Short title: Wu et al.—Heterostyly promotes compatible pollination

Heterostyly promotes compatible pollination in buckwheats: Comparisons of intraflower, intraplant, and interplant pollen flow in distylous and homostylous *Fagopyrum*

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PREMISE OF THE STUDY: Heterostyly, the reciprocal positioning of stigmas and anthers in different floral morphs, has long been thought to promote inter-morph pollination. However, extensive intra-morph pollination occurs commonly in heterostylous species, leading to recurrent questions about the functional and evolutionary significance of heterostyly.

METHODS: To identify the sources of stigmatic pollen [autogamous (intra-flower), geitonogamous (intra-plant), vs. inter-plant], we emasculated either one flower or entire plants in experimental populations of the two closely related buckwheat species, distylous *Fagopyrum esculentum* and homostylous *F. tataricum.* Differences in pollen size allowed unambiguous identification of pollen on stigmas.

RESULTS: Only 2.4% of *F. tataricum* pollen and 1.5% of *F. esculentum* pollen arrived successfully on compatible stigmas of other plants. In the former (homostylous) species, 71.3% of the pollen load on stigmas was autogamous, 10.8% was geitonogamous, and 17.9% was interplant. In the latter (distylous) species, 37.45% of the pollen on stigmas was autogamous, 13.8% was geitonogamous, 17.0% was intra-morph, and 31.75% was inter-morph. The amount of incompatible pollen arriving on stigmas was greatly decreased by both one-flower and whole-plant emasculations, and thus, the proportion of compatible pollen deposited increased with one-flower emasculation and increased even more with whole-plant emasculation.

CONCLUSIONS: Our quantification of pollen-donor sources in these two species indicated that heterostyly in *Fagopyrum esculentum* provided a nearly 2-fold fitness advantage (in terms of compatible pollination) over expected (random) pollen transfers between morphs. Because of reduced herkogamy, the homostylous *F. tataricum* was highly autogamous.

Heterostyly is a polymorphic system in which plants have flowers of either of two (distyly) or three (tristyly) floral morphs (Webb and Lloyd, 1986; Barrett and Shore, 2008). In nearly all cases, morphs exhibit reciprocal herkogamy where anthers and stigmas are in complementary positions in alternate morphs (Darwin, 1877; Barrett and Cruzan, 1994). For example, in distylous plants, the stigmas and anthers are positioned at different heights within each floral morph (herkogamy, usually refers to the spatial separation of anthers and stigmas within flowers), and the stigma position of one morph roughly (or closely) matches the anther position of the other morph and vice versa (reciprocity). Distyly is usually accompanied by diallelic self-incompatibility, which prevents self- and intra-morph fertilization and generally involves polymorphisms of pollen and stigmas (Ganders, 1979; Barrett, 2002a). Long-styled morphs (hereafter L-morphs) have low anthers and generally produce smaller pollen grains than the short-styled morph (S-morph), which have high anthers (Dulberger, 1992). This pollen dimorphism often permits identification of the source morph for every pollen grain on the stigmas (Darwin, 1877; Ganders, 1976; Costa et al., 2017).

Darwin (1877) hypothesized that heterostyly promotes both transmission of pollen between anthers and stigmas at the same level (male component of fitness) and receipt by stigmas of pollen from anthers at the same level (female component of fitness) (see also Baker, 1964; Lloyd and Webb, 1992; Barrett, 2002a). Reciprocal herkogamy of heterostylous flowers may also reduce physical sexual interference (Webb and Lloyd, 1986; Barrett, 2002b), i.e., stigmatic receipt of incompatible pollen, which also potentially increases the female component of fitness. Reciprocal herkogamy effects these potential advantages in two ways: (1) herkogamy by itself is expected to reduce autogamous (intra-flower) self-pollination, and (2) the reciprocity component is expected to reduce arrival of geitonogamous pollen and pollen from other plants of the same morph (intra-morph) (Ganders, 1979; Lloyd and Webb, 1992; Barrett, 2002a, b; Armbruster et al., 2017). The hypothesis that heterostyly promotes efficient compatible pollination assumes pollen grains coming from high and low anthers are placed in different locations on the pollinators (Darwin, 1877). If these

locations correspond with the contact position of the corresponding compatible stigmas, the floral dimorphism should promote inter-morph pollen transfer (Barrett, 2002a; Matsumura and Washitani, 2002; Cesaro and Thompson, 2004; Keller et al., 2014; Armbruster et al., 2017).

The promotion of efficient compatible pollination (Darwin 1877; Barrett, 2002a, b) has not, however, been especially well supported by studies measuring stigmatic pollen loads in heterostylous species. Most studies have generally shown that the majority of pollen grains deposited on stigmas of the two morphs are incompatible. Without emasculations, S-morphs are often observed with high proportions of pollen from the opposite morph (although generally much low pollen loads) than L-morphs (Liu et al., 2016). For example, compatible pollen generally comprised much less than 30% of the total pollen load on stigmas of various *Primula* species, particularly in the case of the L-morph: 12.7% for *P. vulgaris* (Ornduff, 1979), 3–8% for *P. veris* (Ornduff, 1980), 7–8% for *P. sieboldii* (Nishihiro et al., 2000), and less than 15% for *P. poissonii* and *P. secundiflora* (Liu et al., 2016). However, most studies have not been able to ascertain the sources of incompatible pollen, which turns out to be critically important.

