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RESEARCH ARTICLE

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Floral traits differentiate pollination syndromes and species but fail to predict the identity of floral visitors to *Castilleja*

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Abstract

Premise: Animal pollination is critical to plant reproduction and may cause convergent evolution of pollination syndromes. Pollination syndromes in *Castilleja* have been distinguished based on floral traits and historical observations of floral visitors. Here we addressed these questions: (1) Can pollination syndromes be distinguished using floral morphological traits or volatile organic compound emissions? (2) Is there significant variation in floral traits within a pollination syndrome at the level of populations or species? (3) Do pollination syndromes predict the most frequent floral visitor to *Castilleja*?

Methods: Floral traits and visitation were measured for five co-occurring *Castilleja* species (*C. applegatei*, *C. linariifolia*, *C. miniata*, *C. nana*, and *C. peirsonii*), representing three pollination syndromes (bee, fly, and hummingbird), at four sites in the Sierra Nevada Mountains. We used nonmetric multidimensional scaling (NMDS) and multiple linear regressions to address key questions in the differentiation of *Castilleja* and floral visitors.

Results: Our analyses revealed that both morphological traits and floral VOCs can be used to distinguish between some pollination syndromes and *Castilleja* species. Morphological traits defined pollination syndromes reliably, but within the hummingbird syndrome, there was also significant variation among populations and species. Pollination syndrome was a poor predictor of visitors to *Castilleja*.

Conclusions: Floral trait differentiation among *Castilleja* individuals reflects both taxonomy and pollination syndromes. Differentiation was generally more evident in morphological traits compared to VOCs. Furthermore, a priori notions of pollination syndromes in this system are overly simplistic and fail to predict which animals most frequently visit *Castilleja* in natural populations.

KEYWORDS

bee, floral morphology, fly, hummingbird, mutualism, Orobanchaceae, pollination syndrome, scent, volatile organic compounds

Pollination is a critical process to the survival and reproduction of angiosperms. Floral adaptation in response to animal pollinators is thought to be a primary mechanism by which plant lineages have diversified through time (Grant and Grant, 1965; Stebbins, 1970; Schluter, 2000; Johnson, 2006; Schiestl and Dötterl, 2012; Sun et al., 2014; Caruso et al., 2019). Extensive research including descriptive study (e.g., Darwin, 1862; Delpino, 1867), theoretical study (e.g., Grant and Grant, 1965; Stebbins, 1970), and experimental manipulation (e.g., Schemske and Bradshaw, 1999; Gervasi and Schiestl, 2017) have yet to adequately describe the relationships between floral traits and the identity of animals that visit them. Since the 19th century, the idea that suites of traits could be used to discern the identity of pollinators (i.e., pollination syndromes) has continued to spur debate among plant biologists (Grant, 1949; Stebbins, 1970; Waser et al., 1996; Fenster et al., 2004; Johnson, 2006; Ollerton et al., 2009; Rosas-Guerrero et al., 2014). While the concept of a pollination syndrome is relatively simple, challenging specific predictions requires careful consideration of plant traits and their evolutionary context (Whittall and Hodges, 2007).

Pollination syndromes depend on floral traits influencing the behavior of pollinators or on a set of morphologically related pollinator species that constitute a pollinator guild (Fenster et al., 2004). Particular syndromes are thought to arise in response to similar selective pressures on independently evolving plant lineages, which causes them to converge on similar trait combinations (Armbruster et al., 2000; Fenster et al., 2006). For example, when hummingbirds are considered as a guild, they primarily visit plants with red tubular flowers that produce copious amounts of nectar and lack fragrance (Fenster et al., 2006; Rosas-Guerrero et al., 2014). The association of these traits with hummingbird visitation implies that these traits mediate the visual identification of a reliable food source by individual animals (Grant and Grant, 1965; Schemske and Bradshaw, 1999). Many important traits that influence pollination syndrome are categorical in nature (e.g., sweet vs putrid smell or red vs yellow coloration) or readily measured (e.g., nectar volume or petal length), but more cryptic traits (e.g., specific volatile organic compounds) have begun to receive increasing attention. Strong support for these traits in attracting specific pollinators was found in a large meta-analysis (Rosas-Guerrero et al., 2014) and has also been garnered from experiments in the laboratory and field (Schemske and Bradshaw, 1999; Gervasi and Schiestl, 2017).

Perhaps the best evidence for pollination syndromes has emerged from controlled experiments where traits predict pollinator visitation. In a classic study, Schemske and Bradshaw (1999) studied the visitation of a bird-pollinated species (*Mimulus cardinalis*) and a bee-pollinated species (*Mimulus lewisii*) where both species experienced the same pollinator community in a sympatric zone. Pollinators were highly discriminatory and recombinant phenotypes in artificial hybrids revealed the primary pollinator was strongly predicted by specific floral traits expressed by individuals (Schemske and Bradshaw, 1999). In 2017, Gervasi and Schiestl exposed *Brassica rapa* to divergent pollinator environments (bee and fly) resulting in divergent adaptation of floral phenotypes, especially their scent, within 11 generations. The resulting changes affected visitation by pollinators, significantly increasing bee visitation to those plants that had evolved in a bee environment. Importantly, under divergent pollinator communities, the most rapid and striking differences in floral traits were observed in specific volatile organic compound (VOC) emissions. These manipulative experiments demonstrate that pollinators can serve as strong selective forces and that certain traits can increase visitation by particular pollinator guilds in subsequent generations. Such results imply that pollination syndromes should be commonplace in nature.

While the above cases are empirically tractable given the simplicity of the pollinator community, most plants are visited by multiple pollinators and even pollinators from different pollinator guilds (Waser et al., 1996; Johnson, 2006). Theory suggests that natural selection will favor traits that maximize the import and export of pollen (Aigner, 2001). However, analysis of pollination syndromes relies on a multivariate summary of traits under selection by multiple pollinator guilds that can vary across time and space. In addition, the utility of pollination syndromes also depends on the degree to which populations and species in the same pollinator syndrome vary. Significant variation among populations and species may either complicate pollination syndrome concepts or be largely irrelevant if it has no impact on the attraction of floral visitors. Regardless, it is a testament to the magnitude of convergent selective pressures that we have been able to detect pollination syndromes within the extant trait variation across a wide array of systems (Schemske and Bradshaw, 1999; Fenster et al., 2004; Armbruster et al., 2011; Rosas-Guerrero et al., 2014). However, much of this experimental work has primarily focused on those traits that are easiest to describe for a human observer (e.g., floral size or color), though recent studies have broadened the scope of traits that are investigated (Gervasi and Schiestl, 2017; Chapurlat et al., 2019; Friberg et al., 2019; Sletvold, 2019; Joffard et al., 2020).

