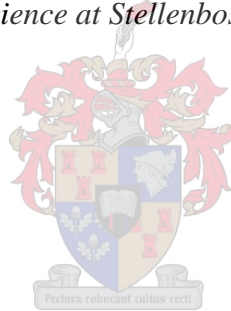


**Flowers with style: The role of pollinators in the origin and maintenance of Proteaceae diversity with a focus on the genus *Leucospermum***

By

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*Thesis presented in fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science at Stellenbosch University*



Promoter: Associate Professor Anton Pauw

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## Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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## Abstract

It is believed that angiosperm diversification can regularly be explained by adaptation to pollinators. This may result from a shift to a new pollinator, recruitment of a supplemental pollinator, employing the traditional pollinator in a new way, or relying less on pollinators altogether and instead placing a stronger emphasis on self-pollination. Diversification can transpire when a population finds itself in a new habitat lacking the traditional pollinator or when taxa overlap in the utilization of a shared pollinator resource. Competition between taxa that utilize the same pollinator ceases once pollinator partitioning is sufficiently achieved if taxa do not compete for pollinator visits. Therefore, pollinators can also be a determinant of angiosperm community composition in addition to drivers of speciation. Throughout this thesis I examine how angiosperms adapt to pollinators as a resource, how pollinators drive speciation, and how pollinators maintain communities by focusing on the southern African genus *Leucospermum* (Proteaceae).

Adaptation to fluctuations in the pollinator mosaic can drive diversification of floral morphology and denotes the onset of speciation. In Chapter 2 we examine pollinator driven adaptive divergence between two closely related *Leucospermum* taxa that have distinct floral morphologies and employ different pollinators. We suggest that these two varieties are ecotypes that originated through adaptation to different pollinators.

Angiosperms in the Cape Floristic Region often evolve elaborate features that allow them to utilize atypical pollinators. In Chapter 3 we explore remarkable adaptations for non-flying mammal pollination in an endangered *Leucospermum* species. We show that unique nectar characteristics accommodate gerbil and mice pollinators, that proximity to the ground does not

influence seed production, and that frequent grooming by non-flying mammals quickly diminishes the pollen available for outcrossing.

In Chapters 2 and 3 we show that pollinators can select for floral traits as well as drive speciation. But how often do pollinator shifts occur? What traits must evolve to utilize specific functional groups of pollinators? What morphological features and/or pollination modes encourage autonomous self-pollination? To answer such questions one must incorporate phylogenetics into analyses to account for relatedness among taxa. In Chapter 4 we construct the first *Leucospermum* phylogeny and use it to test for correlated evolution between floral morphology, pollination mode, and autonomous selfing using 7 floral measures and 10 functional groups of pollinators. We show that floral traits are highly correlated with pollination modes and that the evolution of autonomous selfing is coupled with the bird pollination syndrome.

Along with being drivers of floral diversity, pollinators can also act to shape and maintain floral communities. Since pollinators are a limited resource, pollinators act as ecological filters by restricting certain species from a community while permitting others. When species coexist and utilize the same pollinators there is potential for competition for pollinator visits as well as through interspecific pollen transfer. In Chapter 5 we provide evidence that co-flowering *Leucospermum* and *Mimetes* trees utilize discrete pollen attachment sites on a shared pollinator and density dependent interactions promote a mutualistic interaction between the two species.



## Opsomming

Daar word geglo dat die diversiteit van blomplante verklaar kan word as aanpassing by bestuiwers. Hierdie aanpassings kan die gevolg wees van 'n verskuiwing na 'n nuwe bestuier, werwing van 'n aanvullendebestuier, die gebruik van die voormalige bestuier op 'n nuwe manier, of verskuiwing na self-bestuiering. Diversifikasie kan plaasvind wanneer 'n populasie homself bevind in die afwesigheid van die voormalige bestuier, of as gevolg van kompetisie wanneer taxa oorvleuel in die benutting van 'n gedeelde bestuierings hulpbron. Dus speel bestuiwers ook 'n rol in die samestelling van plant gemeenskappe. In hierdie tesis ondersoek ek hoe blomplante aanpas by bestuiwers as 'n hulpbron, hoe bestuiwers spesiasie aandryf, en hoe bestuiwers gemeenskaps komposisie beïnvloed deur te fokus op die Suider-Afrikaanse genus *Leucospermum* (Proteaceae).

Aanpassing by variasie in die bestuier mosaïek kan diversifisering van blom-morfologie aandryf. In Hoofstuk 2 ondersoek ons bestuier-aangedrewe divergensie tussen twee nouverwante *Leucospermum* taksa wat duidelik verskil in blom-morfologie. Ons stel voor dat die takse twee ekologiese rasse is wat ontstaan het deur aanpassing by verskillende bestuiwers.

Angiosperme in die Kaapse Floristiese Streek ontwikkel dikwels ingewikkelde strukture wat hulle toelaat om van ongewone bestuiwers gebruik te maak. In Hoofstuk 3 ondersoek ons merkwaardige aanpassings vir nie-vlieënde soogdier-bestuiering in 'n bedreigde *Leucospermum* spesie. Ons wys dat unieke nektar eienskappe haarpootnagmuise en streepveldmuise as bestuiwers akkommodeer, dat die nabyheid van blomme aan die grond nie saad produksie beïnvloed nie, en dat die deeglike skoonmaak gewoontes van hierdie knaagdier vinnig die hoeveelheid stuifmeelkorrels beskikbaar vir kruisbestuiering verminder.

In hoofstukke 2 en 3 wys ons dat bestuiwers natuurlike seleksie uitoefen op blom eienskappe en dus spesiasie aandryf. Maar hoe dikwels vind dit plaas? Watter plant eienskappe word geassosieer met spesifieke funksionele groepe van bestuiwers? Watter morfologiese kenmerke en / of metodes van bestuiwing word met self-bestuiwing geassosieer? Om sulke vrae te beantwoord moet 'n mens filogenetiese informasie in die analise inkorporeer om verwantskap tussen taksa in ag te neem. In Hoofstuk 4 bou ons die eerste *Leucospermum* filogenie en gebruik dit om te toets vir gekorreleerde evolusie tussen blom-morfologie en bestuiwing. Die analise sluit 7 blom-eienskappe en 10 funksionele groepe van bestuiwers. Ons wys dat blom eienskappe hoogs gekorreleed is met bestuiwings-metodes en dat die evolusie van outonome self-bestuiwing geassosieer is met die voël bestuiwing sindroom.

Buiten hulle rol in die oorsprong van plant diversiteit speel bestuiwers ook 'n rol in die ekologiese strukturering van plant gemeenskappe. Wanneer spesies saamleef en dieselfde bestuiwers gebruik is daar potensiaal vir kompetisie vir bestuiwer besoeke, sowel as kompetisie deur interspesifieke stuifmeel oordrag. In Hoofstuk 5 wys ons dat mede-blommende *Leucospermum* en *Mimetes* bome kompetisie vermy deur afsonderlike stuifmeel aanhegtingsplekke op suikervoëls te gebruik, en dat hierdie gedeelde bestuiwer dus 'n mutualistiese interaksie tussen hierdie twee plantspesies bewerkstellig.

## Acknowledgements

The importance of Anton Pauw in supervising this research cannot be understated. Along with providing ample guidance and collaboration he has allowed me to use my imagination and at all times encouraged academic freedom. No one could have brought out a better researcher in me. I would also like to thank my collaborators, Tianhua He, Byron Lamont, Sim Lin Lim who sequenced and aligned DNA and constructed the phylogenetic trees contained in this thesis, and the many individuals who helped with fieldwork, most notably Di and Bill Turner, Tony Rebelo, Phoebe Barnard, Brian du Preez, Martin Capraro, Hannes Wiese, Marinus de Jager, Ethan Newman, Dave and Sue Whitelaw, Ishamail Ebrahim, and the custodians of rare and endangered wildflowers. I want to especially thank my family and friends for their encouragement while I lived and worked overseas. I know it was hard on them at times and I would not have been able to succeed without their love and support.

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# Chapter 1

## Introduction



The Cape Floristic Region exhibits one of the most diverse temperate floras in the world, containing more than 9,000 species, approximately 70% of which are endemics, confined to an area of only ~90,000 km<sup>2</sup> (Linder 2003; Schnitzler *et al.* 2011). Explaining this high floral diversity has been of great interest to evolutionary ecologists, who attribute the high diversity to both abiotic and biotic factors (Johnson 1996; van der Niet and Johnson 2009; Schnitzler *et al.* 2011). Within this diversity is a vast collection of angiosperms that display a wide range of floral morphologies. The presence of this floral diversity, along with studies of ecological shifts between sister taxa, point towards diversity of the Cape Floristic region as being strongly influenced by adaptation to pollinators (Johnson 2006; van der Niet and Johnson 2009).

Stebbins introduced the Most Effective Pollinator Principle (Stebbins 1970), which hypothesizes angiosperms will evolve to specialize on the most frequent, effective pollinator in the region where the population is evolving (Stebbins 1970). However, in many instances generalized pollination represent a better strategy for reproduction (Aigner 2001). Although opinions on whether specialization or generalization is more common remains contentious (Waser *et al.* 1996; Vazquez and Aizen 2003) what can be agreed upon is that there is a continuous spectrum of adaptation between the two extremes (Johnson and Steiner 2000). Plant species in angiosperm rich communities often utilize the same pollinator. When this is the case, pollination acts as a niche trait such that co-occurring species that share a primary pollinator exhibit pollinator partitioning. If pollination modes are not unique, competition should lead to the displacement of one of the species from the community. In this way, pollinators can be thought of both as floral architects as well as community engineers.

Throughout this thesis I explore how pollinators drive floral diversification, speciation, and maintain communities. I primarily focus on the southern African endemic genus

*Leucospermum*, one of 16 Proteaceae genera occurring in South Africa and one which exhibits astounding floral diversity amongst its 52 described taxa (see accompanying Appendix 1 on CD). The high floral diversity within *Leucospermum* suggests an important role of pollinators in the evolutionary process, making this an ideal genus to study plant-pollinator interactions. Most species are restricted to the Cape Floristic Region and taxa occur in a variety of habitats ranging from grassland to mountain fynbos and sandveld.

Plant-pollinator relationships are valuable to the field of evolutionary ecology as a whole and have become a common medium for studying the evolutionary process because these relationships provide examples of pollinator mediated selection on plant traits as well as plant mediated selection on pollinator traits. In addition, pollination biology provides us with an ideal system for studying ecological speciation because pollinator shifts can simultaneously bring about phenotypic divergence and reproductive isolation (Servedio *et al.* 2011; Pauw 2013). The merging of pollination biology with evolutionary theory dates back to Darwin (1859) and has stood the test of time as it remains a dynamic, well represented field (Johnson and Steiner 2000) with still much to be revealed.

## **Chapter background and objectives**

### **Chapter 2**

Evolutionary change in floral morphology is often due to selection exerted by pollinators (Grant and Grant 1965; Stebbins 1970; Johnson; Hapeman and Inoue 1997; Johnson *et al.* 1998; Pérez *et al.* 2006; Harder and Johnson 2009) with the early stages of pollinator driven speciation evident in the form of ecotypes. Population ecotypes are drawn along different evolutionary

paths and once divergence reaches the level of reproductive isolation it can be said that distinct species have formed. Here we focus on recently diverged taxa to gain insight into how pollinators can generate diversity. We explore whether two morphologically distinct varieties of *Leucospermum tottum* might have originated by pollinator mediated adaptive divergence by testing if floral traits between the varieties vary in accordance with their different pollination modes.

### Chapter 3

Environmental pressures often drive plants to specialization on unique pollinators such as bats (Muchhala 2003), rodents (Johnson *et al.* 2001; Wester 2011), marsupials (Steiner 1981) and primates (Nilsson *et al.* 1993), with unusual floral features that arise as a result of adaptation. While studies of pollinators selecting on floral traits such as nectar tube length (Schemske and Horvitz 1989; Hodges 1997; Johnson 1997; Johnson and Steiner 1997; Anderson *et al.* 2010) and flower colour (Bradshaw and Schemske 2003; Newman *et al.* 2012) are common, novel pollination modes provide the opportunity to study the role of pollinators in selecting for less ubiquitous floral features, thereby broadening our understanding of plant-pollinator relationships. Preliminary evidence suggested that *Leucospermum arenarium*, a critically endangered sandveld endemic, is pollinated by rodents. In this study we seek to affirm the pollination mode of *L. arenarium* as well as explain its novel mechanism for nectar secretion, to test if geoflory is an adaptation for rodent pollinators, and, for the first time, quantify the consequences of pollinator grooming on the rate of pollen loss.

## Chapter 4

As we have demonstrated in the preceding chapters, pollinators can drive speciation and select for astounding morphological features in *Leucospermum*. However, there are still many questions that remain regarding the influence of pollinators on *Leucospermum* diversification. How frequently can pollinator shifts explain diversification? Which morphological traits show repeated convergence to pollinators? What traits distinguish pollination modes? What pollination modes and morphological features promote autonomous selfing? These questions can be answered through phylogenetic studies of correlated evolution (Armbruster 1996, 2002; Pérez *et al.* 2006; Smith *et al.* 2007; Martén-Rodríguez *et al.* 2010; Rosas-Guerrero *et al.* 2010; Waterman *et al.* 2011; Sakai *et al.* 2013), which have been vital to our understanding of the role of pollinators in the diversification of angiosperms. In this chapter we construct a complete genus level phylogeny and test for correlated evolution between floral traits, pollination mode, and autonomous selfing.

## Chapter 5

Along with being drivers of diversification, pollinators can also act to shape floral communities. The Cape Floristic Region of Southern Africa is characterized by vast floral diversity (Goldblatt and Manning 2002) coupled with low pollinator abundance. This discrepancy leaves many angiosperms vulnerable to the consequences of pollinator sharing, such as competition for pollinator visits and competition through interspecific pollen transfer. Although we would expect the displacement of the less competitive species from a community, mutualisms (Chesson 2000; Bever 2002; Lee and Inouye 2010; Johnson and Amarasekare 2013) and density dependent interactions (Gause and Witt 1935; Feinsinger *et al.* 1991; Bruno *et al.*

2003) can provide an explanation for species coexistence. In this study we address competition for pollinator and nectar thief visits and interspecific pollen transfer to see how two ecologically equivalent Proteaceae species, *Leucospermum conocarpodendron* and *Mimetes fimbriifolius*, can specialize on the same pollinator yet still co-flower.

# Chapter 2

Floral divergence in closely related *Leucospermum*  
*tottum* (Proteaceae) varieties pollinated by birds and  
long-proboscid flies

Christopher Michael Johnson, Tianhua He and Anton Pauw

This chapter has been published in *Evolutionary Ecology*

## Abstract

The Proteaceae are renowned for their floral diversity, but surprisingly the role of pollinators in driving evolutionary divergence in this family has been underexplored. Here we focus on recently diverged taxa to gain insight into the processes that generate diversity by testing whether two varieties of *Leucospermum tottum* might have originated by pollinator mediated adaptive divergence. *L. tottum* var. *tottum* has pale salmon-coloured horizontally-oriented flowers, long nectar tubes, and small volumes of concentrated nectar. *L. tottum* var. *glabrum* has red and yellow vertically-oriented flowers, short nectar tubes, and large volumes of dilute nectar. Despite the morphological divergence, the varieties are indistinguishable using eight molecular markers, indicating a very early stage of differentiation. Consistent with their morphologies, *L. tottum* var. *tottum* is pollinated by long-proboscid flies (*Philoliche rostrata* and *Philoliche gulosa*), Cape sugarbirds (*Promerops cafer*), and, to a lesser extent, by Orange-breasted sunbirds (*Anthobaphes violacea*), whereas, *L. tottum* var. *glabrum* is pollinated only by Orange-breasted sunbirds. *A. violacea* visits both varieties, but makes more frequent contact with pollen presenters when foraging on *L. tottum* var. *glabrum*. The exclusion of birds caused a steeper reduction in seed production in *L. tottum* var. *glabrum* than in *L. tottum* var. *tottum*, consistent with specialization for bird-pollination in this variety. Additionally, *L. tottum* var. *glabrum* exhibits autogamy, whereas *L. tottum* var. *tottum* does not. Floral divergence between the two *L. tottum* varieties corresponds with divergence in pollinator use.

## Introduction

Since Darwin (1862), it has been recognized that angiosperms and their pollinators both possess morphological traits that mechanically fit one another. From a plant's perspective, these traits exist primarily to improve the accuracy and precision of pollen deposition and uptake (Armbruster et al. 2004). When pollinator availability is reliable, angiosperms should adapt to the most abundant and efficient pollinator available (Johnson and Steiner 2000). However, angiosperms with large ranges are likely to encounter high spatial variation in the pollinator fauna, which can lead to divergence in floral traits (Galen 1989; Robertson and Wyatt 1990) and ultimately speciation (Johnson *et al.* 1998; Beardsley *et al.* 2003; Pérez *et al.* 2006; Rymer *et al.* 2010; Waterman *et al.* 2011).