To evaluate the efficacy of heterostyly to promote compatible inter-morph pollination, Ganders (1974, 1979) and Lloyd and Webb (1992) pointed out that it is necessary to discount intra-flower (autogamous) and intra-plant (geitonogamous) self-pollination (pollen transfer between flowers on the same plant) because such transfers are mechanistically expected to exceed random probabilities and prevent detection of any trend toward disassortative pollination facilitated by heterostyly. One way to deal with this problem is to emasculate the study flower (eliminating autogamy) and to emasculate all flowers on the study plant (eliminating geitonogamy; see Lau and Bosque, 2003). This approach has been used in several heterostylous species to illustrate the functional significance of heterostyly (Ganders, 1974; Barrett and Glover, 1985; Nishihiro and Washitani, 1998; Lau and Bosque, 2003). Lloyd and Webb (1992) introduced useful equations for measuring pollen transfer between morphs relative to pollen transfer between-plants of the same morph, which are used

here to assess the relative efficacy of reciprocal herkogamy in heterostylous flowers.

Combined emasculations at the flower and plant levels allow decomposition of self-pollination into its two components: intra-floral pollination and intra-plant pollination. Without such data for a variety of species, it is difficult to assess the mechanics of how well heterostyly promotes compatible pollination. To begin filling this gap, we emasculated single flowers and whole plants of two buckwheat species: the distylous *Fagopyrum esculentum* (Appendix S1, see the Supplemental Data with this article) and its homostylous relative *F. tataricum* (Wu et al., 2017). A comparison of pollen movement in a distylous species (with reciprocal herkogamy) with pollen movement in a homostylous relative allows us to elucidate better the reproductive advantages of the reciprocal herkogamy, while comparisons of inter-morph with intra-morph (among-plant) pollination allow us to assess the effectiveness of reciprocity in promoting compatible pollination. In turn, comparison of a species with herkogamy with one lacking herkogamy provides information about the role of herkogamy in reducing sexual interference in self-incompatible species.

Although the deleterious effects of autogamy and geitonogamy are well known (Waser and Price, 1991; Lloyd, 1992; Harder and Barrett, 1995), it is challenging to evaluate the extent to which bisexual flowers suffer from costs associated with pollen wastage, because it requires partitioning pollination events into intra-flower and intraplant self-pollination, as well cross pollination (de Jong et al., 1992; Schoen and Lloyd, 1992; Snow et al., 1996; Cesaro et al., 2004). Thus, the pollen on a stigma may have come from anthers in the same flower ("intra-flower"), from anthers in other flowers on the same plant ("intra-plant"), or from anthers in flowers on other plants in the population ("inter-plant"). To our knowledge, the full decomposition of pollen on stigmas into donor components (intra-flower, intra-plant, interplant) has been performed very rarely, and its importance remains underappreciated. For these reasons, we assessed pollen fate as an indicator of the male component of fitness in both distylous *Fagopyrum esculentum* and homostylous *F. tataricum*.

MATERIALS AND METHODS

Two annual species of *Fagopyrum* (Polygonaceae) were used in this study: *F. esculentum* and *F. tataricum* are commonly cultivated buckwheat crops and widely distributed in Asia and Europe, especially around the study site (described later). While *F. esculentum* is distylous and has relatively large, open-shaped, white or reddish flowers exhibiting reciprocal herkogamy, *F. tataricum* is homostylous with smaller, open-shaped, greenish flowers with low herkogamy (anthers and stigmas are at similar heights) (see Floral traits in Table 1,). The axillary or terminal inflorescences are racemose, with 5–10 hermaphroditic flowers. Single flowers of these two species last only 1 day. Flowers comprise five petals, eight stamens, one pistil with three stigmas, and eight globose nectaries surrounding the uniovulate ovary. Pollen grains of *Fagopyrum* species are spherical or ellipsoidal. We measured the length of the longitudinal axis as the pollen diameter and found that the L-morph pollen was distinctly smaller than the S-morph pollen in *F. esculentum* (Table 1; see also Björkman, 1995). Pollen diameter also differed significantly between homostylous *F. tataricum* and the two morphs in *F. esculentum* (Table 1). These differences allowed us to identify the morph source of every pollen grain on the stigmas. Pollinators of both species are generalist flower-visiting insects, including hover flies, other flies, and honeybees.

Both study species have open, cup-shaped flowers, making it relatively straightforward to emasculate the flowers in the field without damaging the gynoecium or corolla. The tubular flowers of most heterostylous species make removal of concealed anthers difficult, at least without damaging the corolla, particularly in L-morph flowers.

