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Research paper

Elevational and temporal patterns of pollination success in distylous and homostylous buckwheats (*Fagopyrum*) in the Hengduan Mountains

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ABSTRACT

Reproductive strategies of sexually dimorphic plants vary in response to the environment. Here, we ask whether the sexual systems of *Fagopyrum* species (i.e., selfing homostylous and out-crossing distylous) represent distinct adaptive strategies to increase reproductive success in changing alpine environments. To answer this question, we determined how spatial and temporal factors (e.g., elevation and peak flowering time) affect reproductive success (i.e., stigmatic pollen load) in nine wild *Fagopyrum* species (seven distylous and two homostylous) among 28 populations along an elevation gradient of 1299 −3315 m in the Hengduan Mountains, southwestern China. We also observed pollinators and conducted hundreds of hand pollinations to investigate inter/intra-morph compatibility, self-compatibility and pollen limitation in four *Fagopyrum* species (two distylous and two homostylous). We found that *Fagopyrum* species at higher elevation generally had bigger flowers and more stigmatic pollen loads; late-flowering individuals had smaller flowers and lower pollen deposition. Stigmatic pollen deposition was more variable in distylous species than in homostylous species. Although seed set was not pollen-limited in all species, we found that fruit set was much lower in distylous species, which rely on frequent pollinator visits, than in homostylous species capable of autonomous self-pollination. Our findings that pollination success increases at high elevations and decreases during the flowering season suggest that distylous and homostylous species have spatially and temporally distinct reproductive strategies related to environment-dependent pollinator activity.

1. Introduction

Plant-pollinator interaction is one of the most important mutualisms on Earth. Animal pollination is a critical ecosystem service that ~90% of angiosperms rely on animals for pollen transfer (Ollerton et al., 2011; Ollerton, 2017; Tong et al., 2023). However, pollen limitation in fruit or seed set is widespread in flowering plants (Ashman et al., 2004). Loss of pollinators has become a century challenge to maintain the plant-pollinator mutualisms, as human activity since Anthropocene has brought in unprecedented changes to the planet, reshaping the pattern of many ecological interactions (González-Varo et al., 2013; Settele et al., 2016). Documenting the spatial and temporal patterns of plant reproductive success across populations, may provide a better understanding of the dynamic plant-pollinator interactions at a finer scale (Kunin, 1997; Knight et al., 2005; Cosacov et al., 2008; Delgado-Dávila et al., 2016; Rodríguez-Rodríguez et al., 2017; Zych et al., 2019). Species in biodiversity hotspots are believed to be vulnerable to global change, particularly in mountainous areas (Midgley et al., 2002; Malcolm et al., 2006; Ohlemüller et al., 2008; Bellard et al., 2014; Mu et al., 2015; Trew and Maclean, 2021; Xu et al., 2023). Till now, handful studies of pollination have quantified spatial and temporal variations, while pollinator types, visitation rates, self-pollination and floral morphology often vary with altitude and flowering time across multiple populations in mountainous biodiversity hotspots. Studies on multiple closely related species with

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sexual dimorphism at large spatial and temporal scale in populations remained scarce.

Plant species with specialized reproductive system are prone to environmental changes at large scales. For example, heterostylous species, which rely on pollinator-mediated reciprocal pollination between different floral morphs within populations, are sensitive to environmental changes that affect their pollinators. Furthermore, studies have shown that seed production in specialized sexual system associated with intra-morph self-incompatibility are pollen-limited (Waites and Ågren, 2004). However, it remains unclear how biotic and abiotic factors affect the fitness of different flower morphs. Homostylous species may have sufficient self-pollen deposition due to delayed autonomous selfing, suggesting that the ability to self-fertilize provides reproductive assurance at places and times where pollinators are scarce (e.g. Porche and Lande, 2005; Aizen and Harder, 2007; Vaughton and Ramsey, 2010). Homostylous species have, however, been observed to outcross (Wu, 2017), which might provide compensation for the loss of male gametes. A wide-spread transition from heterostyly to homostyly in numerous lineages suggests that the reproductive strategy of the two sexual systems may differ, which allows for comparisons at a macro spatial and temporal scale. Regardless, few studies have examined how environmentally-sensitive factors affect the reproductive success of closely related species with specialized reproductive systems.

Several factors, including elevation and peak flowering time, are know to affect species with specialized flowers (Memmott et al., 2007). The reproductive success of plants growing along an elevation gradients varies, as variation in both flowering resources and pollinators may alter seed production (Totland, 2001; Stone and Jenkins, 2008). At high elevations, where pollinators are scarce, plants may allocate more resources to floral display to attract pollinator visits (Fabbro and Körner, 2004; Arroyo et al., 2006). Reproductive success may also vary in response to peak flowering time, as pollinators become less active late in the flowering season, likely due to a decrease in temperature (Hegland et al., 2009). Although previous studies have reported that pollinator type, visitation rate, self-pollination and floral morphology often vary with elevation and flowering time across multiple populations in mountainous biodiversity hotspots, few studies have quantified how pollinator activity varies across space and time.

Wild *Fagopyrum* species provide an opportunity to understand how spatial and temporal patterns of pollination success vary across species with distinct reproductive strategies. In the Hengduan Mountains region to the southeast of Qinghai-Tibet Plateau, which is one of the global biodiversity hotspots of the world (Myers et al., 2000; Mittermeier et al., 2011; Boufford, 2014), *Fagopyrum* species are insect-pollinated wild plants (or cultivated as crops) are widely distributed from 250 to 4000 m, flowering time of *Fagopyrum* species varies, generally from June to September (Zhao et al., 2007; Wu, 2017; Wu and Huang, 2018). Previous studies have reported that *Fagopyrum* reproduction is susceptible to alpine environments. Specifically, development and fertility of *Fagopyrum* reproductive structures have been shown to be influenced by temperature (Björkman, 2000), and their yield has been shown to be sensitive to various climatic factors (Morishita and Tetsuka, 2001; Kalinová et al., 2005; Jacquemart et al., 2012). Wild *Fagopyrum* species have two morphs (i.e., homostyly and heterostyly) that may have distinct strategies for reproduction. Additionally, it is easy to infer pollen limitation or resource limitation in *Fagopyrum* species from pollen deposition to fruit set, as flowers only have one ovule, and flowers are open for only one day.