Floral scent traits present an opportunity to potentially clarify pollination syndromes in angiosperms and other plant groups (Rosenstiel et al., 2012). The influence of these traits was famously noted by Stebbins (1970), who suggested that scent may be one of the most important traits in driving pollinator visitation, especially by insects. Volatile organic compounds (VOCs) are those chemicals that comprise scent and embody an important axis of trait variation in flowering plants. Although categorical classifications of scent (i.e., presence/absence) have been common in the literature regarding pollination syndromes, only in the last 50 years have researchers begun isolating and analyzing the individual VOCs that comprise floral scent (Dodson and Hills, 1966; Holman and Heimermann, 1973; Raguso, 2008a). With a growing body of literature, it has become clear that, while VOCs are extremely complex, they are vital to understanding how animals interact with plants (Dufay and Anstett, 2003; Schiestl, 2004; Raguso, 2008a, 2008b; Gervasi and Schiestl, 2017; Rosas-Guerrero et al., 2014; Joffard et al., 2020).

Castilleja (Orobanchaceae) is a genus of herbaceous plants with many closely related species that often co-occur in the same environment and share a similar floral structure (Figures 1 and 2). Previous studies have documented pollinators including primarily hummingbirds and bees (Grant and Grant, 1965; Crosswhite and Crosswhite, 1970; Cariveau et al., 2004; Hersch-Green and Cronn, 2009), though visitation by flies or moths is prevalent in some species. Floral traits such as flower size and color vary widely among species, with syndromes historically defined by flower color and corolla length (Grant and Grant, 1965; Cariveau et al., 2004; Hersch-Green and Cronn, 2009; Baldwin et al., 2012). Here we used color and corolla length as indicators of a species' pollination syndrome because these traits have generally agreed with natural historical observations in these species (Grant and Grant, 1965; Cariveau et al., 2004, Baldwin et al., 2012), and we expected that morphological characters would reflect these differences if pollinators were important drivers of morphological trait differentiation. While scent has been previously detected in few *Castilleja* species (E. T. Hilpman, unpublished data), this work represents the first analysis of

FIGURE 1 *Castilleja* species of diverse pollination syndromes. Top row: Three species in the hummingbird syndrome (left to right: *C. applegatei*, *C. linariifolia*, and *C. miniata*). Bottom row: *C. peirsonii* (bee syndrome, left) and *C. nana* (fly syndrome, right). Photos by Mark Egger, WTU

floral VOC traits in the genus. The conservative nature of floral shape combined with variation in floral color, VOC production, and floral visitors make *Castilleja* an ideal system to test a priori predictions of pollination syndromes. By comparing floral VOCs and morphological traits in close relatives with differing pollination syndromes in sympatry, four questions are addressed: (1) Can pollination syndromes be distinguished using floral morphological traits? (2) Can pollination syndromes be distinguished using VOC emission? (3) Is there significant variation in floral traits within proposed hummingbird syndrome plants, at the level of populations or species? (4) Do pollination syndromes predict the most frequent visitor to *Castilleja*?

MATERIALS AND METHODS

Study system

Castilleja is a genus of approximately 200 hemiparasitic species native to the western United States (Tank et al., 2009). Data was collected from five species of *Castilleja* that represent three pollination syndromes at three sites in the Sierra Nevada Mountains of California: *C. applegatei* Fernald, *C. linariifolia* Benth., *C. miniata* Douglas ex Hook., *C. nana* Eastw., and *C. peirsonii* Eastw. (Table 1, Figure 3). Each *Castilleja* species was represented by populations at two sites with the exceptions of *C. peirsonii* and *C. linariifolia*, which were found at a single site, respectively (Table 1). Sites were sampled in July and early August of 2017. Populations of species that co-occurred at a single site were sampled within a week of each other, and all populations were observed midway through their flowering season. At each site, 10 individuals of each available *Castilleja* species were sampled for morphological and VOC traits.

Morphological data

Morphological measurements were used based on their relevance in determining what pollinator guilds have access to floral rewards and sexual parts. The largest flower with a

