

**Friendly neighbours? Investigating ecological facilitation between
Thunbergia atriplicifolia (Acanthaceae) and *Exochaenium grande*
(Gentianaceae)**

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Submitted in fulfilment of the academic requirements for the degree of Master of Science

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PREFACE

The research contained in this dissertation was completed in the School of Life Sciences, of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal (Pietermaritzburg), South Africa. It was conducted from January 2015 to December 2017, under the supervision of Professor Steven D. Johnson and Dr Adam Shuttleworth.

The work presented in this dissertation represents the original work of the author and has not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

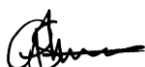
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DECLARATION 1: PLAGIARISM

I, Simangele M. Msweli, declare that:

- (I) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work
- (II) this dissertation has not been submitted in full or in part for any degree or examination to any other university
- (III) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons
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 - a) their words have been re-written but the general information attributed to them has been referenced
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ABSTRACT

Wildflowers that co-exist, share similar floral signals and flower simultaneously, may interact with the same pool of pollinators, therefore sharing pollinator species and individuals. Floral similarity could be a result of a common ancestor, convergent floral syndrome or mimicry. Pollinator sharing may have consequences for the pollination success and fecundity of one or more of the interacting wildflowers. This study was conducted in grassland vegetation of southern KwaZulu-Natal to investigate an observed floral similarity between *Thunbergia atriplicifolia* and *Exochaenium grande*, document pollinator sharing between the two species and investigate the consequences of pollinator sharing on (1) interspecific pollen transfer between the two species and (2) seed set of individuals of *E. grande* that grow within sparse and dense populations of *T. atriplicifolia*. It was hypothesized that *E. grande* is a mimic of *T. atriplicifolia*. Investigated aspects of floral similarity include: flowering phenology, flower colour, flower size and nectar production. Weekly surveys indicated that both species flower simultaneously but *E. grande* occurs at much lower density and peaks in flowering later than does *T. atriplicifolia*. Flowers of the two species have similar spectral reflectance and in terms of a bee vision model can be considered indistinguishable to bees. There is also overlap in flower size of the two species. They provide similar amounts of nectar but nectar of *E. grande* has a higher sugar concentration. Both species are dependent on pollinators for seed production and are pollinated by generalist solitary bees belonging to the Apidae and Hactilidae family, with some species shared by both plant species. Stigmas of *T. atriplicifolia* and *E. grande* carried heterospecific pollen in varying degrees. Estimates of pollen production indicated that *E. grande* produces more pollen than *T. atriplicifolia*. Although interspecific pollen transfer was confirmed, it is not yet clear whether this improper pollen transfer affects seedset. Plant density of *E. grande* and *T. atriplicifolia* was not a significant predictor of seed set in *E. grande*. However, there were very high levels of seed predation in *E. grande*, and that hinders our ability to rule out the any effects of pollinator sharing in seed set. These findings reveal a striking similarity and pollinator sharing between the two species, but despite this, there appears to be no negative or positive effects of this on seed set. Seed set only accounts for half of fitness, however, and the effects of pollinator sharing may therefore be evident on other measures of fitness. The role of trait similarity for pollinator attraction still requires further experimental investigation before the hypothesis of ecological facilitation (or mimicry) can be accepted.

ACKNOWLEDGMENTS

This work was made possible by the contribution of the following organisations, and people whom I extend my gratitude to:

- a) The National Research Foundation for funding in the first two years of the degree
- b) The Mandela Rhodes Foundation (MRF) for financial assistance in the third year and for leadership workshops that developed me on a personal level
- c) Professor Steven D. Johnson for supervising the project, offering guidance where needed and monitoring my progress
- d) Dr Adam Shuttleworth for co-supervising the project and providing technical assistance where needed
- e) Dr Karl Duffy, Dr Dara Stanley and Dr Timo Van der Niet for proof reading my drafts. As a non-native English speaker, I relied on their assistance in checking all language related mistakes
- f) Several postgraduate students, especially Zwelibanzi Mdlalose, Mthokozisi K. Zuma and Sachin Doorsamy for driving me to the study sites and assisting with data collection
- g) My friends (Naledi Zama, Rerani Ramaano, Abongile Mbande, Sithembile Ndlovu, Lutendo Mugwedi and Hloniphani Mthunzi) for their generous support
- h) And lastly but not least, my family. As a first person to do an MSc degree at home, they had less understanding of what exactly I was doing, but still supported me wholeheartedly. Thanks to my mom; Nombuso T. Msweli, my aunt; Hlengiwe T. Msweli, my brothers; Sibusiso Nxumalo, Ntuthuko Nxumalo and Siboniso Msweli and my sister; Samukelisiwe Sukazi.

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CHAPTER 1: LITERATURE REVIEW

Pollinator diversity, flower diversity and pollinator sharing

Approximately 87.5% of all flowering plants rely on animal vectors for pollination but a minority rely on abiotic vectors such as wind and water (Ollerton et al. 2011). Animals are good pollinators since they are mobile and can carry pollen to distant conspecific flowers, therefore promoting outcrossing and ensuring increased seed set and fruit set. Insects are the dominant group of animal pollinators (Ollerton et al. 2011). Animal pollinators are a key driver in diversification of flowering plants (Grant and Grant 1965; Stebbins 1970; Ollerton 1999). This is because flowering plants have evolved a plethora of strategies to attract pollinators which are often geographically localized, thus creating ecological gradients over which diversification takes place (Johnson 2006). To attract and maintain pollinators, plants provide rewards and exhibit floral traits that are attractive to pollinators. Examples of floral traits include colour, shape, orientation, olfactory and tactile cues that are used by pollinators to either locate food or brood sites. Particular combinations of these traits are attractive to locally available and effective pollinators (Dyer et al. 2012).

Plants often occur in communities where they co-exist with other flowers sharing similar floral traits. This creates potential for pollinator sharing. As a result, a pollinator may have interactions with various co-occurring flowers. These interactions then make up a plant-pollinator network of specialized and generalized pollinators (and flowers). Pollinator sharing may be detrimental (i.e. competition), beneficial (i.e. facilitation and mimicry) or have no effects on fitness of the plant species concerned.

Since animal vectors are major pollinators, their foraging behavior is very important in understanding plant-plant interactions. In this study, I investigate the interaction between *Exochaenium grande* (Gentianaceae) and *Thunbergia atriplicifolia* (Acanthaceae) which have strikingly similar flowers to the human observer (Figure 1). Preliminary observations showed that the two species are pollinated by bees. Bees detect and discriminate flowers using signals such as colour, size, shape, patterns and odour (Gumbert 2000; Burger et al. 2010). Amongst the above mentioned floral signals, colour and scent are said to be the most important for flower

recognition (Burger et al. 2010), and this has led to a research focus on bee learning and foraging behaviour.

Floral signals and foraging decisions

Newly emerged naïve bees have innate preferences for certain floral cues whereas experienced bees make use of associative learning in order to forage on more rewarding flowers (Gumbert 2000). For instance, experimental studies by Seguin and Plowright (2007) and Simonds and Plowright (2004) have respectively shown that flower naïve bumblebees prefer radial patterns (over concentric patterns or unpatterned discs), and blue or yellow flowers (over red and white flowers).

Some bees are oligolectic (restrict pollen gathering to species in same genus or same family) while others are polylectic (collect pollen from species in different families). Since oligolectic bees use mainly color and scent to locate their hosts, the presence of extremely similar looking (or similar scented) flowers in a community may lead to foraging inconsistency (Gross 1992). Oligolectic bees may also visit other flower species (other than their host) to collect nectar (Burger et al. 2010). Floral similarity can now not only be inferred by human vision, but with biophysical models that have been developed to allow objective comparisons of colours as perceived by a particular pollinator, for example, the colour hexagon model for Hymenoptera, developed by Chittka (1992). Using such models, Gumbert and Kunze (2001), showed that floral similarity between a deceptive orchid *Orchis boryi* and other co-occurring species at different sites, encouraged non-selective foraging by *Apis mellifera* and *Bombus* spp. This current understanding of bee foraging behavior shows how floral signals, in both specialized and generalist bees, can promote pollinator sharing which can be detrimental (i.e. competition), beneficial (i.e. facilitation and mimicry) or have no effects on the involved flowers.

Competition

Co-occurring flowers may compete for pollinators, resulting in reduced plant fecundity (Brown and Kodric-Brown 1979). Competition may therefore select for floral divergence in plant-pollinator coevolution. Competition may occur between individual plants of the same species (intraspecific competition) or between individuals of different species (interspecific

competition) or both. Competition occurs in different ways and these include: (1) competition to attract pollinators and (2) interference with pollen removal and deposition (Waser (1978, 1983) and Rathcke (1983)). Pollinators visit flowers solely for rewards and the flowers that are perceived as more rewarding may be visited more frequently, therefore causing flowers to compete for pollinators. In pollen interference, pollinators might visit flowers that occur in small or low-density patches, but because of increased abundance of heterospecifics, pollen is not deposited on conspecific stigmas (Armbruster et al. 1994). Instead, it ends up on heterospecific stigmas or other non-stigmatic surfaces of intervening flowers (Muchhala and Thomson 2012). The lost pollen no longer contributes to paternal fitness (pollen discounting). In addition, conspecific stigmas might also be clogged with heterospecific pollen, reducing stigma space for the deposition of conspecific pollen (Rathcke 1983). This Improper Pollen Transfer (IPT) has been shown to reduce maternal fitness through production of hybrids and through chemical and physical interference with proper (conspecific) pollen (Rathcke 1983). In a recent study of bat pollination, Muchhala and Thomson (2012) found that competition (in the form of pollen misplacement) was greater for flowers that use similar pollen-placement locations, implying that pollen placement may increase or decrease the degree of competition between flower species.

To avoid reduced per capita fecundity as a result of inter-specific competition, plants have adopted various strategies. Firstly, different plant species that share the same pollinator may flower at different times, so that the pollinator does not visit species simultaneously (Heinrich 1975; Brown and Kodric-Brown 1979; Armbruster and Herzig 1984). Difference in pollen placement is another strategy that reduces competition since flowers utilise the pollinator body in different ways (Armbruster et al. 1994). These differences in pollen placement may result from character displacement for traits that are involved in reproduction (Heinrich 1975; Waser 1983; Armbruster et al. 1994). Lastly, there is evidence for divergence in floral characters to promote species specific pollinator interactions (specialization) (Heinrich 1975; Brown and Kodric-Brown 1979). At its most extreme, specialization involves a single species of pollinator (Johnson and Bond 1994; Johnson and Steiner 2000).

Some of these strategies have been observed in *Acacia macracantha*, *A. angustissima*, *A. hindsii* and *A. farnesiana* studied by Raine et al. (2007). In this study, they investigated heterospecific pollen transfer in the above-mentioned *Acacia* species. Among the four species,

there was partial seasonal separation in flowering, with *A. hindsii* and *A. farnesiana* flowering in the dry season while *A. macracantha* and *A. angustissima* flowers in the wet season. Since *A. macracantha* and *A. angustissima* frequently co-flowered, and were both mainly pollinated by bees, they exhibited another strategy to minimise heterospecific pollen transfer. *A. angustissima* was shown to dehisce later in the day, around 13h00. At sites where *A. macracantha* populations occurred alone, they were shown to dehisce throughout the day. However, when they co-flowered with *A. angustissima*, they dehisced earlier in the day (about 09h00). Since *A. hindsii* and *A. farnesiana* also co-flowered, heterospecific pollen transfer was reduced by specialization to different pollinators, with *A. hindssi* being pollinated mainly by bees and *A. farnesiana* being pollinated mainly by flies. Although the above-mentioned strategies reduce IPT, they may have evolved independently but, because they reduce IPT, they allow this particular assemblage of species to co-occur due to ecological sorting.

The opposite side of the coin exists, where similarity in appearance and flowering simultaneously are beneficial, and that is the main focus of this study. This positive outcome of the two above mentioned conditions can happen through ecological facilitation, which in some cases is due to the special case of floral mimicry.

Ecological facilitation

Ecological facilitation is a very broad phenomenon but in pollination, it refers to a situation where pollinator visitation and reproductive success (seed set) of a plant species is increased by the presence of other plant species that share the same pollinators (Waser and Real 1979; Rathcke 1983; Callaway 1995; Moeller 2004; Ghazoul 2006). This positive interaction could be unidirectional or bidirectional. It could occur between species with similar floral displays or species with distinct floral displays. Such ecological facilitation may result from the “magnet species effect” (Thomson 1978) whereby a rewarding species (the “magnet” species) increases the abundance of pollinators in a patch and consequently increases the pollination success of neighbouring plants that are less attractive.

Thomson (1978) investigated the effect of stand composition on insect visitation on two hawkweed species, namely: *Hieracium florentinum* (yellow flowers) and *Hieracium aurantiacum* (orange flowers) from the Asteraceae family. He found that *H. aurantiacum*

received more visits than its counterpart in populations where each species grew in isolation (or in populations where either of them was dominant). However, when they co-occur, *H. florentinum* received more visits, suggesting that it was getting an advantage from the presence of *H. aurantiacum* which is a “magnet” species. Although Thomson didn’t make any nectar measurements, we can infer from his discussion that both species indeed produce nectar. He also found that a large number of bees collected from *H. florentinum* also carried *H. aurantiacum* pollen whereas bees collected from *H. aurantiacum* contained little or no *H. florentinum* pollen. This therefore suggested that bees visiting *H. florentinum* had first been attracted to *H. aurantiacum*, emphasizing that the latter is a magnet species. This is evidence of facilitation between two rewarding species.

Johnson et al. (2003) provided evidence for facilitation between a rewarding and a non-rewarding flower species. They studied the pollination success of the deceptive *Anacamptis morio* (Orchidaceae) and co-occurring nectar producing flowers. They found that *A. morio* experienced increased pollination success (pollen removal, pollen deposition and pollinator visitation) when it was translocated to patches with nectar producing flowers, namely: *Geum rivale*, *Anthyllis vulneraria* and *Allium schoenoprasum*. Another interesting finding of this study was that pollination success of *A. morio* was not affected by *Lotus corniculatus* (a nectar producing plant also tested in the experiment), suggesting that not all rewarding species act as magnet species.

Anacamptis morio does not closely resemble all the magnet species and Nilsson (1984) characterized its pollination system as generalized food deception. Generalized food deception is a system whereby a flower exploits non-specific food-seeking responses in their pollinators; this is often achieved by exhibiting colour, scent or any other floral display that the pollinator associates with food (Schaefer and Ruxton 2009; Peter and Johnson 2013). Johnson et al. (2003) did indeed find that amongst the three observed magnet species, *A. schoenoprasum* had the most influence in increasing pollination success of the non-rewarding orchid and it was the only species that had similar colour (purple-pink) to the orchid. This suggests that colour plays an important role in facilitation and similar looking flowers are more likely to facilitate each other.

Facilitation may also occur via joint attraction of highly mobile pollinators or joint maintenance of resident pollinators, which occurs as a result of a convergent floral syndrome (Grant 1966;

Rathcke 1983). As opposed to the “magnet species effect”, facilitation via joint attraction or maintenance of resident pollinators does not necessarily require a more rewarding flower. All the involved flowers could be providing the same reward, but because one or all of them have populations at low density, their attractiveness is increased by co-occurring with flowers pollinated by similar species (Moeller 2004). Highly mobile pollinators such as birds, hawkmoths and social bees may fly over low density patches, while looking for more suitable patches to forage, hence co-occurrence of similar looking flowers and presence of increased resources may attract such pollinators. Some plant populations have resident pollinators who, as long as there are enough rewards available, will stay in one area i.e. some hummingbird species (Sazima et al. 1996). Facilitation might be due to one or all of the above explained mechanisms.

A number of studies have provided evidence for facilitation between flowers with different floral displays, but the mechanisms resulting in this phenomenon remain poorly understood. Ghazoul (2006) proposed the “complementary resource provision” as a possible mechanism. This occurs if the pollinators involved do not discriminate or partially discriminate between display types, resulting in indiscriminate foraging by pollinators. In this situation, any additional flowers in the patch would increase the pool of available resources, making the patch more attractive to pollinators. The benefits of increased pollinator activity in the patch could then outweigh the costs of competing with heterospecifics. It can also occur if pollinators are looking for more than one reward i.e. pollen and nectar simultaneously (Ghazoul 2006). The latter possibility is less likely to occur since most pollinators are often looking for one reward. In facilitation, the resemblance between the involved plant species is not necessarily adaptive whereas in mimicry (which is the special case of the broader concept of facilitation) it is adaptive.

