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Divergence in pollen performance between *Clarkia* sister species with contrasting mating systems supports predictions of sexual selection

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Animal taxa that differ in the intensity of sperm competition often differ in sperm production or swimming speed, arguably due to sexual selection on postcopulatory male traits affecting siring success. In plants, closely related self- and cross-pollinated taxa similarly differ in the opportunity for sexual selection among male gametophytes after pollination, so traits such as the proportion of pollen on the stigma that rapidly enters the style and mean pollen tube growth rate (PTGR) are predicted to diverge between them. To date, no studies have tested this prediction in multiple plant populations under uniform conditions. We tested for differences in pollen performance in greenhouse-raised populations of two *Clarkia* sister species: the predominantly outcrossing *C. unguiculata* and the facultatively self-pollinating *C. exilis*. Within populations of each taxon, groups of individuals were reciprocally pollinated ($n = 1153$ pollinations) and their styles examined four hours later. We tested for the effects of species, population, pollen type (self vs. outcross), the number of competing pollen grains, and temperature on pollen performance. *Clarkia unguiculata* exhibited higher mean PTGR than *C. exilis*; pollen type had no effect on performance in either taxon. The difference between these species in PTGR is consistent with predictions of sexual selection theory.

KEY WORDS: *Clarkia exilis*, *Clarkia unguiculata*, gametophytic selection, mating system evolution, pollen competition, pollen tube growth rate.

Within plant species, intrasexual selection has been the focus of many empirical and theoretical investigations since the late 1970s, when the parallels between sexual selection in animals and plants were first recognized (Charnov 1979; Willson 1979; Bawa 1980a,b; Thomson and Barrett 1981; Lloyd and Yates 1982; Queller 1983; Stephenson 1983; Stephenson and Bertin 1983; Willson and Burley 1983; Bell 1985; Couvet et al. 1985; Galen et al. 1986). As in animals, competition among pollen-producing individuals may occur both before and after physical contact between mates. Prior to physical contact (i.e., pollination), pollen-producing plants compete for pollinator visitation, pollen removal, and the successful transfer of pollen to stigmas. After physical contact, between pollination and fertilization, competition among male gametophytes (mature, sperm-containing pollen) and selection favoring rapid pollen germination or growth can occur in any flower in which pollen grains must compete for access to ovules.

The conditions necessary for postpollination intrasexual selection in plants can be common even in populations in which the mean number of pollen grains per stigma does not exceed the mean number of ovules per ovary because when the variance among flowers in pollen receipt is high, many stigmas receive more pollen than the number of ovules available (Snow 1986; Herrera 2002, 2004). Consequently, in spite of widespread pollen

limitation of seed production detected in studies that compare the mean seed production of open-pollinated vs. pollen-supplemented flowers or individuals (Ashman et al. 2004; Knight et al. 2005), natural selection may favor pollen genotypes that germinate or grow faster on any receptive stigmas on which pollen is nearly simultaneous (late-arriving pollen may not effectively compete with previously deposited pollen) and exceeds the number of ovules available for fertilization.

It is now evident that, within many plant species, the rates of pollen germination and pollen tube growth can be influenced by paternal genotype and positively affect siring success or seed quality (Quesada et al. 1991, 1993; Jóhannsson and Stephenson 1997; Winsor et al. 2000; Swanson et al. 2016), both of which are necessary (but not sufficient) for intrasexual selection to occur. Female choice may, in principle, also operate in plants after pollination, whereby pollen recipients discriminate among competing pollen genotypes, allowing differential access to their ovules. However, because haploid male gametophyte genotypes and diploid female genotypes are necessarily in physical contact within a style, it is problematic to determine unambiguously whether the differential success of pollen genotypes after pollination is due to intrinsic differences among them in competitive ability, pollen-pistil complementarity (Carlson et al. 2009), or discrimination (i.e., "choice") by the pollen recipient (Marshall and Diggle 2001; but see Waser et al. 1987; Waser and Price 1993 for a potential example of female choice).

CONSEQUENCES OF MATING SYSTEM FOR SEXUAL SELECTION

The study of mating system evolution among flowering plant taxa has revealed many instances in which suites of morphological or life-history traits have coevolved with self-fertilization: the well-known "selfing syndrome" (Darwin 1876; Ornduff 1969; Richards 1986; Sicard and Lenhard 2011). Self-fertilization has evolved independently within many families, genera, and species, and may be associated with evolutionary changes in sex allocation (Delesalle et al. 2008; Delesalle and Mazer 2009; Mazer et al. 2009), genetic architecture (Mazer et al. 2007), floral development rate or life span (Wyatt 1986; Runions and Geber 2000; Mazer et al. 2004, 2009; Dudley et al. 2007; Delesalle et al. 2008), physiological rates (Mazer et al. 2010a; Dudley et al. 2012), flower size (Ornduff 1969; Lyons and Antonovics 1991; Goodwillie et al. 2006; Sicard and Lenhard 2011; Doubleday et al. 2013; Tedder et al. 2015; Carleial et al. 2017), floral scent (Doubleday et al. 2013), flower brightness (Button et al. 2012), style length (Duncan and Rausher 2013), habitat preference (Anderson et al. 2015; Schneider and Mazer 2016), and life history (Mazer et al. 2004; Dudley et al. 2007; Shimizu and Tsuchimatsu 2015; Schneider and Mazer 2016). Few studies have investigated the effects of mating system on pollen performance (Smith-Huerta 1996; Kerwin and Smith-Huerta 2000; Taylor and Williams 2012; Hove and Mazer 2013), but it has been proposed that intrasexual selection should cause traits that affect the competitive ability of male gametophytes to diverge between outcrossing versus regularly self-pollinating taxa (Mazer et al. 2010b).

Several differences between habitually outcrossing and self-pollinating taxa result in a greater opportunity for selection among pollen genotypes in the former (Mazer et al. 2010b). First, due to their higher heterozygosity, individuals of outcrossing taxa typically produce pollen that is more genetically diverse than that of highly selfing taxa; consequently, the stigmas of outcrossers receive pollen of higher diversity than those of closely related selfers even when receiving pollen from a single donor. Second, flowers adapted for outcrossing typically stay fresher and receptive longer than those of self-pollinating taxa (Primack 1985; Weber and Goodwillie 2013). This greater longevity can promote multiple pollinator visits and the receipt of pollen from multiple donors, as evidenced by studies showing that seeds within individual fruits often represent multiple fathers (Ellstrand and Marshall 1986; Bernasconi 2003; Mitchell et al. 2005; Teixeira and Bernasconi 2007). Third, relative to closely related selfing taxa, the flowers of outcrossers are often larger, with larger stigmas and longer styles (Snell and Aarssen 2005; Sicard and Lenhard 2011; Duncan and Rausher 2013; but see Carleial et al. 2017). Consequently, the potential number of competing pollen grains and the distance over which their pollen tubes may compete are also greater, intensifying both gametophytic competition and selection against slow germination and pollen tube growth rate (PTGR) (Travers and Shea 2001).

As a result of these differences between selfers and outcrossers in the genetic diversity of pollen produced and received, in floral longevity, and in floral phenotype, the opportunity for postpollination sexual selection among pollen genotypes is higher in outcrossers. Accordingly, in pairs of angiosperm sister taxa with highly divergent outcrossing rates, selection among pollen genotypes based on their competitive ability is expected to be comparatively strong in the more highly outcrossing taxon, assuming that its receptive stigmas regularly receive, within a short time period, more pollen than there are ovules available to fertilize (a common condition: Ashman et al. 2004; Herrera 2004; Knight et al. 2005; Vamosi et al. 2006). The inference that the efficacy of selection on pollen competitive ability is (or has been) greater in outcrossing taxa than in closely related selfers requires that the former harbor sufficient genetic variation in pollen performance traits for them to evolve, an attribute reported in many studies (Snow and Spira 1996; Marshall 1998; Pasonen et al.1999; Kerwin and Smith-Huerta 2000; Nikkanen et al. 2000; Lankinen et al. 2009). For populations to respond to selection on such traits depends, in turn, on a strong and positive relationship between pollen competitive ability and male fertility or seed

quality, which has been observed in several species (Snow and Spira 1991, 1996; Marshall et al. 1996; Delph et al. 1998; Marshall and Diggle 2001; Pasonen et al. 2001; Baskin and Baskin 2015).

The hypothesis that a taxon's mating system influences the evolution of traits expressed during the haploid phase of the life cycle has been tested and corroborated in animals. In rodent, fish, bird, and primate species experiencing relatively intense sperm competition (e.g., polyandrous species), sperm have evolved to swim faster than those of closely related taxa experiencing weaker competition among males (Gomendio and Roldan 1991; Birkhead and Immler 2007; Fitzpatrick et al. 2009; Kleven et al. 2009; Alvarez 2017). The analogous prediction in plants—that highly outcrossing (i.e., relatively "promiscuous") taxa will evolve faster-growing male gametophytes than closely related self-fertilizing taxa—has been reported in a few taxa (Smith-Huerta 1996; Diaz and Macnair 1999; Kerwin and Smith-Huerta 2000; Taylor and Williams 2012; Hove and Mazer 2013), but the interpretation of these studies is somewhat problematic because several factors other than mating history may influence pollen performance and must be accounted for when comparing taxa.

First, the amount of pollen deposited on a stigma (i.e., the "pollen load") may affect the probability that a given pollen grain will penetrate the style (the stigma penetrance rate, or SPR) and/or the mean PTGR. For example, negative interactions among pollen genotypes may reduce pollen germination or growth rates, particularly when pollen deposition is high. Physical interference, competition for nutrients, or allelopathic interactions among pollen grains may occur in the stigma or style when space or resources provided by these organs are limited (Cruzan 1986; Holm 1994; Niesenbaum and Schueller 1997; Kerwin and Smith-Huerta 2000; Németh and Smith-Huerta 2002; Parantainen and Pasonen 2004; Mazer et al. 2016; Swanson et al. 2016). Alternatively, positive interactions among male gametophytes may occur if a minimum number of pollen grains must be deposited to induce the allocation of maternal resources to stigmas or styles, or if pollen grains or pollen tubes interact in a way that promotes their germination or growth (cf. Cruzan 1986, 1990b; Ganeshaiah and Shaanker 1988; Holm, 1994; Niesenbaum and Schueller 1997; Pasonen and Käpylä 1998; Niesenbaum 1999; Parantainen and Pasonen 2004). The number of pollen grains deposited on a stigma may affect the observed mean PTGR in an additional way. As the pollen load increases, selection among competing pollen genotypes will intensify, potentially favoring those with a higher PTGR (Cruzan 1986; Pasonen and Käpylä 1998; Niesenbaum 1999). As a result of this selective process, the mean PTGR of the pollen tubes that successfully enter the style may increase as the pollen load increases simply because the slower tubes are excluded from stylar entry. Analyses designed to detect significant differences between taxa in either SPR or PTGR should therefore control for variation in the pollen load.

Second, the condition of pollen-producing plants may affect pollen performance (Delph et al. 1997; Travers 1999; Stephenson et al. 2003; Smith-Huerta et al. 2007; Lankinen 2008) just as the stylar environment or genotype may affect the performance of pollen received (Cruzan 1990a,b; Smith-Huerta et al. 2007). In addition, pollen-pistil interactions (Herrero and Hormaza 1996; Diaz and Macnair 1999; Swanson et al. 2004), temperature (Jóhannsson and Stephenson 1998; Lankinen 2001; Hove and Mazer 2013), and inbreeding depression (Stephenson et al. 2001) may affect pollen performance. Consequently, interspecific comparisons of pollen performance should control for pollen type (self vs. outcross), pollen load, and temperature, and also test individual pollen donors across a variety of pollen recipients in a common environment.

The current study was designed to test the prediction that closely related taxa with contrasting mating systems differ in the SPR and PTGR observed shortly after pollination. To date, no studies have compared pollen performance between species with contrasting mating systems in a uniform environment while controlling for variation among populations and for the effects of pollen load, SPR, temperature, and pollen type (self vs. outcross); here, we examined all of these sources of variation. In addition, unlike previous studies (Smith-Huerta 1996; Diaz and Macnair 1999; Kerwin and Smith-Huerta 2000; Taylor and Williams 2012; Hove and Mazer 2013), we tested for species differences in PTGR using two estimates of this trait. First, we estimated the mean PTGR based on the distance traveled by each pollen tube visible in each style and, second, we estimated PTGR from the length of the longest tube (LLT) in each style.

For the current study, we compared the performance of pollen donors representing multiple wild populations of two putative sister species in the genus *Clarkia* to address the following questions:

(1) Has pollen performance diverged between the predominantly outcrossing *C. unguiculata* and the highly self-fertilizing *C. exilis*? We predicted that *C. unguiculata* would exhibit higher SPR and a higher mean PTGR than *C. exilis*.

