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Evolutionary biology

Diplostigmy in plants: a novel mechanism that provides reproductive assurance

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Differentiation of female sexual organs in flowering plants is rare and contrasts with the wide range of male reproductive strategies. An unusual example involves diplostigmy, the possession of spatially and temporally distinct stigmas in *Sebaea* (Gentianaceae). Here, the single pistil within a flower has an apical stigma, as occurs in most flowering plants, but also a secondary stigma that occurs midway down the style, which is physically discrete and receptive several days after the apical stigma. We examined the function of diplostigmy in *Sebaea aurea*, an insect-pollinated species of the Western Cape of South Africa. Floral manipulations and measurements of fertility and mating patterns provided evidence that basal stigmas function to enable autonomous delayed self-pollination, without limiting opportunities for outcrossing and thus avoiding the costs of seed discounting. We suggest that delayed selfing serves as a mechanism of reproductive assurance in populations with low plant density. The possession of dimorphic stigma function provides a novel example of a flexible mixed-mating strategy in plants that is responsive to changing demographic conditions.

1. Introduction

The hermaphrodite condition of most angiosperms has led to diverse morphological, physiological and phenological solutions for reducing the harmful effects of self-fertilization on progeny fitness, and limiting interference between female and male function [1]. As a result, the reproductive organs and mating biology of angiosperms exhibit greater variety than those of any other group of organisms [2]. Much of this diversity is associated with structural and numerical variation in male organs (stamens), resulting in various pollen packaging and dispensing mechanisms that promote outcrossed siring success [3]. By contrast, female organs (pistils) usually display less structural diversity, and most variation involves style length, orientation and the elaboration of the stigma and stigmatic papillae. Here, we examine a novel example of structural differentiation in female sexual organs of a hermaphroditic flowering plant and investigate its function in pollination and mating.

Diplostigmy, the presence of a primary stigma (apical) at the terminus of the style and a secondary stigma (basal) midway down the style (figure 1*a*) is a rare condition in angiosperms reported only from *Sebaea* (Gentianaceae), a genus of insect-pollinated mostly African species [4–6]. Although highly restricted in its taxonomic occurrence, diplostigmy is widely distributed within *Sebaea* [6]. The rarity of diplostigmy in angiosperms is explained by unusual ontogenetic features such as the post-genital fusion of the two carpels that occur within a flower in *Sebaea*, and the occurrence of flat rather than folded upper carpels allowing the development of secondary basal stigmas (for further details, see [6]). Kissling *et al.* [6] proposed that diplostigmy functions to provide reproductive

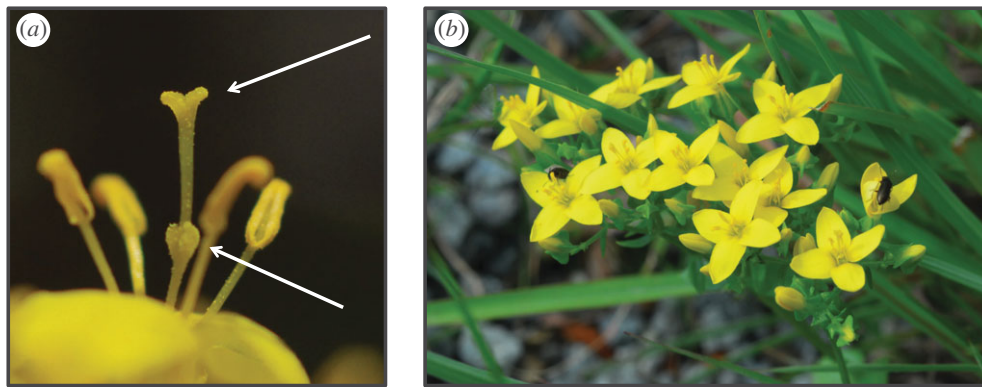


Figure 1. (a) Flower and (b) inflorescence of *S. aurea*, white arrows in figure 1a indicate the two stigmatic regions.

assurance when pollinator service is unreliable, potentially resulting in mixed mating. Apical stigmas may receive mostly outcrossed pollen when pollinator service is reliable, whereas self-pollen could be autonomously deposited on basal stigmas when pollinator service is insufficient to fertilize all ovules; such a situation is likely to occur in small and/or low-density populations, or when flowers remain closed during inclement weather. If diplostigmaty functions as a flexible mixed-mating strategy, then it is of general relevance to the broader question of the evolutionary maintenance of mixed mating [7]. Here, we examine the hypothesis of dual pollination and mating function in *Sebarea aurea* through field experiments, floral manipulations and the use of genetic markers.