This study was conducted at a field station of Central China Normal University, Shangri-La Alpine Botanical Garden (27°54′5″N, 99°38′17″E, 3300–3350 m a.s.l.), Yunnan Province, southwest China from July to August 2015. Seeds of the two species were sown in early April in two separate fields, each 20×20 m^2. By July, the study plots contained thousands of flowering individuals with similar population densities in both fields. The ratios of L- to S- morph in the distylous species did not

deviate from 1:1 based on a random sample within three 2×2 m^2 quadrats (23 and 26, 24 and 24, and 25 and 26 L- and S-morph plants, respectively). The plant density of *F. tataricum* was similar to that of *F. esculentum*, as assessed by comparing densities in three random plots (containing 48, 50, and 53 plants) from each field.

Four floral traits were also measured to test for differences among the two floral morphs of *F. esculentum* (one flower from 30 plants each morph) and homostylous *F. tataricum* (one flower from 30 plants), using digital calipers (0–150 mm). Stigma and anther heights were measured as the distance from the tip of the stigma or anther to the base of the ovary. The stigma–anther distance (herkogamy) was calculated by subtracting the height of the low reproductive organ from the height of the high reproductive organ in each flower.

Emasculation treatments

To quantify intra-flower, intra-plant, and interplant pollination in both buckwheat species, we used three treatments in three separate experimental plots for each species: intact flowers (unmanipulated), one-flower emasculation, and whole-plant emasculation. All plants were exposed to natural pollination. Experimental plots contained only one flowering buckwheat species and were spatially isolated from other flowering species by at least 30 m (to exclude unwanted pollen transfer). (In this alpine meadow, heterospecific pollen was generally found on stigmas of coflowering plants only within a 10-m radius from the source [Fang and Huang, 2016]). Using thin forceps, we gently removed undehisced anthers of one flower in early morning after the flower had just opened. No pollen was observed on 10 stigmas examined microscopically immediately after emasculation, indicating that the emasculation procedure did not lead to pollen deposition on stigmas. In the afternoon (6 h after emasculation) when flowers began to close, stigmas from the emasculated and control flowers were collected. Each stigma was separately stored in FAA solution (formalin–acetic acid–70% alcohol, 1:1:18) in a 1-mL tube. In the laboratory, each stigma was placed on a slide and then squashed under a coverslip. We identified pollen by source morph and counted pollen grains on each stigma under a light microscope (at 400× magnification, Eclipse E100, Nikon, Tokyo, Japan) using a

micrometer to determine pollen source.

In distylous *F. esculentum*, flowers of 44 L- and 44 S-morph plants were collected in the plot with the one-flower emasculation treatment, allowing us to estimate the joint sum of intra-plant (geitonogamy) and interplant pollination, without the confounding effect of intra-flower self-pollination. In a second plot, all just-opened flowers (usually five flowers) on each treatment plant were emasculated. From these whole-plant emasculations, 78 flowers from 40 L-morph plants and 87 flowers from 45 S-morph plants were sampled to measure pollen deposition on the stigmas, allowing us to estimate interplant pollination without any intra-flower or geitonogamous pollination. The two emasculation treatments were spread equally over two consecutive 3-d periods because of the effort required for these manipulations. As controls, stigmas of one intact flower from each of 20 plants of each morph in the unmanipulated plot were collected on the same day as each emasculation treatment (i.e., in total 20 plants × 6 days = 120 plants per morph).

In homostylous *F. tataricum*, (1) 42 plants with one flower emasculated were sampled; (2) two flowers per plant were randomly collected from 34 plants (68 flowers in total) with whole-plant emasculation (all flowers emasculated on plants with 4–7 open flowers); (3) 30 open-pollinated flowers (one from each of 30 plants) were sampled as controls to estimate the amount of natural pollen deposition.

Assessment of pollen sources

The mean stigmatic pollen load of intact flowers was considered the total pollen load. In *F. esculentum*, intra-flower pollination for each morph was calculated as (same-morph pollen load on stigmas of intact flowers) − (same-morph pollen load on stigmas of one-flower-emasculation flowers). Intra-plant pollination was calculated as (same-morph pollen load on stigmas of one-flower-emasculation flowers) − (same-morph pollen load on stigmas of whole-plant-emasculation flowers). Intra-morph pollination (among plants) was estimated as the same-morph pollen load on stigmas of whole-plant-emasculation flowers. Inter-morph pollination was estimated as the opposite-morph pollen load on stigmas of intact (control) flowers. Finally, interplant pollination was calculated as the sum of intra-morph and

inter-morph pollinations.

Because there is only one morph in *F. tataricum*, intra-flower pollination was calculated as (intact-flower pollen load) – (one-flower-emasculation pollen load). Intra-plant pollination was calculated as (one-flower-emasculation pollen load) – (whole-plant-emasculation pollen load). Finally, interplant pollination was estimated as the whole-plant-emasculation pollen load.