Pollinator driven divergence is thought to be a potent mechanism of ecological speciation because adaptation to different pollinators can simultaneously bring about phenotypic divergence and reproductive isolation (Servedio *et al.* 2011; Pauw 2013). Divergent selection by pollinators may act on a variety of floral traits, including flower colour (Bradshaw and Schemske 2003; Newman *et al.* 2012), nectar tube length (Schemske and Horvitz 1989; Hodges 1997; Johnson 1997; Johnson and Steiner 1997; Whittall and Hodges 2007; Anderson *et al.* 2010), perianth traits (Pérez-Barrales *et al.* 2007) and flowering phenology (Olsson and Ågren 2002). However, selection by pollinators can not only bring about trait divergence, but also convergence leading to “syndromes” of floral traits that are shared amongst plant species that utilize similar pollinators (Baker 1959; Faegri and van der Pijl 1970). A well-known example of this is that bird-pollinated flowers are often reddish in colour and produce large volumes of dilute nectar (Faegri and van der Pijl 1970). In contrast, the 20 plant species from the Cape Floral Region that are pollinated by long-proboscid flies (*Moegistorhynchus longirostris*, *Philoliche rostrata*, and



*P. gulosa*), have white or pale salmon-coloured flowers, extremely long, narrow nectar tubes and smaller volumes of more concentrated nectar (Manning and Goldblatt 1997).

Although the pollination syndrome concept is widely used in pollination biology there has been much debate about the link between pollinators and floral traits (Ollerton 1996, 1998). Some angiosperms possess what appear to be specialized floral structures adapted for a particular pollinator species, but attract additional pollinators that facilitate pollination without a fitness trade-off (Macior 1986; Sahley 1996; Aigner 2004; Devoto *et al.* 2006; Muchhala *et al.* 2008; Chalcoff *et al.* 2012). This often occurs when multiple pollinators select for the same floral traits, leading to specialization for a functional group of pollinators such as those with long-proboscises (Waser 1998; Fenster *et al.* 2004).

Although pollinator driven floral divergence is thought to have been particularly important in the Cape Floral Region of South Africa (Johnson 2010), virtually all studies of the phenomenon are restricted to monocotyledons (Johnson 1997). Here we focus on the Proteaceae, which are eudicotyledons that dominate most of the Fynbos vegetation in this region. Composed of roughly 360 South African species, the family exhibits spectacular phenotypic variation, which includes adaptations for bird (Hargreaves *et al.* 2004), rodent (Weins and Rourke 1978; Fleming and Nicolson 2002; Biccard and Midgley 2009; Johnson and Pauw 2014), insect (Steenhuisen and Johnson 2011; Johnson *et al.* 2012; Steenhuisen *et al.* 2012) and wind pollination (Friedman and Barrett 2008).

Our study centres on the two varieties of *Leucospermum tottum* (Proteaceae) that occur allopatrically on sandstone slopes in the Cape Fold Mountains. The typical variety, *L. tottum* var. *tottum* (L.) R. Br., is widespread in the mountains of the South-Western Cape of South Africa, while *L. tottum* var. *glabrum* E. Phillips occurs in a single population of approximately 50-75

plants in the Hex River Mountains. *L. tottum* var. *glabrum* had been suggested to be a natural hybrid between *L. tottum* var. *tottum* and *Leucospermum vestitum* (Rourke 1971), but this was precluded by the discovery of a relatively large natural population. Up until now no attempt at phylogenetic analysis had been conducted to test the hybridization hypothesis.

A pilot study suggested that both long-proboscid flies (*Philoliche spp.*) and birds interact with *L. tottum* var. *tottum* in the field, whereas *L. tottum* var. *glabrum* only utilizes bird pollinators (Manning 2004). However, the pollination biology of neither variety has been studied systematically. *L. tottum* var. *tottum* inflorescences are comprised of horizontal, widely spread, pale salmon coloured flowers with maroon tips and possess long nectar tubes (Fig 2.1a). The flowers of *L. tottum* var. *glabrum* are bright yellow with red tips and are curved and erect, forming a cage around the terminal end of the inflorescence and possess short nectar tubes (Fig 2.1b). The horizontal, spreading styles of *L. tottum* var. *tottum* appear to accommodate hovering pollinators; whereas the vertical, curved styles of *L. tottum* var. *glabrum* would fit perching pollinators. These morphological variations suggest pollinator driven differential adaptation to long-proboscid flies versus birds in the two varieties. Evidence for such an evolutionary shift would be intriguing, because the role of pollinators in driving floral divergence in this highly diverse genus has not previously been investigated. Additionally, long-proboscid fly pollination has been studied in great detail in South Africa (Goldblatt and Manning 1995; Manning and Goldblatt 1996, 1997; Johnson and Steiner 1997; Combs and Pauw 2009; Pauw *et al.* 2009; Zang *et al.* 2013), but its role in the Proteaceae has been overlooked until recently (Manning 2004; Johnson *et al.* 2012).

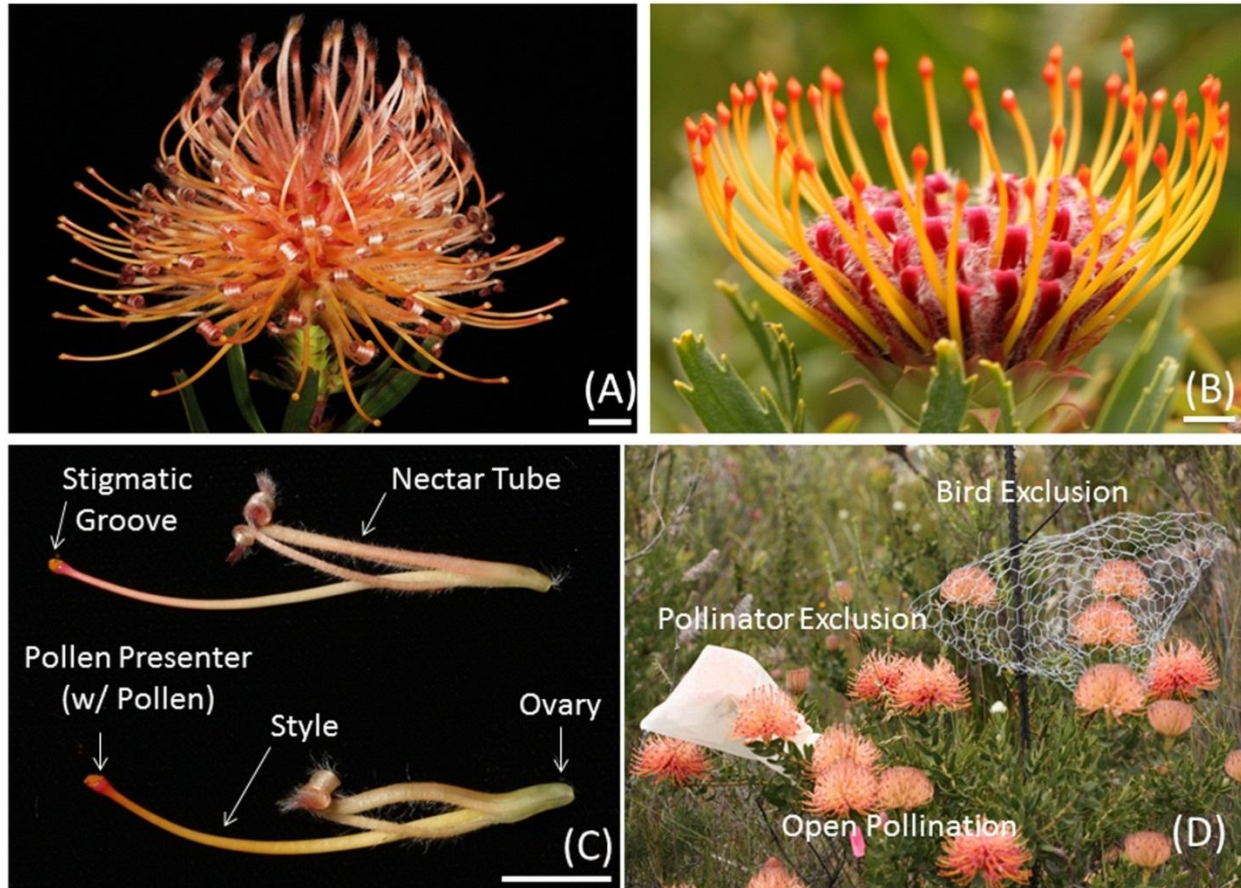


Figure 2.1. *L. tottum* varieties in the field. a) *L. tottum* var. *tottum* inflorescence. b) *L. tottum* var. *glabrum* inflorescence. c) Morphology of a *L. tottum* var. *tottum* flower. d) Three treatments applied to a *L. tottum* var. *tottum* plant. Scale bars = 1 cm.

The incidence and efficiency of autonomous self-pollination within *Leucospermum* differs at the species level (Lamont 1985), but has not been tested in either *L. tottum* variety. Divergence in mating system can be driven by adaptation to a variable pollination environment, and although these adaptations (such as the loss of self-incompatibility) may be cryptic, they have profound ecological consequences. In particular, small or isolated plant populations that experience a low pollinator visitation rate have been shown to resort to an increased reliance on selfing for reproductive assurance (Baker 1955; Kalisz *et al.* 2004; Morgan and Wilson 2005; Moeller 2006). Differences in pollinator composition and density between the two *L. tottum*

varieties may have led to a divergence in mating systems, with the localized variety exhibiting increased autogamy.

We hypothesize that pollinator shifts have driven the morphological divergence between the two varieties of *L. tottum*. To test this hypothesis we evaluate the following predictions: 1) varieties will be each other's closest relatives; 2) the pollinator fauna will differ between the varieties such that long-proboscid flies are frequent visitors of *L. tottum* var. *tottum* and birds are frequent visitors of *L. tottum* var. *glabrum*; 3) morphological variation between varieties will match the morphology of pollinators; 4) the exclusion of birds will have a stronger effect on *L. tottum* var. *glabrum* than on *L. tottum* var. *tottum*, which appears adapted for insect pollination; 5) when all pollinators are excluded, autogamy will be higher in the localized *L. tottum* var. *glabrum*.

## Materials and methods

**Study site**- The Proteaceae are a dominant element of fynbos communities and are often associated with South Africa and the Cape Floral Kingdom. In this family the genus *Leucospermum* is noticeable for its long styles protruding outward from inflorescences, rendering the common name "Pincushion Protea." Both varieties of *L. tottum* have dense inflorescences that contain ~50-75 flowers each. The style has a stigmatic groove on its apex and also acts as a pollen presenter. While in the bud, the swollen tip of the style is enclosed in the four perianth segments. The four anthers are attached to the insides of the perianth segments and deposit their pollen in a ring around the tip of the style just below the stigmatic groove. During development the style elongates more than the perianth segments, and is forced to bend until its tip is released through a rupture in the segments. The pollen-loaded style springs erectly outward,

leaving the perianth segments behind to serve as the mature flower's nectar tube (Fig 2.1c). The anthers roll out of the way and do not make contact with pollinators. All species of *Leucospermum* produce only one ovule per flower.

*L. tottum* var. *tottum* was studied at Dasklip Pass (S 19°02'36.2", E 32°54'15.4"), du Toit's Kloof Pass (S 19°04'08.8", E 33°42'46.8") and Bain's Kloof Pass (S 19°06'04.8", E 33°38'07.5"). *L. tottum* var. *glabrum* was studied in the Hex River Mountains, its only known population (S 19°19'42.82", E 33°32'21.77"). All sites contained natural mountain fynbos void of major agricultural or other human impacts and are likely to contain the full range of plants and pollinators historically available. At the end of this study we discovered individuals of what would be morphologically categorized as *L. tottum* var. *tottum* (N=7) approximately 100 meters from the *L. tottum* var. *glabrum* population. Because these individuals were discovered at the tail end of the flowering season they are not included in the observational portion of our study, but were included in the molecular analysis. This marked the first time that both varieties were recorded in the same locality. Voucher specimens are stored at the Stellenbosch University Herbarium.

***Analysis of genetic differentiation between varieties-*** *L. tottum* samples were taken from Dasklip Pass (*L. tottum* var. *tottum* occurring only), du Toit's Kloof Pass (*L. tottum* var. *tottum* occurring only), Bain's Kloof Pass (*L. tottum* var. *tottum* occurring only) and the Hex River Mountains (*L. tottum* var. *tottum* and *L. tottum* var. *glabrum* co-occurring). Sampled plants were at least 5 meters apart, except for at Bain's Kloof Pass and the Hex River Mountains, which only contained a few individual plants. Six other species of *Leucospermum* and one species of each *Diastella* and *Mimetes* (Proteaceae) were also collected. Leaf tissue was harvested and stored in activated silica gel before further processing. Eight DNA sequences from the nuclear ribosomal

internal transcribed spacers (ITS) and plastid *matK*, *rbcL*, *trnL* intron, and *trnL-trnF* intergenic spacer, *atpB*, *atpB-rbcL* intergenic spacer, and *rpl16* intron, were produced following standard protocols (Mast *et al.* 2005; Barker *et al.* 2007; Sauquet *et al.* 2009). All sequences were aligned using ClustalX (Larkin *et al.* 2007) and ambiguous regions were removed before combining all datasets into a NEXUS matrix of 80 taxa and 7,111 characters.

Phylogenetic relationship among the collected samples was explored using Bayesian inference in BEAST v. 1.7.2 (Drummond *et al.* 2012). Bayesian inference used a coalescent tree prior that assumes a constant (unknown) population size back through time, as this tree prior is most suitable for trees describing the relationships between individuals in the population/species (Drummond *et al.* 2012). We searched for divergence time by applying a normal prior distribution and the following secondary calibration points extracted from (Sauquet *et al.* 2009) with the crown minimum age of common ancestor of *Leucospermum*, *Diastella* and *Mimetes* at 12.3 million years. Five Monte Carlo Markov chains (MCMC) of 10 million generations each were run, with parameters sampled every 2,000 generations. A maximum clade credibility tree was then generated. For Bayesian analyses, posterior probabilities  $P > 0.98$  were considered good support. Final trees were viewed and edited in FigTree v. 1.3.1 (<http://tree.bio.ed.ac.uk>). Voucher information and GenBank accession numbers are provided as Supplementary Information (Table S2.1).

Genetic differentiation between varieties was measured by calculating  $F_{ST}$  based genetic distances using *Arlequin* ver, 3.5 (Excoffier and Lischer 2010). In addition, an exact test of population differentiation based on haplotype frequencies under the hypothesis of panmixia was implemented in *Arlequin*. Exact test of population differentiation extends Fisher's exact test on a 2x2 contingency table to a contingency table, with hypothesis of a random distribution of  $k$



different haplotypes among  $r$  populations (Raymond and Rousset 1995). All potential states of the contingency table are explored with a Markov chain (Excoffier and Lischer 2010). During random walk between the states of the Markov chain, the probability of observing a table less or equally likely than the observed sample configuration under the null hypothesis of panmixia was estimated. The exact differentiation test for all populations defined in the project was performed by constructing a table listing populations (rows) against haplotypes (columns), in which 100,000 Markov steps were run. Significance was taken at  $p < 0.05$ .

***Floral measurements***- We measured floral traits at Dasklip Pass, du Toit's Kloof Pass and Bain's Kloof Pass for *L. tottum* var. *tottum* and in the Hex River Mountains for *L. tottum* var. *glabrum* in order to determine if morphology differs among locations, choosing traits that could influence interactions with pollinators. Traits included nectar volume and concentration, which was measured with a hand-held refractometer from newly opened flowers in the lab (ECLIPSE hand-held refractometer, Bellingham & Stanley, Basingstoke, United Kingdom), style and nectar tube length, flower orientation, as well as the number of flowers per inflorescence. Style length was measured as the straight-line distance from the proximal end of the ovary to the stigmatic groove. Flower orientation was determined using a protractor, which was placed parallel with the stem and centred on the middle of the inflorescence. A flower that pointed directly downward in line with the stem would be  $0^\circ$  and one pointing upward in line with the stem would be  $180^\circ$ . For each inflorescence the minimum and maximum flower orientation was recorded. Data were analysed with one-way ANOVAs followed by a Tukey's Post-hoc test in R (R Development Core Team 2009).

***Pollinator observations***- To test whether the varieties differ in their pollinator fauna, pollinator observations were conducted at the three populations of *L. tottum* var. *tottum* and the

single population of *L. tottum* var. *glabrum*. Three separate days of observations (October-December 2011) were conducted at each site from the early morning until the afternoon to coincide with peak pollinator activity. Each observation was conducted continuously by a single observer and took place on warm, clear days with little wind in order to standardize weather conditions. Sites were visited alternately on different days. Nocturnal observations were not conducted. During each observation we recorded the visiting species, number of inflorescences probed, visitor orientation and whether or not pollen presenter contact was observed. We attempted to record all visitors present during each observation. Since pollen presenters also contain the stigmatic groove, contact with the pollen presenter can result in pollen deposition or uptake. Thus, pollinator effectiveness was measured indirectly as the proportion of visits in which pollen presenter contact was observed. Stands were described by the number of inflorescences of the *L. tottum* variety flowering. This was done by counting a portion of inflorescences open on a few individual plants and from that estimating how many could be viewed. Visitation rate was calculated as visits per inflorescence per hour. Insect visitors were netted and proboscis and extended proboscis length were measured. Flies extend their proboscises while feeding, so the extended length is the functional length. In cases where the extended proboscis was not directly measured, it was calculated using the following derived calibration curve:  $y = 1.06896(x) + 3.73266$  ( $N=10$ ,  $R^2=0.8595$ ,  $p > 0.001$ ) where “x” represents non-extended proboscis length and “y” the extended length.