Here, we determined the spatial and temporal patterns of pollination success (indicated as stigmatic pollen load) in *Fagopyrum* species with different reproductive strategies (i.e., homostylous with autonomous selfing; distylous with outcrossing). For this purpose, we conducted population surveys and hand-pollination experiments to specifically answer the following questions: (1) How do pollinator activities change along an elevation gradient? (2) Is elevation related to stigmatic pollen loads across populations of *Fagopyrum* species? (3) Is the time of stigma collection related to pollen deposition across populations of *Fagopyrum* species?

2. Materials and methods

2.1. Study species

Wild *Fagopyrum* (Polygonaceae) species are essential genetic sources for buckwheat crops, mainly distributed in southwestern China (Ohnishi and Matsuoka, 1996; Zhao et al., 2007). Pollen records indicate that buckwheat cultivation originated in southwestern China based on pollen records (Yao et al., 2023). Phylogenetic analyses suggest this mountainous region in China is the centre of the origin of the genus *Fagopyrum* (Ohnishi and Konishi, 2001; Tsuji and Ohnishi, 2001; Fan et al., 2021). The open-shaped hermaphroditic flowers of *Fagopyrum* species are white, reddish, or greenish (Fig. 1). Individual flowers have five petals, eight anthers, one pistil with three stigmas, and eight globose nectaries emerging between the filaments and one central single ovary (Fig. 1). A few *Fagopyrum* species in the study region are homostylous, while most are distylous. Distyly is a sexually dimorphic system in which one species has two floral morphs, long-styled (L-morph) and short-styled (S-morph), with reciprocal placement of anthers and stigmas in hermaphroditic flowers, while homostyly is the derived condition which has only one floral morph with stigmas and anthers at the same position (Fig. 1). Distyly has been hypothesized to promote cross-pollination between different floral morphs through accurate placement of pollen on the pollinator body (Darwin, 1877; Nishihiro et al., 2000; Cesaro and Thompson, 2004; Zhou et al., 2015; Barrett, 2019; Costa et al., 2019). At least three losses of distyly as well as self-incompatibility, have been observed in 13 studied *Fagopyrum* species, a typically distylous genus primarily pollinated by flies and bees (Wu et al., 2017). Single flowers of *Fagopyrum* species in the study region last only one day.

2.2. Population investigations

2.2.1. Population sampling

Following previous collection records of *Fagopyrum* species in China (Zhao et al., 2007), we revisited field populations in Sichuan and Yunnan Provinces, southwestern China from late June to mid-August in 2012 and successfully sampled nine *Fagopyrum* species. These included seven distylous species (*F. dibotrys* (D. Don) Hara, *F. esculentum* Moench, *F. gilesii* (Hemsl.) Hedb., *F. leptopodum* (Diels) Hedb., *F. lineare* (Sam.) Harald., *F. statice* (Lévl.) H. Gross, *F. urophyllum* (Bur. et Franch.) H. Gross) and two homostylous species (*F. gracilipes* (Hemsl.) Damm. ex Diels and *F. tataricum* (L.) Gaertn. In these species, *F. esculentum* and *F. tartaricum* can be cultivated as crops. To examine the effects of elevation and flowering time on pollination success, we sampled multiple populations for each species at different flowering periods. A total of 28 populations at peak flowering time was investigated with each species in 2–5 populations, while a total 787 distylous and 184 homostylous individuals, and 1122 distylous and 204 homostylous flowers were sampled for a series of days from June 20th to August 14th in 2012 (Table 1). The elevation of each population was recorded. Stigmatic pollen load was used to assess pollination success. Stigma
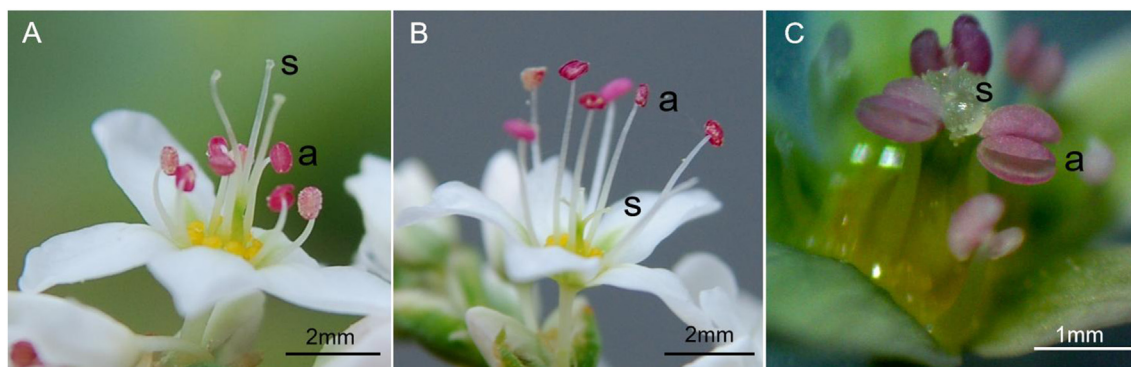
Fig. 1. Floral morphs of long-styled (A) and short-styled (B) flowers in distylous *Fagopyrum esculentum* and homostylous *F. tataricum* (C). Flowers are characterized by five petals, eight anthers (marked as "a"), one pistil with three stigmas ("s"), and eight yellow globose nectaries surrounding the uniovulate ovary. Bar length is given.

Table 1
The number of sampled individuals and flowers of different populations in nine species of *Fagopyrum*, including the long-styled (L), short-styled (S) and homostylous (H) morph in seven distylous species (*F. dibotrys, F. esculentum, F. gilesii, F. leptopodum, F. lineare, F. statice, F. urophyllum*) and two homostylous species (*F. gracilipes* and *F. tataricum*). Each species was sampled 2−5 populations. In the locality column, abbreviations indicate provinces in China: YN for Yunnan, SC for Sichuan, TB for Tibet.