(2) Does the difference between species in pollen tube growth rate (PTGR) depend on whether it is estimated as the mean PTGR of all pollen tubes visible in each style or as the LLT? We expected that, due to recombination and segregation during meiosis, the variance in PTGR among the pollen produced by individuals of *C. unguiculata* would be higher than among the pollen produced by individuals of *C. exilis*. If so, then even if the mean PTGR of the two taxa are similar, the mean LLT observed shortly after pollination would be greater in *C. unguiculata* than in *C. exilis*. Hence, we predicted that the difference between taxa in mean LLT would exceed the difference between them in mean PTGR.

(3) If these species differ in pollen performance, is the difference between them independent of the type of pollen used? We expected *C. unguiculata* to have evolved greater discrimination against self pollen than *C. exilis* to avoid the negative effects of inbreeding depression (among the resulting offspring), which should be higher in outcrossers due to their higher genetic load. By contrast, *C. exilis* is routinely fertilized by its own pollen and has purged much of its genetic load (Lowry 2007). Therefore, we predicted that self and outcross pollen will either perform similarly in *C. exilis* or that self pollen will outperform outcross pollen.

(4) Do other pollination conditions affect pollen performance? We predicted that, in both species, as pollen load increases, SPR will decrease due to interference competition among pollen grains. We also expected that mean PTGR will increase with pollen load due to the greater opportunity for selection to filter out slower-growing pollen tubes, and that both pollen performance traits would increase with temperature.

(5) Does intraspecific variation in pollen performance differ between species? If, relative to highly selfing taxa, outcrossing taxa have experienced stronger and more consistent directional selection favoring rapidly germinating pollen and faster growing pollen tubes, then contemporary populations of *C. unguiculata* should exhibit less variation among pollen donors in pollen performance traits than *C. exilis*.

Materials and Methods

STUDY SYSTEM

Clarkia is a genus of 40 annual species for which the center of diversity is the western United States. *Clarkia* species are self-compatible, and self-fertilization has evolved at least 10 times in the genus (Lewis and Lewis 1955; Vasek 1958, 1964). In this study, we cultivated seeds from wild populations of two diploid ($2n = 18$) sister taxa that differ in their geographic ranges, mating system, and life history; while they are occasionally sympatric, they do not hybridize.

Clarkia unguiculata Lindl. is endemic to and widespread in California, with outcrossing rates in the southern Sierra Nevada reported to range from 0.64 to 0.88 (Ivey et al. 2016), to 0.80 to 0.98 (Hove, 2012), to 0.96 (Vasek, 1965). Populations occur on oak woodland slopes, disturbed soils, and roadside embankments of the Coastal, Transverse, and Sierra Nevada ranges, at elevations from sea level to 1500 m. For the current study, we sampled four populations of *C. unguiculata* along an elevation gradient from 443 to 1006 m in the foothills of the Sierra Nevada (Fig. 1, Table 1).

Clarkia exilis H. Lewis and Vasek is putatively descended from one or more populations of *C. unguiculata* (Vasek 1958) and is geographically restricted to a small portion of *C. unguiculata's* range in and near the Kern River Valley (in Kern and Tulare Counties) in the Sierra Nevada foothills (Fig. 1). Populations range in elevation from 270 to 550 m. This species is facultatively autogamous, with low dichogamy and herkogamy promoting self-pollination (Vasek 1964). *Clarkia exilis* typically completes its flowering before *C. unguiculata* begins to flower, precluding heterospecific pollination. Observed outcrossing rates of *C. exilis* populations have been reported as 0.19 (Hove, 2012), 0.03–0.70 (Vasek 1964), and 0.38–0.89 (Vasek and Harding 1976). Under greenhouse conditions (in the absence of pollinators), *C. exilis* exhibits 100% fruit set (Mazer, pers. obs.). *Clarkia exilis* exhibits more rapid development than *C. unguiculata* with respect to the rate of sequential flower production as well as the number of days between floral bud opening and both anther dehiscence and stigma receptivity (Dudley et al. 2007). In addition, *C. exilis* flowers are less protandrous, have shorter styles (Fig. S1) and have shorter floral life spans than those of *C. unguiculata* (Knies et al. 2004; Dudley et al. 2007). For this study, we sampled seeds from four populations of *C. exilis* ranging from 365 to 543 m elevation and from 35.47 to 36.02° N (Table 1); one of these populations is sympatric with *C. unguiculata* (Stark Creek, 443 m elevation). In 2008 and 2010, seeds were collected from 20 to 30 maternal plants in each field population, placed in coin envelopes (one maternal family per envelope), stored in plastic zip-lock bags, and frozen until use in this study.

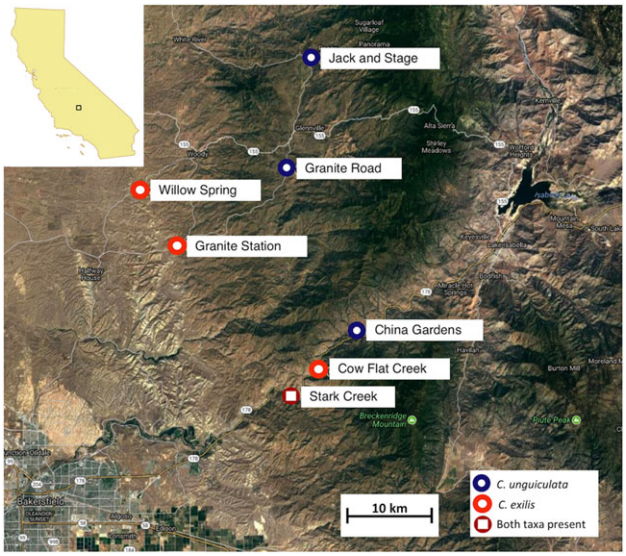

Figure 1. Map of locations of populations sampled. See Table 1 for GPS coordinates and elevations.

CULTIVATION OF GREENHOUSE-RAISED PLANTS

Seeds were germinated in agar and then raised in a temperature-controlled greenhouse prior to hand pollination. On 31 October 2014, to simulate the cool, moist conditions under which seeds

Table 1. Populations used as seed sources for this study.

Population Name	Date of Seed Collection	Taxon	Elevation (m)	Latitude (N)	Longitude (W)
Willow Spring (WS)	25 June 2010	*C. exilis*	365	35.670	−118.902
Stark Creek (SCCE)	24 June 2010	*C. exilis*	443	35.475	−118.726
Stark Creek (SCCU)	19 July 2010	*C. unguiculata*	443	35.475	−118.726
Cow Flat Creek (CFC)	20 May 2008	*C. exilis*	518	35.499	−118.694
Granite Station (GS)	10 May 2008	*C. exilis*	543	35.617	−118.859
China Gardens (CG)	19 July 2010	*C. unguiculata*	641	35.537	−118.649
Granite Road (GR)	19 July 2008	*C. unguiculata*	869	35.691	−118.732
Jack and Stage (JS)	19 July 2008	*C. unguiculata*	1006	35.796	−118.703

See Figure 1 for map of locations. *Clarkia unguiculata* is predominantly outcrossing; *C. exilis* is highly self-pollinating to mixed mating.

naturally germinate in the fall, seeds from each maternal family were placed on agar-filled Petri dishes, vernalized in the dark at 10 °C for two weeks, and then moved to room temperature under ambient light, where they germinated over the following week. Following germination, healthy 4- to 11-day-old germinants were transferred into soil-filled growing tubes (4 × 20 cm "Cone-tainers," Stuewe and Sons, Inc., Tangent, Oregon). We used a soil mix composed of 9:1 Sunshine #4 mix (sphagnum peat moss, perlite, dolomitic limestone, and endomycorrhizae; Sun Gro Horticulture, Agawam, MA) and worm castings, respectively. In each cone-tainer, four Osmocote slow-release fertilizer pellets (14-14-14 NPK; Scotts-Sierra Horticultural Products Co., Marysville, OH) were placed into a 3-cm deep hole into which seedlings were inserted. Three seedlings per family were placed into each of three cone-tainers. The cone-tainers were then moved into a greenhouse and, one week later, new young seedlings were added to any cone-tainers in which there were no surviving seedlings. Plants were bottom-watered with deionized water to maintain soil moisture. When plants were ∼15 cm tall, each cone-tainer was thinned to one healthy seedling. When plants exceeded 0.5 m in height, they were stabilized by attaching the primary stems (using small plastic rings) to a vertical, narrow gauge steel wire taped to the outer surface of each cone-tainer. In the greenhouse, day length was extended to 13-h days using 300 W LED grow lights (PAR approximately 1147 μmol/s/m^2).

HAND POLLINATION: EXPERIMENTAL DESIGN

Hand pollinations of virgin stigmas were conducted within replicates of a diallel matrix design. Within each population, as individual plants matured, they were divided into groups (replicates) of five to eight individuals that began to flower at approximately the same time. Each group contained unique maternal families (and no known siblings), and each population was represented by three or four groups. Within each replicate, reciprocal crosses among all pairs of individuals, including both self- and cross-pollinations, were conducted over a ∼one-month period (across all populations, hand pollinations occurred between 5 Jan to 16 Mar 2015). In both species, prior to anther dehiscence, flowers were emasculated to prevent autonomous self-pollination. As stigmas became receptive, flowers on the primary stem of each maternal plant were hand pollinated using pollen from one of the other members of its replicate. Among the flowers of each recipient, pollen donors were used on an alternating basis to prevent any effects of floral position from being confounded with donor identity. To identify the pollen donor used for each flower, pollinated flowers were each labeled with a small strip of tape wrapped around the internode beneath it. Hand pollinations were conducted between 8:30 a.m. and 12:00 p.m., during which time the greenhouse temperature ranged from 16.25 °C to 29.81 °C (mean: 25.57 °C; SD = 2.47). The greenhouse temperature was recorded every 15 min, and for each pollinated flower, the mean temperature during the four hours following pollination was calculated. The four-hour interval commenced at the start of the hour during which a flower was pollinated, so a flower pollinated at 10:45 a.m. was associated with the mean temperature from 1000 to 1400 h. This variable is referred to as "temperature," below.

As pollen-producing flowers became limiting, not all plants could be used as pollen donors in reciprocal crosses. In *C. exilis*, a total of 77 maternal plants were pollinated and 73 plants were used as pollen donors; in *C. unguiculata*, 64 plants were pollinated and 63 plants were used as pollen donors. Within each replicate, 30–65 flowers were pollinated and could be unambiguously scored for the number of pollen tubes visible in each successive 1-mm interval (as required for estimates of mean PTGR, described below). A total of 106–173 flowers were pollinated (and scoreable) per population, resulting in a total of 1153 pollinated styles.

SAMPLE PREPARATION AND MEASUREMENT OF POLLEN PERFORMANCE

Style preparation

Four hours after pollination, the stigma and style of each pollinated flower was severed at the base and placed in a labeled

microcentrifuge tube filled with formalin-acetic acid until prepared for viewing. To prepare samples for microscopy, each style was gently rinsed several times with distilled water within the microcentrifuge tube, which was then filled with a 1 M NaOH solution to soften the stigma and style. Approximately 30 h later, the NaOH was removed from the tube and the sample was rinsed again, taking care to avoid the removal of any pollen grains from the stigma. The tube was then filled with a solution of 0.1% aniline blue (weight/volume) dissolved in 0.1 M K_3PO_4; ~18 h later, the sample was placed on a glass slide (keeping the style as straight as possible) and gently squashed underneath a coverslip. Samples were then viewed under an epifluorescence microscope (Olympus BX61) using a DAPI excitation filter.

Estimation of SPR

Each squashed stigma was observed using a dissecting microscope to record the number of pollen grains retained by the stigma (defined here as the pollen load). Pollen grains that did not germinate would have been washed off during style preparation because they were not anchored to the stigma by their pollen tubes. We then calculated the ratio of the number of pollen tubes observed in the first millimeter of the style to the number of pollen grains observed on the stigma to estimate the proportion of pollen grains adhering to the stigma whose tube reached the style, that is, the SPR. This is distinct from the pollen germination rate, which we cannot estimate because we did not count the number of pollen grains originally deposited on the stigma.

