



University of Cape Town

*Variation in Breeding Systems
and Consequences for
Reproductive Traits in Erica*

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Plagiarism declaration

I, Brittany Arendse,

know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own.

Signed by candidate

Brittany Arendse

November 2014

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Abstract

Erica makes up 7% of all species in the Cape flora. It is the most species-rich genus in the Cape Floristic Region (CFR), which has an area of 90 000km². *Erica* species have great inter- and intra-specific variation in floral form as well as in post-fire regeneration strategies. Previous studies of other plant groups (Barrett et al. 1996; Button et al. 2012) have illustrated changes in floral traits with a shift from outcrossing to selfing. The aim of this thesis was to determine whether similar changes occur in *Erica*. I thus analysed breeding systems in *Erica* in relation to floral traits, pollinators, and fire survival strategies.

Seed and fruit set data obtained from hand-pollination treatments suggested that self-fertilization occurs rarely. Autonomous selfing did not generally yield significant seed set. When comparing changes in floral traits with increased selfing ability and pollination syndromes weak relationships were observed. Average plant height showed a positive relationship with selfing ability but this correlation was not significant. Comparing traits within small and large flowered species separately yielded significant relationships between corolla size and selfing ability of small-flowered species.. Furthermore, general trends indicate that small-flowered species have increased ability to self-fertilise when corolla sizes are larger, corolla apertures larger and herkogamy reduced. Large-flowered species employ the same strategy but average corolla aperture is reduced. A trade-off between the size and the number of flowers was seen within the *Erica* genus. Small-flowered species had significantly more flowers compared to larger-flowered species that had markedly fewer flowers per unit height. This finding has implications for the selfing potential of small-flowered species as increased self-incompatibility may have evolved in order to reduce the effects of increased geitonogamy due to increased floral number.

The prediction that self-fertilisation would be increased in seeders compared to resprouters, on the basis that seeders are more reliant on seeds for persistence than resprouters, was not realised when comparing the selfing ability of different fire-survival strategies. *Erica mammosa*, a species with morphs possessing both fire-survival strategies, shows no significant differences in selfing ability, this includes differences in pollen-ovule (P/O) ratios. However, indices suggest the resprouting form of *E.*

mammosa to have an increased ability to self-fertilize while the seeder form has an increased ability for autonomous selfing. The 29 species analysed were divided into outcrossers and facultative outcrossers based on selfing indices but these did not fit neatly within Cruden's proposed P/O ranges (facultative autogamy: P/Os= 32-397; facultative outcrossers: P/Os= 160.7 - 2258.6; outcrossers: P/Os= 1062 - 19525). This may be due to his classification of species into breeding systems being based on relatively few distantly related species per category with extremely variable P/O ratios per category. For example, outcrossers ranged an order of magnitude (from 1000+ to 20 000). It is also true that this ratio can be influenced by a variety of different factors, these include: habitat, evolutionary history and pollination syndrome. Consequently, P/O ratios in *Erica* do not seem to reflect pollination syndromes very well. Presumably, sex allocation theory may explain the relationship of breeding system with P/O ratios better.

Histological studies of pollen tube growth for self- and cross-pollinated flowers of eight species suggested that *Erica* has late acting self-incompatibility (LSI). LSI is a barrier to selfing that occurs in the ovary. However, I could not determine if the rejection process occurs pre- or post-fertilization.

Although, a large amount of knowledge is still lacking, this preliminary study provided insight into the reproductive biology of *Erica*.

General Introduction

The process of plant reproduction, from pollination, through seed production, to the recruitment of a new generation of reproductive individuals, is extremely variable across the angiosperms. First, there is large variation in whether a pollen vector is required, whether seed production requires fertilization of ovules by pollen (e.g. fertilization versus apomixis) (Richards, 2003), whether plants can be fertilised by self-pollen (self-compatibility versus self-incompatibility) (Arroyo, 1976.). To help conceptualise these factors, a distinction can be made between a plant's breeding system (defined as the potential the individual has for different types of mating) and mating system (defined as the realised level of mating that actually occurs in nature). Second, whether seeds develop properly and grow to maturity to become reproductive individuals themselves can depend on the presence of inbreeding depression (decrease in survival rate of offspring due to mating with a genetically similar partner) (See Figure1).

Angiosperms possess an exceptional level of versatility when it comes to mating systems. Mating systems are largely determined by three characteristics of flowering plants: 1) their immobile nature – this sessile habit has necessitated pollen-transporting vectors for successful fertilisation (Barrett and Harder 1996); 2) their hermaphroditic flowers – having sexual structures in such close proximity provide opportunities for selfing, often at the expense of outcrossing (Barrett and Harder 1996; Barrett 1998). 3) Pollen and ovules are packaged in different structural and temporal combinations at different levels, from the individual flower to the level of the population (Barrett and Harder 1996). Pollination is fundamental as a determinant of fitness (Barrett 2002). It also generates mating patterns by determining mating opportunities (Barrett and Harder 1996). However, not all floral diversification is due to pollinator action; floral modification can also arise in response to reducing the often fatal (for progeny) consequences of self-fertilisation and promoting outcrossing (Barrett 2003). Long term survival of plant populations rely on both biotic and abiotic factors but ultimately the goal is to reproduce and sire successful offspring (Schneemilch and Steggle 2010).

Plants have multiple ways of ensuring seed set in differing circumstances. Most plants cross-pollinate (Mahy and Jacquemart, 1998; Herlihy and Eckert, 2002), whereby pollen

is removed from the anthers of one individual and deposited on the stigma of a different individual. Cross-pollination ensures genetic variability of offspring and thus promotes the persistence of populations. Alternatively, self-pollination, the movement of pollen within an individual plant, if it leads to self-fertilization reduces genetic variability, perpetuating deleterious alleles and results in inbreeding depression. Autonomous selfing, automatic self-fertilization without the use of pollination vectors, may be useful in environments where pollinators are scarce; this mode of fertilization promotes reproductive assurance (Johnston and Schoen 1996; Runions and Geber 2000; Fausto et al. 2001; Elle and Hare 2002; Herlihy and Eckert, 2002; Goodwillie and Ness 2005). There are three types of selfing and although all may provide reproductive assurance, they differ in the cost they incur. Prior selfing (occurs prior to outcrossing) or competing selfing (occurs concurrently with outcrossing) can increase pollen and ovule discounting, reducing the pollen and ovules available for cross-fertilization. Delayed selfing (occurs after opportunities for cross-fertilization has passed) does not increase pollen and ovule discounting and can provide reproductive assurance (Elle and Hare, 2002). Self-fertilisation has an additional cost, other than inbreeding depression, termed pollen discounting (see Barrett 1998). This phenomenon reduces parental fitness; the pollen used for self-pollination is lost and cannot be used for cross-pollination. Consequently, there are mechanisms that reduce or prevent self-fertilisation (Silva and Goring 2001; Barrett 1998).

Self-incompatibility (SI) occurs in 39% of species in over 100 families across the angiosperms (Igic et al. 2008). Two types of SI systems are recognised (Lewis 1979): 1) Heteromorphic incompatibility, entails morphological disparity in structures resulting in distyly or tristly as well as genetically-controlled physiological mechanisms that prevent pollen from a different morph from fertilising ovules; and 2) homomorphic incompatibility entails genetically-controlled physiological mechanisms that prevent successful pollen germination on the stigma (sporophytic self-incompatibility (SSI)) (Barrett 1988; Sage et al. 1999) or tube growth down the style (gametophytic self-incompatibility (GSI)) (Seavey and Bawa, 1999; Gibbs and Bianchi, 1999). Late-acting self-incompatibility is a third self-pollen rejection mechanism that operates in the ovary (Sage et al. 1994; Lipow and Wyatt 1999; Sage et al. 1999). These mechanisms along with inbreeding depression are believed to cause self-sterility (Sage et al. 1999). Theoretically, distinguishing the different mechanisms of homomorphic SI is simple but

in practice it become more difficult, especially in instances where rejection occurs in the ovary (Sage et al. 1999). This is probably due to the similarities in the consequences of LSI and early-acting inbreeding depression (EAID), both resulting in a reduced seed set when selfing occurs (Bittencourt and Semir, 2005). However, criteria have been developed to assist in identifying LSI from EAID. Initially, late-acting self-incompatibility (LSI), SI systems that operate in the ovary, were believed to be quite rare in angiosperms, but more and more instances of this mechanism have been documented in recent years (Kenrick et al. 1986; Sage et al. 1994; 1999; Gibbs and Bianchi 1999; Bittencourt et al. 2003; Sage and Sampson, 2003; Bittencourt and Semir, 2005; Vaughton et al. 2010). Lewis (1979) suggested that LSI is a rare phenomenon in nature because it is such a costly way to prevent selfing; it causes ovule discounting when self-pollen tubes fertilize ovules and these ovules are ultimately lost due to reduced fitness (Vaughton et al. 2010). Alternatively, Kenrick et al. (1986) and Sage et al. (1994), argued that the reason for LSI being rare is not due to a scarcity of the phenomenon in nature but rather due to researcher preference for studying SI systems in short-lived herbaceous species, in which the mechanism is not that prevalent. Additional to SI systems, many floral traits evolve to reduce self-pollination as well as promote pollen export (Barrett 1998).

Plants display substantial inter- and intraspecific variation in floral traits that influence mating patterns (Barrett et al. 1996). Evolutionary shifts in mating systems from predominantly outcrossing to obligate selfing has occurred numerous times in angiosperm history and accompanying these shifts are changes in floral characteristics, ecology and life history strategies (Barrett et al. 1996; Button et al. 2012). Increased herkogamy (spatial separation in anther and stigma) and dichogamy (temporal separation in anther and stigma) are some of the main features that signify a shift from self-pollination to cross-pollination. Furthermore, outcrossing species are often self-incompatible, large and have large showy flowers. Alternately, selfers have small flowers and are largely self-compatible and less attractive than outcrossers (Lyons and Antonovics 1991; Runions and Geber 2000; Jacquemart 2003). Pollen-ovule (P/O) ratios are another such trait that is suggested to be reduced in selfing species compared to outcrossers (Cruden 1977). Cruden (1977) hypothesised that P/O ratios reflect the pollination efficiency - the probability of sufficient pollen reaching the stigma to result in maximum seed set of a species. Pollination is easily realized for self-pollinating

species and therefore, requires few pollen grains per ovule, whereas outcrossing is uncertain due to the reliance on pollinators and may require more pollen per ovule. However, this 'efficiency' hypothesis is still very controversial, with many studies either supporting (Cruden 1977; Cruden and Jensen 1997; Spira 1980; Cruden and Miller-Ward 1981; Preston 1986; Vasek and Weng 1988; Jürgens et al. 2002; Schneemilch and Kokkinn 2011) or opposing (Schlising et al. 1980; Arnold 1982; Böcher and Philipp 1985; Pellmyr 1985; Gallardo et al. 1994) this tentative indicator of plant breeding systems. Charnov (1982) provided the most compelling opposition for the 'efficiency' hypothesis. He argued that one cannot equate a plant's reproductive success with seed production (i.e. female function) alone and interpreted the relationship between P/O ratios and breeding systems as one of optimal resource allocation to sex. The sex allocation theory suggests that hermaphroditic plants that set seed autonomously will reduce resources allocated to the production of pollen and increase allocation to seeds. Similarly, plants that are predominantly outcrossing will assign more resources to pollinator attraction, as outcrossing is heavily dependent on pollinators for seed. The P/O ratio is thus an approximate indicator of differing resource allocation to the sexual function and indirectly correlates with breeding systems. Floral traits influence pollinator activity and visitation and concurrently with post-pollination mechanisms, like SI systems, comprise the key elements of mating systems in angiosperms (Barrett 2003).

Johnson (1996) suggests that adaptations to pollinators have been the driving force behind the radiation of some of the Cape's major plant clades, which are florally extremely diverse. If this is the case for *Erica*, we can use variables such as floral form as a proxy for different pollination systems. The genus *Erica* could be a model system for investigating patterns of variation in breeding systems and their correlates, as well as the post-pollination mechanism, such as self-incompatibility systems, that operate to ensure out-crossing. In the Cape Floristic Region (CFR) *Erica* makes up approximately 7% of the total flora and consist of approximately 680 species out of a global count of about 840 (Pirie et al. 2011). *Erica* is a large clade consisting of closely related sisters with an extreme diversity of pollination guilds.

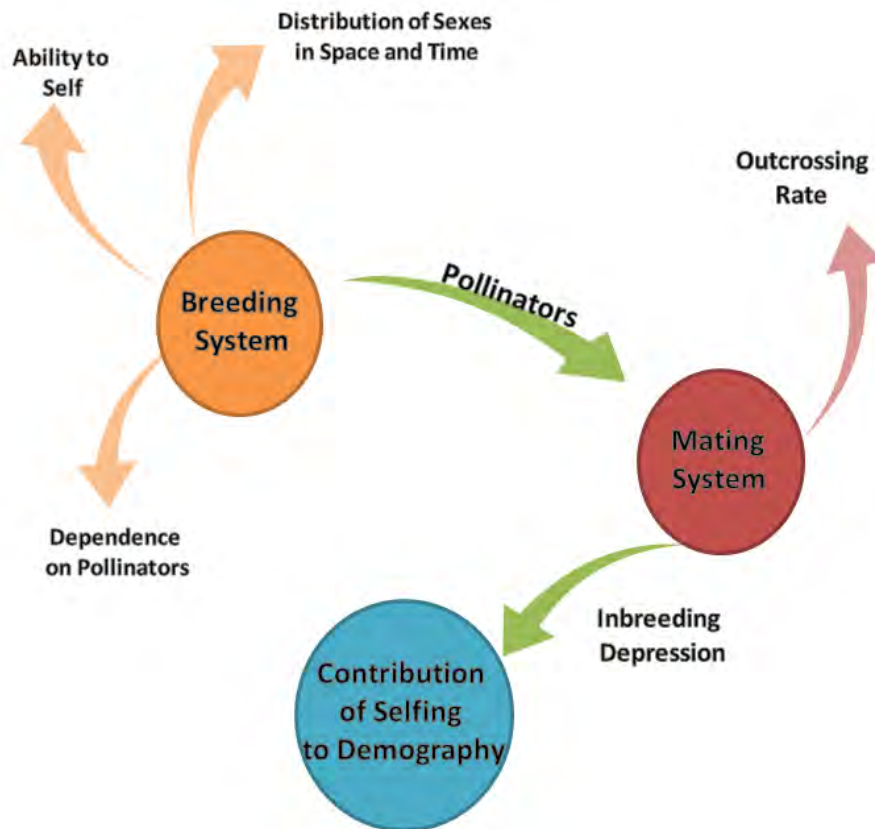


Figure 1: Interaction between breeding systems, mating systems and inbreeding depression for the persistence of individual plants.

Thesis Structure

This thesis is arranged in three chapters. Some unavoidable repetition will occur as my aim is to publish each separately. All the data were collected and analysed by myself, with input from Professors Jeremy Midgley (University of Cape Town) and Steve Johnson (University of Kwa-Zulu Natal).

Chapter 1: Breeding system variation and ecological correlates in the genus Erica

Research Questions

- 1) What is the spectrum of selfers to outcrossers in a section of Cape *Erica* species?
- 2) Do shifts in the breeding system coincide with changes in floral form?
- 3) Does the breeding systems of a species influence its pollination syndrome and fire-survival strategy?

Procedure

This chapter was based on a field study of a variety of species located on the Cape Peninsula. I performed hand-pollination treatments (self-pollination, cross-pollination

and autonomous self-pollination) and obtained fruit and seed set data. The data was used to draw conclusions about the degree of selfing within different species and the level of autonomous selfing. To ascertain if increased selfing would cause shifts in different variables, an index of self-incompatibility was calculated and compared to traits, pollination systems and post-fire responses.

Chapter 2: Late-acting self-incompatibility in *Erica*?

Research Questions

- 1) Is self-incompatibility early or late in *Erica*?
- 2) Is there a difference in the rate of pollen tube growth between selfed and crossed pistils?

Procedure

Flowers of different *Erica* species were self-pollinated and cross-pollinated by hand and pollen tubes were left to grow down styles for differing periods. The gynoecia were then collected and prepared for staining. After mounting the gynoecia they were viewed with a fluorescent microscope to determine positive tube growth between treatments.

Chapter 3: Pollen-ovule ratios in the genus *Erica*

Research Questions

- 1) Are P/O ratios a dependable indicator of breeding system?
- 2) Are there any correlations between P/O ratios, pollination syndromes and fire survival strategy?
- 3) Which theory best explains the data obtained, the 'efficiency' hypothesis (Cruden, 1977) or sex allocation theory (Charnov, 1982)

Procedure

Acetolysis was performed on flowers of *Erica* species to obtain pollen number. By dissecting ovaries, ovule number could be obtained. The P/O ratio along with a morphological outcrossing index (MOI), based on floral traits, was used to ascertain the relatedness between P/O ratios and breeding systems pollination syndromes and fire-survival strategies.

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Chapter I

Breeding System Variation and Its Floral and Ecological Correlates in *Erica*

Variation in breeding and mating systems and the presence of inbreeding depression is not scattered randomly across the angiosperms. Breeding systems, for instance, can depend on whether the species is rare or widespread; or whether species are pollinator specialists or generalists. In theory, as an outcrossing plant species becomes more rare it should experience a reduction in pollinators and in conspecifics to mate with. Changes such as these can also select for alterations in the plant's usual breeding systems (Tepedino 1979; Sipes and Tepedino 1995). This is especially true for species with specialised pollination systems (Tepedino 1979). Similarly, the mating system is also likely to change because of reduction in the number of mating partners and altered behaviour of pollen vectors.

Flowering plants have diverse traits that enhance the chances of pollinator-mediated outcrossing (Button et al. 2012). Evolutionary shifts in mating systems from xenogamy (outcrossing) to geitonogamy (self-fertilisation) have occurred numerous times in flowering plants and are expected to be associated with a change in floral biology, life history and ecology (Barrett et al. 1996; Button et al. 2012). Outcrossing plants are self-incompatible, protandrous, with noticeable stigma-anther distances; they are large individuals with large showy flowers and high pollen ovule (P/O) ratios (Lyons and Antonovics 1991; Goodwillie and Ness 2005). Alternatively, self-fertilising plants may show reduced temporal (dichogamy), as well as spatial separation (herkogamy) of male and female function, flower size and attractiveness and low P/O ratios (Cruden 1977; Lyons and Antonovics 1991; Runions and Geber 2000; Jacquemart 2003). According to Button et al. (2012), this reduction of traits most likely occurs due to the high cost to produce and maintain them and the fact that when individuals self-pollinate the gain in fitness with investment in attraction is reduced. As the ability to self-fertilise increases, pollinator attraction is not required as pollination occurs in the absence of pollinators, any investment in attraction would thus be wasteful and reduce the fitness gain of the species. Many studies have shown that outcrossing is a positive co-variate of floral size (Schoen, 1982; Morgan and Barrett 1989; Lyson and Antonovics 1991; Johnston and

Schoen 1996; Armbruster et al. 2002; Goodwillie and Ness 2005; Tomimatsu and Ohara 2006) and floral display size (Schoen 1982, Morgan and Barrett 1989; Tomimatsu and Ohara 2006; Goodwillie et al. 2010). Furthermore, floral attractiveness is a function of flower size, number and arrangement on the plant and thus plays a major role in pollinator attraction, mating patterns and reproductive success (Worley et al. 2000). However, the benefits of producing multiple flowers occur at the expense of flower number due to resource limitation (Worley and Barrett 2000). Morgan (1998) conducted an empirical study of the trade-off between flower size and number in *Claytonia virginica*. Along with other studies (Stanton 1988) Morgan (1998) failed to find a significant relationship between the two traits. However, Sato and Yahara (1999) called upon the fact that *C. virginica* is a perennial species to explain the absence of such a trade-off. Alternatively, many studies also support a trade-off between flower size and number (Schemske and Argen 1995; Sato and Yahara 1999; Worley 2000; Worley and Barrett; Sargent et al. 2007). Sargent et al. (2007) argues that trade-offs between flower size and number are not always seen due to the complexity of these traits, and because they are influenced by various life history and ecological factors. For instance, species that are predominantly self-fertilising, may select for smaller flowers (Lyons and Antonovics, 1991; Goodwillie and Ness, 2005), furthermore changes in pollination mode may also affect flower size as well as number (Weller et al. 2005).

There are many other plant traits that are important components of pollinator attraction and very little consideration has been given to the evolutionary fate of these traits during shifts in the mating system from outcrossing, for which pollinator attraction is essential, to predominant selfing, for which it is much less important (Button et al. 2012). Lloyd (1987) produced a quantitative selection model trying to explain the decreasing expenditure on pollination mechanisms as self-fertilisation increases. It was found that as autogamous self-fertilisation increases, the evolutionary stable strategy allocation to seeds increased while the allocation to pollen and pollination mechanisms simultaneously decreased. Furthermore, Button et al. (2012) analysed attractiveness traits and their relationship with the mating systems of *Camissoniopsis cheiranthifolia*. Their results were consistent with the consequences of selection having favoured reduced investment in petal colour in populations that were mainly self-pollinating.

A particularly powerful approach to studying patterns of variation in breeding-/mating systems and their correlates is by looking at a large clade, and assessing repeated instances of associations between certain breeding/mating systems and other ecological correlates (Igic and Kohn 2006; Button et al. 2012; see also Goodwillie et al. 2010). Subsequently, one can determine if and/or where these shifts may occur. *Erica* would be a model genus for this investigation as it consists of a large number of closely related species with extremely diverse pollination systems. The genus *Erica* makes up about 7% of the Cape Flora and is extremely florally diverse but not well studied at all in terms of reproductive biology. Pirie et al. (2011) recently published the first phylogenetic analysis of *Erica*. This preliminary phylogeny indicates widespread floral convergence and divergence with adaptations to various pollinators, across the phylogeny.

When pollinator are lacking in an environment, breeding system shifts may occur in order to ensure reproductive success (Goodwillie and Ness 2005; Johnston and Schoen 1996). Elle and Hare (2003) and Goodwillie and Ness (2005), suggest that type and timing of selfing as well as the environment in which it occurs are important when weighing the benefits and costs of shifting to a self-fertilizing mating system. Type is important because there are different modes of self-pollination. Geitonogamy (self-pollination) will not provide reproductive assurance, as pollen movement within individuals requires a vector to transfer pollen from the male to female sex organs. Autonomous selfing, fertilization without the physical transfer of pollen by a pollen vector, provides reproductive assurance when pollinators are few but the timing of this fertilization is crucial as it involve different costs (Elle and Hare 2002). Prior selfing (occurs before opportunities for outcrossing) or competing selfing (occurs concurrently with outcrossing) can increase pollen and ovule discounting, reducing the pollen and ovules available for cross-fertilization. Delayed selfing (occurs after opportunities for cross-fertilization has passed) does not increase pollen and ovule discounting and can provide reproductive assurance. Consequently, the benefits of selfing may be dependent on the mode and timing of self-fertilization. The environment also plays an important role as it can affect pollinator availability and floral morphology, which in turn can affect pollinator visitation and the resulting mating systems (Elle and Hare 2002).

Reproductive correlates such as seed production and the potentially associated ability of plants to autonomously self-fertilise may rely on life history traits. Runions and Geber (2000) stated that related outcrossers and selfers might often differ in life history and ecology. Ojeda (1998) found that most of the *Erica* species in the CFR are seeders, with post-fire resprouting being uncommon. Seeders and resprouters allocate resources to growth differently: seeders allocate their resources to aboveground growth and development of an extensive root system, while resprouters mainly allocate their resources to production of costly storage tissue for survival in a fire-prone environment (Bell and Ojeda 1999). Although unexplored, a similar difference can be expected in the investment in seed production, as survival of a seeder population depends heavily on post fire seed production, each fire resulting in a complete generation turnover. Conversely, resprouters are less dependent on seeds for survival due to the large lignotubers that allow for resprouting and persistence after fires. Consequently, we predict that if self-fertilisation does occur in *Erica*, it would be more prevalent in seeders than it would be in resprouters. As mentioned before there is a significant gap in the knowledge we possess for this genus and further study is needed.

In this study I investigate the breeding systems of *Erica* species and possible correlates. Specifically, I aim to, 1) investigate if species from the genus can self-pollinate and can do so autonomously. I also intend to, 2) determine if different breeding systems have different floral correlates. Furthermore, I ask: is there a trade-off between flower number and flower size; and are pollinators more likely to visit large or small flowered species? I also aim to 3) ascertain if the breeding system of a species influences its pollination syndrome and fire-survival strategy or vice versa.