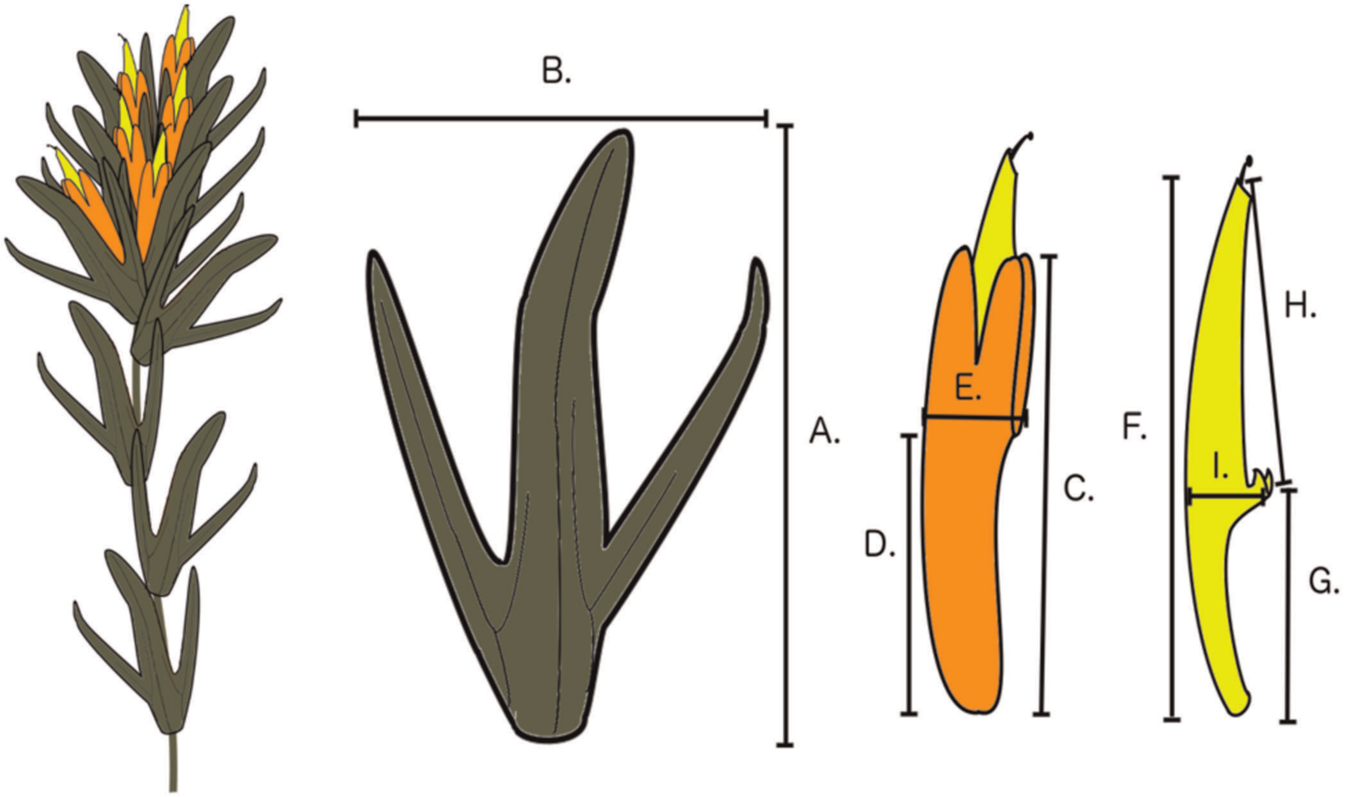

FIGURE 2 From left to right, a generalized *Castilleja* inflorescence, bract (green), calyx (orange) surrounding corolla (yellow), and exposed corolla (yellow). Floral measurements included (A) bract length, (B) bract width, (C) calyx length, (D) calyx cleft, (E) calyx width, (F) adaxial corolla length, (G) abaxial corolla length, (H) mouth length, (I) mouth width

receptive stigma was measured on each inflorescence for which VOCs were collected. For each flower, measurements included bract length, bract width, calyx length, calyx cleft, calyx width, adaxial corolla length, abaxial corolla length, mouth length, mouth width (Figure 2), and stigma–anther distance. Additional measurements included nectar volume and nectar sucrose content but low nectar volumes yielded little usable data. Although a priori assignment of pollination syndrome was based on floral color and corolla length, measures of floral morphology were expected to differ based on the selective pressures of pollinator guilds. Hummingbird-syndrome flowers were expected to exhibit the longest corolla tubes and largest flowers (Fenster, 1991), with bee-syndrome flowers intermediate and fly-syndrome flowers the smallest (Dafni and Kevan, 1997). Where relevant pollination studies have been conducted, pollination syndromes were assigned based on these data, such as *C. miniata* (Grant and Grant, 1965) and *C. linariifolia* (Cariveau et al., 2004). However, few studies have detailed pollination in *Castilleja* species. Therefore, other syndrome assignments were made in accordance with corolla color, corolla length, and published materials related to species identification for *C. applegatei* (Baldwin et al., 2012), and the generally supported pattern of bee pollination in yellow flowers with intermediate-sized flowers like *C. peirsonii* (Stebbins, 1970; Schemske and Bradshaw, 1999; Fenster et al., 2004). Finally, fly pollination was predicted for the smaller generally green flowers of *C. nana*, especially since their flowers are not shades of red, pink, or yellow (Kastinger and Weber, 2001; Fenster et al., 2004).

Floral VOC samples

Samples were collected on individual plants from an inflorescence with at least one receptive flower. The VOCs were collected from an inflorescence enclosed in heat-resistant nylon bags using dynamic headspace sampling at a flow rate of 200 mL/min with a Spectrex PAS-500 personal air sampler (Spectrex Corp., Redwood City, CA, USA) through 20 mg of Porapak-Q housed in a ¼ inch (6.35 mm) borosilicate tube (traps purchased from Volatile Collection Trap [VCT] LLC, Gainesville, FL, USA) and eluted with 200 μL high purity hexane (modified from Raguso and Pellmyr, 1998). Samples were stored on ice, then transferred to −80°C as soon as possible for storage until analysis by gas chromatography/time-of-flight mass spectrometry (GC-TOFMS). All analyses were done using split injections on a polar GC column (diameter 0.25 mm, length 30 m, film thickness 0.25 μm, Rtx-WAX Cap by Restek Corp., Bellefonte, PA, USA) on a GC-TOFMS system. The heating program was modified from the protocol of Raguso et al. (2003) and started with an injection at 60°C holding for 3 min, followed by an increase of

TABLE 1 *Castilleja* species sampled at various sites and their a priori pollination syndromes (bee, fly, and hummingbird [Hum]), general corolla color, and sample size (*N*). Trait means are reported for 10 morphological traits measured in millimeters, with standard deviation in parentheses below. Traits include bract length (Bract L), bract width (Bract W), calyx length (Calyx L), calyx cleft (Calyx C), calyx width (Calyx W), corolla adaxial length (Corolla DL), corolla abaxial length (Corolla BL), mouth length (Mouth L), mouth width (Mouth W), all corresponding to measures A–I on Figure 2 and stigma–anther distance or herkogamy (Herk). At each site, generally 10 individuals were sampled for each species present

Species	Site	Syndrome	Color	*N*	Bract L	Bract W	Calyx L	Calyx C	Calyx W	Corolla DL	Corolla BL	Mouth L	Mouth W	Herk
C. applegatei	CL, LW	Hum	Red	19	18.69	10.65	14.56	6.43	3.84	22.41	14.73	4.50	4.20	1.66
					(3.30)	(3.56)	(1.78)	(1.04)	(0.62)	(2.99)	(1.59)	(0.68)	(0.72)	(0.97)
C. linariifolia	RC	Hum	Red	10	18.65	13.61	21.71	13.38	4.13	29.64	15.11	5.16	4.88	2.56
					(6.58)	(4.00)	(3.63)	(2.51)	(0.90)	(3.61)	(2.25)	(0.83)	(0.55)	(0.76)
C. miniata	CL, LW	Hum	Red	20	27.47	7.59	28.20	15.79	5.21	36.46	19.16	5.14	4.81	1.48
					(4.60)	(1.35)	(3.00)	(1.68)	(0.66)	(3.78)	(3.12)	(0.82)	(0.60)	(1.39)
C. nana	LW, RC	Fly	Green	20	17.98	13.70	15.00	7.13	3.64	15.93	14.37	4.52	3.58	0.57
					(3.63)	(2.89)	(1.74)	(2.12)	(1.41)	(1.92)	(1.70)	(0.93)	(0.90)	(0.68)
C. peirsonii	CL	Bee	Yellow	10	19.16	9.90	(15.43	8.43	4.14	22.79	15.20	4.97	4.65	1.75
					(5.31)	(3.25)	(1.94)	(1.32)	(0.96)	(2.33)	(2.00)	(0.80)	(0.68)	(0.85)