2. Material and methods

(a) Study system

Sebarea aurea (L.f.) Roem. & Schult. is a diploid, annual, self-compatible species endemic to the Western Cape of South Africa. The yellow flowers are actinomorphic with four petals united basally into a floral tube with four free lobes (figure 1b). The apical stigma when mature is situated above the anthers, whereas the basal stigma matures 2–4 days later [5,6]. Flowers open only when weather conditions are sunny and warm and suitable for pollinators. Inflorescence sizes vary from a single flower to approximately 350 flowers depending on ecological conditions; most commonly, inflorescences contain 20–30 flowers (figure 1b). Populations occur in fynbos vegetation and their appearance and size is tightly coupled to fire cycles, being very large immediately after fire, but diminishing dramatically in size as succession proceeds to the extent that only a few scattered individuals are evident in mature vegetation.

(b) Field experiment

To evaluate the facility for autonomous self-pollination, we bagged flowers of *S. aurea* in five populations during spring 2009. Entire plants were bagged, because flowers are too small to be bagged individually. We randomly chose one flower per individual to evaluate seed set in eight individuals per population. In 2010, we performed floral manipulations in these populations. We chose large populations (more than 1000 individuals) to conduct our experiments to enable sufficient replication of treatments. Pollinators observed included monkey beetles, flies and solitary bees. Five treatments were performed in each population: (i) open pollination of intact flowers, (ii) emasculation of anthers prior to anthesis, (iii) apical stigma removed before receptivity, (iv) emasculation and apical stigma removed and (v) pollen supplementation using outcross pollen applied to apical stigmas. Manipulated plants had a mean of 25.4 flowers per inflorescence. We used

a mean of 23 flowers per treatment, with the exception of treatment 1 (mean of 275 flowers). We analysed variation in seed set among treatments using generalized mixed models, with treatment as a fixed effect and population, individual and flower position as nested random effects using the lme4 package in R. We fitted models by the Laplace approximation, specifying a Poisson distribution and using a log link function. To account for overdispersion, the number of observations was added as an additional random effect.

We determined the mating patterns resulting from the five experimental treatments using six polymorphic microsatellite loci as described in Kissling *et al.* [8]. Amplification products were analysed on an automated sequencer (Applied Biosystems 3130xl) with an internal size standard (LIZ 500) and sized using GENEIOUS. We dried leaf tissue of maternal parents collected in the field and seedling tissue of offspring in silica gel before DNA extraction. Germination rates of seed families were close to 100% for each population, and we used a mean of 12 families per treatment, and a mean of six offspring per family, to estimate the mean and standard error of the multilocus outcrossing rate per treatment implemented in the MLTR program [9], using the family as the unit of resampling and 500 bootstraps. To investigate the effect of small population size on mating patterns, we estimated the outcrossing rate from nine families (mean of four offspring per family) from an additional population (LI) sampled in 2010. The population was large in 2005 after fire but progressively declined in size with succession, and the few plants remaining were at low density when we sampled families.

3. Results

Flowers of *S. aurea* can self-pollinate autonomously, as seeds were set in all bagged flowers in 2009 (mean 16 seeds per flower, range 3.83–31.6). Preventing autonomous self-pollination by emasculation had no significant effect on open-pollinated seed set in any of the five large populations in 2010 (figure 2a and table 1). Furthermore, outcrossing rates in intact and emasculated flowers were not significantly different and were close to $t = 1.0$ in all populations (figure 2b and table 2). This indicates that pollinator service was sufficient to mediate high levels of outcrossing in all five populations. By contrast, in the small population (LI), considerable selfing was detected ($t = 0.33 \pm 0.05$).

Removal of apical stigmas reduced seed set significantly, by 49% on average, compared with open pollination. The fact that seed set was not zero demonstrates that basal stigmas are functional. Outcrossing rates were also significantly reduced by removal of apical stigmas (mean $t = 0.63$ range 0.56–0.70), consistent with the hypothesis that basal stigmas can function