Methods

Site, Phylogeny and Species

Breeding systems experiments were performed on 15 *Erica* populations, located on the Cape Peninsula of South Africa. A population of *Erica baccans* was sampled above Rhodes Memorial within the Table Mountain National Park range. Five *Erica* species were sampled on Chapman's Peak: *E. hirtiflora*, *E. viscaria* ssp. *viscaria*, *E. abietina* ssp. *constantiana*, *E. nevillei* and *E. pulchella*. Two of the species sampled were located within the Silvermine Nature Reserve (Gate2): *E. urna-viridis* and *E. imbricata*. A further seven species were sampled within Kommetjie: *Erica mammosa* (seeder), *E. corifolia* and *E. brachialis* were sampled on the flats of the Slangkop Reserve, *E. plukenetii* and *E. subdivaricata* were sampled at the base of the mountains opposite Slangkop and *E. mammosa* (resprouter) and *E. abietina* ssp. *atrorsea* were sampled in the mountainous area surrounding Kommetjie.

Table 1: Study areas from which *Erica* species were sampled and collected.

Locality	Latitude	Longitude
Table Mountain National Park	33° 57' 15" S	18° 27' 36" E
Chapman's Peak	34° 05' 06" S	18° 21' 52" E
Silvermine	34° 05' 26" S	18° 25' 36" E
Kommetjie	34° 09' 26" S	18° 20' 14" E
Kommetjie:	33° 09' 37" S	18° 21' 21" E



Map 1: Illustration of the Cape Peninsula of the Western Cape, South Africa. Red markers are indicative of the locations of *Erica* population that were investigated.

All populations were located in stands of mature fynbos and all sampling was done during each species' flowering period. Species selection was made based on what was available within a practical geographic distance. The existing phylogeny is very unresolved (Pirie et al. 2011) at this stage and there are little signs of phylogenetic constraints (for example bird pollination appears all over the phylogeny). For the purpose of this thesis, I assumed phylogenetic independence amongst taxa.

Floral Traits

To investigate variation in floral traits, three fully developed flowers were collected from each of 10 to 20 individual plants per species. For all plant species used in the breeding systems experiments, I measured corolla tube length, corolla tube width, corolla aperture width, stigma-anther separation, total flowers per plant and nectar (volume and concentration). Floral measurements were made with digital callipers and replicated three times per sampled plant. Corolla tube width was measured at the widest point along the corolla. Stigma-anther separation was measured as the

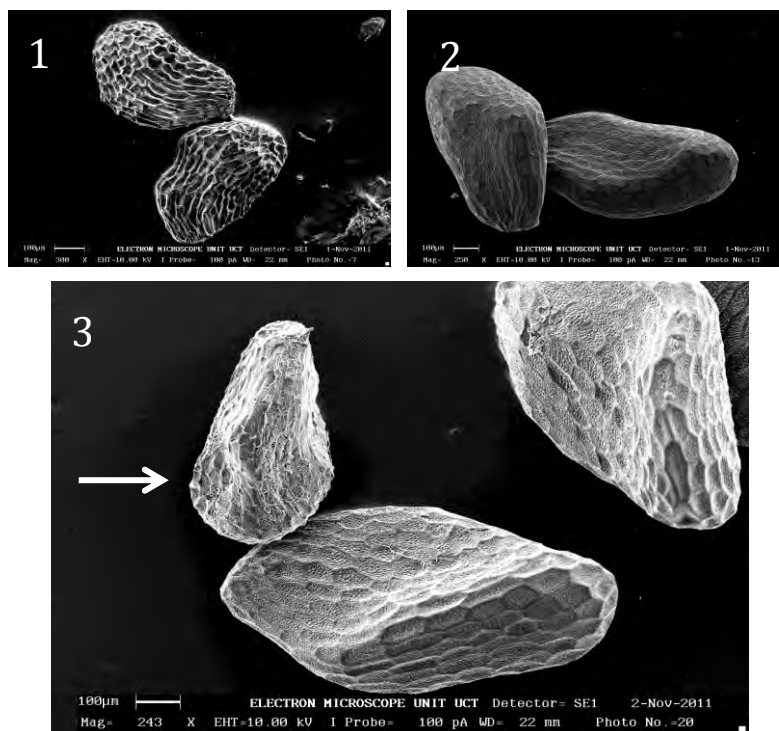
difference between the length of the stigma and the length of the anther. Negative numbers indicate that the stamens were longer than the stigma. To adjust for the interspecific variation in floral morphology and pollinator type, stigma-anther separation was calculated as a proportion of corolla tube length, yielding the relative stigma-anther distance. Counts of the number of flowers for each species were also collected as a measure of the display size. Due to the varying heights of species (fire-related), display size of each species was calculated as a function of that species' height. In order to determine the amount of reward offered to pollinators by *Erica* species, nectar samples were extracted from unvisited (intact anther-ring) flowers of ten *Erica* species using a capillary tube. The capillary tubes were used to estimate volume, while an Eclipse handheld refractometer (Bellingham and Stanley Ltd.) (Morrant et al. 2009) was used to estimate nectar concentration.

Pollinators of thirteen of the fifteen species studied could be established (Oliver and Oliver 2000; R. Turner, pers. comm. 2013). Where the pollination syndrome could not be determined, it was inferred from the floral morphology on the basis that floral form could be used as a proxy for pollination syndrome, if pollinators have driven the floral radiation within *Erica*. A size of 4 cm was used as the cut-off point between small- and large-flowered species, mainly because small-flowered species clustered below this point while large-flowered species did so above 4 cm. Generally, larger flowered species are bird-pollinated while smaller flowered species are insect pollinated. Consequently, for instances where there was a weak overall correlation between traits and ISI, small and large flowered species were analysed separately. Resprouting individuals were identified by swollen lignotuber and multiple stem growth from the rootstock. Seeders were single stemmed and lacked sizeable rootstocks.

Hand-pollination

I examined the seed set potential of each species by applying three treatments: 1) self-pollination, 2) cross-pollination and 3) autonomous selfing (no manipulation). To hand-pollinate a plant, the stamens were disturbed over a petri-dish and the collected pollen applied to the stigma with dissecting forceps and the help of a 20x handlens. For the self-fertilization treatment, flowers were pollinated with pollen from the same individual plant, while for the cross-pollination treatment flowers are pollinated with pollen from a completely different individual. To test whether the selected species are

capable of autonomous seed set, i.e. set seed without a pollinator, I bagged unmanipulated flowers. Each treatment was replicated once per plant therefore the level of replication depends on the number of plants sampled per species. A minimum of 10 individual plants were sampled per species. Flowers were re-bagged after hand-pollinations as to control against subsequent fertilisation by pollinators. Treated flowers were left to mature within the bags for 7-8 weeks before collection of the fruits. Flowers were scored for fruit set and seeds were counted for all treatments and all species. Both total seeds and viable seeds were noted – on the basis that non-viable seeds looked shrivelled and appeared to have little content, while viable seeds were plump and healthy (See SEM image). Although, further germination experiments or tests for viability need to be conducted to establish if this can be used as a proxy for viability – due to time constraints, this could not be done within the scope of this dissertation. Additionally, between ten and twenty (depending on availability and accessibility) immature flowers were collected for each species to determine average ovule number.



Scanning electron microscope images 1: Seeds produced by the different hand-pollination treatments. A. Seeds from self-pollination treatment. B. Seeds from cross-pollination treatment. C. Seeds from both treatments, showing the size difference. The smaller, shrivelled seed from self-pollination is highlighted with an arrow. These images were taken for *E. plukenetii* but all species studied appeared to have reduced size for the self-pollination treatment.

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 average number of seeds per flower of self-pollinated fruit divided by the average number of seeds per flower 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-fertilization (IAS) was obtained by dividing the percentage fruit set of unmanipulated flowers by that of cross-pollinated flowers. These values also range from zero to one, where zero indicates complete dependence on pollinators for fruit set, while one indicates 100% fruit set through autogamy.

Statistical Analyses

Seed- and fruit-set data

All statistical analyses were performed using SPSS Version 21 (IBM Corp.). I used generalized estimating equations (GEEs) to analyse the hand-pollination (including the unmanipulated treatments) experiments. Dependent variables included proportion fruit set, viable seed set per flower, viable seed set per fruit and viable seed set per ovule, while 'Treatment' was used as the within subject variable. These were chosen in order to establish the overall trends in fruit- and seed set between the different treatments. Proportion of fruit set per treatment for each species as well as viable seeds per ovule were analysed using GEE models with a binomial error distribution model and logit link function. When analysing the proportion fruit set, per species, I substituted one fruit for species that experienced no fruit set. This provided a statistically conservative solution to the problem of undefined logits when a set of binomial data has no variance. The results of the substitution was corrected for post analysis. For seed set per flower a negative binomial distribution model and log link function was used. Seed set per fruit was analysed with GEE models using a Poisson loglinear distribution model with a log link function. GEE models used an exchangeable correlation matrix and significance was assessed using Score statistics. To assess the significance of differences among means, we used the Dunn-Šidák method. For graphical presentation of means in the original measurement scale, we back-transformed log or logit data resulting in asymmetrical

standard errors. Marginal means and standard errors were plotted using SigmaPlot version 9.0 (Systat Software, Inc. 2005)

Traits data

Tests for homogeneity and normality were performed in STATISTICA 12 (StatSoft, Inc. 2013). Transformations of the data were not necessary to improve the normality of the data. Simple regressions (correlations) comparing average plant height, corolla size, corolla aperture and stigma-anther distance to ISI were performed in STATISTICA 12 while the graphs for these were plotted using EXCEL 2010 (Microsoft Excel 2010, Redmond, Washington).

Two-way (factorial) ANOVAs tested the effect of the three treatments and fifteen species on fruit- and seed set data. One-way ANOVAs were used to test for differences in traits between pollination syndromes (bees/other insects/birds) and fire-survival strategies (seeder/resprouter). Tukey post-hoc tests were performed where significant differences were found in order to make multiple comparisons of mean differences.

Results

Selfing and Autogamy in Erica

Fruit set, viable seed set, total viable seed set for all species were significantly influenced by the effect of treatment, species and the interaction between the two factors (Table 2).

Table 2: Two-way (factorial) ANOVA illustrating the effect of treatment, species and the interaction between the two factors, on fruit set, viable seed set and total seed set (viable and non-viable seed). The degrees of freedom, F- statistic and p-value for each factor are given.

Statistics	Treatment			Species			Treatment x Species		
	df	F	p	df	F	p	df	F	p
Fruit Set	2	245.15	0.0001	14	4.33	0.0001	28	2.27	0.0003
Viable Seed Set	2	79.22	0.0001	14	17.73	0.0001	28	2.94	0.0001
Total Seed Set	2	121.63	0.0001	14	11.86	0.0001	28	6.4	0.0001

For most species, fruit set was significantly lower in the unmanipulated treatment but did not differ between self- and cross-pollination treatments. Approximately half the species sampled (46.7%) yielded no fruit set for the autonomous selfing treatment (Appendix 1a, Figure 1).

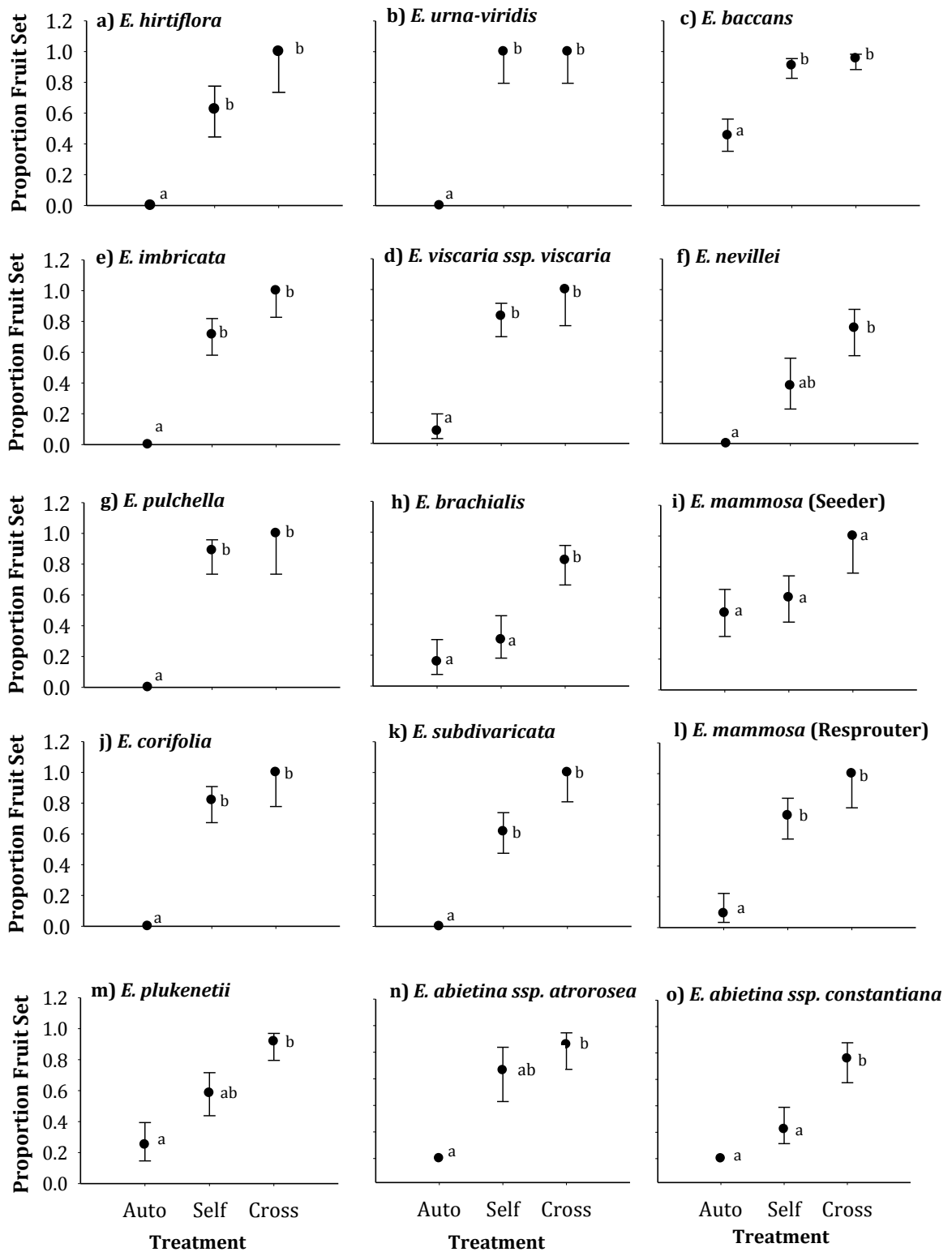


Figure 1: Proportion fruit set of 15 *Erica* species, subjected to three different pollination treatments: self-pollination, cross-pollination and autonomous selfing. Lower case letters indicate significant differences between treatments at the 0.05 level, which were determined using GEE models. See Appendix 1a for full results of statistical analyses.

Across all species, cross-pollination yielded an average of 49.7 ± 9.9 viable seeds per flower. Comparatively, autonomous selfing (2.23 ± 1.2) as well as the self-pollination treatment (13.4 ± 4.2) are very low in terms of their viable seeds per flower. Seed set per flower for the autonomous selfing treatments was zero or near zero for most species, except, *E. baccans* and *E. brachialis* (Appendix 1b). Furthermore, this treatment yielded seed set that was significantly different to the cross-pollination treatment in all species studied (Figure 2).

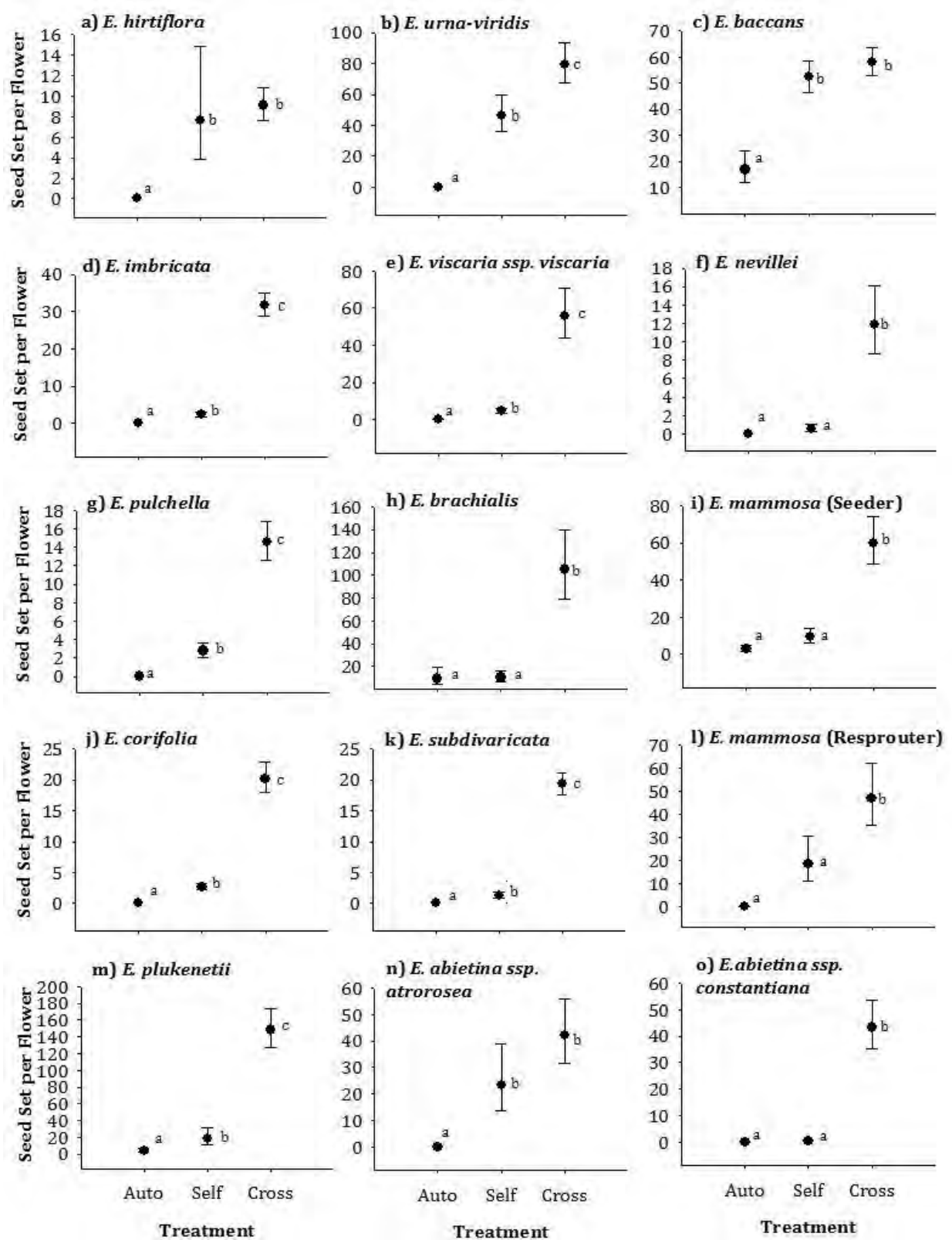


Figure 2: Seed set per flower for 15 ericas subject to different pollination treatments: self-, cross and autonomous self-pollination. Lower case letters indicate significant differences between treatments at the 0.05 level. See Appendix 1b for full results of statistical analyses.

The autonomous selfing treatments yielded no seeds for most species. The self-pollination treatment, in most cases, yielded a viable seed set significantly lower (Appendix 1c) than that of the cross-pollination treatment. The exceptions are *E. baccans*, in which all three treatments were similar, and *E. abietina* ssp. *atrorosea*, in which cross- and self-pollination treatments yielded similar viable seed set, while there was no seed set for the autonomous selfing treatment (Figure 3).

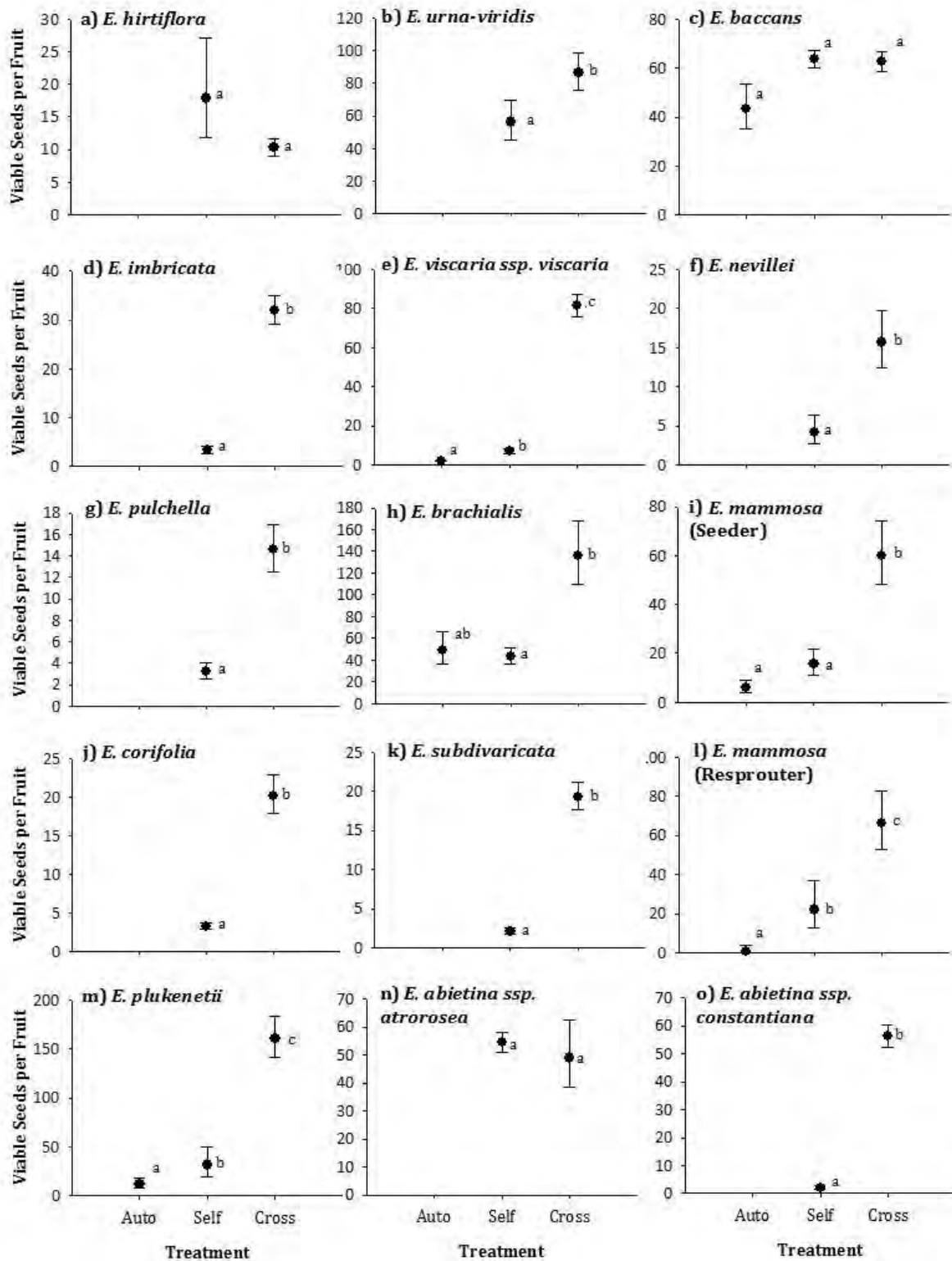


Figure 3: Counts of viable seed set per fruit for 15 *Erica* species, subjected to three different pollination treatments: self-pollination, cross-pollination and autonomous selfing. Lower case letters indicate significant differences between treatments at the 0.05 level. See Appendix 1c for full results of statistical analyses.

Viable seed set per ovule was highest for the cross-pollination treatment for all 15 species, ranging from 0.3 to approximately 0.7. Self-pollination treatments yielded a proportion of viable seeds per ovule that was significantly different to cross-pollination treatments for twelve of the fifteen species. Proportion viable seed set for the autonomous treatments was relative low throughout the sampled species (Figure 4, Appendix c).