Estimation of pollen fates

To measure pollen production, we collected a ready-to-open floral bud from 20 L- and 20 S-morphs of *F. esculentum* and 20 individuals of *F. tataricum*. The eight anthers were opened, and pollen grains per flower were counted using a light microscope. To estimate the fates of pollen produced by distylous *F. esculentum* and homostylous flowers of *F. tataricum*, we counted the stigmatic pollen load of intra-flower, intra-plant, interplant (intra-morph and inter-morph for *F. esculentum*) according to the assessment of pollen sources in the previous section and calculated the percentage of different pollen fates. Pollen loss (pollen not deposited on stigmas) was calculated by subtracting the number of pollen grains successfully reaching stigmas from pollen production.

Statistical analyses

Generalized linear models (GLM) were used to determine the effects of treatment and floral morph on stigmatic pollen loads in *F. esculentum*. To test whether the total, compatible and incompatible pollen loads differed significantly among treatments and between morphs, we used a model with a Poisson distribution and log-link function. To test the effects on percentage compatible pollen, we used GLM with a binomial distribution and logit-link function. We used treatment, morph and their interactions as fixed effects influencing pollen load or percentage compatible pollen. The sequential Bonferroni procedure was used to correct for multiple comparisons. We used independent-sample *t*-tests to test the differences in stigmatic pollen loads among different treatments in *F. tataricum*.

We used the χ^2 test of independence to test for differences in percentage of pollen from different sources across the two species or the two morphs within

distylous *F. esculentum*. In distylous *F. esculentum*, we used independent-sample *t*-tests to compare the differences in pollen production between two morphs. SPSS version 19.0.0 (IBM, Armonk, New York, USA) was used for all statistical analyses .

Pollen transfer proficiencies

We used Lloyd and Webb's (1992) method for comparing compatible and incompatible pollen transfers from the viewpoints of both pollen donation and pollen receipt. We thus calculated the probabilities of single pollen grains being transported to compatible or incompatible stigmas and for stigmas receiving compatible pollen. By this method the probability (T_{ij}) of transfer of a single pollen grain of type *i* to any of the stigmas of type *j* is the total load of pollen of type *i* on all stigmas of type *j* divided by the total pollen production of type *i*, or

$$T_{ij} = (S_{ij} \times F_j)/(P_i \times F_i), \qquad \text{Eq. 1}$$

where S_{ij} is the number of pollen grains of morph *i* per stigma of morph *j*, F_j is the number of flowers of morph *j*, P_i is mean number of pollen grains produced per flower of morph *I*, and F_i is the number of flowers of morph *i*.

Because our experimental population had equal morph frequencies and the same number of L- and S-morph flowers opening daily, F_i / F_j reduces to unity. To gain a summary estimate of the total fitness consequence of the floral dimorphism of *F. esculentum*, we used Lloyd and Webb's (1992) method for calculating the average extent of the proficiency of compatible pollen transfers (*Pc* in *F. esculentum*, employing the geometric mean of the legitimate proficiencies divided by the geometric mean of the incompatible proficiencies:

$$Pc = [(d_{SL} \times d_{LS})/(d_{SS} \times d_{LL})]^{1/2}, \qquad \text{Eq. 2}$$

where d_{SL} is the probability of an S-morph grain being deposited on an L-morph stigma, d_{LS} the probability of an L-morph grain being deposited on an S-morph stigma, d_{SS} the probability of S-morph grain being deposited on a S-morph stigma, and d_{LL} the probability of L-morph grain being deposited on a L-morph stigma.

RESULTS

Floral measurements indicated that *Fagopyrum esculentum* exhibited typical distyly,

with reciprocal herkogamy and pollen dimorphism; *F. tataricum* was monomorphic with nonherkogamous flowers (small anther–stigma distances; Table 1). The corolla diameter and anther-stigma distance were smaller in L-morph flowers than in S-morph flowers in *F. esculentum* (Table 1).

Pollen production and fates

In the distylous *F. esculentum*, the number of pollen grains produced by L-morph flowers (1072 ± 62, mean ± SE) was significantly greater (t = 4.42, df = 38, $P < 0.001$) than the number produced by S-morph flowers (758 ± 35), while pollen production by homostylous *F. tataricum* flowers was very much lower (273 ± 13).

In *F. tataricum*, 13.5% of the pollen reached conspecific stigmas successfully, higher than in *F. esculentum*, where only 4.9% of L-morph ($\chi^2 = 21.58$, df = 1, $P < 0.001$) and 4.7% of S-morph pollen ($\chi^2 = 20.67$, df = 1, $P < 0.001$) reached conspecific stigmas (Fig. 1), indicating that pollen loss in *F. tataricum* was lower than in *F. esculentum*. However, the proportion of pollen moving intraflorally was higher in *F. tataricum* (9.6%) than in *F. esculentum* (1.7% for L-morph, $\chi^2 = 37.96$, df = 1, $P < 0.001$ and 1.9% for the S-morph, $\chi^2 = 28.39$, df = 1, $P < 0.001$). The proportions of intra-plant vs. interplant (inter-morph + intra-morph) pollen movement did not differ significantly between species (all homostylous transfer versus those in each distylous morph: χ^2 tests of independence, $P > 0.1$). The percentages of intraflower ($\chi^2 = 0.07$, df = 1, $P = 0.79$), intra-plant ($\chi^2 = 0.60$, df = 1, $P = 0.44$), intramorph ($\chi^2 = 1.34$, df = 1, $P = 0.25$), and inter-morph ($\chi^2 = 0.50$, df = 1, $P = 0.48$) pollen reaching stigmas did not differ significantly between L- and S-morphs of *F. esculentum*.

