



Can and do ericas self-pollinate?

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Abstract

Within the Cape Floristic Region many lineages are characterised by large floral diversity. The genus *Erica* is one of these lineages, making up ~7% of the CFR. Surprisingly, even though pollinators have been suggested to be a driving force of floral morphology, the role of pollinators in the floral diversification and speciation of this genus is not yet well understood. Therefore the aim of this paper was to establish if *Erica* species can and do self-pollinate. Two *Erica* species, *E. plukenetii* and *E. urna-viridis*, were obtained from Kirstenbosch nursery, where hand-pollinations were performed on 15-20 flowers of each of three treatments (self-pollination, cross-pollination and autogamous self-pollination). Additionally pollen tube analyses were performed on seven *Erica* species, which were collected from the Constantia Mountain. In *E. plukenetii*, self-incompatibility seems to be the predominant breeding system. It appears that, like *E. urna-viridis*, most of the other species analysed, via pollen tube analyses, have the potential to self-pollinate. However, more experiments are required to establish if these species are truly self-compatible. Autogamy, on the other hand, does not appear to set seed in the species studied. Therefore it would be fair to say that some ericas *can* self-pollinate but none actually *do* self-pollinate. These results indicate that ericas have a strong dependence on pollinators for seed set and in the past speciation may have occurred due to adaptation to different pollinators, when pollinators were scarce.

Introduction

The flora of the Cape Floristic Region (CFR) is extremely distinct from that of the surrounding region, in its exceptional level of diversity (~9000 species) and its degree of endemism (68% of species, 19.5% of genera and 6 families) (Linder, 2003). These features have led to the area being recognised as one of the six floristic kingdoms on Earth (Takhtajan, 1986). For areas of similar size, whether it is temperate or tropical, the CFR has one of the highest recorded species density in the world (Cowling, 1992).

Within the CFR many large lineages are characterized by large floral diversity. Lineages that show floral rather than vegetative diversification are likely to have radiated mainly through pollinator-mediated processes rather than selection from herbivores or environmental factors (Johnson, 1996). Johnson (1996) thus suggests that adaptations to pollinators have been the driving force behind the radiation of some of the major clades which are florally extremely diverse, among others, these include *Iridaceae* (1050 species), *Ericaceae* (804 species), *Apocynaceae* (700 species), and *Orchidaceae* (470 species). Within the CFR the genus *Erica* is one of extraordinary diversity (Rebello and Siegfried, 1985) and makes up approximately 7% of the entire Cape flora. Surprisingly, the extremely florally diverse ericas have not yet been well investigated. This lack of knowledge is severely impeding our understanding of the driving forces of speciation in the Cape flora. Therefore, the logical next step is to explore the role of pollinators in the floral diversification and speciation of *Erica*.

Prior to investigating the role of pollinators within a genus, it is important to have a firm understanding of the genus' modes of reproduction. These modes (self-pollination or cross-pollination) may explain the enormous diversity in the floral structure within *Erica* and ultimately also in other genera in the CFR, in two different ways. If ericas are largely self-incompatible, it means that they need out-crossed pollen to reproduce and survive. Pollinators supply this out-crossed pollen to the flowers but if pollinators are scarce this will lead to vulnerability of the plant species. Pollen limitations may force pollinator shifts (Johnson, 1996, Johnson, 1997). Consequently, this may lead to radiation, driven by adaptation to different pollinators (Johnson et al. 1998) and may explain the diversification seen in *Erica* floral structure. Conversely, if *Erica* turns out to be largely self-compatible, there might be different processes in play, especially if species reproduce autogamously. In the case of autogamy, pollinator availability is not a

limiting factor for plant dispersal and species are free to disperse into new habitats (Levin, 2010), where species can adapt to different local environments and eventually diversify and speciate. If the latter is confirmed, this would shed new light on the speciation processes in the CFR, which thus far have been dominated by pollinator-driven mechanisms (but see Ellis and Johnson, 2010).

The main objective of this paper is, therefore, to determine if *Erica* species can and do self-pollinate. The critical information needed to support either idea outlined above is whether ericas are self-compatible and if they are, do they reproduce autonomously.

Methods

Two *Erica* species, *Erica plukenetii* and *E. urna-viridis* were obtained from Kirstenbosch nursery. The nursery has an extensive record of the history of each of their plant species. As most plants are grown from cuttings, individuals that were grown from different cuttings that originate back to the same plant cannot be used for out-crossing as this would technically constitute self-pollination. To control for this, out-crossed pollen was collected from the appropriate species in the wild. Each treatment was applied to a different individual, therefore one plant had flowers that were cross-pollinated, another only had self-pollinated flowers and the third plant had flowers that were left unmanipulated. Moreover, each species also had one individual with all three treatments (self-pollination, cross-pollination and autogamy) performed on it.