Species	Locality	Lon. (E)	Lat. (N)	Elev. (m)	Collect. time	No. of individ.			No. of flowers		
						L	S	H	L	S	H
F. dibotrys	Chengjiang (YN)	102.877°	24.700°	1888	Jun. 29	10	12		20	20	
	Yangbi (YN)	99.966°	25.662°	1520	Jul. 10	14	4		35	13	
	Jianchuan (YN)	99.860°	26.588°	2375	Jul. 12	15	25		30	29	
	Dali (YN)	100.133°	25.696°	2230	Jul. 8	18	32		30	31	
F. esculentum	Liangshan (SC)	102.840°	27.995°	2049	Jun. 20	20	20		20	20	
	Tonghai (YN)	102.758°	24.097°	2015	Jul. 2	5	15		10	17	
F. gilesii	Deqin (YN)	98.797°	28.968°	2115	Jul. 19	30	30		29	28	
	Mangkang (TB)	98.651°	28.820°	2248	Jul. 20	20	14		28	21	
F. leptopodum	Deqin (YN)	98.797°	28.968°	2115	Jul. 19	19	22		18	21	
	Dongchuan (YN)	103.138°	26.164°	1351	Aug. 5	30	30		30	30	
	Qiaojia (YN)	102.893°	26.968°	2156	Aug. 7	30	30		30	29	
	Yongsheng (YN)	100.426°	26.827°	2141	Jul. 26	31	30		30	29	
	Lijiang (YN)	99.199°	28.266°	2278	Jul. 25	30	30		30	30	
F. lineare	Heqing (YN)	100.274°	26.099°	1656	Jul. 29	15	15		28	29	
	Yongsheng (YN)	100.470°	26.793°	2162	Aug. 1	31	30		29	30	
F. statice	Gejiu (YN)	103.250°	23.515°	1299	Aug. 12	7	8		30	29	
	Jiangchuan (YN)	102.818°	24.388°	1769	Aug. 14	25	25		29	30	
	Mengzi (YN)	103.461°	23.368°	1449	Aug. 9	7	9		28	29	
F. urophyllum	Anning (YN)	102.457°	24.963°	1893	Jul. 4	16	23		28	28	
	Fumin (YN)	102.431°	25.232°	1955	Jul. 6	1	1		29	28	
	Luquan (YN)	102.800°	25.762°	2003	Aug. 3	4	4		30	30	
F. gracilipes	Chengjiang (YN)	102.880°	24.696°	1881	Jun. 29			11			30
	Fumin (YN)	102.428°	25.231°	1947	Jul. 6			30			30
	Jianchuan (YN)	99.873°	26.587°	2353	Jul. 12			31			30
	Tonghai (YN)	102.760°	24.095°	2006	Jul. 3			31			30
F. tataricum	Liangshan (SC)	102.840°	27.995°	2049	Jun. 29			21			21
	Tonghai (YN)	102.098°	24.098°	2017	Jul. 3			30			30
	Shangri-La (YN)	99.638°	27.900°	3315	Jul. 17			30			33

collection time was transformed from original collection date to the nth day after the first day's stigma collection.

2.2.2. Floral measurements and stigmatic pollen deposition counting

To estimate flower size, we measured corolla diameter with a digital caliper (0−150 mm) to the accuracy of 0.01 mm. About 30 flowers were sampled from the individuals of L-morph, S-morph or homostyly (Table 1). To estimate stigmatic pollen loads, flowers were sampled on a sunny day after the cessation of pollinator activity (at about 16:00), as single flowers generally last only one day. Then, all stigmas per flower were carefully collected and stored in a 0.5-mL centrifuge tube with FAA. To test whether stigmatic pollen deposition was related to sampling time or elevation, we identified pollen morph and counted pollen grains from L- or S-morph on the

three stigmas on each flower with a micrometer (at 400 × magnification) under a light microscope (Wu et al., 2018).

2.3. Field observations and manipulations

To compare how spatial and temporal patterns of pollination success vary between species with distinct reproductive systems, we examined two distylous species, *Fagopyrum dibotrys* and *F. esculentum*, and two homostylous species, *F. gracilipes* and *F. tataricum*. The two distylous species have stable and suitable populations with multiple open flowers, representative of distyly. When analyzing the effect of flowering time on reproductive success, we excluded the possible effect of space by comparing pairs of sites at similar elevations. For the cases, the distylous species *F. dibotrys* and homostylous *F. gracilipes* from Chengjiang were

compared, whereas the distylous *F. esculentum* and homostylous *F. tataricum* from Shangri-La were compared.

2.3.1. Pollinator observation

On sunny days, pollinators were observed in field populations with abundant flowers from 10:00 to 16:00 in distylous *Fagopyrum dibotrys* and *F. esculentum*, and in homostylous *F. gracilipes* and *F. tataricum*. We used 15 min as a census interval. Before observation, we recorded the number of open flowers in the observed patch to calculate pollinator visitation frequency (Wu, 2017).

2.3.2. Hand pollination

To investigate whether fruit/seed set is pollen-limited in different *Fagopyrum* species, we hand-pollinated either L- or S-morph flowers of two distylous species, *F. dibotrys* and *F. esculentum*, with inter-morph pollen (compatible pollination), intra-morph pollen (incompatible pollination) or intra-flower self-pollen at the same sites where pollinator observations were conducted. In addition, we hand-pollinated two homostylous species, *F. gracilipes* and *F. tataricum*, with cross pollen and self-pollen. Note these two species are not wind-pollinated. Open pollination served as control for all species. Before hand-pollination, mesh bags were used to exclude pollinators (Wu et al., 2018). Undehisced anthers of flowers were removed in early morning when the flowers just opened. Natural pollination (open-pollinated flowers) and different artificially pollinated flowers were conducted on the same plant to reduce confounding effects of sampled individual plants on the variation in resource condition. Pollen donors for artificial pollination were derived from at least three different individuals. Each pollination treatment had over 30 to more than 300 samples based on the availability of plant materials in field populations. Labeled flowers that experienced pollination treatments above were collected in half a month to estimate fruit or seed set (Wu, 2017). As *Fagopyrum* flowers have only one ovule, the fruit set is equal to seed set in the these species.