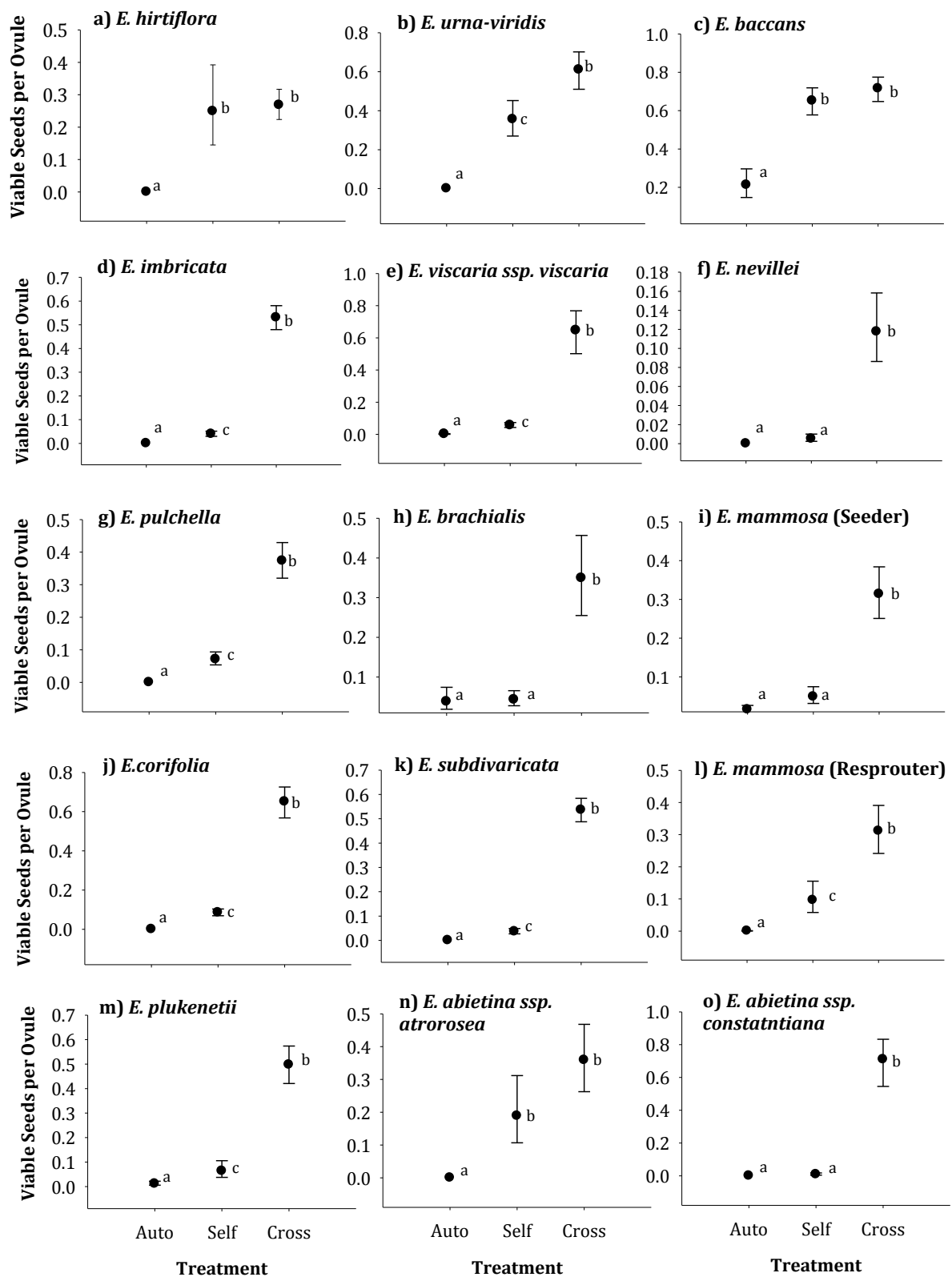


Figure 4: Proportion of viable seeds per ovule for 15 *Erica* species, subjected to three different pollination treatments: self-pollination, cross-pollination and autonomous selfing. Lower case letters indicate significant differences between treatments at the 0.05 level. See Appendix 1c for full results of statistical analyses.

Self-compatibility and Autonomous Selfing

Majority of species (10/15) appear to be self-incompatible ($0 < \text{ISI} < 0.3$) and unlikely to set seed autonomously ($\text{IAS} \leq 0.3$). Large-flowered, *E. mammosa* (resprouter) is partly self-compatible ($\text{ISI} > 0.3$), while *E. hirtiflora*, *E. urna-viridis*, *E. abietina* ssp. *atrorosea* and *E. baccans* seem to approach full self-compatibility. *Erica baccans* and the seeder form of *E. mammosa* appear to be partially autogamous.

Table 3: Index of Self-incompatibility (ISI) based on seed set data and Index of Autonomous Selfing (IAS), based on fruit set data for 15 *Erica* species.

Species	ISI	IAS
<i>E. abietina</i> ssp. <i>constantiana</i>	0.01	0
<i>E. nevillei</i>	0.05	0
<i>E. viscaria</i> ssp. <i>viscaria</i>	0.07	0.09
<i>E. subdivaricata</i>	0.07	0
<i>E. imbricata</i>	0.07	0
<i>E. brachialis</i>	0.10	0.16
<i>E. plukenetii</i>	0.13	0.27
<i>E. corifolia</i>	0.13	0
<i>E. mammosa</i> (Seeder)	0.16	0.50
<i>E. pulchella</i>	0.19	0
<i>E. mammosa</i> (Resprouter)	0.32	0.09
<i>E. hirtiflora</i>	0.59	0
<i>E. urna-viridis</i>	0.64	0
<i>E. abietina</i> ssp. <i>atrorosea</i>	0.66	0
<i>E. baccans</i>	0.86	0.48

Correlates of Selfing Ability

The correlation shows a non-significantly positive relationship between height and selfing ability ($r = 0.449$; $n = 15$; $p = 0.093$) for species of the *Erica* genus.

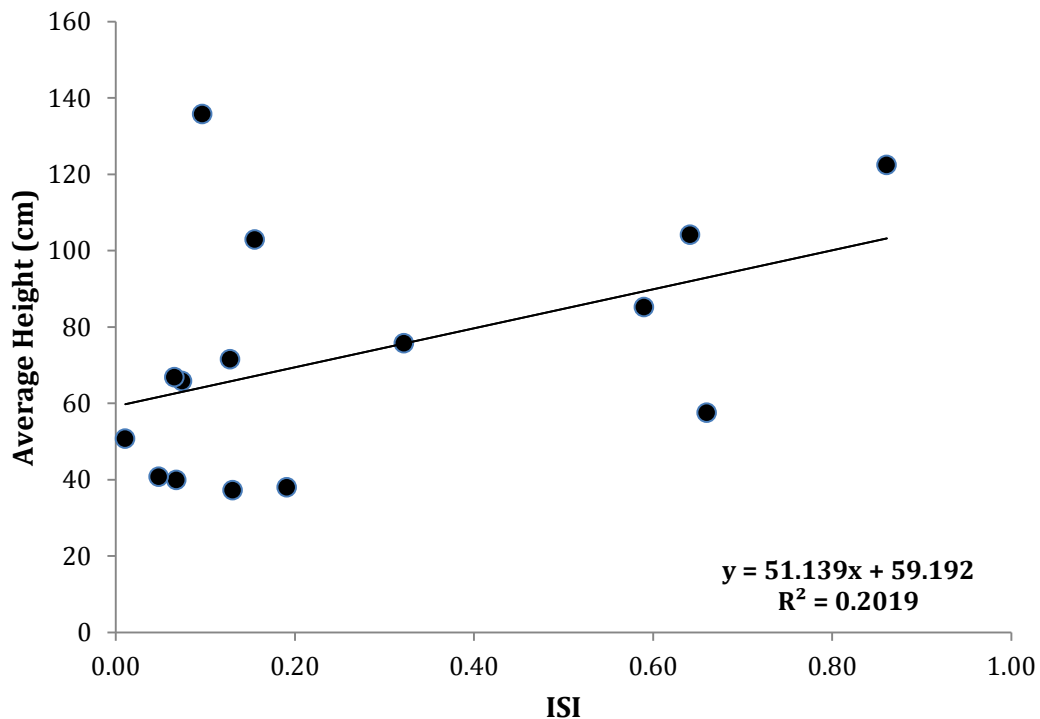


Figure 5: Relationship between the degree of selfing and the average height (cm) of 15 *Erica* species. The linear equation and R^2 values are indicated on the graph.

Corolla size shows a negative logarithmic relationship with floral display size (number of flowers per unit of height). Indicating that as flower size increases, the number of flowers in the display decrease ($r = -0.700$; $n = 15$; $p = 0.004$). Species with smaller corolla sizes ($1.19\text{cm} \pm 0.241\text{cm}$) and increased flower number (3841.1 ± 1215.0) clump together, while those with large corollas ($7.04\text{cm} \pm 0.69\text{cm}$) and reduced flower number (873.6 ± 339.6) cluster together. The difference between the size classes are significant ($F = 11.33$; $df = 1$; $p = 0.005$). Hereafter, the two sizes classes will be analysed separately.

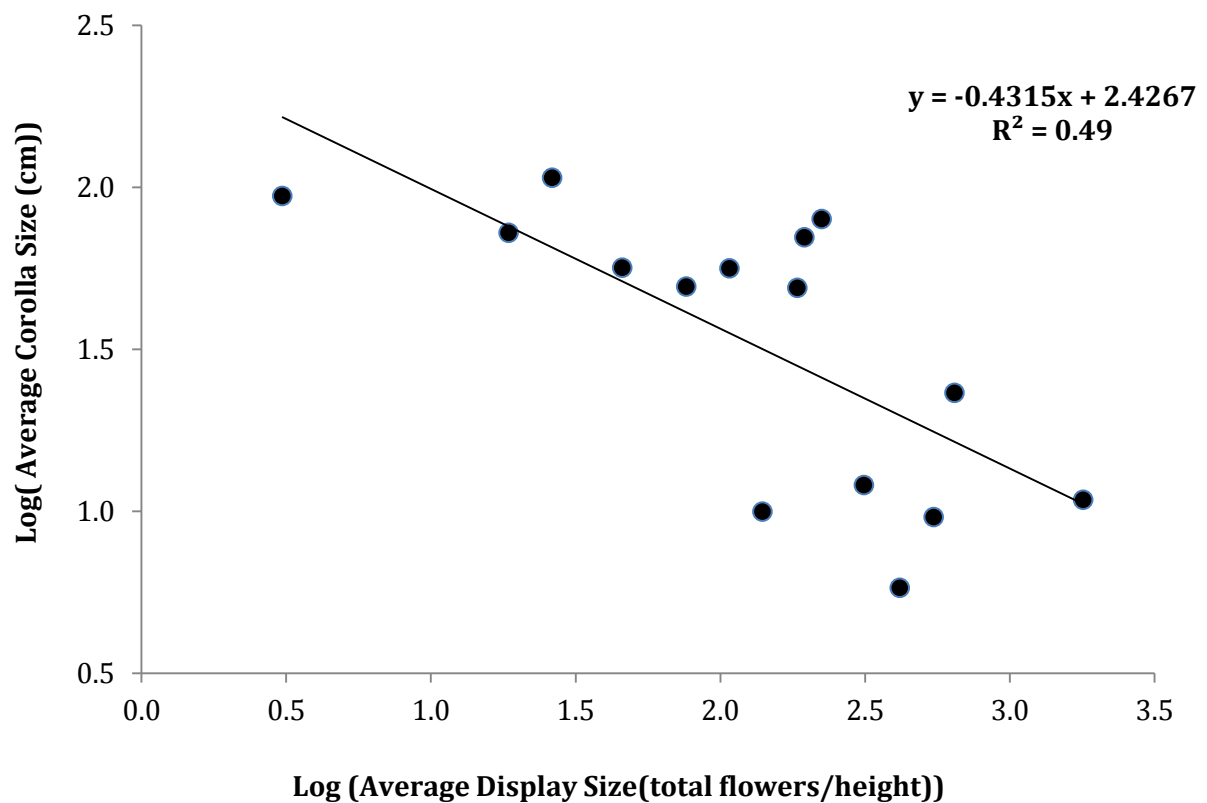


Figure 6: Relationship between average corolla size (cm) and average display size (Number of flowers/cm) on a logarithmic scale for each of 15 species. The linear equation and R^2 values are indicated on the graph.

Corolla size does not appear to have a significant relationship with the degree of selfing species employ; species are arranged randomly with no obvious trend ($r= 0.017$; $n= 15$; $p= 0.953$) visible. Similar-sized species continue to cluster together. Large flowered species exhibited a non-significant positive relationship between average corolla size and selfing ability ($r= 0.270$; $n= 9$; $p= 0.482$). Small-flowered species yielded a significant positive correlation between the two variable ($r= 0.883$; $n= 6$; $p= 0.020$). *Erica baccans*, *E. hirtiflora*, *E. abietina ssp. atroflorea* and *E. urna-viridis*, have ISI values that are above the norm of the dataset but are included in all analyses to present a more holistic result as they represent the facultative outcrossing breeding system.

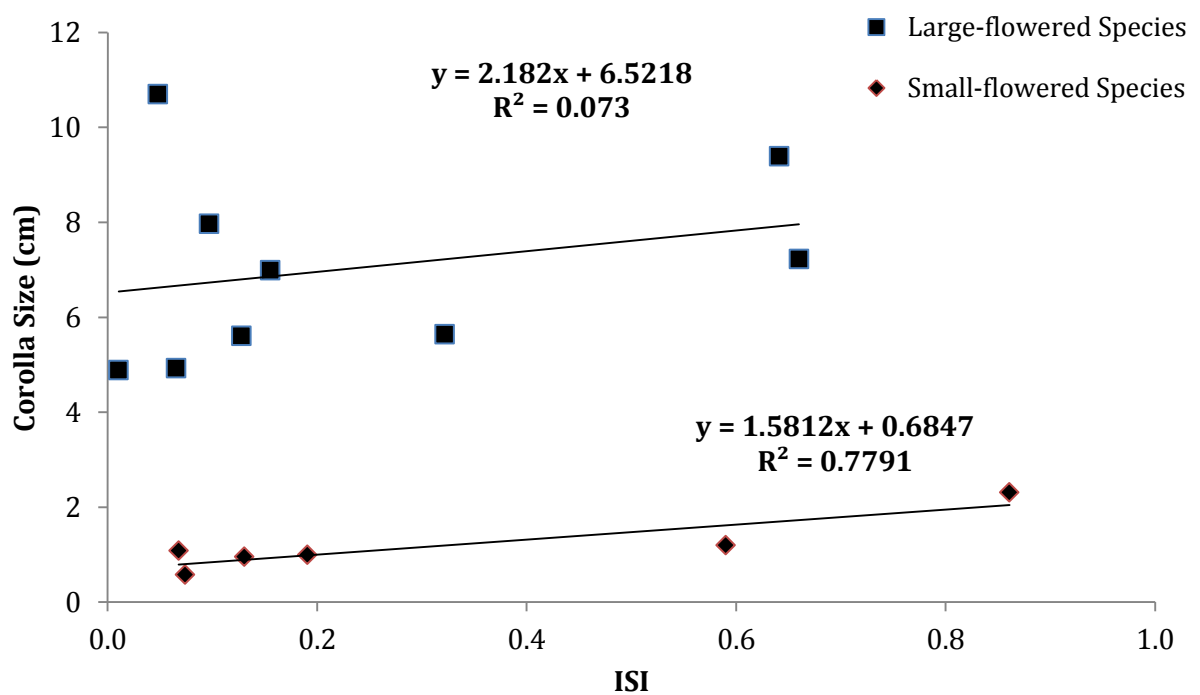


Figure 7: Relationship between average corolla size and degree of selfing of 15 *Erica* species. The linear equations and R^2 values are indicated on the graph. Diamonds (◆) signify small-flowered species, with corolla size less than 4 cm, while squares (■) signify large-flowered species, with corolla size larger than 4 cm.

A non-significant relationship exists between the average corolla aperture size and selfing ability of the sampled *Erica* species ($r = -0.130$; $n = 15$; $p = 0.644$). Large-flowered species exhibit a non-significant negative relationship between average corolla aperture and selfing ability ($r = -0.370$; $n = 9$; $p = 0.328$). Small-flowered species, on the other hand, had a positive non-significant relationship between variables ($r = 0.349$; $n = 6$; $p = 0.498$). For small-flowered species this relationship was not significant. However, when removing the effects of *E. baccans* and *E. hirtiflora* the relationship becomes negative ($r = -0.251$; $n = 4$; $p = 0.749$), tending towards a more expected result.

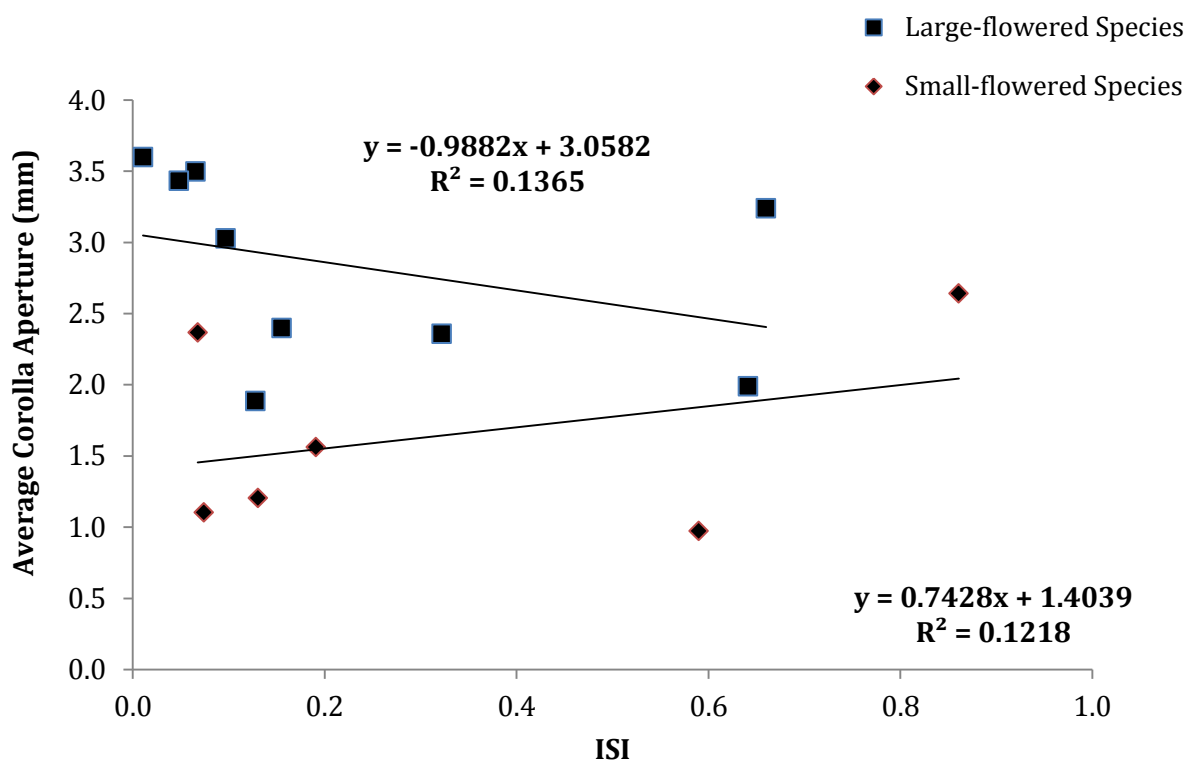


Figure 8: Relationship between increasing selfing ability and the average corolla tube aperture of 15 *Erica* species. Marker shapes indicate differently sized corollas: diamonds (◆) symbolize small-flowered species, while squares (■) symbolize large-flowered species. The linear equation and R^2 values are indicated on the graph.

Generally there is a very weak negative relationship between the degree of selfing and stigma-anther separation ($r = -0.234$; $n = 15$; $p = 0.402$). Analysing large and small corolla size classes separately did not significantly change the finding. Both large ($r = -0.109$; $n = 9$; $p = 0.780$) and small-flowered ($r = -0.467$; $n = 6$; $p = 0.350$) species yielded non-significant relationships between stigma-anther separation and ISI and removing the outliers did not improve the relationships.

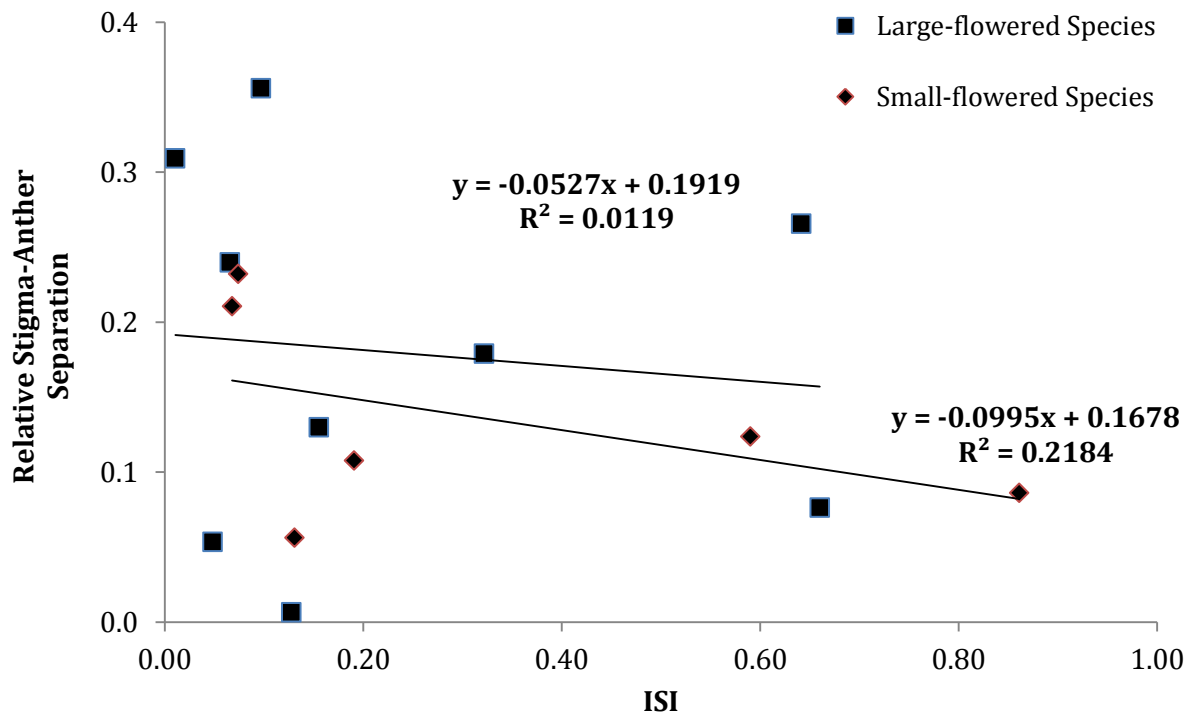


Figure 9: Relationship between the selfing ability and relative stigma-anther separation of 15 sampled *Erica* species. Diamonds (◆) symbolize small-flowered species (<4cm), while squares (■) symbolize large-flowered (>4 cm) species. The linear equation and R^2 values are indicated on the graph.

Pollination Syndrome and the Ability to Self-fertilize

No significant differences were found when comparing pollination syndromes in terms of selfing ability ($F= 0.092$; $df= 2$, $p= 0.912$). Nonetheless, species that are predominantly bee pollinated appear to have a higher selfing capability than bird-pollinated species, and other insect pollinated species.

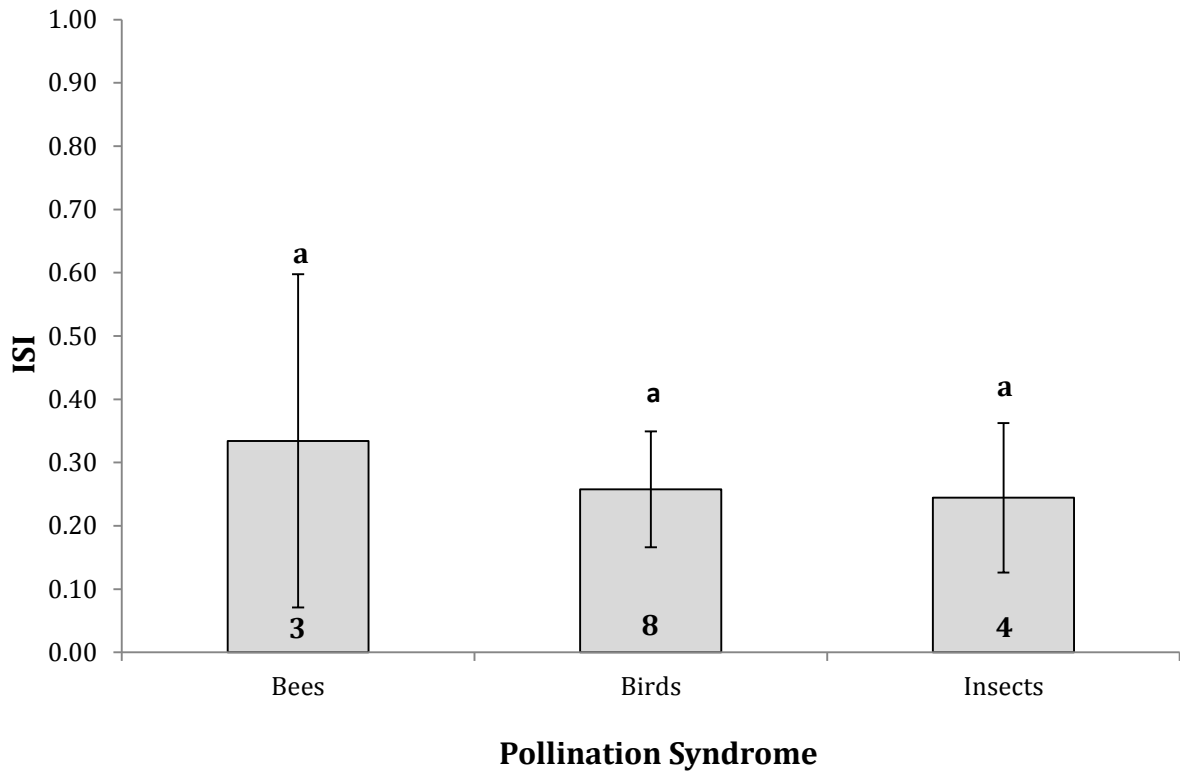


Figure 10: Average selfing capabilities of three different pollination syndromes. Letters indicate the significance level between pollination syndromes – similar letter indicate no significant differences, while different letter indicate significant differences. Standard error ranges are indicated on the plot and samples sizes are depicted at the bottom of each bar.