Stigmatic pollen loads

In *Fagopyrum esculentum*, stigmatic pollen loads were significantly affected by both emasculation treatments (Table 2). Both one-flower and whole-plant emasculations significantly reduced the total-pollen and incompatible-pollen loads, but not the compatible-pollen loads (Fig. 2A, B). Not surprisingly, the decreases in incompatible-pollen deposition were generally more pronounced with whole-plant emasculations than with one-flower emasculations. Also as expected, the percentage of compatible (inter-morph) pollen on stigmas was significantly higher in whole-plant

emasculations than in one-flower emasculations; in both cases, it was much higher than in the control (intact) flowers (Table 2; Fig. 2D). There were significant morph × treatment interaction effects on the distribution of pollen loads and the percentages of compatible pollen (Table 2), suggesting that the two morphs had differential responses to emasculation treatments.

Overall, S-morphs received more compatible pollen, but less incompatible pollen, and consequently a higher percentage of compatible pollen than L-morphs (Table 2; Fig. 2). The total-pollen and incompatible-pollen loads on stigmas were significantly reduced by one-flower emasculations, and reduced even more by whole-plant emasculations, although there were no significant differences in total-pollen load between one-flower and whole-plant emasculations in the S-morph (Table 2; Fig. 2A, B). Emasculations did not significantly influence the number of compatible pollen grains on L-morph stigmas. However, in the S-morph, the number of compatible pollen grains on stigmas was greater in flowers subjected to whole-plant emasculations than in intact flowers (Fig. 2C). The percentage of compatible pollen on stigmas increased slightly, but significantly, from intact flowers, to one-flower, and to whole-plant emasculations in both morphs (Fig. 2D).

In homostylous *F. tataricum*, stigmatic pollen loads were significantly reduced by both one-flower (mean ± SE = 10.6 ± 1.4, $t = 10.361$, df = 70, $P < 0.001$) and whole-plant emasculations (6.6 ± 0.9, $t = 14.856$, df = 95, $P < 0.001$) relative to intact flowers (36.9 ± 2.1). The stigmatic pollen loads with one-flower emasculations were higher than with whole-plant emasculations ($t = 2.362$, df = 107, $P = 0.020$).

<H2>*Comparison of arrival on stigmas of pollen from different sources*

One-flower and whole-plant emasculations permitted us to disentangle sources of pollen in both species (Fig. 3). Intra-floral pollination accounted for 71.3% of the average pollen load in open-pollinated homostylous *F. tataricum*, significantly more than in both L- (35.3%; $\chi^2 = 126.024$, df = 1, $P < 0.001$) and S-morphs of distylous *F. esculentum* (39.6%; $\chi^2 = 76.219$, df = 1, $P < 0.001$). The percentage of intraplant pollination (geitonogamous pollination) in homostylous *F. tataricum* was significantly lower than in the L- (17.9%; $\chi^2 = 20.677$, df = 1, $P < 0.001$) but not the

S-morph (9.7%; $\chi^2 = 0.730$, df = 1, $P = 0.393$) of *F. esculentum*. The percentages of interplant pollination in *F. tataricum* were lower than in both the L- ($\chi^2 = 130.930$, df = 1, $P < 0.001$) and S-morphs ($\chi^2 = 143.104$, df = 1, $P < 0.001$) of *F. esculentum* (inter-morph + intra-morph; Fig. 3).

In both morphs of *F. esculentum*, intraflower pollen accounted for most of the incompatible pollen load, while self-pollen (intra-flower + intra-plant) accounted for around half of the total pollen load (Fig. 3). There was no detectable difference in percentage intra-flower pollen between the L- and S-morphs ($\chi^2 = 3.160$, df = 1, $P = 0.075$) (Fig. 3). The percentage of compatible pollen on L-morph stigmas was significantly lower than on S-morph stigmas ($\chi^2 = 38.029$, df = 1, $P < 0.001$), while the percentage of intra-plant ($\chi^2 = 35.065$, df = 1, $P < 0.001$) and intra-morph ($\chi^2 = 37.823$, df = 1, $P < 0.001$) pollen on L-morph stigmas was significantly higher than on S-morph stigmas (Fig. 3).