2.4. Statistical analysis

To investigate the general effects of elevation, flower time and floral morph (distylous or homostylous) on the stigmatic pollen loads, we constructed generalized linear mixed models (GLMM) with Poisson distribution and a log-link function, assigning elevation, time, floral morph and the interaction between elevation and time as a fixed effect, and species as a random effect. To test the effect of elevation, flower time and floral morph on flower size, a GLMM with normal distribution and linear function was used, assigning elevation, time, floral morph and the interaction between elevation and time as fixed, setting species as a random effect. A GLMM with the same factor settings as above was also conducted separately for homostylous species and distylous species to investigate the effect of elevation and time in homostylous or distylous

species. To compare the ratio of stigma deposited pollen, number of pollen grains on stigma and the coefficient variation (CV) of pollen deposition between homostylous species and distylous species, a t-test was used. CVs were calculated as the standard deviation divided by the mean (Sokal and Rohlf, 1981). We calculated CV for each population, and grouped CVs by floral morph for comparative analysis. To compare the pollinator frequency between distylous and homostylous species, a generalized linear model (GLM) with a normal distribution and linear function was used, assigning site and morph as fixed factors. To test the effects of different pollination treatments on seed set, we used GLM with a binomial distribution and a logit-link function. Seed set was included as a dependent variable and treatments as a fixed factor. To test the effect of flower size on stigmatic pollen load, a GLMM with flower size, collection time, elevation, and the interaction between elevation and collection time as fixed factor was used, and species as a random effect. All statistics were performed in SPSS 27.0 (IBM Inc., New York, USA).

3. Results

3.1. Population macro trends

3.1.1. Elevational trends of pollination success

GLMM analysis pooling distylous and homostylous species showed that elevation affected flower size (F = 138.842, $df_{(1, 2)}$ = 1, 1321, $P < 0.001$) and pollen deposition (F = 13.042, $df_{(1, 2)}$ = 1, 1321, $P < 0.001$) (Table 2), with plants individuals at higher elevation having larger flowers (Fig. 2A) and higher pollen deposition (Fig. 2C). GLMM analysis separating distylous and homostylous species showed that distylous flowers at higher elevation were larger in size (F = 239.003, $df_{(1, 2)}$ = 1, 1118, $P < 0.001$), and received more pollen grains (F = 65.018, $df_{(1, 2)}$ = 1, 1118, $P < 0.001$) (Table S1), whereas homostylous flowers at higher elevation were slightly larger (F = 13.96, $df_{(1, 2)}$ = 1, 200, $P < 0.001$) but did not have more pollen deposition (F = 3.393, $df_{(1, 2)}$ = 1, 200, $P = 0.067$) (Table S2).

3.1.2. Temporal trends of pollination success

GLMM analysis pooling homostylous and distylous species showed that late-collected plant individuals had smaller flowers in size (F = 55.465, $df_{(1, 2)}$ = 1, 1321, $P < 0.001$) (Table 2, Fig. 2B), and lower pollen deposition (F = 31.720, $df_{(1, 2)}$ = 1, 1321, $P < 0.001$) (Table 2, Fig. 2D). GLMM analysis separating homostylous and distylous species showed that early-blooming distylous flowers were larger (F = 125.074, $df_{(1, 2)}$ = 1, 1118, $P < 0.001$) but did not have more pollen deposition (F = 1.543, $df_{(1, 2)}$ = 1, 1118, $P = 0.214$) (Table S1), whereas floral size of late-blooming individuals did not significantly differ from early-blooming individuals (F = 2.166, $df_{(1, 2)}$ = 1, 200, $P = 0.143$), but pollen deposition was slightly lower (F = 11.724, $df_{(1, 2)}$ = 1, 200, $P = 0.001$) (Table S2) in homostylous species.

Table 2

Effect of collection time, elevation, floral morph, and the interaction of collection time and elevation on flower size and stigmatic pollen deposition in all nine *Fagopyrum* species by using generalized linear mixed models (GLMM). $P < 0.05$ indicates significant effects and are in bold.