On average larger flowered species are bird-pollinated while smaller flowered species are insect pollinated. The difference in corolla sizes were significant between pollination syndromes ($F= 17.207$; $df= 2$; $p< 0.001$). Bee and other insect pollinated species have corolla sizes that are similar ($F= 17.207$; $df= 2$; $p= 0.876$) while birds and other insects ($F= 17.207$; $df= 2$; $p= 0.002$) as well as birds and bees ($F= 17.207$; $df= 2$; $p= 0.001$) have significantly different corolla sizes. *Erica abietina* ssp. *constantiana* has been observed to be visited by both birds and insect with equal measure (R. Turner, pers. comm., 2013), however, average corolla sizes indicate that it is more similar to that of bird-pollinated species. *Erica viscaria* ssp. *viscaria* appear to have a corolla size similar to bird-pollinated species but insects predominantly visit this species (R. Turner, pers. comm., 2013).

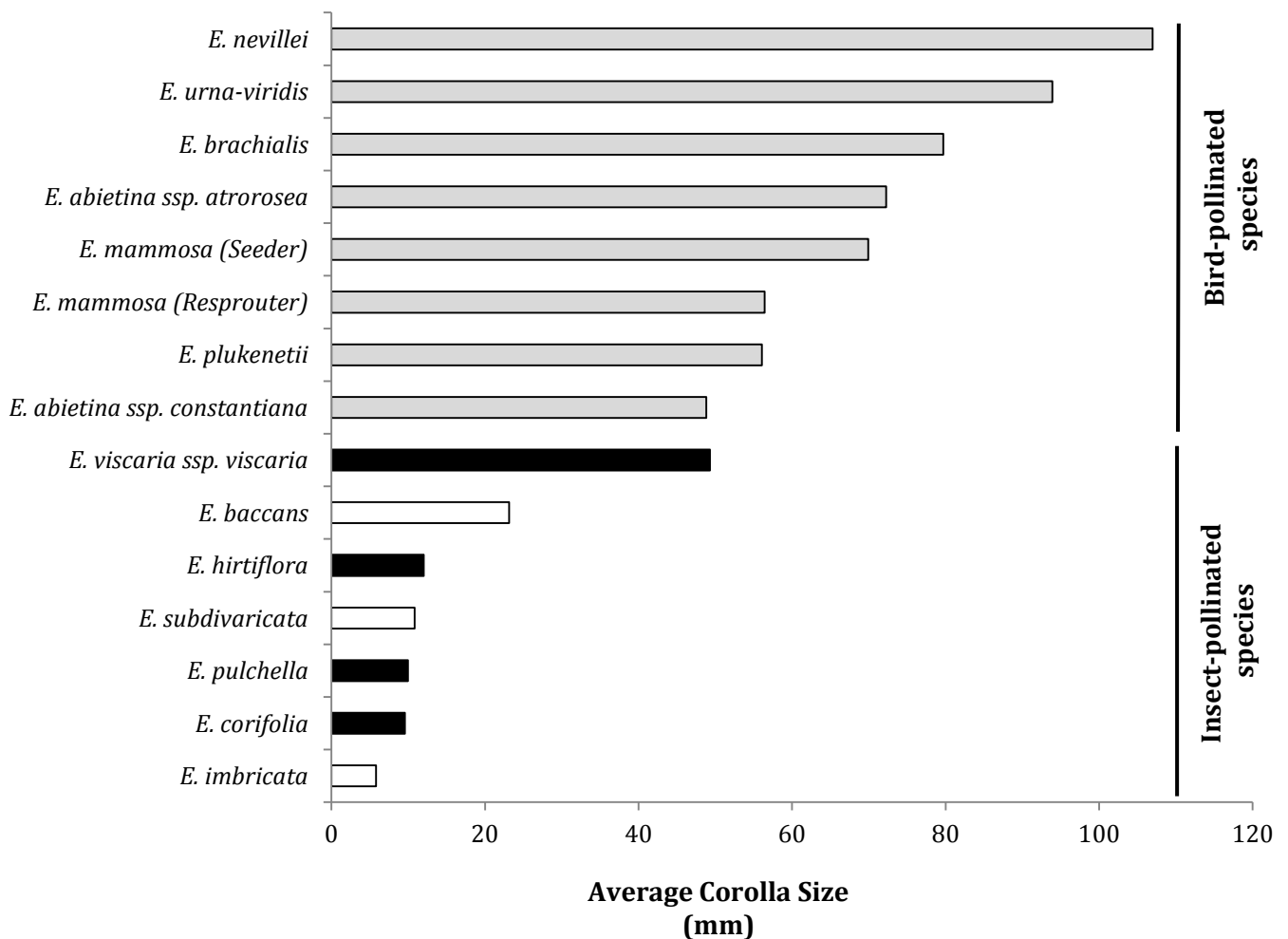


Figure 11: Average corolla sizes of different pollination syndromes. Differently coloured bars indicate the different pollination syndromes: grey – bird-pollination; white –bee-pollination; black – other insects (insects other than bees).

Large-flowered species are significantly different from small-flowered species in both nectar concentration ($F= 18.46$; $df= 1$; $p< 0.01$) and volume ($F= 18.46$; $df= 1$; $p< 0.01$).

Table 4: Comparison of mean nectar volume (μl) and mean nectar concentration (%) between six large-flowered and four small-flowered *Erica* species. Different letter indicate significant differences at the 1% level.

Floral Size	Small-flowered Species	Large-flowered Species	F	df	p
Average Volume (μl)	3.32 ^a	14.45 ^b	134.8	1	<0.01
Average Concentration (%)	17.97 ^a	22.28 ^b	18.46	1	<0.01

Small-flowered species have less flowers per unit height compared to large-flowered species but this difference is not significant ($F= 1.955$; $df= 1$; $p= 0.185$). There is also no significant differences when comparing pollination syndrome in terms of display size ($F= 2.360$; $df= 2$; $p= 0.137$).

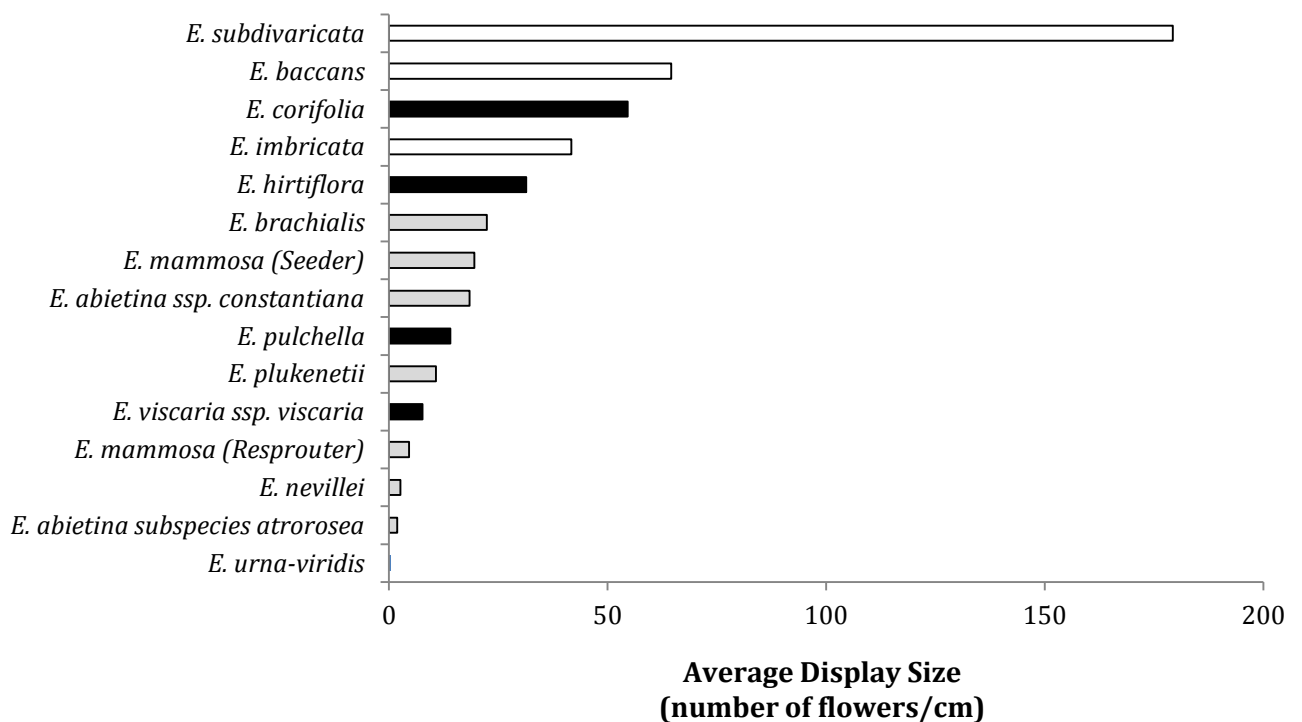


Figure 12: Average display size (number of flowers/cm) of *Erica* species belonging to different pollination syndromes. Differently coloured bars indicate the different pollination syndromes: grey – bird-pollination; white – bee-pollination; black – other insects (insects other than bees).

Fire-survival Strategy and the Ability to Self-fertilize

Majority of species sampled were seeders, with only three having the ability to resprout (either epicormically or from below ground rootstocks). The epicormic resprouter, *Erica brachialis*, resprouts from the stem after fire but acts as a seeder in fireless years – the species was thus added to the seeder category during analyses. No obvious trends were seen when comparing species of differing fire survival strategies with their index of self-incompatibility. No significant differences were found between fire-survival strategies ($F= 0.204$; $df= 1$; $p= 0.659$).

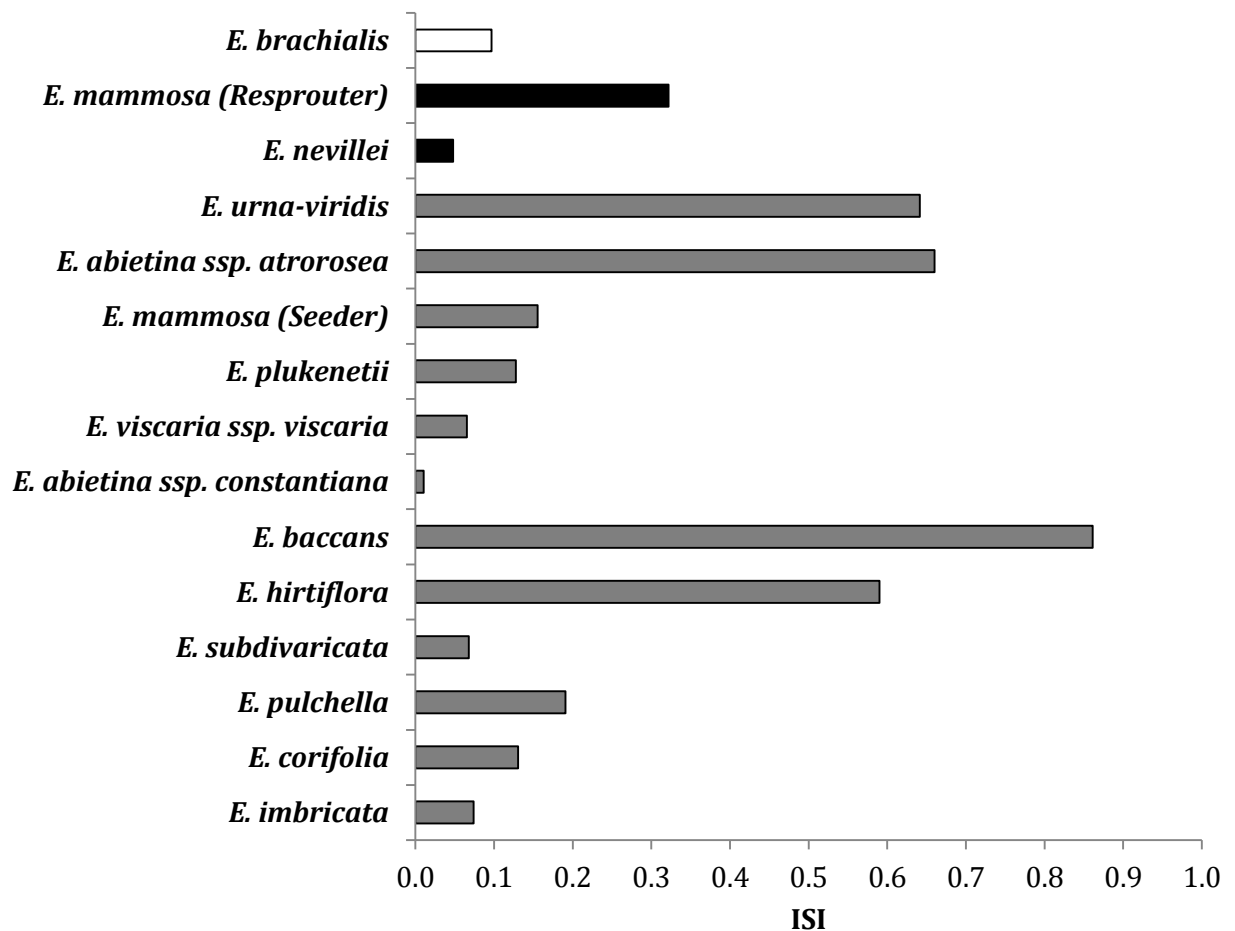


Figure 13: Average selfing capabilities of species with differing fire-survival strategies. Differently coloured bars indicate the different fire-survival strategies: grey – seeders; white – seeder/epicormic resprouting; black – resprouter (from belowground root stocks).

There was no significant differences between resprouter and seeder forms of *E. mammosa*, in viable seed set for the three different treatments: cross-pollination ($F=0.091$; $df=1$; $p=0.7566$), self-pollination ($F=1.599$; $df=1$; $p=0.222$) and autonomous selfing ($F=3.116$; $df=1$; $p=0.094$). These findings are reflected in the ISI values below.

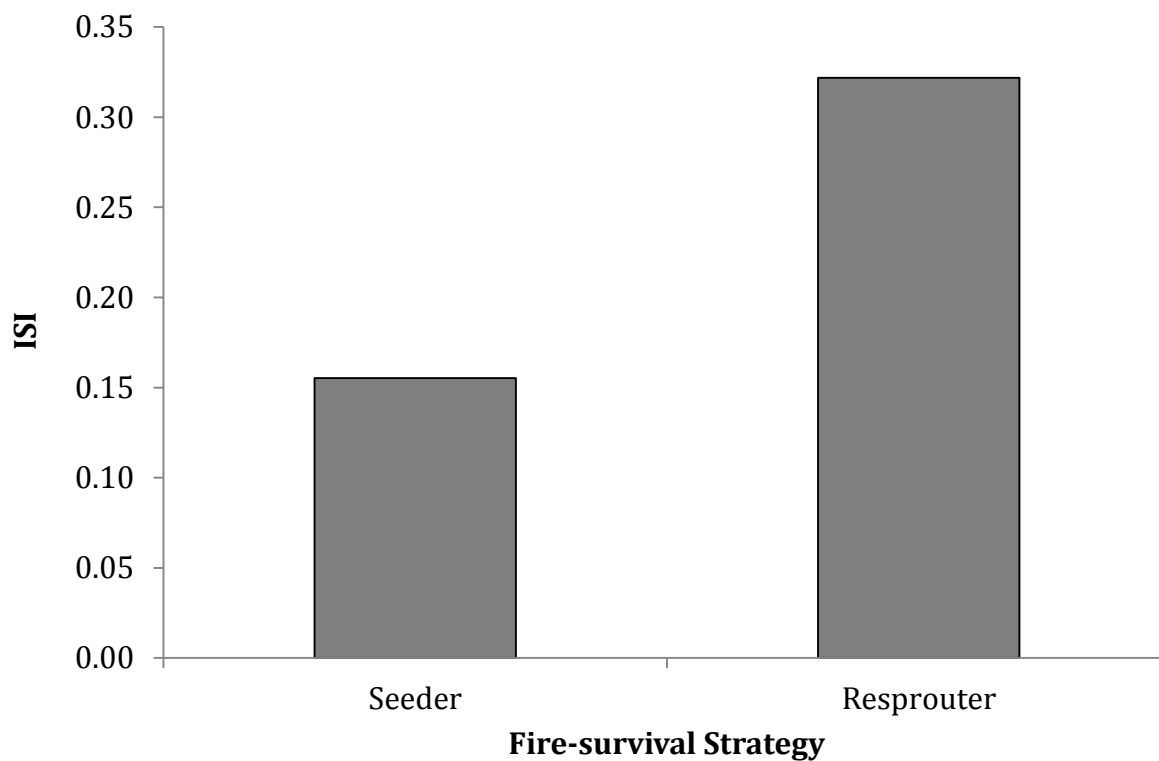


Figure 14: Average selfing capability based on ISI for *Erica mammosa*, a species with both resprouting and seeder life-history strategies.

Discussion

Self-incompatibility and Autonomous Selfing in Erica

Given the limitation of the phylogeny, species were sampled within the geographic distance available and species and traits were regarded to be independent. The results of this study indicate that *Erica* species of the Cape Peninsula are able to self-fertilise to some extent (Figure 1). However, the capacity to produce viable seeds through autonomous selfing was low (Figure 2-4). For all treatments, I encountered a low seed production per ovule, surprisingly also for the cross-pollination treatment, of which only approximately 50% of ovules developed into viable seeds (Figure 4). A few reasons for this finding may include: 1) pollen limitation, but this seems unlikely as flowers were hand-pollinated and ample pollen was added to stigmas to ensure successful growth to ovary. Alternatively, 2) ovule discounting, Verdú et al. (2006) has shown that reduction in available ovules is not always a consequence of interference from self-pollen but is more general and may be due to outcrossing with inferior fathers. It appears that self-fertilisation does in fact yield viable seeds (Figure 2) and for most ericas, where fruits do occur, seed set of self-pollinated species are significantly ($p < 0.05$, Appendix 1) different from that of the cross-pollination treatments.

Indices based on seed and fruit set data supported the above findings. The index of self-incompatibility (ISI) suggested most species studied are self-incompatible ($0 < \text{ISI} \leq 0.3$). *Erica mammosa* (resprouter) has an increased level of self-compatibility ($0.3 < \text{ISI} < 1$), while *E. hirtiflora*, *E. urna-viridis*, *E. abietina* ssp. *atrorosea*, and *E. baccans* approached full self-compatible ($0.6 < \text{ISI} > 1$). Furthermore, the ability to set seed autonomously was also weak among species, *E. mammosa* (seeder) and *E. baccans* had the largest capacity for autonomy, reaching an IAS of approximately 0.5 on a scale from zero to one (Table 3).

Correlates and the Increased Ability to Self-fertilise

Generally, I observed weak correlations of traits with gradients of increased self-compatibility. However, what was evident from these associations is that species that produce similar sized flowers tended to cluster together; large- and small-flowered species clustered separately. What was unexpected from this trend is the location of these two size groups along the ISI axis. I expected small-flowered species to be on the

higher end of the scale, i.e. more self-compatible. The rationale behind this involves the fact that small-flowered species have reduced separation of male and female function (herkogamy) allowing for a higher probability of self-pollination (Johnston and Schoen, 1996). However, this was not observed, majority of small-flowered species are situated on the lower level of the ISI, suggesting species to be self-incompatible and predominantly outcrossing. Similarly, larger flowered species are distributed at the lower end of the selfing scale. However, both large- and small-flowered species appear to have some species that have increased selfing ability, above the rest of its size class. Small-flowered *Erica baccans* and *E. hirtiflora*, as well as large-flowered *E. abietina* ssp. *atorosea* and *E. urna-viridis*, were the only species that had relatively high self-compatibility. Species with increased plant height appeared to have increased selfing ability. This relationship is not significant (Figure 5, $p= 0.093$) as other larger and similar sized species often have very weak selfing abilities.

When investigating the relationships between the correlates and ability to self-fertilise no significant differences were observed. This holds true for differences within different corolla size classes, except average corolla size of small-flowered species (Figure 7, $p= 0.020$). Trends were generally very weak when comparing the correlates with selfing ability and in some cases this appeared to be due to the facultative outcrossers, *E. hirtiflora*, *E. abietina* ssp. *atorosea*, *E. baccans* and *E. urna-viridis*, skewing the relationship. However, these species were retained in the analyses to give a better understanding of the effects of breeding systems on the correlates, as they were the only facultative outcrossers among the 11 obligate outcrossers. Although differences for most of the correlations were not significant, general trends could be established. Small-flowered species have increased selfing ability when corolla sizes are larger, corolla apertures larger and herkogamy reduced. Large-flowered species have increased selfing ability when corolla sizes are large, apertures small and herkogamy reduced. It appears that both large and small flowered species employ much the same strategy, except for selfing ability to be increased, apertures in large-flowered species need to be smaller, while it is bigger for small-flowered species.

A trade-off between floral size and floral number exist (Sato and Yahara 1999; Goodwillie et al. 2010) among the *Erica* species sampled. Small-flowered species tend to have significantly more flowers than large-flowered species (Figure 6, $p= 0.005$). In

Eichhornia paniculata (Pontederiaceae), inflorescence size along with mean number of flowers was negatively related to increasing selfing (Morgan and Barrett, 1989). An implication for the selfing potential in small-flowered species may be due to the increased flower number. The potential for self-fertilization increases with increased number of flowers. Consequently, increased self-incompatibility (Table 3) in small-flowered species may have evolved to reduce geitonogamy.

Pollination Syndrome and the Ability to Self-fertilize

At least for the species investigated here, different pollination syndromes were not significantly different in their ability to self-pollinate (Figure 10, $p = 0.912$). However, a clear trend was seen when comparing average corolla size with pollination syndrome (Figure 11; $p < 0.001$) - bird-pollinated species having significantly larger average corolla sizes than bees and other insects. When comparing average display size to the different pollination syndromes this trend was not mirrored, resulting in an overlap of pollination syndrome throughout the range of average display sizes (Figure 12; $p = 0.137$). Bell (1985) found that both the increase in corolla size and larger inflorescences stimulate more visits by pollinators. Similarly, this study suggests larger pollinators, birds, preferred larger flowered *Erica* species, while smaller pollinators, bees and other insects, preferred species with smaller corolla sizes. This may be as a result of larger flowers offering a significantly higher nectar volume (Table 4, $p < 0.01$) and concentration (Table 4, $p < 0.01$) as reward to birds that need large amounts of sugar/energy for sustenance. It is also true that birds cannot fit into small-flowered plants without expending more energy than needed to collect rewards. It appears that the breeding system does not influence pollinator visitation behaviour. It is, however, clear that flower size and possibly the amount and concentration of rewards do influence pollinator choice.

Fire-survival Strategy and the Ability to Self-fertilize

The ability to self-fertilize does not appear to influence fire survival strategy (Figure 13, $p = 0.659$). *Erica mammosa*, a species with both resprouter and seeder form, also showed no significant differences when comparisons of viable seeds were made (Figure 14, $p > 0.05$; see also Figures 1-4) for each treatment applied. Although the difference is not significant, the indices suggest that the resprouting form of *E. mammosa* has an increased ability to self-fertilize compared to the seeder form (Table 3). However, the

seeder form has a higher score on the index of autonomous selfing (IAS) compared to the resprouter. This finding may be driven by the fact that seeders have much higher dependence on seeds for persistence than does the resprouter, as the resprouter has a rootstock to survive on if, for instance, pollinator visitation decreases. Seeders may have evolved the ability to set seed autonomously when outcrossing opportunities are low, due to a lack of pollination vectors. However, more extensive sampling of resprouting species, in which this study was lacking, is needed and a different outcome may be achieved. This will allow a much clearer perspective on how shifts in the breeding systems may affect or be affected by, if at all, survival strategies of various *Erica* species.

