South African Journal of Botany 111 (2017) 387–391

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Selfing versus out-crossing in six *Erica* species of the southern Cape

S.Y.J. Angoh [a],*, M. Brown [b], J. Midgley [a]

[a] Department of Biological Sciences, University of Cape Town, Rondebosch 7701, South Africa
[b] School of Life Sciences, University of KwaZulu-Natal, Durban 4041, South Africa

ARTICLE INFO

Article history:
Received 19 September 2016
Received in revised form 16 March 2017
Accepted 3 May 2017
Available online xxxx

Edited by J Van Staden

Keywords:
Erica
Pollination
Self-incompatibility
Autogamy
Geitonogamy
Cape Floristic Region

ABSTRACT

Despite *Erica* being the most diverse genus in the Cape Floristic Region, very few studies have focused on their breeding systems. The ability to self-fertilise autonomously and to tolerate self-pollen was assessed in three bird-pollinated obligate seeder (*E. densifolia*, *E. sessiliflora* and *E. discolor*) and three insect-pollinated obligate seeder (*E. penicilliformis*, *E. scabriscula* and *E. formosa*) *Erica* species in the southern coastal part of the Cape Floristic Region. None of the species successfully reproduced through autogamy and only the bird-pollinated *E. sessiliflora* produced a significant proportion of viable seeds per fruit when selfed. With the other five *Erica* species examined, cross-pollination yielded a significantly higher proportion of viable seeds per fruit compared to other treatments (autogamy and self-pollination). This suggests that the role of pollinators is essential for the reproductive success of *Erica* regardless of their pollination system (bird- versus insect-pollination) and that most are out-crossers.

1. Introduction

The genus *Erica* is the largest genus in the Cape Floristic Region, consisting of more than 680 species (Oliver and Oliver, 2002; Pirie et al., 2011). This spectacular speciation appears to be driven by floral innovation, given the diversity of floral forms present. Three major pollination systems (namely; insect, bird and wind) and a minor system (rodent) have been identified in the Cape *Erica* species (Oliver and Oliver, 2002; Turner, 2012). It has been put forward that high levels of pollen limitation due to widespread self-incompatibility and pollinator limitation, has set the scene for adaptive radiation and speciation in the Cape (Johnson et al., 1998; Goldblatt et al., 2000; Manning and Goldblatt, 2005). Despite the reproductive advantages that self-compatibility could confer when pollinators are few, many plant traits have nevertheless consistently evolved mechanisms to prevent self-pollination (Knight et al., 2005). Records of autogamous species have been rare in *Erica*, probably due to widespread protogyny (Rebelo and Siegfried, 1985). Understanding the prolific and relatively recent speciation in *Erica* will benefit from descriptive data on whether they are an obligate out-crosser and in relation to pollination mode, yet only a few observational and experimental breeding system studies have focussed on *Erica* species to date.

Adopting geitonogamy in addition to cross-pollination as a reproductive strategy could be advantageous for some plant species (Roberts et al., 2014). However, geitonogamy still requires a vector and thus cannot provide reproductive assurance in times of low pollinator abundance, for instance post-fire (Elle and Hare, 2002; Geerts, 2011). Nevertheless, geitonogamy might form part of a bet-hedging strategy that permits even small plant populations to generate a substantial seedbank (Roberts et al., 2014). In this study, we asked: are *Erica* species typically self-incompatible and does this vary with pollination mode (insect- versus bird-pollination)?

2. Methods

Species selection for the breeding system experiments was based on which *Erica* species were in flower in and around Nature's Valley (lat. 33.976754°S, long. 23.562155°E) from September to December 2015. The assumed pollination syndrome of the six species under investigation was established by use of literature and local botanical experts (Oliver and Oliver, 2002; R. Turner, Unpublished Data; Smuts, 2012). In this study, species with long tubular flowers (15 to 30 mm) – *E. densifolia*, *E. sessiliflora* and *E. discolor* (Fig. 1) – were considered to be pollinated by sunbirds (Smuts, 2012). The plant species with small corollas (2 to 4 mm) were all considered to be insect-pollinated species (pollinated by a suite of insect species within different functional types) — *E. penicilliformis*, *E. scabriscula* and *E. Formosa* (Fig. 2; Smuts, 2012). All *Erica* species examined in this study were obligate seeders (R. Turner, Unpublished Data).