Estimation of mean PTGR and the LLT

As *Clarkia* pollen tubes grow down a style, they deposit callose plugs at approximately 1-mm intervals. This feature provides a way of estimating the mean PTGR among all tubes observed in a given style after a given time period (here, four hours following pollination). By recording the number of callose plugs visible in each 1-mm interval (starting from the base of the stigma and extending down the style), and assuming that each callose plug within a 1-mm interval represents a different pollen tube, the mean PTGR of all tubes in a given style can be estimated from equation (1), which provides the estimated mean length of all observed pollen tubes at the time that the style is fixed.

$$\bar{l} = \sum_{i=1}^{n} (p_i - p_{i+1})\, l_i, \tag{1}$$

where n is the number of 1-mm intervals in a given style in which pollen tubes are visible, and p_i is the proportion of pollen tubes entering the style that are visible in interval i. The distance between the base of the stigma and the midpoint of interval i is assumed to be the mean length (l_i) of all pollen tubes that reached interval i but did *not* grow beyond it. So, $l_1 = 0.5$ mm; $l_2 = 1.5$ mm, etc. The mean pollen tube length in a given style ($\bar{l}$) is then calculated as the mean of a discrete random variable, where each discrete length (l_i) is multiplied by the proportion of pollen tubes that reach interval i but fail to grow beyond it ($p_i - p_{i+1}$). Dividing $\bar{l}$ by the number of hours since pollination yields the mean PTGR per hour. By example, if the first five 1-mm intervals of a style contain 55, 42, 20, 15, and 0 callose plugs, respectively, then, for $i = 1$: $p_1 = 55/55$, $l_1 = 0.5$ mm; $i = 2$: $p_2 = 42/55$, $l_2 = 1.5$ mm; $i = 3$: $p_3 = 20/55$, $l_3 = 2.5$ mm; $i = 4$: $p_4 = 15/55$, $l_4 = 3.5$ mm; and $i = 5$: $p_5 = 0/55$, $l_5 = 4.5$ mm. In this case,

$$\bar{l} = \frac{55-42}{55}0.5\text{ mm} + \frac{42-20}{55}1.5\text{ mm} + \frac{20-15}{55}2.55\text{ mm} + \frac{15-0}{55}3.5\text{ mm} = 1.9\text{ mm},$$

and the mean PTGR of all tubes in the style would equal 1.9/4 = 0.475 mm/h. In this example, the LLT would be estimated as 3.5 mm, or the midpoint of the 1-mm interval reached by the longest tube.

STATISTICAL ANALYSES

To test for differences between species in pollen performance independent of the effects of population of origin, replicate, type of pollination (self- vs. cross-), pollen load, and temperature, three datasets were analyzed. In the first dataset, each hand-pollinated flower was included as a record. The second dataset was composed of the pollen donor means for each pollination type. The third dataset was composed of pollen donor means estimated from the means of the two pollination types. The analyses of donor means provide a more conservative test for differences between species than the analysis of individual styles, preventing potential bias due to unequal sample sizes among donors, populations, or between pollination types. For all models described below, standard least squares analyses of covariance (ANCOVA) were conducted. All independent variables were treated as fixed, except for replicate, which was treated as a random factor. Least squares means of the categorical variables were compared using Student's t (for two groups) or Tukey's HSD (for multiple groups) to detect significant differences among group means. JMP Pro 13.0.0 (2016, SAS Institute, Inc.) was used for all analyses.

Analyses of individual styles

Using the dataset of individual styles ($n = 1153$), we first assessed whether mean PTGR (mm/h) and LLT (mm) provided equivalent estimates of PTGR by estimating the correlation between them within and across populations. We then used these data to conduct ANCOVAs to detect effects of species, population (nested within species), pollen load, temperature at pollination type, and the species × pollination type interaction on each pollen performance variable (SPR, PTGR, and LLT). The variance component associated with replicate was nonsignificant in all cases, so this

factor was excluded from the models presented below. To compare analyses across variables, this dataset was constrained to include only those styles ($n = 1136$) of which all three traits were measured.

Analyses of pollen donor means

The individual flower measurements were then used to create a dataset composed of pollen donor means for SPR and mean PTGR. Pollen donor means for self- and for cross-pollinated flowers were calculated separately ($n = 254$ pollen donor means). Pollen donor means for the pollen load, SPR, and temperature were also included. The ANCOVAs of these pollen donor means (described below) detected no significant difference within either species between self- and cross-pollinations in either SPR or mean PTGR (see Results; Tables 3 and 4). Consequently, this dataset was used to create a third dataset composed of 136 donor means, with each donor mean calculated using the means of its self- and cross-pollinated flowers; ANCOVAs of these data excluded the effects of pollination type and the species × pollination type interaction.

Bivariate analyses to detect effects of pollen load and temperature on pollen performance

To detect evidence of interference among pollen grains in each species, we conducted bivariate linear regressions among pollen donor means ($n = 73$ donors in *C. exilis*; $n = 63$ donors in *C. unguiculata*) to detect effects of the number of pollen grains per stigma on SPR, on the number of pollen tubes entering the style, and on mean PTGR. To evaluate the need to control for temperature when testing for the difference between species in pollen performance, we examined the effect of temperature on SPR and PTGR among pollen donor means.

Factors influencing the SPR

ANCOVAs were conducted on pollen donor means ($n = 254$) to detect the effects of species, population (nested within species), replicate, pollination type, the species × pollination type interaction, temperature, and the number of pollen grains per stigma on SPR. The replicate variance component was nonsignificant in all models and was therefore excluded from all of the models presented here. As these models detected no significant effect of pollination type, ANCOVAs (excluding the effect of pollination treatment) were also conducted on the pollen donor means ($n = 136$) for which each donor's mean SPR was calculated from the means of the two pollination treatments. Alternative models were compared with respect to the second-order Akaike Information Criterion (AICc) to identify the consequences of excluding different variables for each model's fit (Burnham and Anderson 2002).

Factors influencing mean PTGR

ANCOVAs for PTGR were conducted as above, with the exception that SPR was also included as a covariate to control for the possibility that pollen donors with relatively high SPR would appear to have higher PTGR simply because their pollen grains germinated sooner (giving them more time to grow before the style was harvested). Similar to the analysis of SPR, reduced models (excluding temperature and SPR) were constructed to test for the effects on model fit of eliminating variables that were nonsignificant in the full model.

Intraspecific variation in pollen performance

To determine whether *C. unguiculata* exhibits less genetically based variation in pollen performance traits than *C. exilis* when grown under controlled conditions, we compared species with respect to the frequency distributions of pollen donor means. For each species, we examined the distributions of (a) the raw values of SPR and mean PTGR, where each donor was represented as the mean of the means of its selfed and its outcrossed flowers; (b) the residuals of SPR derived from the ANCOVA of these donor means (in which species, population, pollen load, and temperature were the independent variables); and (c) the residuals of mean PTGR derived from the ANCOVA of these donor means (in which species, population, pollen load, temperature, and SPR were the independent variables). The residuals provide the pollen donor means of each trait, controlling for the independent variables in the model.

Results

CORRELATION BETWEEN TWO ESTIMATES OF POLLEN TUBE GROWTH

Mean PTGR and the LLT per style are positively correlated in both species (populations pooled: *C. exilis*: $r = 0.60$, $n = 512$, $P < 0.0001$; *C. unguiculata*: $r = 0.52$, $n = 624$, $P < 0.0001$; Fig. 2). The correlation between these traits is also positive in all populations ($r = 0.54$–0.68 in the populations of *C. exilis*, $n = 4$; $r = 0.45$–0.57 in *C. unguiculata*, $n = 4$ populations; $P < 0.0001$ in all populations; data not shown). The two traits differed, however, with respect to the degree of divergence between species (see below).

DIVERGENCE IN POLLEN PERFORMANCE BETWEEN SPECIES

Analyses of individual styles

Stigma penetrance rate: The habitually selfing *C. exilis* and the predominantly outcrossing *C. unguiculata* do not differ in SPR (Table 2: $F_{1,1124} = 0.27$; $P = 0.6022$).

Two estimates of pollen tube growth (PTGR vs. LLT): Although PTGR and LLT are strongly correlated, they differ with

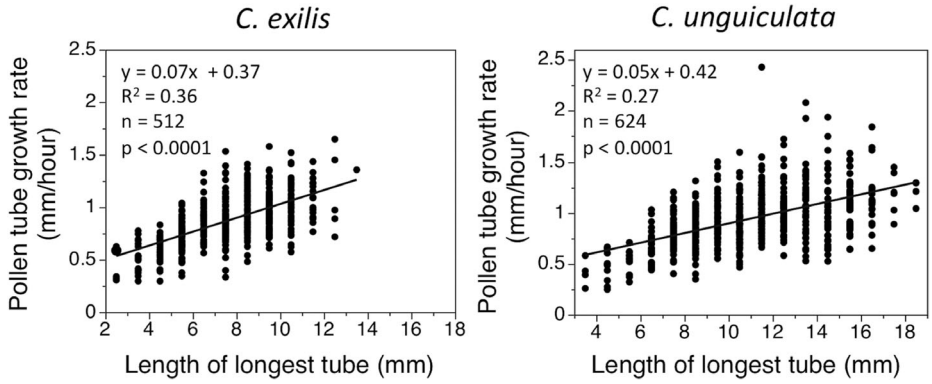

Figure 2. **Bivariate regression of mean pollen tube growth rate (PTGR: mm/h) on the length of the longest tube (LLT: mm) in *C. exilis* and *C. unguiculata*. In both species, LLT predicts the mean PTGR, but the proportional difference between species in LLT greatly exceeds the difference between them in PTGR (Table 2).**

respect to the proportional difference between species. *Clarkia exilis* has a lower mean PTGR than *C. unguiculata* (Table 2: $F_{1,1124} = 5.53$; $P = 0.0189$); based on the least squares means, the mean PTGR is 5.5% lower in *C. exilis* ($\bar{x} \pm \text{SE} = 0.88 \pm 0.014$ mm/h) than in *C. unguiculata* ($\bar{x} \pm \text{SE} = 0.92 \pm 0.013$ mm/h). Populations also differ significantly with respect to mean PTGR, and temperature at pollination has a positive effect on this trait has a positive effect on this trait ($b \pm \text{SE} = 0.0077 \pm$

Table 2. **Summary of standard least squares analysis of variance among individual styles to detect the independent effects of species, population (nested within species), pollination type (self- vs. cross-pollination), species × pollination type, pollen load, and temperature on the stigma penetrance rate (SPR), the pollen tube growth rate (PTGR), and the length of the longest tube (LLT). Bold-faced values indicate statistically significant effects.**

		SPR			PTGR			LLT		
Source	DF	Sum of Squares	*F*-Ratio	*P*-Value	Sum of Squares	*F*-Ratio	*P*-Value	Sum of Squares	*F*-Ratio	*P*-Value
Species	1	0.01	0.27	0.6022	0.34	5.53	**0.0189**	1053.67	172.33	**<0.0001**
Population (species)	6	1.35	5.11	**<0.0001**	4.24	11.36	**<0.0001**	703.81	19.19	**<0.0001**
Pollination type	1	0.04	0.94	0.3335	0.10	1.56	0.2116	2.22	0.36	0.5470
Pollen grains/stigma	1	8.00	181.57	**<0.0001**	0.11	1.71	0.1918	155.28	25.40	**<0.0001**
Temperature	1	0.67	15.25	**<0.0001**	0.29	4.73	**0.0299**	96.14	15.72	**<0.0001**
Species × pollination type	1	0.15	3.30	*0.0696*	0.01	0.24	0.6250	0.00	0.00	0.9846
Model	11	10.29	21.23	**<0.0001**	6.00	8.75	**<0.0001**	3780.99	56.22	**<0.0001**
Error	1124	45.51			69.99			6872.31		
Corrected total	1135	59.80			75.99			10,653.30		

	n	Adjusted $R^2 = 0.17$ Least Squares Means for SPR (SE)	Adjusted $R^2 = 0.08$ Least Squares Means for PTGR (mm/h) (SE)	Adjusted $R^2 = 0.35$ Least Squares Means for LLT (mm $\pm$ SE)
C. unguiculata	512	0.64 (0.011)	0.92^{b} (0.013)	10.57^{b} (0.127)
C. exilis	624	0.63 (0.012)	0.88^{a} (0.014)	7.92^{a} (0.144)
Self-pollination	249	0.63 (0.014)	0.89 (0.016)	9.19 (0.160)
Cross-pollination	904	0.64 (0.007)	0.91 (0.009)	9.30 (0.084)
C. unguiculata selfed	139	0.64 (0.019)	0.92 (0.022)	10.51 (0.218)
C. unguiculata outcrossed	498	0.63 (0.010)	0.93 (0.012)	10.62 (0.118)
C. exilis selfed	110	0.61 (0.021)	0.86 (0.025)	7.86 (0.243)
C. exilis outcrossed	406	0.65 (0.011)	0.89 (0.014)	7.97 (0.134)

Among the least squares means (SE), distinct superscripts (a vs. b) indicate significant differences between species means, based on Student's *t* ($\alpha = 0.05$).