Concluding Remarks

In this preliminary study of breeding systems in *Erica*, it appears that species can self-fertilize but none achieves significantly high seed set through autonomous selfing. Globally, self-compatibility (SC) occurs in 61% of all angiosperms (Iqbal et al. 2008). The indices indicated that few of the species studied are fully self-compatible, although all species can self-fertilise to some degree. Four of the fifteen (27%) species studied here, appear to be strongly self-fertilising with ISI values >0.5 . Consequently, this leads me to doubt the frequency of naturally occurring selfing-fertilization in populations of *Erica*. Another finding supporting this notion involves the sampling method. Very little is known about *Erica* breeding behaviour; through sampling somewhat randomly across the phylogeny within one geographic area, Table Mountain National Park (TMNP), I expected to obtain species with a variety of distinct breeding systems. The breeding systems were not as clear as expected but it appears that 73% of the sampled species have existing incompatibility mechanisms while 27% does not.

Generally, species that have larger corollas and reduced herkogamy appear to have a higher potential for self-fertilisation. Large-flowered species, in contrast to smaller flowered species, have a greater ability to self-fertilise when corolla apertures are reduced. Although the relationship is weak, this association of increased selfing ability with larger corolla sizes and larger apertures is unusual as the literature suggests this to be associated with outcrossing (Lyons and Antonovics 1991; Goodwillie and Ness 2005). It is possible that small-flowered species invoke larger corolla apertures in order to counteract the detrimental effects of self-fertilisation on offspring. Different mechanisms may be at play in large- and small-flowered species and further

investigation with larger sample sizes may give rewarding insights. Floral size is weakly associated with breeding system and the amount and concentration of rewards offered appear to influence pollinator visitation choice. Fire-survival does not show any significant trends with increased selfing ability of species. The indices, however, suggest the seeder form of *E. mammosa* to have increased ability for autonomous selfing compared to the resprouter. This aids our conjecture that seeders are more inclined to self-fertilise (autonomously) to set seed in pollinator limited years/areas as they do not have a rootstock to support them in those conditions.

The next step would be to perform growth experiments to ascertain the level of inbreeding depression and use molecular markers to determine if selfing does in fact occur as a natural mating system. Lyons and Antonovics, (1991) found species to be predominantly facultative outcrossers with mixed mating systems in both American and Belgian *Erica*. If selfing is found to be prevalent, it would be worthwhile to recreate this study with obligate selfers and obligate outcrossers, as most of the Cape *Erica* species investigated here appear to be facultative outcrossers, with some degree of self-compatibility. This along with a comprehensive phylogeny may allow us to see clear shifts in correlates with increased selfing and permit us to clarify some uncertainty in the results.

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Appendix 1a: Species-specific Chi-squared (χ^2) tests comparing fruit set between treatments. Significant differences are indicated in **bold** font.

Species	Comparison of Fruit Set Between Species								
	Comparing Three Treatments			Cross- vs. Self-pollination		Cross-pollination vs. Autonomy		Autonomy vs. Self-pollination	
	Wald Chi-squared (χ^2)	p	df	p	df	p	df	p	df
<i>E. hirtiflora</i>	5.602	0	2	0.241	1	0.001	1	0.006	1
<i>E. urna-viridis</i>	5.271	0.022	2	0.064	1	0.001	1	0.001	1
<i>E. baccans</i>	10.59	0.005	2	0.570	1	0.021	1	0.022	1
<i>E. nevillei</i>	7.102	0.029	2	0.312	1	0.032	1	0.326	1
<i>E. viscaria</i> ssp. <i>viscaria</i>	11.965	0.003	2	0.431	1	0.003	1	0.003	1
<i>E. imbricata</i>	10.558	0.005	2	0.182	1	0.018	1	0.004	1
<i>E. pulchella</i>	8.785	0.012	2	1.000	1	0.050	1	0.009	1
<i>E. brachialis</i>	8.194	0.017	2	0.024	1	0.016	1	0.273	1
<i>E. mammosa</i> (S)	3.309	0.191	2	0.194	1	0.219	1	0.560	1
<i>E. mammosa</i> (R)	11.087	0.004	2	0.146	1	0.003	1	0.007	1
<i>E. subdivaricata</i>	7.678	0.022	2	0.120	1	0.034	1	0.017	1
<i>E. corifolia</i>	10.003	0.007	2	0.568	1	0.005	1	0.005	1
<i>E. plukenetii</i>	11.143	0.004	2	0.170	1	0.006	1	0.170	1
<i>E. abietina</i> ssp. <i>atrorosea</i>	6.604	0.037	2	0.239	1	0.030	1	0.103	1
<i>E. abietina</i> ssp. <i>constantiana</i>	7.942	0.19	2	0.140	1	0.025	1	0.635	1

Appendix 1b: Species-specific Chi-squared (χ^2) tests comparing seed set per flower between treatments. Significant differences are indicated in **bold** font.

Species	Comparison of Seed Set per Flower Between Species								
	Comparing Three Treatments			Cross- vs. Self-pollination		Cross-pollination vs. Autonomy		Autonomy vs. Self-pollination	
	Wald Chi-squared (χ^2)	p	df	p	df	p	df	p	df
<i>E. hirtiflora</i>	22.785	0.001	2	0.761	1	0.001	1	0.002	1
<i>E. urna-viridis</i>	52.667	0.001	2	0.020	1	0.001	1	0.001	1
<i>E. baccans</i>	11.215	0.004	2	0.449	1	0.002	1	0.003	1
<i>E. nevillei</i>	47.081	0.001	2	0.001	1	0.001	1	0.277	1
<i>E. viscaria</i> ssp. <i>viscaria</i>	201.758	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. imbricata</i>	114.973	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. pulchella</i>	44.266	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. brachialis</i>	48.321	0.001	2	0.001	1	0.003	1	0.858	1
<i>E. mammosa</i> (S)	43.05	0.001	2	0.001	1	0.001	1	0.103	1
<i>E. mammosa</i> (R)	46.839	0.001	2	0.050	1	0.001	1	0.001	1
<i>E. subdivaricata</i>	112.529	0.001	2	0.001	1	0.001	1	0.006	1
<i>E. corifolia</i>	93.707	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. plukenetii</i>	34.66	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. abietina</i> ssp. <i>atorrosea</i>	39.229	0.001	2	0.238	1	0.001	1	0.001	1
<i>E. abietina</i> ssp. <i>constantiana</i>	83.465	0.001	2	0.001	1	0.001	1	0.322	1

Appendix 1c: Species-specific Chi-squared (χ^2) tests comparing seed set per fruit between treatments. Significant differences are indicated in **bold font**.

Species	Comparison of Seed Set per Fruit Between Species								
	Comparing Three Treatments			Cross- vs. Self-pollination		Cross-pollination vs. Autonomy		Autonomy vs. Self-pollination	
	Wald Chi-squared (χ^2)	p	df	p	df	p	df	p	df
<i>E. hirtiflora</i>	1.773	0.183	1	0.183	1				
<i>E. urna-viridis</i>	4.564	0.033	1	0.033	1				
<i>E. baccans</i>	3.579	0.167	1	0.848	1	0.172	1	0.172	1
<i>E. nevillei</i>	8.442	0.004	1	0.004	1				
<i>E. viscaria</i> ssp. <i>viscaria</i>	8104.441	0.001	1	0.001	1	0.001	1	0.001	1
<i>E. imbricata</i>	86.763	0.001	1	0.001	1				
<i>E. pulchella</i>	38.896	0.001	1	0.001	1				
<i>E. brachialis</i>	12.967	0.002	1	0.002	1	0.084	1	0.721	1
<i>E. mammosa</i> (S)	31.28	0.001	1	0.001	1	0.001	1	0.114	1
<i>E. mammosa</i> (R)	6.865	0.032	1	0.027	1	0.027	1	0.027	1
<i>E. subdivaricata</i>	112.653	0.001	1	0.001	1				
<i>E. corifolia</i>	110.798	0.001	1	0.001	1				
<i>E. plukenetii</i>	68.835	0.001	1	0.001	1	0.001	1	0.001	1
<i>E. abietina</i> ssp. <i>atrorosea</i>	0.203	0.652	1	0.652	1				
<i>E. abietina</i> ssp. <i>constantiana</i>	97.301	0.001	1	0.001	1				

Appendix 1d: Species-specific Chi-squared (χ^2) tests comparing seed set per ovule between treatments. Significant differences are indicated in **bold font**.

Species	Comparison of Seed Set per Ovule Between Species								
	Comparing Three Treatments			Cross- vs. Self-pollination		Cross-pollination vs. Autonomy		Autonomy vs. Self-pollination	
	Wald Chi-squared (χ^2)	p	df	p	df	p	df	p	df
<i>E. hirtiflora</i>	23.226	0.001	2	0.865	1	0.001	1	0.001	1
<i>E. urna-viridis</i>	59.938	0.001	2	0.013	1	0.001	1	0.001	1
<i>E. baccans</i>	16.969	0.001	2	0.492	1	0.001	1	0.001	1
<i>E. nevillei</i>	47.4	0.001	2	0.001	1	0.001	1	0.277	1
<i>E. viscaria</i> ssp. <i>viscaria</i>	80.488	0.001	2	0.001	1	0.001	1	0.002	1
<i>E. imbricata</i>	140.665	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. pulchella</i>	43.95	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. brachialis</i>	41.178	0.001	2	0.001	1	0.001	1	0.833	1
<i>E. mammosa</i> (S)	41.971	0.001	2	0.001	1	0.001	1	0.103	1
<i>E. mammosa</i> (R)	150.417	0.001	2	0.002	1	0.001	1	0.001	1
<i>E. subdivaricata</i>	128.893	0.001	2	0.001	1	0.001	1	0.005	1
<i>E. corifolia</i>	91.703	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. plukenetii</i>	40.746	0.001	2	0.001	1	0.001	1	0.001	1
<i>E. abietina</i> ssp. <i>atrorosea</i>	42.539	0.001	2	0.194	1	0.001	1	0.001	1
<i>E. abietina</i> ssp. <i>constantiana</i>	52.592	0.001	2	0.001	1	0.001	1	0.327	1

Chapter II

Late-acting Self-incompatibility in *Erica*?

Over 250 000 angiosperm species are in existence today (Campbell and Reece 2005) and a high proportion of these are hermaphroditic (having both male and female sexes within the same flower). Having the male and female reproductive structures in such close proximity enhances the chances of the deposition of pollen on a stigma and removal of pollen from anthers, in a single visit (Gibbs and Bianchi 1999). This may also increase the probability of self-pollination, possibly leading to unfit offspring through inbreeding depression (Seavey and Bawa 1986). Many hermaphroditic angiosperm species have evolved mechanisms which prevent self-fertilisation and promote genetic variability (Silva and Goring 2001). These phenomena are referred to as self-incompatibility (SI) systems and there are two known types (Lewis 1979). Heteromorphic incompatibility entails morphological disparity in structures resulting in distyly or tristyly, while homomorphic incompatibility, the system this study will be focussed on, includes genetically-controlled physiological mechanisms that prevent successful pollen germination (Barrett 1988; Sage et al. 1999) on the stigma (referred to as sporophytic self-incompatibility (SSI)) or tube growth down the style (referred to as gametophytic self-incompatibility (GSI)) (Seavey and Bawa, 1999; Gibbs and Bianchi, 1999).

Lewis (1979) suggested that late-acting self-incompatibility (LSI) systems, which operate in the ovary, are relatively rare in angiosperms. He argued that incompatibility that functions after fertilization would cause ovule discounting, thus explaining scarcity of the strategy. Dulberger (1964) and Wase and Price (1991) also proposed LSI to be wasteful of ovules. Kenrick et al. (1986) suggested the reason for LSI being rare in the literature is not due to a lack of the phenomenon in nature, but merely due to a preference of authors to study short-lived herbaceous species (Sage et al. 1994). Alternatively, Sage et al. (1999) suggested that if plants were to minimize resource investment into selfed-seeds, plants with LSI might actually save resources as opposed to those that invest in selfed-seeds but experience loss due to inbreeding depression (ID). Morgan et al. (1997) propose that these saved resources can be used later to increase lifetime fitness.

Additional negative effects of self-pollination have been noted (Cope 1962; Dulberger 1964; Wase and Price 1991). Chase experiments indicated that stylar clogging by selfed pollen tubes may also affect seed set negatively due to prevention of subsequent cross-pollination (Sage et al. 1999). However, Sage et al. (1999) concluded that the reduced seed set for the self-pollination treatment might be due to a lack of hormonal cues from ovular tissue for ovule development rather than physical blockage. In a series of studies, O'Neil (1997) showed that pollen tubes provide important stimuli necessary for several reproductive processes, including ovule development. Furthermore, Seavey and Bawa (1986) suggested that maternal ovarian tissue retards the growth rate of selfed pollen tubes and/or restricts penetration of ovules.

Although LSI is conceptually straightforward, it is experimentally very difficult to discern from early-acting inbreeding depression (EAID) (Sage et al. 1999). This is mainly because LSI and EAID have different causes but both manifest with low seed set, even with apparently normal pollen tube growth (Bittencourt and Semir, 2005). The distinction is important due to the view that SI is a pre-zygotic mechanism and that post-zygotic sterility and rejection of selfed-ovules is more likely due to EAID (Seavey and Bawa, 1986 Sage et al., 1994).

Over the last few decades great strides have been made investigating ovular rejection as a potential SI system, resulting in a set of about seven criteria to discern LSI from EAID (Lipow and Wyatt 1999; Sage and Sampson 2003; Bittencourt and Semir 2005): 1) The timing of abortion. SI is identified by uniform failure at a single stage of life, whereas ID demonstrates continual failure throughout life. 2) The degree of variability in selfed seed set among individuals of a genetically diverse population. Inbreeding depression would result in a variable selfed seed set, while SI would yield seed set of zero or close to zero for most individuals. 3) Dependence of abortion on the paternal genotype. Cross-incompatibility between siblings or parents and offspring, with a limited number of cross-compatible groups indicating segregation of S-alleles, might be evidence of genetically based LSI. 4) Embryos carrying lethal homozygous genes would not survive, whereas those aborting due to incompatibility might survive if their incompatible nature does not affect their physiology (Sage and Sampson 2003). 5) Failure of SI due to mutation. Genetically altering plants that are presumed to have a LSI

system, by means of mutation, would cause a shift from self-incompatible to self-compatible, whereas pistil abortion might increase with mutation if ID is involved. 6) Verification of the ovule penetration. In a study by Gibbs and Bianchi (1999) pollen tube growth down the style was assessed and in two species of Bignoniaceae that were found to enter the ovary and penetrate many ovules providing visual support of selfed tubes penetrating ovules. 7) Histological analysis of post pollination events after cross and self-pollination treatments (Bittencourt et al. 2003). Lower effectiveness of ovule penetration, fertilization and embryo initiation as well as an absence of embryos or structural abnormalities in ovules of self- vs. crossed treatments indicate a delayed SI mechanism, while this would not be expected for ID (Bittencourt and Semir 2005). Bittencourt et al. (2003) assessed ovule penetration, fertilization and endosperm initiation in *Spathodea campanulata* (Bignoniaceae). They found clear indications for self-incompatibility and successful penetration of ovules by selfed pollen tubes and concluded LSI for the species.

Breeding systems analyses in Cape *Erica* are severely lacking. In an attempt to start filling the gaps I aimed to investigate the incompatibility systems in *Erica*, my main focus was on whether they exhibit the classic stigmatic or stylar incompatibility systems or an ovular self-incompatibility system. Moreover, I qualitatively determined if differences in the speed of tube growth occurred between selfed vs. crossed pollen tubes and whether this correlates with LSI.

Methods

Histological Analyses - Pollen tubes

Each species belonged to a population of at least ~50 individuals and each population was located in stands of mature fynbos and species selection was based on geographical availability. Two branch samples from two separate individuals were collected (cut from adult plants) before anthesis (to ensure anther-ring was undisturbed) for each of the eight species. Hand-pollinations were performed, both self-pollination and cross-pollination, on each branch for each time interval. Hand-pollinated flowers were harvested at different time interval: 15 hours, 24 hours and 48 hours, after hand pollinations. *Erica urna-viridis* was not harvested at 48hours; the species lacked flowers with intact anther-rings and consequently we collected flowers after 15 and 24 hours only. Flowers of *E. mammosa* and *E. brachialis* were harvested after 15h, 24h and 111h. Unforeseen circumstances resulted in flowers not being collected in time (48h) and left to grow over a weekend, for an additional 2.5 days. Overall, two hand-pollinated flowers could successfully be viewed for each species per time interval and for both treatments (see Table1).

At the different time intervals, after hand-pollination, (as indicated in chapter 1) 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 over 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 in distilled water again, twice over an hour, after which 20% H₂O₂ (hydrogen peroxide) was applied for 4 hours. After sufficient time had lapsed, the tissues were rinsed in distilled water again, twice over an hour. Staining of the styles and ovaries enables better visualization of the pollen tubes. To prepare the stain, 21ml of 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. Gynoecia were left in the stain for four hours before mounting in glycerine and a drop of the stain, and examined using a Zeiss Laser Scanning Microscope

510 META. Images were viewed in Zen 2012 at 20x magnification, with a green filter (MaiTai) across a manually adjusted wavelength of 480nm to 780nm. When analysing the pollen tubes successful germination (stigma), pollen tube growth (down the style) and fertilization (in ovary) were noted by visual inspection.

Compatibility Indices

Following Zapata and Arroyo (1978), ratios based on seed set were calculated for each species in order to determine the degree of self-compatibility. The index of self-incompatibility (ISI) was calculated as the average seed set of self-pollinated flowers divided by the average seed set of cross-pollinated flowers. The values obtained from this index range from 0 (fully self-incompatible) to 1 (fully self-compatible).

Results

Generally, self-fertilised flowers appear to experience slower pollen tube growth compared to cross-fertilized flowers, regardless of species. The results indicate successful pollen tube growth to the ovary for both the cross-pollination and self-pollination treatments of all eight species (table 1). In some cases, as for *E. pulchella*, *E. imbricata*, and *E. plukenetii* pollen tubes of both treatments reached the ovary after only 15 hours of growth. For both cross- and self-pollination treatments, pollen tubes of *Erica baccans* and *E. viscaria* ssp. *viscaria* reached the ovary within 24 hours of growth. *E. urna-viridis*, *E. mammosa* and *E. brachialis* experienced the slowest growth of pollen tubes for the self-pollination treatment. Cross-pollinated tubes reached the ovary within 24 hours of growth, while self-pollinated tubes reached the ovary after 24 hours (for *E. urna-viridis* this occurred between 24 and 48 hours).

Table 1: Pollen tube presence in different parts of the gynoecia (stigma, style, ovary and ovule) after set tube growth periods for eight *Erica* species. Two replicates for each tube growth period per treatment (cross-pollination vs. self-pollination) were analysed. Zeroes indicate absence of tubes at the specific region in the gynoecia, whereas twos indicate successful tube presences in both samples. The ‘Inconclusive’ column pertains to instances where pollen tubes successfully reached the ovary, but ovule penetration was unclear.

Species	Hand-pollination Treatment	Growth Period	Pollen Tube Presence								
			Stigma		Style		Ovary		Ovule		
			Yes	No	Yes	No	Yes	No	Yes	No	Inconclusive
<i>E. pulchella</i>	Cross	15	2	0	2	0	2	0	0	0	2
	Self	15	2	0	2	0	2	0	0	0	2
	Cross	24	2	0	2	0	2	0	0	0	2
	Self	24	2	0	2	0	2	0	0	0	2
	Cross	48	2	0	2	0	2	0	0	0	2
	Self	48	2	0	2	0	2	0	0	0	2
<i>E. imbricata</i>	Cross	15	2	0	2	0	2	0	0	0	2
	Self	15	2	0	2	0	2	0	0	0	2
	Cross	24	2	0	2	0	2	0	0	0	2
	Self	24	2	0	2	0	2	0	0	0	2
	Cross	48	2	0	2	0	2	0	2	0	0

	Self	48	2	0	2	0	2	0	2	0	0
<i>E. plukenetii</i>	Cross	15	2	0	2	0	2	0	0	0	2
	Self	15	2	0	2	0	2	0	2	0	0
	Cross	24	2	0	2	0	2	0	0	0	2
	Self	24	2	0	2	0	2	0	2	0	0
	Cross	48	2	0	2	0	2	0	0	0	2
	Self	48	2	0	2	0	2	0	0	0	2
<i>E. urna-viridis</i>	Cross	15	2	0	2	0	2	0	0	0	2
	Self	15	2	0	0	2	0	2	0	0	2
	Cross	48	2	0	2	0	2	0	0	0	2
	Self	48	2	0	2	0	2	0	0	0	2
<i>E. baccans</i>	Cross	15	2	0	2	0	0	2	0	2	0
	Self	15	2	0	0	2	0	2	0	2	0
	Cross	24	2	0	2	0	2	0	0	0	2
	Self	24	2	0	2	0	2	0	0	0	2
	Cross	48	2	0	2	0	2	0	0	0	2
	Self	48	2	0	2	0	2	0	0	0	2
<i>E. viscaria ssp. viscaria</i>	Cross	15	2	0	2	0	0	2	0	2	0
	Self	15	2	0	0	2	0	2	0	2	0
	Cross	24	2	0	2	0	2	0	2	0	0
	Self	24	2	0	2	0	2	0	0	0	2
	Cross	48	2	0	2	0	2	0	0	0	2
	Self	48	2	0	2	0	2	0	2	0	0
<i>E. mammosa</i>	Cross	15	2	0	2	0	0	2	0	2	0
	Self	15	2	0	0	2	0	2	0	2	0
	Cross	24	2	0	2	0	2	0	0	0	2
	Self	24	2	0	2	0	0	2	0	2	0
	Cross	111	2	0	2	0	2	0	0	0	2
	Self	111	2	0	2	0	2	0	0	0	2
<i>E. brachialis</i>	Cross	15	2	0	2	0	0	2	0	2	0
	Self	15	2	0	2	0	0	2	0	2	0
	Cross	24	2	0	2	0	2	0	0	0	2
	Self	24	2	0	2	0	0	2	0	2	0
	Cross	111	2	0	2	0	2	0	0	0	2
	Self	111	2	0	2	0	2	0	2	0	0

Based on seed set data the Index of Self-incompatibility (ISI) predicted the majority of the study species to experience some degree of self-compatibility (table 2), although in most cases this was very low. Species with an index value of less than or equal to 0.3 were classified as mostly self-incompatible, while those with a value between 0.3 and 1 were classified as incompletely self-incompatible. *Erica baccans* and *E. urna-viridis* scored the highest index values (ISI>0.6) and appear to approach full self-compatibility.

Table 2: Index of Self-incompatibility (ISI) based on seed set data obtained from hand-pollination treatments (cross-pollination, self-pollination and autonomous selfing) for eight *Erica* species.

Species	ISI	Compatibility
<i>E. imbricata</i>	0.07	Self-incompatible
<i>E. viscaria ssp. viscaria</i>	0.07	Self-incompatible
<i>E. brachialis</i>	0.10	Self-incompatible
<i>E. plukenetii</i>	0.13	Self-incompatible
<i>E. mammosa</i> (Seeder)	0.16	Self-incompatible
<i>E. pulchella</i>	0.19	Self-incompatible
<i>E. urna-viridis</i>	0.64	Increased self-compatibility
<i>E. baccans</i>	0.86	Increased self-compatibility

Across all species, pollen tubes were able to successfully germinate and grow through the style and penetrate the ovary regardless of the treatment (Table1; Figure 1-4). Due to unclear sample slides, it was not always possible to capture images of ovular penetration. Consequently, only species where penetration was observed and captured for both self- and cross-pollination treatments were represented in figures 1 and 2. For figures 3 and 4 no clear images of the cross-pollinated pollen tube penetrating an ovule could be taken and consequently these images are missing from the figure.