Hand pollinations were performed on 15-20 flowers of each individual for both the self-pollination and cross-pollination. To ensure that tests were done on unfertilised flowers, hand-pollination was only performed on newly opened buds, with undisturbed anthers.

Hand-pollination

To hand-pollinate a plant, the stamens were disturbed over a petri-dish and the collected pollen were applied to the stigma with forceps. Pollinated flowers were marked by means of coloured tape. Wilted flowers were scored for fruit set and seeds were counted for both self- and cross-pollinated flowers. For *E. plukenetii* it was possible to distinguish between viable and non-viable seeds, while for *E. urna-viridis* this was not possible. Scanning electron microscope (SEM) images were prepared in

order to distinguish between seeds of the self- and cross-pollination treatments. The seeds were sprinkled onto an aluminium stub coated with carbon glue, they were then sputter coated with gold/palladium alloy to make them conducive. They were then loaded into a Leo S440 scanning electron microscope and examined with the secondary detector at 243-300 magnification, a working distance (wd) of > 22mm and Kv of 10.00.

The second aspect would be to test whether the selected species are autogamous, i.e. set seed without a pollinator. This can be done by bagging flowers, and then scoring fruit and seed set when fruits have matured. However, bagging the plants may cause bag-induced pollination, which constitutes as self-pollination but not autogamy. Therefore, these tests were performed in the greenhouse, in a cage with 0.5mm mesh size, consequently excluding all major insects and thereby, bypassing the problem of bag-induced pollination.

Pollen tubes

Species were sampled across the phylogeny by collecting species from the slopes of Constantia Mountain. Additionally, pollen tube growth was analysed in seven *Erica* species (Table 2). Twenty-four hours after hand-pollination (as stipulated above) flowers were collected and the gynoecia were then removed and submerged in Carnoy's solution (1 glacial acetic: 3 95%ethanol) for two hours to arrest metabolic processes. They were then left in 70% ethanol to preserve them until staining and images could be taken using a fluorescent microscope. The stigma, style and ovary were rinsed in distilled water twice for one hour to remove all excess ethanol. This was followed by treatment with 8M NaOH (sodium hydroxide) for three hours to soften the tissue. The tissue was then rinsed again, twice for an hour, after which 20% H₂O₂ (hydrogen peroxide) was applied for 4 hours. After sufficient time had lapsed, the tissues were rinsed again, twice for an hour. Staining of the styles and ovaries enables better visualization of the pollen tubes. To prepare the stain, 21ml 1% aniline blue (0,2g Gurr aniline blue), 7ml K₃PO₄ (potassium phosphate) (1,4g grains or pellets in 7ml) and 182ml distilled H₂O were added together to make 200ml 1% aniline blue stain. The stain was left to decolour in the fridge for 12 hours before applying to the tissues. They tissues were then mounted in glycerine and a drop of the stain, and examined using a Diaphot-tmd Nikon Inverted fluorescent microscope, model Diaphot-tmd. Images were taken with the Zeiss axiocam camara, attached to the microscope. When analysing the

pollen tubes successful germination (stigma), pollen tube growth (down the style) and fertilization (in ovary) were recorded by visual inspection under the microscope.

Compatibility Indices

Following Zapata and Arroyo (1978), ratios were calculated for each species in order to determine the degree of self-compatibility and the capacity for autogamy. The index of self-incompatibility (ISI) was calculated as the fruit set of self-pollinated flowers divided by the fruit set of cross-pollinated flowers. The values obtained from this index range from 0 (fully self-incompatible) to 1 (fully self-compatible). The index of autogamous self-pollination (IAS) was obtained by dividing the fruit set from unmanipulated flowers by that of cross-pollinated flowers. These values also ranges from zero to one, where zero indicates complete dependence on pollinators for fruit set, while one indicates 100% fruit set through autogamy.

Statistical Analyses

All data collected were analysed using the statistical program, Statistica10 (StatSoft 2011). Tests for homogeneity and normality were performed on the data. Because in several cases the data were not normally distributed, and transformation did not improve this, non-parametric tests were applied to the data. Man-Whitney U tests were run comparing the seed set of the varying treatments with one another, for each species. Chi-squared (χ^2) analyses were performed on the absolute counts of fruit set, comparing each treatment of each of the two species.