Proficiencies of compatible pollination in F. esculentum

Our calculation showed that the proficiencies of pollen dispersal to stigmas of the opposite morph were higher than to the same morph (Table 3). These data suggest that distyly promotes the dispersal of S-morph pollen to L-morph stigmas over S-morph stigmas and vice versa. An S-morph pollen grain is 2.95 (0.0172 / 0.0058) times as likely to be carried to an L-morph stigma as to an S-morph stigma. Similarly, an L-morph pollen grain is 1.23 (0.0130 / 0.0105) times as likely to be carried to an S-morph stigma as to an L-morph stigma. In turn, an L-morph stigma is 1.63 times as likely to receive S-morph pollen as L-morph pollen. An S-morph stigma is 2.23 times as likely to receive L-morph pollen as S-morph pollen.

Application of Eq. 2 (Lloyd and Webb, 1992) to the values in Table 3 reveals that the overall fitness advantage (in terms of compatible pollen-arrival probabilities) of heterostyly in *F. esculentum* of 1.92; i.e., heterostyly creates a nearly 2-fold advantage for compatible pollination, after the confounding effects of within-flower self-pollination and geitonogamy have been excluded by the experimental emasculation.

DISCUSSION

Our quantification of pollen sources in the two buckwheat species showed that autogamous (intra-flower) pollen accounted for the largest portion of the stigmatic pollen loads in both study species, but that intra-flower pollen was lower in the heterostylous than in the homostylous species, as expected from the greater degree of herkogamy in the former (Table 1). In the heterostylous *F. esculentum*, significantly less compatible pollen arrived on stigmas than total incompatible pollen, but when intra-flower self-pollination is excluded, compatible pollen exceeded incompatible pollen on stigmas. Incompatible pollination greatly decreased and the percentage of compatible pollination increased following one-flower and whole-plant emasculations (Fig. 2). These results, together with our calculation of pollination proficiency based on Lloyd and Webb's (1992) formulae, support the functional hypothesis that heterostyly promotes compatible pollination.

Adaptive significance of heterostyly

After Darwin's (1877) research, heterostyly has generally been thought to be a floral mechanism promoting disassortative (inter-morph) pollination and reducing intra-morph pollination (Ganders, 1979; Webb and Lloyd, 1986; Barrett, 2002a). We observed that the proportion of incompatible pollen on stigmas of both floral morphs was high (>60%) in the distylous species, despite being lower than the proportion of self-pollen on stigmas of the homostylous species (Fig. 3). However, as discussed below, this relationship reverses when analyzing emasculated plants. Conducting one-flower and whole-plant emasculations allowed us to test accurately the functional hypothesis of heterostyly, because, unlike in most studies, we were able estimate the amount of compatible and incompatible interplant pollen flow, without the confounding effects of autogamous and geitonogamous pollination.

Darwin's hypothesis for heterostyly in *Fagopyrum esculentum* was previously assessed in a cultivated plot at an agricultural experimental station in New York State, where honeybees were the main pollinators (Björkman, 1995). Björkman (1995) found 23% compatible pollen on L-morph stigmas and 69% on S-morph stigmas; the low proportion of compatible pollen on L-morph stigmas led Björkman to conclude

that heterostyly was ineffective in promoting inter-morph pollination. A similar pattern was observed in our own investigations, showing that proportions of compatible pollen were higher on S-morph stigmas than on L-morph stigmas. The proportions of compatible pollen that we observed on S-morph stigmas ($13.9/36.1 = 38.5\%$) were even lower than that observed by Björkman ($17/24 = 70.8\%$). In contrast, the observed ratios in the L-morph were very similar ($13.0/51.9 = 25.0\%$ here vs. $14/60 = 23.3\%$ by Björkman [1995]).

Intra-flower self-pollination in a hermaphroditic flower can be mediated by pollinators, even in the case of heterostylous species with reciprocal herkogamy (Ganders, 1974). Ganders' results can be considered in light of Darwin's functional hypothesis about heterostyly. Ganders counted pollen on stigmas from 20 emasculated flowers and 10 intact flowers of each morph in distylous *Jepsonia heterandra* (Saxifragaceae) and calculated the percentages of compatible pollen loads. In emasculated flowers, these figures were 13.2% and 43.5% in the L and S-morphs, respectively. His one-flower emasculation reduced both incompatible and compatible pollen loads and resulted in estimates of 49.4% and 9.7% intra-plant pollinations (self and geitonogamous) in L- and S-morphs, respectively (Ganders, 1974). In a separate study, Ganders (1976) conducted one-flower and whole-plant emasculations in the S-morph of *Amsinckia douglasiana* (Boraginaceae; removal of concealed anthers in L-morph was considered impractical). Because the proportions of compatible and incompatible pollen were not significantly different between the one-flower and whole-plant emasculations, Ganders concluded that geitonogamy did not significantly impair disassortative pollination in this species. However, unlike in our system, intra-flower self-pollination was relatively low in this species, contributing only about 10% of the total pollen load.