Dependent variable	Source	d.f. $_{(1, 2)}$	F	P
Flower size	Collection time	1, 1321	55.465	**< 0.001**
	Elevation	1, 1321	138.842	**< 0.001**
	Floral morph	1, 1321	2.191	0.139
	Collection time × elevation	1, 1321	68.976	**< 0.001**
Stigmatic pollen deposition	Collection time	1, 1321	31.720	**< 0.001**
	Elevation	1, 1321	13.042	**< 0.001**
	Floral morph	1, 1321	0.000	0.998
	Collection time × elevation	1, 1321	0.569	0.451
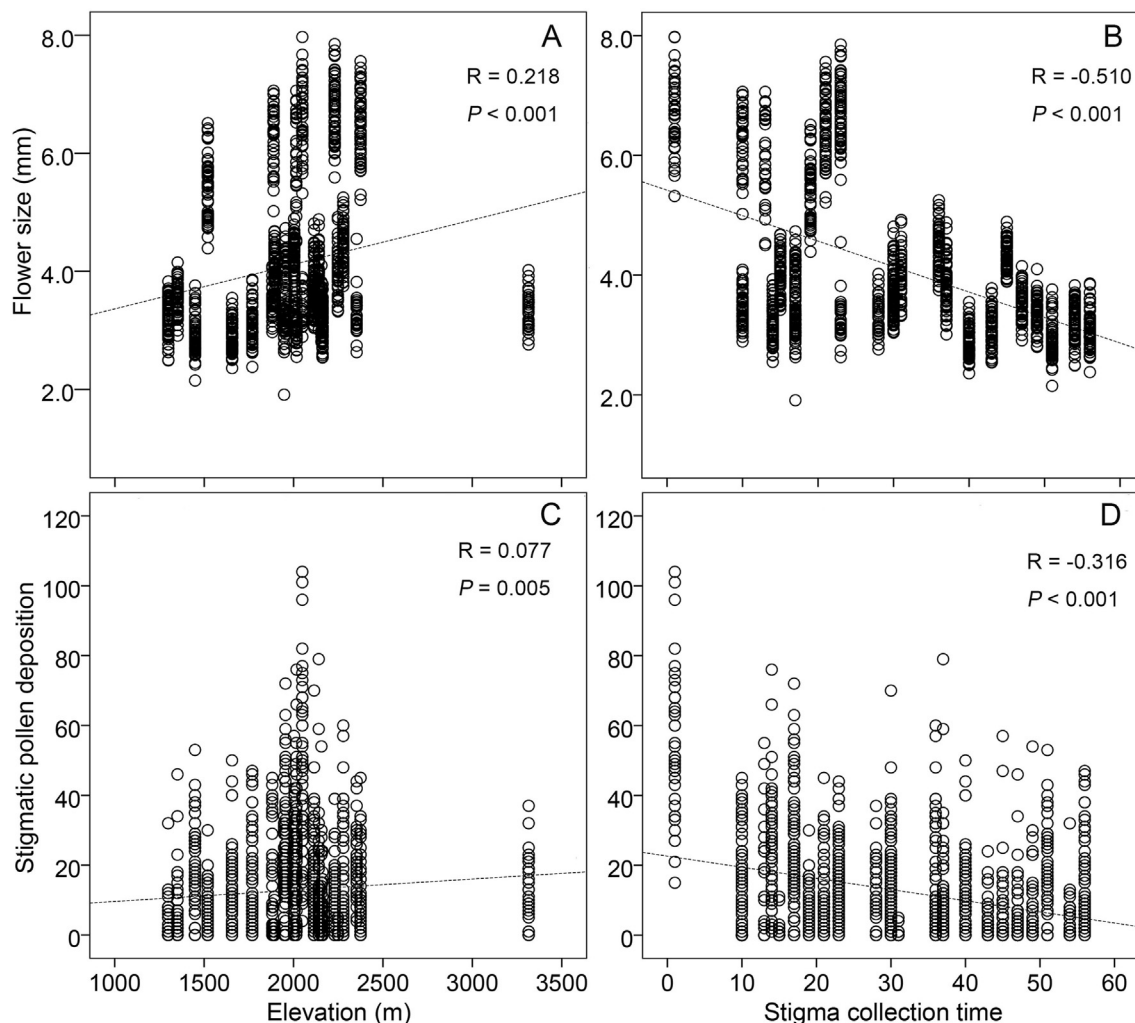
Fig. 2. Relationship between elevation and flower size (represented as corolla diameter) (A), and stigmatic pollen deposition (C). Relationships between stigma collection time (represented as the sequential number of days since the first collection) and flower size (B), and stigmatic pollen deposition (D). Coefficient value R and significance value P are given.

3.1.3. Stigmatic pollen deposition pattern

Across the 28 populations, the percentage of stigmatic pollen deposition in homostylous species ranged from 96 ± 4% to 98 ± 1% (mean ± s.e.), while that on distylous species ranged from 52 ± 40% to 83 ± 17% (Table 3). Homostylous species had a higher proportion of stigma deposited with pollen than did distylous species (t = 4.158, P < 0.001, Fig. 3A). On the stigmas with pollen deposited,

the amount of stigmatic pollen grains of distylous species (8−38 grains per flower) was significantly lower (t = −8.587, P < 0.001) than that on homostylous (20−22) species (Table 3).
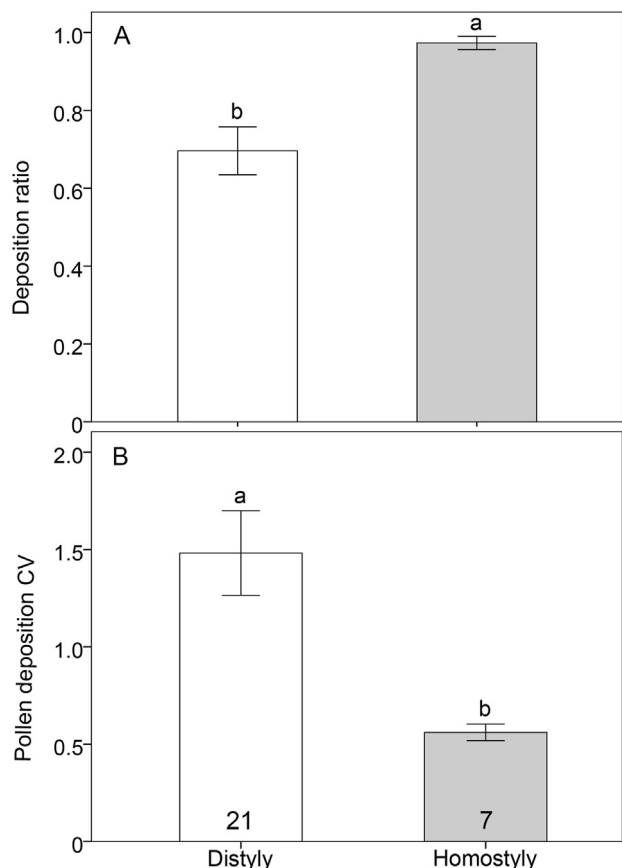
The CV of stigmatic pollen number was lower in homostylous than in distylous species (t = −4.350, P < 0.001, Fig. 3B), suggesting a more stable pollen deposition pattern in the homostylous rather than in the distylous species.

Furthermore, neither the proportion of stigma deposited with pollen (t = 0.760, P = 0.452, Fig. S1A) nor the CV of stigma pollen number (t = −0.643, P = 0.524, Fig. S1B) differed significantly between L- and S-morph. Similarly, flower size did not differ significantly between the L- and S-morph (t = −1.471, P = 0.141, Fig. S2A); however, pollen loads on stigma of L-morph were higher than on S-morph (t = 4.586, P < 0.001, Fig. S2B).