Results

Fruit set

Fruit set for *E. plukenetii* and *E. urna-viridis* showed the same trend in terms of their percentage fruit set (figure 1). For both *E. plukenetii* and *E. urna-viridis* fruit set was highest for cross pollination, intermediate for self, and almost 0 for autogamy (figure 1), these differences were significant among all treatments and between cross and self (table 1). The Index of Self-incompatibility (ISI) was lower for *E. plukenetii* (0.45) compared to *E. urna-viridis* (0.75). Furthermore, it was also found that the Index of autogamous self-compatibility (IAS) denoted both species to be unable to set seed via autogamous selfing.

Seeds set

Seed set was significantly higher for cross-pollination than self-pollination in absolute number of seeds ($U= 143$, $p= 0.02$) (figure 2). There is a clear difference in the appearance between self-pollinated and cross-pollinated seeds in *E. plukenetii* (figure 4). The self-pollinated seeds appeared shrivelled and porous, while the cross-pollinated seeds appeared more inflated and firm. Seeds from cross-pollinated treatments are also larger than seeds from self-pollinated treatments (figure 3). Seed set was significantly higher for cross-pollination than self-pollination in the proportion of viable seeds ($U= 0.0$, $p< 0.00$) (figure 4). Seed set for the self-pollination treatment was significantly higher than that of the cross-pollination treatment for *E. urna-viridis* (figure 5) ($U=131$, $p>0.01$).

Pollen tubes

Generally it was found that pollen tube were able to successfully germinate and grow through the ovary regardless of the treatment (Table2; Figure 5 and 6). The only exception being, *E. hispidula*, which displayed no pollen tube growth for either treatment. Generally the small-flowered species, *E. hirtiflora*, *E. hispidula*, and *E. lutea* had the least success in pollen tube growth for the self-pollination treatment (table2).

Discussion

This is one of the first studies to quantify the ability of southern African *Erica* species to self-pollinate. The two main species looked at in this study, *E. plukenetii* and *E. urna-viridis*, had very different levels of self-compatibility. From the pollen tube data it appears that most of the *Erica* species studied can to some degree successfully self-pollinate, with the exception of *E. hispidula*. The findings indicate that the potential for self-pollination in the Cape *Erica* species could be a common phenomenon. Autogamy, on the other hand, does not appear to be a successful way of setting seed, at least for the two species studied with more detail.

Pollen tube analyses indicate that at least six of seven species can self-pollinate. The exception being, *E. hispidula*, exhibiting no pollen tube growth for either of the two treatments. It is peculiar that this species did not even show pollen tube growth for the cross-pollination treatment as this is effectively a control treatment and should show pollen tube growth if the experiment was done correctly. This may be an indication that

some error in preparation of the slides may have occurred if not during the hand-pollination of the stigmas. It may be possible that staining of the gynoecia was not successful but this seems unlikely as all stains were prepared on the same day, under the same conditions and all other species showed pollen tube growth. Another possibility is that due to the small, delicate nature of gynoecia of this species placing the cover slip may have corrupted the tissue to a degree where pollen tube recognition was impossible. Lastly, it is possible that stigmas were not yet receptive at the time of pollination; however, stigmas were assessed for maturity before application of self or cross pollen. Additionally, due to the lack of studies done with aid of pollen tubes, there is some uncertainty about the pollen tube actually fertilising the ovule. Nonetheless, in many of the species analysed there appears to be successful fertilisation of the ovaries, regardless of the treatment. It therefore appears that the hypothesis that most Cape *Erica* species cannot self-pollinate has been falsified and that most species have the potential to set seed via self-pollination.

Although self-pollination appears to be possible, fruit set data from hand pollination suggested that fruit set was somewhat reduced in both species for the self-pollination treatment. Cross-pollination treatment possibly had a higher fruit set due to self-incompatibility allowing for the avoidance of inbreeding depression and consequently reduced gene flow. Inbreeding depression results from a loss of fitness due to increased inbreeding (Petanidou *et al.*, 1995).

An anomaly in the data did occur, with *E. urna-viridis* exhibiting a significantly higher seed set for the self-pollination treatment compared to the cross-pollination treatment. This gives rise to the possibility that bias occurred in the sampling method. Due to the lack of specimen available for the study, each treatment was allocated to an individual plant rather than applying all treatments to multiple individuals. This in fact resulted in the flowers being pseudoreplicates instead of independent statistical replicates. This can severely affect the data obtained from the experiments, for instance, if one of the individuals plant were of poor health this would affect all the replicates on that specific plant, possibly resulting in lower seed set for all flowers on that plant. Consequently, the patterns illustrated in the results may not reflect the true population mean of each species.