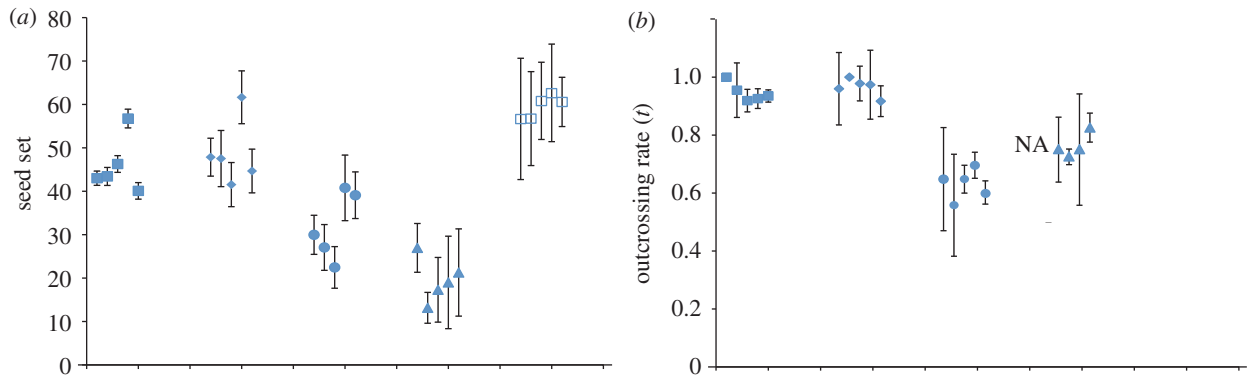


Figure 2. The mean and standard error of (a) seed set and (b) outcrossing rate (t) for experimental treatments conducted on five populations of *S. aurea*. Within each treatment, populations are ordered as follows: HE, PA, SO, THA and THB (localities in electronic supplementary materials). Filled squares, intact; diamonds, emasculated; circles, apical stigma removed; triangles, emasculaton and apical stigma removed and open squares, supplemental pollen. (Online version in colour.)

Table 1. Generalized linear mixed models (with Poisson error distribution and population, individual and flower position as nested random effects) of the effect of experimental treatments on seed set in *S. aurea*.

	estimate	s.e.	z-score	p-value
intercept (intact flowers)	3.432	0.075	45.65	<0.0001
emasculaton	0.002	0.072	0.04	0.967
apical stigma removed	-0.665	0.084	-7.84	<0.0001
emasculaton and apical stigma removed	-1.161	0.121	-9.53	<0.0001
supplemented pollen	0.269	0.107	2.52	0.0119

Table 2. Linear models (with normal error distribution) of the effects of experimental treatments on outcrossing rate in *S. aurea*. Multiple $R^2 = 0.9385$, Adjusted $R^2 = 0.9262$, F -statistic = 76.31 with 3 and 15 d.f., p -value = 2.6×10^{-9} .

	estimate	s.e.	t-value	p-value
intercept (intact flowers)	0.947	0.018	52.11	<0.0001
emasculaton	0.018	0.025	0.73	0.476
apical stigma removed	-0.316	0.025	-12.32	<0.0001
emasculaton and apical stigma removed	-0.184	0.027	-6.76	<0.0001

Table 3. Linear model (with normal error distribution) comparing the effect of emasculaton and apical stigma removal versus apical stigma removal alone on outcrossing rate in *S. aurea*. Multiple $R^2 = 0.6995$, adjusted $R^2 = 0.6566$, F -statistic = 16.29 with 1 and 7 d.f., p -value = 0.004954.

	estimate	s.e.	t-value	p-value
emasculaton and apical stigma removed	0.762	0.024	31.21	<0.0001
apical stigma removed	-0.132	0.032	-4.03	0.00495

to provide reproductive assurance. Seed set was further reduced by removal of both apical stigmas and anthers (mean reduction 69%), indicating that self-pollen can contribute towards seed set when basal stigmas function alone. There were moderate increases in outcrossing rate when self-pollen was removed as a source of pollen for basal stigmas (figure 2b and table 3). In three (HE, PA and SO) of the five populations, there was no evidence of pollen limitation of seed set; however, in populations THA and THB open-pollinated flowers produced approximately 20% less seed than those receiving supplemental pollen ($0.1 > p$ -value > 0.05).

4. Discussion

Our study confirms that in *S. aurea* the unusual basal stigmas that characterize the diplostigmatic condition are functional and enable seed set from self-pollen falling from anthers. When apical stigmas were removed experimentally, the selfing rate increased significantly in all five populations. The increase in selfing rate probably resulted from autonomous intrafloral self-pollination rather than geitonogamous pollination, because excision of stigmas and emasculaton resulted in an increase in outcrossing rate in comparison with the

removal of stigmas alone. Basal stigmas become receptive several days after apical stigmas and their development can vary. Our observations confirm those of Hill [5] that removal of apical stigmas leads to a substantial increase in the size of basal stigmas. By contrast, when apical stigmas received pollen supplementation, the basal stigmas usually remain much smaller in size. The flexible development and delayed receptivity of basal stigmas suggest that their main function is to enable delayed selfing and serve as a mechanism of reproductive assurance. The spatial and temporal differentiation of stigma receptivity is significant because it does not diminish opportunities for outcrossing. Thus, diplostigmy is a novel mechanism by which plants can avoid the costs of seed discounting, the reduction in outcrossed seed set because selfing pre-empts ovules that could have been outcrossed [10,11].