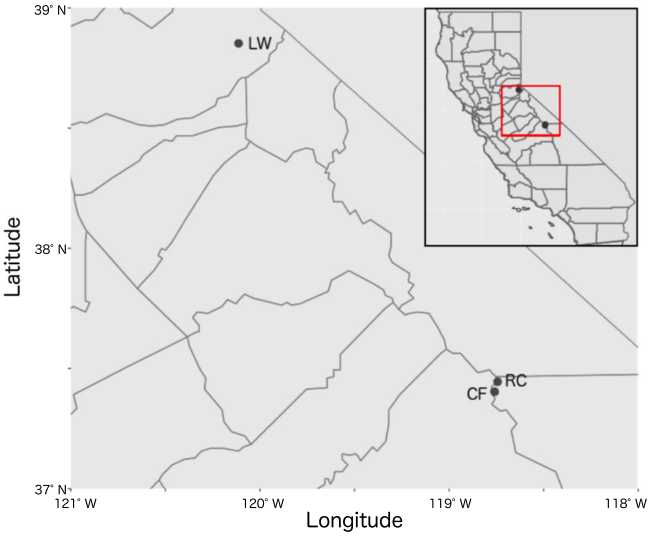

FIGURE 3 Map of study sites in California: Chickenfoot Lake (CF), Lake of the Woods (LW), and Rock Creek (RC)

10°C/min to a maximum of 220°C where it was held for 11 min. Chemical compositions of samples were left as analytes, identifiable across samples, but not identified to chemical species through nuclear magnetic resonance or external standards. Although most of these analytes were not identified to chemical species, those that were identified using external standards were terpenes (e.g., limonene). Ambient and vegetative controls were used to identify and analyze only those analytes that were produced exclusively or in larger quantities in the inflorescences (Raguso et al., 2003). However, because bracts are integrated among the flowers throughout inflorescences in *Castilleja*, any VOCs produced by these bracts would have been analyzed as part of the inflorescence.

Observations of floral visitors

Visitors were observed concurrently with trait measurements, up to four times per day using five AKASO EK7000 digital cameras (Akaso, Frederick, Maryland, USA). Observation periods began when insect activity was observed (typically ~08:00 hours) and observation length averaged 55 min. More than 120 total hours of observations were collected on individuals for which floral morphology and VOCs were also collected. At the beginning of an observation period, weather conditions were recorded (temperature in the sun and in the shade, relative humidity, average wind speed, and maximum wind speed) using a Kestrel 3000 pocket wind meter (Kestrel Instruments, Boothwyn, PA, USA) and light intensity using an Apogee Quantum PAR meter MQ-100 (Apogee Instruments, Logan, UT, USA). Pollinator guild abundance and community composition was recorded by visual observation periods of 15 min. Pollinator videos were downloaded to external hard drives and reviewed. Visitation was defined as a floral visitor contacting the corolla or any sexual parts as visitation has frequently been cited as a proxy for pollination because visitation has been shown to correlate positively with effective pollination (Galen and Stanton, 1989; Young and Stanton, 1990; Jones and Reithel, 2001; Reynolds et al., 2009; Engel and Irwin, 2003). Floral visitors were pooled into these guilds: flies, bees, hummingbirds (Hum), lepidopterans (Lep), and other. This pooling was largely for simplicity of data presentation and partly due to the reliability of identifying visitors from video observations. While this standard approach may obscure some patterns (Ollerton et al., 2009), infrequent visitation over the 120 h of data required such pooling to ensure meaningful sample sizes. Flies included all visitors of the order Diptera, all of which were likely members of the Muscidae and Tachinidae families (no recorded visits from syrphid or bombyliid flies). Bees included all members of the Apidae family, but were represented in this data set only in small halictid bee visits (family Halictidae, most identified specimen belonging to the *Halictus* and *Lasioglossum* genera). Lepidopterans included two total floral visitors (one small moth [likely Noctuidae] and a blue butterfly [Lycaenidae], which together visited 3 flowers). "Other" included four floral visitors (two beetles, a wasp, and an unidentified small insect, which together visited eight flowers).

Statistical analyses

All analyses were conducted using R (R Core Team, 2019), and figures were produced using the package ggplot2 version 3.1.0 (Wickham, 2016). To test that a priori assignment of pollination syndromes conformed with expectations of floral morphological differences, we performed a series of one-tailed *t*-tests. Specifically, we tested that hummingbird-syndrome corollas were longer than bee-syndrome corollas and that bee-syndrome corollas were longer than fly-syndrome corollas. Nonmetric multidimensional scaling (NMDS) analyses were conducted using the package vegan version 2.5.6 (Oksanen et al., 2019). This method allows for the ordination of individual *Castilleja* using 10 morphological variables to demonstrate whether they clustered by pollination syndrome (Figure 4) or by species (Figure 5). Differences arise between the pollination syndrome and species level NMDS analyses due to the expected number of clusters (three syndromes vs. five species) and the "stress" statistic, which decreases when the data better fits the model.

Significant inter- and intraspecific variation within pollination syndromes potentially complicates their application. To test for these components of variation, we restricted morphological analyses to the hummingbird syndrome, which had fully nested sampling of populations within species. Specifically, a nested ANOVA was used to test for differences in corolla adaxial length in *C. miniata* and *C. applegatei* at both the population and species levels. A single morphological variable was used for this nested ANOVA due to the positive correlation among most morphological characters (Table 2). Among-syndrome variation in floral VOCs was assessed by first identifying those VOCs that differed significantly among syndrome by one-way ANOVA, then further filtering out variables that displayed high covariance (≥0.95). The remaining 51 VOCs were analyzed for among syndrome differences using NMDS.