Mimicry

Floral mimicry is one of the classic examples of adaptation through natural selection (Johnson and Schiestl 2016). Mimicry is the adaptive resemblance of one organism to another (or to an inanimate object such as faeces), so that their resemblance is indistinguishable by a third organism (Barrett 1987; Schaefer and Ruxton 2009). It is a well-documented process in both animals and plants. In pollination systems, mimicry involves a model (plant or object being mimicked), a

floral mimic (organism that resembles the model) and the operator (the pollinator organism that is duped and therefore cannot distinguish between the mimic and the model) (Little 1983). Mimicry is particularly common in the Orchidaceae (comprising about 20 000 species in 850 genera (Cameron et al. 1999); with approximately 33% of orchids being non-rewarding and relying on mimicry to attract their pollinators (Johnson 2000). The main difference between facilitation and mimicry is that mimicry entails adaptive resemblance whereas in facilitation, resemblance of involved flower species is not necessarily adaptive. There are two main types of floral mimicry, namely Batesian mimicry and Müllerian mimicry.

Batesian mimicry is a case in which a non-rewarding flower has evolved an advertising style similar to that of a rewarding flower and gains reproductive advantage from mimicking the rewarding flower (Dafni 1984). For a pollination system to be considered a Batesian mimicry system, the following conditions must be met: (i), the model and the mimic should flower in the same place at the same time (Johnson 1994; Johnson 2000). (ii), The mimic should occur at low frequency relative to the model (Johnson 1994; Johnson 2000). This is mainly because, pollinators visit flowers to get rewards. Making visits to non-rewarding flowers costs energy and time that is spent handling and manipulating flowers. If the pollinator encounters more non-rewarding than rewarding flowers, it may leave the patch and forage somewhere else. There are however rare cases where the mimic is at high density (De Jager et al. 2016), provided the costs of visiting a mimic are low (iii), The mimic should resemble the model such that the pollinator is unable to distinguish between the two (Dafni and Ivri 1981; Johnson et al. 2003) or such that the pollinator makes mistakes some of the time (De Jager et al. 2016). The mimic exploits the signals which are used by the pollinator to locate the flowers. These include colour, scent, shape of flowers, and orientation of flowers or a combination of various signals. (iv), The fitness of the mimic should be higher in populations where it co-occurs with the model compared to in populations that grow without the model (Johnson 1994; Johnson 2000). (v), it must be demonstrated that the resemblance between the mimic and the model is adaptive for the mimic only (resulting in divergent evolution) in order to rule out coincidental similarity (Johnson et al. 2003; Benitez-Vieyra et al. 2007). In this case divergent evolution means the mimic has evolved to look similar to the model (but not vice versa). Amongst these conditions, the last one is the most challenging to test because evolution of floral traits can be induced by many selection pressures. However, it can be tested using experimental and phylogenetic techniques.

In Batesian mimicry systems, the pollinator and the model flower can potentially experience negative effects on their fitness (Anderson et al. 2005). The pollinator may lose energy and time without getting rewards and the rewarding flower may lose visits that could have been made to it, in the absence of the mimic.

Müllerian mimicry is a case in which two or more species of similar character or behaviour, at comparable levels of abundance evolve a common “advertising style” to their mutual advantage (Proctor and Yeo 1973). It shares some conditions with Batesian mimicry (condition i and iii) with the exception of two factors. Firstly, all the participating flowers experience increased fecundity when occurring at high frequency (Sherratt 2008). This is mainly because all involved flowers are rewarding but have reduced advertisement if they occur alone. Secondly, the resemblance between plants must be due to adaptive convergent evolution (Johnson et al. 2003; Sherratt 2008). Adaptive convergence is different to coincidental similarity and similarity due to a shared ancestor (Johnson et al. 2003; Schaefer and Ruxton 2009). The classic example of a Müllerian mimicry is the case of nine hummingbird-pollinated flowers (from 7 different families) in Arizona, United States of America, namely *Ipomopsis aggregata* (Polemonaceae), *Penstemon barbatus* (Polemonaceae), *Castilleja austromontana* and *C. integra* (Orobanchaceae), *Lonicera arizonica* (Caprifoliaceae), *Aquilegia triternata* (Ranunculaceae), *Silene laciniata* (Caryophyllaceae), *Echinocereus triglochidiatus* (Cactaceae) and *Lobelia cardinalis* (Campanulaceae) (Brown and Kodric-Brown 1979). These hummingbird-pollinated flowers are red in colour, have overlapping distributions and overlapping flowering periods (Kodric-Brown and Brown 1978). Most of them are similar in corolla length, arrangement of flowers in the inflorescence and nectar characteristics. Brown and Kodric-Brown (1979) discovered that these similarities resulted in non-selective foraging by hummingbirds. Another interesting observation was that pollen placement on the pollinator body was different amongst species, therefore reducing pollen interference. These species are from different families and genera, therefore the similarity is not due to common ancestry. Also, they are very different from their close relatives who are insect-pollinated, suggesting that their similarity is adaptive, at least in terms of modifications for bird pollination, although it is less certain that these plants species have influenced each other’s evolution as expected from the Müllerian mimicry hypothesis.

Amongst the nine species, *Lobelia cardinalis* secreted no nectar but closely resembled two of the nine species and thus appears to benefit from co-occurring with the other species (Brown and Kodric-Brown 1979). Subsequent evidence suggested that *Lobelia cardinalis* may be a Batesian mimic and therefore that this population of hummingbird-pollinated flowers involves both Müllerian and Batesian mimicry (Brown and Kodric-Brown 1979). Although there are established conditions of both types of floral mimicry, some flower systems have been observed not to comply with all of them. This was shown in a study by Benitez-Vieyra *et al.* (2007). They investigated floral mimicry between *Turnera sidoides* ssp. *pinnatifida* (Turneraceae) and *Sphaeralcea cordobensis* (Malvaceae). The two flowers are morphologically similar and pollinated by pollen collecting bees. Although they are both rewarding, only *Turnera sidoides* seems to have evolved to be more similar to *Sphaeralcea cordobensis*. Although there is divergent evolution, it also defies the expectation that in Batesian mimicry one of the species must be non-rewarding since both these species have pollen and the pollinator mainly engages in pollen collection. As emphasized by Little (1983), the known “requirements” for mimicry may apply to most species, but there are exceptions. The above-mentioned system clearly shows the necessity to evaluate any observed system independently. It also brings a question of whether rewarding species can be Batesian mimics or not.

Syndromes versus mimicry

There is a historical problem of distinguishing between syndromes and mimicry, particularly when similar species are both rewarding (Müllerian). In floral syndromes, convergence among flowers arises through a process of adapting to similar pollinators, while for mimicry, it arises through selection for resemblance among organisms (Schiestl and Johnson 2013; Johnson and Schiestl 2016). In Batesian mimicry, the mimic and the model belong to the same floral syndrome (since they are adapted to pollination by the same type of pollinator), but the similarity extends beyond that expected from a floral syndrome (Johnson and Schiestl 2016) and there is a non-rewarding mimic and a rewarding model. In Müllerian mimicry, the rewarding species mimic each other’s signals as this similarity enforces positive associative learning by operators and thus pollination success. This usually involves coevolutionary convergence, whereas in floral syndromes, the patterns of convergence do not involve coevolution among plant species (Johnson and Schiestl 2016). Plant species that are in different

regions and do not interact directly can develop a common floral syndrome if both are adapted for a functionally similar pollinator. In mimicry, on the other hand, floral similarities arise directly from pollinator-mediated ecological interactions among the plant species.

Distinguishing between adaptive and coincidental similarity introduces the importance of comparative biology, especially the use of dated phylogenetic comparisons among interacting species. Numerous studies have shown that mimics tend to look more like their models than their congeners. Although we expect that in Müllerian mimicry floral convergence occurs simultaneously (through a process of coevolution) (Johnson and Schiestl 2016), studies in butterflies have shown that one species is often much younger than its Müllerian partner (s) and therefore the evolution of similarity may be more advergent than convergent (Mallet 1999). This is more akin to Batesian mimicry. In floral syndromes, evolution of similarity between species is independent and therefore follows no order (traits between species are not evolving simultaneously, neither are they evolving subsequently). Phylogenetic comparisons therefore give insight on the order of evolution of traits that are involved in enforcing pollinator sharing. Although phylogenetic analyses are extremely important in distinguishing between mimicry and syndromes, they are not always possible if phylogenies are unresolved or lack a dating component, and as a result, distinguishing between mimicry and syndrome remains a difficult problem in biology.

The study species and aims

Exochaenium grande (E. Mey.) Griseb (Gentianaceae) and *Thunbergia atriplicifolia* E.Mey. ex. Nees (Acanthaceae) have been observed to frequently co-occur in South African grasslands. They look similar to the human eye, both in colour and in shape, and as a result, may interact with each other in terms of pollination. Studies have shown that in any positive or negative interaction, a flowering plant that occurs at low density and is allogamous (highly depended on pollinators) is usually the one that is most affected by interacting with other species (Johnson et al. 2003). *Exochaenium grande* occurs at low density in populations where the two species co-occur and we know from literature that it has a heteromorphic incompatibility system (Wolfe et al. 2009). In this system, a short-styled morph can only mate with a long-styled morph and as a result, not all available flowers in the population are potential mating partners. Therefore, the flower is highly dependent on pollinators for its reproduction. *Thunbergia atriplicifolia* on

the other hand, occurs at high density and is not distylous. Owing to the above-mentioned observations, it was hypothesized that *E. grande* is a Mullerian mimic of *T. atriplicifolia* and this study was conducted to investigate the floral similarity, pollinator sharing and consequences of pollinator sharing in *T. atriplicifolia* and *E. grande*.

Exochaenium grande, commonly known as primrose gentian, is an annual herb that grows up to 350mm in length. This species is mainly found in grasslands, and is widely distributed in South Africa and other tropical African countries. Its petal colour ranges from white to creamy yellow (Pooley 1998). Kissling (2012) described one population with salmon petals. Each plant can produce one to seven flowers that remain in flower for about 20 days. *Exochaenium grande* is distylous i.e. there are long-styled and short styled floral morphs in populations (Wolfe et al. 2009).

Thunbergia atriplicifolia, commonly known as the natal primrose, is a perennial shrublet that grows up to 400mm and inhabits grasslands. This species is mainly distributed in South Africa and other tropical countries (Pooley 1998). The petals of this species can be pointed or rounded with white to creamy yellow flowers. In contrast to *E. grande*, the floral tube of these flowers is more yellow, relative to the petal colour (in *E. grande*, both petals and floral tube are similar in color). Each plant can have a lot of branches that have a high flower turnover with flowers remaining in anthesis for approximately 5 days. This plant species (along with other closely related plants) has been included in studies of the evolution of Acanthaceae, specifically the Thunbergioideae subfamily (Blackwill and Campbell-Young 1999; Borg and McDade 2008) but has received little attention in terms of pollination biology and ecology.

Based on anecdotal observations along with previous work, to explore the pollination ecology of these two species and to test the mimicry hypothesis, I address the following questions:

- (1) Is there overlap in the flowering periods of the two species?
- (2) As perceived by pollinators, are there similarities in the assumed functional floral traits (i.e. colour and size) of these species that may elicit similar responses in animal pollinators?
- (3) Are similarities in functional floral traits coincidental, phylogenetic or adaptive?
- (4) Do the two species share pollinator species and pollinating individuals?

- (5) Are the observed pollinators specialists or generalists in their local community?
- (6) Is there improper pollen transfer between the two species?
- (7) Does *E. grande* experience increased fecundity with increasing aggregation of *T. atriplicifolia*?

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CHAPTER 2: Sharing of floral signals and bee pollinators in two grassland flowers: syndrome, mimicry or coincidence?

Abstract

Co-existing, morphologically similar flowers that flower simultaneously, may interact with the same pool of pollinators. A key challenge when studying floral similarity of rewarding species is to distinguish between floral syndromes, Müllerian floral mimicry (both representing pollinator-mediated evolutionary convergence in functional traits) and non-adaptive similarity due to chance. I investigated the evolutionary basis for the remarkable similarity in flowers of the South African grassland plants *Exochaenium grande* (Gentianaceae) and *Thunbergia atriplicifolia* (Acanthaceae). Weekly surveys indicated that both plants flower simultaneously but *E. grande* occurred at much lower density and peaked in flowering later than *T. atriplicifolia*. Flowers of the two species have similar spectral reflectance and in terms of a bee vision model can be considered indistinguishable to bees. There is also overlap in flower size of the two species. They provide similar amounts of nectar but nectar of *E. grande* had a higher sucrose concentration. Both species are dependent on pollinators for seed production and are pollinated by generalist solitary bees belonging to the Apidae and Halictidae families. Some bees carry pollen of both species. However, the pollinators show generalist foraging behaviour in the studied wildflower community and the role of trait similarity for pollinator attraction requires further experimental investigation before the hypothesis of mimicry can be accepted.

Keywords: floral signals, pollinator sharing, floral syndrome, mimicry.

Introduction

About 87.5% of flowering plants are pollinated by animals (Ollerton et al. 2011) which, in the process, usually gain access to nectar, pollen, brood sites and other rewards (Simpson and Neff 1981). These pollinators may be generalist (visiting a range of plant species) or specialists (only visiting one species, or a few species, or only species from a certain genus or family). Plants use floral signals such as colour, size, shape, orientation and scent to attract pollinators (Gumbert 2000; Burger et al. 2010). If these signals are similar, plants may be indistinguishable to a pollinator resulting in pollinator sharing. Pollinator sharing therefore remains of great interest in pollination biology since it represents an example of indirect plant-plant interactions in ecology and evolution.

In distantly related flowers (from different families), similarity in floral signals such as colour and scent may be adaptive or coincidental (Starrett 1993). Flowers may look similar because they are adapted to pollination by the same pollinator (or same type of pollinators), a phenomenon known as a floral syndrome (Fenster et al. 2004). This convergent evolution can be attributed to the sensory systems, morphologies and behaviours of particular pollinator functional groups (Benitez-Viera et al. 2007; Johnson and Schiestl 2016). As opposed to adapting directly to the sensory systems of pollinators, flowers of plants can evolve adaptive resemblance to the flowers of other species (i.e. mimicry) (Johnson and Schiestl 2016). There are two main types of food-based floral mimicry, namely Batesian mimicry and Müllerian mimicry.

Batesian floral food-source mimicry is a case in which a non-rewarding flower has evolved an “advertising style” similar to that of a rewarding flower and gains reproductive advantage from mimicking the rewarding flower (Dafni 1984). This type of deception was referred to by Little (1983) as “two taxa deceit mimicry” owing to the fact that the model and the mimic belong to different species. Model and mimic are indeed different species in most examples of mimicry, not just Batesian. The involved species may belong to different genera or different families. Batesian mimicry is characterised by the following conditions: (i) the model and the mimic should flower in the same place, at the same time and interact with the same pollinators (Johnson 1994; Johnson 2000), (ii) the mimic should occur at low frequency relative to the model (Johnson 1994; Johnson 2000), (iii) the mimic should resemble the model such that the

pollinator is unable to distinguish between the two (Dafni and Ivri 1981; Johnson et al. 2003), (iv) the response of pollinators should be shaped by their experience with the model, (v) the fitness of the mimic should be higher in the presence of the model than in its absence and (vi) it must be demonstrated that the resemblance between the mimic and the model is adaptive for the mimic. Since the adaptive resemblance occurs on the side of the mimic, but not the model, Batesian food-source mimicry evolves through a process of divergent evolution. In this context, divergent evolution means the mimic has evolved to look similar to the model (but not vice versa).

Müllerian floral food-based mimicry is a case in which two or more species evolve a common “advertising style” to their mutual advantage (Proctor and Yeo 1973). Here all the involved species provide a reward (i.e. nectar). As a result, there is no one “model”. Species A is a selecting pressure acting on B and species B is the selective pressure acting on species A. Müllerian mimicry shares condition (i) and (iii) with Batesian mimicry and differ with a few factors. Firstly, when co-occurring, all the participating flowers benefit through a facilitative effect from increasing frequency and therefore experience increased fecundity (Sherratt 2008). Secondly, the resemblance between plants must occur through a process of co-evolution resulting in mutual adaptive resemblance between species (Johnson et al. 2003; Sherratt 2008). Thirdly, participating flowers can occur at comparable frequencies. The existence of floral Müllerian mimicry is contentious and there are no compelling published examples. Most studies have documented floral similarity, pollinator sharing and its advantages on interacting species, but don’t have enough evidence of the historical sequence of trait evolution to argue for a Müllerian mimicry hypothesis.