***Differential pollinator exclusion experiments***- In order to assess the relative contribution of different classes of pollinators and autogamy to seed production in the two varieties we conducted differential pollinator exclusion experiments. Our study was conducted from September-December 2011 at Dasklip Pass (*L. tottum* var. *tottum*) and in the western Hex River



Mountains (*L. tottum* var. *glabrum*). Three treatments were applied to individual plants (Fig 2.1d).

- 1) Pollinator exclusion: inflorescences covered with fine gauze bags to test for autogamy.
- 2) Bird exclusion: chicken wire cage around inflorescence, excluding bird pollinators but not excluding insects such as long-proboscid flies.
- 3) Open pollination: inflorescences tagged, but not manipulated.

*Philoliche* spp. (Diptera) hover while feeding. We therefore constructed a chicken wire cage with hexagonal openings with a maximum diameter of 2.9 cm and a spherical design that allowed sufficient lateral space for long-proboscid flies to manoeuvre in. The cage was then attached to a planted steel rod in order to stay in place.

All three treatments were applied to 30 plants of *L. tottum* var. *tottum* (Dasklip Pass) and 25 plants of *L. tottum* var. *glabrum* (Hex River Mountains). Two inflorescences were lost giving a total of 163 inflorescences. Each treatment was commenced while inflorescences were in the bud stage. Later in the season, when inflorescences began wilting, treatments were bagged as described in Treatment “1” to ensure that no seeds were lost. Inflorescences were collected three months later and the seeds were counted to be used as a measure of female fitness.

To test whether the two varieties responded differently to bird exclusion we constructed a generalized linear mixed model of seed production with variety (*tottum*; *glabrum*), treatment (open; bird excluded), and their interactions as predictors. Plant individual was included as a random factor to account for variation in seed production among individuals. The model was run in R (R Development Core Team 2009). To test whether the two varieties responded differently to complete pollinator exclusion we compared seed production in pollinator-excluded inflorescences using a Mann-Whitney U-test in STATISTICA. This treatment was not included

in the generalized linear mixed model because pollinator-excluded seed production for one of the varieties had zero variance.

## Results

*Analysis of genetic differentiation between varieties* - Phylogenetic re-construction using Bayesian inference demonstrated *L. tottum* var. *tottum* and *L. tottum* var. *glabrum* are indistinguishable using eight gene regions (Fig 2.2). Although the two varieties did not form separate lineages, individuals of the same location more or less clustered with each other. Bayesian analyses has shown strong support (with posterior probabilities greater than 0.98) for the node where species are divergent, while poor or no support (posterior probabilities < 0.75) for the divergence below species level, further suggesting the *Leucospermum tottum* var. *tottum* and *L. tottum* var. *glabrum* are not differentiated phylogenetically. Exact test of population differentiation revealed no significant differentiation among the five populations (Exact  $p > 0.05$ ). Analysis on  $F_{ST}$  suggested that genetic distance between the two co-occurring varieties of *L. tottum* was not significantly different from zero (Table 2.1). Four individuals of *L. vestitum* form a single clade, which is sister to *L. grandiflorum* and *L. gueinzii*, ruling out the possibility that *L. tottum* var. *glabrum* was derived through hybridization between *L. vestitum* and *L. tottum* var. *tottum*.

Table 2.1.  $F_{ST}$  based genetic distance between pairwise populations of the two varieties of *L. tottum*

|   | <i>L. tottum</i> var.<br><i>tottum</i><br>Dasklip Pass | <i>L. tottum</i> var.<br><i>tottum</i><br>du Toit's Kloof<br>Pass | <i>L. tottum</i> var.<br><i>tottum</i><br>Hex River Valley | <i>L. tottum</i> var.<br><i>tottum</i><br>Bain's Kloof<br>Pass |
|---|--|---|--|--|
| <i>L. tottum</i> var.<br><i>tottum</i><br>du Toit's Kloof<br>Pass | 0.228*   |   |  |  |
| <i>L. tottum</i> var.<br><i>tottum</i><br>Hex River Valley        | 0.631*   | 0.195*  |  |  |
| <i>L. tottum</i> var.<br><i>tottum</i><br>Bain's Kloof Pass       | 0.640*   | 0.251*  | 0.568*   |  |
| <i>L. tottum</i> var.<br><i>glabrum</i><br>Hex River valley       | 0.780*   | 0.402*  | -0.002   | 0.728*   |

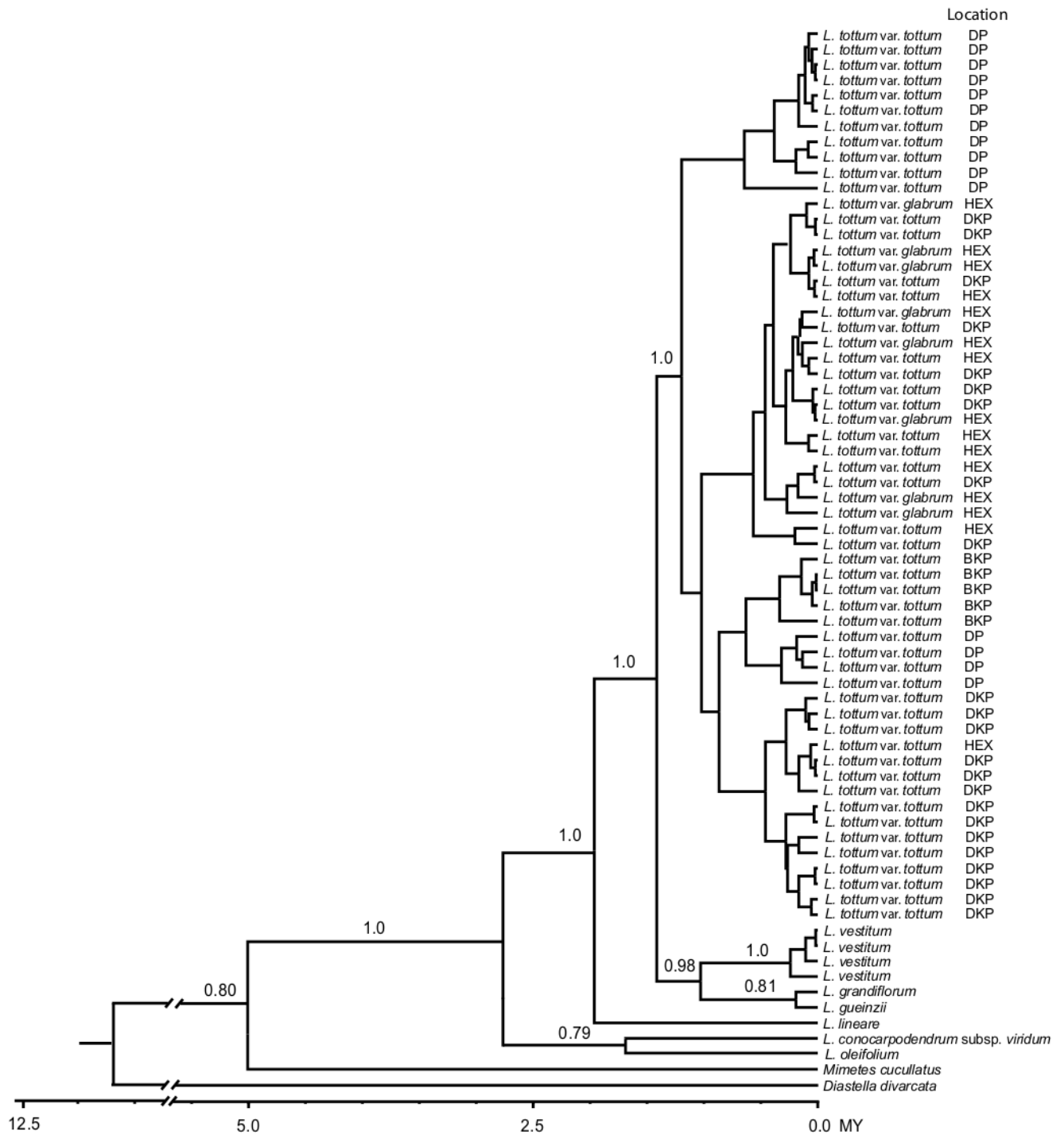


Figure 2.2. Bayesian analyses of phylogenetic relationship between the two varieties of *L. tottum*. Numbers above the lineage indicate posterior probability. Posterior probability < 0.75 was not shown.

**Floral morphology**- The number of flowers per inflorescence was found to be similar among all populations except for at Dasklip Pass ( $F(3,58)=8.838$ ,  $p<0.001$ ), which has significantly more flowers per inflorescence ( $\bar{x}=72.73$ , S.D =16.14, N=15) than all other populations ( $\bar{x}=56.43$ , S.D.= 9.45, N=45). Nectar concentration did not vary significantly among *L. tottum* var. *tottum* populations, but both Dasklip Pass and Bain's Kloof Pass populations have significantly higher nectar concentrations than *L. tottum* var. *glabrum* ( $F(3,39)=4.437$ ,  $p=0.009$ , Fig 2.3a). Nectar volume similarly did not vary significantly among the *L. tottum* var. *tottum* populations, but volume is significantly higher in *L. tottum* var. *glabrum* ( $F(3,39)=12.2$ ,  $p<0.001$ , Fig 2.3b). Style length in *L. tottum* var. *tottum* at Dasklip Pass was found to be greater than at Bain's Kloof Pass and du Toit's Kloof Pass, and all three of these populations possess significantly longer styles than *L. tottum* var. *glabrum* ( $F(3,58)=35.36$ ,  $p<0.001$ , Fig 2.4a). Nectar tube length differed significantly between the northern (Dasklip Pass) and the Southern (du Toit's Kloof Pass) *L. tottum* var. *tottum* populations, and were of intermediate length at the *L. tottum* var. *tottum* population with intermediate locality (Bain's Kloof Pass). *L. tottum* var. *glabrum* had shorter nectar tubes than any of the *L. tottum* var. *tottum* populations ( $F(3,58)=198.7$ ,  $p<0.001$ , Fig 2.4b). There was little variation among *L. tottum* var. *tottum* populations in flower orientation, but flowers of *L. tottum* var. *glabrum* were more vertically oriented with a higher maximum ( $F(3,58)=15.89$ ,  $p<0.001$ , Fig 2.4c) and minimum angular deviation ( $F(3,58)=70.76$ ,  $p<0.001$ , Fig 2.4d). Extended proboscis length differed between the two sites where long-proboscid flies were captured, with *Philoliche rostrata* at Dasklip Pass having significantly longer proboscises than at Bain's Kloof Pass ( $t=4.6035$ ,  $df=8$ ,  $p<0.001$ ,  $N_1=13$ ;  $N_2=9$ ). Dasklip Pass flies had an average extended proboscis lengths of 40.86 mm (S.D.=5.40, N=13) and Bain's Kloof 31.17 mm (S.D.=4.01, N=9) corresponding approximately to the nectar

tube length in their respective populations (Fig 2.4b). The analysis of proboscis length excluded *P. gulosus* because only one specimen was captured in total (at Dasklip Pass).

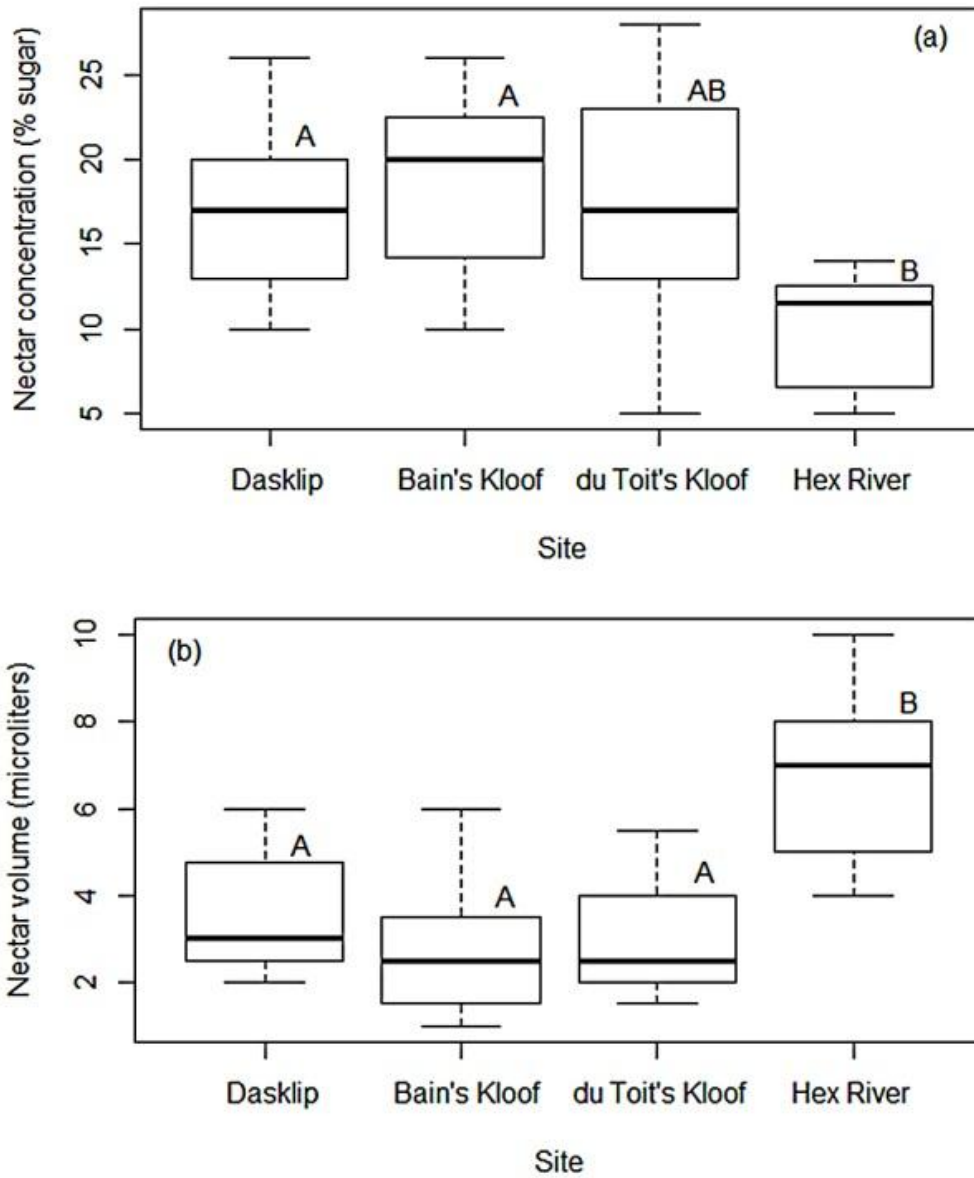


Figure 2.3. Nectar properties among sites. (a) Nectar concentration (% sugar) measurements for each site. (b) Nectar volume (microliters) for each site.

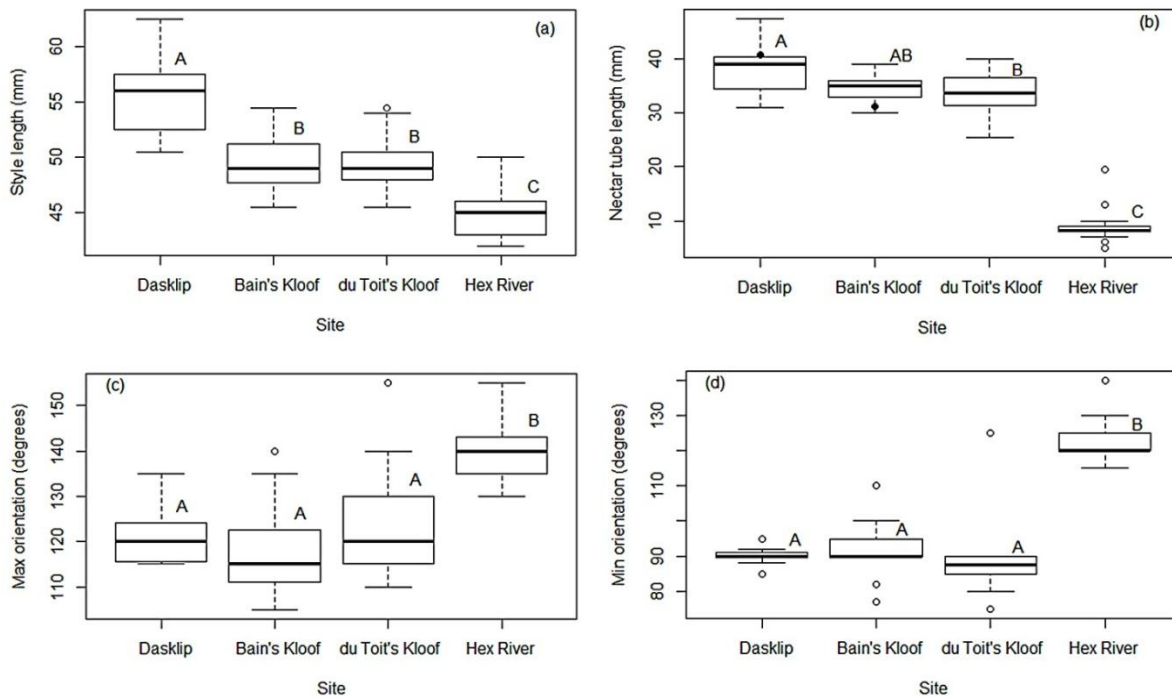


Figure 2.4. Floral measurements among sites. (a) Straight-line style length (mm) for each site. (b) Nectar tube length (mm) for each site. The black circles on “Dasklip” and “Bain’s Kloof” reflect corresponding mean proboscis lengths for flies captured at those sites. (c) Maximum flower orientation for each site. (d) Minimum flower orientation for each site.