3.2. Comparison of reproductive strategy between homo/distylous species

3.2.1. Pollinator activity

During 33.75 h of observations at two sites for field observations and manipulations, a total of 724 insects visited the four *Fagopyrum* species, including individuals of 60 bees (including honeybee, solitary bee), 658 flies (including muscoids, syphilis), four butterflies,

Table 3
Proportion of flowers with pollen deposited on stigmas, and pollen amounts deposited per flower in the seven distylous and two homostylous species in *Fagopyrum*. Sample size (N) was given.

Species	Sexual system	Stigmatic pollen		
		deposition (%)	pollen number	N
F. dibotrys	Distyly	66.6 ± 18.4	8 ± 1	208
F. esculentum	Distyly	83.3 ± 16.7	38 ± 3	67
F. gilesii	Distyly	51.7 ± 39.5	9 ± 1	106
F. leptopodum	Distyly	75.4 ± 6.9	9 ± 1	277
F. lineare	Distyly	79.5 ± 11.7	8 ± 1	116
F. statice	Distyly	70.4 ± 18.5	11 ± 1	175
F. urophyllum	Distyly	59.2 ± 22.8	11 ± 1	173
F. gracilipes	Homostyly	98.3 ± 1.0	22 ± 1	120
F. tataricum	Homostyly	96.0 ± 4.0	20 ± 2	84

Fig. 3. Pollen deposition features in 28 populations. (A) The ratio of stigma with deposited pollen and (B) CV of pollen deposition. Error bars indicate standard error (s.e.). Numbers in the histogram indicate sample size (N). Different letters above error bars indicate significant difference ($P < 0.05$).

and two beetles. GLMM analysis showed that homostylous species (from 0.1802 ± 0.2177 to 0.2311 ± 0.2903 times/flower/h, mean ± s.e.) were visited less frequently by pollinators than were distylous species (from 0.3522 ± 0.1124 to 2.6137 ± 0.1232) (Wald $\chi^2 = 40.927$, $P < 0.001$). In addition, the site affected visitation (Wald $\chi^2 = 33.537$, $P < 0.001$); however, the effect of sites was eliminated by experimental design.

3.2.2. Pollen limitation and reproductive system

In the distylous *Fagopyrum dibotrys*, seed set of hand-pollinated flowers with compatible pollen ($22.8 \pm 3.5\%$, mean ± s.e.) and open-pollinated flowers ($28.3 \pm 3.3\%$) did not significantly differ (mean difference = 0.055, $P = 0.212$), suggesting no pollen limitation (Fig. 4A). Incompatible pollination with the intra-morph or self-pollen did not set any seeds, indicating that *F. dibotrys* is typically self-incompatible. In the distylous *F. esculentum*, seed set of hand pollination with compatible pollen ($24.8 \pm 2.4\%$) and open pollination ($25.9 \pm 2.5\%$) (mean difference = 0.011, $P = 0.704$) did not significantly differ, suggesting no pollen limitation (Fig. 4B). Seed set of incompatible pollination ($3.3 \pm 1.0\%$ for intra-morph, $3.0 \pm 0.9\%$ for self) was significantly lower than compatible (mean difference = 0.215, $P < 0.001$) and open pollination (mean difference = 0.225, $P < 0.001$) in distylous *F. esculentum*, indicating it is partially intra-morph self-compatible. Furthermore, in distylous *F. dibotrys*, seed set did not differ between L- and S-morph in four treatments (all $P > 0.05$) (Fig. S3A), whereas in distylous *F. esculentum*, seed set of open ($P = 0.025$) and intra-morph

($P = 0.041$) pollination in S-morph were slightly higher in the S-morph than in the L-morph (Fig. S3B).

In two homostylous species, seed set under self-pollination of bagged flowers and open pollinated flowers (control) did not significantly differ in *Fagopyrum gracilipes* (Fig. 4C) and in *F. tataricum* (Fig. 4D), suggesting that these two species were self-compatible and seed set was not limited by pollinator visits. Interestingly, hand pollination with cross pollen was significantly lower than under natural pollination (mean difference = 0.332, $P < 0.001$) in *F. gracilipes* (Fig. 4C) but not in *F. tataricum* (Fig. 4D). This inconsistent result from our emasculation of cross-pollinated flowers might suggest that removing anthers could bring confounding effects of damaging floral tissues during seed development.