It is extremely difficult to distinguish between floral syndromes and Müllerian floral mimicry in the case of floral similarity among rewarding species (Johnson and Schiestl 2016). In floral syndromes, similarity among flowers arises through a process of adapting directly to pollinators, while in mimicry it arises through selection for resemblance among organisms (Schiestl and Johnson 2013; Johnson and Schiestl 2016). Community context is critical for the evolution of mimicry, but is not considered critical for the evolution of floral syndromes. In Müllerian mimicry, the rewarding species mimic each other, provided, the similarity will enforce learning of each other’s signals by operators. This usually involves coevolutionary

convergence, whereas in floral syndrome, the pattern of convergence does not require coevolution among plant species (Johnson and Schiestl 2016).

Distinguishing between adaptive and coincidental similarity requires the use of dated phylogenetic comparisons among interacting species. Numerous studies have shown that mimics tend to look more like their models than their congeners. Although it is generally expected that in Müllerian mimicry, floral convergence occurs through a process of coevolution (Johnson and Schiestl 2016), studies in butterflies have shown that one species is often much younger than its Müllerian partner (s) and therefore that evolution of similarity may be more advergent than convergent (Mallet 1999). This means that these systems may be more characteristic of Batesian mimicry in which, the evolution is always advergent. The problem with this is that coevolution can be more asymmetric, and look almost like advergence or it can be more symmetric, but both are still coevolution. In floral syndromes, evolution of similarity is independent and therefore follows no particular order. Phylogenetic comparisons therefore may potentially give two important insights: 1) the novelty of traits that are involved in signal similarity between species and 2) the historical sequence of the evolution of signal similarity.

Preliminary observations indicated that *Exochaenium grande* (Gentianaceae) and *Thunbergia atriplicifolia* (Acanthaceae) often co-occur in South African grasslands, flower simultaneously and look similar to humans. This study was conducted to objectively determine the degree of floral similarity and to document the extent of pollinator sharing between the two species. If resemblance is due to Mullerian mimicry and not chance or syndrome, then I predict that (1) the two species will be indistinguishable by pollinators, (2) they will both provide a reward to their visitors (i.e. nectar) and (3) the pattern of the evolution of similarity, will be coevolution.

Materials and Methods

Study site

The study was conducted in South Africa, KwaZulu-Natal Province, Vernon Crookes Nature Reserve (S30.2882°; E30.5621°). Vernon Crookes Nature Reserve is 2,189-hectare located 150 to 610 meters above sea level and is characterized by a matrix coastal grasslands and forests. The reserve has co-existing populations of *E. grande* and *T. atriplicifolia*.

Study species

Thunbergia atriplicifolia E. Mey. ex Nees and *Exochaenium grande* (E. Mey) Griseb have white to cream-white flowers (Figure 1). *Exochaenium grande* is distylous, and has a system of heteromorphic incompatibility (Wolfe et al. 2009). Flowers of *T. atriplicifolia* are open for up to five days, while flowers of *E. grande* live up to 21 days. *E. grande* is distributed throughout tropical Africa (Kissling et al. 2012). In South Africa, the two species are mainly distributed in the eastern part (Figure 2).

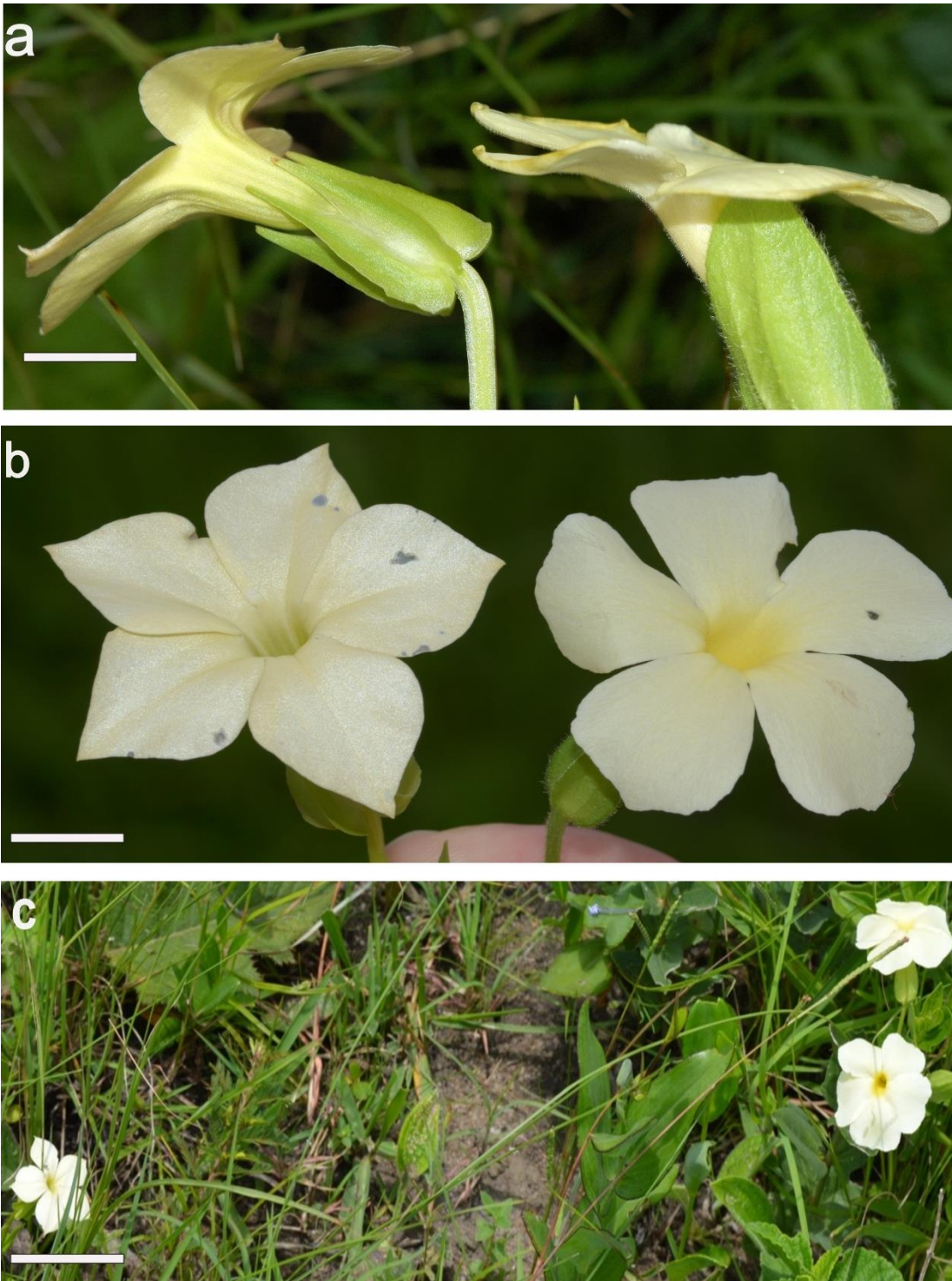


Figure 1: (a) Lateral view of *E. grande* (left) and *T. atriplicifolia* (right) flowers. (b) Top view of *E. grande* (left) and *T. atriplicifolia* (right) flowers. (c) Flowers of *E. grande* (left) and *T. atriplicifolia* (right) co-occurring in the field. Scale bars: a, b = 1 cm, c = 5 cm. Photo credits: a to c = Steven D. Johnson

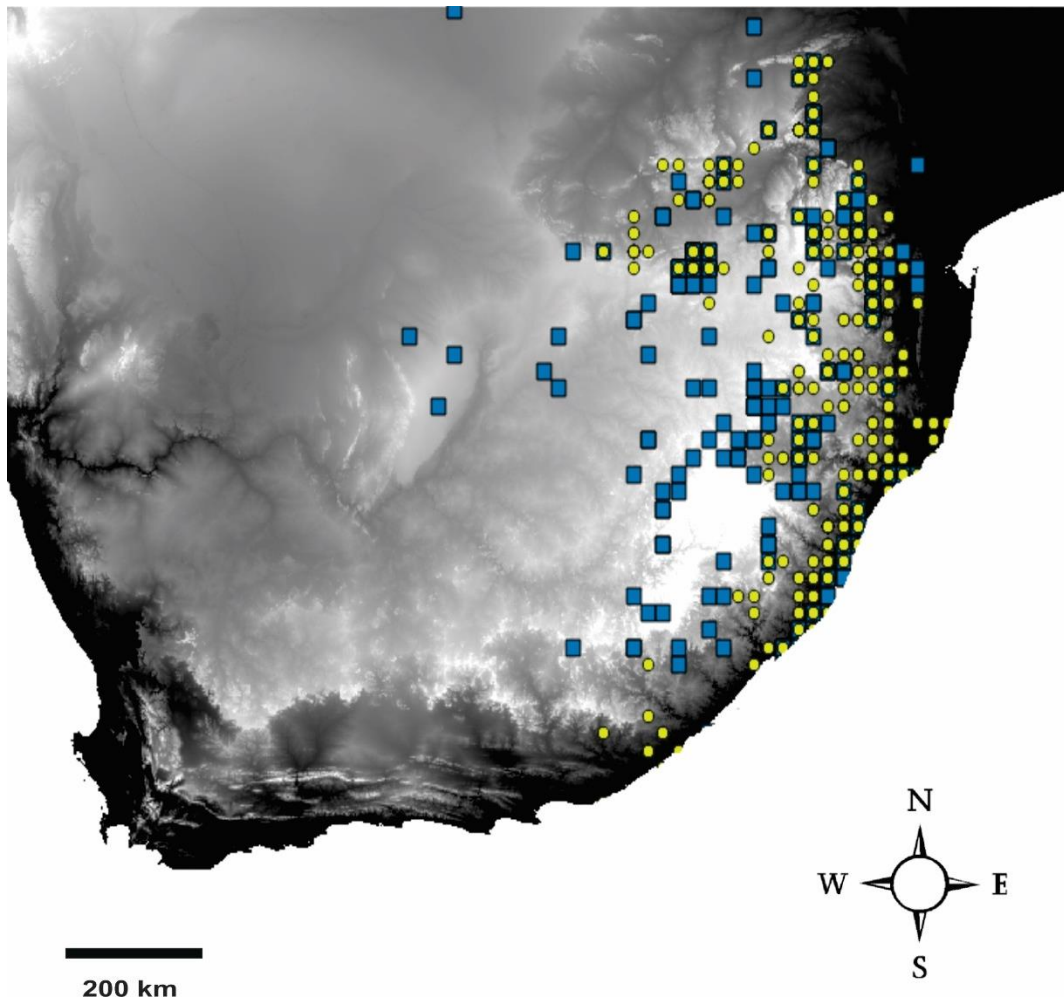


Figure 2: Distribution of *T. atriplicifolia* (yellow circles) and *E. grande* (blue squares) in South Africa. This figure is based on presence records from SIBIS.

Floral signals

Flowering phenology

To determine the flowering phenology of the two species, five independent patches (at least one kilometre away from each other) were identified where the two species co-occur. In each patch, a 50 metre transect was made and marked with poles. The number of flowering plants of *E. grande* and *T. atriplicifolia* within two metres of the transect line was quantified every two weeks, from January to May 2016. Flowering phenology was further investigated by examining records of *T. atriplicifolia* and *E. grande* from the Bews Herbarium (University of KwaZulu-Natal, Pietermaritzburg).

Colour

To determine whether flower colours of the two species would be distinguishable by bees, 15 flowers of each species were collected and the spectral reflectance of petals was measured within 4 hours of collection. Spectral reflectance was determined using an Ocean Optics S2000 spectrometer (Ocean Optics Inc., Dunedin, Fla.) and fibre optic reflection probe (UV–VIS 200 μm) held at 45° to the petal surface. The light source used was an Ocean Optics DT-mini-2-GS deuterium tungsten halogen light source with a $\sim 200\text{--}1100$ nm spectral range. Colour patterns as perceived by pollinators were measured by plotting the reflectance of the outer and central parts of the flower as separate loci in a bee colour hexagon model (Chittka 1992), divided into six partitions (B, BG, G, UG, U and UB) according to the colours perceived by bees. Spectral reflectance of the (green) background vegetation was taken from Shuttleworth and Johnson (2009). Shuttleworth and Johnson (2009) measured spectral reflectance of the upper surfaces of leaves for 16 different plant species (three replicates for each species) from Vernon Crookes Nature Reserve. To determine how unique the colours of the study species are in their community context, five flowers of each of 31 flowering plant species that flower in sympatry, were collected and mapped as described above. Flower colour of *E. grande* is not a derived trait (it is a trait novel in the *Exochaenium* genus). Flower colour of congeners were obtained from Kissling and Barrett (2013) and mapped onto a phylogeny obtained from Kissling and Barrett (2013). They were mapped using MESQUITE, a software for phylogenetic analyses. Flower colours of *T. atriplicifolia*'s congeners were obtained from Schonenberger (1999).

Flower size

To determine the extent of the overlap in flower size of *T. atriplicifolia* and *E. grande*, 102 pictures of flowers of *T. atriplicifolia* and 105 of *E. grande* were taken in the field using a Nikon D3100 camera. For each picture, a line gauge (ruler) was placed beside the flower and later used to determine the size of each flower. Flower size was measured as diameter. A frequency distribution was then used to compare the flower size of both species. To determine if flower size of *E. grande* is unusual relative to its congeners, I compared my data on flower sizes with data on the sizes of congeners presented in Kissling and Barrett (2013). Flower sizes of *E. grande* and congeners were mapped into a phylogeny obtained from Kissling and Barrett (2013), using MESQUITE.

Nectar properties

Twenty flowers of *T. atriplicifolia* and *E. grande* were bagged overnight to exclude visitors. Nectar from these flowers was collected at 10 a.m. A 5µl micro-pipette was used to measure nectar volume. Bellingham and Stanley hand held refractometers designed for sugar concentration of either 0-45% or 50-90% were used to measure nectar concentration. Nectar collecting time was chosen based on the basis that pollinator activity was observed to be highest between 8:00 to 10:00 and plants were assumed to produce nectar during the peak activity period of their pollinators. The data was normally distributed. Statistical comparisons of mean nectar volume and concentration for the two species was done using simple t-tests in SPSS 25 (IBM Corp).

Breeding system

The breeding system of *E. grande* is already known from literature (Wolfe et al. 2009) and therefore was not investigated in this study. The breeding system of *T. atriplicifolia* was investigated in the field using controlled hand pollinations. Ninety buds of *T. atriplicifolia* were bagged with pollinator exclusion bags just before the buds could open to mature flowers. Once anthesis occurred, 30 flowers were randomly allocated to each of three different pollination treatments (cross-pollination, self-pollination and no pollination). Cross-pollination served as a positive control while self-pollination served to test for self-incompatibility and no manual pollination served to test for autonomous selfing. The flowers were immediately re-bagged after pollination. Pollen used for cross pollinations was collected from surrounding flowers which were not part of the experiment. Fruit set and seed set were scored 14 days after controlled pollinations. For each treatment, we counted the proportion of flowers that set fruit, the average number of seeds per fruit and average number of seeds per flower. Fruit set and seed set data were analysed using generalized linear models incorporating a binomial distribution and logit link function and Poisson distribution and log link function, respectively. Post-hoc comparisons of means were done using the sequential Sidak method. These statistical analyses were carried out using SPSS 25 (IBM Corp.)

Pollinator sharing

Pollinator observations

To determine the pollinators of the study species and the extent of pollinator sharing, field observations were conducted from January to May in 2015 and 2016. Visitors that entered the floral tube were collected and identified in the lab. To directly determine whether pollinators moved between flowers of the two study species, individual pollinators were followed as they made visits. To determine which other species in the community were visited by pollinators of the study species, focal observations were made for 61 co-flowering species. A total of eight observation periods each lasting 15 minutes, were made for each species, therefore adding up to two hours for each species over a period of 90 days. The observed plant-pollinator interactions were mapped using the bipartite package, in the R statistical environment (Dormann et al. 2017).

Pollen loads

To indirectly determine whether there is pollinator sharing by the study species, pollen loads were collected from the bodies (especially in the legs, head and the back) of collected visitors using fuchsin gel. This pollen was put on a slide and identified under a microscope. Pollen reference slides of *E. grande* and *T. atriplicifolia* were prepared using pollen from anthers of both species. Pollen was identified to be either from (1) *T. atriplicifolia*, (2) *E. grande* or (3) other (if it matched neither of the focal species).