**Pollinator observations-** At *L. tottum* var. *tottum* populations we found that Cape sugarbirds (*Promerops cafer*) and long-proboscid fly species (*Philoliche rostrata* and *Philoliche gulosa*) made regular contact with pollen presenters while foraging. Cape sugarbirds perched on top of *L. tottum* var. *tottum* inflorescences while accessing nectar tubes (Fig 2.5c) making pollen presenter contact in 100% (N=813) of observations (Table 2.2; 2.3). Long-proboscid flies were also 100% successful (N=95) in making pollen presenter contact in all instances (Table 2.2; 2.3). Long-proboscid flies foraged on *L. tottum* var. *tottum* nectar while hovering and contact was made with pollen presenters on their ventral thorax (Fig 2.5d). The slender proboscis enters into

the perianth tube via the narrow distal entrance. Orange-breasted sunbirds (*Anthobaphes violacea*), on the other hand, exhibited two foraging orientations of which only one made pollen presenter contact (Table 2.2; Fig 2.5a,b). In instances when an Orange-breasted sunbird forages from underneath a *L. tottum* var. *tottum* inflorescence there is no contact with pollen presenters (Fig 2.5a). Even when sunbirds forage from atop *L. tottum* var. *tottum* inflorescences pollen presenter contact is not assured. For example, pollinator presenter contact was not made in 100% of visits (N=16) at du Toit's Kloof Pass and 28% of visits (N=292) at Dasklip Pass. Since we did not capture and mark birds, our observations do not measure bird density.

*L. tottum* var. *glabrum* differed greatly in pollinator fauna from *L. tottum* var. *tottum*. Both Cape sugarbirds and long-proboscid flies were completely absent from *L. tottum* var. *glabrum* and Orange-breasted sunbirds became the only pollinator available. Additionally, when visiting *L. tottum* var. *glabrum*, Orange-breasted sunbirds foraged from atop inflorescences (Fig 2.5b), displaying the successful pollination orientation and contacting pollen presenters more frequently (76%, N=103) than on *L. tottum* var. *tottum* (29%, N=1060, Table 2.2).

Table 2.2. Instances of pollinator orientations at each site. Birds can visit flowers by perching above inflorescences (where pollen presenter contact can occur) or below inflorescences (where nectar thefts are imminent). Long-proboscid flies hover while feeding and consistently contact pollen presenters. Values are the total number of inflorescence visits observed displaying each possible orientation.

| Site                 | <i>L. tottum</i> variety | Orange-breasted sunbird |       | Cape sugarbird |       | Long proboscid flies |
|----------------------|--------------------------|-------------------------|-------|----------------|-------|----------------------|
|                      |                          | Above                   | Below | Above          | Below | Hover                |
| Hex River Mountains  | <i>glabrum</i>           | 78                      | 25    | 0              | 0     | 0                    |
| Dasklip Pass         | <i>tottum</i>            | 292                     | 290   | 35             | 0     | 35                   |
| du Toit's Kloof Pass | <i>tottum</i>            | 16                      | 462   | 778            | 0     | 0                    |
| Bain's Kloof Pass    | <i>tottum</i>            | 0                       | 0     | 0              | 0     | 86                   |



Table 2.3. Pollinator observation data. For each site and day of observations inflorescence density is given as well as duration of observation (Time), the number of inflorescence visits and frequency of pollen presenter contact (success rate) for each visiting species. Visitation rate (# of successful visits/hour\*# of inflorescences at the site) for each visitor is also given.

| Site            | Variety        | Obs. date  | # of inflorescences | Time (hours) | <i>A.violacea</i> |              | <i>P.cafer</i>  |             | <i>Philoliche spp.</i> |                 |             |              |                 |
|-----------------|----------------|------------|---------------------|--------------|-------------------|--------------|-----------------|-------------|------------------------|-----------------|-------------|--------------|-----------------|
|                 |                |            |                     |              | # of visits       | success rate | visitation rate | # of visits | success rate           | visitation rate | # of visits | success rate | visitation rate |
| Hex River Mnts  | <i>glabrum</i> | 14/11/2011 | 80                  | 4.5          | 54                | 0.6111       | <b>0.0917</b>   | 0           | -                      | <b>0</b>        | 0           | -            | <b>0</b>        |
|                 |                | 6/12/2011  | 45                  | 4            | 4                 | 1            | <b>0.0222</b>   | 0           | -                      | <b>0</b>        | 0           | -            | <b>0</b>        |
|                 |                | 14/12/2011 | 70                  | 5            | 45                | 0.78         | <b>0.1171</b>   | 0           | -                      | <b>0</b>        | 0           | -            | <b>0</b>        |
| Dasklip Pass    | <i>tottum</i>  | 27/10/2011 | 125                 | 7            | 227               | 0.3656       | <b>0.0949</b>   | 27          | 1                      | <b>0.0309</b>   | 6           | 1            | <b>0.0057</b>   |
|                 |                | 18/11/2011 | 135                 | 5            | 277               | 0.3538       | <b>0.1452</b>   | 0           | -                      | <b>0</b>        | 0           | -            | <b>0</b>        |
|                 |                | 20/11/2011 | 152                 | 6            | 78                | 0.3590       | <b>0.0307</b>   | 8           | 1                      | <b>0.0088</b>   | 29          | 1            | <b>0.0318</b>   |
| du Toit's Kloof | <i>tottum</i>  | 30/11/2011 | 4200                | 7            | 302               | 0            | <b>0</b>        | 599         | 1                      | <b>0.0204</b>   | 0           | -            | <b>0</b>        |
|                 |                | 10/12/2011 | 3000                | 7            | 96                | 0            | <b>0</b>        | 142         | 1                      | <b>0.0068</b>   | 0           | -            | <b>0</b>        |
|                 |                | 12/12/2011 | 3200                | 5            | 80                | 0            | <b>0</b>        | 37          | 1                      | <b>0.0023</b>   | 0           | -            | <b>0</b>        |
| Bain's Kloof    | <i>tottum</i>  | 5/12/2011  | 85                  | 5.5          | 0                 | -            | <b>0</b>        | 0           | -                      | <b>0</b>        | 51          | 1            | <b>0.1091</b>   |
|                 |                | 15/12/2011 | 75                  | 5.5          | 0                 | -            | <b>0</b>        | 0           | -                      | <b>0</b>        | 6           | 1            | <b>0.0145</b>   |
|                 |                | 17/12/2011 | 80                  | 5.5          | 0                 | -            | <b>0</b>        | 0           | -                      | <b>0</b>        | 29          | 0            | <b>0.0659</b>   |



Figure 2.5. Visitors to *L. tottum* varieties. (a) Orange-breasted sunbird foraging from underneath *L. tottum* var. *tottum*. Note no pollen presenter contact. (b) Orange-breasted sunbird foraging atop *L. tottum* var. *glabrum*. Pollen presenter contact is possible with this orientation. (c) Cape sugarbird on *L. tottum* var. *tottum*. This orientation is extremely efficient as pollen is deposited on the bird's head. (d) *Philoliche rostrata* on *L. tottum* var. *tottum*. Contact places pollen directly on the fly's ventral thorax.

**Differential pollinator exclusion experiment** - Our manipulative experiments showed that the two *L. tottum* varieties responded differently to the exclusion of birds from their inflorescences as indicated by the significant interaction between “Treatment” and “Variety” (Table 2.4). For *L. tottum* var. *glabrum*, bird exclusion caused a significant reduction in seed

production ( $\bar{x}$ =2.125 seeds/ inflorescence, S.D.=1.56, N=24) compared to open treatments ( $\bar{x}$ =6.80 seeds/ inflorescence, S.D.=3.46, N=25), whereas *L. tottum* var. *tottum* saw no significant change in seed production between bird exclusion treatments ( $\bar{x}$ =3.17 seeds/ inflorescence, S.D.=2.44, N=30) and open treatments ( $\bar{x}$ =3.77 seeds/ inflorescence, S.D.=2.28, N=30, Fig 2.6). Additionally, the varieties differed in their capacity for autogamy when all pollinators were excluded (U=90.000,  $p < 0.0001$ , Mann-Whitney *U*-test). Notably, *L. tottum* var. *tottum* set no seeds via autogamy (S.D.=0, N=30), whereas 19/24 *L. tottum* var. *glabrum* inflorescences set at least one seed with a mean of 1.54 seeds/ inflorescence (S.D.=1.44, N=24, Fig 2.6).

Table 2.4. Significance tests for the effect of bird exclusion on seed set in *Leucospermum tottum* var. *tottum* and *L. tottum* var. *glabrum* by means of a Generalized linear mixed model with plant individual as a random factor.

|  | df | Residual df | F-value   | P-value  |
|--|----|-------------|-----------|----------|
| Intercept                                    | 1  | 53          | 180.68849 | < 0.0001 |
| Variety ( <i>glabrum</i> : <i>tottum</i> )   | 1  | 53          | 3.88412   | 0.054    |
| Treatment (Open pollination: Bird Exclusion) | 1  | 52          | 36.46022  | <0.0001  |
| Variety: Treatment                           | 1  | 52          | 25.71536  | <0.0001  |

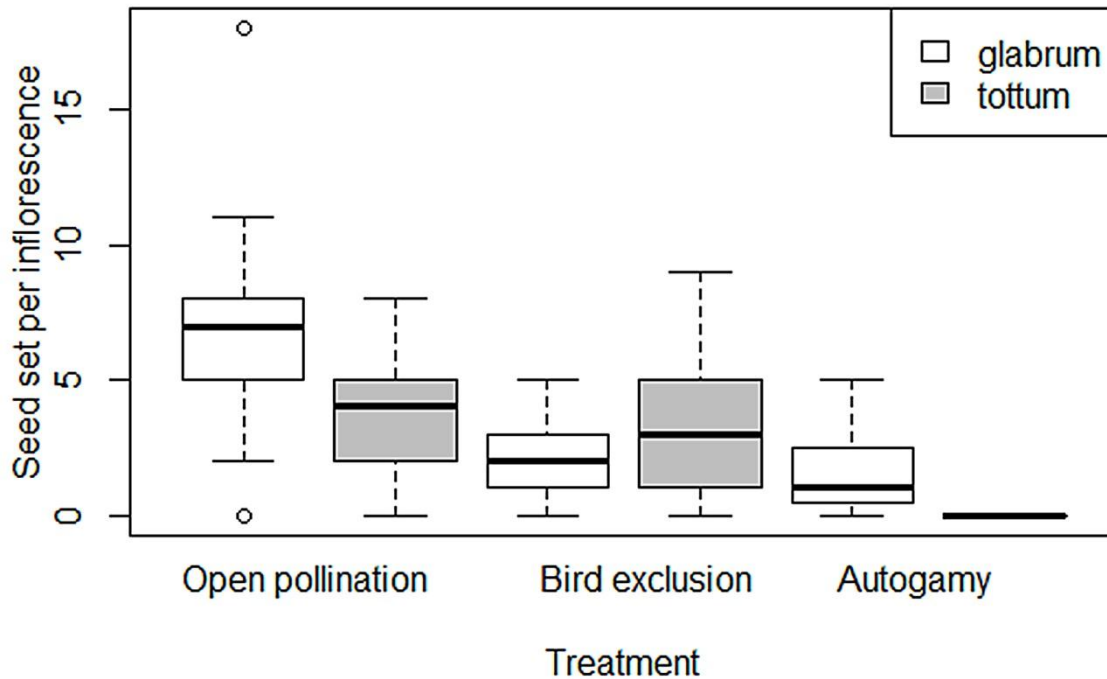


Figure 2.6. Pollinator exclusion treatments for *L. tottum* var. *tottum* and *L. tottum* var. *glabrum*. For *L. tottum* var. *tottum*, both open pollination and bird exclusion treatments (insects included) yielded similar seed set, providing evidence that long-proboscid flies alone can facilitate pollination. There is no autogamy in this variety. For *L. tottum* var. *glabrum* there is a strong reduction in seed set when birds are excluded as pollinators, providing evidence that their role in pollination is greater than in the *L. tottum* var. *tottum*. Seed set in bird exclusion treatments may be explained by autogamy, as the bagged treatments (all pollinators excluded) set comparable quantities of seed.

## Discussion

***Analyses of genetic differentiation between varieties*** – The results support the conclusion that the two *L. tottum* varieties are not independent species. In the Bayesian phylogenetic reconstruction, the two varieties did not form separate lineages, which is likely the result of insufficient resolution of the used DNA sequences, evident by the low support for many

internal nodes. We expect that a high polymorphic DNA marker (such as microsatellite DNA) or more DNA sequences than currently used would allow separating the two varieties. Although hybridization between the two varieties (which were found in the same area) could mix the genetic information, and therefore lead to the results of a lack of molecular divergence, hybridization would also have led to the disappearance of morphological divergence, which is marked. We also suggest that *L. tottum* var. *glabrum* did not originate as a hybrid between *L. tottum* var. *tottum* and *L. vestitum*, since the two varieties of *L. tottum* var. *tottum* together form a monophyletic clade whereas *L. vestitum* resides in a distinct clade alongside *L. grandiflorum* and *L. gueinzii* (Fig 2.2). The monophyly of *L. tottum* varieties is consistent with the classification of pollination ecotypes (Turesson 1922; Van der Niet *et al.* 2014).

### ***Pollinator mediated divergence***

*Visitors to L. tottum* var. *tottum*- Our results indicate that the floral traits of *L. tottum* varieties match their different pollinator fauna. In the case of *L. tottum* var. *tottum*, floral morphology seems to reflect a good “fit” with long-proboscid flies (Fig 2.5d). Flowers of *L. tottum* var. *tottum* are horizontally presented to accommodate hovering pollinators (Fig 2.3c,d) and the high nectar concentration and low nectar volume is suitable for insects (Fig 2.2). Most noticeably, the long, straight nectar tubes, which are unique in the genus, match the length of the proboscises of tabanid flies. There is even preliminary evidence for population level trait matching between mean fly proboscis and mean floral tube length (Fig 2.4b), a pattern that has previously been detected in long-proboscid fly pollination systems (Pauw *et al.* 2009). A possibility worth considering is that fly proboscis length does not vary across sites, but within sites, and that the apparent match between floral tube length and fly proboscis length results because, at each site, *L. tottum* var. *tottum* filters out only those flies from the variable population

that match the local floral tube length. There are two reasons why this seems unlikely. Firstly, our sample of fly proboscis length is not derived only from flies captured on *L. tottum* var. *tottum*, but includes individuals captured on other nectar producing plant species in the communities and thus is likely to represent the range of variability in fly population in the area. Secondly, *L. tottum* var. *tottum* will not filter out individuals with proboscises that are longer than the floral tube because these will still be able to access the nectar. Thus, the lack of very long proboscid individuals at Bain's Kloof is likely to result from a true shift in the population mean, rather than a filtering effect.

Despite these recognizable adaptations for pollination by long-proboscid flies, Cape sugarbirds are also seen successfully pollinating *L. tottum* var. *tottum* and were the only pollinators observed at the du Toit's Kloof population (Table 2.3). Since Cape sugarbirds have been seen visiting *L. tottum* var. *tottum* populations at the du Toit's Kloof population in the past (Manning 2004) this occurrence is unlikely the result of a single good season for Cape sugarbirds and they are instead consistent visitors from season to season. The morphology of the nectar tube, which is slit along its entire length, allows access to pollinators with broad mouth parts, including the Cape sugarbird whose bill would otherwise be too wide to fit inside the floral tube (Fig 2.2c). The slit allows the tube to expand and accommodate these large bills without being damaged and to rebound to keep the structure needed in guiding long-proboscid flies. However, long nectar tubes and flowers seem to come with a trade-off, as Orange-breasted sunbirds, which possess short, hooked beaks, are not well matched with the morphology of *L. tottum* var. *tottum*, despite being frequent visitors at some sites (Table 2.3). As a result, Orange-breasted sunbirds exhibit nectar thievery more often than actually facilitating pollination (Fig 2.5a; Table 2.2).



Several floral traits of *L. tottum* var. *tottum*, in particular, flower colour, flower orientation, nectar tube length, nectar volume, and nectar concentration conform to the long-proboscid fly pollination syndrome of the region (Manning and Goldblatt 1997; Goldblatt and Manning 2000) and suggests an important role for long-proboscid flies as agents of selection (Fig 2.3;2.4;2.5). Additionally, experimental exclusion of birds, but not insects, showed that long-proboscid flies alone can facilitate pollination (Fig 2.6; Table 2.4). However, since the pollinator exclusion experiments were conducted at one site, extrapolation of the results to the entire variety should be done with caution.