The incompatibility index suggests that although *E. plukenetii* can set seed via self-pollination, the proportion of viable seed produced are severely reduced. This is most probably due to the ability of an individual to prevent self-fertilisation by recognising and subsequently rejecting pollen with the same genetic make-up as itself (Wright *et al.*, 2010) to prevent inbreeding depression. Alternatively, this may also be explained by early acting inbreeding depression but distinguishing between the two is very difficult (Seavey and Bawa, 1986).

The adaptive radiation of *Ericaceae* in the Cape region is one of the more striking features of speciation in the Cape. Many scientists maintains that it is the low nutrient quantity of the soils found in the region that drives this high level of diversity (Cowling *et al* 1996, Goldblatt and Manning 2002, Linder 2003, Pauw 2007). Nevertheless, Cowling *et al* (1990) suggested it is difficult to reconcile the theory that speciation in *Erica* occurred solely based on adaptation to soil types due to the uniform nature of the vegetative structures but high floral diversity exhibited by the genus. In some species, such as *E. plukenetii*, self-incompatibility seems to be the predominant breeding system. It appears that, like *E. urna-viridis*, most of the other species analysed have the potential to self-pollinate. However, more experiments are required to establish if these species are truly self-compatible. Autogamy, on the other hand, appears to be less effective for setting seed in the species studied. Therefore it would be fair to say that some ericas *can* self-pollinate but none actually *do* self-pollinate.

How do these results affect our view on pollinator-driven speciation in Erica?

There is a strong dependence on pollinators for successful seed set. Consequently, any radiation of floral form that has occurred would most probably be due to adaptation to different pollinators caused by scarcity of these vectors. Being able to self-pollinate rather than setting seed autogamously would result in even sparsely distributed individuals experiencing successful fertilisation. Pollinators do not need to transport pollen from another individual but instead are only required to collect and deposit pollen on the same individual. This would possibly allow small populations to persist in remote places, promoting isolation and consequently resulting in speciation. If selfing but not autogamy is possible in a species, speciation could perhaps also have occurred with a single dispersal event, where one selfed seed could have developed in a genetically uniform population, whereas for cross-pollination to be successful at least

two genetically different individuals are needed. If such a dispersal event is rare enough to maintain the population in isolation and local adaptation were to take place this could result in differentiation, consequently, promoting speciation and resulting in the amazing diversity seen in *Erica* species in the Cape.

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Figures and Tables

Table1: Results of the Chi-squared (χ^2) test for associations between treatments. Significant differences are indicated in bold font.

Species	Comparing Three Treatments			Cross-pollination vs. Self-pollination			Indices	
	Chi-squared (χ^2)	p	d f	Chi-squared (χ^2)	p	df	ISI	IAS
<i>E. plukenetii</i>	27.31	1.18x10⁻⁶	2	12.21	0.000	1	0.45	0.00
<i>E. urna-viridis</i>	80.00	5.26x10⁻¹⁹	2	7.07	0.004	1	0.75	0.04

ISI=index of self-incompatibility, IAS=index of autonomous self-pollination.