Results

Floral signals

Flowering phenology

Flowering of *T. atriplicifolia* occurs from November to the first week of May, whereas for *E. grande*, it starts at the end of January but ends at the same time as *T. atriplicifolia* (Figure 3). The flowering period of the two species therefore overlaps. *E. grande* flowers at a lower density than *T. atriplicifolia* (Figure 3).

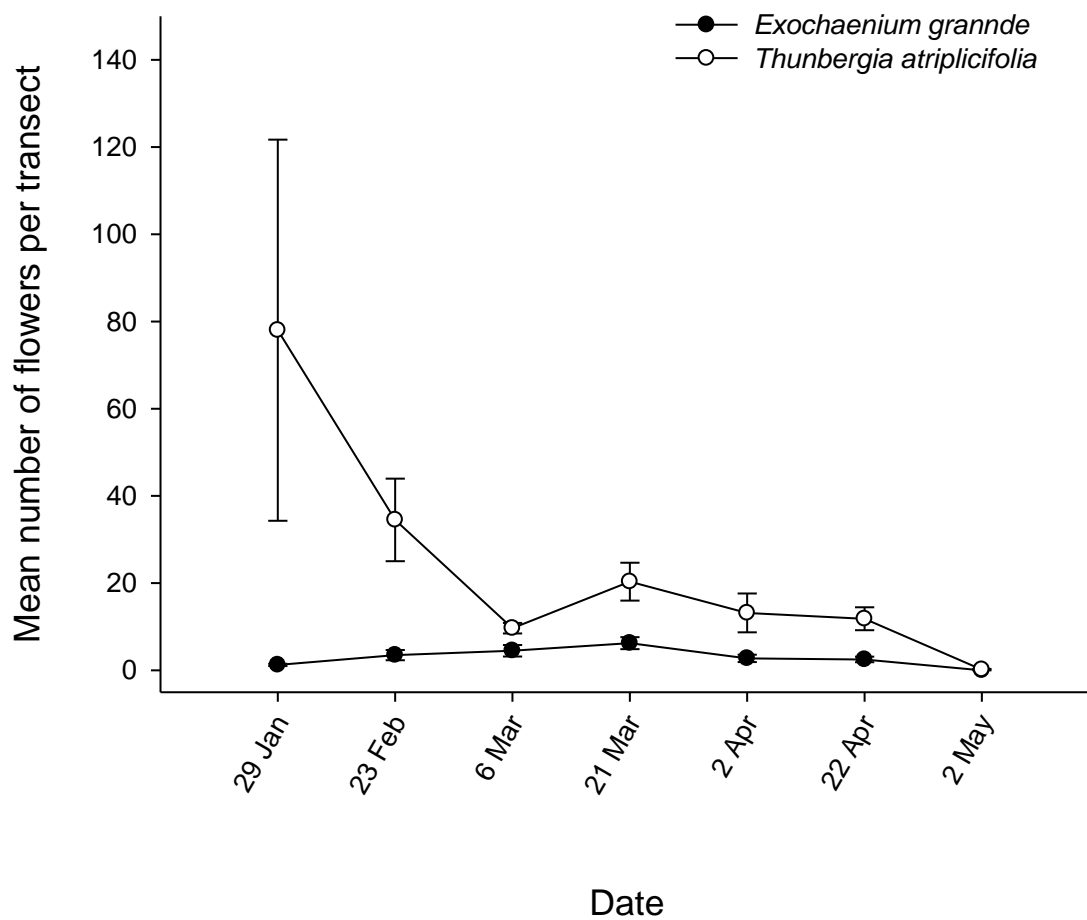


Figure 3: Flowering phenology of *T. atriplicifolia* and *E. grande*

Additionally, specimen records from the Bews herbarium further indicate the overlap in flowering season of *T. atriplicifolia* and *E. grande* in KwaZulu-Natal (Figure 4). Specimens of flowering plants of *T. atriplicifolia* were collected between September to June whereas specimens of *E. grande* were collected between November to April.

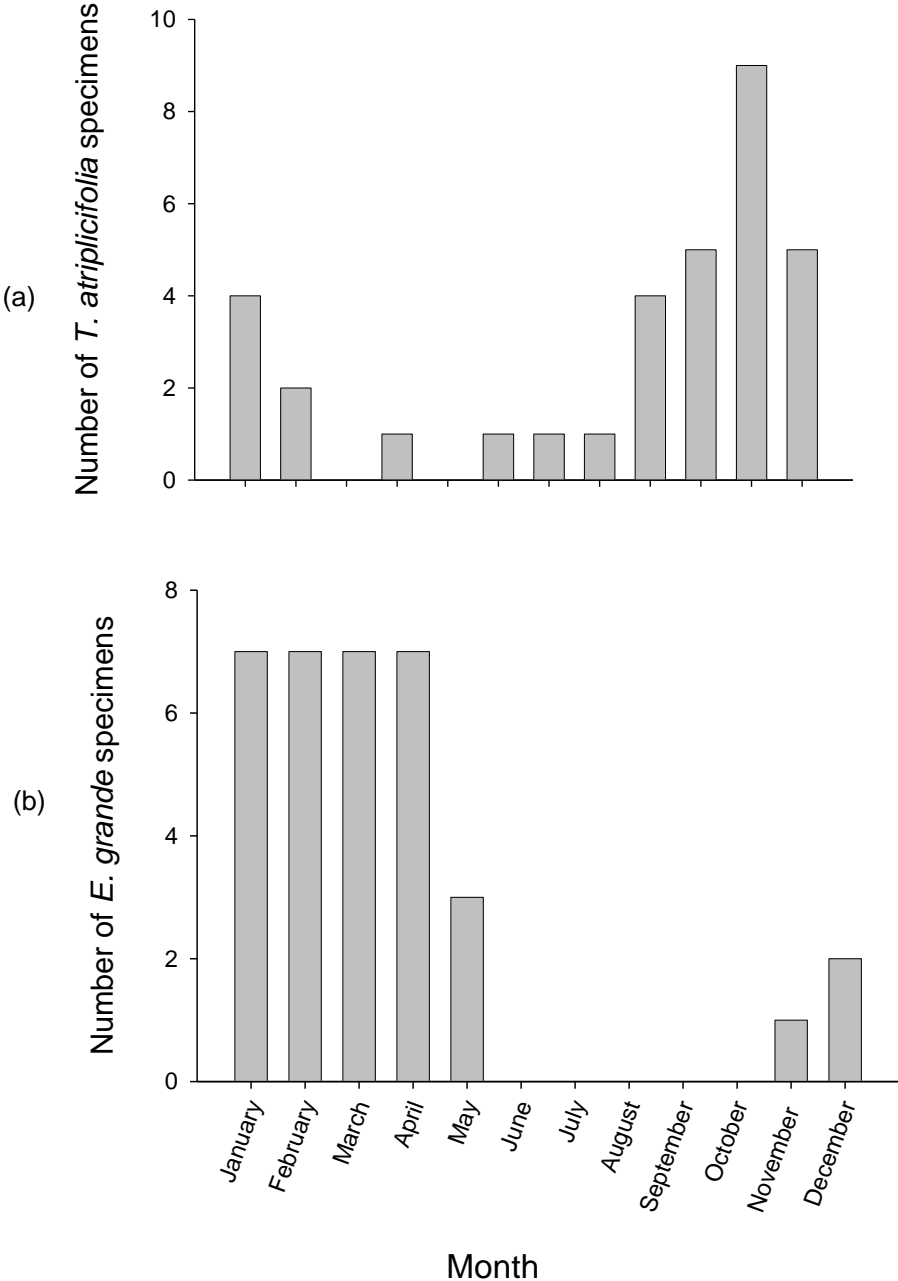


Figure 4: Frequencies of of *T. atriplicifolia* (a) and *E. grande* (b) collections in different months for specimens collected in KwaZulu-Natal and housed in the Bews Herbarium.

Colour analyses

The spectra of petals of *E. grande* and *T. atriplicifolia* have similar inflection points (corresponding to hue), but flowers of *E. grande* show higher overall brightness levels (Figure 5). According to Dyer and Chittka (2004a,b) bee colour hexagon model, a distance below 0.1 units between loci of colour indicates that colours are indistinguishable by bees while a distance above 0.1 units, indicates that colours are distinguishable by bees. The colour hexagon distance between the loci of *E. grande* and *T. atriplicifolia* petals is less than 0.1 units, thereby establishing that the two species are probably indistinguishable by colour to the bee pollinators (Figure 6). *Thunbergia atriplicifolia* is distinguishable from two congeners (*T. alata* and *T. natalensis*) that were flowering in the same community but indistinguishable from the much rarer congener *T. dregeana*. In the colour hexagon including other flowers from the community, *T. atriplicifolia* and *E. grande* are closer to each other than to flowers of other co-occurring species (Figure 7). In the phylogeny of Exaceae where *E. grande* is mapped, the colour white is the most common in the genus and therefore not derived (Figure 8).

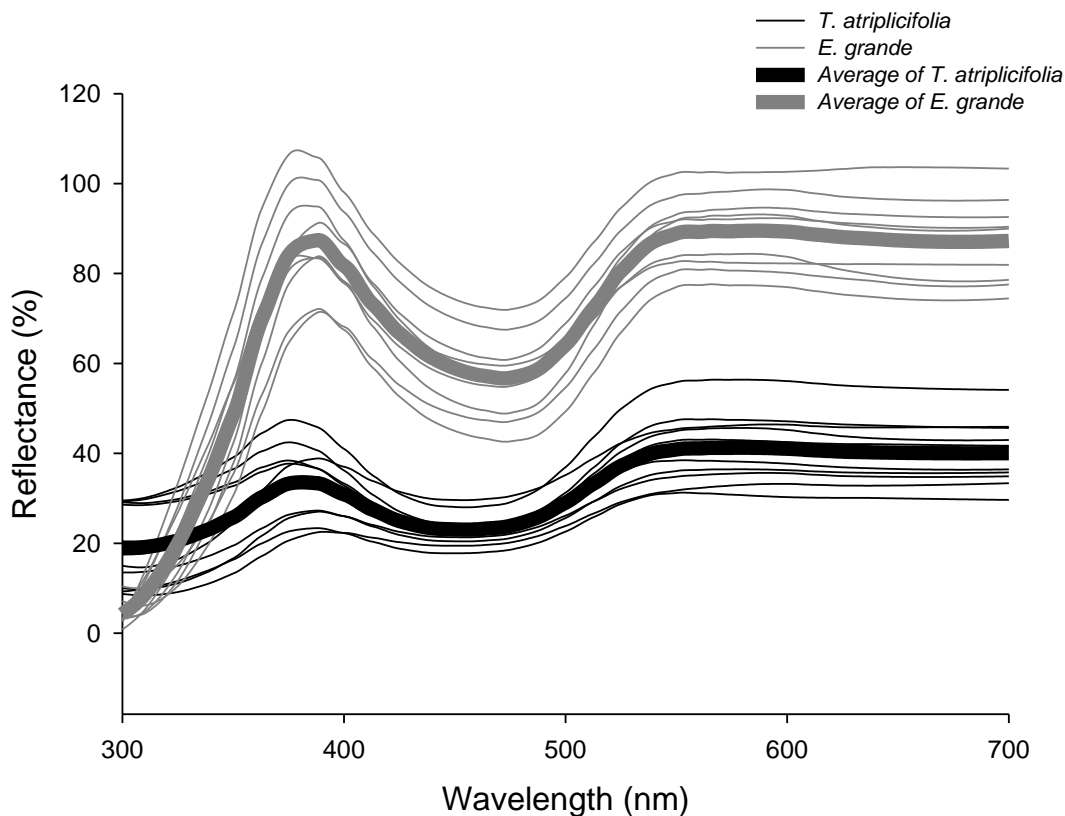


Figure 5: Spectral reflectance of *T. atriplicifolia* and *E. grande*

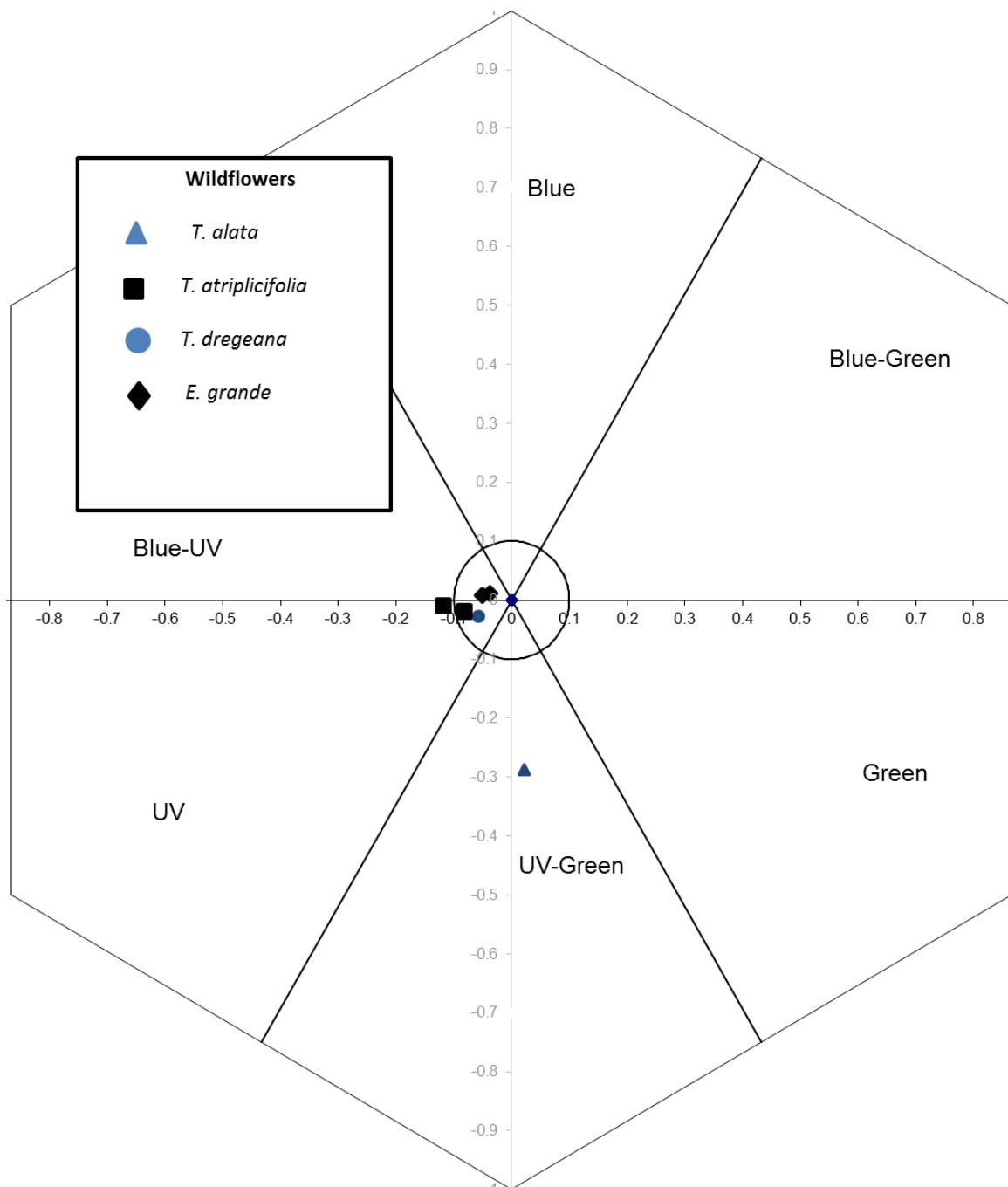


Figure 6: Colour hexagon showing loci of *T. atriplicifolia*, *E. grande* and *Thunbergia* congeners