Although it appears that long-proboscid flies are selecting for the floral morphology of *L. tottum* var. *tottum* we see Cape sugarbirds visiting this variety at high frequencies (Table 2.3) and with a high probability of pollen presenter contact (Table 2.2). One explanation for this would be that *L. tottum* var. *tottum* is exhibiting an intermediate stage of double function in a shift from one pollination type to another (Stebbins 1970; Steiner 1998). Alternatively, a phenotypically specialized flower may appear ecologically generalized if multiple pollinators select for the same functional traits (Macior 1986; Gómez and Zamora 1999; Fenster and Martén-Rodríguez 2007; Ollerton *et al.* 2007). In this case, long-proboscid flies and Cape sugarbirds, which both possess long mouthparts, may be selecting for plants with long flowers and nectar tubes. If true, *L. tottum* var. *tottum* is unlikely to show an adaptive response to the observed spatial fluctuations in the relative abundance of Cape sugarbirds and long-proboscid flies among the three populations of *L. tottum* var. *tottum* (Herrera 1988; Fenster *et al.* 2004; Table 2.3). This theory seems unlikely since other Cape sugarbird pollinated *Leucospermum* species such as *L. conocarpodendron* and *L. lineare* have short nectar tubes (pers. obs.). We suggest that long-proboscid flies strongly influenced floral traits, but these adaptations that

accommodate long-proboscid flies do not deleteriously affect Cape sugarbird foraging behaviour.

Observations of pollinator visits (Table 2.3) and behaviour (Table 2.2) provide strong evidence that both long-proboscid flies and Cape sugarbirds can effectively pollinate *L. tottum* var. *tottum*. However, in the present study we were unable to directly measure the pollen transfer efficiency of these two pollinator types. Therefore, it is unknown if there are fitness trade-offs when utilizing multiple pollinators. Such trade-offs have been detected in *Dudleya greenei* in southern California (Aigner 2004), *Aphelandra acanthus* in the Cloud Forest of Ecuador (Muchhala *et al.* 2008) and *Embothrium coccineum*, a hummingbird and long-proboscid fly pollinated South American Proteaceae (Devoto *et al.* 2006; Chalcoff *et al.* 2012). However, birds have been seen to pollinate insect-syndrome flowers almost as effectively as insects (Castellanos *et al.* 2003).

*Visitors to L. tottum* var. *glabrum*- The morphology of *L. tottum* var. *tottum* and the way in which it influences Orange-breasted sunbird behaviour provides insight into what morphological modifications could optimize Orange-breasted sunbird pollination in *L. tottum* var. *glabrum*. The long flowers and nectar tubes, which are horizontally presented in *L. tottum* var. *tottum*, result in Orange-breasted sunbirds frequently foraging from underneath inflorescences (Fig 2.5a; Table 2.2) causing a high incidence of nectar thefts. Favourable modifications that improve Orange-breasted sunbird effectiveness are clearly displayed in *L. tottum* var. *glabrum*, where flowers and nectar tubes are reduced in length and styles are curved to match the shorter, curved bills of Orange-breasted sunbirds. The low flower density and vertical flower orientation in *L. tottum* var. *glabrum* produces a morphology that force Orange-breasted sunbirds to perch on top of inflorescences, feed downwards and contact pollen



presenters (Fig 2.5b; Table 2.2). Flower orientation has previously been shown to influence pollinator behaviour in other plant species (Ushimaru and Hyodo 2005; Ushimaru *et al.* 2009) and its importance has specifically been addressed with nectariferous birds in the Cape Floral Kingdom (Geerts and Pauw 2009). Although we did not measure colour differences objectively, there is a noticeable colour difference between the varieties, with *L. tottum* var. *glabrum* possessing a red-orange colour that conforms to the bird pollination syndrome (Faegri and van der Pijl 1970). Nectar volume and concentration in *L. tottum* var. *glabrum* are also typical of bird-pollinated plants in general (Fig 2.3).

Experimental data confirms the importance of birds as *L. tottum* var. *glabrum* pollinators. Inflorescences experienced a drastic reduction in seed production when birds, but not insects, were excluded (Fig 2.6; Table 2.4). Additional indirect evidence for reliance on a single pollinator species is the finding that *L. tottum* var. *glabrum* can set seed in the absence of pollinators. This is consistent with the theory that plants with specialized pollinators have compensatory mechanisms that offer reproductive assurance when pollinators are scarce or absent (Bond 1994; Fenster and Martén-Rodríguez 2007; Pérez *et al.* 2009). However, inbreeding depression would have to be measured in order to conclude if autogamy is indeed offering reproductive assurance.

At the *L. tottum* var. *glabrum* population Orange-breasted sunbirds were the only pollinators seen in three days of observations (Table 2.3), and long-proboscid flies and Cape sugarbirds were not observed in the general area. However, the pollinator fauna was not systematically censused away from the study species, so pollinator fauna data remains dependent on site differences as well as morphological differences between the varieties. Reciprocal transplant experiments can potentially resolve this, but were precluded by the extreme rarity of *L.*

*tottum* var. *glabrum* coupled with the large number of inflorescences that would have been needed in order to entice bird visits. This shortcoming is to some extent addressed by the observation that Orange-breasted sunbirds, which visit both varieties, are effective pollinators of *L. tottum* var. *glabrum*, but not of *L. tottum* var. *tottum*. Despite these and other shortcomings, such as the lack of replicates of *L. tottum* var. *glabrum*, there is multiple evidence that morphological differences between *L. tottum* varieties have evolved in response to a geographical shift in the composition of the pollinator fauna, with one dominated by long-proboscid flies and Cape sugarbirds, and one dominated by Orange-breasted sunbirds. We suspect that Cape sugarbirds are present in the Hex River Valley but do not temporally occur when *L. tottum* var. *glabrum* is in flower. There were large populations of Proteaceae species that would support Cape sugarbirds in the Hex River Valley but they had long ceased flowering by the time *L. tottum* var. *glabrum*'s flowered (pers. obs.). We also suggest that long-proboscid flies are absent from the Hex River Valley entirely, as we neither saw them nor plants that would suggest their presence. At the sites where long proboscid flies were observed, several other long-proboscid fly pollinated plants were present in the community. At Dasklip Pass these included *Geissorhiza confusa*, *Lepeirousia anceps*, *Pelargonium elongatum* and *Pelargonium longicaule*; and at Bain's Kloof Pass *G. confusa*, *P. longicaule* and *Gladiolus carneus* were present. The absence of these nectar sources is consistent with the apparent absence of the flies from these sites. An alternative hypothesis would be that Cape sugarbirds and long-proboscid flies occur in the study area, but do not visit *L. tottum* var. *glabrum* inflorescences due to morphological incompatibilities. Although we believe these visitors to be absent from the study site, this explanation cannot be ruled out without site observation for multiple seasons, as pollinators can be highly variable from season to season.

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# Chapter 3

Adaptation for rodent pollination in *Leucospermum arenarium* (Proteaceae) despite rapid pollen loss during grooming

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## Abstract

Plants are adapted for rodent pollination in diverse and intricate ways. Here we explore an extraordinary example of these adaptations in the pincushion *Leucospermum arenarium* (Proteaceae) from South Africa. We used live trapping and differential exclusion experiments to test the role of rodents versus birds and insects as pollinators. To explore the adaptive significance of geoflory we raised inflorescences above ground level and compared seed production. We used captive rodents and flowers with artificial stigmas to test the effect of grooming on the rate of pollen loss. To investigate the bizarre nectar production and transport system we used microscopy, nectar composition analysis and manipulative experiments. Differential exclusion of rodents, birds and insects demonstrated the importance of rodents in promoting seed production. Live trapping revealed that hairy-footed gerbils *Gerbillurus paeba* and striped field mice *Rhabdomys pumilio* both carry *L. arenarium* pollen on their forehead and rostrum, but much larger quantities end up in feces as a result of grooming. Terrarium experiment showed that grooming exponentially diminished the pollen loads that they carried. The nectar of *L. arenarium* is unusually viscous and is presented in a novel location on the petal tips where rodents can access it without destroying the flowers. Nectar is produced inside the perianth, but is translocated to the petal tips via capillary ducts. In common with many other rodent-pollinated plants, the flowers are presented at ground level, but when raised to higher positions seed production is not reduced, indicating that selection through female function does not drive the evolution of geoflory. Despite the apparent cost of pollen lost to grooming, *L. arenarium* has evolved remarkable adaptations for rodent pollination and provides the first case of this pollination system in the genus.

## Introduction

Although non-flying mammal pollination was first described nearly 80 years ago (Porsch 1934) it remains poorly understood how and why plants utilize these unusual pollinators. Early studies considered non-flying mammals to be incidental visitors (Hopper 1980; Paton and Turner 1985). Currently, however, non-flying mammal pollination is known to be a specialization accompanied with unique floral and vegetative traits (Carthew and Goldingay 1997). Despite the unique features of this pollination system, the research field is still at an early descriptive stage, and the ecology of rodent-flower interactions has received little attention (Hackett and Goldingay 2001; Wooller and Wooller 2003; Kleizen *et al.* 2008; Letten and Midgley 2009; Wester *et al.* 2009; Turner *et al.* 2011; Wester 2011).

The Proteaceae offer many opportunities for studying non-flying mammal pollination. The pollination system occurs in multiple Australian genera (Rourke and Wiens 1977; Carpenter 1978; Hackett and Goldingay 2001), and in South Africa is known from several species in the genus *Protea* (Wiens and Rourke 1978; Fleming and Nicolson 2002; Biccard and Midgley 2009). Common convergent traits shared by non-flying mammal pollinated *Protea* species include: geoflorous and/or downward-facing inflorescences, flowers with hooked styles; production of copious amounts of nectar; a nutty or yeasty odor; late winter or early spring flowering; as well as inflorescences that are smaller in diameter and in flower length compared to congeneric bird-pollinated species (Rourke and Wiens 1977).

The South African Proteaceae genus *Leucospermum*, commonly known as pincushions, contains approximately fifty taxa (Rourke 1971), most of which are apparently bird or insect pollinated. One unusual member of the genus is *Leucospermum arenarium* Roycroft, an

endangered shrub that grows in Fynbos vegetation at one locality on the low-lying, sandy plain along the southwest coast. Morphology suggests that *L. arenarium* belongs to a clade of insect-pollinated species, however, whereas the other clade members have short, straight, bright yellow flowers (Rourke 1971), the flowers of *L. arenarium* are considerably longer, curved, and dull-colored (Fig 3.1d; 3.2d). These features are analogous to those of rodent-pollinated *Protea* species, and led us to hypothesize that this member of the genus *Leucospermum* is also adapted for rodent pollination. However, from the outset, it was not clear how rodents would interact with the flowers. In common with its clade members, *L. arenarium* has very narrow nectar tubes, so a question that needed answering was how rodents would access nectar without destroying flowers.

One of the features that *L. arenarium* has in common with other rodent-pollinated plant species is the presentation of the flowers at ground level – so-called “geoflory.” Although this trait is often listed among the adaptations for rodent pollination (Rourke and Wiens 1977), the adaptive significance of geoflory has been brought into question by the finding that some flower-visiting rodents are adept climbers that will visit flowers well above ground level (Biccard and Midgley 2009). *L. arenarium* inflorescence height ranges from 0-45cm above ground level, allowing us to test (using natural and experimentally induced variation) whether ground level (geoflorous) inflorescences produce more seeds than inflorescences displayed at greater heights.





Figure 3.1. *L. arenarium* in the field and one of its pollinators, *G. paeba*, feeding on flowers. A) Pollen presenter contact on *G. paeba*. B) *G. paeba* foraging on *L. arenarium*. C) Flowering *L. arenarium* with dense mat forming inflorescences. D) Geoflorous inflorescences. E) Pendulous inflorescences above ground level.





Figure 3.2. The odd nectar secretion of *L. arenarium*. A) Nectar near the end of the petals. B) Nectar accumulation on *L. arenarium* inflorescence. C) Single *L. arenarium* flower with nectar present. D) *L. arenarium* inflorescence. Scale bars =5mm.

A consistent feature of studies of rodent pollination is that more pollen is found in the feces than on the fur. Many studies conclude that pollen appears in feces because it is ingested while grooming rather than directly consumed, but this idea has not been tested. It is important to know how effective rodents are at removing pollen by grooming, because this will influence their efficiency as pollinators. The question of what happens to pollen once it leaves the anther (pollen fate), is of general interest. Many studies have focused on pollen losses that result when

pollen is improperly transferred to heterospecific plants through shared pollinators (reviewed in Morales and Traveset 2008; Muchhala and Thomson 2013). Despite the probable importance of the latter mechanism, it is often concluded that the bulk of pollen lost during animal mediated pollination must be due to grooming (Harder and Thomson 1989; Holsinger and Thomson 1994; Johnson *et al.* 2005; Flanagan *et al.* 2009; Mitchell *et al.* 2009; Muchhala *et al.* 2010). A study conducted by Castellanos *et al.* (2003) suggested that dissimilarities in pollen-carryover curves between birds and bees were likely the result of differences in grooming behavior between the two pollinator types, although grooming behavior was not directly measured. Direct examination of the effects of pollinator grooming on pollen loss are rare (Thomson 1986), because most studies fail to distinguish between pollen lost from grooming behavior and pollen lost passively (Morris *et al.* 1995; Rademaker *et al.* 1997; Harder and Thomson 1989; Holmquist *et al.* 2012). By focusing on a non-flying mammal pollinator, which can be observed continuously, we were able to directly measure the rate of pollen loss due to grooming.

In summary, we tested the following predictions: 1) *L. arenarium* is specialized for pollination by non-flying mammals; 2) geoflorous inflorescences will be more accessible to rodents and therefore produce more seeds than inflorescences higher above the ground; 3) unique nectar properties and presentation will facilitate rodent pollination; 4) pollen lost between uptake and deposition will be directly related to time spent grooming.

## **Materials and methods**

**Study site-** *L. arenarium* is only known to occur on three farms between the towns of Redelinghuys and Aurora, South Africa. This study was conducted on the Witwater Farm (32°37'31.80"S, 18°30'16.20"E) which has a large area of natural Sandveld habitat.

***The role of rodents as pollinators-*** Diurnal pollinator observations were conducted over three days (27/9/2012-29/9/2012) from 730-1500 h during which floral visitors were recorded as well as if visitors made contact with pollen presenters. Pollen presenter contact can conveniently be used to estimate pollinator effectiveness in *Leucospermum* because the pollen presenter not only presents pollen, but also houses the stigmatic groove. Plants were observed at close proximity and at a distance of 20 meters in order to survey insects and birds. The presence of nocturnal pollinators (rodents) was determined using live traps baited with rolled oats and peanut butter. Trapping was conducted on four days (18/9/2012, 27/9/2012-29/9/2012). On each day thirty live traps were baited and set out before sunset and collected at 700 h. The entire forehead region of captured rodents were swabbed with fuchsin gel (Beattie 1974) and checked for pollen. Rodent droppings were removed from traps and kept frozen for later examination. Pollen samples were extracted from three haphazardly chosen fecal pellets using the methods described in Wester *et al.* (2009).

We applied four differential pollinator exclusion treatments each to 10 *L. arenarium* plants in order to determine if *L. arenarium* is reliant on non-flying mammals for pollination: 1) un-manipulated, geoflorous inflorescences that allowed access to all potential visitors; 2) caged, geoflorous inflorescences that allowed access to insects only; 3) caged geoflorous inflorescences with small, ground-level “doors” cut in (~5.5x 5.5 cm) that allowed access to rodents and insects, but not birds, and 4) bagged inflorescences that tested for autogamy. Inflorescences were bagged and caged once wilting occurred to prevent seed loss. Seed production was used to measure pollinator effectiveness. Data was analyzed using a Friedman ranked sum test followed by post-hoc Wilcoxon signed rank tests in R (R Development Core Team 2009).

***The importance of geoflory for accessibility to rodent pollinators-*** *L. arenarium* is a semi-geoflorous shrub with inflorescences at various heights above the ground (Fig 3.1c,d,e). In order to determine the distribution of inflorescences in vertical space we sampled a portion of *L. arenarium* plants (N=5) and measured the heights of each inflorescence within that portion. These measurements were then assigned to height classes (5 cm intervals from 0-50 cm) to determine the abundance of inflorescences at different heights.

In order to test if seed production varies in geoflorous vs. non-geoflorous inflorescences we compared seed production from inflorescences at the two height class extremes. We chose inflorescences that occur 31-45 cm above the ground as our maximum height class (non-geoflorous) since we had found that the top 3% of *L. arenarium* inflorescences occur at this height (see results). This was compared to inflorescences occurring directly on the ground (geoflorous) and to a third treatment, where we raised the flexible branches of naturally geoflorous inflorescences to match the maximum height class by fixing them to steel rods with a cable tie. This treatment was conducted to control for heterogeneity in resource allocation. Inflorescences were bagged and caged once wilting occurred to prevent seed loss to rodents and other predators and dispersers. Each of the 10 plants received all three treatments. Data was analyzed using a Friedman ranked sum test in R-statistical software (R Development Core Team 2009).

***Nectar Properties and Nectary Morphology-*** Nectar sugar concentration was measured in the field using a handheld refractometer (ECLIPSE hand-held refractometer, Bellingham & Stanley, Basingstoke, United Kingdom). Nectar volume was not measured in the field because nectar was often too viscous to be syphoned with capillary tubes. Instead nectar volume was measured by removing all nectar from a flower (N=5) onto filter paper and measuring the added

mass (Mettler PJ400 Precision Balance, Mettler-Toledo International, Columbus, Ohio, USA). Nectar volume was then calculated by converting nectar mass to volume (0.001g =1.0 µl). This was done with newly opened flowers in the lab.