4. Discussion

4.1. Strategy of distylous and homostylous species in reproduction

4.1.1. Pollinator visitation

We found that pollinators to *Fagopyrum* species were generally flies and bees. Bees and flies are common flower visitors in the alpine environment under investigation (Yang and Sun, 2009; Fang and Huang, 2013; Song et al., 2014). Our observations are in accordance with previous reports on *Fagopyrum* species that showed the most common visitors belong to bees and syrphid flies (Jacquemart et al., 2012).
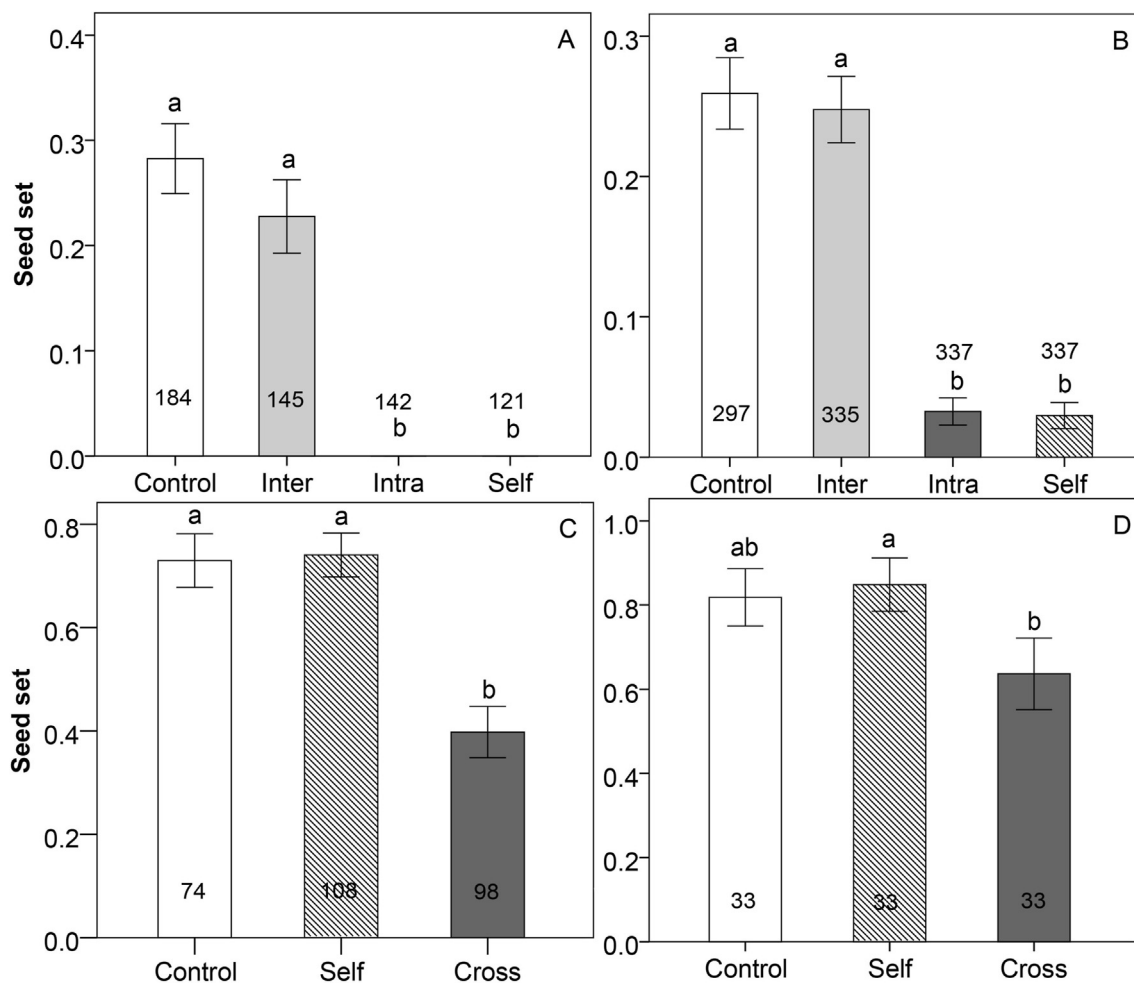
The two distylous species *Fagopyrum dibotrys* and *F. esculentum* received much more pollinator visits, whereas fewer pollinators visited the two homostylous species *F. gracilipes* and *F. tataricum*. This difference may be because distylous species have larger flowers and provide larger rewards (Wu 2017) (Fig. 4A and B), while homostylous species rely less on outcross pollen and more on self-pollen (Fig. 4C and D).

4.1.2. Hand pollination

Hand pollination showed that outcross pollination can result in seed set of all four *Fagopyrum* species studied here, which indicates pollinators can contribute to reproductive success in all these species. Moreover, in our study system, it seems that seed productions was not pollen-limited in either distylous or homostylous species, but the seed set of both natural controls and hand-pollinated distylous species were generally low (< 30%), much lower than that of homostylous species (> 70%). This may be related to resource limitation and an unstable pollination environment. We found that the proportion of stigmas pollinated, the amount of pollen deposited on stigmas, and the stability of pollen deposition were lower in pollinator-dependent distylous species than in homostylous species, which are less pollinator-dependent. Combined with the possible ambient effects of resource limitation, a general low seed set in distylous species is not surprising.

Our finding that seed set is low in distylous species is consistent with previous studies. For example, in Belgium, natural control of *Fagopyrum* led to only 15% seed set, which does not increase after hand cross pollination (Cawoy et al., 2006). In a greenhouse batch experiment, hand-pollination of *Fagopyrum* led to seeds in only 9%−24% of plants (Taylor and Obendorf, 2001). These findings suggest that distylous *Fagopyrum* flowers are not pollen limited; instead, other stages of reproduction must be limited (Bjorkman, 1995; Taylor and Obendorf, 2001; Cawoy et al., 2006, 2007).

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Fig. 4. Comparisons of seed set under four pollination treatments in two distylous species (A: *Fagopyrum dibotrys*, B: *F. esculentum*) and three pollination treatments in two homostylous (C: *F. gracilipes*, D: *F. tataricum*) species of *Fagopyrum*. Control = open pollination, Inter = inter-morph cross pollination, Intra = intra-morph cross pollination, Self = self-pollen pollination, Cross = cross pollen pollination. Numbers on the columns are the sample sizes for each treatment. Different letters indicate significant difference at $P < 0.05$ under generalized linear models (GLMs).

9%−24% of plants (Taylor and Obendorf, 2001). These findings suggest that distylous *Fagopyrum* flowers are not pollen limited; instead, other stages of reproduction might be limited (Bjorkman, 1995; Taylor and Obendorf, 2001; Cawoy et al., 2006, 2007). In *Fagopyrum,* the time between delivery of the first pollen grain and the later entry into the ovule has been reported to be about an hour, which seems more than enough for fertilization to have occurred (Björkman and Pearson, 1995). Thus, three post-pollination processes may explain lower set seed in *Fagopyrum*. One possible explanation is male sterility. Although male sterility is a marginal event, it would explain why pollen deposition does not always lead to higher seed set (Björkman, 1995). Alternatively, low set seed may arise from female deficiencies of flowers. These deficiencies depend on the location on the plant and on plant age (Asako et al., 1980; Taylor and Obendorf, 2001; Halbrecq et al., 2005; Cawoy et al., 2007). Flowers at unfavorable positions or times are more vulnerable to resource limitation, and endure "early abortion" in response (Taylor and Obendorf, 2001; Halbrecq et al., 2005; Cawoy et al., 2007). A third explanation is resource competition between different organs. This type of competition is independent of the timing of pollination; however, the fate of flowers appears to depend on a mechanism internal to the raceme, which controls the percentage of flowers able to set a seed (Gang and You, 1998; Halbrecq et al., 2005; Cawoy et al., 2007).