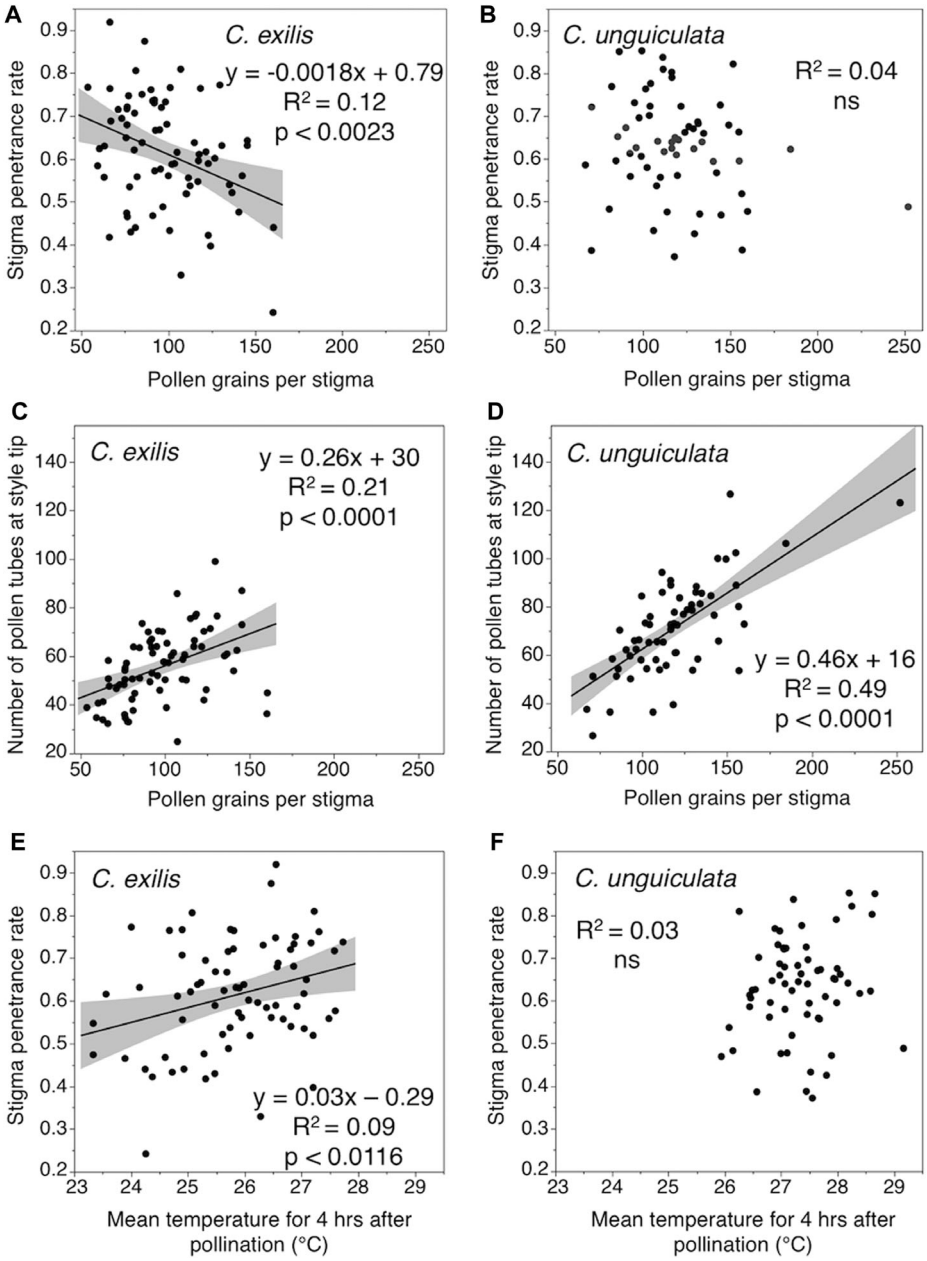

Figure 3. **Linear regressions between pollen performance traits, pollen load, and temperature; points represent pollen donor means, each calculated from the means of the self- and cross-pollination treatments. The shaded region represents the 95% confidence interval of the slope. *Clarkia unguiculata* is predominantly outcrossing; *C. exilis* is highly selfing to mixed mating. (A and B) SPR versus pollen grains per stigma. (A) *Clarkia exilis* (*n* = 73); (B) *C. unguiculata* (n = 63). (C and D) Number of pollen tubes visible in the first 1 mm of the style versus the number of pollen grains per stigma. (C) *Clarkia exilis*; (D) *C. unguiculata*. (E and F) SPR versus mean temperature during the four hours following pollination. (E) *Clarkia exilis*; (F) *C. unguiculata*.**

0.0035). There is no significant effect of pollination type, pollen load, or species × pollination type interaction on mean PTGR.

The mean LLT is also significantly lower in *C. exilis* than in *C. unguiculata*, and the proportional difference between species is much higher for this trait than for mean PTGR (Table 2: $F_{1,1124} = 172.33$; $P < 0.0001$). Based on the least squares means, the mean LLT of *C. exilis* is 33.45% lower than of *C. unguiculata* (*C. exilis*: $\bar{x} \pm \text{SE} = 7.92 \pm 0.14$ mm; *C. unguiculata*: $\bar{x} \pm \text{SE} = 10.57 \pm 0.13$ mm). Similar to mean PTGR, populations differ significantly with respect to LLT, and temperature has a positive effect on this trait ($b \pm \text{SE} = 0.1387 \pm 0.0350$). There is also a significant positive effect of pollen load on LLT, but no significant effect of pollination type or a species × pollination type interaction.

Table 3. Summary of analysis of variance among pollen donor means to detect significant differences between species in stigma penetrance rate (SPR) independent of the effects of population (nested within species), pollination type (self- vs. cross-pollination), species × pollination type interaction, pollen load, and temperature. Bold-faced values indicate statistically significant effects.

Source	DF	Sum of Squares	*F*-Ratio	*P*-Value
Species	1	0.030	1.50	0.2220
Population (species)	6	0.292	2.42	**0.0275**
Pollination type	1	0.016	0.77	0.3808
Species × pollination type	1	0.065	3.21	*0.0743*
Pollen grains/stigma	1	0.742	36.86	<0.0001
Temperature	1	0.069	3.45	*0.0646*
Model	11	1.274		
Error	242	4.872		
Corrected total	253	6.146		
Adjusted $R^2 = 0.17$				
AICc = −255.94				
Number of observations = 254				

The AICc value is provided for comparison with another model applied to the same data (Supporting Information).

Table 4. Least squares means. Within each group (species, pollination type, and species × pollination type), no significant differences were detected between means (α = 0.05), based on Student's *t* (for two means) or Tukey HSD (for > two means).

	n (Number of Pollen Donor Means)	Least Squares Means for SPR	Standard Error
C. unguiculata	123	0.64	0.015
C. exilis	131	0.61	0.014
Self-pollination	135	0.62	0.013
Cross-pollination	119	0.63	0.012
C. unguiculata (self-pollinated)	60	0.65[a]	0.020
C. unguiculata (cross-pollinated)	63	0.63[a,b]	0.019
C. exilis (self-pollinated)	59	0.59[b]	0.020
C. exilis (cross-pollinated)	72	0.64[a,b]	0.018

Among the least squares means (SE), distinct superscripts (a vs. b) indicate significant differences between species means, based on Student's t (α = 0.05).

Analyses of pollen donor means

Bivariate analyses: Pollen load size has a negative effect on SPR in *C. exilis* but not in *C. unguiculata* (Fig. 3A and B). In both species, pollen load size has a positive effect on the number of pollen tubes that enter the style, but the slope of the relationship is much less than 1.0 in both species (*C. exilis*: slope $\pm$ SE = 0.26 ± 0.06, $n = 73$, $P < 0.0001$; *C. unguiculata*: slope $\pm$ SE = 0.46 ± 0.06, $n = 63$, $P < 0.0001$) (Fig. 3C and D). Among pollen donor means, pollen load size does not affect mean PTGR in either species (*C. exilis*: $r = -0.11$, *C. unguiculata*; $r = -0.018$, data not shown).

Among pollen donors, temperature positively affected SPR in *C. exilis* but not in *C. unguiculat*a (Fig. 3E and F). Mean temperature did not significantly affect PTGR in either species (*C. exilis*: $r = -0.008$, *C. unguiculata*; $r = 0.218$, data not shown).

Factors influencing the SPR: The full model detected no difference between species in SPR (α = 0.05; Tables 3 and 4, Fig. 4A; see Tables S1–S6 for statistical parameters of alternative models) when controlling for variation in pollen load size and temperature, both of which had independent effects on SPR (Table 3, Fig. 4C and D, and Tables S1–S6). The reduced model that excluded temperature (Table S6) detected a significant difference between species in SPR due to a difference between them in the mean temperature following pollination; when variation in temperature was controlled statistically, no difference between species was detected.

Across the two species, there was no significant difference in SPR between self- and cross-pollinated flowers (Tables 3 and 4; $P < 0.05$). The marginally nonsignificant effect of the species × pollination type interaction detected in the full model (Table 3) reflects a difference between taxa in the SPR of selfed relative to outcrossed flowers, as detected by the (less conservative) Tukey's HSD (Fig. 4B, Table 3). In *C. exilis*, selfed flowers had 8.5% lower SPR than outcrossed flowers, whereas in *C. unguiculata*, selfed flowers had 3.2% higher SPR than outcrossed flowers. In addition, among self-pollinated flowers, *C. unguiculata* had higher SPR than *C. exilis* (Fig. 4B).

Factors influencing mean PTGR: All models detected that *C. exilis* exhibits lower mean PTGR than *C. unguiculata* (Tables 5 and 6, Fig. 5; see Tables S7–S18 for statistical parameters of alternative models). Neither pollination type (selfed vs. outcrossed), pollen load, SPR, nor temperature significantly affected mean PTGR in any of the models ($P > 0.05$ in all cases). The model presented in Table 5 has a slightly higher AICc than the model from which SPR is excluded (AICc = −207.89 vs. −208.47; see Models 2 vs. 3, Table S7). This model (Table 5), however, indicates that the difference between species in PTGR is not due to any difference between them in SPR.

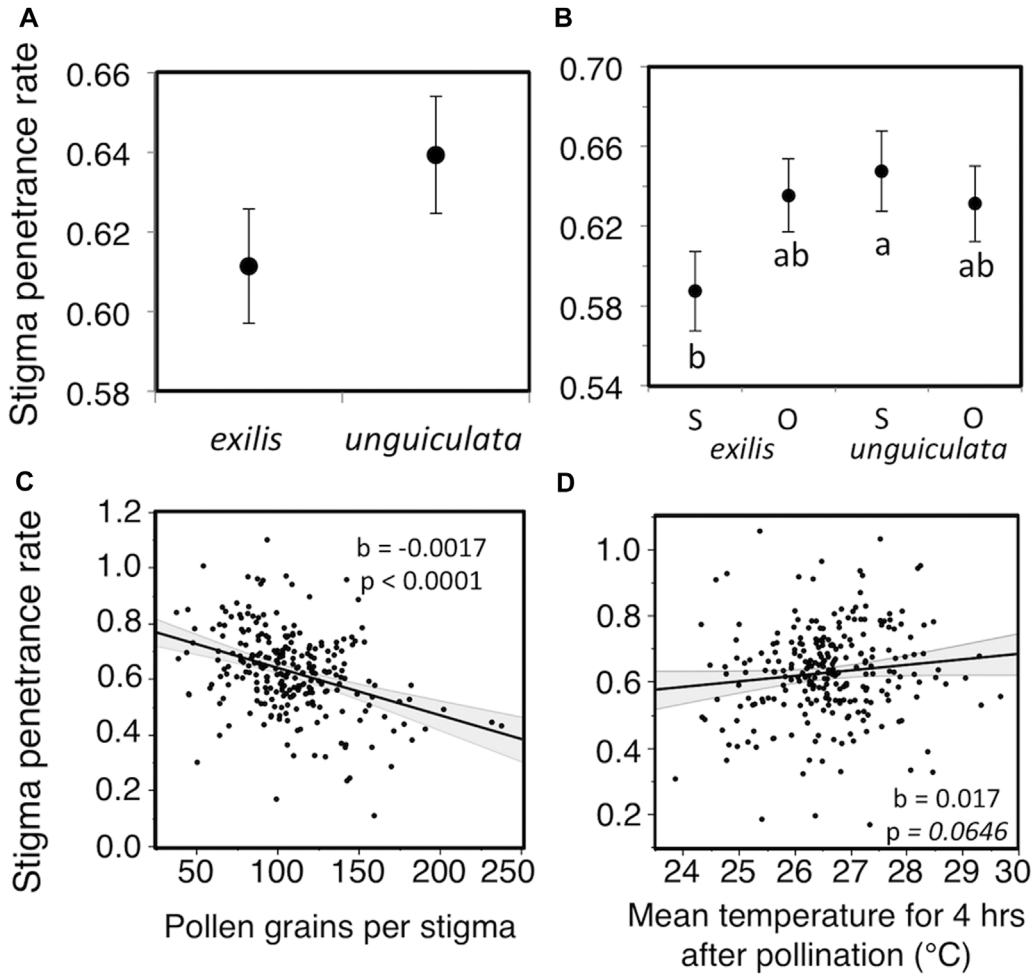

Figure 4. **Factors affecting the SPR: see Table 3 for analysis of variance. (A and B) Least square means (± standard error) of the SPR by (A) species and (B) species × pollination type (S = self-pollinations vs. O = cross-pollinations). In (B), means associated with distinct letters are significantly different at $P < 0.05$ (Tukey's HSD). (C) Leverage plot showing the effect of pollen grains per stigma on SPR, independent of the effects of species, population, and temperature (data derived from Table 3). (D) Leverage plot showing the effect of temperature on the SPR, independent of the effects of species, population, and the number of pollen grains per stigma (data derived from Table 3). In (C and D), b represents the slope of the standard least squares regression and the shaded regions represent the 95% confidence interval of the slope.**