Floral visitation data were analyzed to address whether pollination syndrome predicts the most frequent visitor to *Castilleja* species. Differences in the visitation rates of bees, flies, and hummingbirds were evaluated for plants from each pollination syndrome using one-way ANOVAs. These models tested the prediction that plants from a given pollination syndrome would be visited most often by a specific pollinator guild (i.e., hummingbird-syndrome individuals would be most frequently visited by hummingbirds). Additionally, the effect of environmental variables on visitation was evaluated with multiple linear regression. This model included visitation rate as a response variable and relative humidity, temperature, light intensity, average wind speed, and maximum wind speed as predictors. This analysis did not implicate any particular environmental factors in predicting rates of floral visitors (Table 3), so observations were treated independent of environmental conditions. Furthermore, pollinator guild abundance and community composition were analyzed for their effects on floral visitation through multiple linear regression; these factors did not affect visitation and observations were treated independent of pollinator community (Table 4).

RESULTS

Can pollination syndromes be distinguished using floral morphological traits?

Castilleja were highly variable in morphological traits (Table 1), and morphological traits were positively correlated except for bract width and herkogamy (Table 2). Hummingbird-syndrome plants had significantly larger flowers than bee-syndrome plants (Welch's *t*-test, $t = 5.03$, $df = 46$, $P < 0.001$) and fly-syndrome plants (Welch's *t*-test, $t = 11.60$, $df = 52$, $P < 0.001$) (Table 5, Figure 4A). Furthermore, the flowers of bee-syndrome plants were significantly larger than those of fly-syndrome plants (Welch's *t*-test, $t = 8.51$, $df = 14$, $P < 0.001$) (Table 5, Figure 4A). Results of these *t*-tests are robust to classification of *C. applegatei*, regardless of placement in bee or hummingbird syndrome. Data from 10 floral morphological traits were consolidated using NMDS to create a two-dimensional ordination that shows individuals that belong to the same pollination syndrome typically cluster together (Figure 4A). This ordination also demonstrates morphological overlap among individuals of the hummingbird and bee syndromes (Figure 4A). Combined analysis of these morphological traits supports highly significant among-syndrome differentiation (Bray–Curtis distance, stress = 0.103, $P = 0.001$). However, due to unequal within-syndrome variance, this analysis violates the assumption that multivariate dispersion is homogeneous across pollination syndromes. When tested at the species-level,

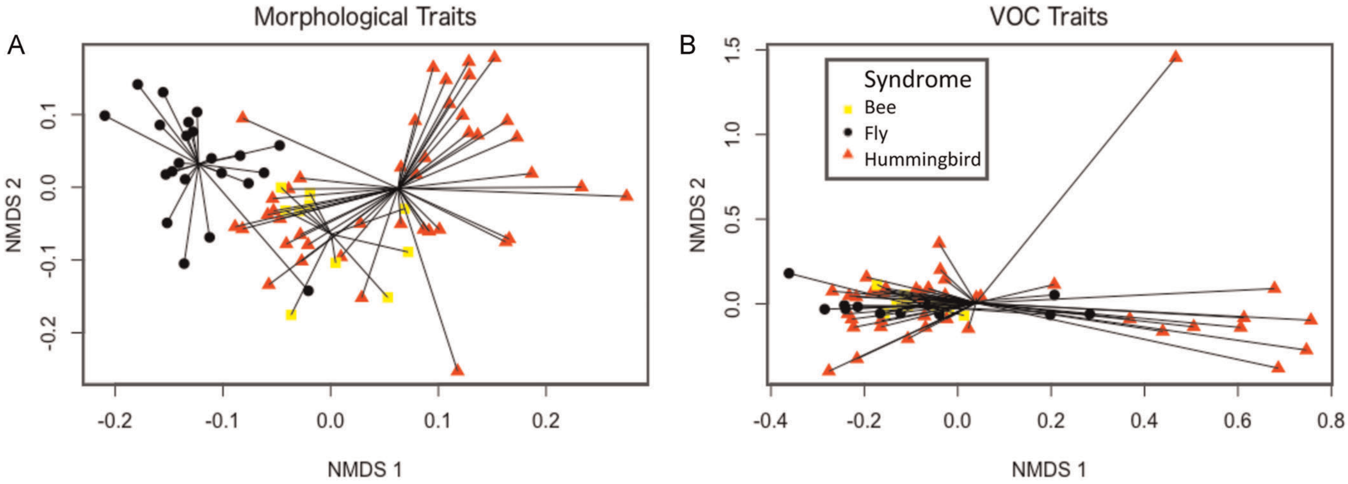

FIGURE 4 NMDS analyses of morphological traits (A) and floral volatile organic compound (VOC) traits (B) grouped by pollination syndrome; bee (yellow), fly (black), and hummingbird (red)