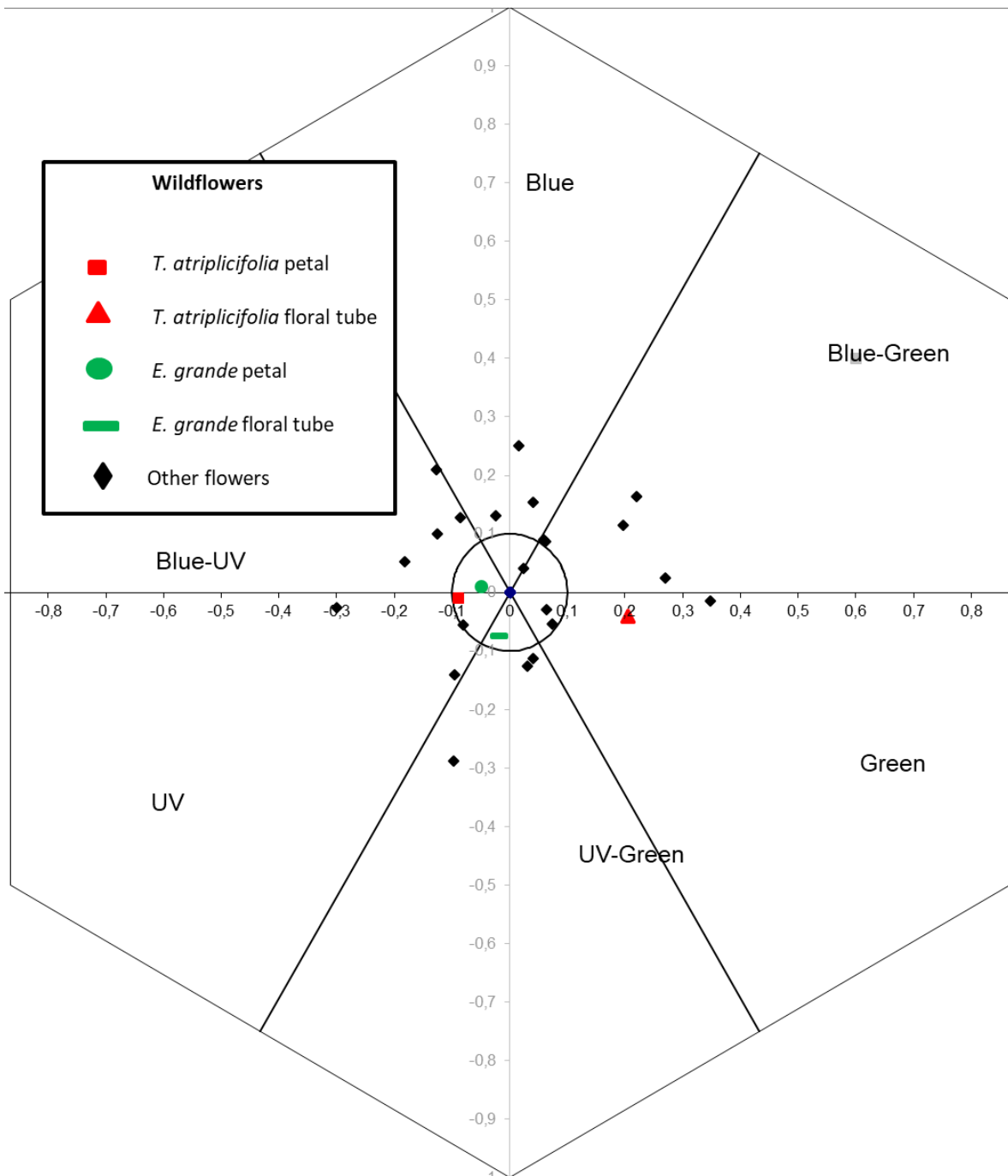


Figure 7: Colour hexagon of *T. atriplicifolia*, *E. grande* and co-flowering species



Figure 8: Phylogeny of species in the *Exochaenium* genus, showing flower colours.

Flower size

The diameter of *E. grande* flowers ranges from 1.6 to 4.0 cm whereas that of *T. atriplicifolia* ranges from 2.3 to 5.4 cm (Figure 9). There is therefore overlap in flower size distribution of both species. In the phylogeny of *Exochaenium* genus (where *E. grande* is placed), the observed flower size is relatively larger in comparison to those of its congeners (Figure 10).

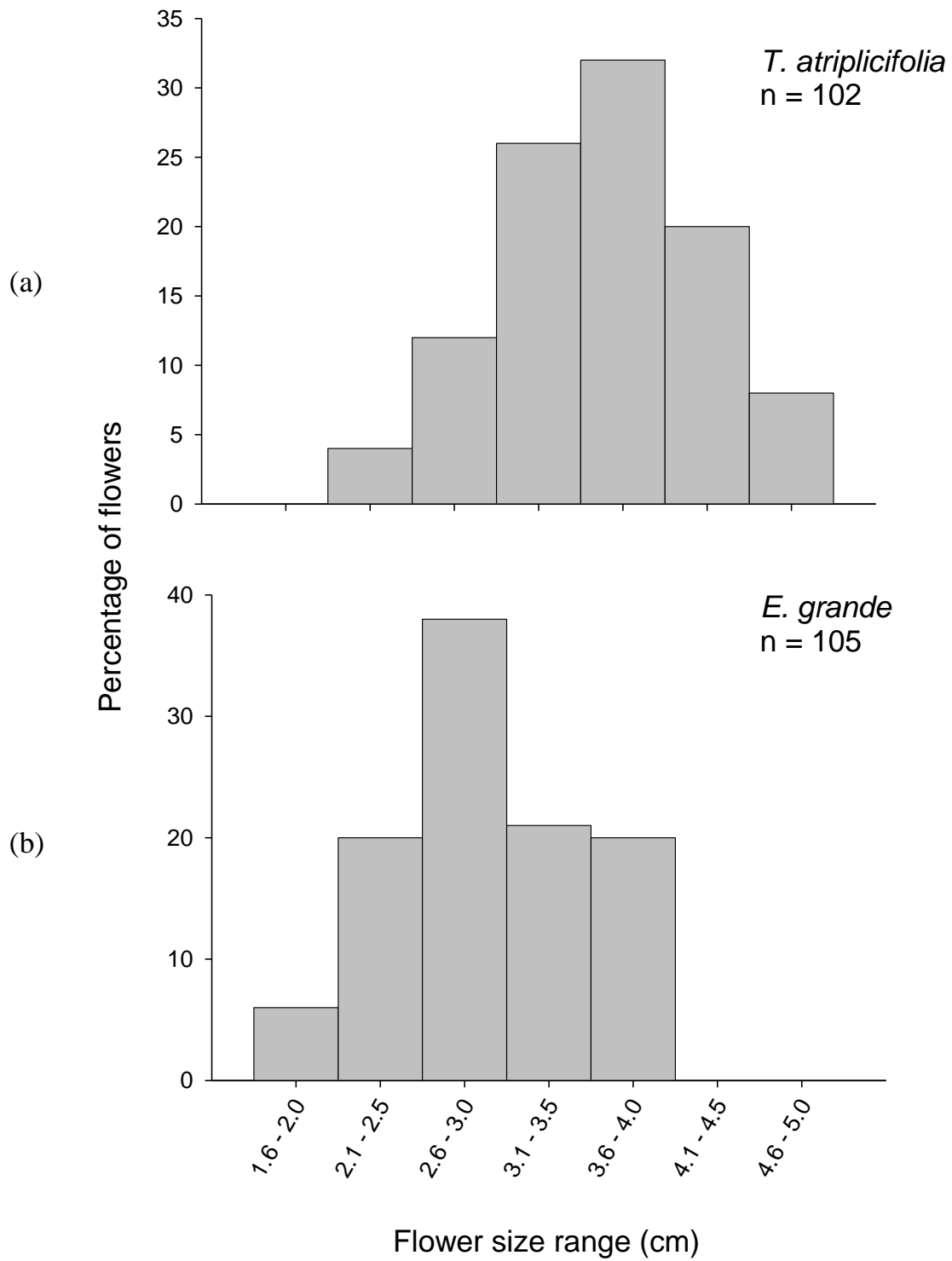


Figure 9: Flower size (= diameter of the corolla) distribution of *T. atriplicifolia* and *E. grande*.

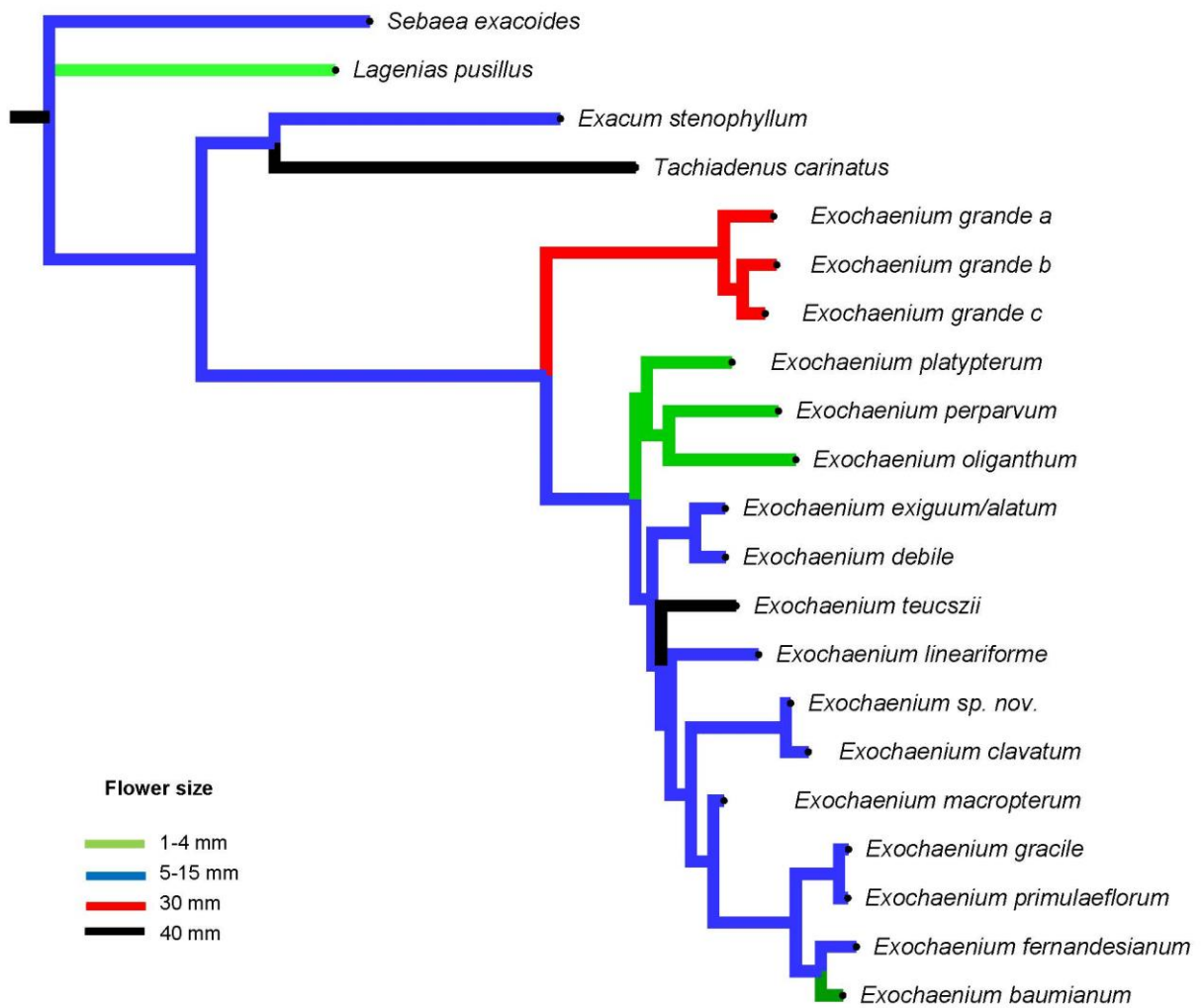


Figure 10: Phylogeny of *Exochaenium* species, showing flower size.

Nectar

Both flowers provide nectar to their visitors. Mean nectar volume was $0.4 \pm 0.08 \mu\text{l}$ for *E. grande* and $0.3 \pm 0.05 \mu\text{l}$ for *T. atriplicifolia*. Although nectar volume was not significantly different ($p=0.33$) (Table 1), there was a significant difference ($p=0.003$) in sugar concentration of the two species, with *E. grande* having a mean of $53 \pm 2.83 \%$ and *T. atriplicifolia* having a mean of $33 \pm 2.48 \%$.

Breeding system

The mean proportion of flowers that set fruit was significantly lower for *T. atriplicifolia* plants that were not hand-pollinated (0.19 ± 0.04) than for those that were hand-pollinated with self-pollen (0.55 ± 0.011 ; $p=0.005$) or cross pollen (0.60 ± 0.11) ($\chi^2=18.81$; $p=0.001$) (Figure 11a). The mean proportion of flowers that set fruit was not significantly different for self-pollinated and cross-pollinated plants (Figure 11a). Mean seeds per fruit was two-fold higher for cross pollinated flowers (2.58 ± 0.36), compared to those that were hand pollinated with self-pollen (1.25 ± 0.32) and the ones that were only bagged (1.45 ± 0.26) ($\chi^2=9.32$; $p=0.001$) (Figure 11b). Unpollinated (bagged) flowers set the least number of seeds per flower (0.28 ± 0.06), followed by self-pollinated (0.7 ± 0.24) and cross pollinated flowers (1.25 ± 0.37). The mean seeds per flower for self-pollinated and cross pollinated flowers was not significantly different ($\chi^2=16.89$; $p=0.001$) (Figure 11c). Overall, this indicates that *T. atriplicifolia* is self-compatible and largely allogamous (pollinator dependent).

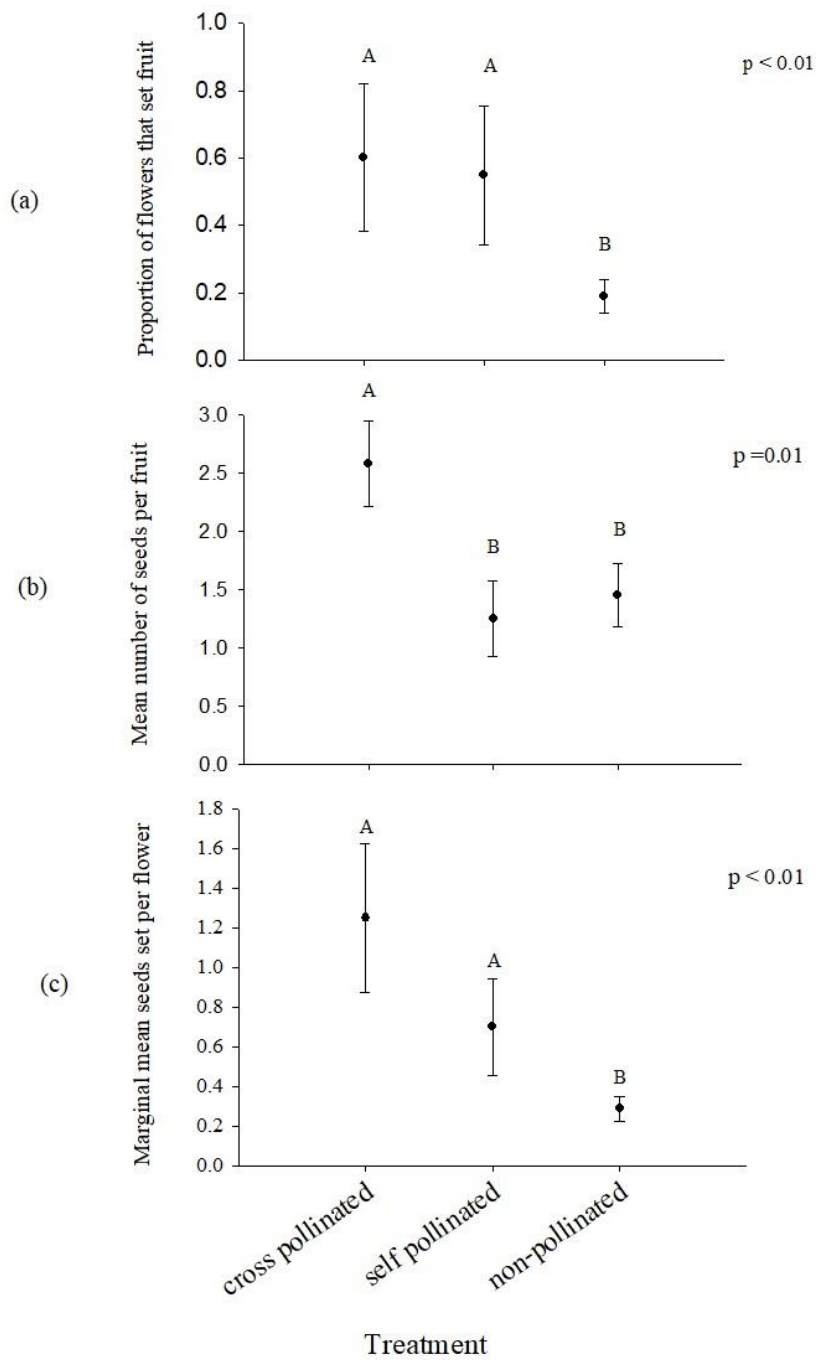


Figure 11: Results of controlled hand-pollinations to determine the breeding system of *T. atriplicifolia*. Treatments with the same letter are not significantly different. Marginal means are means for seed set, adjusted for other factors in the model, i.e. fruits per flower. Error bars represent standard error.