Intraspecific variation in pollen performance: We found no evidence that pollen performance traits exhibit less variation among pollen donor means in *C. unguiculata* than *C. exilis*. The standard deviations of both SPR and PTGR among pollen donor means (both the residuals and raw values; Figs. 6 and 7) are statistically identical (Bartlett's test for equal variances: SPR: F-ratio = 0.3361, $P = 0.5621$; PTGR: F-ratio = 0.0034, $P = 0.9535$).

Discussion

DIVERGENCE IN POLLEN PERFORMANCE BETWEEN SPECIES

The divergence in pollen performance between *C. unguiculata* and *C. exilis* is consistent with the hypothesis that differences between them in the opportunity for selection to favor rapid pollen tube growth (PTGR) have led to evolutionary divergence in the competitive ability of male gametophytes—a predicted outcome of sexual selection (Smith-Huerta 1996; Mazer et al. 2010b).

To our knowledge, this is the first study designed specifically to compare taxa with different mating systems with respect to mean PTGR independent of variation in SPR soon after pollination, and to control for variation in the pollen load, which determines the number of pollen grains competing for access to the style. In addition, our estimate of mean PTGR was based on the mean of the distances reached by every pollen tube visible in each style rather than on the distance achieved by the fastest-growing pollen tube (cf. Taylor and Williams 2012), on the distance reached by a majority of pollen tubes (cf. Diaz and Macnair 1999), or on the number of tubes reaching a given

Table 5. Summary of analysis of variance among pollen donor means to detect significant differences between species in mean pollen tube growth rate (PTGR) independent of the effects of population (nested within species), pollination type (self- vs. cross-pollination), species × pollination type interaction, pollen load, and SPR. Boldfaced values indicate significant effects. In this analysis, the value for temperature was that observed exactly at the time of pollination.

Source	DF	Sum of Squares	*F*-Ratio	*P*-Value
Species	1	0.230	9.53	**0.0023**
Population (species)	6	0.572	3.95	**0.0009**
Pollination type	1	0.001	0.03	0.8634
Species × pollination type	1	0.011	0.45	0.5029
Pollen grains/stigma	1	0.011	0.44	0.5084
Stigma penetrance rate	1	0.038	1.57	0.2119
Model	11	0.948	3.57	**<0.0001**
Error	240	5.797		
Corrected total	251	6.745		
Adjusted $R^2 = 0.10$				
AICc = −207.89				
Number of observations = 252				

The AICc value is provided for comparison with another model applied to the same data (Supporting Information).

Table 6. Least squares means. Within each group (species, pollination type, or species × pollination type), distinct superscripts indicate significant differences between means (α = 0.05), based on Student's *t* (for two means), or Tukey's HSD (for > two means).

	n (Number of Pollen Donor Means)	Least Squares Means for PTGR	Standard Error
C. unguiculata	122	0.94[a]	0.015
C. exilis	130	0.87[b]	0.014
Self-pollination	117	0.90	0.015
Cross-pollination	135	0.91	0.013
C. unguiculata (self-pollinated)	60	0.94[a]	0.021
C. unguiculata (cross-pollinated)	63	0.93[a,b]	0.020
C. exilis (self-pollinated)	59	0.87[b]	0.021
C. exilis (cross-pollinated)	72	0.88[a,b]	0.019

Among the least squares means (SE), distinct superscripts (a vs. b) indicate significant differences between species means, based on Student's t (α = 0.05).

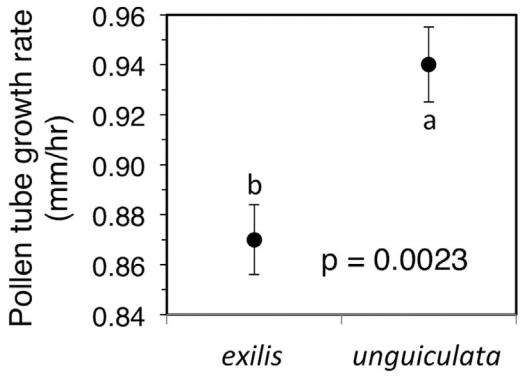

Figure 5. Least squares means (± standard error) of PTGR by species. Data and significance test derived from Table 4. *Clarkia unguiculata* is predominantly outcrossing; *C. exilis* is highly selfing to mixed mating.

distance within a given time period (cf. Smith-Huerta 1996; Kerwin and Smith-Huerta 2000). This study also provides the first assessment of the difference between using LLT versus mean PTGR when comparing taxa with respect to PTGR.

EVIDENCE FOR INTERFERENCE AMONG POLLEN GRAINS

The bivariate relationships among pollen donor means between pollen performance and the pollen load (Fig. 3) demonstrate that pollen grains or pollen tubes interfere with one another in both species. In the absence of space limitation, resource limitation, or negative interactions among pollen grains, SPR should be independent of pollen load size. In *C. exilis*, the relationship is significantly negative; in *C. unguiculata*, we detected no effect of pollen load on SPR, potentially due to its larger stigma. In the absence of negative interactions among pollen grains or pollen tubes, we would expect to observe a positive relationship (and a slope of 1.0) between the number of pollen tubes entering the style and pollen load size. In both species (Fig. 3C and D), the slope of this relationship, while positive, is significantly less than 1.0. These negative interactions indicate that, in both species, when pollen loads are larger, earlier-germinating and faster-growing pollen should enhance male fitness by increasing the probability of stylar entry.

ESTIMATING POLLEN COMPETITIVE ABILITY FROM THE MEAN PTGR VERSUS THE LLT IN THE STYLE

Comparing species with respect to the LLT is most appropriate for species in which there is only one ovule per ovary or per carpel (if each carpel is accessed by its own style) (e.g., Taylor and Williams 2012). In species with many ovules per fruit (e.g., *Clarkia*), however, the length of a pollen donor's longest tube does not predict with very high accuracy its competitive ability against other

donors ($R^2 = 0.38$ in *C. exilis* and 0.29 in *C. unguiculata;* Fig. 2). Although the two traits are highly correlated, the proportional difference between species' means differs between these traits. *Clarkia unguiculata* exceeded *C. exilis* in LLT by 35.5%, but only by 5.5% with respect to PTGR (Table 2). The larger difference between species in LLT may in part reflect the difference between them in mean style length (*C. unguiculata*: $\bar{x} = 14.3$ mm + 2.71; *C. exilis*: $\bar{x} = 8.84$ mm + 1.76; Fig. S1). Given that, among styles in which the longest tube reached the style base, the LLT could not exceed style length, the shorter styles of *C. exilis* necessarily constrained its LLT. Comparing species in the mean PTGR of all visible pollen tubes in the style provides, in this case, a much more conservative measure of their evolutionary divergence.

EFFECTS OF POLLINATION TYPE AND POLLEN LOAD ON POLLEN PERFORMANCE

Several results ran counter to our predictions. First, we found no significant difference between the performance of self versus outcross pollen within either species. Contrary to our predictions, outcross pollen did not perform better than self pollen in *C. unguiculata*, and self pollen did not outperform outcross pollen in *C. exilis* (Fig. 4B). The similarity between self- and cross-pollination treatments in *C. unguiculata* observed here is consistent with previous work that detected no evidence of cryptic postfertilization self-incompatibility in *C. unguiculata* (Travers and Mazer, 2000), whereas the similarity between pollination types in *C. exilis* is a new observation for this species. Second, we detected no evidence that higher numbers of pollen grains competing for access to the style filtered out slow-growing pollen tubes; the mean PTGR was independent of the pollen load (Table 5, and all alternative models presented in the Supporting Information).

INTRASPECIFIC VARIATION IN POLLEN PERFORMANCE

Greenhouse-raised populations of both taxa exhibit high (and similar) levels of variation among pollen donors in both the SPR and mean PTGR. This variation was observed both among the raw values (Figs. 6C, D and 7C, D) of these traits and when controlling for variation associated with population of origin, pollen type, temperature, and SPR (in the case of PTGR) (Figs. 6A, B and 7A, B). Either directional selection on pollen performance in *C. unguiculata* has not been sufficiently strong (relative to the rates of gene flow or mutation) to reduce variation among pollen donors relative to *C. exilis*, or other factors have contributed to the maintenance of genetic variation in pollen performance in *C. unguiculata* and/or to its reduction in *C. exilis*. At least three factors could contribute to the higher-than-expected variation in pollen performance observed in *C. unguiculata* relative to *C. exilis*. First, *C. exilis* is putatively derived from *C. unguiculata* and may have harbored less genetic variation than its progenitor when they diverged. Second, if outcrossing taxa exhibit a quantitative tradeoff between functional genders (with some genotypes functioning more as males and others as females), then their populations may retain higher variance in male function than selfing taxa, in which individuals contribute equally to their offspring as males and females. Third, if selection on gender function varies seasonally in outcrossing taxa, then their populations may maintain high levels of genetic variation in pollen performance.

PREVIOUS COMPARATIVE STUDIES OF POLLEN PERFORMANCE

Four previous studies compared pollen performance in predominantly selfing vs. outcrossing species, corroborating the primary results presented here. Smith-Huerta (1996) conducted the first experiment designed to test whether pollen donors sampled from a wild population adapted to outcrossing (based on its relatively protandrous and long-styled flowers) had evolved faster-germinating pollen or faster-growing pollen tubes than those sampled from a population adapted to selfing. In a growth chamber experiment, she tested two populations of *C. tembloriensis*; flowers within each population were self- and cross-pollinated, and then observed for differences in the timing of pollen tube entry into the style and in PTGR. Pollen from the outcrossing population germinated more rapidly and synchronously, and exhibited higher PTGR, than that of the selfing population. Smith-Huerta (1996), however, did not estimate mean PTGR based on all of the pollen tubes visible within each style, but rather as the mean numbers of tubes visible 1 cm below the stigma and at the style base at two-hour intervals following pollination. This method may have generated estimates of PTGR that were biased upwards in the outcrossing population because slower-growing genotypes may not have been included in the estimates; the estimate for the selfing population may have been less biased if its pollen was more genetically uniform, as would be expected in autogamous selfers.