Controlled hand-pollination experiments were conducted from September to December 2015. Closed floral buds were bagged (bags made

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* Corresponding author.
E-mail address: jenniferangoh@gmail.com (S.Y.J. Angoh).

http://dx.doi.org/10.1016/j.sajb.2017.05.001
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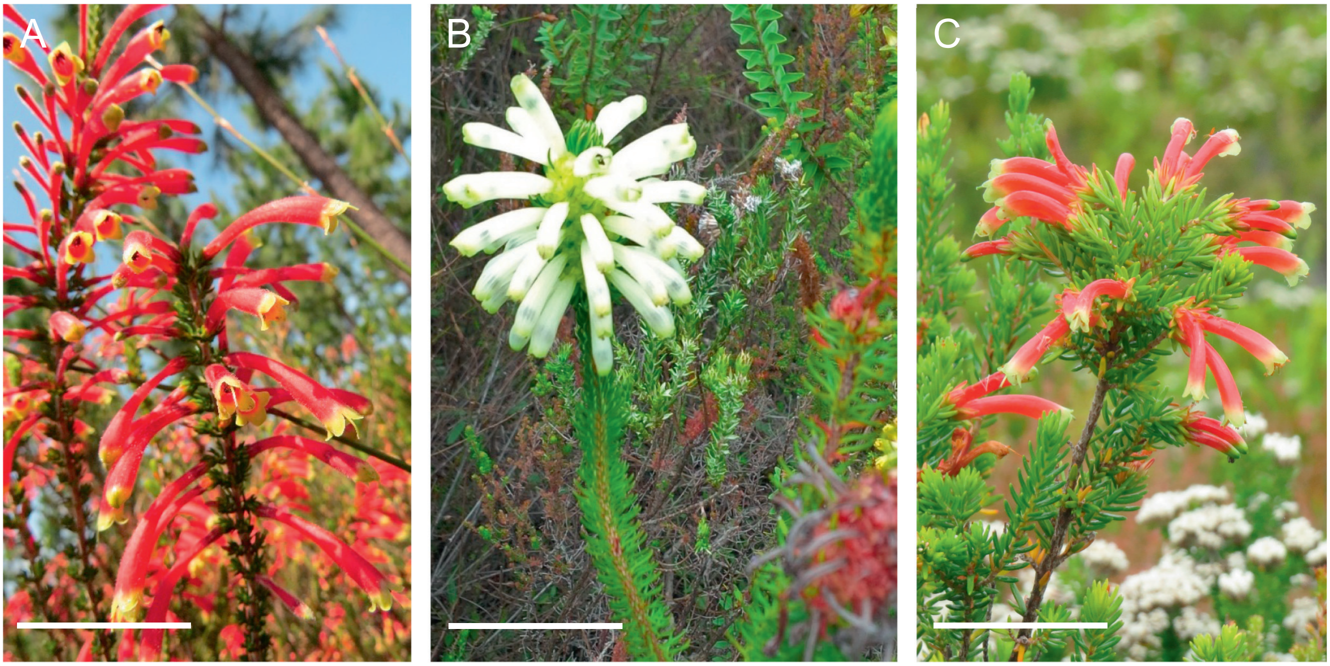

Fig. 1. Bird-pollinated *Erica* species. (A) *Erica densifolia*. Scale 40 mm. (B) *Erica sessiliflora*. Scale 30 mm. (C) *Erica discolor*. Scale 40 mm.