Nectar was kept in filter paper and sugar composition was determined by gas chromatography. The samples were reconstituted in 80 µl methoxyamine hydrochloride (30mg/ml in pyridine) and incubated in the oven for 2 hours at 30°C. After two hours samples were derivatised with 140 µl of MSTFA (*N*-Methyl-*N*-trimethylsilyltrifluoroacetamide) for 1 hour at 37°C. One µl of each of the derivatised samples were then injected onto a gas chromatography column. Analyses were carried out by the Central Analytical Facility at Stellenbosch University, South Africa.

Plant material for microscopy was stored in 70% ethanol before processing. Superficial examination of the flower petal surface was done with a LEO<sup>®</sup> 1400VP Scanning Electron Microscope. Samples were dehydrated in a graded series of ethanol, critical-point dried with an acetone dehydrant and liquid CO<sub>2</sub> transitional fluid, mounted on aluminum stubs with double-sided copper tape, coated with gold, observed and photographed.

We cut off the petal tips (where nectar ordinarily accumulates) on fifteen flowers to determine whether nectar is secreted by nectaries on the petal tips or if nectar is produced within the flower and then translocated. Flowers were also dissected to look for nectaries and flower measurements were taken to the nearest 0.1 mm (N=5).

***The effects of pollinator grooming on pollen transfer-*** We conducted controlled terrarium experiments with three hairy-footed gerbils (*Gerbillurus paeba*, Fig 3.1a,b) and two striped field mice (*Rhabdomys pumilio*) in order to test the effects of pollinator grooming on

pollen loss. The experiment had two stages: pollen uptake by rodents and pollen deposition on stigmas.

*Pollen uptake by rodents*- An individual rodent was placed in an empty terrarium and presented with one nectar rich *L. arenarium* inflorescence that had all but one pollen presenter removed. We ensured that the single flower that was left intact had a complete pollen load in order to standardize the amount of pollen available for each trial. Rodents were allowed to forage on inflorescences until they fed on the intact flower and made contact with its pollen load. The inflorescence was then immediately removed so that only one visit occurred. In each trial the intact flower was positioned at the top of the inflorescence so that contact was made on the same area of the forehead of each rodent.

*Pollen deposition on stigmas*- After pollen uptake, the inflorescence was replaced with another that also had all but one pollen presenter removed. The remaining pollen presenter had all pollen removed and was capped with a 10 x 4mm piece of double sided tape. Similar to the first treatment, rodents were allowed to forage until they fed on the experimental recipient flower and made contact with the double-sided tape after which the inflorescence was removed. Each inflorescence was presented as in the first stage so pollen presenter contact was consistently made on the rodent's forehead. The time from pollen uptake to deposition (N=10) and the amount of time spent grooming (N=19) between uptake and deposition were kept with separate stopwatches. After each experiment (N=19) the tape was removed, stained, and examined under a compound microscope to count total pollen grains. Replicates occurred at least 24 hours apart to ensure no pollen carryover between experiments. Data was analyzed using generalized linear mixed models in R (R Development Core Team 2009).

## Results

*The role of rodents as pollinators*- Diurnal observations recorded no bird visitors and only nine insect individuals (6 Hymenoptera, 2 Coleoptera, 1 Diptera) visiting *L. arenarium* flowers during 22.5 hours of observation. Of these visitors, none made contact with the pollen presenters or removed a substantial amount of nectar. At night, six hairy-footed gerbils (*G. paeba*), two striped field mice (*R. pumilio*), and one un-identified mouse were caught in live traps. Small quantities of *L. arenarium* pollen was found on rodents' heads (mean=11.44 pollen grains, S.D.=6.13, N=9 rodents), but considerably more occurred in fecal pellets (mean=58.22 pollen grains per microscope field, S.D.=39.09, N=9).

Differential pollinator exclusion experiments demonstrated that seed production in *L. arenarium* is impacted by access to certain pollinator groups ( $\chi^2(3)=25.598$ ,  $p < 0.0001$ , Friedman ranked sum test). Bagged inflorescences that excluded all pollinators yielded no seed production (Fig 3.3), indicating that *L. arenarium* cannot autogamously self-pollinate. To test the role of insects as pollinators we compared seed production from bagged inflorescences (which allowed no visitors) to inflorescences that were caged and only allowed access to insects. Allowing insects did not significantly influence seed production above bagging ( $W=0$ ,  $Z=-1.7321$ ,  $p=0.25$ ,  $r=0.3873$ , Wilcoxon signed rank test, Fig 3.3). Rodents were included as visitors along with insects by cutting ground-level doors in cages. This treatment led to a significant increase in seed production compared to inflorescence that only allowed access to insects ( $W=0$ ,  $Z=-2.8196$ ,  $p=0.002$ ,  $r=0.6305$ , Wilcoxon signed rank test, Fig 3.3). Un-manipulated inflorescences allowed access to bird visitors along with rodents and insects. This treatment, which tested the role of birds as pollinators, yielded no significant change in seed production when compared to



inflorescences that were allowed only rodent and insect visitors ( $W=20.5$ ,  $Z=-0.722$ ,  $p=0.5391$ ,  $r=0.1614$ , Wilcoxon signed rank test, Fig 3.3).

***The importance of geoflory for accessibility to rodent pollinators-*** Fig 3.4 shows a representative distribution of inflorescences in vertical space for *L. arenarium*. In the five *L. arenarium* plants sampled we found that the majority of inflorescences appear within five cm above the ground (mean=69.8%, S.D.=3.53%) with the highest ~3% located in the 31-45cm range (Fig 3.4). Surprisingly, there was no significant difference in seed production between inflorescences in the geoflorous ( $\bar{x}=3.50$ , S.D.=1.12, N=10) and 31-45 cm height class ( $\bar{x}=2.91$ , S.D.=1.50, N=10) or in inflorescences that were raised from ground level to match the 31-45 cm height class ( $\bar{x}=2.60$ , S.D.=1.80, N=10;  $\chi^2(2)=0.2353$ ,  $p=0.889$ , Friedman ranked sum test).



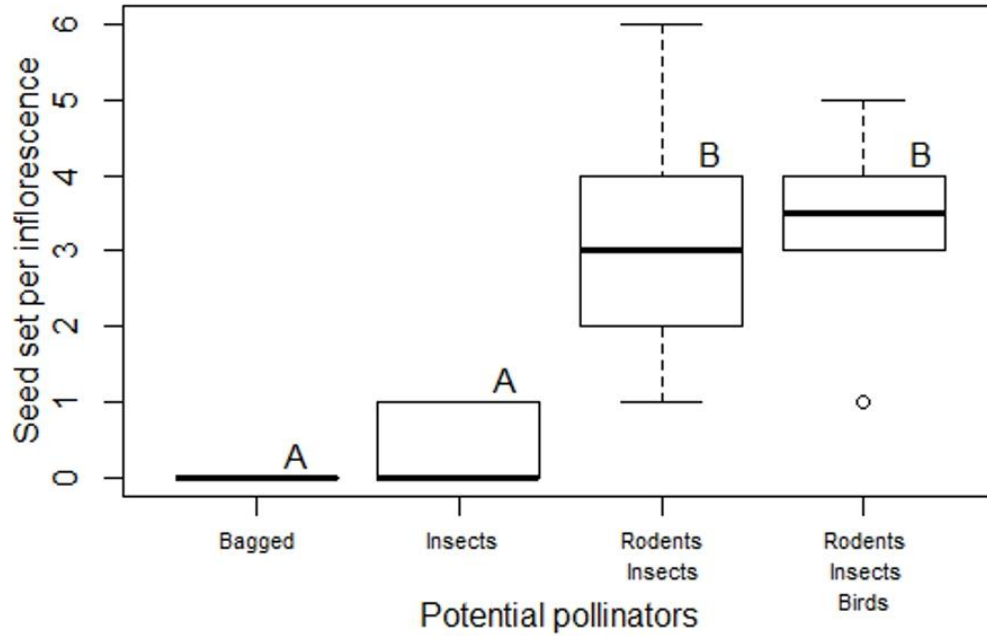


Figure 3.3. Seed production from differential pollinator exclusion experiments. All four treatments were applied to 10 plants.

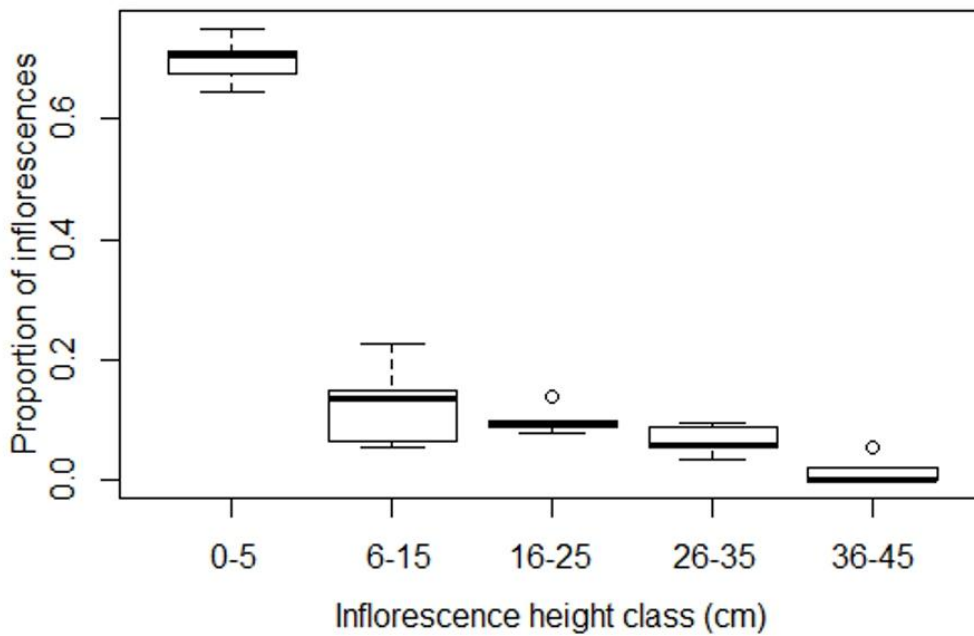


Figure 3.4. The number of *L. arenarium* inflorescences at different height classes for five sampled plants.

***Nectar properties and location of nectaries-*** *L. arenarium* flowers produce large volumes of nectar ( $\bar{x}$ =9.9  $\mu$ l, S.D.=1.884, N=5) with relatively high sugar concentrations ( $\bar{x}$ =26.1%, S.D.=12.017, N=5). Nectar sugar is comprised almost entirely of sucrose (97.832%, S.D.=1.059, N=6) with small traces of fructose (1.630%, S.D.=0.790) and glucose (0.538%, S.D.=1.059, N=6) and no xylose (N=6). Cross-sectioning of the petal shows capillary channels (Fig 3.5a) leading up to where the nectar is held (Fig 3.5b). These canals are also revealed by the SEM in Fig 3.5c. When petal tips were cut off nectar welled out of the channels at the location where the perianth was severed. Dissections revealed four hypogynous scales (nectaries) located between the perianth and the ovary, with one scale contained in each petal. Flower length (straight-line distance from ovary to stigmatic groove) measured 28.2 mm (N=5, S.D.=0.927) and stigma-nectar distance measured 14.1 mm (N=5, S.D.=0.663).

***The effects of pollinator grooming on pollen loss and outcrossing-*** Pollen deposition declines exponentially as the amount of time spent grooming increases (Estimate=-0.053133, Std. Error= 0.001881, z-value= -28.24,  $p < 0.001$ , Fig 3.6) whereas there is no relationship when simply considering time between visits (Estimate= 0.0002386, Std. Error= 0.0007121,  $z=0.335$ ,  $p=0.738$ , Fig 3.7). The identity of rodents used in each trial was included as a random factor in both models to control for pseudoreplication.

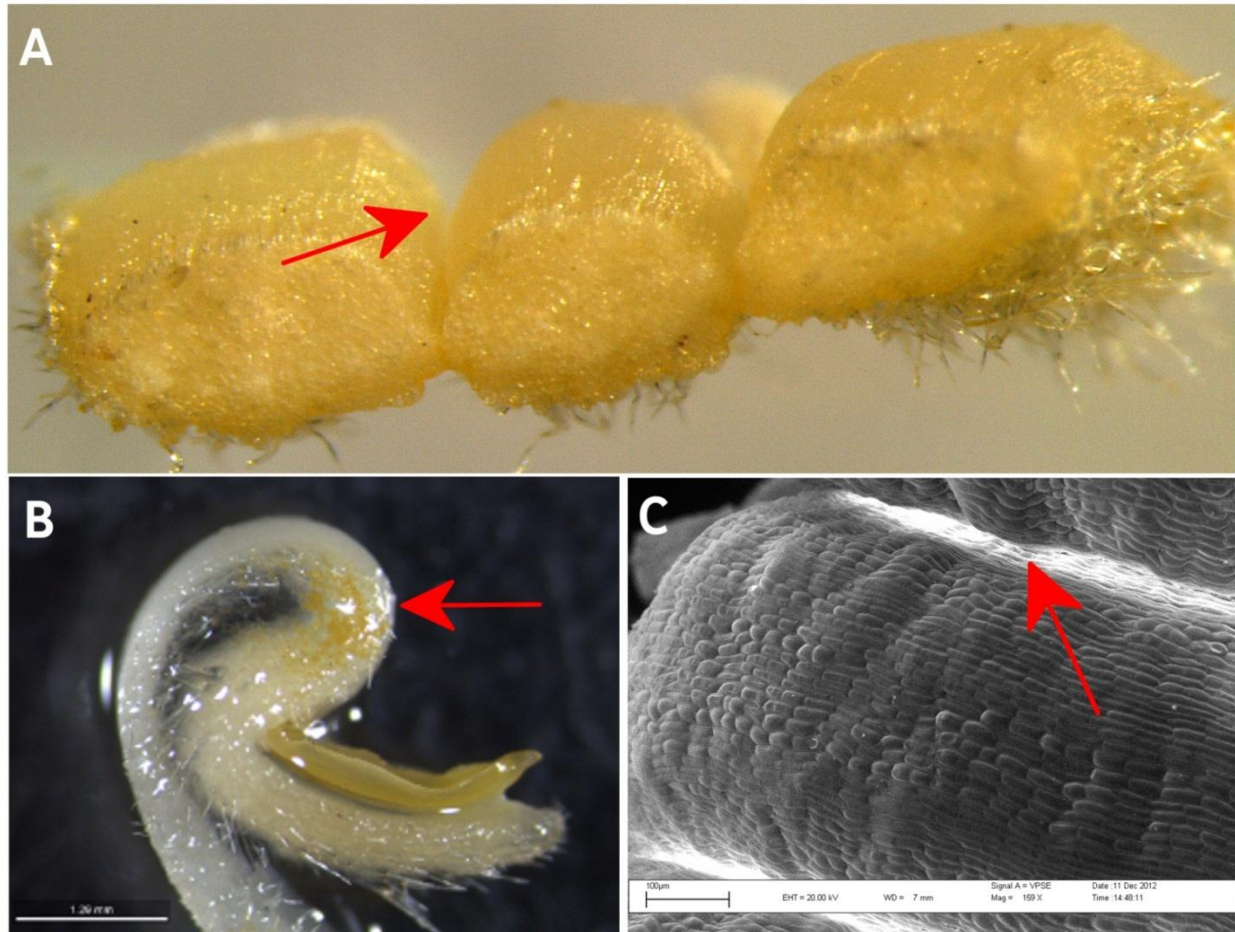


Figure 3.5. Dissections and scanning electron micrograph depicting the capillary channels for nectar transport. A) Capillary channels shown by a longitudinal section of a *L. arenarium* perianth. B) Site of nectar accumulation in *L. arenarium* taken with a dissecting microscope. C) Electron microscope scan showing capillary channels formed from fused perianth segments.

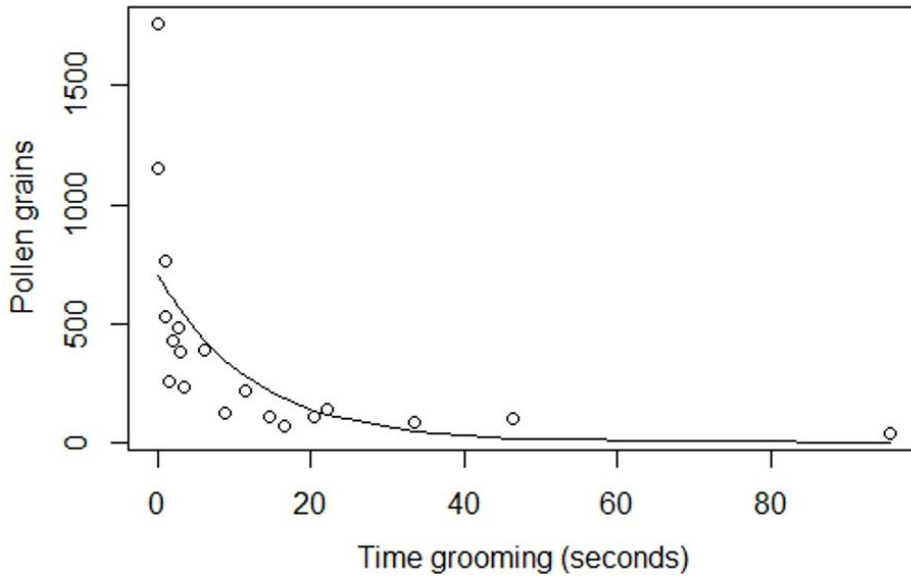


Figure 3.6. The amount of pollen transferred to recipient flowers with an increase in time spent grooming (seconds) between pollen uptake and deposition. The solid line represents the model prediction.