Diaz and Macnair (1999) conducted reciprocal crosses between the partially cleistogamous self-pollinating *Mimulus nasutus* and its putative progenitor, the bee-pollinated *M. guttatus*. PTGR was estimated as the distance reached by ~70% of the pollen tubes (the "wavefront"). Consistent with the predictions presented here, PTGR of *M. nasutus* pollen was slower than that of *M. guttatus* when the pollen of both species was used to pollinate the latter, but the two species' pollen grew at similar rates when tested in the styles of *M. nasutus*. The observed difference in PTGR also resulted in low production of hybrids when both species' pollen was mixed and used to pollinate *M. guttatus* (most seeds produced were *M. guttatus*), but ~50% hybrid production when pollen mixtures were used to pollinate *M. nasutus*. This study demonstrated that the two species have diverged with

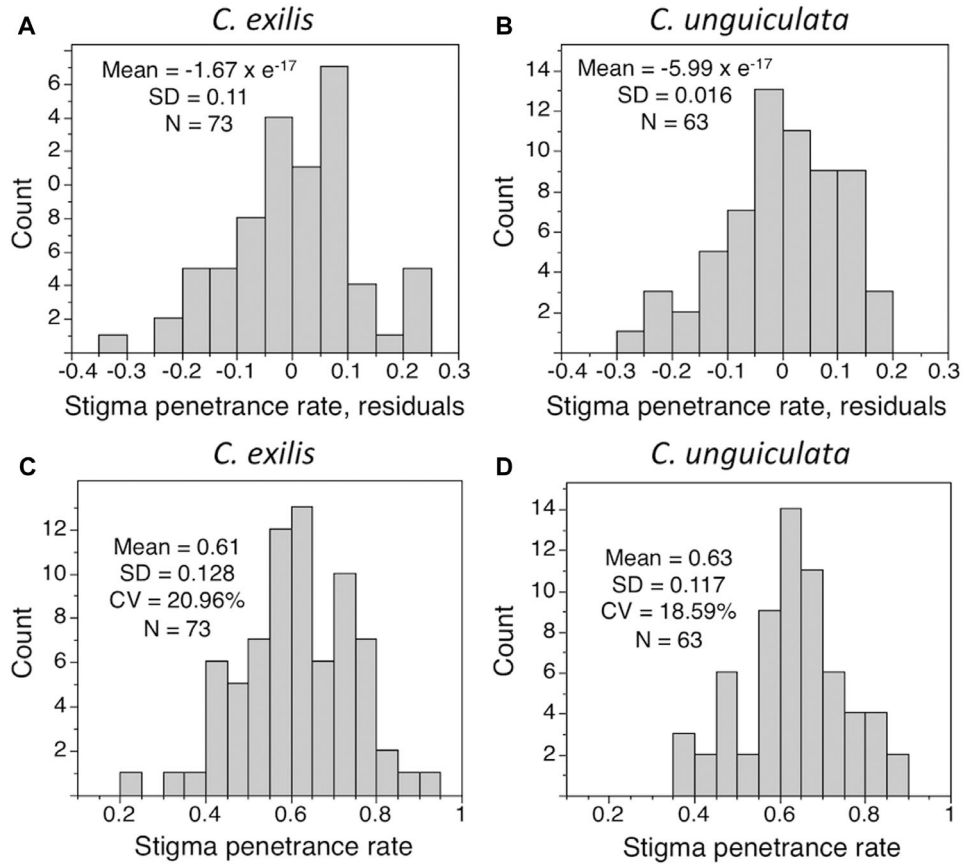

Figure 6. **Frequency distributions of the SPR among pollen donor means for *C. exilis* (A and C) and *C. unguiculata* (B and D). *Clarkia unguiculata* is predominantly outcrossing; *C. exilis* is highly selfing to mixed mating. (A and B) Residuals of the SPR were estimated from the model in which species, population (nested with species), pollen load, and temperature are the independent variables; (C and D) frequency distributions of the raw values of the SPR among pollen donor means.**

respect to the competitive ability of their pollen, consistent with the predictions of sexual selection.

Taylor and Williams (2012) compared two species of the early divergent genus, *Trithuria* (Hydatellaceae; Nymphaeales): the dioecious (obligately outcrossing) *T. austinensis* and a highly selfing congener, *T. submersa*. The uniovulate carpels were hand pollinated and then fixed at various intervals up to three hours after pollination. The styles and ovules were then examined to compare taxa with respect to the proportion of microgametophytes that had entered the style by each time period and mean PTGR. Pollen grains germinated more slowly in the outcrossing *T. austinensis* than in selfing *T. submersa*, a pattern inconsistent with the prediction presented here. The difference between these taxa in PTGR, however, supported our prediction: in *T. austinensis*, the mean maximum PTGR was 2165.9 mm/h, whereas that of *T. submersa* was 321.1 mm/h. Taylor and Williams (2012) based their estimate of PTGR on the length of the longest pollen tube within each style rather than on the distances reached by all of the pollen tubes; similar to Smith-Huerta (1996), this method may have resulted in an overestimate of the mean PTGR in the outcrossers.

Hove and Mazer (2013) conducted self- and cross-pollinations within two field populations each of *C. unguiculata* and *C. exilis*, harvesting and fixing styles 2.5 h after pollination. Similar to the current study, Hove and Mazer (2013) estimated PTGR based on the distances reached by all of the pollen tubes within each style, and they detected no significant differences across taxa in pollen performance following self- vs. cross-pollinations. After adjusting for variation in temperature at the time of pollination, Hove and Mazer (2013) found that *C. unguiculata* pollen germinated more rapidly and exhibited less variation among donors in mean PTGR than *C. exilis*, consistent with the predictions presented here. However, they detected no difference between *C. unguiculata* and *C. exilis* in mean PTGR. A parallel experiment in field populations of the predominantly insect-pollinated *C. xantiana* ssp. *xantiana* and its autogamous sister subspecies, *C. xantiana* ssp. *parviflora*, detected no difference between these subspecies in germination rate (Hove and

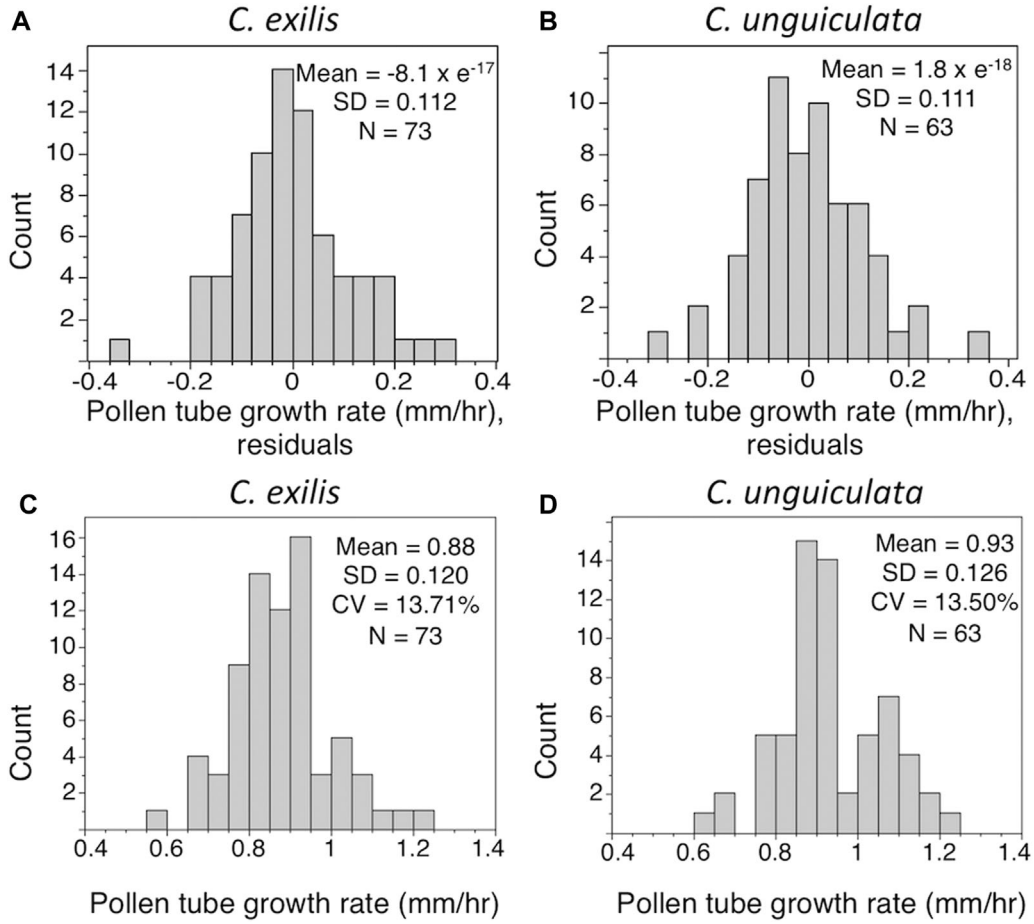

Figure 7. Frequency distributions of mean PTGR among pollen donor means for *C. exilis* (A and C) and *C. unguiculata* (B and D). *Clarkia unguiculata* is predominantly outcrossing; *C. exilis* is highly selfing to mixed mating. (A and B) Residuals of mean PTGR were estimated from the model in which species, population (nested within species), pollen load, stigma penetrance rate, and temperature were the independent variables. (C and D) Frequency distributions of the raw values of mean pollen tube growth rate among pollen donor means.

Mazer 2013); in addition, contrary to the predictions presented here, the mean PTGR of ssp. *parviflora* was faster than that of ssp. *xantiana*. This experiment was conducted in natural populations, and did not control for variation among experimental plants or taxa with respect to water or nutrient availability, limiting the ability to test directly for evolutionary divergence between taxa. In addition, Hove and Mazer (2013) did not control statistically for the potential effects of pollen load size on pollen performance; given the negative effect of large pollen load size on SPR (at least in *C. exilis*; Fig. 3A), this may have obscured differences in pollen performance between taxa.

Although most of these investigations supported the hypothesis that facultatively outcrossing species or populations evolve more competitive pollen than closely related mixed-mating or selfing taxa, the current study addressed several limitations of these studies in two ways: first, by estimating mean PTGR based on the growth of all visible pollen tubes, and, second, by controlling statistically for the effects of temperature, self- vs. outcross pollination, population of origin, and the number of pollen grains competing for access to the style.

UNRESOLVED QUESTIONS

Future studies designed to test predictions derived from sexual selection theory in plants should consider the following concerns. First, although the results presented here and in the studies reviewed above are largely consistent with sexual selection theory, we caution against the assertion that intrasexual selection necessarily accounts for the difference in pollen performance observed between *C. unguiculata* and *C. exilis* or between the taxa reviewed above. If, for example, outcrossing taxa produce flowers with longer styles than their self-pollinating sister taxa, then selection may favor faster pollen germination and growth in the former because these features may ensure that pollen tubes reach the ovary before the flower begins to senesce.

Second, the data presented here cannot be used to infer the direction of evolutionary change in pollen performance traits that

generated the divergence between taxa. Comparative studies of pollen performance among many species with contrasting mating systems and among which the phylogenetic relationships are known are needed to identify unambiguously the direction of evolutionary change. Consequently, we cannot conclude that the difference in pollen performance between the taxa observed here is due to an evolutionary reduction in mean PTGR in *C. exilis* since the time of its divergence from *C. unguiculata*, an increase in PTGR in *C. unguiculata*, or both. The divergence between these species is, however, consistent with the hypothesis that *C. unguiculata* experienced a longer history of selection favoring faster pollen germination and PTGR. If, instead of (or in addition to) PTGR increasing in *C. unguiculata* since its divergence from *C. exilis*, mean PTGR declined in *C. exilis*, then one must ask why selection favored this reduction. Perhaps there is a fitness cost to high PTGR, causing mean PTGR to decline when (as proposed here) direct selection on this trait was relaxed under self-fertilization in *C. exilis*. Artificial selection experiments conducted to detect fitness costs associated with high rates of PTGR, particularly in selfers, could resolve this question.

Third, it has not yet been demonstrated that higher SPR or faster PTGR in *C. unguiculata* necessarily results in higher fertility or seed quality, so we cannot conclude that directional selection in the stigma and style causes the evolution of faster PTGR in this species. However, numerous studies have detected positive associations between pollen germination, the intensity of pollen competition or PTGR, and siring success or seed quality in other taxa, supporting this inference (Mulcahy and Mulcahy 1975; Snow 1986; Stephenson et al. 1986; Davis et al. 1987; Winsor et al. 1987; Bertin 1990; Snow and Spira 1991; Pasonen et al. 1999; Quesada et al. 2001; Armbruster and Rogers 2004; Swanson et al. 2016).

Fourth, we estimated PTGR based on single-donor pollinations; stigmas in wild populations of *C. unguiculata* may typically receive pollen from multiple donors. If so, and if interactions among pollen grains from different donors negatively influence their PTGR (as observed in *Erythronium grandiflorum*, Cruzan 1990b), then our estimates of PTGR for *C. unguiculata* may have been biased upwards.

Fifth, while the evolutionary divergence between the taxa studied here was statistically significant, the difference between them might have been greater if the contrast between their mating systems was starker and more consistent. The variation in outcrossing rates among field populations (Vasek, 1964, 1965; Vasek and Harding, 1976; Hove, 2012; Ivey et al. 2016) suggests that the opportunity for selection on pollen performance traits may at times be quite high in both species.