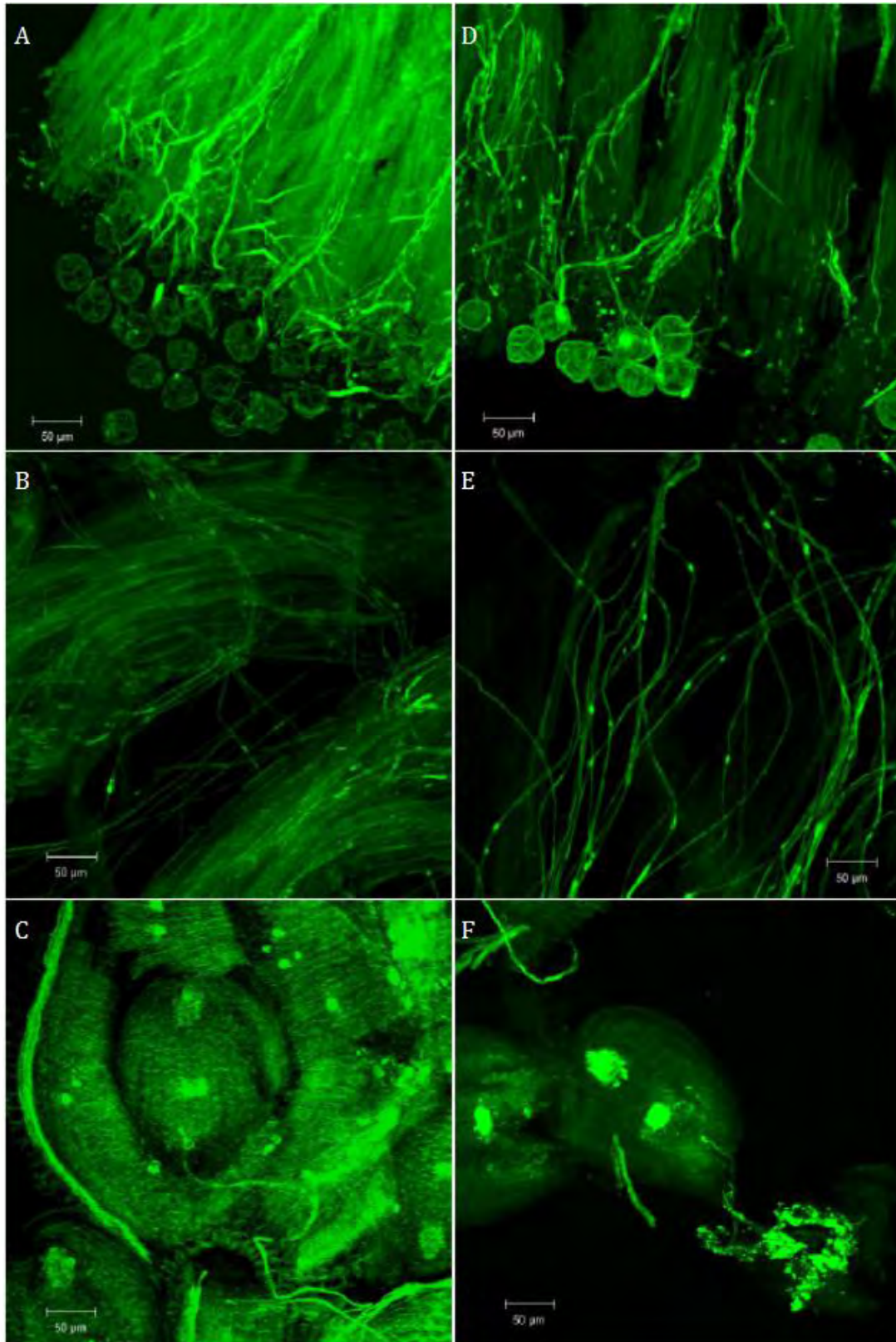


Figure 1: Fluorescent images illustrating the different stages of pollen tube growth for *Erica imbricata*. A-C represents the self-pollination treatment, while D-F represents the cross-pollination treatment. A and D denote the germination stage on the stigma, B and E show the growth of the pollen tube down the style and C and F indicate fertilisation of the ovules within the ovary.

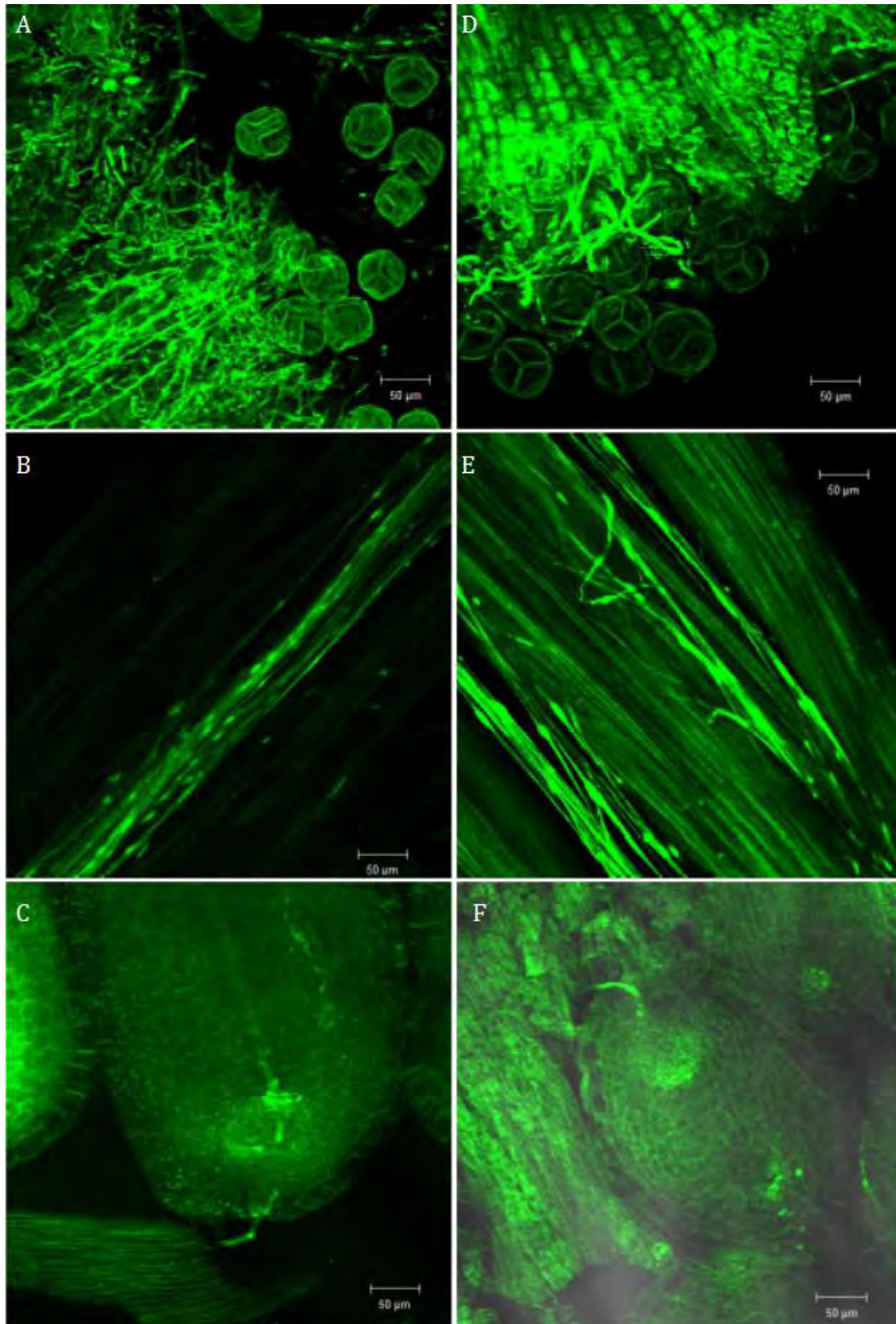


Figure 2: Fluorescent images illustrating the different stages of pollen tube growth for *Erica viscaria ssp. viscaria*. A-C represents the self-pollination treatment, while D-F represents the cross-pollination treatment. A and D denote the germination stage on the stigma, B and E show the growth of the pollen tube down the style and C and F indicate fertilisation of the ovules within the ovary.

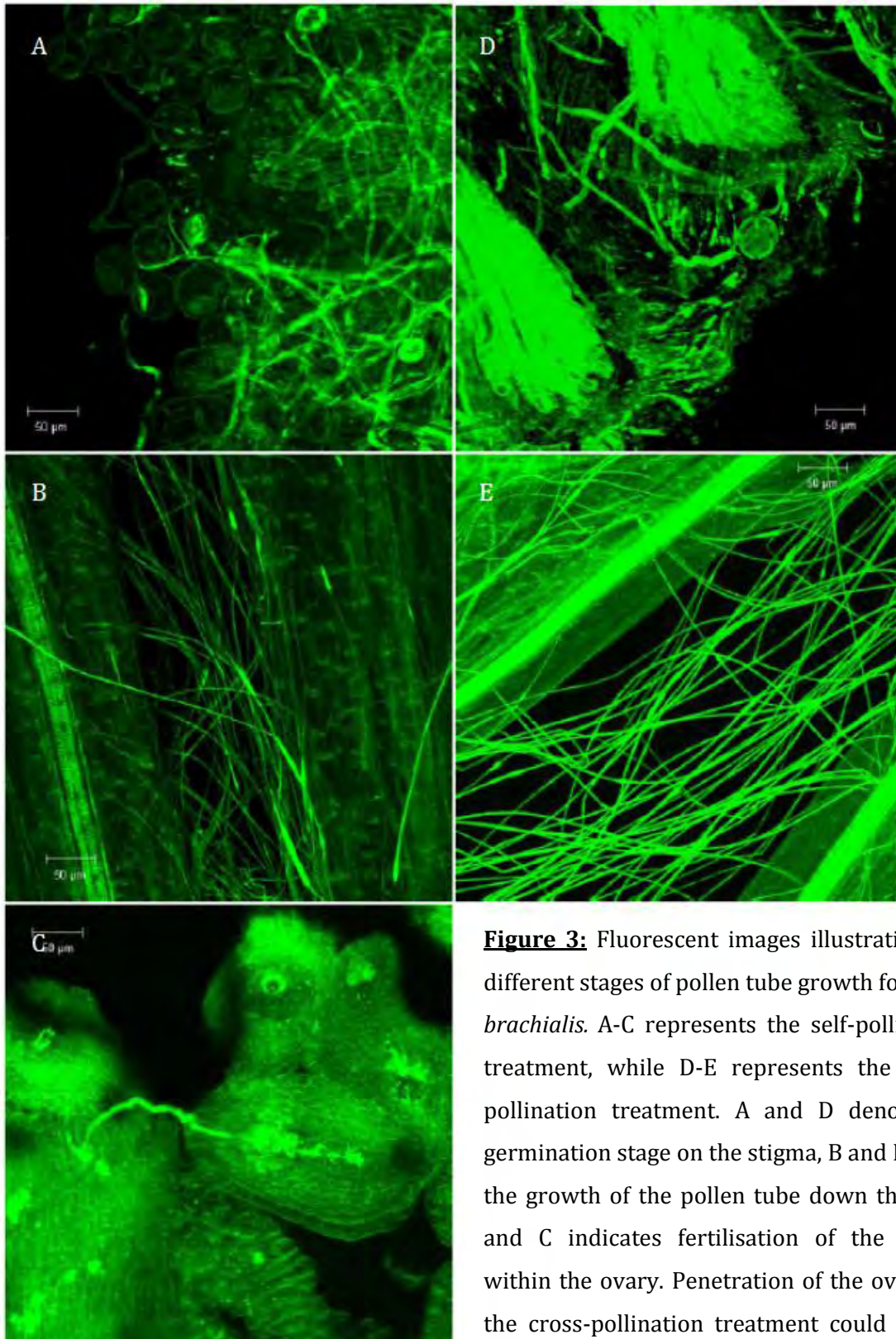


Figure 3: Fluorescent images illustrating the different stages of pollen tube growth for *Erica brachialis*. A-C represents the self-pollination treatment, while D-E represents the cross-pollination treatment. A and D denote the germination stage on the stigma, B and E show the growth of the pollen tube down the style and C indicates fertilisation of the ovules within the ovary. Penetration of the ovary for the cross-pollination treatment could not be observed.

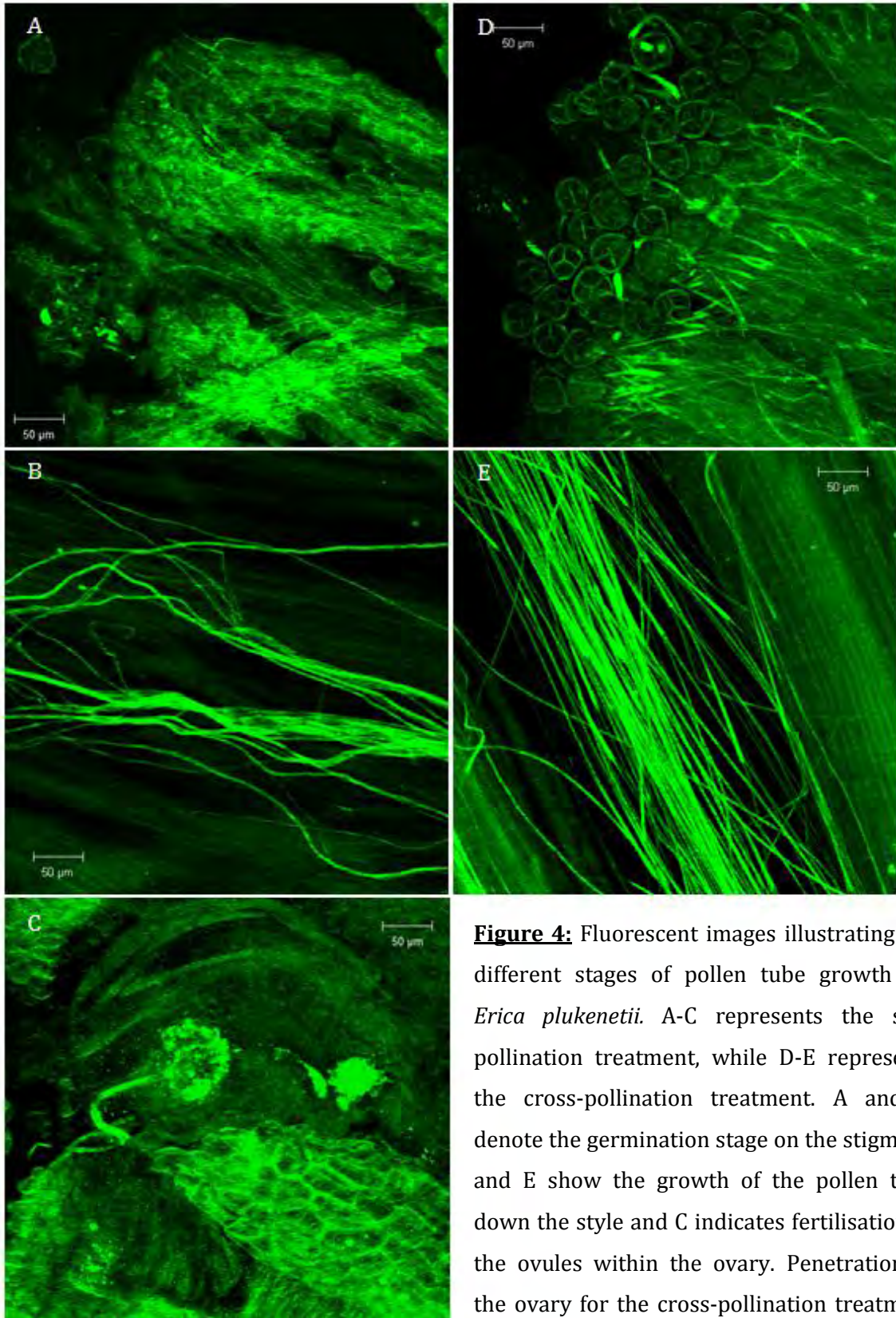


Figure 4: Fluorescent images illustrating the different stages of pollen tube growth for *Erica plukenetii*. A-C represents the self-pollination treatment, while D-E represents the cross-pollination treatment. A and D denote the germination stage on the stigma, B and E show the growth of the pollen tube down the style and C indicates fertilisation of the ovules within the ovary. Penetration of the ovary for the cross-pollination treatment could not be observed.

Discussion

Histological analyses suggested that the *Erica* species studied were self-compatible. Pollen tubes germinated on the stigma, grew down the style and penetrated the ovary regardless of the treatment (Table 1; Figure 1-4). However, in some species slower pollen tube growth was detected for the self-pollination treatment (Table 1). Although selfed tubes grew slower, they still reached the ovary and, when I was able to discern, penetrated ovules normally. Studies interpreted this slower growth of the selfed tubes as a distance signalling process, whereby miscommunication between the pollen tubes and the ovular tissue results in retarding the growth of the tube (Sears 1937; Seavey and Bawa 1986; Sage et al. 1994; 1999; Bittencourt and Semir 2005; Vaughton et al. 2010).

The results from the previous chapter indicate that most *Erica* species have some level of self-compatibility, although, seed set is usually reduced. This along with the current chapter's indication of a late acting mechanism may point toward a SI mechanism that is distinct from the classic stigmatic or stylar barriers to selfing, one that functions within the ovule. Whether this mechanism is pre- or post-zygotic is uncertain and requires further study. However, post-zygotic barriers to selfing have been reported in tropical woody species (Ellis and Sedgely 1992).

A late-acting self-incompatibility (LSI) system may explain the reduced seed set and fruit set after hand self-pollination compared to cross-pollination treatments I encountered in most species; because SI is delayed, rejection occurs after selfed tubes enter the ovule resulting in loss of those ovules and possible future seeds. *Erica baccans* and *E. urna-viridis* were the only sampled species that illustrated high self-compatibility ($0.6 > ISI < 1$) with a seed set result for self-pollination that mirrors that of cross-pollination. These two species may have experienced a breakdown of their barriers to selfing and can set seed of equal proportions whether it cross-pollinates or self-pollinates. However, whether the selfed progeny will have equal fitness to the crossed progeny is unknown and would be an interesting future prospect to investigate.

Although reduced seed set may also be typical of inbreeding depression, I favour LSI due to the timing of the abortion of seeds. One measure of determining if a system is

experiencing self-incompatibility (SI) rather than early-acting inbreeding depression (EAID) suggests uniform failure at a single stage of life. Inbreeding depression (ID) is characterised by failure at different stages during seed development (Sage and Sampson 2003; Bittencourt and Semir 2005; Hao et al 2012). Consequently, all species except *E. baccans* and *E. urna-viridis* experienced low fruit set after self-fertilisation, while approximately 73% of species have low seed set after self-fertilisation. Self-incompatibility is further reflected in the uniformly low seed set between individuals of each species (Sage and Sampson 2003; Bittencourt and Semir 2005).

The relevance of this study is that ovule discounting could be significant in *Erica*. Selfed seeds are presumably lost due to the late rejection system, reducing seed set dramatically. This is supported by previous findings (Chapter 1), which suggests that in most species (60%) less than half of the available ovules develop into seeds for the cross-pollination treatment, while for self-pollination 73% of species experience less than 20% seed set per ovule. This suggests that additional mechanisms to prevent selfing may exist, for instance protogyny. The scope of this study did not allow for genetically based analyses but the mechanisms of genetic control would be well worth investigating for such an evolutionarily interesting genus. Additionally, analyses of seed development and growth success after selfing would also contribute to a clearer picture of LSI in *Erica*.

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Chapter III

Pollen-ovule Ratios in the Genus *Erica*

The knowledge of plant breeding systems is pivotal in understanding the evolutionary trends and life history of species, due to the different genetic and ecological consequences of xenogamy (outcrossing) and autogamy (selfing) (Preston, 1986). Barrett et al. (1996) suggest that evolutionary shifts in mating systems, from outcrossing to autogamy, have occurred numerous times in flowering plants and coincide with a change in floral biology, life history and ecology. This shift has been correlated with decreased floral display size, floral size, and other morphological changes (Cruden 1977; Jacquemart 2003; Button et al. 2012), which include modifications in pollen grain number and, more specific to this study, to pollen-ovule (P/O) ratios (Cruden 1977).

Studies have illustrated that outcrossing taxa produce more pollen grains than closely related autogamous taxa (Gibbs et al. 1975; Cruden 1977; Wyatt 1984; Preston 1986; Vasek and Weng 1988; Gallardo et al. 1994; Jürgens and Gottsberger 2002; Cruden 2000). Furthermore, P/O ratios decrease with a shift from predominantly outcrossing to predominantly autogamous breeding systems (Baker 1967; Cruden 1973; Gibbs et al. 1975). Since these findings emerged, P/O ratios have been increasingly used to as an indicator of plant breeding systems. Cruden (1977) was the first to link P/O ratios with breeding systems. He compiled data from a range of species and concluded that P/O ratios reflect pollination efficiency, described as the likelihood of a pollen grain reaching the stigma without the loss of fecundity. Consequently, lower P/O ratios are indicative of more efficient pollen transfer. Following this logic, cleistogamous (closed-flowered) species should have the lowest P/O ratios, followed by autogamous species; outcrossing species should have the highest P/O ratios due to reliance on inefficient external vectors to transfer pollen. Subsequently, many other studies agree with this 'efficiency' hypothesis and found a positive correlation between P/O ratios and the degree of outcrossing (Preston 1986). This association occurs between species (Cruden 1977; Cruden and Jensen 1979; Jürgens et al. 2002; Spira 1980; Wyatt 1984) as well as within species (Cruden 1976; Schoen 1977). In another study conducted on 19 outcrossing species, Cruden and Miller-Ward (1981) found that as the stigmatic area increases, the

pollen-bearing area of the pollinator ($r = -0.81$) decreases, and more relevant for this study, they found a negative correlation between P/O ratios and pollen grain surface area ($r = -0.86$). The latter suggests that P/O ratios might reflect pollen grain size as well as number. Cruden and Miller-Ward (1981) explained this relationship by referring to the enzymes necessary for germination and penetration of the stigma by the pollen tube. Larger pollen grains will have more of these essential enzymes and consequently, fewer large grains are required per seed. Conversely, a greater number of small grains are required to yield enough enzymes for successful pollen tube growth, resulting in a higher P/O ratio in such species.

Charnov (1982) criticized Cruden's efficiency hypothesis due to its bias towards seeds as the means to gain fitness. He argued that pollen does not solely exist to ensure seed set, but in fact as an equivalent means to fitness gain. Charnov (1982) used Cruden and Miller-Ward's (1981) data to perform a regression analysis, in which he found a negative correlation between the log P/O ratio and log pollen grain size. He, however, interpreted this relationship not as one of pollination efficiency but in terms of optimal resource allocation to sexual function (Charnov 1982; Charlesworth and Charlesworth 1981; Queller 1984; Lloyd 1987). Sex allocation theory argues that hermaphroditic plants with a degree of autogamy will experience reduced resource allocation to the amount of pollen and increased allocation to seeds; less pollen is needed due to reduced reliance on pollinators for pollen movement and consequently resources can be diverted from pollen to ovules. Along this line of logic, plants that are predominantly outcrossing will allocate more resources to pollen as this is their main means of dispersing their genes. This explains why P/O ratios (Charnov 1982; Charlesworth and Charlesworth 1981; Queller 1984) - as an approximate indicator of the ratio of resources allocated to pollen and seeds - in many instances correlate with breeding systems (Gallardo et al. 1994). Charnov (1982) and Queller (1984) stress the importance of considering both the resource allocation to the amount of pollen grains and ovules as well as the investments per pollen grain and ovule.

In the above model, the P/O reflects the relative allocation of resources to male and female function. Since size is a component of both effort and quantity, Charnov's model predicts that, species with larger pollen grains will have a lower P/O ratio. Also, species with larger seeds will have higher P/O ratios (Preston, 1986), given that seed size

shows no variation with pollen size and allocated resources remain constant (Charnov, 1982). Preston (1986) found a significant positive correlation between mean seed weight and P/O ratios among different species of Cruciferae. Uma Shanker and Ganeishaiah (1984) explored this in *Phyllanthus* species and found a positive correlation between seed size and P/O ratio. Mione and Anderson (1992) found similar results for species from Solanaceae. After removing the effects of ovule cost and the degree of self-compatibility, Gallardo et al. (1994), found a negative correlation between P/O ratios and pollen grain size for taxa of the genus *Astragalus*. He claims this supports the predictions of Charnov's model as it suggests a negative relationship is likely if breeding system and cost per ovule remain constant. However, in the *Astragalus* taxa analysed neither of these were, in fact, constant and the correlations were not statistically significant. Galloni and others (2007) examined 32 species of the subfamily, Faboideae (Fabaceae), and, as in Gallardo et al. (1994), failed to obtain a significant correlation between P/O ratio and pollen grain volume. More recently, Götzenberger (2006) examined 299 floral species and found positive correlations between P/O ratio and seed size, particularly so within the families Brassicaceae and Fabaceae, when statistically controlling for ovule number. He also took into account the degree of disturbance in the habitat of the study species and selection on seed size for establishment, but this did not affect the correlation. Furthermore, Götzenberger (2006) found the expected negative relationship between P/O ratio and pollen grain size, although he noted the exceptions seem more frequent for this relationship than for seed size.