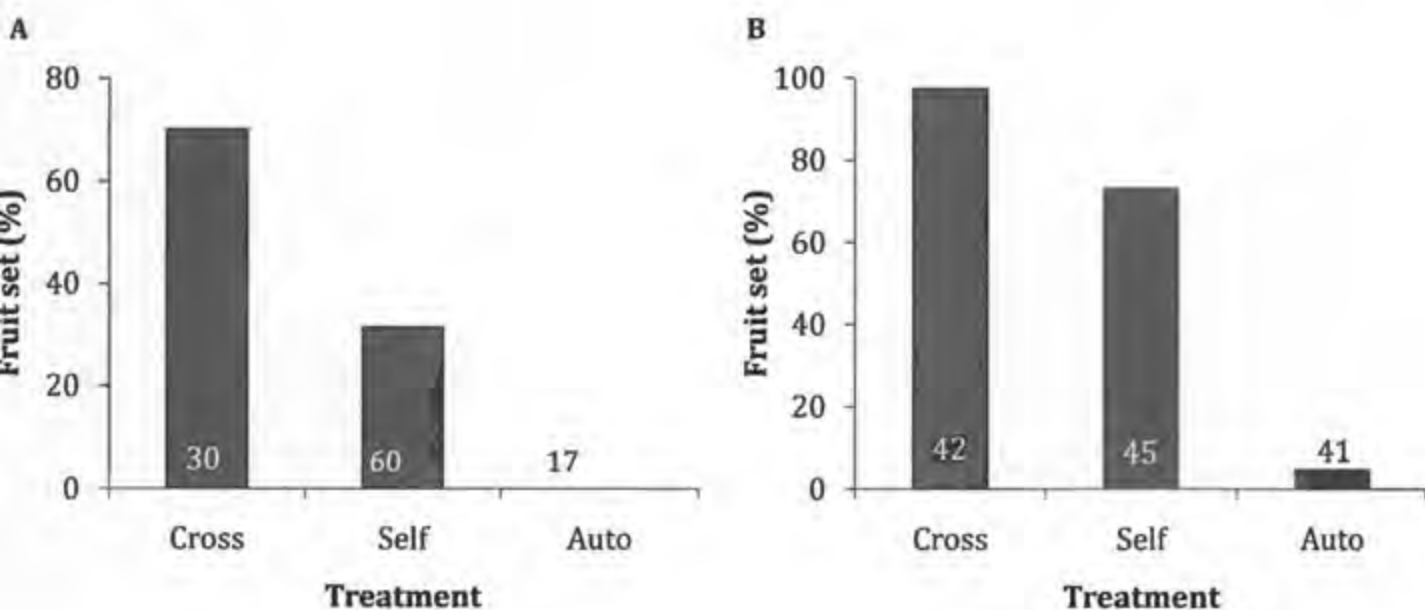


Figure 1:

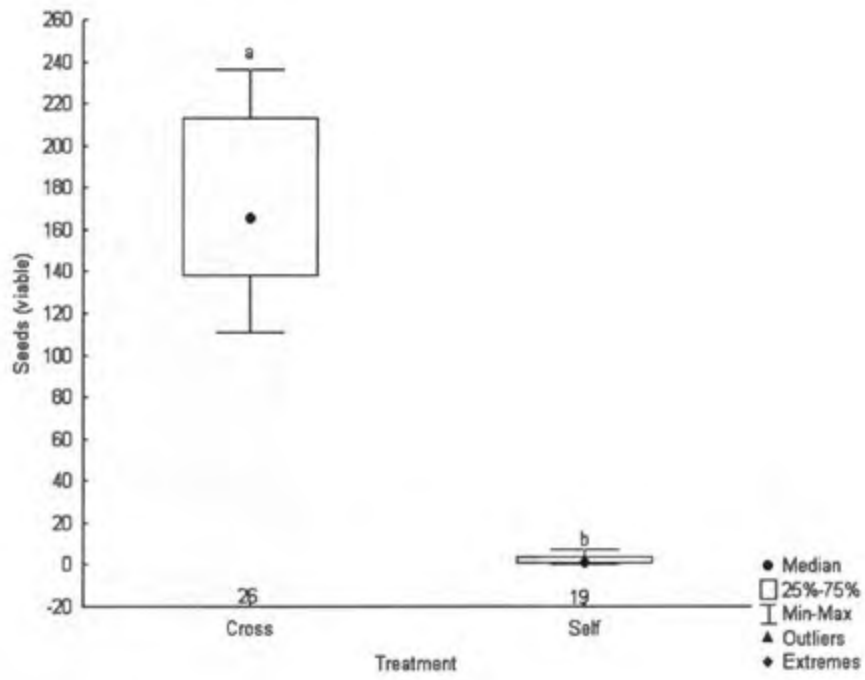


Figure 2

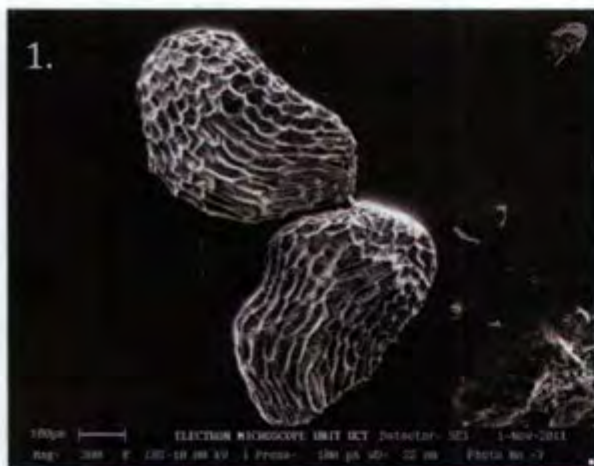


Figure 3

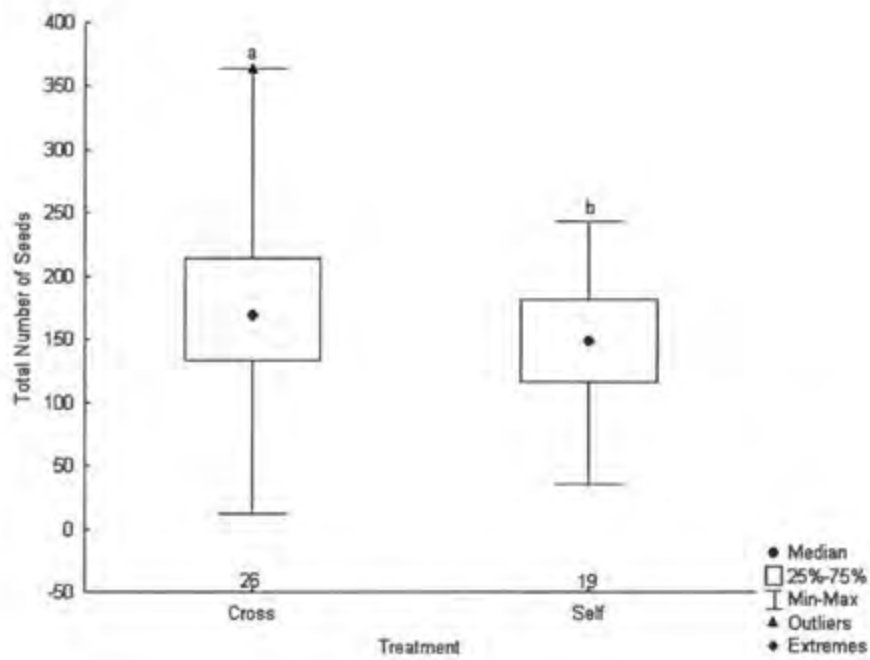


Figure 4:

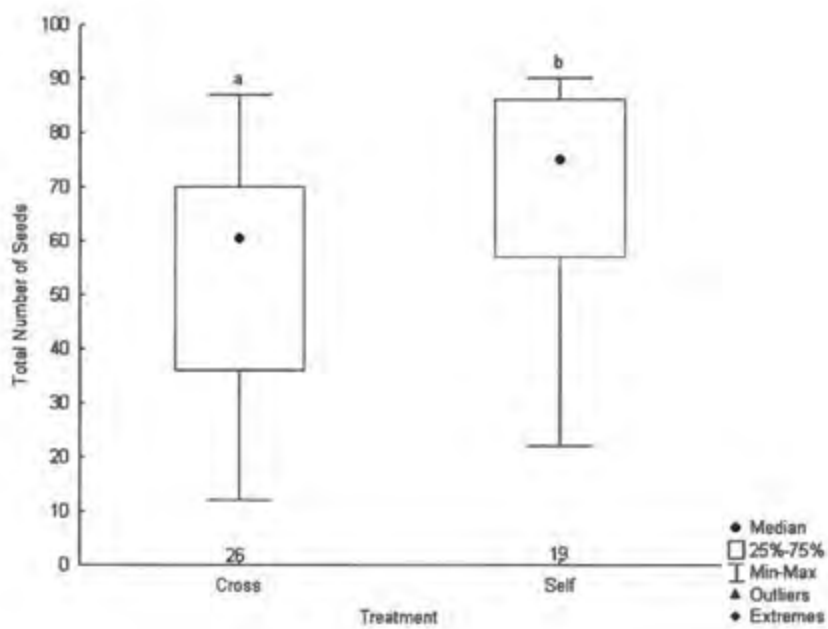


Figure 5:

Table 2: Results for the analyses of pollen tubes of seven different *Erica* species. Different stages of pollen tube growth in the stigma, style and ovary was scored 'Yes' for presence of pollen tube or 'No' for absence.

Species	Treatment	Pollen tube presence						
		Stigma		Style		Ovary		
		Yes	No	Yes	No	Yes	No	Inconclusive
<i>E. abietina</i>	Cross	3	0	3	0	3	0	0
	Self	2	0	2	0	2	0	0
<i>E. coccinea</i>	Cross	4	0	4	0	1	3	0
	Self	2	0	2	0	2	0	0
<i>E. hirtiflora</i>	Cross	4	0	4	0	0	1	3
	Self	1	2	1	2	1	2	0
<i>E. hispidula</i>	Cross	0	2	0	2	0	2	0
	Self	0	2	0	2	0	2	0
<i>E. lutea</i>	Cross	4	0	4	0	3	0	1
	Self	2	2	2	2	1	3	0
<i>E. plukenetii</i>	Cross	2	0	2	0	2	0	0
	Self	2	0	2	0	1	0	1
<i>E. urna-viridis</i>	Cross	5	0	5	0	5	0	0
	Self	2	0	2	0	1	0	1