In a study of another distylous member of the Polygonaceae, one-flower and one-inflorescence emasculations of *Persicaria japonica* did not reduce incompatible pollen loads in either morph (Nishihiro and Washitani, 1998). Using a fluorescent dye in *Linum tenuifolium* (Linaceae), Nicholls (1985) labeled one flower per population and compared intra-flower pollination in one dimorphic population (two floral

morphs with reciprocal herkogamy) and one monomorphic population (single floral morph but with separation of stigmas and anthers). He showed that 42% and 49% of the pollen on stigmas was from intra-flower pollinations in these two populations, respectively. Baena-Díaz et al. (2012) measured legitimate and illegitimate pollen flow with fluorescent dyes and found that compatible pollen-transfer efficiency was increased in the transition from tristyly to distyly in *Oxalis alpina*, suggesting that patterns of pollen transfer and capture could be affected by changes in the level of herkogamy during the tristyly–distyly transition.

To date, relatively few studies indicate that reciprocal herkogamy (heterostyly) reduces interference by incompatible pollen. Heterostyly might reduce self- and intramorph pollination in *Primula elatior* and *P. vulgaris* (Keller et al., 2014). Armbruster et al. (2006) suggested very low rates of intra-morph pollen flow in *Linum suffruticosum* because of the difference in pollen placement on pollinators (ventral vs. dorsal surfaces), although they only quantified anther and stigma contact by pollinators, not stigmatic pollen loads, and the system of herkogamous reciprocity they described is not characteristic of most heterostylous plants.

Darwin's hypothesis proposed that pollination accuracy could be promoted by differential pollen placement on the pollinator body. One might expect that less intra-morph pollen and more inter-morph pollen would be present on the emasculated flowers. We did find that incompatible pollen on stigmas significantly decreased and compatible pollen significantly increased, at least in the S-morph (Fig. 2B, C). The differences in stigmatic pollen loads associated with emasculation were unlikely to have been the result of decreased pollinator visitation, because compatible pollen did not decrease in emasculated flowers compared to intact flowers in *F. esculentum* (this result also indicates that our emasculation approach is appropriate). Although we did not quantify pollinator visits in the experimental plots, insect visitors seemed primarily interested in nectar and emasculation treatments did not affect rates of pollinator visitation (L.-Y. Wu and S.-Q. Huang, unpublished data), which was also observed in a recent study of tristylous *Lythrum salicaria* (Costa et al., 2017).

<H2>*Pollen sources on stigmas*

Relatively few studies have quantified the sources of the pollen on stigmas or compared the quantities across related heterostylous and homostylous species. Piper and Charlesworth (1986) compared one-flower emasculated and intact flowers in *Primula vulgaris* in a population that had distylous and homostylous plants; they found that the percentage of intra-flower pollen was 83.2% in L-morph flowers, 52.2% in S-morph flowers, and 90.4% in homostylous flowers. While they did not conduct whole-plant emasculations, their results showed that self-pollen (intra-flower) accounted for a substantial proportion of the stigmatic pollen load and that S-morph flowers experienced much less self-pollination than homostylous flowers. Our measurements in distylous *F. esculentum* documented that about 37% (L-morph 35.3% and S-morph 39.6%) of the stigmatic pollen load was intra-flower pollen, while in homostylous *F. tataricum* (with minimal herkogamy), intra-flower self-pollen accounted for 71.3% of stigmatic pollen load. Heterostyly thus reduces the stigmatic load of autogamous self-pollen because it involves herkogamy. Compared to the related distylous buckwheat, the total pollen loss (86.5%) was lower and interplant pollination (2.4%) higher in the homostylous buckwheat (Fig. 1), suggesting that homostyly could be beneficial from lower pollen loss and be favored by a scarcity of pollinators.

Proficiencies of compatible pollination of heterostyly

The average fitness advantage of heterostyly in *F. esculentum*, as reflected in the geometric mean of the probabilities of compatible pollination in relation to incompatible pollination (Eq. 2), was 1.92, a little higher than the mean fitness advantage of distyly reported by Lloyd and Webb (1992) for *Jepsonia heterandra* (1.78), based on Ganders' (1974) data. Our result was very similar to the mean fitness advantage of tristyly reported by Lloyd and Webb (1992) for *Pontederia cordata* (1.94), based on Barrett and Glover's (1985) data. Similar values were also reported by Ree (1997), with a mean compatible-proficiency advantage of 2.17 in *Palicourea padifolia* (Rubiaceae). However, less-supportive results were reported by Nishihiro and Washitani (1998), with compatible transfer (dis)advantages of 0.54–1.44 in

Persicaria japonica (Polygonaceae) and by Pailler et al. (2002), with compatible transfer (dis)advantages of 0.77–2.69 in *Gaertnera vaginata* Poir. (Rubiaceae). More recently, Costa et al. (2017) reported that compatible-pollen transfer probabilities were greater than incompatible-pollen transfer probabilities for only two of the three morphs of tristylous *Lythrum salicaria* (Lythraceae).