There is clearly still a significant amount of uncertainty surrounding the correlates of pollen-ovule ratios; two well-known theories exist, both attempting to shed light on the apparent positive relationship between P/O ratios and increased xenogamy. This study surveyed species from the genus *Erica* to investigate this gradient and to determine if P/O ratio can be a dependable indicator of breeding systems. I chose this genus for two main reasons: 1) the genus *Erica* is, in most respects, relatively understudied (this is a surprising fact, as within the Cape Floristic Region of South Africa, *Erica* makes up a significant 7-8% of the floral composition), and 2) studies focussing on a group of congeneric species seem to be severely lacking. The main objectives of this study were: 1) to ascertain whether there is any correlation between the P/O ratio and the pollination syndrome, fire-survival strategy and breeding system, and 2) to assess the

extent to which Cruden's efficiency theory and Charnov's sex allocation theory best explains the data obtained.

Methods

Pollen-ovule Ratios

Acetolysis of plant material

All species studied were collected in and around the Cape Peninsula area. Acetolysis (modified from Erdtman 1943) was performed on 10-21 flowers for each of 29 species of *Erica*. In most cases, each replicate was collected from a separate individual. This is true for all species except *E. occulta*; this population was severely burnt and had a maximum of six individual that could be sampled. Fresh flowers were picked in one of two states of maturity for the acetolysis process: they were either, 1) open, but had an intact anther-ring (indicating unvisited flowers), or 2) picked just before anthesis, when pollen is mature but the flower not yet open to visitors. For large-flowered species, the corolla was snipped just above the ovary and for small-flowered species the whole flower was used. Fresh pollen bearing material was placed in Eppendorf tubes containing 0.5-1ml of glacial acetic acid. The tubes are placed into a centrifuge and rotated at 850rpms for 10 minutes. The acetic acid is then carefully decanted and the material crushed against the wall of the tube with a glass rod or forceps. In a measuring cylinder one part concentrated sulphuric acid was added to nine parts of acetic anhydride (Erdtman 1943). Approximately 1ml of the mixture was added to each eppendorf and stirred with a glass rod. The eppendorfs were then transferred to a water bath (100°C) for 5-10 minutes and stirred occasionally. Heating was done in fume cupboard. The eppendorfs were moved to the centrifuge for 10 minutes (rotated at 1250rpms) and then the liquid was decanted in to a waste container. A series of rinses then followed by adding approximately 1ml of water to each eppendorf, centrifuging and then decanting. This was repeated until the solutions in each eppendorf looked clear. Lastly, 200 µl of alcohol (70%) is added to each eppendorf to preserve the polliniferous fluid.

Haemocytometer

Each eppendorf was vortexed at for 5 seconds until the pollen was equally distributed throughout the alcohol solution. Additionally, a pipette was used to mix the liquid further by pumping the liquid inside the eppendorfs 50 times. Both sides of the haemocytometer were filled with 15µl of the polliniferous fluid. The pollen grains in each of the nine small chamber of the haemocytometer were counted and recorded

separately. This count was converted to a count per small chamber (average of nine counts) to give the concentration of pollen grains per 0.1µl. It was then multiplied by ten to give the concentration per 1µl, and this was multiplied by the total volume of solution in the eppendorf to give the number of pollen grains per flower. The haemocytometer was rinsed with distilled water and then 99% alcohol after each count, and it was completely dried before the next count (if the haemocytometer is wet then it is difficult to get the pollen suspension drawn into the counting chamber correctly). The number of ovules was determined by dissecting the ovary and counting the number of ovules inside. It was assumed that all ovules would develop into seeds if fertilised.

Breeding systems vs. Pollen-ovule Ratios

Morphological Outcrossing Index (MOI)

An "Index of Outcrossing" (MOI) was determined for 22 of the 29 species from their morphological attributes. Each MOI class is the sum of assigned values for five characteristics of the each species' flowers: 1) flower aperture i.e. the target area. Flowers were assigned to one of three classes; corolla with no aperture (0 mm) = 0; 0 - 3mm wide =1; more than 3mm wide = 2. 2. Cleistogamous (closed-flowered) species do not rely on pollinators for pollen transfer but rather set seed either via self-pollination or autonomous selfing. This reduces the possibility for outcrossing and results in a low index score. Alternatively, large chasmogamous (open-flowered) species would have reproductive parts spaced further apart and require pollinating vectors for pollen deposition and removal. Subsequently, allowing for greater outcrossing ability and therefore receiving a larger score on the index. Relative stigma-anther distance - indication of spatial relationship and calculated as:

$$= \frac{\text{Stigma length} - \text{Anther length}}{\text{Length of tube}}$$

For a stigma-anther distance less than or equal to zero, where contact between the stigma and anther seemed possible, the value given was 0. For an average stigma-anther distance between 0 and 0.25, the value was 1, and for distance larger than 0.25, the value was 2. Logically, a smaller stigma-anther distance would increase the probability of self-fertilisation occurring and such a species would therefore receive a smaller index score, while those with larger distances between the stigma and anthers would have increased capacity for outcrossing and score a higher index value. The last three morphological characters were tube width, with classes: 0–0.39mm = 1; 0.4–0.59mm = 2

and $>0.6\text{mm} = 3$, tube length, with classes: $0.5\text{-}0.79\text{mm} = 1$; $0.80\text{-}1.19\text{mm} = 2$ and $>1.2\text{mm} = 3$, and size, with classes: $0.5\text{-}0.99\text{mm} = 1$; $1.0 - 1.49\text{mm} = 2$; $1.50\text{-}1.89\text{mm} = 3$, $>1.9\text{mm} = 4$. These floral measurements are concerned with the size dimensions of the flowers. In theory, flowers that are larger will offer more rewards to pollinators and enhance outcrossing potential, resulting in a larger index value.

Data Analysis

All statistical analyses were performed using the STATISTICA 10 package (StatSoft, Inc. 2013). Tests for homogeneity and normality were performed on the data. Log transformations of the average pollen grain size, average pollen number and pollen-ovule ratios for each species were necessary to improve the normality of the data. Correlations depicting the relationship between pollen number and ovule number; ISI and P/O ratios; pollen grain size and P/O ratios; and pollen grain size and pollen number were performed in STATISTICA 10 while the graphs for these were plotted using EXCEL 2010 (Microsoft Excel 2010, Redmond, Washington).

To determine the classes for the Morphological Outcrossing index (MOI) we performed a one-way ANOVA comparing the raw traits values between species and a Tukey post-hoc test for multiple comparisons of species mean differences. This allowed us to see significant differences between groups of species and allocate values accordingly. The compatibility indices used in Table 1 were calculated in Chapter 2.

One-way ANOVAs were also used to disclose possible significant differences within pollination syndromes (in terms of pollen-ovule ratios and pollen number) and fire survival strategy (in terms of pollen-ovule ratios), respectively. Furthermore, a t-test based on pollen-ovule ratios was used to analyse differences between breeding systems.

Results

Breeding Systems vs. Pollen-ovule Ratios

The Morphological Outcrossing Index (MOI) classified all of the species as class 5 or higher, indicating that species are most likely outcrossers (Cruden 1977). However, the incompatibility indices (index of self-incompatibility (ISI) and index of autonomous selfing (IAS)), calculated for 15 of 22 species using seed-and fruit set data do not corroborate this finding. The indices predict some species to be outcrossers, while *Erica baccans*, *E. hirtiflora*, *E. abietina* ssp. *atorosea* and *E. urna-viridis*, are facultative outcrossers. According to Cruden's classification (inferred from Appendix 1, Cruden 1977), five of the 15 species were facultatively autogamous, while 10 were facultative outcrossers. The outlier, *E. riparia*, had a pollen ovule ratio of 3800 and was therefore, classified as an outcrossing species.

Table 1: Classes, assigned to 22 of 29 *Erica* species, based on a Morphological Outcrossing Index (MOI), relative to pollen-ovule (P/O) ratios (\pm sdev) for each. Classification of species into breeding systems based on compatibility indices. When comparing the outcrossing and facultative outcrossing breeding systems, in terms of P/O ratios, no significant ($F= 0.223$, $df= 1$, $p= 0.637$) difference was found.

Species Name	Number of flowers	Average P/O ratios	\pm Sdev	MOI	Classification Based on Indices (ISI and IAS)
<i>Erica mammosa (Resprouter)</i>	10	68	20.9	10	Outcrosser
<i>Erica mammosa (Seeder)</i>	10	86	25.4	13	Outcrosser
<i>Erica occulta</i>	19	104	77.7	7	-----
<i>Erica viscaria ssp. viscaria</i>	21	109	46.9	12	Outcrosser
<i>Erica brachialis</i>	6	136	59.8	14	Outcrosser
<i>Erica excavata</i>	12	150	71.6	11	-----
<i>Erica baccans</i>	15	158	52.3	8	Facultative Outcrosser
<i>Erica grisbrookii</i>	11	167	66.3	11	-----
<i>Erica abietina ssp. atrorosea</i>	9	167	83.2	11	Facultative Outcrosser
<i>Erica hirtiflora</i>	13	210	77.8	8	Facultative Outcrosser
<i>Erica pulchella</i>	8	221	35.6	7	Outcrosser
<i>Erica corifolia</i>	10	227	63.9	5	Outcrosser
<i>Erica nabea</i>	16	227	41.4	6	-----
<i>Erica curviflora</i>	10	227	42.3	13	-----
<i>Erica urna-viridis</i>	7	253	56.6	12	Facultative Outcrosser
<i>Erica abietina ssp. constantiana</i>	5	253	121.2	13	Outcrosser
<i>Erica imbricata</i>	10	254	38.4	6	Outcrosser
<i>Erica plukenetii</i>	15	320	110.6	9	Outcrosser
<i>Erica nevillei</i>	8	329	118.1	13	Outcrosser
<i>Erica subdivaricata</i>	15	335	191.0	8	Outcrosser
<i>Erica irregularis</i>	19	466	109.7	8	-----
<i>Erica riparia</i>	15	3800	1044.5	9	-----

Average pollen and ovule counts suggest a positive correlation ($r=0.81$, $F= 248.12$; $p= 0.001$). This holds true for all species except *E. riparia*, which had an average pollen number, similar to other insect-pollinated species, but a comparatively low ovule number, yielding an above average pollen-ovule ratio of 3800 (Table 1). Due to this disparity with the general trend, the data representing *E. riparia* was not included in the analysis. The trend suggests pollen/ovule ratios to be similar across the species sampled.

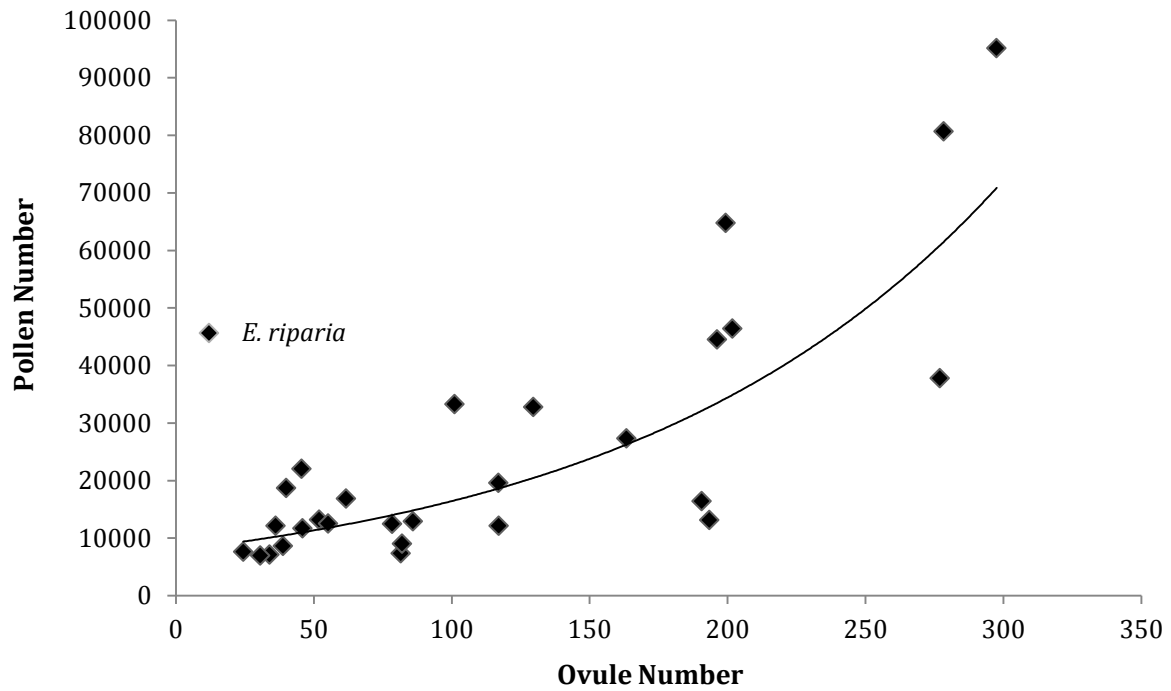


Figure 1: Relationship between the number of pollen and ovules for 29 species of *Erica*.

The relationship between selfing ability and P/O ratio is weak, indicating that the pollen-ovule ratio has no significant ($r = -0.153$; $F = 0.313$; $p = 0.586$) effect on the level of self-compatibility.

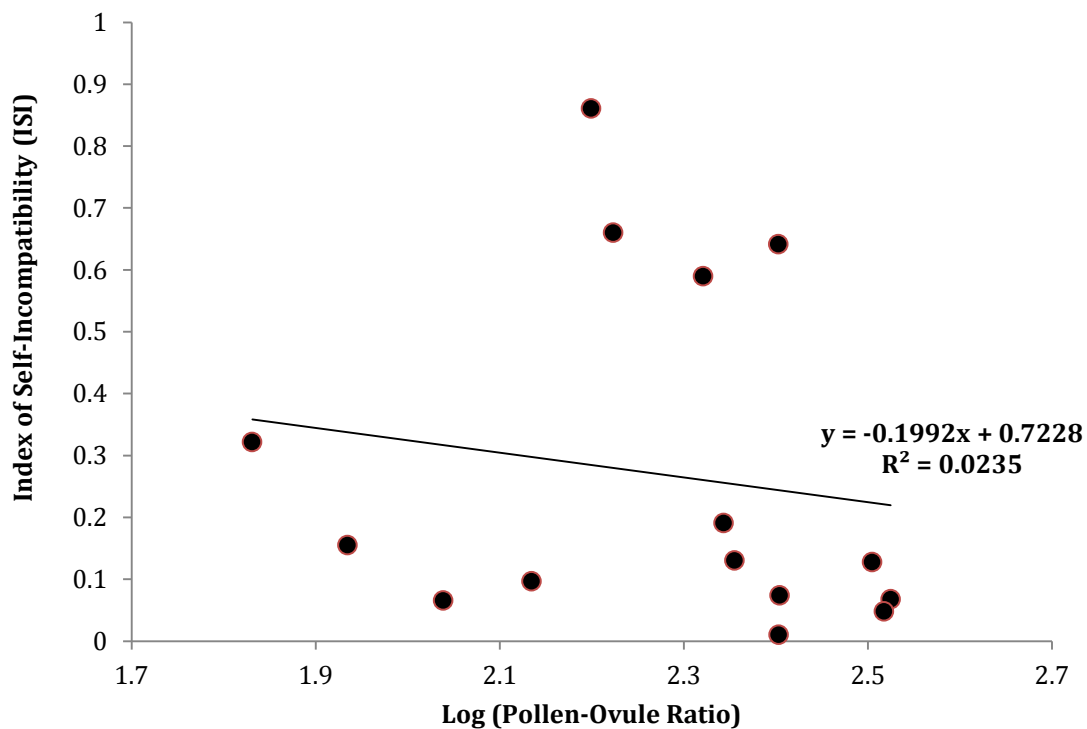


Figure 2: Relationship between the logarithm of pollen ovule ratio and an Index of Self-incompatibility (ISI) for 15 *Erica* species. The y-axis shows increasing self-compatibility from zero to one.

Pollination Syndrome and Fire-survival Strategy

In terms of pollen-ovule (P/O) ratios, significant differences were found when comparing other insect-pollinated species with bird-pollinated ($F= 10.01$; $df= 3$; $p< 0.001$) species and other insect-pollinated species with bee-pollinated ($F= 10.01$; $df= 3$; $p= 0.001$) species. *Erica riparia*, an insect-pollinated species, has a significantly higher ($F= 39.85$; $df= 28$; $p< 0.001$) pollen-ovule ratio than all other species. *E. lasciva*, (wind-pollinated) had the second highest pollen ovule ratio. Overall the wind-pollinated species does not appear to be significantly different ($F= 10.01$ $df= 3$; $p= 0.699$) from any other pollination syndromes.

No significant differences, in P/O ratios ($F= 0.926$; $df= 1$; $p= 0.337$), when comparing different fire-survival strategies (seeders vs. resprouters). The epicormic resprouter, *Erica brachialis*, resprouts from the stem after fire but acts as a seeder in fireless years – the species was thus added to the seeder category during analyses. Fire-survival strategies (Figure 1b) do not separate pollen-ovule ratios into distinct groups; there is overlap across the range. This is supported by *E. mammosa*: both the resprouting and seeder form have similar pollen-ovule ratios, 68 and 86, respectively. *Erica riparia*, once again, has a pollen-ovule ratio far above the rest of the seeders group ($p< 0.01$). *E. brachialis*, an epicormic resprouter, is significantly different to seeders: *E. sudivaricata* ($p< 0.05$), *E. lasciva* ($p< 0.01$), *E. riparia* ($p< 0.01$), *E. pudens* ($p< 0.05$) and *E. irregularis* ($p< 0.01$), and to resprouter: *E. recurvata* ($p< 0.01$) (Appendix 1).

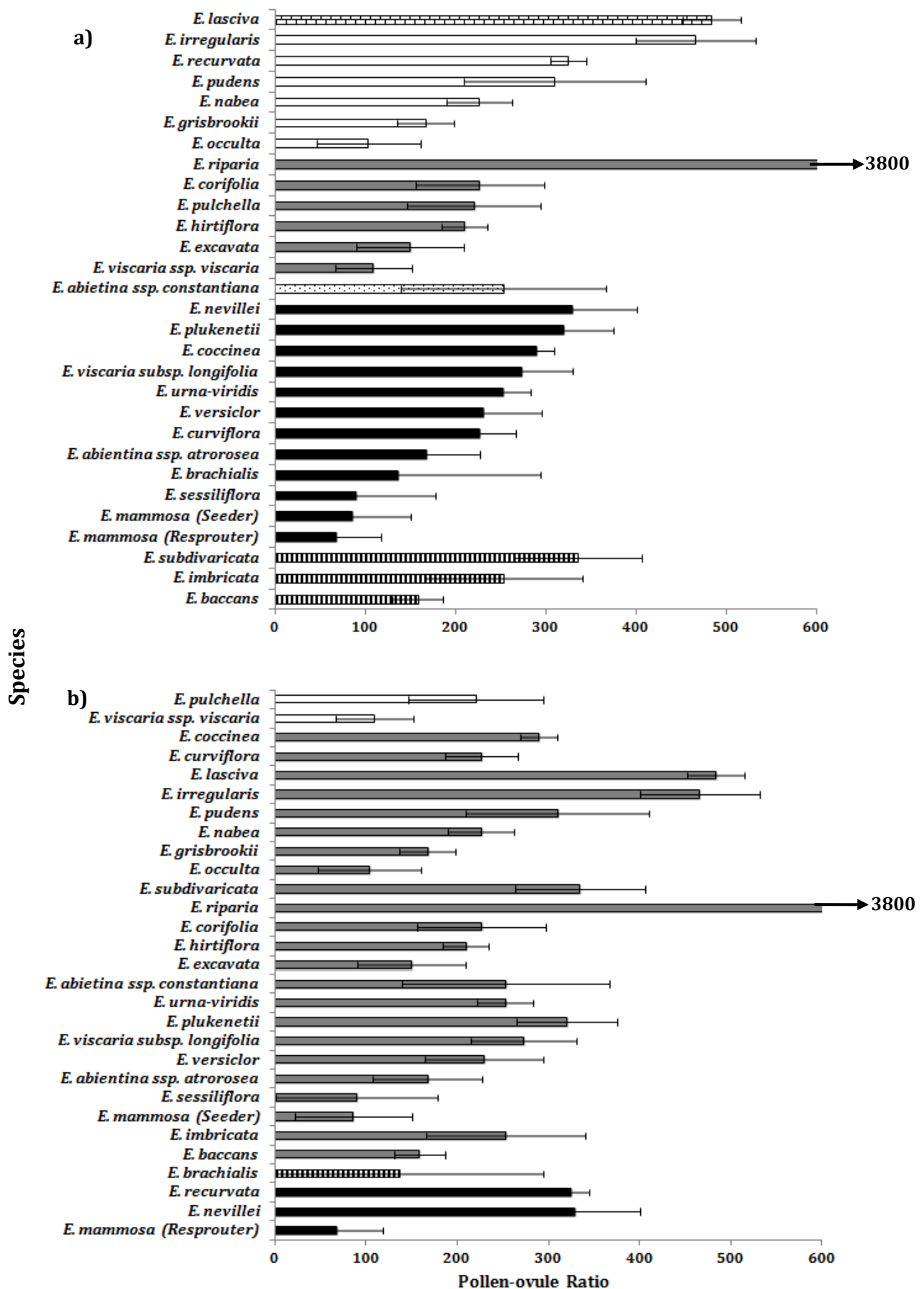


Figure 3: the relationship between pollen-ovule ratios and (a) pollination syndrome and (b) fire-survival strategy. Standard errors are indicated with bars. For (a) the differently coloured bars indicate different pollination syndromes: bricks – wind; white – unknown; grey – other insects (including moth/butterfly pollination); dots – insects/birds; black – birds; vertical

stripes – bees. For (b) the differently coloured bars indicate fire-survival strategy: white – unknown; grey – seeder; vertical stripes – seeder/epicormic resprouter; black – resprouter.

Pollen Number as Predictor of Pollination Syndrome

Statistically significant differences in pollen number were found between bird and insect-pollinated species, as well as bird and bee-pollinated species ($F= 21.68$; $df= 3$; $p< 0.001$). Insect and bee-pollinated species were not significantly different ($F= 21.68$; $df= 3$; $p= 0.999$). Average pollen number increases from insect- to bird-pollinated species but this is not seen in average ovule number. There are some anomalies in both pollination syndromes: *E. mammosa* (resprouter) and *E. sessiliflora* are bird-pollinated but pollen number reflects insect-pollination, while *E. riparia* is insect-pollinated but pollen number reflects bird-pollination. *Erica abietina* ssp. *constantiana* is both bird ($F= 17.97$; $df= 4$; $p= 0.124$) and insect-pollinated ($F= 17.97$; $df= 4$; $p= 1.00$), it is not significantly different to either.

