Pauw et al. 2009; Anderson and Johnson, 2009; Thompson *et al.* 2013).

In conclusion, *D. caeruleum* can screen for efficient insects using a complex mechanism based on a combination of length screening and biomechanical screening. The differences in flower traits among different populations and the matching relationships with local pollinator traits are probably the result of flower traits response to the traits of visitors. Our results suggest that visitors have played significant roles in selecting for flower traits. Pollinators make important contributions to promoting the convergence and divergence of flower traits through the adaptation of plants to the morphology of pollinators. The strong correlations between plant traits and pollinators demonstrate the power of flower morphology for predicting the morphological characteristics of pollinators and vice versa, thereby supporting the pollination syndrome concept. The biomechanical method used in our study allowed us to address evolutionary questions and to assess the ecological importance of plant–pollinator interactions, as well as providing the possibility of further exploring functional modules in the flower structures of other plants.

Declarations

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Code availability: Not applicable.

Authors' contributions: HQZ and SK originally formulated the idea, SWJ developed methodology, NE, SWJ, YG, and ZYP conducted fieldwork, NE performed statistical analyses, NE and HQZ wrote the manuscript.

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Figure 1

Maps of the three populations.



Schematic diagrams showing morphological structures in *Delphinium caeruleum*. A, Inflorescence of *D. caeruleum*. B, Front view of *D. caeruleum*. C, Stamens and pistils covered under the "gate" staminodes. D, Side view of *D. caeruleum*.



Simple devices and methods used for measuring the strength of insects and operative strength required to open the staminodes in *Delphinium caeruleum*.



Correlations between flower traits. Abbreviations: Cs, calyx spur, Sp, nectar spur, Lf, length of flower, Wf, width of flower, Ls, length of sepals, Ws, width of sepals, Lst, length of staminodes, Wst, width of staminodes, St, operative strength of staminodes. The size of the circle represents the degree of significance and * indicates a significant correlation. The color represents the degree of correlation, where red represents a positive correlation and blue represents a negative correlation. The number represents the Pearson's correlations coefficient (r).



Pollinator assemblages and number of visits to *Delphinium caeruleum*at the three populations.



Visitors to *Delphinium caeruleum*. a,*Bombus pyrosoma*, b, *B. lepidus*, c, *B. impetuosus*, d, *B. filchnerae*, e, *Apis mellifera*, f, *B. rufofasciatus*.



Mean amount of pollen grains deposited on stigmas and pollen removed by visitors in *Delphinium caeruleum*. Boxplots represent the amounts of pollen deposited and pollen removed (red for pollen deposited and dark cyan for pollen removed by visitors), where the medians, quartiles, interquartile ranges, and outliers are shown.



Operative strength of staminodes and strengths of insects at the three populations. The line represent the operative strength of staminodes and boxplots represent the strength of insects, where the medians, quartiles, and interquartile ranges are shown (red for visitors and dark cyan for non-visitors). The different letters for an item indicate significant differences at P < 0.05.



Spur length and proboscis length in insects at the three populations. The line represents the spur length and boxplots represent the proboscis lengths in insects, where the medians, quartiles, and interquartile ranges are shown (red for visitors and dark cyan for non-visitors). The different letters for an item means indicate significant differences at P < 0.05.