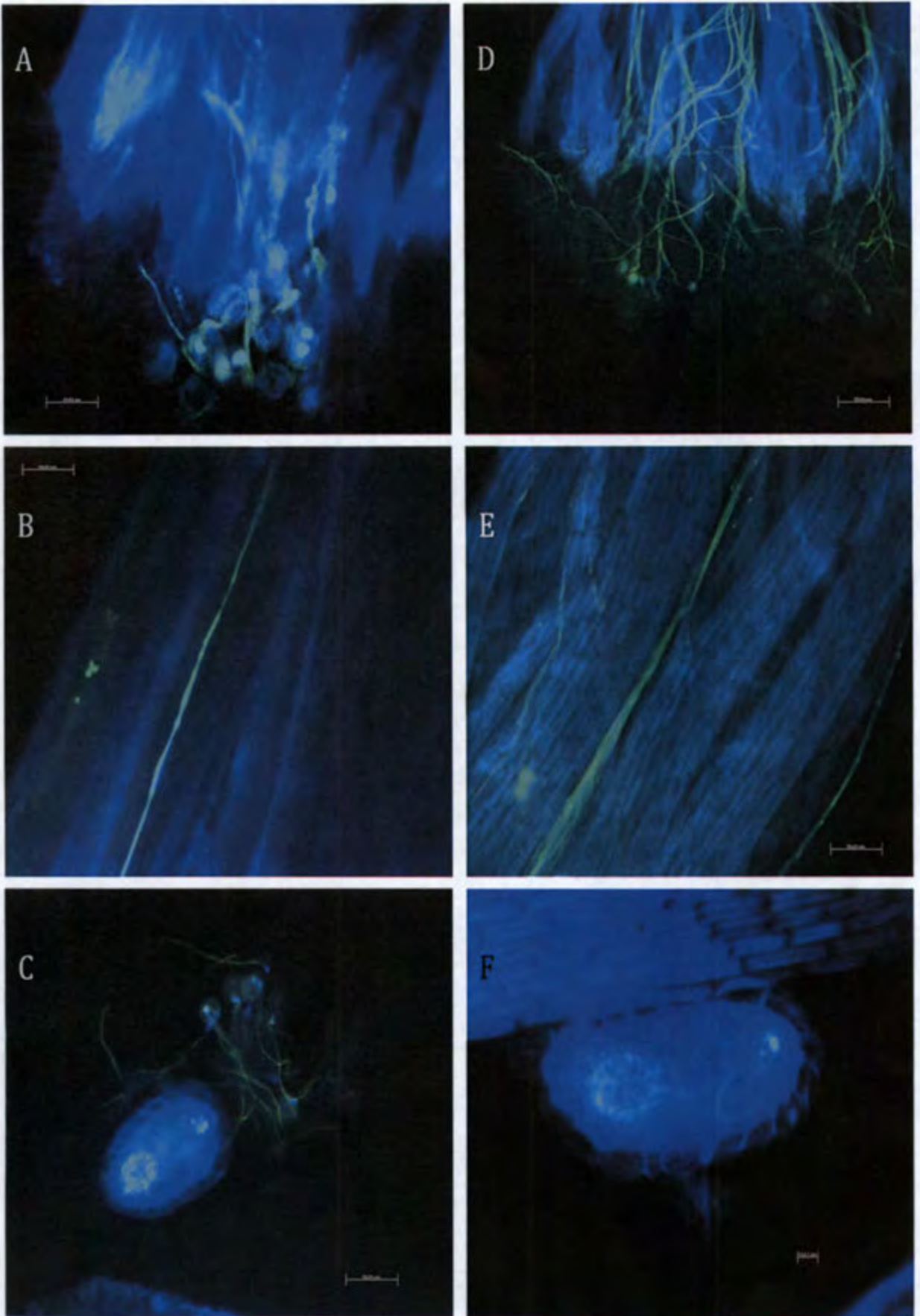


Figure 6:

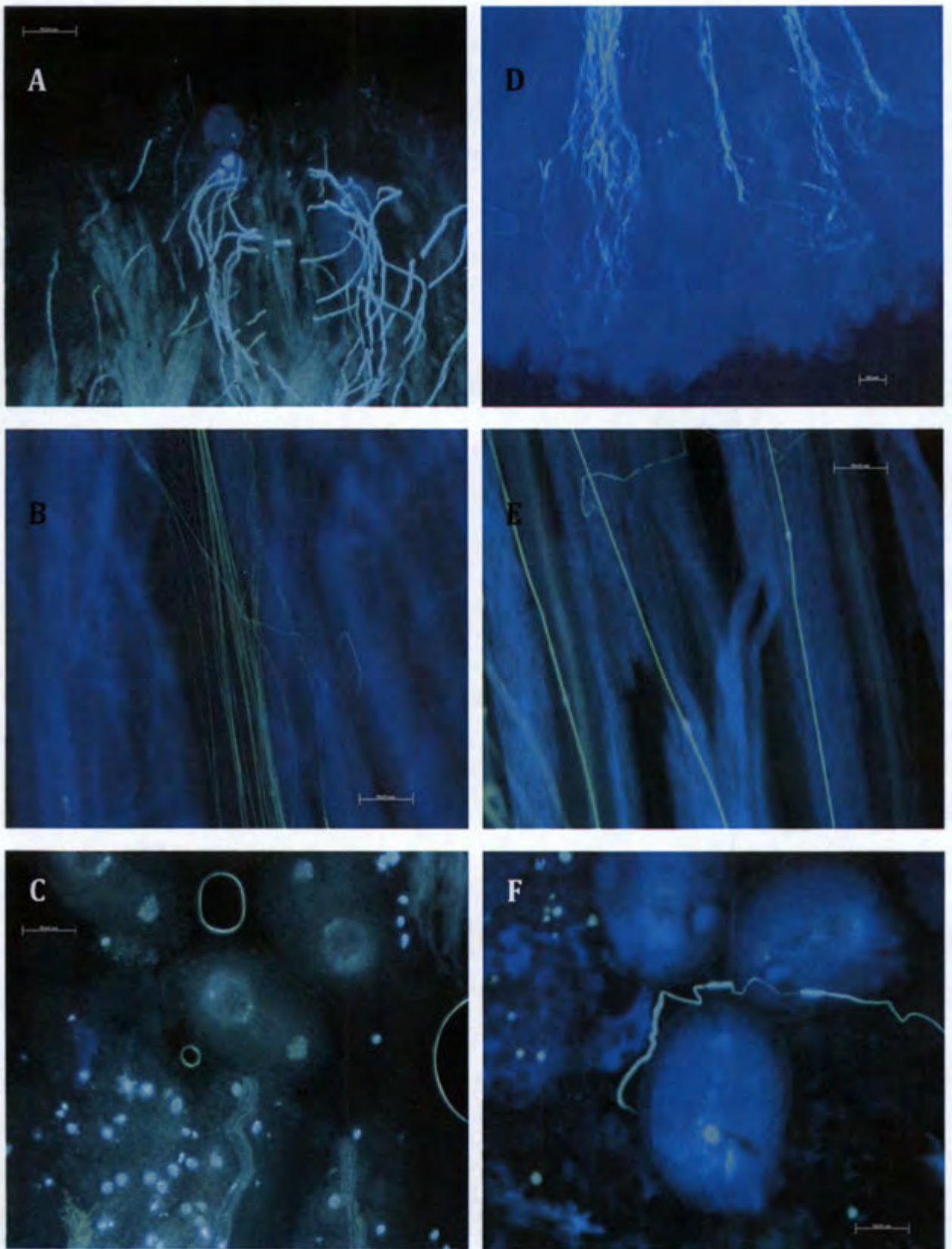


Figure 7:

Figure 1: Comparisons of fruit set frequencies of different treatments (cross-pollination, self-pollination and autogamy) for A. *E. plukenetii* and B. *E. urna-viridis*. Sample size (N) of each treatment is indicated at the bottom of the bars.

Figure2: box plots illustrating the proportions of the total number of seeds obtained from the fruits of *E. plukenetii*, for both Cross- and Self-pollination. Letters were used to show the differences in seed set for each treatment. Different letters indicate a significant

Figure 3: Scanning electron microscope images of the seeds produced by the different treatments. A. Seeds from self-pollination. B. Seeds from cross-pollination. C. Seeds from both treatments, showing the size difference. The smaller seed from self-pollination is highlighted with an arrow.

Figure 4: box plots expressing the viable seeds as raw counts obtained from the fruits of *E. plukenetii*, for both Cross- and Self-pollination. Letters were used to show the differences in seed set for each treatment. Different letters indicate a significant

Figure 5: Seeds set of *E. urna-viridis*, for both Cross- and Self-pollination. Different letters allocated above the boxes indicate a significant difference between the treatments. Sample sizes (N) are indicated below the boxes.

Figure 6: illustration of the different stages of pollen tube growth for *E. lutea*. A-C represents the self-pollination treatment, D-F represents the cross-pollination treatment. A and D denotes the germination stage on the stigma, B and E show the growth of the pollen tube down the style and C and F indicate fertilization of the ovules within the ovary.

Figure 7: illustration of the different stages of pollen tube growth for *E. urna-viridis*. A-C represents the self-pollination treatment, D-F represents the cross-pollination treatment. A and D denotes the germination stage on the stigma, B and E show the growth of the pollen tube down the style and C and F indicate fertilization of the ovules within the ovary.