Finally, the difference in mean PTGR observed between *C. unguiculata* and *C. exilis*—as well as between the other pairs of taxa cited above—could have been due partly or wholly to differences between them in the quality of their pistils rather than in the competitive abilities of their pollen. Due to the lack of cross-compatibility between *C. unguiculata* and *C. exilis*, we could not conduct reciprocal crosses to assess whether the stylar environment of *C. unguiculata* was more favorable to pollen tube growth than that of *C. exilis*. Nevertheless, while the prediction that outcrossing taxa should evolve higher SPR and faster PTGR than their selfing counterparts is derived from the clear effects of mating system on the opportunity for selection on pollen performance, there is no complementary argument for why the stigmas and styles of outcrossers should inevitably promote more rapid pollen germination and growth than those of selfers. Moreover, previous work in *Clarkia* supports the interpretation that the divergence between taxa observed here reflects an intrinsic difference in the performance of their pollen. Kerwin and Smith-Huerta (2000) found that, among pollinations conducted within and between a selfing and an outcrossing population of *C. tembloriensis*, pollen germination rate was determined more by the properties of the pollen rather than by the pistil, while PTGR was influenced by both the genotype of the pollen donor and the pollen recipient.

CONCLUSIONS

The differences between *C. unguiculata* and *C. exilis* in mean PTGR and LLT reported here are consistent with the hypothesis that sexual selection leads to evolutionary divergence in PTGR between closely related outcrossers and chronic selfers (even those with a more mixed mating system). Support for this hypothesis would be strengthened by additional studies conducted in multiple populations of numerous, independently evolved pairs of sister taxa with mating systems that differ even more starkly than the taxa investigated here. The study of sister species that can be reciprocally pollinated would be particularly amenable to such work, as this would allow the independent measurement of paternal versus maternal effects on pollen performance and the detection of pollen-pistil interactions that may influence pollen performance.

AUTHOR CONTRIBUTIONS

SJM designed this study, trained co-authors on empirical methods, conducted all analyses, and wrote and prepared the manuscript; MVS and SJM supervised the greenhouse and lab research; BTH, JPC, LJK, and JS conducted lab and greenhouse work, participated in literature review, and discussed all results. JWL participated in the literature review. All co-authors edited the manuscript.

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DATA ARCHIVING

The datasets will be archived in Dryad (https://doi.org/10.5061/dryad.qs4nh).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Comparison of alternative models for the standard least squares ANCOVAs among pollen donor means conducted to detect significant differences between species in the stigma penetrance rate (SPR), independent of the effects of population (nested within species), pollination type (self- vs. cross-pollination), the species × pollination-type interaction, pollen load, and temperature.

Table S2. Model 1: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), pollination type (selfing vs. outcrossing), species × pollination type, the number of pollen grains per stigma, and temperature for the four hours following pollination on the proportion of gametophytes that reach the style (stigma penetrance rate, or SPR).

Table S3. Model 2: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), pollination type (selfing vs. outcrossing), species × pollination type, and the number of pollen grains per stigma on the proportion of gametophytes that reach the style (stigma penetrance rate, or SPR).

Table S4. Model 3: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), the number of pollen grains per stigma, and temperature for the four hours following pollination on the stigma penetrance rate.

Table S5. Model 4: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), and temperature for the four hours following pollination on the stigma penetrance rate.

Table S6. Model 5: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), and the number of pollen grains adhering to the stigma on the stigma penetrance rate.

Table S7. (a) Summary of alternative models for ANCOVAs of pollen donor means conducted to detect significant differences between species in pollen tube growth rate (PTGR), controlling for other factors.

Table S8. Model 1: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), pollination type (selfing vs. outcrossing), species × pollination type, the number of pollen grains adhering to the stigma, stigma penetrance, and temperature on mean PTGR.

Table S9. Model 2: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), pollination type (selfing vs. outcrossing), species × pollination type, the number of pollen grains adhering to the stigma, and stigma penetrance on mean PTGR.

Table S10. Model 3: Summary of standard least ANCOVA among pollen donor means to detect the effects of species, population (nested within species), pollination type (selfing vs. outcrossing), species × pollination type, the number of pollen grains adhering to the stigma, and stigma penetrance on mean PTGR.

Table S11. Model 4: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), the number of pollen grains adhering to the stigma, and stigma penetrance on mean PTGR.

Table S12. Model 5: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), stigma penetrance, and temperature on mean PTGR.

Table S13. Model 6: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), the number of pollen grains adhering to the stigma, stigma penetrance, and temperature on mean PTGR.

Table S14. Model 7: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), and the number of pollen grains adhering to the stigma, and temperature during pollen germination and growth on mean PTGR.

Table S15. Model 8: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), and the number of pollen grains adhering to the stigma on mean PTGR.

Table S16. Model 9: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), and stigma penetrance on mean PTGR.

Table S17. Model 10: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species, population (nested within species), and temperature during pollen germination and growth on mean PTGR.

Table S18. Model 11: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of species and population (nested within species) on mean PTGR.

Figure S1. Frequency distributions of style length among sampled flowers of *C. exilis* and *C. unguiculata*.

Supporting Information

Table S1. Comparison of alternative models for the standard least squares ANCOVAs among pollen donor means conducted to detect significant differences between species in the stigma penetrance rate (SPR), independent of the effects of Population (nested within species), Pollination Type (self- vs. cross-pollination), the Species x Pollination Type interaction, Pollen Load and Temperature. Shaded cells indicate the variables included in a given model. Models 1 and 2 were applied to the data set that included pollen donor means of both the self-pollinated and cross-pollinated flowers as separate records. The exclusion of temperature led to the detection of significant differences between species in SPR (Models 2 and 5) because there was a small difference between species in the temperature at which pollinations were conducted; controlling for temperature therefore eliminated the difference in the least squares means between species. Models 3-5 are based on pollen donor means estimated from the means of the self- and cross-pollinated treatments.

Source	df	Model 1	Model 2	Model 3	Model 4	Model 5
Species	1	ns	0.0056	ns	ns	**0.0172**
Population (Species)	6	**0.0275**	0.0045	ns	ns	**0.0154**
Pollination type	1	ns	ns			
Species x Pollination Type	1	*0.0743*	0.0560			
Pollen grains/stigma	1	**<0.0001**	**<0.0001**	**0.0002**		**0.0002**
Temperature	1	*0.0646*		**0.0499**	**0.0477**	
Model p-value		**<0.0001**	**<0.0001**	**0.0003**	**0.0332**	**0.0007**
Model df		11	10	9	8	8
Error df		242	243	126	127	127
Corrected total df		253	253	135	135	135
Number of Observations		254	254	136	136	136
Adjusted R^2		0.17	0.16	0.16	0.07	0.14
AICc		-255.94	-254.57	-192.84	-180.3	-191.05

Table S1 (continued)

Least Squares Means. Within each group (species, pollination type, and species x pollination type), distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t (for two means) or Tukey HSD (for > two means). LS means estimated from Model 1.

	n	Least Squares Means for SPR	Standard Error
C. unguiculata	123	0.64^{a}	0.015
C. exilis	131	0.61^{a}	0.014
Self pollination	135	0.62	0.013
Cross pollination	119	0.63	0.012
C. unguiculata (self-pollinated)	60	0.65^{a}	0.020
C. unguiculata (cross-pollinated)	63	0.63^{ab}	0.019
C. exilis (self-pollinated)	59	0.59^{b}	0.020
C. exilis (cross-pollinated)	72	0.64^{ab}	0.018

Table S2. Model 1. Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), Pollination Type (selfing vs. outcrossing), Species x Pollination Type, the number of pollen grains per stigma, and temperature for the four hours following pollination on the **proportion of gametophytes that reach the style (stigma penetrance rate, or SPR)**.

a. Model 1 (Table 3 of the manuscript)

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.030	1.50	0.2220
Population (Species)	6	0.292	2.42	**0.0275**
Pollination type	1	0.016	0.77	0.3808
Species x Pollination Type	1	0.065	3.21	*0.0743*
Pollen grains/stigma	1	0.742	36.86	< 0.0001
Temperature	1	0.069	3.45	*0.0646*
Model	11	1.274	5.75	<0.0001
Error	242	4.872		
Corrected Total	253	6.146		
Adjusted $R^2 = 0.17$				
AICc = -255.94				
Number of observations=254				

b. Least Squares Means. Within each group (species, pollination type, or species x pollination type), distinct superscripts indicate significant differences between means ($\alpha = 0.05$) based on Student's t (for two means) or Tukey's HSD (for > two means).

	n	Least Squares Means for SPR	Standard Error
C. unguiculata	123	0.64	0.015
C. exilis	131	0.61	0.014
Self pollination	135	0.62	0.013
Cross pollination	119	0.63	0.012
C. unguiculata (self-pollinated)	60	0.65^{a}	0.020
C. unguiculata (cross-pollinated)	63	0.63^{ab}	0.019
C. exilis (self-pollinated)	59	0.59^{b}	0.020
C. exilis (cross-pollinated)	72	0.64^{ab}	0.018

Table S3. Model 2. Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), Pollination Type (selfing vs. outcrossing), Species x Pollination Type, and the number of pollen grains per stigma on the **proportion of gametophytes that reach the style (stigma penetrance rate, or SPR)**.

a. Model 2

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.159	7.81	**0.0056**
Population (Species)	6	0.394	3.23	**0.0045**
Pollination type	1	0.014	0.70	0.4041
Species x Pollination Type	1	0.075	3.69	*0.0560*
Pollen grains/stigma	1	0.725	35.65	**< 0.0001**
Model	11	1.205	5.92	**<0.0001**
Error	242	4.941		
Corrected Total	253	6.146		
Adjusted $R^2 = 0.16$				
AICc = -254.56				
Number of observations=254				

b. Least Squares Means. Within each group (species, pollination type, or species x pollination type), distinct superscripts indicate significant differences between means ($\alpha = 0.05$) based on Student's t (for two means) or Tukey's HSD (for > two means). For the difference between species' means: t = 1.97.

	n	Least Squares Means for SPR	Standard Error
C. unguiculata	123	0.65^{a}	0.013
C. exilis	131	0.60^{b}	0.013
Self pollination	135	0.62	0.013
Cross pollination	119	0.63	0.012
C. unguiculata (self-pollinated)	60	0.66^{a}	0.020
C. unguiculata (cross-pollinated)	63	0.64^{a}	0.019
C. exilis (self-pollinated)	59	0.57^{b}	0.020
C. exilis (cross-pollinated)	72	0.62^{ab}	0.018

Table S4. Model 3. Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), the number of pollen grains per stigma, and temperature for the four hours following pollination on the **stigma penetrance rate**.

a. Model 3

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.004	0.32	0.5748
Population (Species)	6	0.130	1.69	0.1298
Pollen grains/stigma	1	0.187	14.60	**0.0002**
Temperature	1	0.050	3.92	**0.0499**
Model	9	0.435	3.77	**0.0003**
Error	126	1.615		
Corrected Total	135	2.050		
Adjusted $R^2 = 0.16$				
AICc = -192.84				
Number of observations=136				

b. Least Squares Means

	n	Least Squares Means for SPR	Standard Error
C. unguiculata	63	0.63	0.018
C. exilis	73	0.61	0.016

Table S5. Model 4. Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), and temperature for the four hours following pollination on the **stigma penetrance rate.**

a. Model 4

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.005	0.33	0.5654
Population (Species)	6	0.110	1.30	0.2637
Temperature	1	0.057	4.00	**0.0477**
Model	8	0.247	2.18	**0.0332**
Error	127	1.802		
Corrected Total	136	2.050		
Adjusted $R^2 = 0.07$				
AICc = -180.3				
Number of observations=136				

b. Least Squares Means.

	n	Least Squares Means for SPR	Standard Error
C. unguiculata	63	0.61	0.018
C. exilis	73	0.63	0.017

Table S6. Model 5. Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), and the number of pollen grains adhering to the stigma on the **stigma penetrance rate.**

a. Model 5

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.076	5.83	**0.0172**
Population (Species)	6	0.216	2.74	**0.0154**
Pollen grains/stigma	1	0.19	14.77	**0.0002**
Model	8	0.384	3.66	**0.0007**
Error	127	1.665		
Corrected Total	135	2.050		
Adjusted $R^2 = 0.14$				
AICc = -191.05				
Number of observations=136				

b. Least Squares Means

	n	Least Squares Means for SPR	Standard Error
C. unguiculata	63	0.65	0.015
C. exilis	73	0.60	0.014

Table S7. a. Summary of alternative models for ANCOVAs of pollen donor means conducted to detect significant differences between species in pollen tube growth rate (PTGR), controlling for other factors. Shaded cells indicate the variables included in a given model. Detailed analyses appear in tables S7-S17. Models 1 and 2 are based on the data set in which donor means for self- and cross-pollinations are included as separate records. Given that pollination type did not affect PTGR, Models 3-10 are based on donor means estimated from the means of the self- and cross-pollinated treatments and excluded the effects of pollination type.