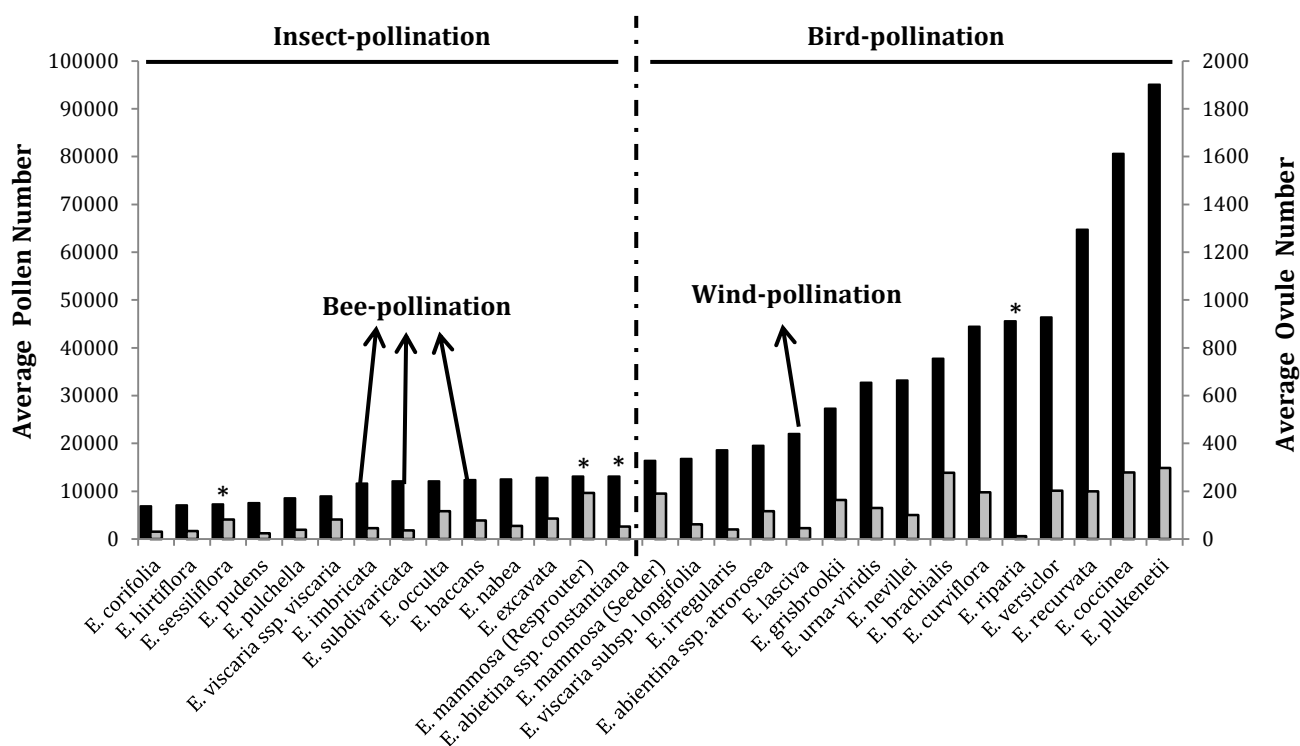


Figure 4: Comparison of Pollen Number (black bars) and Ovule number (grey bars) with 29 *Erica* species of different pollination syndromes. Species with unexpected results are indicated with an asterisk (*). Separation of the different pollination syndromes are indicated with lines.

Sex Allocation

Pollen grain size and pollen ovule ratio has a strong negative correlation ($r= 0.71$; $F= 27.2$; $p= 0.001$), with a slope of -1.24 (Figure 5a). This relationship may be influenced, to some degree, by *E. riparia* pulling the trend towards a greater pollen-ovule ratio to pollen grain size (Figure 5a). Figure 5b indicates a weak relationship ($r= 0.335$; $F= 3.41$; $p= 0.076$) between pollen grain size (μm^2) and pollen grain number, suggesting that the pollen number does not significantly influenced pollen size or vice versa.

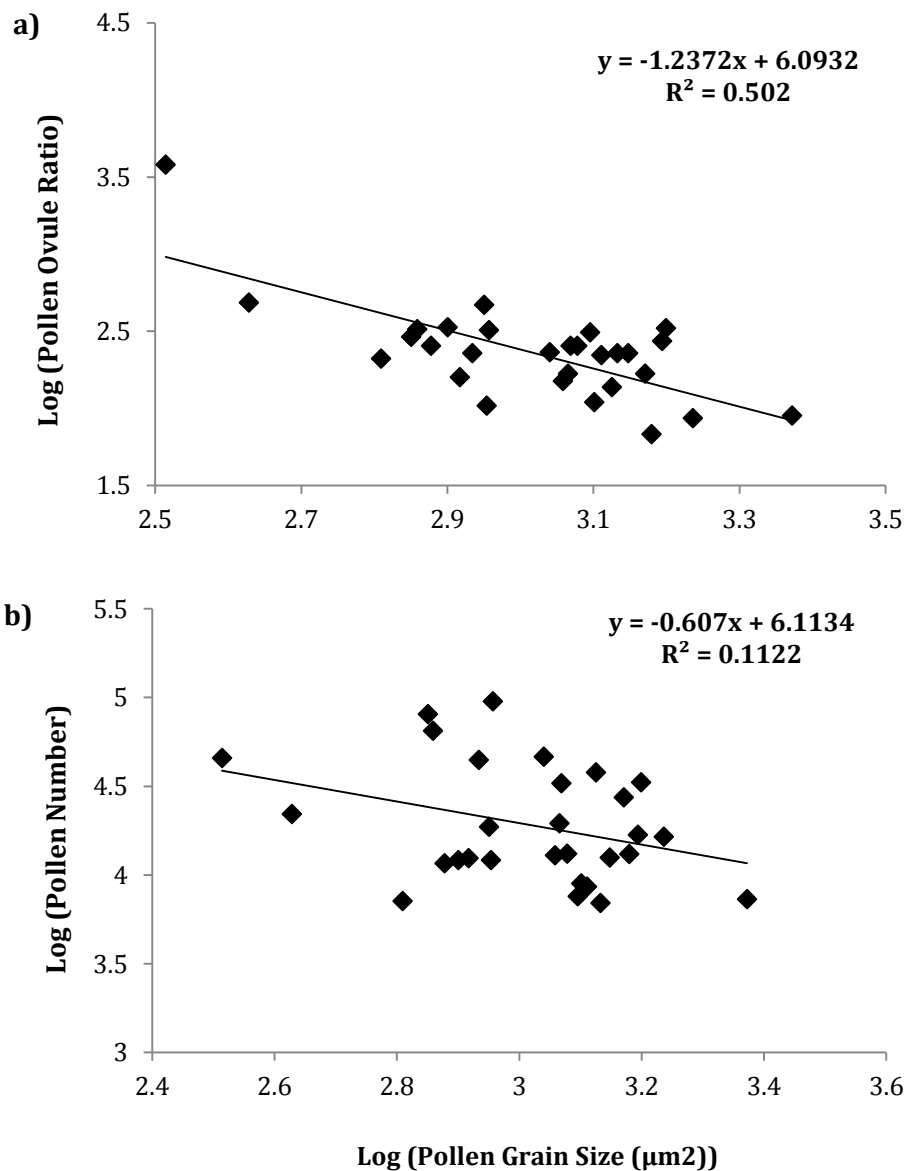


Figure 5: Relationship between the logarithm of pollen grain size (μm^2) and (a) Log(Pollen-Ovule ratio) and (b) Log(Pollen Number), for 29 outcrossing *Erica* species.

Discussion

Breeding Systems and Pollen-ovule Ratios

The Morphological Outcrossing Index (MOI) allocated 5 or higher to each species, which indicates that all species are outcrossers. This is not supported by the indices, which is based on seed set data of a subset of the species. This finding suggests that, within this genus, differences in morphological traits may not be the best measure to confirm different breeding systems. The indices (ISI and IAS) further suggested that an index based on morphological features may be helpful when comparing significantly different breeding systems, such as obligate autogamy and xenogamy, but not mixed systems where differences are not as clear-cut. Among the 15 species, P/O ratios were not significantly different between breeding systems (Table 1; $p = 0.637$).

These findings do not conform to Cruden's predictions; species do not fit within his P/O ranges. According to his classification, 32% species analysed in this study have average P/O ratios which indicate facultative autogamy (P/O ratio of approx. 32-397), while the rest are facultative outcrossers (P/O ratio of approx. 160 to 2558), with the exception of *E. riparia* (3800) which is an obligate outcrosser (P/O ratio approx. 1062 to 19525). Ideally, the 'efficiency' theory would have been more accurately tested if I sampled species with a more diverse range of breeding systems. However, since Cruden (1977) suggests marked differences in P/O ratios for these classes, I consider my result an adequate preliminary test of the theory for this genus. Furthermore, if Cruden's 'efficiency' theory holds true for this genus, one would expect a very strong negative correlation between the self-incompatibility index (ISI) and the P/O ratio, the more self-compatible a species, the lower the P/O ratio will be. Figure 2 ($p > 0.05$) weakly reflects this relationship.

Pollinations Syndrome and Pollen-ovule Ratios

When comparing pollination syndromes in terms of P/O ratios, wind-pollination was not significantly different to any other pollination syndrome (Figure 3b, $p > 0.05$). The 'efficiency' theory predicts wind-pollinated species to have an exceptionally high P/O ratio due to its inefficient means of transporting pollen (Cruden 1977). Tomlinson et al. (1979) supported this by showing wind-pollinated species to produce significantly more pollen (up to an order of magnitude higher) than insect- and bird-pollinated

plants. Erbar and Langlotz (2005) found inconsistent results with wind-pollinated species in some instances having much higher P/O ratios than animal-pollinated species but in other instances lower than expected. They argue that species are able to adapt to inefficient wind-pollination with broad exerted stigmas to provide greater surface area with which to catch airborne pollen. However, in this study only one wind-pollinated species was sampled and the trend seen here may not adequately represent the wind pollination syndrome within the *Erica* genus. Furthermore, the significant difference found is most likely due to a small sample size as well as weak replication compared to other pollination syndromes. Erbar and Langlots (2005) suggested species that provide pollen as a reward, to have higher P/O ratio as they produce extra pollen to compensate for pollen consumption by bees, but I could not confirm this (Figure 3a and 4). The results showed no significant different in P/O ratios between bee and other animal-pollinated species (birds + insects) (Figure 4, $p > 0.05$).

Fire-survival Strategy and Pollen-ovule Ratios

I extended Cruden's 'efficiency' theory to fire-survival strategies, expecting seeders to produce more ovules/seeds compared to resprouters, but no significant difference was found between the different fire-survival strategies (Figure 3b, $p > 0.05$). As with resprouters, seeders appear to have a wide range of P/O ratios, although only three species of below-ground (lignotuber) resprouters and one epicormic (stem) resprouter were sampled. *Erica brachialis* has an interesting survival strategy, making use of seedling recruitment during inter-fire intervals and epicormic resprouting when fire strikes. Regardless of this unique approach, *E. brachialis* is not significantly different from either survival strategy in terms of P/O ratios. I conclude that fire-survival strategies do not influence P/O ratio, although it would be worthwhile to sample resprouting species more extensively to draw a more confident conclusion.

Anomalous Species

A peculiarity in the data concerns *E. riparia*, an insect-pollinated species with a significantly (Figure 3, $p < 0.01$) higher P/O ratio than all other species (Figure 4). This may be due to its rarity (R. Turner, pers. comm., 2013); currently only found in three localities in South Africa, two of which are threatened by alien invasion and the third by coastal development (SANBI: Threatened Species Programme, 2013). If I assume this species, as with the eight other *ericas* in Chapter 2, to have a late-acting self-

incompatibility systems (LSI), another explanation may be that due to its rarity its gene pool is small and it often encounters genetically similar pollen. As barriers to selfing only operate in the ovary it may experience extensive ovule discounting and clogging of the style. Assuming pollinators are not a limiting factor, *E. riparia*, may be coping with this by reducing resource allocation to ovules production and instead is allocating the extra resources to increased pollen production.

Based on the premise that the study species would conform to Cruden's 'efficiency' theory, I anticipated a capacity to allocate 'unknown' species, to a breeding system and fire-survival strategy based on their P/O ratios. However, this goal was not met and it appears that Cruden's (1977) theory is not endorsed in this preliminary study of the *Erica* genus.

Pollen number as predictor of Pollination Syndrome

Pollen number predicts the pollination syndrome of *Erica* species better than the P/O ratios. Species are neatly divided in to insect- and bird-pollinated groups with some slight overlap at the boundary and a few exceptions. Bird-pollinated species have a significantly higher (Figure 4, $p < 0.001$) average pollen number than insect-pollinated species. This may be due to two reasons: 1) birds are believed to cover a wider range and consequently have the potential to pollinate more individuals and, 2) pollen is placed on specific areas of the bird, surplus pollen that does not fall within this region may be lost. Although, the latter is also true for insects, the target area/s on the insect body are smaller and, therefore, generally less pollen can adhere. One irregularity to the trend is *E. sessilifora*: this is a bird-pollinated species with average pollen number similar to that of insect-pollinated species. One explanation for this may be production of large pollen grains, possibly suggesting a size-number trade-off. The resprouting species, *E. mammosa*, has an exceptionally low average pollen count compared to other bird-pollinated species; its average pollen number is more similar to insect-pollinated species. This may be due to its fire-survival strategy, but why then do other resprouters such as: *E. nevillei*, *E. recurvata* and *E. brachialis* reflect much higher pollen production? This suggests that pollen number is not entirely related to fire-survival strategy.

Sex Allocation

The negative relationship between pollen grain size and P/O ratios (Figure 5a, $p < 0.01$) suggests that P/O ratios reflect allocation of resources to reproduction (Charnov, 1982) as opposed to the efficiency of pollination (Cruden 1977). Götzenberger et al. (2007) suggested this relationship only exists due to an underlying positive relationship between P/O ratio and pollen number and a negative relationship between pollen size and pollen number. There is no doubt that P/O ratio and pollen number are positively related; the latter is after all the numerator of the former. However, when comparing pollen grain size to average pollen number I found a weak relationship, indicating little or no trade-off (Figure 5b, $p > 0.05$). Cruden and Miller-Ward (1981) analysed 19 bee-pollinated species and also found no pollen size-number trade-off. However, a later study removed the phylogenetic relatedness of Cruden and Miller-Ward's data and found that this trade-off does exist (Vonhof and Harder 1995).

Concluding remarks

It appears that Charnov's sex allocation theory pertaining to P/O ratios is upheld for species from the genus *Erica*. As the sex allocation theory suggests, species that are more autogamous have a reduced reliance on pollen and can thus allocate more resources to ovules and seed production – acting as an indicator of sex allocation instead of breeding systems as Cruden (1977) suggests. Furthermore, since size is suggested to be a component of both effort and quantity the negative relationship between grain size and P/O ratio is in support of Charnov's model (1982). However, a more extensive assessment should be completed to obtain a more holistic view. One in which more species are sampled and phylogenetic relatedness is accounted for, as this may affect the results dramatically. P/O ratios do not seem to reflect pollination syndrome or fire survival strategies to any significant degree. There is also no significant difference between index-based breeding systems (Table 1, $p > 0.05$). This is an interesting find as many studies use Cruden's table (1977) as a standard of comparison to predict breeding systems. This is particularly alarming, as his classes comprise of relative few unrelated species with variable average P/O ratios. It is also prevalent from this investigation that not all species fall within his predetermined ranges and this, in part, may be due to the variety of different factor that are suggested to influence P/O ratios: habitat, evolutionary history, type of pollination syndrome

(insect, wind, bird, etc.), pollen packaging (tetrads, pollinia, etc.), pollen-rewarding versus nectar rewarding species. This gives rise to inconsistency in results, with many studies either supporting (Cruden 1977; Cruden and Jensen 1997; Spira 1980; Cruden and Miller-Ward 1981; Preston 1986; Vasek and Weng 1988; Jürgens et al. 2002; Schneemilch and Kokkinn 2011) or opposing (Schlising et al. 1980; Arnold 1982; Böcher and Philipp 1985; Pellmyr 1985; Gallardo et al. 1994) the 'efficiency' theory. On this basis alone, I suggest that Cruden's theory cannot be used as a standard of predicting breeding systems. The study suggests that P/O ratio may be helpful if a reliable basis is developed for the specific taxa under investigation.

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Appendix 1: Post Hoc test, showing multiple comparisons of 29 *Erica* species (with different pollination syndromes) in terms of their pollen-ovule ratios. MS = .03897, df = 337. Significant differences have been indicated in bold.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1. <i>E. imbricata</i>																												
2. <i>E. viscaria</i> ssp. <i>viscaria</i>	0.02																											
3. <i>E. urna-viridis</i>	1.00	0.00																										
4. <i>E. abietina</i> ssp. <i>constantiana</i>	1.00	0.11	1.00																									
5. <i>E. baccans</i>	1.00	0.35	0.89	1.00																								
6. <i>E. curviflora</i>	1.00	0.00	1.00	1.00	0.96																							
7. <i>E. pulchella</i>	1.00	0.00	1.00	1.00	0.99	1.00																						
8. <i>E. nevillei</i>	0.92	0.00	1.00	1.00	0.10	1.00	1.00																					
9. <i>E. mammosa</i> (S)	0.01	1.00	0.00	0.04	0.18	0.00	0.00	0.00																				
10. <i>E. brachialis</i>	0.99	1.00	0.57	0.98	1.00	0.73	0.81	0.05	1.00																			
11. <i>E. plukenetii</i>	0.99	0.00	1.00	1.00	0.13	1.00	1.00	1.00	0.00	0.08																		
12. <i>E. corifolia</i>	1.00	0.00	1.00	1.00	0.99	1.00	1.00	1.00	0.00	0.83	1.00																	
13. <i>E. mammosa</i> (R)	0.00	0.92	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.61	0.00	0.00																
14. <i>E. subdivaricata</i>	0.96	0.00	1.00	1.00	0.07	1.00	1.00	1.00	0.00	0.05	1.00	1.00	0.00															
15. <i>E. abietina</i> ssp. <i>atorosea</i>	1.00	0.72	0.96	1.00	1.00	0.99	1.00	0.26	0.42	1.00	0.39	1.00	0.01	0.26														
16. <i>E. nabea</i>	1.00	0.00	1.00	1.00	0.86	1.00	1.00	1.00	0.00	0.56	1.00	1.00	0.00	1.00	0.97													
17. <i>E. recurvata</i>	0.41	0.00	1.00	0.99	0.00	0.97	0.93	1.00	0.00	0.00	1.00	0.91	0.00	1.00	0.01	0.86												
18. <i>E. grisbrookii</i>	1.00	0.43	0.97	1.00	1.00	1.00	1.00	0.26	0.22	1.00	0.38	1.00	0.00	0.24	1.00	0.98	0.01											
19. <i>E. versicolor</i>	1.00	0.00	1.00	1.00	0.89	1.00	1.00	1.00	0.00	0.59	1.00	1.00	0.00	1.00	0.97	1.00	0.95	0.98										
20. <i>E. sessiliflora</i>	0.00	1.00	0.00	0.02	0.06	0.00	0.00	0.00	1.00	1.00	0.00	0.00	1.00	0.00	0.26	0.00	0.00	0.09	0.00									
21. <i>E. viscaria</i> ssp. <i>longifolia</i>	1.00	0.00	1.00	1.00	0.46	1.00	1.00	1.00	0.00	0.24	1.00	1.00	0.00	1.00	0.74	1.00	1.00	0.75	1.00	0.00								
22. <i>E. lasciva</i>	0.01	0.00	0.62	0.34	0.00	0.16	0.11	0.99	0.00	0.00	0.60	0.10	0.00	0.74	0.00	0.07	0.98	0.00	0.12	0.00	0.45							
23. <i>E. coccinea</i>	0.97	0.00	1.00	1.00	0.11	1.00	1.00	1.00	0.00	0.06	1.00	1.00	0.00	1.00	0.31	1.00	1.00	0.30	1.00	0.00	1.00	0.84						
24. <i>E. occulta</i>	0.00	0.76	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.48	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00			
25. <i>E. excavata</i>	0.98	0.99	0.39	0.97	1.00	0.52	0.64	0.01	0.88	1.00	0.01	0.68	0.09	0.00	1.00	0.27	0.00	1.00	0.32	0.75	0.07	0.00	0.01	0.02				
26. <i>E. riparia</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
27. <i>E. pudens</i>	0.96	0.00	1.00	1.00	0.04	1.00	1.00	1.00	0.00	0.04	1.00	1.00	0.00	1.00	0.23	1.00	1.00	0.20	1.00	0.00	1.00	0.62	1.00	0.00	0.00	0.00		
28. <i>E. irregularis</i>	0.00	0.00	0.34	0.16	0.00	0.02	0.01	0.97	0.00	0.00	0.18	0.01	0.00	0.31	0.00	0.00	0.81	0.00	0.01	0.00	0.12	1.00	0.52	0.00	0.00	0.00	0.00	0.15
29. <i>E. hirtiflora</i>	1.00	0.00	1.00	1.00	1.00	1.00	1.00	0.91	0.00	0.97	0.99	1.00	0.00	0.96	1.00	1.00	0.31	1.00	1.00	0.00	1.00	0.01	0.97	0.00	0.91	0.00	0.95	0.00

Synthesis

I have shown that the genus *Erica* is a very unpredictable and diverse taxon, in terms of its reproductive biology. The primary aim of this thesis was to investigate the patterns of variation among breeding systems and how shifts in these correlate with floral traits, pollination syndrome and fire-survival strategies. Secondly, I investigated the self-incompatibility mechanisms that operate in the genus and how P/O ratios relate to breeding systems.

The investigation into the variation in breeding system indicated that most *Erica* species are able to set limited seed via self-fertilisation; viable seed set is significantly lower than that of cross-pollination. Furthermore, autonomous selfing does not seem to yield a substantial seed set for any of the species, except *E. mammosa* (seeder), suggesting they need to be pollinated. Indices of self-compatibility indicated that selfing is not that prevalent among species. However, histological analyses of pollen tube growth indicated that both selfed and outcrossed pollen tubes germinate on the stigma, grows down the style and penetrates the ovules. This indicates that the self-incompatibility mechanism in the genus is most likely late acting, operating in the ovule. This possibly also explains the reduced seed set observed after self-fertilisation. Due to the delayed action of LSI, rejection occurs only after ovules have already been penetrated, these ovules are wasted and cannot be fertilised by subsequent cross-tubes resulting in reduced seed set (and fruit set). Another interesting finding concerning selfed pollen tube growth is the reduced rate at which they grow. Various researchers have considered this to be due to miss-communication in the distance signalling process that acts on the selfed tube while it is in the style and rejects it, even if growth continuously to the ovary (Sears 1937; Seavey and Bawa 1986; Sage et al. 1994; 1999; Bittencourt and Semir 2005; Vaughton et al. 2010).

When correlating traits and selfing ability of species, trends were generally very weak and this may be due to the lack of species representing the facultative outcrossers (as well as other breeding systems). *Erica baccans*, *E. hirtiflora*, *E. abietina* ssp. *atrorosea* and *E. urna-viridis* had an ISI value much larger than the other species within their respective size groups, coming across as outliers in the results. I chose to retain these

species within the analyses, as they were the only facultative outcrosser, among the obligate outcrosser. Species with increased selfing, generally, showed no differences in floral traits from outcrossing taxa. However, we did observe a clustering of small and large flowered species along the selfing gradient. Surprisingly, the arrangement of species along the selfing gradient indicated the probability of selfing to be similar in large and small-flowered species. However, when testing trends in floral changes with degree of selfing, within the different size groups, interesting results were generated. Increased selfing ability in small-flowered species was accompanied by larger corolla sizes, larger apertures and reduced herkogamy. Large-flowered species have increased selfing ability when corolla sizes are large, apertures small and herkogamy reduced. These preliminary results are interesting, and further study with larger sample sizes is required. A trade-off between floral size and display size (number of flowers) was evident across all species studied, as larger flowered species tended to be taller and had fewer flowers when compared to small-flowered species. This trade-off may explain self-incompatibility among small flowered species. With increased number of flowers, the potential for self-fertilisation increases. Self-incompatibility may thus have evolved to reduce geitonogamy in these species.

There were no significant differences between P/O ratios of outcrossers and facultative outcrossers, as Cruden (1977) suggested. This is partly attributed to the variety of different factors that influence pollen and ovule number, as well as the fact that Cruden's (1977) P/O ranges were based on relatively few unrelated species with variable averages. Furthermore, pollen grains size was negatively related to P/O ratios. This along with the weak relationship between pollen number and pollen grain size indicates that the ratio better reflects resource allocation to reproduction (Charnov 1982).

Pollination syndrome did not show a strong correlation with the selfing gradient but it was evident that larger pollinators prefer larger flowered species possibly due to greater rewards provided. Resprouters and seeders did not display any significant differences in selfing ability, in fact analysing a species that has both fire responses showed no significant differences in viable seed set, consequently, also the ISI. Although, ISI between the two *E. mammosa* variants were not significant, the seeder had a greater

ability to set seed autonomously than the resprouter, suggesting a possible adaptation to pollinator-scarce environments.

In conclusion, *Erica* is such a reproductively and florally diverse and interesting taxon that the modicum of knowledge we have is quite sobering. This preliminary investigation into the reproductive biology of *Erica* has been illuminating as to how distinctly species respond to changes in breeding systems and pollinator influence. *Erica* is an understudied genus in South Africa and deserves to be dissected more thoroughly in future, especially, because it makes up such a large percentage of the Cape flora.

Future Direction

It would be worthy endeavour to replicate this study with more species, pollination guilds and fire guilds, as well as a more extensive set of mating systems in order to see a clearer view of the underlying processes at work. This may also reduce the strong impact species such as *E. riparia*, *E. hirtiflora*, *E. abietina* ssp. *atrorosea*, *E. baccans* and *E. urna-viridis* have on trends in the data.

In future, a genetic perspective may provide answers concerning the internal mechanisms of late-acting self-incompatibility. As well as the extent to which self-fertilisation may occur in the natural environment. A more resolved phylogenetic tree would provide a chance to analyse *Erica* data taking relatedness into account as this may, in some cases, have profound effects on interpretation of the data.

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