Plants in all five populations of *S. aurea* were highly outcrossed, as might be expected for large post-fire populations, and we observed frequent pollinator visits to flowers owing to their occurrence in sunny open habitats. Apical stigmas probably received sufficient outcross pollen in these populations that basal stigmas may have played only a limited role in mating. Population sizes of *S. aurea* decline rapidly with succession because of shade caused by shrubby fynbos vegetation. As the density of *S. aurea* decreases, basal stigmas probably become more important in providing reproductive assurance owing to a reduction in pollinator service. Evidence based on the analysis of progeny arrays from

scattered individuals from a small population (LI) supports this hypothesis as selfing rates increased to approximately 70%, indicating that mating patterns are context-dependent. Mechanisms providing reproductive assurance causing mixed mating are commonplace but need not operate consistently in space and time to be maintained and this is particularly the case with delayed self-pollination [12,13].

The evolutionary stability of mixed mating is a central question in mating system biology [7], and thus information on the ecology and evolutionary history of diplostigmy is of interest. Comparative analyses of *Sebaea* indicate that diplostigmy is ancestral and of monophyletic origin [6], although the morphological expression of diplostigmy can vary among the numerous species that exhibit this condition [5]. If, as we suggest, diplostigmy functions as a flexible mixed-mating strategy, then this may have promoted diversification into variable ecological and demographic conditions not accessible to related lineages with monostigmy. Future demographic studies of *Sebaea* species with one versus two stigmas would be useful to determine whether fluctuating population sizes can explain the evolution and maintenance of diplostigmy.

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References

- Barrett SCH. 2002 The evolution of plant sexual diversity. *Nat. Rev. Genet.* **4**, 274–284. (doi:10.1038/nrg776)
- Harder LD, Barrett SCH. 2006 *Ecology and evolution of flowers*. Oxford, UK: Oxford University Press.
- Harder LD, Thomson JD. 1989 Evolutionary options for maximizing pollen dispersal in animal-pollinated plants. *Am. Nat.* **133**, 323–344. (doi:10.1086/284922)
- Marloth R. 1909 A diplostigmatic plant, *Sebaea exacoides* (L.) Schinz (*Belmontia cordata* L.). *Trans. R. Soc. South Africa* **1**, 311–314. (doi:10.1080/00359190909520037)
- Hill AW. 1913 The floral morphology of the genus *Sebaea*. *Ann. Bot.* **27**, 479–489.
- Kissling J, Endress PK, Bernasconi G. 2009 Ancestral and monophyletic presence of diplostigmy in *Sebaea* (Gentianaceae) and its potential role as a morphological mixed mating strategy. *New Phytol.* **184**, 303–310. (doi:10.1111/j.1469-8137.2009.03000.x)
- Goodwillie C, Kalisz S, Eckert CG. 2005 The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical expectations, and empirical evidence. *Annu. Rev. Ecol. Syst.* **36**, 47–79. (doi:10.1146/annurev.ecolsys.36.091704.175539)
- Kissling J, Bachmann O, Thali MR, Segarra-Moragues JG. In press. Novel microsatellite loci for *Sebaea aurea* (Gentianaceae) and cross-amplification in related species. *Applications in Plant Sciences*.
- Ritland K. 2002 Extension of models for the estimation of mating systems using *n* independent loci. *Heredity* **88**, 221–228. (doi:10.1038/sj.hdy.6800029)
- Lloyd DG. 1992 Self- and cross-fertilization in plants. II. The selection of self-fertilization. *Int. J. Plant Sci.* **153**, 370–380. (doi:10.1086/297041)
- Herlihy CR, Eckert CG. 2002 Genetic cost of reproductive assurance in a self-fertilizing plant. *Nature* **416**, 320–323. (doi:10.1038/416320a)
- Schoen DJ, Brown AHD. 1991 Whole-flower and part-flower self-pollination in *Glycine clandestina* and *G. argyrea* and the evolution of autogamy. *Evolution* **45**, 1651–1664. (doi:10.2307/2409786)
- Kalisz S, Vogler DW, Hanley KM. 2004 Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* **430**, 884–887. (doi:10.1038/nature02776)