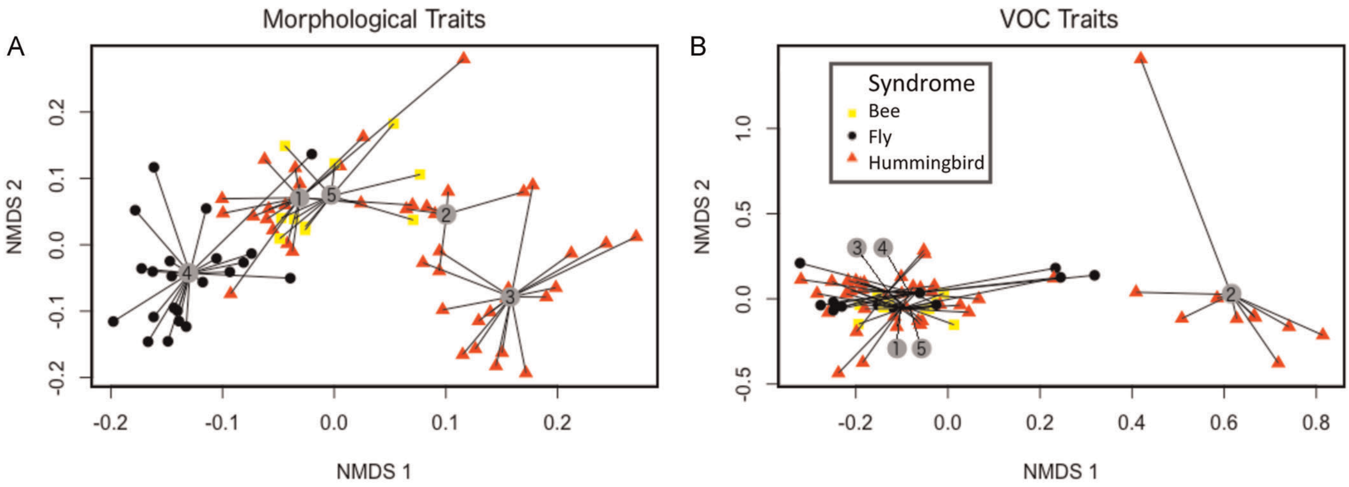

FIGURE 5 NMDS analyses of morphological traits (A) and floral volatile organic compound (VOC) traits (B) grouped by species. 1: *Castilleja applegatei*, 2: *C. linariifolia*, 3: *C. miniata*, 4: *C. nana*, 5: *C. peirsonii*. Sample points are colored by pollination syndrome; bee (yellow), fly (black), and hummingbird (red)

those trends evident at the syndrome level become clearer, and the NMDS analysis is robust to the smaller variation in sampling among taxa (Figure 5A, Bray–Curtis distance, stress = 0.057, $P = 0.001$).

Can pollination syndromes be distinguished using VOC emission?

Although it may be unclear given the two-dimensional ordination of these data (Figure 4B), VOCs demonstrated significant differentiation among syndromes (Bray–Curtis distance, stress = 0.099, $P = 0.013$). At the species-level, again, differences remain statistically significant (Figure 5B, Bray–Curtis distance, stress = 0.054, $P = 0.014$), groupings become clearer, stress on the model is reduced, and the clustering of points tells a different story. Neither NMDS analysis of VOC data is in violation of test assumptions.

Is there significant variation in floral traits within the hummingbird syndrome, at the level of populations or species?

In this study, significant morphological variation was present among species within the hummingbird syndrome. Flowers of *C. miniata* had longer corollas than those of *C. applegatei*, a difference that was statistically significant (Table 6, Figure 5A). Similarly, significant variation in floral morphology was also detected among populations within species of the hummingbird syndrome (Table 6).

Do pollination syndromes predict the most frequent floral visitor to *Castilleja*?

Bee syndrome flowers were visited more frequently than hummingbird or fly syndrome flowers (Figure 6).

TABLE 2 Relationships among floral traits. Values in the upper right triangle denote Pearson correlation coefficients, while others denote *P* values. Trait abbreviations are defined in Figure 2

Trait	Bract L	Bract W	Calyx L	Calyx C	Calyx W	Corolla DL	Corolla BL	Mouth L	Mouth W	Herk
Bract L	—	−0.0646	0.7412	0.6139	0.4191	0.6432	0.7130	0.4112	0.3764	−0.0943
Bract W	0.5981	—	−0.2890	−0.2748	−0.0798	−0.4230	−0.2134	−0.0471	−0.1445	−0.1762
Calyx L	**<0.0001**	**0.0160**	—	0.9336	0.5390	0.8976	0.7346	0.4077	0.4328	0.0705
Calyx C	**<0.0001**	**0.0223**	**<0.0001**	—	0.6195	0.8565	0.6483	0.4397	0.4561	0.1273
Calyx W	**0.0003**	0.5147	**<0.0001**	**<0.0001**	—	0.5057	0.4075	0.4402	0.5299	0.0476
Corolla DL	**<0.0001**	**0.0003**	**<0.0001**	**<0.0001**	**<0.0001**	—	0.7584	0.4252	0.5451	0.2230
Corolla BL	**<0.0001**	0.0783	**<0.0001**	**<0.0001**	**0.0005**	**<0.0001**	—	0.5535	0.4846	−0.0147
Mouth L	**0.0004**	0.7009	**0.0005**	**0.0002**	**0.0002**	**0.0003**	**<0.0001**	—	0.6658	−0.0612
Mouth W	**0.0014**	0.2363	**0.0002**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	—	0.1995
Herk	0.4407	0.1475	0.5649	0.2972	0.6979	0.0655	0.9043	0.6173	0.1002	—

Note: Significant ($P < 0.05$) results are in bold.

TABLE 3 Values for *t* and *P* from a multiple linear regression of environmental covariates: relative humidity (rH), temperature (temp), light intensity (sun.PAR), maximum wind speed (wmax), and average windspeed (wave)

Factor	*t*	*P*
rH	1.634	0.122
temp	−1.345	0.197
sun.PAR	0.925	0.369
wmax	0.54	0.596
wave	−1.132	0.274

TABLE 4 Values for *t* and *P* from a multiple linear regression of pollinator community abundance. Factors included the abundances of flies, bees, hummingbirds (Hum), lepidopterans (Lep), and other guilds present during pollinator observation periods

Factor	T	*P*
Fly	−0.321	0.7526
Bee	−1.387	0.1845
Hum	−1.379	0.1869
Lep	0.345	0.7348
Other	0.201	0.8432

TABLE 5 Results of Welch's *t*-tests comparing adaxial corolla lengths among hummingbird syndrome plants (HB) and bee syndrome plants [HB: Bee], HB and fly [HB: Fly], and fly and bee [Fly: Bee]

Comparison	df	t	*P*
HB: Bee	46	5.03	**<0.001**
HB: Fly	52	11.60	**<0.001**
Fly: Bee	14	8.51	**<0.001**

Note: Significant ($P < 0.05$) results are in bold.

TABLE 6 Nested ANOVA results of corolla adaxial length within hummingbird syndrome plants. Populations were nested within species: *Castilleja applegatei* (populations at sites CL and LW) and *C. miniata* (populations at sites CL and LW)

Source of variation	df	*F*	*P*
Species	1,33	246.047	**<0.001**
Population(Species)	2,33	5.886	**<0.007**

Note: Significant ($P < 0.05$) results are in bold.