from bridal veil), hand-pollinated when they opened, re-bagged and thereafter their ripe fruit were collected six to eight weeks later depending on the *Erica* species. Four treatments were applied to each of the selected mature plants; 1) cross-pollination, 2) self-pollination with manual pollen transfer, 3) autonomous self-pollination, and 4) open-pollinated control. Each of these four treatments was applied on all selected plants. There were a minimum of 20 replicates (individual plants) per species. Pollen used for outcrossing was obtained from neighbouring plants that were at least 5 m distant. Anther rings of un-pollinated flowers were disturbed over a clean petri-dish to collect pollen. Using a micro dissecting spatula, pollen was applied to the receptive stigma (sticky to the feel and shiny surface) of a marked flower. The spatula surface was rinsed with clean water and dried after each pollen application. Flowers ($n = 20$ per species) were bagged but left un-manipulated to test for autogamy in the different species. Treated flowers were left in the bags to allow for fruits to mature for a minimum of six weeks. Toward the end of the ripening period, fruits were checked

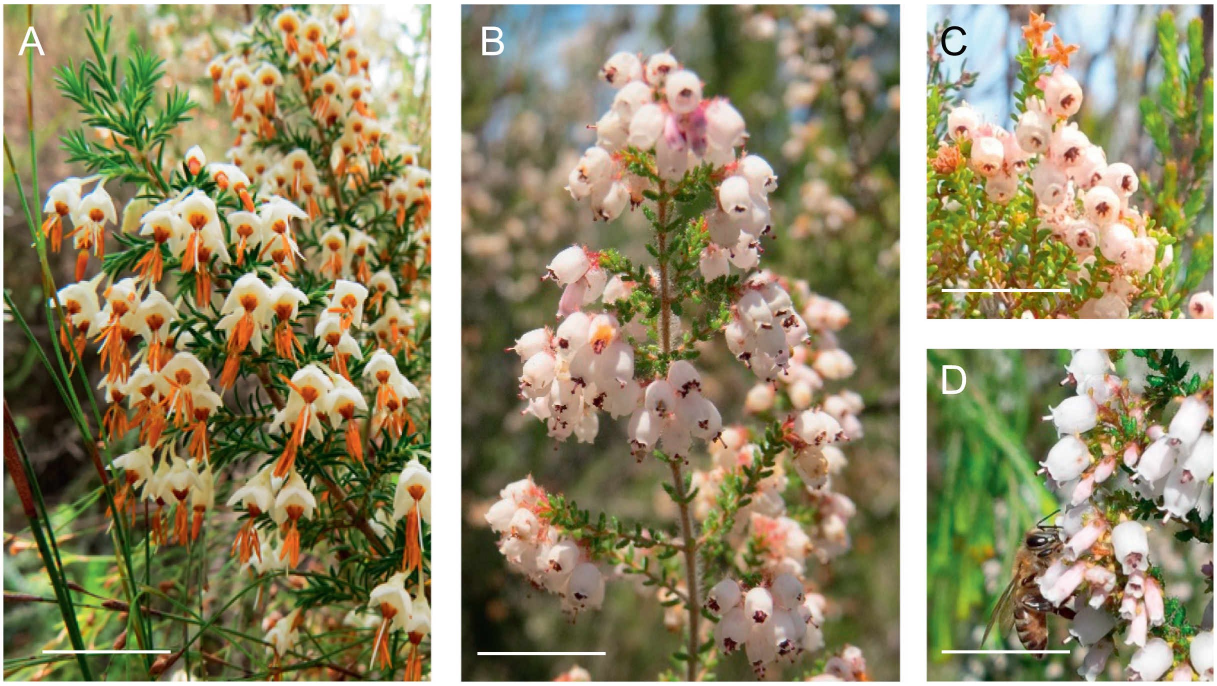

Fig. 2. Insect-pollinated *Erica* species. (A) *Erica penicilliformis*. Scale 8 mm. (B) *Erica scabriscula*. Scale 8 mm. (C) *Erica formosa*. Scale 8 mm. (D) *Apis mellifera* pollinating *Erica scabriscula*. Scale 8 mm.

periodically to ensure that harvesting was done before natural dispersal of the seeds occurred (seeds expelled when fruit is completely mature).

Since the serotinous fruits and seeds of *E. sessiliflora* do not dehisce after each flowering season, they were left to ripen for a longer period (approximately 10 weeks) compared to the other species.