In the two homostylous species, *Fagopyrum gracilipes* and *F. tataricum*, self-pollination yielded similar seed set in open pollinated flowers, which were both similar to or even higher than after cross pollination (Fig. 4C and D). Taken together with the observation that homostylous species received many fewer pollinator visits, it seems that self-compatible homostylous species have taken a path to a more ensured reproductive strategy than distylous species. Additionally, previous studies have reported that hand pollination leads to less reproduction than natural controls in various species (Waser, 1978; Stephenson, 1979; Willson, 1979; Wheelwright et al., 2006; Lara and Ornelas, 2008; Chautá-Mellizo et al., 2012). Some studies have found that hand pollination, especially emasculation, may have detrimental effects (Bierzychudek, 1981), and plants may submit to resource limitation or other biological procedures. Resource limitation may cause abortion of some developing ovules or ovaries (Stephenson, 1981) and can provide a logistical basis for mating choice in plants (Janzen, 1977; Willson, 1979) and sibling competition between developing embryos (Kress, 1981).

4.2. Spatial pattern of pollen deposition

Stigmatic pollen load could reflect pollination success. We found that pollen deposition increased along an elevation gradient

(Fig. 2C). This may due our general findings that flowers at higher elevation are larger (Fig. 2A), which indicate plants may invest more resources in flower size to attract pollinators. Moreover, we found that larger flowers have generally higher stigmatic pollen deposition (Table S3). The increase of pollen deposition with elevation may be due to the distribution of high-efficiency pollinators at higher elevation. For example, in high-elevation alpine regions, bees are much more active and more efficient than are temperature-susceptible flies (Fang and Huang, 2013; Inouye et al., 2015). Although numerous studies have examined on pollinator performance over an elevation gradient (e.g. Warren et al., 1988; Hargreaves et al., 2015; Baumann et al., 2021; Minachilis et al., 2021; Abdusalam et al., 2022; Klomberg et al., 2022), few studies have directly evaluated pollen deposition in an elevation and time framework (but see Alonso, 2005; Brito and Sazima, 2012; Hargreaves et al., 2015), Although it is laborious to harvest stigmas across multiple populations in the field. We recommend that future studies evaluate pollen deposition at both spatial and temporal scales.

4.3. Temporal patterns of Fagopyrum pollen deposition

During the late-phase of the flowering season, flowers were smaller and less pollen was deposited (Table 2, Fig. 2B, D). This pattern can be explained in two ways. Firstly, smaller flowers are likely to be less attractive to pollinators. Flower size is positively related with pollinator visitation (e.g. Conner and Rush, 1996); thus, small flowers in late-collected populations would receive fewer visitors and have fewer stigmatic pollen loads. Secondly, floral visitor activities may gradually cease over time. For example, Jacquemart et al. (2007) reported that the abundance of most-effective honeybee to Fagopyrum decreased at the late-phase of the flower season, which could be linked to the decrease of nectar production observed after the flowering peak (Naumkin, 1998; Alekseyeva and Bureyko, 2000; Cawoy et al., 2006). Although more pollen deposition would not always result in more seeds in Fagopyrum (e.g. Taylor and Obendorf, 2001; Cawoy et al., 2006), it has been reported that more pollen deposition than natural pollen deposition increases seed quality and progeny vigor in Fagopyrum (Bjorkman, 1995). Thus, the time factor between Fagopyrum flowers and pollinator activity would still limit reproductive success. Broadly, in biodiverse alpine regions, such a mismatch between late-active pollinators and late-anthesis flowers would reduce plant reproductive success. Further, the time synchrony of flowers and pollinators is vital to both partners (Waser, 1979), and being affected by changeable climate environments, the mismatch between movement of pollinators and phenology of plants can be destructive (Parmesan, 2007; Hegland et al., 2009; Willmer, 2012). In the study region, a 35-year dataset of seasonal biomass dynamics in a Tibetan alpine grassland showed that climate change promoted both earlier phenology and faster growth, providing an environment for plant phenology change in the alpine regions (Wang et al., 2020). Our findings on stigmatic pollen load, which are indicative of pollinator activity in a biodiversity hotspot affected by global climate change, extend these previous findings.

4.4. Trends in flower size

The relationship between elevation and flower size or reproduction effort has long been discussed. Our study across 28 populations shows that flowers at higher elevation are larger (Fig. 2A). This increase in flower size at higher elevations may be a way for plants to attract or compete for scarce pollinators (Cuartas-Hernández et al., 2019). Generally, bigger flower may attract more pollinators, leading to more stigmatic pollen load. For example,

across a dozen bumblebee-pollinated Pedicularis rex populations in the Hengduan Mountains, flowers with larger floral lower lip would have more stigmatic pollen load (Sun et al., 2016). In Argentina anserina (Rosaceae), plants with larger bullseyes tended to receive more pollen at highest-elevation sites (Koski and Ashman, 2015). Our study shows that bigger flowers receive more pollen (Table S3), which may also support the fact that flower size is related to pollination success.

For example, previous research on the bee-pollinated perennial Campanula rotundifolia (Campanulaceae) found that flower size increased with elevation, and that the increase in flower size was associated with a mechanical fit between plants and bigger bumblebees at higher elevations (Maad et al., 2013). Another study found that the spring-flowering shrub Elaeagnus umbellata (Elaeagnaceae) had longer floral tubes at higher elevations, and that these longer floral tubes may mechanically fit bird pollinators at higher elevations (Pi et al., 2021). In our study, flowers are disk-like, and not as specialized as bilateral flowers; thus, the relationship between flower size and elevation may due to comprehensive strategies of plants allocating resources at different elevations.

5. Conclusion

Our study of Fagopyrum species with distinct reproductive systems revealed that both elevation and time affect plant pollination success at large geographical scales in both distylous and homostylous species. Although our study did not show pollen limitation in Fagopyrum species, a limitation in seed set was detected, especially in distylous species that rely greatly on pollinators. This indicates that climate changes that disrupt plant-pollinator interactions have the potential to affect reproductive success of alpine plants and wild crop sources with sexual polymorphism, especially in biodiversity hotspots.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2023.10.001.

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