Pollinator sharing

Pollinator observations

In a period of seven months (February to April in 2015 and February to May in 2016) 49 bees were observed on flowers of *T. atriplicifolia* and 17 bees were observed on flowers of *E. grande*. I caught 39 bees representing 13 species on *T. atriplicifolia* and nine bees representing four species on *E. grande* (Table 1). All the four species caught on *E. grande* were also found on *T. atriplicifolia*. These are: *Allodape rufogastra*, *Ceratina nyassensis*, *Braunsapis bouyssoui* and *Lasioglossum* sp. Since these flowers are tubular, the visitors crawl in to collect nectar at the base of the floral tube (Figure 12 (a-b)). The tiny bees, i.e. *Ceratina nyassensis* crawl into the base of the floral tube, move around the gynoecium and androecium, and emerge head first before flying away. Bigger bees, i.e. *Lasioglossum* sp, crawl in, since they are not small enough (in relation to floral tube) to turn around inside the flower, they emerge backwards from the flowers. Once the abdomen is out and the rest of the body is in the wide part of the floral entrance, they turn their head up and fly away. During observations, 14 individuals made visits to both species. All the 13 bee species that were observed on the study species, were also observed making visits to at least one of the 26 other flowers in the community (Figure 13). The four shared pollinators of the study species; *Allodape rufogastra*, *Ceratina nyassensis*, *Braunsapis bouyssoui* and *Lasioglossum* sp. each visited nine, one, six and seven other flower species respectively.

Table 1: Floral visitors and number of visits observed on the focal species and congeners of *T. atriplicifolia*

Family	Floral visitor	<i>T. atriplicifolia</i>	<i>E. grande</i>	<i>T. alata</i>	<i>T. natalensis</i>	<i>T. dregeana</i>
Apidae	<i>Allodape rufogastra</i> Lepeletier & Serville	10	2	0	6	0
	<i>Allodape exoloma</i> Strand	6	0	0	0	0
	<i>Ceratina nigriceps</i> Friese	1	0	0	0	0
	<i>Braunsapis albipennis</i> Friese	1	2	0	0	0
	<i>Braunsapis bouyssoui</i> Vachal	5	0	0	0	0
	<i>Ceratina nyassensis</i> Strand	1	2	0	0	0
	<i>Allodape ceratinoids</i> Gribodo	2	0	0	0	0
	<i>Ceratina barbarae</i> Eardley & Daly	0	0	4	0	0
Halictidae	<i>Lasioglossum</i> sp.	7	3	2	5	3
	<i>Lasioglossum</i> sp. 2	3	0	0	0	0
	<i>Lasioglossum</i> sp. 3	2	0	0	0	0
	<i>Thrincostruma</i> sp.	1	0	0	0	0
	<i>Patellapis</i> sp	1	0	1	0	0
Syrphidae	<i>Fly</i> sp. 1	0	0	0	8	0
Bombyliidae	<i>Fly</i> sp. 2	0	0	0	9	0

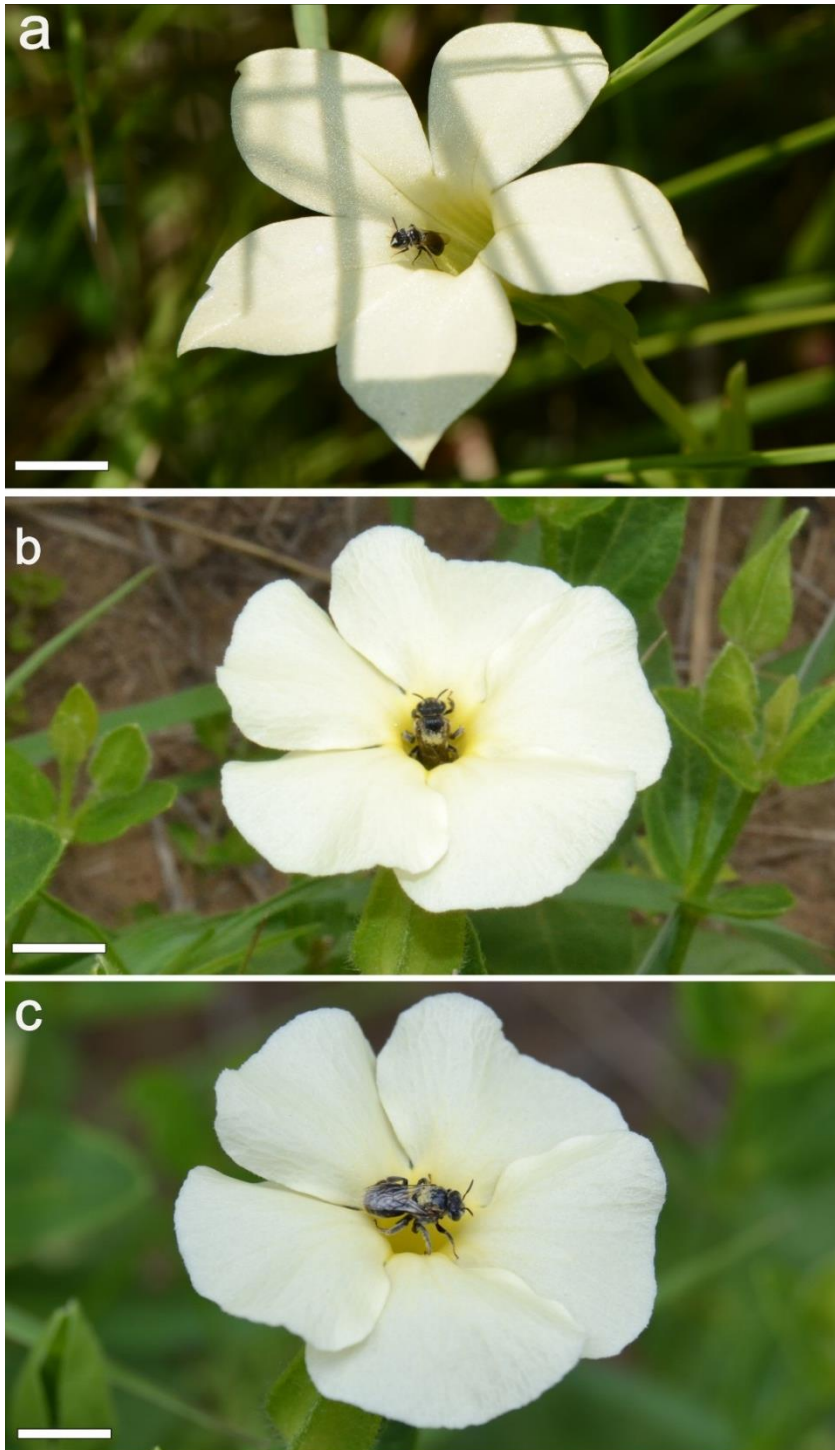


Figure 12: (a) *Ceratina* sp. (Apidae) visiting *E. grande* flower and (b-c) *Lasioglossum* sp. (Halictidae) visiting *T. atriplicifolia* flower. Note the pollen visible on the dorsal surface of the thorax. Scale bars = 1 cm. Photo credits: a = Adam Shuttleworth, b and c: Simangele M. Msweli

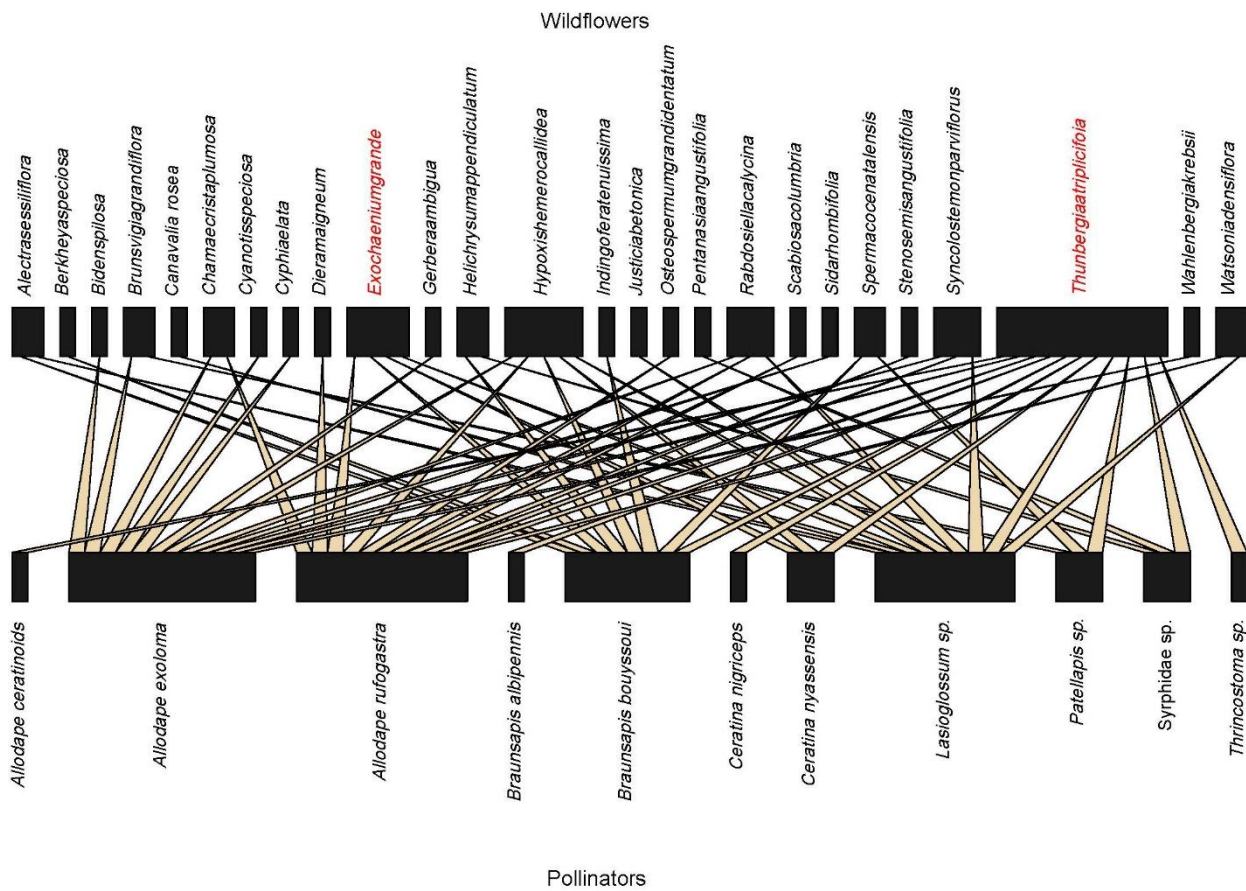


Figure 13: Other flowers which were also visited by floral visitors of *T. atriplicifolia* and *E. grande*. The width of bars represents the number of interactions. Focal species are highlighted in red.

Pollen loads

Among the bees collected on *T. atriplicifolia* and *E. grande*, 47% of them carried pollen of both species (Table 2).

Table 2: Number of pollen grains counted on bees collected on *E. grande* and *T. atriplicifolia* flowers

Bee	host	<i>E.grande</i> pollen grains	<i>T. atriplicifolia</i> pollen grains	Other species pollen grains
1	<i>E. grande</i>	0	0	0
2	<i>E. grande</i>	120	0	0
3	<i>E. grande</i>	135	32	14
4	<i>E. grande</i>	70	0	0
5	<i>E. grande</i>	0	0	0
6	<i>T. atriplicifolia</i>	66	53	18
7	<i>T. atriplicifolia</i>	22	32	0
8	<i>T. atriplicifolia</i>	0	0	0
9	<i>T. atriplicifolia</i>	0	0	0
10	<i>T. atriplicifolia</i>	37	26	43
11	<i>T. atriplicifolia</i>	9	29	9
12	<i>T. atriplicifolia</i>	0	51	0
13	<i>T. atriplicifolia</i>	26	21	11
14	<i>T. atriplicifolia</i>	0	0	52
15	<i>T. atriplicifolia</i>	43	0	0
16	<i>T. atriplicifolia</i>	13	41	7
15	<i>T. atriplicifolia</i>	0	24	0

Discussion

Exochaenium grande and *T. atriplicifolia* flower simultaneously (Figure 3 and 4), are perceived to be similar (in colour and size) by bee pollinators (Figure 6 and Figure 9), produce similar amounts of nectar and share bee pollinators (Table 1). Both direct observations and indirect analyses affirm pollinator sharing between *T. atriplicifolia* and *E. grande* (Table 2), and further shows that the bee visitors are generalists in the community (Figure 13). Direct observations of bees and analysis of pollen on the collected bees show significant levels of interspecific visits between the two species and other co-occurring wildflower species.

Among sympatric species, where bees are major pollinators, similarities in flower morphology may influence pollinator infidelity (Gross 1992). Interspecific visits and identical pollen placement may lead to competition via pollen interference. In other species, competition via pollen interference has been reported to reduce pollination success and fecundity (Free 1968; Waser 1978; Pleasants 1980). *Thunbergia atriplicifolia* and *E. grande* share pollinators and pollen is placed on the same parts of the body. This suggests that there is a scope for interference. More work is required to document the extent of interspecific pollen transfer and understand whether pollinator sharing in these species results in any competition.

Thunbergia atriplicifolia flowers first, followed, three months later by *E. grande* which also flowers at a much lower density than does *T. atriplicifolia*. The herbarium records show a broader flowering period than that in the floral survey. This is more likely due to the fact the floral survey graph depicts flowering in one site whereas the herbarium records depict flowering in different sites in the KwaZulu-Natal province. Similar differences in flowering time and abundance are evident at other sites where the two species co-exist. This suggests that *E. grande* is more likely to be a mimic in this system. Various studies have reported situations in a model-mimicry system, where late flowering, pollinator-dependent and nectar-less (or poorly rewarding) species mimic the flowers of an earlier flowering and nectar producing species (Johnson 1994; Johnson et al. 2003; Johnson and Morita 2006; Johnson and Peter 2008). Although *T. atriplicifolia* and *E. grande* produce the same amount of nectar, the lower frequency of *E. grande* and its later flowering period, suggests that this species may depend more on *T. atriplicifolia* than vice versa. Phylogenetic analysis of *E. grande* showed that the white floral colour is novel in the respective genera, and the flower size of *E. grande* is more similar to *T. atriplicifolia* than it is to its congeners. However, the phylogenies were not dated and therefore do not provide insight into the relative sequence of trait evolution in the two species, and thus whether the observed floral similarity in colour and size is likely to have evolved through divergent or convergent evolution.

Although petal colour of both species should be perceived similarly by bees (Figure 6), the mouth of the floral tube of *T. atriplicifolia* has a distinctive reflectance spectrum. A floral tube mouth with color different to that of the petals has been shown to act as a nectar guide and acts to increase effective pollination in *Lapeirousia oreogena* (Hansen et al. 2012). *Exochaenium grande* had anthers that are bright yellow. Such anthers have been reported to act as nectar

guides that increase pollen transfer in *Commelina communis* (Ushimaru et al. 2007). However, in *E. grande*, anthers were well-hidden and pollinators were less likely to see them from a distance. To determine whether bee pollinators have preference for either of the two species, more focal observations and specific choice tests are required. In this system, the visiting bees are tiny and highly mobile and thus, they can only be observed making two or three visits and cannot be observed when they are in the floral gullet. Therefore, flower preference or constancy could not be verified in a quantitative manner. For future studies, flight cage experiments may be useful for testing more detailed aspects of pollinator behaviour.

In this study, emphasis was placed on flower size and flower colour. Lab based experimental studies by Zhang et al. (1995), Campan and Lehrer (2002) and Sanchez and Vandam (2012) have demonstrated that bees can distinguish between shapes. There are now techniques available to test whether bees can distinguish between flower shapes. In *T. atriplicifolia* and *E. grande*, there is shape variation within each species. There is therefore possibility that these species may be distinguishable/indistinguishable to bee pollinators and geometric morphometric analyses may be employed to test this. A colour hexagon inferred that the two species are non-distinguishable to bees. This could be further investigated by doing choice test experiments.