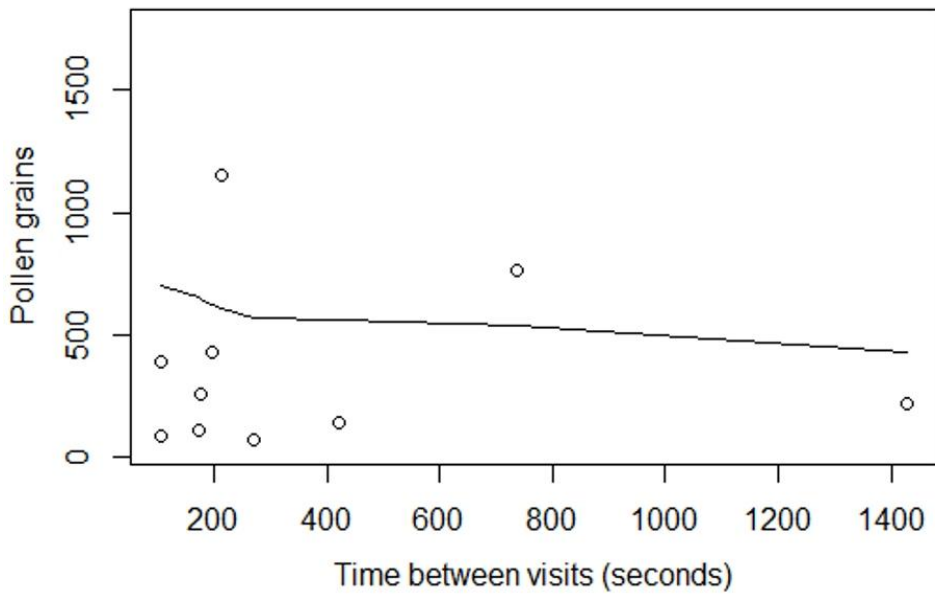


Figure 3.7. The amount of pollen transferred to recipient flowers with an increase in time (seconds) between pollen uptake and deposition. The solid line represents the model prediction.

## Discussion

***Evidence for rodent pollination in L. arenarium-*** During diurnal observations no visitors were seen pollinating *L. arenarium*, however, nocturnal trappings and microscopic investigation revealed *Leucospermum* pollen on the heads and in the feces of two rodent species (*G. paeba* & *R. pumilio*). The number of pollen grains recorded is comparable to the number reported in other rodent pollination studies (Johnson *et al.* 2001; Kleizen *et al.* 2008; Biccard and Midgley 2009; Wester *et al.* 2009). Furthermore, differential pollinator exclusion experiments showed that seed production is only significantly increased when rodents are allowed access. Overall we found strong evidence that *L. arenarium* is pollinated by rodents.

***The importance of geoflory for accessibility to rodent pollinators-*** Geoflory is the most conspicuous and perhaps the most common trait among rodent pollinated plant species. Since many pollinating rodents spend the bulk of their time on the ground, geoflorous inflorescences could increase the likelihood of being visited. However, we saw no reductions in seed production when comparing the highest natural occurring height class to ground level inflorescences, providing evidence that inflorescences up to 45cm above ground level are accessible to rodents. Our study, along with that of Biccard and Midgley (2009), places doubt on the widely held belief that rodent pollinated plants exhibit geoflory as an adaptation to exploit rodents (Rourke and Wiens 1977). A possible explanation is that the height classes selected in these studies do not vary enough to detect selection. Another possibility is that selection acts via the male component of fitness (pollen export), which was not measured in this study.

***Nectar properties and nectar translocation-*** The most distinguishing features of *L. arenarium* are its novel nectar properties and presentation. The nectar is uniquely viscous and is

completely exposed outside of the flower, whereas all other *Leucospermum* species produce typical liquid nectar that is held within the nectar tube. Similarly viscous nectar has been seldom documented and in both recorded cases is associated with vertebrate pollination (Johnson *et al.* 2001; Sazima *et al.* 2001).

Our analysis shows that *L. arenarium* nectar is sucrose dominant (~97%), with just ~2% comprising hexoses. Both hexose and sucrose rich species have been described in *Leucospermum*, but the only members of *L. arenarium*'s proposed clade that have been analyzed (*L. hypophylloconocarpodendron* subs. *canaliculatum* and *L. rodolentum*) are hexose dominant (Nicholson and Van Wyk 1998). This could be an indication of adaptation in *L. arenarium*, or that the species is phylogenetically misplaced. Ongoing genetic work should resolve this dilemma. Although we were unable to determine what causes the high nectar viscosity of *L. arenarium* we can conclude that sugar concentration alone cannot explain it (mean=26.1%, S.D.=12.017, N =5). Unfortunately, no conclusions were made in other studies that describe a similarly high nectar viscosity, although they suggest that it may be caused by the presence of a glucomannan (Sazima *et al.* 2001) or a mucopolysaccharide (Johnson *et al.* 2001) respectively. Additionally, high viscosity could be caused by the presence of a macromolecule solute (Mathlouthi and Génotelle 1995) or a natural deep eutectic solvent (Choi *et al.* 2011). This wide range of explanations highlights the need for more research on secondary nectar compounds, particularly compounds that directly impact viscosity.

Because nectar held outside of the nectar tube is vulnerable to theft (Pacini *et al.* 2003), many plants with open flowers produce unpalatable nectar that deters thieves (Stephenson 1981 1982; Adler 2000; Johnson *et al.* 2006; Shuttleworth and Johnson 2006, 2009). Nectar in *L. arenarium* is palatable to humans, so it is unlikely to be unpalatable to potential thieves.

However, viscosity alone may deter thieves because nectar ingestion rate declines with increasing viscosity in suction feeding birds and insects (Baker 1975; Harder 1986; Tezze and Farina 1999; Josens and Farina 2001; Nicolson and Thornburg 2007; Köhler *et al.* 2010).

In addition to foiling potential nectar thieves, high nectar viscosity may benefit *L. arenarium* by decreasing evaporative water loss from the exposed nectar droplets in an arid environment. Our nectar samples kept on filter paper did not evaporate even after weeks in the open air; whereas nectar samples taken from various other *Leucospermum* species dried in a matter of minutes. Lastly, the adhesiveness of *L. arenarium* nectar may be beneficial by helping to keep nectar in place, since many inflorescences are pendulous and less viscous nectar would drip out of flowers. It is likely that one or a combination of these theories can explain the benefits of possessing nectar of high viscosity.

An additional unusual feature of nectar secretion in *L. arenarium* is that the nectar is held in a unique cup near the tips of the petals rather than inside the perianth tube as is typical for members of the Proteaceae (Fig 3.2 a,b,c). Our experiments and anatomical investigations make it clear that the nectar is not secreted at the petal tips, but is produced by the four hypogynous scales located deep inside the flower around the ovary base. The perianth tube fits very snugly around the ovary, leaving no space for nectar accumulation at its site of production. As a result the nectar is shunted up the two capillary channels that run along the sutures of the petals until it wells out near the tips of the petals (Fig 3.5). This scenario is consistent with the findings by Rao (1967) that the location of nectaries (hypogynous scales) in the Proteaceae is highly conserved. Nectar translocation also occurs in *Asclepias syriaca* (Kevan *et al.* 1989) and many species of Solanaceae (Vogel 1998).

Appropriate positioning of the reward ensures that the pollinator makes consistent contact with anthers and/or stigmas (Vogel 1998). In *L. arenarium* the translocation of the nectar from the base of the perianth tube to the distal ends of the petals reduces the nectar-stigma distance from 28.2 mm (N=5, S.D.=0.927), which would be too large to allow contact with the rodent's rostrum, to 14.1 mm (N=5, S.D.=0.663), which ensures a good fit between rostrum and flower. This reduced distance conforms to the nectar-stigma distance observed in many other non-flying mammal pollinated Proteaceae (Wiens *et al.* 1983). In addition, placing nectar on the petal tips raises it well above the level of ovaries and may reduce damage caused during foraging. In our trials, we never saw rodents foraging destructively.

***The effects of pollinator grooming on pollen loss and outcrossing-*** Nearly all rodent pollination studies involve swabbing captured rodents for pollen as well as sampling feces, and in all cases there is substantially more pollen in fecal samples than on the rodents themselves. These studies inadvertently point to a fundamental and overlooked aspect of pollination biology; active grooming by pollinators and the subsequent loss of pollen is detrimental to male fitness. Since the efficiency of grooming likely varies among pollinator species, plants should either avoid pollinators that groom effectively or evolve mechanisms that lessen these impacts. Given a rodent's expert grooming ability it seems likely that virtually all pollen will be lost during an extended rest period in the burrow, so that there will be no pollen carry-over from one day to the next. In contrast, pollen may potentially stay on other species, such as birds and long-proboscid flies, for multiple days, but data for such comparisons are still lacking.

***Conclusions-*** *Leucospermum arenarium* relies on rodents for pollination and provides the first case of rodent pollination in the genus. Its flowers are modified for rodents in interesting ways: the nectar is viscous and is translocated from the nectaries deep inside the perianth tube to



the petal tips via capillary ducts. Thus, the nectar is presented in an exposed position where rodents can access it without destroying the flowers. In common with many other rodent-pollinated plants the flowers are presented at ground level, but when flowers are experimentally raised to higher positions, seed production is not reduced, indicating that selection through female function does not drive the evolution of geoflory. Differential exclusion of rodents, birds and insects shows the importance of rodents in promoting seed production. This reliance on rodent pollination has apparently evolved despite the fact that rodents are very adept at removing pollen through grooming.

### **Acknowledgements**

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# Chapter 4

Correlated evolution between floral morphology,  
pollination mode and breeding system in the southern  
African genus *Leucospermum* (Proteaceae)

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This chapter will be submitted to *Evolution*

## Abstract

Phylogenetic studies of correlated evolution between floral traits and pollination mode have been vital to our understanding of the role of pollinators in the diversification of angiosperms; however, the potential knowledge we could gather using these methods leaves much to be desired. Current studies are restricted by focusing on few pollinator functional groups, by often focusing on the same morphological traits, and by failing to incorporate floral evolution through divergent use of the same pollinator. Additionally, inclusion of autonomous selfing data into these studies is surprisingly rare. In this study, we search for correlated evolution between 7 morphological traits and 10 functional groups of pollinators using 49/52 *Leucospermum* (Proteaceae) taxa. In addition, we examine if autonomous selfing is correlated with specific pollination modes and/or morphological features in 45/52 taxa. Our results support the following pollinator-trait correlations: 1) Insects and actinomorphic, short-styled, straight flowers; 2) moths and horizontal flowers; 3) long-proboscid flies and long nectar tubes; 4) non-flying mammals and highly curved flowers with short or modified nectar tubes; 5) birds and zygomorphic, long-styled flowers with long nectar tubes and high flower density. We also examined functional groups of bird pollinators based on variation in beak length and found that 6) short-billed bird pollinator importance is negatively correlated with style length and long-billed bird pollinator importance is positively correlated style length and flower curvature. We present evidence that divergent use of pollen transfer sites on long-billed birds have driven floral diversification in *Leucospermum* by showing that style length increases and flower orientation decreases when taxa utilize body feathers to transfer pollen instead of crown feathers. Additionally, we found autonomous selfing to be negatively correlated with insect and long-proboscid fly pollinator importance and positively correlated with bird pollinator importance.

Within long-billed bird pollinated taxa, long-billed birds were positively correlated with selfing rate and within long-billed bird pollinated *Leucospermum*; taxa that transfer pollen on body feathers have higher selfing rates. We discuss the evolutionary significance of each correlation.

## Introduction

The vast floral diversity of angiosperms is often acknowledged as being the result of adaptation to pollinators (Darwin 1862; Grant and Grant 1965; Stebbins 1970; Johnson 2006). Floral congruencies in unrelated taxa are explained by convergent evolution to employ the same functional groups of pollinators (Fenster *et al.* 2004), with this repeated selection on particular floral traits at the core of the Pollination Syndrome hypothesis (Faegri and van der Pijl 1970). Although contentious (Ollerton 1996, 1998; Ollerton *et al.* 2009), the Pollination Syndrome hypothesis provides a general structure as to what floral traits are valued by pollinators (Pellmyr 2002; Rosas-Guerrero *et al.* 2014). Support for pollinator driven adaptive divergence has been shown in multiple empirical studies (see special issue in *Annals of Botany* 113(2) (2014); Johnson *et al.* 2014). Adding to this knowledge is the amalgamation of phylogenetics with measures of floral phenotypes and pollination modes (Armbruster 1996, 2002; Pérez *et al.* 2006; Smith *et al.* 2007; Martén-Rodríguez *et al.* 2010; Rosas-Guerrero *et al.* 2010; Waterman *et al.* 2011; Sakai *et al.* 2013), which have become popular approaches for testing the role of pollinators in driving floral evolution and diversification (reviewed in Smith 2010; reviewed in van der Niet and Johnson 2012). Since phylogenetic conservatism constrains adaptability (Poisot *et al.* 2011), and data from species are hampered by non-independence due to a shared history (Felsenstein 1985), accounting for relatedness is essential to properly test for convergent evolution (Felsenstein 1985; Grafen 1989; Pagel and Harvey 1989).

In this study, we test for correlated evolution between floral morphology, pollination mode and breeding system in *Leucospermum* (Proteaceae), a hallmark of South Africa's diverse Cape Floristic Region and an endemic to southern Africa. Evidence often points towards recent radiations explaining much of the species diversity in the CFR, with the diversification of some

species rich clades such as the Proteaceae being attributed to the formation of summer-arid habitats established during the Late Miocene (Linder 2005; Verboom *et al.* 2009). The diversification of *Leucospermum*, which is comprised of 52 taxa, has been suggested to be primarily growth-environment-driven (Johnson 1996). Alternatively, diversification of this genus may be pollinator driven. This theory is supported by the immense floral diversity of the genus as well as the recent work focusing on the pollination biology of the genus, which has revealed a wide variety of pollination modes ranging from pollination by various birds and long-proboscid flies (Johnson *et al.* 2014) to pollination by rodents (Johnson and Pauw 2014). In addition, the ability to autonomously self-pollinate varies at the species level (Lamont 1985) as well as between ecotypes that differ in pollinators and floral morphology (Johnson *et al.* 2014). Aside from these few studies, the reproductive strategies for the remainder of the genus, until now, have only been inferred.

*Leucospermum* possess capitulum inflorescences comprised of flowers whose styles vary from completely straight to highly curved. Flowers are either zygomorphic or actinomorphic, although most are zygomorphic. Following anthesis, perianth segments peel away to expose the pollen presenter and remain to house the flower's nectar, most often in the form of a traditional nectar tube located at the base of flowers. *Leucospermum* flowers are hermaphroditic and the pollen presenter is capped with a stigmatic groove to receive pollen. Flowers are presented in a variety of orientations, ranging from vertical to horizontal to downward-facing and the number of flowers per inflorescence (flower density) varies among taxa from 5 to over 100. Our study is the first to include the morphological features flower symmetry, curvature, orientation and flower density into a phylogenetic model of correlated evolution with pollination mode, thereby

broadening our scope of pollinator driven floral adaptations. To our knowledge, this is also the first study to examine the evolution of inflorescence architecture in relation to pollination.

Although phylogenetic studies of correlated evolution between pollination mode and floral morphology have provided valuable insights into the evolutionary process, most have included only a small subset of pollinator functional groups (Pérez *et al.* 2006 (4 functional groups); Smith *et al.* 2007 (4 functional groups); Martén-Rodríguez *et al.* 2010 (3 functional groups); Sakai *et al.* 2013 (3 functional groups)) and have lacked the more unique pollinators such as non-flying mammals and long-proboscid flies. In addition, bird pollinators are almost always categorized as a single functional group (Perez *et al.* 2006; Smith *et al.* 2007; Martén-Rodríguez *et al.* 2010; but see Bruneau 1997). Although this may have been appropriate for some of the studies, omission of beak length variation in the realized pollinator mosaic can bias the role of pollinators in shaping floral diversity and underestimate the role of pollinators as drivers of selection (Fenster *et al.* 2009 a). For this reason we demarcated two distinct functional groups of bird pollinators based on beak length alone; the short-billed birds *Anthobaphes violacea* (30-34.5 mm) and *Cinnyris chalybea* (34.5 mm), and the long-billed birds *Promerops cafer* (45-54 mm) and *Nectarinia famosa* (43.5-51 mm) (Rebelo 1987) and focused on correlations between floral morphology and pollination mode among bird pollinated taxa. Recent work in the CFR has shown that these groups display variable foraging behaviors and select for different floral traits (Geerts and Pauw 2009; Johnson *et al.* 2014; van der Niet *et al.* 2014). We also wanted to study correlations between variations in floral morphology and the pollen attachment sites on long-billed birds. Observations made by Vogel (1954) suggested that the floral morphology of *Leucospermum* can manipulate pollinator orientation and pollen attachment site, but has since been unexplored. We grouped *Leucospermum* taxa pollinated by long-billed



birds into two groups; ones that transfer pollen to crown feathers (the more ubiquitous mode) and ones that place pollen on body feathers (Fig 4.1) and searched for correlations between floral morphology and these varying pollen transfer sites.