Source	df	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7	Model 8	Model 9	Model 10	Model 11
Species	1	**0.0166**	**0.0023**	**0.0010**	**0.0030**	**0.0229**	**0.0125**	**0.0115**	**0.0019**	**0.0057**	**0.0249**	**0.0045**
Population (Species)	6	**0.0012**	**0.0009**	**0.0003**	**0.0008**	**0.0014**	**0.0010**	**0.0005**	**0.0004**	**0.0013**	**0.0008**	**0.0006**
Pollination type	1	ns	ns	ns								
Species x Pollination Type	1	ns	ns	ns								
Pollen grains/stigma	1	ns	ns	ns	ns		ns	ns	ns			
Stigma penetrance rate	1	ns	ns		ns	ns	ns			ns		
Temperature	1	ns				ns	ns	ns			ns	
Model		**0.0002**	**0.0001**	**0.0001**	**0.0002**	**0.0003**	**0.0003**	**0.0002**	**<0.0001**	**0.0001**	**0.0001**	**<0.0001**
Model df		12	11	10	9	9	10	**9**	**8**	**8**	**8**	**7**
Error df		239	240	241	126	126	125	**126**	**127**	**127**	**127**	**128**
Corrected total df		251	251	251	135	135	135	135	**135**	**135**	**135**	**135**
Number of Observations		252	252	252	136	136	136	136	136	136	136	136
Adjusted R^2		0.10	0.10	0.10	0.17	0.16	0.16	0.17	0.17	0.17	0.16	0.17
AICc		-205.73	-207.89	-208.47	-188.90	-187.67	-186.60	-188.85	-191.15	-189.91	-189.37	-191.66

b. Least Squares Means of mean PTGR. Within groups (species, pollination type, and species x pollination type), distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t test (for two means) or Tukey's HSD (for > two ,eams). Least squares means based on Model 2.

	n	Least Squares Means for PTGR (mm/hr)	Standard Error
C. unguiculata	122	0.94^a	0.016
C. exilis	130	0.88^b	0.016
Self pollination	117	0.90	0.015
Cross pollination	135	0.91	0.014
C. unguiculata (self-pollinated)	60	0.94^a	0.020
C. unguiculata (cross-pollinated)	63	0.93^a	0.021
C. exilis (self-pollinated)	59	0.87^a	0.022

Table S8. Model 1: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), Pollination Type (selfing vs. outcrossing), Species x Pollination Type, the number of pollen grains adhering to the stigma, stigma penetrance, and temperature on **mean PTGR**.

a. Model 1

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.141	5.82	**0.0166**
Population (Species)	6	0.554	3.81	**0.0012**
Pollination type	1	0.001	0.03	0.8578
Species x Pollination Type	1	0.010	0.43	0.5126
Pollen grains/stigma	1	0.011	0.47	0.4942
Stigma penetrance	1	0.036	1.47	0.2261
Temperature	1	0.002	0.08	0.7837
Model	12	0.950	3.26	**0.0002**
Error	239	5.795		
Corrected Total	251	6.744		
Adjusted $R^2 = 0.10$				
AICc = -205.73				
Number of observations=252				

b. Least Squares Means. Within each group (species, pollination type, and species x pollination type), distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t (for two means) or Tukey's HSD (for > two means). For the difference between species' means: t = 1.97.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	123	0.94^a	0.016
C. exilis	131	0.88^b	0.016
Self pollination	119	0.905	0.0146
Cross pollination	135	0.908	0.0135
C. unguiculata (self-pollinated)	60	0.94	0.022
C. unguiculata (cross-pollinated)	63	0.93	0.021
C. exilis (self-pollinated)	59	0.87	0.022
C. exilis (cross-pollinated)	72	0.88	0.020

Table S9. Model 2: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), Pollination Type (selfing vs. outcrossing), Species x Pollination Type, the number of pollen grains adhering to the stigma, and stigma penetrance on **mean PTGR**.

a. Model 2 (Table 4 in the manuscript)

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.230	9.53	**0.0023**
Population (Species)	6	0.572	3.95	**0.0009**
Pollination type	1	0.001	0.03	0.8634
Species x Pollination Type	1	0.011	0.45	0.5029
Pollen grains/stigma	1	0.011	0.44	0.5084
Stigma penetrance	1	0.038	1.57	0.2119
Model	11	0.948	3.57	**< 0.0001**
Error	240	5.797		
Corrected Total	251	6.745		
Adjusted $R^2 = 0.10$				
AICc=-207.89				
Number of observations=252				

b. Least Squares Means. Within each group, distinct superscripts indicate significant differences between means, based on Student's t (for two means) or Tukey's HSD (for > 2 means). For the difference between species' means: t = 1.97.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	123	0.94^{a}	0.015
C. exilis	131	0.87^{b}	0.014
Self pollination	119	0.90	0.015
Cross pollination	135	0.91	0.013
C. unguiculata (self-pollinated)	60	0.94^{a}	0.021
C. unguiculata (cross-pollinated)	63	0.93^{ab}	0.020
C. exilis (self-pollinated)	59	0.87^{b}	0.021
C. exilis (cross-pollinated)	72	0.88^{ab}	0.019

Table S10. Model 3: Summary of standard least ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), Pollination Type (selfing vs. outcrossing), Species x Pollination Type, the number of pollen grains adhering to the stigma, and stigma penetrance on **mean PTGR**.

a. Model 3

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.269	11.10	**0.0010**
Population (Species)	6	0.649	4.47	**0.0003**
Pollination type	1	0.001	0.06	0.8076
Species x Pollination Type	1	0.016	0.67	0.4141
Pollen grains/stigma	1	0.033	0.03	0.2463
Model	10	0.910	3.76	**< 0.0001**
Error	241	5.835		
Corrected Total	251	6.745		
Adjusted $R^2 = 0.10$				
AICc=-208.47				
Number of observations=252				

b. Least Squares Means. Within each group, distinct superscripts indicate significant differences between means, based on Student's t (for two means) or Tukey's HSD (for > 2 means). For the difference between species' means: t = 1.97.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	123	0.94^{a}	0.014
C. exilis	131	0.87^{b}	0.014
Self pollination	119	0.90	0.015
Cross pollination	135	0.91	0.014
C. unguiculata (self-pollinated)	60	0.95^{a}	0.021
C. unguiculata (cross-pollinated)	63	0.94^{ab}	0.020
C. exilis (self-pollinated)	59	0.86^{b}	0.021
C. exilis (cross-pollinated)	72	0.88^{ab}	0.019

Table S11. Model 4: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), the number of pollen grains adhering to the stigma, and stigma penetrance on **mean PTGR**. Standard least squares analysis was conducted using REML and unbounded variance components.

a. Model 3

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.121	9.17	**0.0030**
Population (Species)	6	0.325	4.11	**0.0008**
Pollen grains/stigma	1	0.017	1.27	0.2622
Stigma penetrance	1	0.001	0.11	0.7379
Model	9	0.476	4.01	**0.0002**
Error	126	1.663		
Corrected Total	135	2.138		
Adjusted $R^2 = 0.17$				
AICc=-188.90				
Number of observations=136				

b. Least Squares Means. Distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t. For the difference between species' means: t = 1.98.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	63	0.94^a	0.015
C. exilis	73	0.87^b	0.014

Table S12. Model 5: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), stigma penetrance, and temperature on **mean PTGR**. Standard least squares analysis was conducted using REML and unbounded variance components.

a. Model 4

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.071	5.31	**0.0229**
Population (Species)	6	0.309	3.86	**0.0014**
Stigma penetrance	1	0.008	0.61	0.4352
Temperature	1	0.002	0.11	0.7357
Model	9	0.460	3.84	**0.0003**
Error	126	1.678		
Corrected Total	135	2.138		
Adjusted $R^2 = 0.16$				
AICc=-187.67				
Number of observations=136				

b. Least Squares Means. Distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t. For the difference between species' means: t = 1.98.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	63	0.94^a	0.017
C. exilis	73	0.87^b	0.016

Table S13. Model 6: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), the number of pollen grains adhering to the stigma, stigma penetrance, and temperature on **mean PTGR**. Standard least squares analysis was conducted using REML and unbounded variance components.

a. Model 5

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.085	6.42	**0.0125**
Population (Species)	6	0.323	4.05	**0.0010**
Pollen grains/stigma	1	0.016	1.24	0.2675
Stigma penetrance	1	0.002	0.15	0.7025
Temperature	1	0.001	0.10	0.7574
Model	10	0.477	3.59	**0.0003**
Error	125	1.661		
Corrected Total	135	2.138		
Adjusted $R^2 = 0.17$				
AICc=-255.94				
Number of observations=254				

b. Least Squares Means. Distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t. For the difference between species' means: t = 1.98.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	63	0.94^{a}	0.018
C. exilis	73	0.87^{b}	0.016

Table S14. Model 7: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), and the number of pollen grains adhering to the stigma, and temperature during pollen germination and growth on **mean PTGR**. Standard least squares analysis was conducted using REML and unbounded variance components.

a. Model 6

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.087	6.58	**0.0115**
Population (Species)	6	0.343	4.33	**0.0005**
Pollen grains/stigma	1	0.023	1.72	0.1921
Temperature	1	0.001	0.06	0.8048
Model	9	0.475	4.00	**0.0002**
Error	126	1.663		
Corrected Total	135	2.138		
Adjusted $R^2 = 0.17$				
AICc=-188.85				
Number of observations=136				

b. Least Squares Means. Distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t. For the difference between species' means: t = 1.98.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	63	0.94^a	0.018
C. exilis	73	0.87^b	0.016

Table S15. Model 8: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), and the number of pollen grains adhering to the stigma on **mean PTGR**. Standard least squares analysis was conducted using REML and unbounded variance components.

a. Model 7

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.132	10.11	**0.0019**
Population (Species)	6	0.353	4.49	**0.0004**
Pollen grains/stigma	1	0.022	1.71	0.1929
Model	8	0.474	4.52	**< 0.0001**
Error	127	1.664		
Corrected Total	135	2.138		
Adjusted $R^2 = 0.17$				
AICc=-191.15				
Number of observations=136				

b. Least Squares Means. Distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t. For the difference between species' means: t = 1.98.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	63	0.94^a	0.015
C. exilis	73	0.87^b	0.014

Table S16. Model 9: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), and stigma penetrance on **mean PTGR**. Standard least squares analysis was conducted using REML and unbounded variance components.

a. Model 8

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.104	7.90	**0.0057**
Population (Species)	6	0.310	3.91	**0.0013**
Stigma penetrance	1	0.007	0.54	0.4621
Model	8	0.459	4.34	**< 0.0001**
Error	127	1.679		
Corrected Total	135	2.138		
Adjusted $R^2 = 0.17$				
AICc=-189.91				
Number of observations=136				

b. Least Squares Means. Distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t. For the difference between species' means: t = 1.98.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	63	0.93^a	0.015
C. exilis	73	0.88^b	0.014

Table S17. Model 10: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species, Population (nested within species), and temperature during pollen germination and growth on **mean PTGR**. Standard least squares analysis was conducted using REML and unbounded variance components.

a. Model 9

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.068	5.15	**0.0249**
Population (Species)	6	0.329	4.13	**0.0008**
Temperature	1	0.001	0.04	0.8379
Model	8	0.452	2.28	**< 0.0001**
Error	127	1.686		
Corrected Total	135	2.138		
Adjusted $R^2 = 0.16$				
AICc=-189.37				
Number of observations=136				

b. Least Squares Means. Distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t. For the difference between species' means: t = 1.98.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	63	0.94^a	0.017
C. exilis	73	0.87^b	0.016

Table S18. Model 11: Summary of standard least squares ANCOVA among pollen donor means to detect the effects of Species and Population (nested within species) on **mean PTGR**. Standard least squares analysis was conducted using REML and unbounded variance components.

a. Model 10

Source	DF	Sum of Squares	F Ratio	P-Value
Species	1	0.110	8.37	**0.0045**
Population (Species)	6	0.339	4.29	**0.0006**
Model	7	0.452	4.90	**< 0.0001**
Error	128	1.686		
Corrected Total	135	2.138		
Adjusted $R^2 = 0.17$				
AICc=-191.66				
Number of observations=136				

b. Least Squares Means. Distinct superscripts indicate significant differences between means ($\alpha = 0.05$), based on Student's t. For the difference between species' means: t = 1.98.

	n	Least Squares Means for PTGR	Standard Error
C. unguiculata	63	0.93^a	0.014
C. exilis	73	0.88^b	0.014

Figure S1. Frequency distributions of style length among sampled flowers of *C. exilis* and *C. unguiculata*.