Here I have provided evidence that *T. atriplicifolia* and *E. grande* are similar in flowering time, flower colour, flower size and nectar production. The similarity is not due to a shared recent ancestor since the two species belong to different families and genera. They share pollinators and place pollen on similar parts of the pollinator's body. The later flowering time and relative rarity of *E. grande*, together with the novelty of cream-white flower colour in its lineage, suggest, but do not confirm, that this species is more likely to function as a mimic than as a model. Ultimately, it is very difficult to distinguish between Müllerian mimicry and a floral syndrome, and as suggested by some researchers, floral syndromes may include elements of mimicry (Johnson and Schiestl 2016). Even if it is accepted that mimicry played some role in the evolution of the floral similarity of these two species, it remains difficult to distinguish between Müllerian and Batesian mimicry. I believe that this case is neither Batesian mimicry, nor is it Müllerian mimicry, but it combines both Müllerian mimicry and Batesian mimicry. More work is required to determine the ultimate evolutionary basis of floral similarity and pollinator sharing and to determine the costs vs benefits of co-occurrence in the two species.

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CHAPTER 3: Implications of pollinator sharing by two grassland wildflowers for interspecific pollen transfer and fecundity

Abstract

Pollinator sharing may have consequences for the pollination success and fecundity of one or more interacting wildflowers. These consequences are unique for each system and may be negative or positive. This study was conducted to determine the implications of pollinator sharing between *Exochaenium grande* (Gentianaceae) and *Thunbergia atriplicifolia* (Acanthaceae) on (1) interspecific pollen transfer between the two species and (2) on seed set of individuals of *E. grande* that grow within sparse and dense populations of *T. atriplicifolia*. The study was conducted in grassland vegetation in southern KwaZulu-Natal, South Africa. To test for the effect of pollinator sharing for pollen transfer, stigmas of both species were collected and analysed for presence of con- and heterospecific pollen. To test for the effect of local plant density on fecundity, focal plants of *E. grande* were randomly selected using point sampling. Number of conspecifics and number of *T. atriplicifolia* plants within a five metre radius were recorded. Five percent of *T. atriplicifolia* stigmas carried *E. grande* pollen while 34% of *E. grande* stigmas carried *T. atriplicifolia* pollen. Although interspecific pollen was confirmed, it is not yet clear whether this improper pollen transfer affects fecundity. Plant density of *E. grande* and *T. atriplicifolia* was not a significant predictor of seed set in *E. grande*. However, there were very high levels of seed predation in *E. grande*, and that hinders our ability to rule out the any effects of pollinator sharing in seed set. These findings imply that although the two species share pollinators, there are currently no known negative or positive effects of this on fecundity.

Keywords: pollinator sharing, pollen interference, facilitation, mimicry

Introduction

Wildflowers often occur in communities where they co-exist with other flowers sharing similar floral traits. Such species often share pollinators and this leads to pollinator-mediated ecological interactions among plant species (Sargent and Ackerly 2008, Schiestl and Johnson 2013). Pollinator sharing may be detrimental (i.e. competition), beneficial (i.e. facilitation and Mullerian mimicry) or have no effects on the involved flowers. Pollinator sharing may thus influence pollination success and affect the resulting fecundity and other indicators of plant reproductive performance (De waal et al. 2015). Understanding the degree and consequences of pollinator sharing is thus important if we are to understand the evolution of co-existing communities of wildflowers.

Competition may occur between individual plants of the same species or between individuals of distinct species. This may arise through competition to attract pollinators and interference with pollen removal and deposition (Waser 1978, 1983; Rathcke 1983). Pollen transport can be affected such that pollen ends on heterospecific stigmas or other non-stigmatic surfaces of intervening flowers (Muchhala and Thomson 2012). The lost pollen no longer contributes to paternal fitness, a phenomenon termed pollen discounting (Harder 1998). Conspecific stigmas might also be clogged with heterospecific pollen, reducing stigma space for the deposition of conspecific pollen (Rathcke 1983). This Improper Pollen Transfer (IPT) has been shown in some studies to reduce maternal fitness through production of hybrids and through chemical and physical interference with conspecific pollen (Rathcke 1983). In a study of bat pollination, Muchhala and Thomson (2012) found that competition (in the form of pollen misplacement) was greater for flowers that use similar pollen-placement locations on a shared pollinator.

The opposite side of the coin exists, where having similar floral signals and flowering simultaneously is beneficial. In pollination, ecological facilitation refers to a case where plant reproductive performance is improved by the presence of other flowering plant species that share the same pollinators (Thomson 1978; Waser and Real 1979; Rathcke 1983; Callaway 1995; Moeller 2004; Ghazoul 2006). This positive interaction could be unidirectional or bidirectional. It could occur within individuals of the same species and between individuals of different species. Such ecological facilitation may result from the “magnet species effect” (Thomson 1978) whereby a rewarding species (the “magnet” species) increases the abundance

of pollinators in a patch and consequently increases the pollination success of neighbouring plants that could be less rewarding or unrewarding.

Facilitation may also occur via joint attraction of highly mobile pollinators or joint maintenance of resident pollinators, which occurs because of a convergent floral syndrome (Feinsinger 1987; Rathcke 1983). In a convergent floral syndrome, certain floral traits look similar because they have independently adapted to pollination by the same pollinator species or pollinator functional group (Fenster et al. 2004). As opposed to the “magnet species effect”, facilitation via the latter mentioned mechanism does not necessarily require a more rewarding flower or greater abundance. All the involved flowers could be providing the same reward, but because each or all of them have populations at low density, they are increasingly attractive by co-occurring with flowers pollinated by similar species and exhibiting similar floral traits (Moeller 2004). Facilitation requires no adaptive resemblance of the involved species, as is the case for mimicry. Mimicry is defined by Johnson and Schiestl (2016) as the adaptive resemblance of one organism (the mimic) to other organisms or their by-products (the models), such that there is cognitive misclassification and behavior by third-party organisms (operators) that enhances the fitness of the mimic. In flowering plants, adaptive resemblance of floral traits may evolve either through convergent evolution or advergent evolution, and this similarity increases plant reproductive performance of either one or all the interaction species.

Exochaenium grande (Gentianaceae) and *Thunbergia atriplicifolia* (Acanthaceae) present a good opportunity to study the consequences of pollinator sharing. The two species have been confirmed to be similar in flowering time, flower colour and flower size (chapter 2). They have also been confirmed to share pollinating species and pollinating individuals (Chapter 2). *Exochaenium grande* occurs at a lower density than *T. atriplicifolia* (Chapter 2) and is self-incompatible (Wolfe et al. 2009), it is therefore more likely to be more affected by pollinator sharing. Specific aims of this study were: (1) to investigate pollen transfer of *T. atriplicifolia* and *E. grande* and (2) to investigate seed set of *E. grande* individuals in relation to their density and the density of *T. atriplicifolia* with which they co-occur. If these species are Mullerian mimics, I predict that pollinator sharing will result in increased seed set.

Materials and methods

Study site

The study was conducted in South Africa, KwaZulu-Natal province, Vernon Crookes nature reserve (S30.2882°; E30.5621°). Vernon Crookes is a 2. 189-hectare nature reserve located 150 to 610m above the sea level and is characterised by coastal grasslands and coastal forests. The reserve has co-existing populations of *E. grande* and *T. atriplicifolia*. It also has patches where the two populations grow in isolation, making it a good site to test for differences in fecundity where the two do not co-occur.

Study species

In the study population, the flowering period of *T. atriplicifolia* and *E. grande* overlaps but *E. grande* occurs at much lower density (Chapter 2). They are indistinguishable to bee pollinators by petal colour (Chapter 2). They show significant overlap in flower size (Chapter 2). They provide similar amount of nectar with nectar of *E. grande* having a higher sugar concentration (Chapter 2). *E. grande* has a heteromorphic incompatibility system (Wolfe et al. 2009) and *T. atriplicifolia* is self-compatible, with very limited autonomous selfing (Chapter 2). The two species share some bee species as pollinators (namely: *Allodape rufogastra*, *Ceratina nyassensis*, *Braunsapis bouyssoui* and *Lasioglossum* sp) (Chapter 2).

Pollen transfer

Seed set is usually reflective of pollination success. To determine whether the pollinators of *T. atriplicifolia* and *E. grande* transfer pollen between flowers of these species (improper pollen transfer), 54 flowers (from 54 different plants) of each species were collected in the field. The stigmas were put on a slide, stained with fuchsin gel and pollen loads were counted under a light microscope. Pollen reference slides of *E. grande* and *T. atriplicifolia* were prepared using pollen from anthers of both species. For each stigma, pollen was identified to be either from (1) conspecific anthers or (2) heterospecific (from the other species) anthers. From the above-mentioned counts, I calculated the percentage of stigmas that carried heterospecific and conspecific pollen. Differences in the mean number of conspecific and heterospecific pollen grains collected on the stigmas were tested using generalized linear models incorporating a binomial distribution and logit link function in IBM SPSS version 25.

Pollen quantity and pollen size

The quantity of pollen deposited on stigmas may also reflect differences in pollen production by different species. As a result, should there be differences in improper pollen transfer of the two species, as investigated in 3.1, measurement of pollen production sheds light on whether this difference may result from higher visitations for any of the species or from differences in pollen production of the two species, or a combination of both these factors. To measure pollen production per flower, buds of 10 individual plants of *T. atriplicifolia* and *E. grande* were respectively bagged. Once the flowers opened and the anthers dehisced, the pollen from each flower was transferred into 1ml of 70% ethanol. The mixture of pollen and alcohol was shaken using a vortex in order to ensure even distribution of the pollen. 50 μ l of this was transferred into a microscope slide, stained with fuschin gel and viewed under a compound microscope. Three subsamples (slides) were made from each sample. The number of pollen grains per 50 μ l for the extracted samples was used to extrapolate the number of pollen grains in 1ml. Differences in mean number of pollen grains produced by both species were tested using a simple t-test in IBM SPSS v25.

The slides used to count pollen were also used to measure the diameter of pollen grains of each species. Pollen grain size was measured using a Zeiss Axio lab.A1 fluorescence microscope fitted with a camera (AxioCam ICc5), using the scaling function in the Zeiss software (ZEN 2012, blue edition). Five pollen grains were measured per slide, making a total of 50 pollen grains per species. Differences in mean number of pollen grains produced by both species were tested using a simple t-test in IBM SPSS v25.

Fecundity analysis

From literature, plants that mainly benefit or suffer from pollinator sharing, are those that are self-incompatible and rare, since they are under pressure to attract and maintain pollinators (Johnson et al. 2003). I only investigated the effect of density on *E. grande*, because *E. grande* has been confirmed to occur at lower density and flower later than *T. atriplicifolia* and has a heteromorphic self-incompatibility system which excludes autonomous self-fertilization. In

this test, density refers to the number of *E. grande* and *T. atriplicifolia* flowers within a five meter radius.

To determine the effects of population density, 54 focal plants of *E. grande* were randomly selected using a point sampling method. For each focal plant, the number of conspecifics and the number of *T. atriplicifolia* plants within a five-metre radius were recorded. Some of the 54 focal plants were well isolated from other plants while some were in dense patches. It was ensured that there was no overlap between the five-metre radii around focal plants. All of the focal plants were tagged and left in the field for four weeks after which, fruit set was recorded. Fruits were harvested and the number of seeds per fruits was also counted. Since *E. grande* seeds are very small and cannot be counted with the naked eye, a microscope with a camera connected to it was used to count the number of seeds per fruit. To test for a relationship between the number of seeds per flower and the local plant densities of *E. grande* and *T. atriplicifolia*, data were analysed using generalized estimating equations implemented in SPSS 25 (IBM Corp). To control for neighbourhood effects, site was treated as the subject in models with an exchangeable correlation matrix. Models used a negative binomial distribution with a log link function. Density of the two species and their interactions were treated as covariates. I also used these covariates in models with a single predictor. Model significance was tested using Wald statistics.

Results

Pollen interference

I found that 5% of *T. atriplicifolia* stigmas had pollen grains of *E. grande* while 34% of *E. grande* stigmas had pollen gains of *T. atriplicifolia* (Figure 14a). For stigmas of both species, the mean number of conspecific pollen grains was significantly higher than that of heterospecific pollen grains (Figure 14b). Stigmas of *T. atriplicifolia* carried a mean number of 9.8 ± 0.42 conspecific pollen grains and 2.18 ± 0.19 *E. grande* pollen grains and stigmas of *E. grande* carried 327 ± 2.49 conspecific pollen grains and 6.35 ± 0.33 of *T. atriplicifolia* pollen grains.

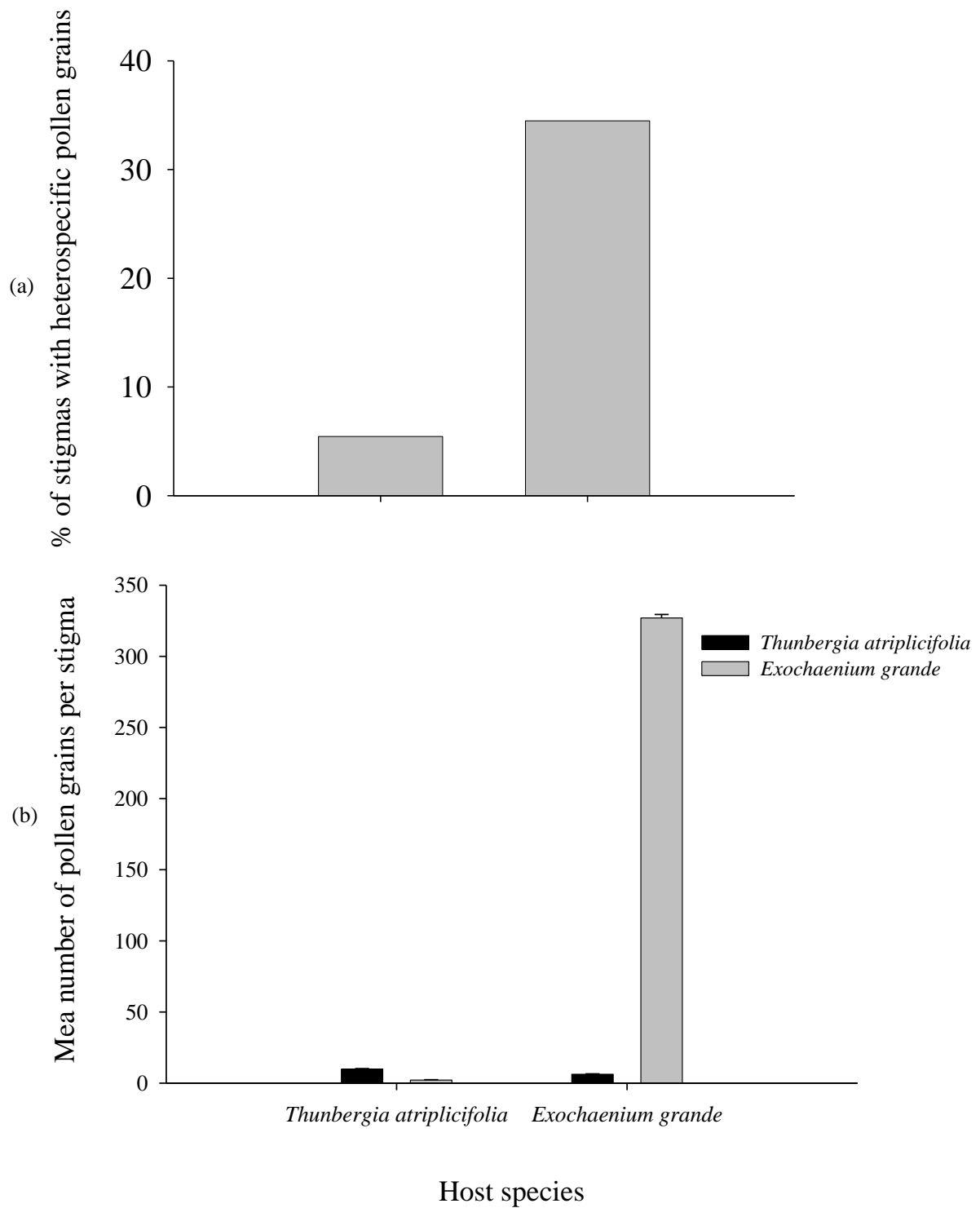


Figure 14: (a) The percentage of stigmas with heterospecific pollen and (b) mean number of pollen grains per stigma in *E. grande* and *T. atriplicifolia*. Error bars represent standard error.

Pollen quantity and size

I found that there was a significant difference in the mean number of pollen grains produced by *E. grande* and *T. atriplicifolia* ($t=4.24$; $p<0.001$). Anthers of *T. atriplicifolia* produced a mean of 935 ± 103.86 pollen grains and anthers of *E. grande* produced $11\ 318\pm 2583.92$ pollen grains. There was also a significant difference in the mean pollen grain size of *E. grande* and *T. atriplicifolia* ($t=42.98$; $p<0.001$). The mean size of pollen grains for *E. grande* was 16.75 ± 0.29 μm and for *T. atriplicifolia*, it was 70.95 ± 1.22 . All means are presented as mean \pm SE. *Thunbergia atriplicifolia* has four anthers and *E. grande* has five anthers (Figure 15).