We were interested in seed set in control flowers that had been pollinated and therefore our control treatment was the analysis of ripe fruits from un-bagged and un-manipulated flowers ($n = 20$ per species), but which had set fruit. Fruit set will occur despite low seed-set (R. Turner, Pers. Comm. 2015, this study) and therefore all we have excluded is flowers lost to herbivory and those that received no visits. The proportion of viable seeds per fruit (viable seed set) was established from the dissection of ripe fruits. Viable seeds are visually different from aborted ones in that they are plump and larger in size compared to their shrivelled and smaller counterparts (Turner et al., 2012). Viable and non-viable seeds per fruit were scored using image processing software (Image-J; Rasband, 2015). In order to test the accuracy of the Image-J results, a comparison between the automated and manual seed set counts was done for 30 fruits of *Erica discolor*. There was no significant difference in viable seed count obtained using the automated or manual counts ($df = 29$, $SE = 0.05$, t-value $= 1.66$, $P > 0.05$), validating the use of this technique. Seeds of insect-pollinated species were counted manually with the help of a light microscope (×10 magnification) because the faint colour or small size of seeds from insect-pollinated plants could not be determined reliably from photographs.

Prior to comparing the proportion of viable seeds per fruit obtained with four hand-pollination treatments (i.e. cross-pollination, self-pollination requiring pollen transfer, autonomous self-pollination, and control), homoscedasticity of the data for each *Erica* species was verified using the Bartlett test (Snedecor and Cochran, 1989). Since the variances were not homogeneous ($P < 0.05$), the nonparametric pairwise multiple-comparison test, Dunn's test with Bonferroni adjustment, was performed for comparisons between paired treatments (Dinno, 2015).

To determine the degree of self-incompatibility for each of the *Erica* species studied here, two indices (1. index of self-incompatibility, ISI; 2. index of autonomous self-pollination, IAS) were calculated (equations used adapted from Steenhuisen and Johnson, 2012).

Equation for index of self-incompatibility (Eq. (1)):

$$ISI = 1 - \frac{\textit{Proportion viable seeds for flowers pollinated with self pollen}}{\textit{Proportion viable seeds for cross pollinated flowers}} \tag{1}$$

An ISI value close to zero indicates full self-compatibility, while an index ≥0.2 indicates self-incompatibility (Zapata and Arroyo, 1978; Steenhuisen and Johnson, 2012).

Equation for index of autonomous self-pollination (Eq. (2)):

$$ISI = 1 - \frac{\textit{Proportion viable seeds for unmanipulated bagged flowers}}{\textit{Proportion viable seeds for flowers pollinated with self pollen}} \tag{2}$$

Proportions for viable seed set obtained from the un-manipulated bagged flowers and self-pollinated flowers were used in the IAS equation. IAS values typically range from zero (completely reliant on a vector for self-pollination) to one (fully capable of setting seeds through autogamy). Any species scoring an IAS >0.2 is considered to be capable of autonomous selfing (Zapata and Arroyo, 1978; Steenhuisen and Johnson, 2012).

3. Results

None of the six *Erica* species we studied is autogamous (IAS < 0.2; Table 1). Five (*E. formosa*, *E. peniciliformis*, *E. scabriscula*, *E. discolor* and *E. densifolia*) of the six species examined showed low self-compatibility (ISI > 0.2; Table 1), with significantly lower proportions of viable seed per fruit (≤0.27 ± 0.08; Bonferroni adj. $P < 0.05$) within the self-pollination treatment than within the cross-pollination treatment (≥0.47 ± 0.06 proportion of viable seeds per fruit; Fig. 3). On the other hand, for *E. sessiliflora*, both self- and cross-pollination treatments yielded similar viable seed proportions per fruit (0.43 ± 0.04 and 0.36 ± 0.06 respectively; $P = 0.82$; Fig. 3D). *E. sessiliflora* having an ISI of roughly 0 (−0.16 ± 0.17), is fully capable of selfing. In all cases (six species), viable seed proportions obtained for the cross-pollination treatment (≥0.36 ± 0.06) were similar (Bonferroni adj. $P <$ 0.05), if not significantly higher (for *E. scabriscula*, Bonferroni adj. $P =$ 0.02; Fig. 3C) compared to that of the control treatment (≥0.29 ± 0.05; Fig. 3). This indicates that crossing predominates in control flowers and that the number of pollen grains carried by pollinators is relatively low.