However, the visitation rates of pollinator guilds were not significantly different for plants with a syndrome expected to attract bees (ANOVA, $F_{3,36} = 2.032$, $P = 0.127$), flies (ANOVA, $F_{3,148} = 0.606$, $P = 0.612$), or hummingbirds (ANOVA, $F_{3,244} = 1.850$, $P = 0.139$). Visitation was not significantly affected by environmental factors measured (Table 3) or by the abundance of pollinators at each site (Table 4). While morphological variation among species could be associated with visitor attraction, visitation did not significantly vary among species (Appendices S1, S2). Fly visitation differed significantly among populations (Appendices S3, S4), but these differences were not associated with any measured trait and when included into a linear model with measured traits, populations did not demonstrate a statistically significant correlation with fly visitation (Appendix S5).

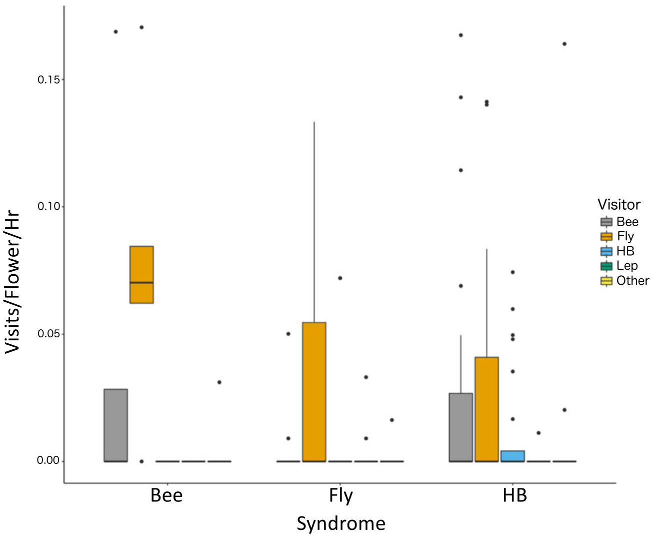

FIGURE 6 Boxplots of visitation by five pollinator guilds (bee, fly, hummingbird [HB], lepidopterans [Lep], and others) to *Castilleja* belonging to three syndromes (bee, fly, and hummingbird [HB]). Differences in visitation were not statistically significant among any *Castilleja* of three syndromes. These data reflect a total of 137 floral visits by bees (39), flies (59), HB (28), Lep (3), and others (8) across 39 independent observations

DISCUSSION

Are *Castilleja* differentiated by pollination syndrome?

A primary aim of this study was to test the hypothesis that plants from distinct pollination syndromes present distinct floral phenotypes. Such a pattern would be consistent with the action of pollinator-mediated selection (Stebbins, 1970; Fenster et al., 2004; Caruso et al., 2019). When considering univariate and multivariate analysis of morphological traits such as bracts, corollas, and calyces, *Castilleja* species hypothesized to be visited by bees, flies, and hummingbirds were significantly differentiated (Figure 4A, Tables 5 and 6). In a similar fashion, volatile organic compounds (VOCs) produced by *Castilleja* from these three hypothesized syndromes were also significantly differentiated (Figure 4B). In this system, assignments of plants to syndromes have been made based on floral color and corolla length. The logic for this classification has been derived from natural history observations (Caruso, 1999; Cariveau et al., 2004) and from previously reported pollination syndromes (Fenster et al., 2004; Rosas-Guerrero et al., 2014). While such an approach depends heavily on a few key traits, color alone has been found to reliably predict the primary pollinators that visit plants in some systems (Fenster et al., 2004; Rosas-Guerrero et al., 2014). Taken at face value, our findings and those of many others are consistent with syndromes likely reflecting the outcome of long-term interactions with pollinators in natural populations (Fenster et al., 2004; Caruso et al., 2019).

Although pollination syndromes conform to patterns of trait variation in *Castilleja*, this trend is confounded by taxonomy. In this study, *C. applegatei*, *C. linariifolia*, and *C. miniata* are all hypothesized members of the hummingbird syndrome (Caruso, 1999; Cariveau et al., 2004). These species identities appear to explain morphological variation within the hummingbird syndrome rather well (Figure 5A), and *C. linariifolia* clearly has a distinct VOC phenotype (Figure 5B). Regarding VOCs, *C. applegatei* and *C. miniata* overlap with plants predicted to belong to other syndromes, while *C. linariifolia* largely clusters outside of this group (Figure 5B). Phylogenetic relationships are not consistent with divergence in floral phenotypes, since *C. linariifolia* and *C. nana* are the most closely related yet have the most distinct morphologies and volatile chemistries (Tank and Olmstead, 2008). In a large meta-analysis, effects of pollination syndromes persisted after removing the potentially confounding effects of taxonomy (Rosas-Guerrero et al., 2014). While questions have been raised regarding the robustness of this conclusion (Ollerton et al., 2015), studies should carefully evaluate expectations based on syndrome while also considering the importance of species-level effects (Rosas-Guerrero et al., 2014; Joffard et al., 2020).

Caveats regarding the utility of pollination syndromes

Syndromes alone do not explain all floral morphological variation between species in *Castilleja* and many other taxonomic groups (Waser et al., 2016). In this study, there was significant variation among species and among populations within the hummingbird syndrome. In fact, variation in morphological and VOC traits more strongly supported differentiating individuals into five species as compared to differentiating three pollination syndromes. Such extensive variation among lineages within a syndrome seems consistent with stochastic convergent evolution of angiosperms in response to shared selection pressures (Stebbins, 1970; Smith and Kriebel, 2017). Moreover, independently evolving lineages have their own unique genetic and environmental constraints that may influence their convergence on a single set of traits (Fenster et al. 2015; Smith and Kriebel, 2017). On the other hand, one might also expect trait distributions among some syndromes to overlap, at least partially, either because those syndromes share visits from multiple pollinator guilds or because of phylogenetic conservatism during plant diversification (Rosas-Guerrero et al., 2014). For example, there was high trait similarity among *C. applegatei* and *C. peirsonii*, even though these species were assigned to hummingbird and bee syndromes, respectively. Similarities between *C. applegatei* and *C. peirsonii* included highly overlapping morphological and VOC traits (Appendix S6), but these two species are generally divergent in their corolla color. Such overlap seems reasonable, given that most syndromes are thought to evolve from an ancestral condition of bee pollination (Grant and Grant, 1965; Stebbins, 1970; Rosas-Guerrero et al., 2014; Wessinger et al., 2019). Regardless, future investigations of syndromes would benefit from considering how pollinator-mediated selection and phylogenetic history complicate simple expectations for the evolution of floral traits (Wessinger et al., 2014; Fenster et al., 2015).