Figure 15: Anthers and pollen of *E. grande* (a-b) and *T. atriplicifolia* (c-d). Note the short styled morph (left) and the long styled morph (right) of *E. grande* (a). Scale bars = 1 cm. Photo credit: a and b = Steven D. Johnson, c and d = Simangele M. Msweli

Fecundity analysis

In univariate analyses, seed set per flower in *E. grande* did not increase with an increase in the density of *E. grande*, *T. atriplicifolia* or the total density of both species (within a 5 metre radius) (Figure 14). Seed set is therefore not explained by the local density of the investigated species. Multiple regression analyses based on generalized linear models further showed that density of *E. grande*, *T. atriplicifolia* and their interaction, is not a predictor of *E. grande* seed set (Table 3).

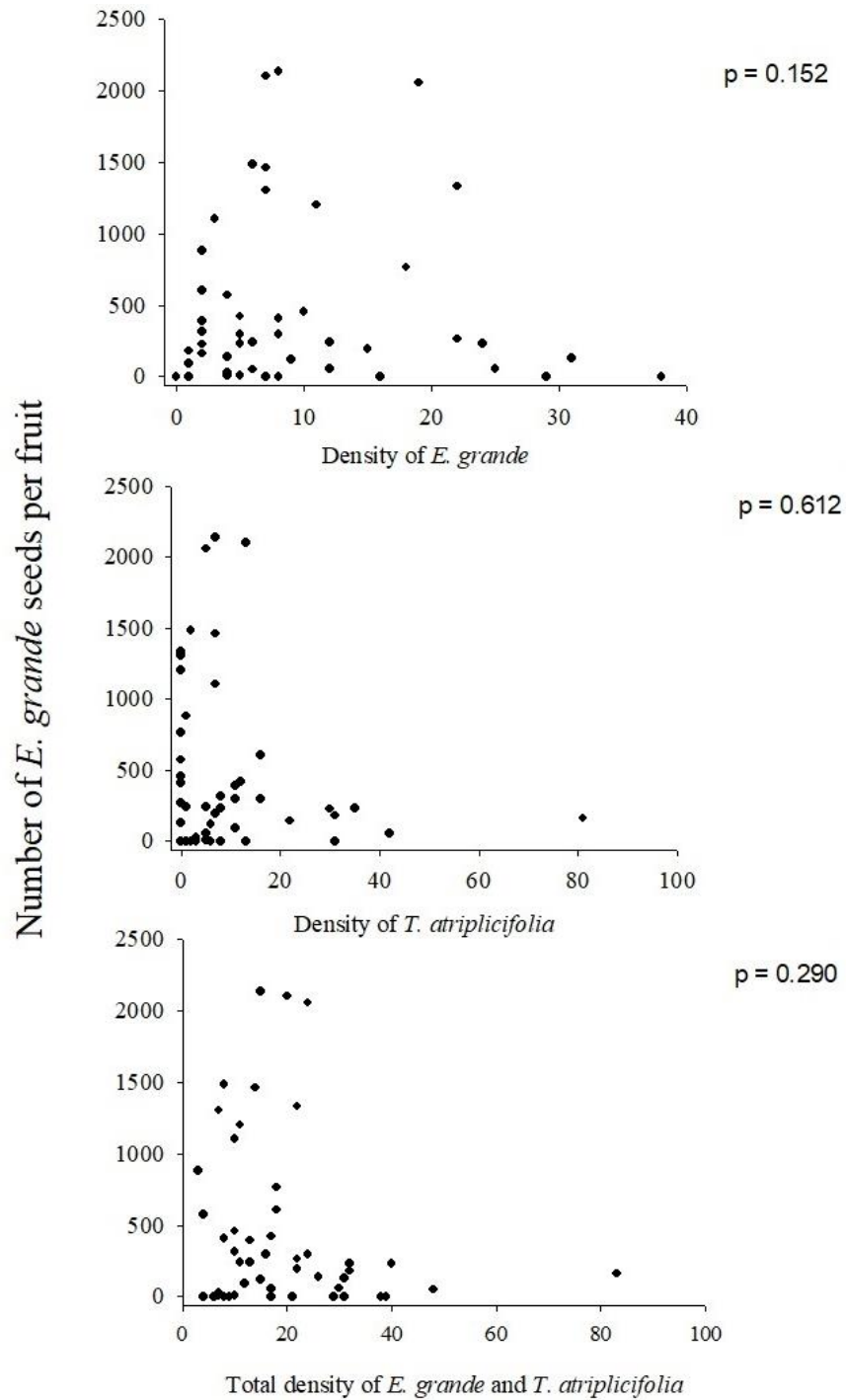


Figure 16: Number of *E. grande* seeds per fruit, in relation to the number of *E. grande* Individuals, *T. atriplicifolia* individuals and sum of the two species within a 5 metre radius of each focal plant. Statistical values are for univariate negative binomial regression models.

Table 3: Multiple negative binomial regression model of the relationship between three predictor variables and seed set of *E. grande*.

	Standardized coefficient B	χ^2	p
<i>E. grande</i> density	-0.029	2.432	0.126
<i>T. atriplicifolia</i> density	-0.027	1.653	0.199
<i>E. grande</i> * <i>T. atriplicifolia</i>	0.001	0.035	0.852

Discussion

Stigmas of both *T. atriplicifolia* and *E. grande* carried pollen of the other species (Figure 14). However, the proportion of stigmas that carried foreign pollen was higher in *E. grande* than in *T. atriplicifolia*. This is probably because *E. grande* occurs at low density (Chapter 2) and therefore more likely to be visited by bees that have been to the more abundant *T. atriplicifolia*. Although both species carried pollen of other species, the quantity of conspecific pollen was still significantly higher than heterospecific pollen. It is important to note that much of the conspecific pollen is probably self-pollen.

Heterospecific pollen has been shown to clog the stigma thereby limiting space that could otherwise be taken by conspecific pollen (Rathcke 1983). It can also interfere with conspecific pollen through physiological and physical interference (Rathcke 1983). Some have argued that pollen deposited on heterospecific stigmas represents the loss of male gametes capable of fertilizing ovules (Brown and Kodrik-Brown 1979). Schemske (1981) demonstrated that although there was intense interspecific visitation (97% of flowers checked had received heterospecific pollen grains) between morphologically similar and pollinator sharing *Costus allenii* and *C. laevis*, the high probability of interspecific pollination did not affect fruit set, which is an indicator of female fitness. Heterospecific pollen detected on the stigmas of *T. atriplicifolia* and *E. grande* was in significantly smaller amounts than conspecific pollen, and therefore it is not clear if the observed improper pollen transfer would have any negative effects in male and female fitness of both species. For future studies, the effect of improper pollen transfer in fecundity of *E. grande* and *T. atriplicifolia* could be tested by controlled hand

pollinations of the two species and thereafter comparing the fecundity of flowers pollinated with only conspecific pollen and those pollinated with a mixture of conspecific and heterospecific pollen.

On average, stigmas of *E. grande* had 327 ± 2.49 legitimate pollen grains, which is higher than the 9.8 ± 0.42 legitimate pollen grains per stigma observed in *T. atriplicifolia*. This enormous difference in number of pollen grains per stigma, may be reflective of the difference in pollen grain size and quantity, as produced by the anthers. *E. grande* anthers have five “pollen sacs” (Figure 15 (a-b)) which produce an average of $11\ 266 \pm 2526.78$ pollen grains while *T. atriplicifolia* has four brush-like anthers (Figure 15 (c-d)) producing an average of 938 ± 95.66 pollen grains. Pollen transfer efficiency (PTE) for *T. atriplicifolia* is 1.2% and 2.8% for *E. grande*. These PTE values are comparable to the range of 0.07% - 2.16%, observed from 11 animal pollinated species with granular pollen (Harder 2000).

Seed set of *E. grande* could not be explained by either the density of *E. grande*, density of *T. atriplicifolia* or total density of both species in Vernon Crookes Nature Reserve (Figure 16). There is therefore no evidence that *E. grande* experiences either within or between species facilitation. It is important to note that in addition to successful pollination, seed set is affected by other conditions such as resource availability and predation as shown by Agren et al. (2008) in *Vincetoxicum hirundinaria*. As a result, supplemental pollination is required in order to determine the possible maximum seed set in a given environment. About 18% of collected *E. grande* fruits, had evidence of predation. Some fruits were found to have larvae in them. Prior to fruiting, weevils were occasionally observed feeding on the flowers and this suggests that on overall, predation affects more than 18% of the *E. grande* population. *Thunbergia atriplicifolia* is self-compatible and can set seeds through autonomously selfing (Chapter 2). Seed set in this species is therefore not entirely reflective of only cross pollination success.

In light of the current results, although *T. atriplicifolia* and *E. grande* are morphologically similar and share pollinators, there is no evidence that pollinator sharing has any negative or positive implications for reproductive performance. This is contrary to the general expectation that rarer, self-incompatible species are usually affected (either negatively or positively), when they co-occur with more abundant, morphologically similar flowers.

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CHAPTER 4: General discussion and conclusions

Co-existing, morphologically similar flowers often share pollinators and this leads to pollinator-mediated ecological interactions among plant species (Sargent and Ackerly 2008, Schiestl and Johnson 2013). Pollinator sharing may be detrimental (i.e. competition), beneficial (i.e. facilitation and mimicry) or have no effects on the involved flowers. Similarity could be due to a shared common ancestor, convergent floral syndrome or mimicry. It remains a great challenge to distinguish between a floral syndrome and mimicry.

In the first research chapter of this dissertation I investigated floral similarity and pollinator sharing between *Thunbergia atriplicifolia* (Acanthaceae) and *Exochaenium grande* (Gentianaceae). Investigated aspects of similarity included: flowering phenology, flower colour, flower size and nectar production. I also used phylogenies to test 1) the novelty of flower colour and size similarity between species and 2) the historical sequence of signal similarity. I also investigated the breeding system of *T. atriplicifolia* and obtained that of *E. grande* from literature. I then documented pollinator sharing through extensive field observations and stigma analysis. In the second research chapter I quantified pollen production and investigated the consequences of pollinator sharing for pollen transfer and for fecundity of *E. grande*. The intention of this chapter is to summarise and discuss the major findings, their significance and opportunities for further research.

Conclusion

Thunbergia atriplicifolia and *E. grande* are quite common in South African grasslands, therefore providing a good model system to test a mimicry hypothesis. Johnson et al. (2003) argued that a rare rewarding *Brownleea* orchid was a mimic of a more common *Scabiosa* species. More studies have shown that in any positive or negative interaction, a flowering plant that occurs at low density, flowers later and is self-incompatible, is usually the one that is most affected from the interaction. Owing to this background knowledge and anecdotal observations (i.e. *T. atriplicifolia* and *E. grande* co-exist in South African grasslands and look similar to the human eye), I hypothesized that *E. grande* is a mimic of *T. atriplicifolia*.

In Chapter 2 I have shown that the flowering period of *E. grande* and *T. atriplicifolia* overlaps, with *E. grande* occurring at low density (Figure 3 & 4). The two species are indistinguishable by colour to bees (Figure 5 & 6), their flower size distribution overlaps (Figure 9), they produce equal amounts of nectar and *E. grande* is self-incompatible (Wolfe et al. 2009) while *T. atriplicifolia* is partially self-compatible (Figure 11). Such floral similarity has been shown to promote pollinator sharing in two bee-pollinated tropical herbs, namely *Costus allenii* and *Costus laevis* (Zingiberaceae) (Schemske 1981) and in bee pollinated distantly related *Turnera sidoides* ssp. *pinnatifida* (Turneraceae) and *Sphaeralcea cardobensis* (Malvaceae) (Benitez-Vieyra et al. 2007). *Thunbergia atriplicifolia* and *E. grande* indeed share pollinating species (Table 1) and individuals (Table 2), namely *Allodape rufogastra*, *Ceratina nyassensis*, *Braunsapis bouyssoui* and *Lasioglossum* sp.

From the above-mentioned results, we can see that there is not enough evidence to accept or reject the hypothesis that *E. grande* is a Mullerian mimic of *T. atriplicifolia*. Batesian mimicry usually involves a non-rewarding species that occurs at low density (relative to its model) whereas Müllerian mimicry involves rewarding species at comparable densities (Johnson and Schiestl 2016). This system shares characteristics from both types of floral mimicry, since both species are rewarding but only one of them occurs at much lower density. In addition to confirming floral similarity, to test a mimicry hypothesis, one must demonstrate that the traits involved in attracting pollinators (i.e. colour and size) are derived and trace the historical sequence of their evolution. Batesian mimicry often involves adverbent evolution whereas Müllerian mimicry involves convergent evolution which may be symmetric or asymmetric (Johnson and Schiestl 2016). From the phylogeny of the potential mimic, *E. grande*, it has been showed that flower colour (white-cream white) is shared by other species in the genus (Figure 8) and that flower size is relatively larger than that of other species in the genus (Figure 10) (Kissling and Barret 2013). Since there are no dated phylogenies of both species, I cannot make any conclusions about the sequence of evolution of similarity in flower colour and size, i.e. whether they evolved through adverbent or convergent evolution.

Two of the predictions for a mimicry system, are that: the fitness of the mimic should be higher in the presence of the model than in its absence and that the mimic performs best when models are relatively abundant. In Chapter 3 I found that the number of *T. atriplicifolia* plants within a

five meter radius was not a significant predictor of *E. grande* seed set. However, 18% of the fruits collected to measure seed set, had evidence of predation. Prior to setting fruits, beetles in the genus *Decapotoma* (Meloidae), were observed feeding on *E. grande*. This suggests that the effects of predation in that population, affect more than 18% of the population. Although predation is a natural phenomenon, unusually elevated levels of predation may hinder our ability to rule out any positive or negative effects of *T. atriplicifolia* density, on the fecundity of *E. grande*.

As opposed to facilitation and mimicry, pollinator sharing may in fact have negative effects (i.e. competition). In Chapter 3, I have shown that there is improper pollen transfer between the two species- with stigmas of either *T. atriplicifolia* or *E. grande* carrying foreign pollen. This however, does not imply that there is competition between the two species. For instance, Schemske (1981) demonstrated that although there was intense interspecific visits (97% of flowers checked had received heterospecific pollen grains) between morphologically similar and pollinator sharing *Costus allenii* and *C. laevis*, the high probability of interspecific pollination did not affect fruit set. More experimental work is therefore required to test for the effect of interspecific pollen on the stigmas of *T. atriplicifolia* and *E. grande*.

Future possibilities

Building on this work, more experiments are required to confirm if this is indeed floral mimicry and if yes, what type of mimicry it is. I found it challenging to investigate the behaviour of pollinators inside the flower and investigate foraging constancy. Firstly, because pollinators are tiny, as a result, once they get into the floral tube, they are hidden from your view. Secondly, in addition to a smaller body size, they are highly mobile and tend to fly out quickly, rendering it hard to observe consecutive visits of one individual. I therefore suggest that foraging constancy be studied in flight cage experiments (as opposed to field observations). Constructing a dated phylogeny for both species will be useful in understanding the evolutionary direction of floral similarity, thereby establishing whether the observed similarity arose through advergent or convergent evolution. *E. grande* has a short-styled and a long-styled morph. When testing pollen production and the effect on density on seed set, data for both morphs was pooled

together. For future work, it may be worth to analyse data for morphs separately. In addition to quantifying pollen grains, pollen grain size for both species may be measured.

Here I have presented results on interspecific pollen transfer. In order to test for the effect of heterospecific pollen on the stigmas of both species, I suggest the use of controlled hand pollinations with hetero- and conspecific pollen grains as this will give more insight on the effects of pollinator sharing in fruit and seed set. Since there was predation on the seeds and flowers of *E. grande*, more controlled hand pollinations will be useful in investigating possible maximum fruit and seed set of these plants in the field. Most of this work was done at one site, it could be interesting to see how floral traits differ between sites where the two species co-occur, and especially where there is a yellow colour morph of *E. grande*. One of the predictions of Batesian and Mullerian mimicry is that the mimic, may vary in its display traits among populations when there may have different models in different sites (Johnson and Schiestl 2016). It will therefore be interesting to see how yellow and orange forms of *E. grande* interact with other plant species and pollinators throughout their distribution.

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