Table 1
Index of autonomous self-pollination (IAS) and of self-incompatibility (ISI) based on mean seed set data of six *Erica* species. Mean number of total seeds (viable and unviable) produced per species regardless of treatment applied. Standard errors provided for both indices and mean number of seeds produced per species.

Species	IAS (±S.E)	ISI (±S.E)	Mean no. of seeds (±S.E)
E. formosa	0 (±0)	0.79 (±0.10)	56.90 (±2.19)
E. peniciliformis	0.11 (±0.07)	0.56 (±0.15)	35.92 (±1.77)
E. scabriscula	0 (±0)	0.91 (±0.05)	72.01 (±3.59)
E. sessiliflora	0.19 (±0.08)	−0.16 (±0.17)	86.00 (±2.47)
E. discolor	0.09 (±0.05)	0.73 (±0.08)	236.11 (±6.58)
E. densifolia	0.14 (±0.01)	0.86 (±0.02)	280.81 (±17.32)

4. Discussion

The sample of six species used in this study, indicates that *Erica* species are reliant on pollinators for effective fertilisation (low autogamy), irrespective of their pollination syndrome (bird- or insect-pollinated). The proportion of viable seeds per fruit yielded with the control flowers was higher than geitonogamous viable seed proportions and closer to cross pollination (Fig. 3). Also the variability in viable seed proportions in control flowers was low. Since low viable seed proportion per fruit is typically due to selfing, the generally high proportion of viable seeds produced by control flowers suggests that seed set was due to outcrossing facilitated by pollinators (Hirayama et al., 2005; J. Midgley, Pers. Comm. 2015). Further experiments are needed to determine the full extent of pollen limitation, in particular the number of flowers that are not visited. Seed numbers per flower were consistently higher in the bird-pollinated species (Table 1), and may compensate for the generally fewer but larger flowers found on bird-pollinated species compared to the more floriferous insect-pollinated species (Lambers et al., 2008).

For many *Erica* species, costs associated with the shift to an autonomous self-fertilising mating system may be higher than the benefits (Elle and Hare, 2002; Arendse, 2014). Favouring outcrossing instead of selfing could moreover increase seed set through higher pollen quality, and through improved seed germination and seedling survivorship (Elle and Hare, 2002). The breeding system experiment in this study reported no significant difference in proportion of viable seeds per fruit between geitonogamy and cross-pollination in one of the *Erica* species studied, *E. sessiliflora* (Fig. 3D). The ISI value further confirmed genetic self-compatibility in *E. sessiliflora* (Table 1; Steenhuisen and Johnson, 2012; Zapata and Arroyo, 1978). This particular bird-pollinated species is the only known serotinous *Erica* species (Bond and Van Wilgen, 2012). It is an obligate seeder which retains its' seeds in canopy stored ‘fruiting bodies’ until after a fire (Marais, 2012). Only in the post-fire environment will seeds of non-sprouting serotinous species be dispersed in a single pulse, taking advantage of the nutrient flush in the soil, light and space for successful seedling recruitment (Van Wilgen and