Do syndromes predict floral visitation?

Pollination syndromes are thought to arise because these multivariate phenotypes reflect persistent pollinator-mediated selection in natural populations (Stebbins, 1970; Fenster et al., 2004; Smith and Kriebel, 2017). In *Castilleja*, most species are predominantly outcrossing (Cariveau et al., 2004), suggesting that fitness returns are heavily reliant on pollinator visitation. In this study, pollination syndromes failed to predict the most frequent floral visitor to *Castilleja* (Figure 6). However, visits to flowers in this study were comparatively infrequent to what has been reported in *Castilleja* (Adler, 2003; Hersch-Green and Cronn, 2009) and many other flowering plants (Robertson and MacNair, 1995; Kay and Schemske, 2003; Reynolds et al., 2009). Given that individual flowers last several days and most flowers produce seeds (E. T. Hilpman, personal observations), these relatively low rates of floral visitation are puzzling. With over 120 h of observations amongst these taxa and with so little variation in floral visitation, differences in visitation rates among pollinator guilds are likely subtle or nonexistent. Moreover, visitation to *Castilleja* was not influenced by abiotic factors (temperature, humidity, wind speed, etc.; Table 3) nor the available pollinator community across observation periods (Table 4). Taken together, our results clearly do not support the idea that pollination syndrome predicts floral visitation.

There is compelling support for the idea that pollination syndrome predicts the identity of visiting pollinators (Darwin, 1862; Reynolds et al., 2009; Armbruster et al., 2011; Rosas-Guerrero et al., 2014). In some cases, studies have clearly linked specific plant traits to their ability to attract pollinators in a community (Schemske and Bradshaw, 1999; Fenster et al., 2015). However, some studies have also failed to support the hypothesis that plant syndrome predicts the identity of visiting pollinators (Waser et al., 1996; Mayfield et al., 2001; Valdivia and Niemeyer, 2006). At least some of these negative results likely suggest that floral visitation does not accurately reflect the effectiveness of those visits resulting in seed production. Previous work has simultaneously studied rates of visitation and the effectiveness of those pollinators as agents of cross pollination in natural populations (Mayfield et al., 2001; Aigner, 2004; Reynolds et al., 2009; Ne'eman et al., 2010). Floral visitation rate is often positively correlated to the effectiveness of those visitors in causing seed production (Galen and Stanton, 1989; Young and Stanton, 1990; Jones and Reithel, 2001; Engel and Irwin, 2003; Reynolds et al., 2009), though not invariably so (Lau and Galloway, 2004; Sahli and Conner, 2007; Madjidian et al., 2008). To robustly evaluate the hypothesis that pollination syndromes arise from pollinator mediated selection, studies must endeavor to link individual variation in fitness to the activity of specific pollinators in a community.

Weak relationships between pollination syndrome and the identity of floral visitors may not be too surprising in *Castilleja*. Phylogenetic and morphometric studies have provided evidence for repeated hybridization between distinct lineages of *Castilleja* (Jacobs et al., 2019), sometimes even between divergent clades within the subtribe Castillejinae, which contains all of the species evaluated in this study (Chuang and Heckard, 1991; Hersch-Green and Cronn, 2009; Tank et al., 2009). Given that the species in this study flower in close physical proximity and attract similar floral visitors, the processes maintaining distinct phenotypes are not yet known. While potentially rare hybridization events are documented in *Castilleja*, there is little known regarding its prevalence in natural populations. The rates of hybrid production depend on pre- and post-zygotic factors that could potentially discourage the formation and reduce the fitness of offspring, respectively (Hersch-Green and Cronn, 2009). Even if hybrids are formed at an appreciable rate, it is possible that environmental filtering occurs such that lineages remain distinct because they inhabit and experience selection in response to finely varying abiotic factors (e.g., soil characteristics; Caruso et al., 2019).

CONCLUSIONS

A major goal in biology is to understand and explain the diversity of traits in natural populations (Darwin, 1862; Fenster et al., 2004). In flowering plants, striking diversity in floral traits has often been attributed to the activity and consequences of pollinator visitation (Stebbins, 1970; Waser et al., 1996; Schemske and Bradshaw, 1999; Armbruster et al., 2011; Gervasi and Schiestl, 2017). While phenotypic differentiation in the morphology and chemical profiles was evident in the flowers of *Castilleja* from multiple pollination syndromes, there was considerable variation among species within the hummingbird syndrome. The relative importance of taxonomy and syndrome is an important unanswered question in this and other systems. In this study, phenotypic differentiation strongly supports an expansive view of pollination syndromes that closely resembles taxonomic distinctions. Perhaps most importantly, floral trait differentiation did not predict the activity of floral visitors to *Castilleja* in natural populations. The long-term maintenance of phenotypically distinct floral phenotypes may reflect local adaptation to abiotic factors or cryptic differences between pollinators in their propensity to affect seed production. Studying these processes in *Castilleja* and similar groups has the potential to shed light on the maintenance of biodiversity, the characteristics critical to attract pollinators, and the distributions of closely related plant lineages.

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AUTHOR CONTRIBUTIONS

E.T.H. was responsible for conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, visualization, and writing the original draft and editing. J.W.B. contributed to formal analyses, funding acquisition, resources, supervision, and writing the original draft and editing.

DATA AVAILABILITY STATEMENT

A single R script including all pertinent to analyses and figures presented in this paper are now available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.v6wwpzgwg (Hilpman and Busch, 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

Appendix S1. Boxplots of visitation by five pollinator guilds (bee, fly, hummingbird [HB], lepidopterans [Lep], and others) to *Castilleja* species.

Appendix S2. A summary of three ANOVAs. In each model, a *Castilleja* species effect was tested.

Appendix S3. Boxplots of visitation by five pollinator guilds (bee, fly, hummingbird [HB], lepidopterans [Lep], and others) to populations of *Castilleja*.

Appendix S4. A summary of three ANOVAs. In each model, population effects were tested.

Appendix S5. Multiple linear regression of fly visitation to plants, with individual traits as predictors.

Appendix S6. NMDS analyses of morphological traits grouped by pollination syndrome; bee (yellow), fly (black), and hummingbird (red).

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