As pointed out by Ganders (1979), Lloyd and Webb (1992), and Costa et al. (2017), the fitness advantage of promotion of inter-morph pollination generated by heterostyly, as reported here for *F. esculentum*, is detectable only when it is possible to distinguish between same-morph pollen on stigmas coming from other plants of the same morph vs. same-morph pollen coming from the same flower or from other flowers on the same plant (self-pollination; i.e., intrafloral plus geitonogamous pollination). Our detection of the fitness advantage of heterostyly was possible because we conducted whole-plant emasculations. Future research based on whole-plant and single-flower emasculations in additional species should provide a wealth of detailed insights into the dynamics of compatible and incompatible pollen transfers, as well as the selective conditions that generate and maintain heterostyly.

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Table 1. Five floral traits (mean ± SE, $n = 20$ for pollen diameter, $n = 30$ for the other four traits) measured in distylous *Fagopyrum esculentum* and homostylous *F. tataricum*. One-way ANOVA was used to test the differences among floral morphs: L-morph, S-morph and homostylous.

| Floral trait | *Fagopyrum esculentum* | | *F. tataricum* | df | *F* |
	L-morph	S-morph	Homostyly		
Corolla diameter (mm)	7.48 ± 0.14^b	7.82 ± 0.08^a	3.11 ± 0.05^c	2, 87	778.993
Stigma height (mm)	3.80 ± 0.06^a	2.29 ± 0.04^b	1.58 ± 0.03^c	2, 87	791.168
Anther height (mm)	2.51 ± 0.05^b	4.10 ± 0.05^a	1.57 ± 0.03^c	2, 87	580.460
Stigma–anther distance (mm)	1.29 ± 0.04^b	1.80 ± 0.04^a	0.08 ± 0.01^c	2, 87	714.141
Pollen diameter (µm)	36.48 ± 0.48^c	49.33 ± 0.76^a	40.08 ± 0.68^b	2, 57	103.175

Notes: Significant differences among L-morph, S-morph, and homostyly are shown by different superscript letters (all $P < 0.001$).

Table 2. Results of generalized linear model (GLM) to test whether stigmatic pollen loads differ significantly between the two morphs, and with emasculation treatments in experimental populations of distylous *F. esculentum*.

Dependent variable	Source	df	Wald χ^2	*P*
Total pollen load	Morph	1	3.498	0.061
	Treatment	2	85.346	**<0.001**
	Morph × treatment	2	18.699	**<0.001**
Incompatible pollen load	Morph	1	115.970	**<0.001**
	Treatment	2	286.279	**<0.001**
	Morph × treatment	2	7.466	**0.024**
Compatible pollen load	Morph	1	33.082	**<0.001**
	Treatment	2	3.234	0.199
	Morph × treatment	2	11.614	**0.003**
% Compatible pollen	Morph	1	161.814	**<0.001**
	Treatment	2	178.191	**<0.001**
	Morph × treatment	2	22.990	**<0.001**

Table 3. Pollen loads and probabilities of single pollen grains being transferred to stigmas of opposite- or same-morph flowers, calculated after removing confounding effects of self-pollination and geitonogamy in *Fagopyrum esculentum*. Rationale and calculations based on method of Lloyd and Webb (1992), assuming equal numbers of morphs in the population. T_{LS} is the transfer probability of an L-morph grain to an S-morph stigma, T_{LL} is the transfer probability of an L-morph grain to an L-morph stigma, T_{SL} is the transfer probability of an S-morph grain to an L-morph stigma, and T_{SS} is the transfer probability of an S-morph grain to an S-morph stigma.

Pollen source	Measurement	Source-flower total	Target stigma	
			L-morph	S-morph
S-morph	No. pollen grains	758	13	4.4
	Transfer probability	--	$T_{SL} = 0.0172$	$T_{SS} = 0.0058$
L-morph	No. pollen grains	1072	11.3	13.9
	Transfer probability	--	$T_{LL} = 0.0105$	$T_{LS} = 0.0130$

Figure legends

Fig. 1. Estimated fates of pollen produced by long- and short-styled flowers of *Fagopyrum esculentum* and homostylous flowers of *F. tataricum*. Percentage of pollen (number in brackets indicates mean pollen number) reached on different stigmas or pollen un-deposited on stigmas.

Fig. 2. Number of (A) total, (B) incompatible, and (C) compatible pollen grains, and (D) percentage of compatible pollen load on stigmas (mean ± SE) under three emasculation treatments in two morphs of distylous *Fagopyrum esculentum*. Bars with different letters indicate significant differences within morph among treatments. See Table 2 for statistical details.

Fig. 3. Proportions of pollen from different sources on stigmas of long- and short-styled morphs of heterostylous *Fagopyrum esculentum* and on stigmas of homostylous *F. tataricum*. Numbers in parentheses indicate mean number of pollen grains from each pollen source on stigmas.

Appendix S1. The distylous flowers on the two morphs of *Fagopyrum esculentum* (Polygonaceae), a buckwheat crop, showing the reciprocal positioning of stigmas and anthers between long- and short-styled flowers.