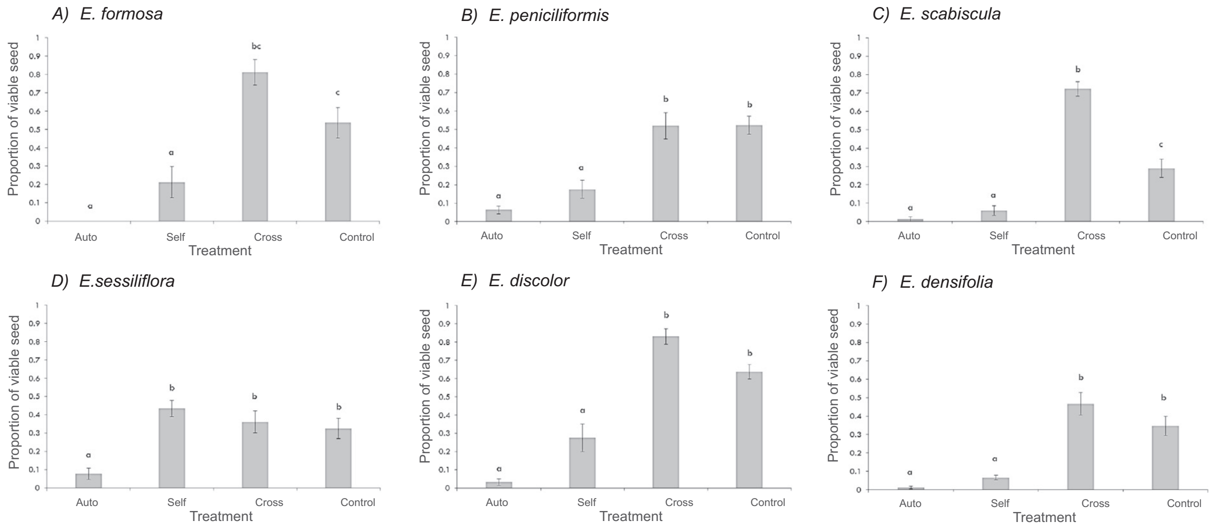

Fig. 3. Proportion of viable seed per flower of six *Erica* species obtained with four different treatments: 1. cross-pollination, 2. self-pollination with manual pollen transfer, 3. autonomous self-pollination and 4. control. Lower case letters indicate significant differences between treatments at the 0.05 level. Standard error bars are shown for each treatment.

Forsyth, 1992; Pausas and Keeley, 2014). Given *E. sessiliflora*'s unique life history trait (i.e. serotiny) and its strong fire dependence, it might have exploited or even favoured traits associated with pre-adaptation for self-compatibility as a bet-hedging strategy (Bond and Van Wilgen, 2012; Roberts et al., 2014). Therefore, increased seed production may reduce the reproductive risks associated with low pollinator abundance and genetically dissimilar conspecific plants abundance in the post fire period (Vaughton, 1995; Geerts, 2011; Roberts et al., 2014). The similar viable seed set obtained with the self- and cross-treatment in the present study (Fig. 3D) may imply a lack of preference for 'self' or 'cross' pollen in *E. sessiliflora*. Moreover, in similar fire-dependent species like the Proteaceous shrub *Grevillea macleayana*, post-germination selection in favour of outcrossed seedlings may compensate for the initial lack of parental mate choice if resources are limited when it comes to germination (Briggs and Leigh, 1996; England et al., 2003; Roberts et al., 2014). Further studies looking at genotype frequencies within seedling cohort would be needed to determine if post-germination selection occurs in *E. sessiliflora*.

While this study only examined a tiny fraction of all *Erica* species, it clearly indicates the importance of pollinators for the successful reproduction of both bird- and insect-pollinated *Erica* species. To date, more than a fourth of all *Erica* species in the CFR (>181 of the approx. 680 extant species) are considered threatened (Rebelo, 1992; Turner, 2012). Due to the interdependence of these species and their pollinators, further loss of species interactions, due to the current elevated rates of anthropogenic disturbance and landscape modifications, could eventually lead to the deterioration of ecosystem functionality (Boyer and Jetz, 2014) if no immediate conservation measures are taken.

Acknowledgements

We are grateful to SANParks, the Southern Crags and Redford conservancies, and Cape Pine for allowing us to conduct this research on their lands while under the Natures' Valley Trust banner. Daniël Cloete provided useful discussion and help in the field. We are also grateful to Alan Lee, Ross Turner, Sandy Steenhuisen and Susie Cunningham for their advice throughout the project. We are grateful to the Nature's Valley Trust for hosting this project. Funding: this work was supported by The Percy Fitz Patrick Institute of African Ornithology, University of Cape Town.

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