Figure 4.1. Morphological variation in long-billed bird pollinated taxa and resulting pollen attachment sites. A) Cape sugarbird *Promerops cafer* feeding on *L. lineare* subsp. *calocephalum*. Pollen is transferred via crown feathers. B) Cape sugarbird feeding on *L. catherinae*. Pollen is transferred via neck and body feathers. C) Malachite sunbird *Nectarinia famosa* feeding on *L. reflexum*. Pollen is transferred via body feathers.



Aside from adaptation to pollinators, floral adaptations may instead reflect a shift towards self-pollination (Darwin 1876; Ornduff 1969; Lande and Schemske 1985; Schemske and Lande 1985), with this “selfing syndrome” characterized by decreasing flower size and floral display (Stebbins 1970; Goodwillie et al. 2010; Sicard and Lenhard 2011). However, showy, specialized flowers often exhibit high selfing rates (reviewed in Fenster and Martén-Rodríguez 2007). One explanation for this is that since showy, specialized flowers can be vulnerable to reproductive failure by relying on a subset of pollinators, selfing facilitates reproduction when outcrossing fails (Darwin 1859; Müller 1883; Lloyd 1992; de Vos *et al.* 2013). Species may be strictly selfing or strictly outcrossing, but selfing should be viewed as a quantitative phenomenon (Becerra and Lloyd 1992) since intermediate levels of self-fertilization are common (Barrett and Eckert 1990) and evolutionarily stable (Johnston 1998). Despite receiving some attention, the few studies that use phylogenetics to test for correlations between selfing and pollination mode are limited either by not focusing on functional groups of pollinators (Schoen *et al.* 1997; de Vos *et al.* 2013), or having large proportions of selfing data missing (Martén-Rodríguez *et al.* 2010). Comparative approaches mapping breeding system and pollination mode on well-supported phylogenies would allow us to address many questions about the evolution of selfing (Fenster and Martén-Rodríguez 2007); yet such studies are surprisingly rare (but see Pérez *et al.* 2009). In this study, we perform phylogenetic generalized least squares (Martins and Hansen 1997; Pagel 1997) to test for correlated evolution between floral morphology, pollination mode, and selfing ability. We produced the first phylogeny for *Leucospermum* to address the following questions: 1) are particular floral traits correlated with different functional groups of pollinators and 2) is autonomous selfing correlated with pollinator functional groups and/or 3) floral morphology?

## Materials and methods

### *Sample collection and phylogenetic construction*

The genus *Leucospermum* is a southern African endemic comprised of 52 taxa, 49 of which reside within the greater Cape Floristic Region (CFR). CM Johnson collected all 49 representatives within the CFR, while the 3 additional taxa (*L. saxosum*, *L. gerrardi*, and *L. innovans*) were collected and sent to us for analysis (see acknowledgements for collector identification) providing a complete genus level phylogeny. Representatives of *Diastella*, *Mimetes*, *Leucadendron*, *Adenanthos*, *Isopogon*, *Aulax*, *Petrophile*, *Protea*, *Synaphea*, *Conospermum* and *Stirlingia* (Proteaceae) were also collected and sequenced as outgroups. Voucher specimens are held at the Stellenbosch University Herbarium.

Leaf tissue was harvested and stored in activated silica gel before further processing. Seven DNA sequences from the mtDNA and cpDNA (matK, rbcL, trnL intron, and trnL-trnF intergenic spacer, atpB, atpB-rbcL intergenic spacer, and rpl16 intron) and one nuclear sequence (ITS) were produced following DNA sequencing protocols described in Sauquet *et al.* (2009). Our tree is therefore based on two independent estimates of phylogeny. All sequences were aligned and edited using MUSCLE (Edgar 2004). The datasets were combined into a NEXUS matrix of 75 taxa and 6,022 characters. The chloroplast and nuclear DNA sequences in all species are available in the NCBI database (Table S4.1).

Phylogenetic relationship and the divergence times among the collected samples were obtained using Bayesian inference in BEAST version 2.1.0 (Bouckaert *et al.* 2014). The dataset partition for cpDNA and mtDNA was unlinked to assume the sequences evolved independently and set to a general time reversible (GTR) model with  $\gamma$ -distributed rate heterogeneity. We used

a Yule prior for rates of cladogenesis and ran analyses of 10 million generations sampling every 1000 generations with a burn-in of 2.5 million generations. We set nine point calibrations in the BEAST analysis based on well-known crown group ages that have been critically evaluated by Sauquet *et al.* (2009) as shown in Table S4.2 (Sauquet *et al.* 2009). The majority of the priors were set to normal as this distribution allows for bidirectional uncertainty in estimates of divergence times (Ho & Phillips 2009). A maximum clade credibility tree was generated and viewed in FigTree v. 1.4.0 (<http://tree.bio.ed.ac.uk/>).

### *Floral morphology*

We recorded floral traits of 49/52 taxa (excluding *L. saxosum*, *L. gerrardi*, and *L. innovans*) measuring traits we considered likely to be selected for by pollinators. These included style length (straight line distance from the tip of the ovary to the pollen presenter), nectar tube length (distance between where nectar is stored and where the flower can be entered), flower symmetry (actinomorphic/zygomorphic scored as 0/1), flower density (number of flowers/inflorescence), minimum and maximum flower orientation (angle of the most upward and downward facing flowers on an inflorescence; see Johnson *et al.* 2014), and flower curvature. We measured the absolute length of styles by straightening them on a ruler and derived an estimate of flower curvature by dividing the straight line style length by the absolute length. Styles with no curvature have a value of 1 and curvature increases as this value approaches zero. All traits were measured on 5 randomly chosen individuals of a population for each taxon and the mean values were used for analysis.

To test which morphological traits separate taxa, we performed a principle component analysis (PCA) using flower symmetry, style length, nectar tube length, style curvature, and

minimum orientation. Maximum orientation was omitted from the PCA because it was correlated with minimum orientation and flower density was omitted because it was correlated with style length. The PCA was performed in R (R Development Core Team, 2009).

### *Reproductive biology*

We studied the pollination biology for all 49 CFR taxa. Pollinators were categorized into the following 10 functional groups: Hymenoptera, Coleoptera, Diptera, butterfly, moth, long-proboscid fly, non-flying mammal (NFM), short-billed bird, long-billed bird crown (pollen is transferred via the crown feathers), and long-billed bird body (pollen is transferred via body feathers). Pollinator observations at a single population of all 49 CFR taxa were conducted between September 2012-September 2014. Diurnal observation times were not standardized, but were carried out for a minimum of two hours and lasted up to 12 hours (over multiple days). Observations were terminated after two hours only in the case of extreme pollinator activity. Each observation was conducted continuously by a single observer (CM Johnson) and took place on warm, clear days with little wind. Two additional hours of dusk observations were conducted for all strong scented taxa that were clearly adapted for insects (10 taxa). During observations we recorded the number of flowers probed by visitors that contacted pollen presenters, thereby acting as true pollinators. Pollinator importance was measured as the number of visits by each functional group as a proportion of the total number of recorded visits (Sakai *et al.* 2013). In addition, either live rodent traps or camera traps were set out for all geoflorous taxa (13 taxa) to test the role of non-flying mammals (NFM) as pollinators. Traps or cameras were never set during a full moon when nocturnal rodents are less active. We inspected captured rodents for the presence of *Leucospermum* pollen by sampling fur and feces with fuchsin gel cubes. All NFM pollinated species were found to be pollinated exclusively by rodents, so the issue of

standardizing visitation rate data with other pollinators was avoided as these taxa all had NFM pollinator importance values of 1.

We measured the ability of all CFR *Leucospermum* taxa to autonomously self-pollinate, excluding *L. harpagonatum*, *L. secundifolium* and *L. cordatum*, all of which are critically endangered. Bags could not be retrieved for *L. glabrum*, which was therefore excluded in the analysis. Autonomous selfing was measured by bagging 10 inflorescences for each taxon while in the bud stage, thereby excluding all potential animal pollinators, and comparing seed production with that of 10 inflorescence that were left open to pollinators. We used these values to calculate the autofertility index (Lloyd 1992; Martén-Rodríguez *et al.* 2010) for each species by dividing the average seed production after pollinator exclusion by the seed production from open pollination experiments.

#### *Comparative analyses*

If models of correlation incorporate related taxa, then observations are no longer independent as values are expected to be more similar in close than distant relatives. One way to account for this relatedness is by using phylogenetic generalized least squares (PGLS), which incorporates the covariance between taxa into the calculation of coefficients using the branch lengths of a phylogeny (Grafen 1989). To examine correlations between floral morphology, pollination mode, and autonomous selfing while accounting for phylogeny, we adopted a strategy that utilizes PGLS and a maximum credibility phylogeny (Rosas-Guerrero *et al.* 2010). A PGLS covariance matrix assumes a Brownian motion model of trait evolution i.e. that variation between tips accrues along the branches of the phylogeny at a rate proportional to the length of the branches (Orme 2013). However, not all variables necessarily correspond to this

assumption (Freckleton *et al.* 2002). Therefore we used maximum likelihood transformation of  $\lambda$  in each analysis to improve the fit of the model to the data. When  $\lambda=0$  branch lengths are ignored and data is modeled as a function of independent evolution,  $\lambda=1$  represents a model under Brownian motion, intermediate values of  $\lambda$  reflect an evolutionary process where the effect of phylogeny is weaker than Brownian motion, and if  $\lambda>1$  data exhibits more covariance than expected under Brownian motion (Pagel 1999).

Correlations between floral morphology and pollination mode were determined using univariate PGLS analyses of each floral trait as an effect of each pollinator importance value. We first looked at correlated evolution using the following broad scale functional groups of pollinators: insects (excluding long-proboscid flies), long-proboscid flies (Tabanidae), birds, and non-flying mammals. Next, we tested for floral shifts in *Leucospermum* taxa pollinated by various insects by breaking the insect functional group down into Hymenoptera, Diptera, Coleoptera, butterfly, and moth (moths and hawkmoths) and used the pollinator importance values of these groups in PGLS analyses. To test if taxa pollinated by different functional groups of bird pollinators (short-billed birds vs. long-billed birds) are correlated with different floral traits we ran PGLS analyses for bird pollinated taxa using pollinator importance values of these two groups. Lastly, we wanted to test if utilization of different pollen transfer sites on long-billed birds leads to divergent floral morphology. We ran PGLS analyses on all *Leucospermum* taxa pollinated by long-billed birds with pollinator importance by treating pollen transfer sites as functional groups. In addition we tested for correlations between autofertility and floral morphology and autofertility and pollination mode at each level. In each analysis, trees were pruned to include only the taxa for which we had data, or which were relevant to the analysis.

To account for phylogenetic uncertainty, we selected 1,000 phylogenetic trees (1 maximum credibility tree, and 999 trees generated from the last 1 million generation samplings) generated from BEAST. For each analysis administered using the maximum credibility tree we constructed a corresponding loop that ran a PGLS on the 1,000 tree dataset. From this, we report the 95% confidence interval of correlation values as well as the number of models that have a p-value of  $<0.05$ .  $\lambda$  values were assigned in each 1,000 tree analysis based on the result of the corresponding maximum credibility tree analysis in order to duplicate the parameters. All analyses were conducted using the *ppls* command in R's *caper* package (Orme *et al.* 2013).

## Results

### *Analysis of genetic differentiation between genera*

Phylogenetic reconstruction using Bayesian inference demonstrated that *Leucospermum* divergence was distinguishable using eight gene regions. The timing of divergence and overall topology of the phylogeny was consistent with previous reports on Proteaceae phylogeny (Sauquet *et al.* 2009). Among 74 nodes in the maximum clade credibility tree with outgroups that were summarized from 1,000 possible phylogenies generated by the Bayesian MCMC procedure, posterior probabilities  $>0.8$  were found for 44 nodes, while 30 nodes were supported by a posterior probability  $<0.8$ . This maximum credibility phylogeny including outgroups is provided as supplementary material (Fig S4.1). Further analysis revealed that the Bayesian inference had shown strong support (with posterior probabilities  $> 0.98$ ) for the nodes in genus divergences, but not for the *Leucospermum* species divergences. A *Leucospermum* phylogeny is shown in the form of a maximum credibility tree (Fig 4.2).

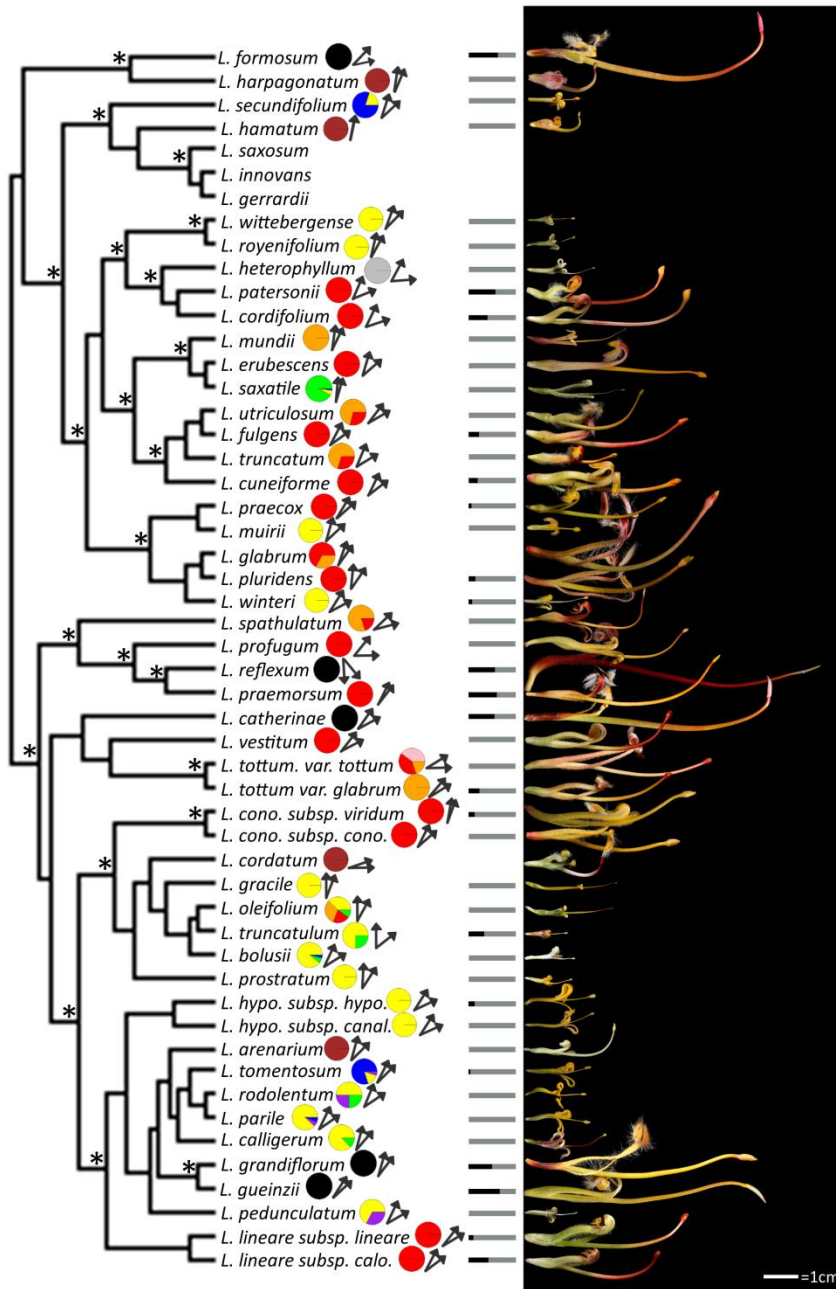


Figure 4.2. Phylogenetic relationship of floral morphology, pollination mode, and autonomous selfing in *Leucospermum*. The phylogeny is a maximum credibility tree from a Bayesian analysis of 8 genes. An asterisk denotes branches with >0.8 posterior probability. Pie-graphs show proportions of pollinator importance as follows: yellow=Hymenoptera, green=Coleoptera, purple= Diptera, Blue=butterfly, grey=moth, pink=long-proboscid fly, orange=short-billed bird, red=long-billed bird crown, black=long-billed bird body, brown=non-flying mammal. Arrows represent minimum and maximum flower orientation. Bars represent the autofertility index (black is the level of selfing).



*Correlations between floral morphology, pollination mode, and autonomous selfing*

Mean values and standard deviations of floral measurements and autofertility (Table S4.3), pollinator importance values (Table S4.4), and species identification for vertebrate pollinators (S4.5; S4.6) are provided as supplementary material. Five distinct morphological clusters emerged from our PCA analysis and are closely matched with the Hymenoptera, NFM, short-billed birds, and both long-billed bird functional groups (Fig 4.3). The only moth pollinated (*L. heterophyllum*) and long-proboscid fly pollinated (*L. tottum* var. *tottum*) taxa also stand alone in the PCA. Component 1 of the PCA is most strongly correlated with style length, nectar tube length, and minimum orientation and component 2 is most strongly correlated with flower symmetry and style curvature (Fig 4.3).

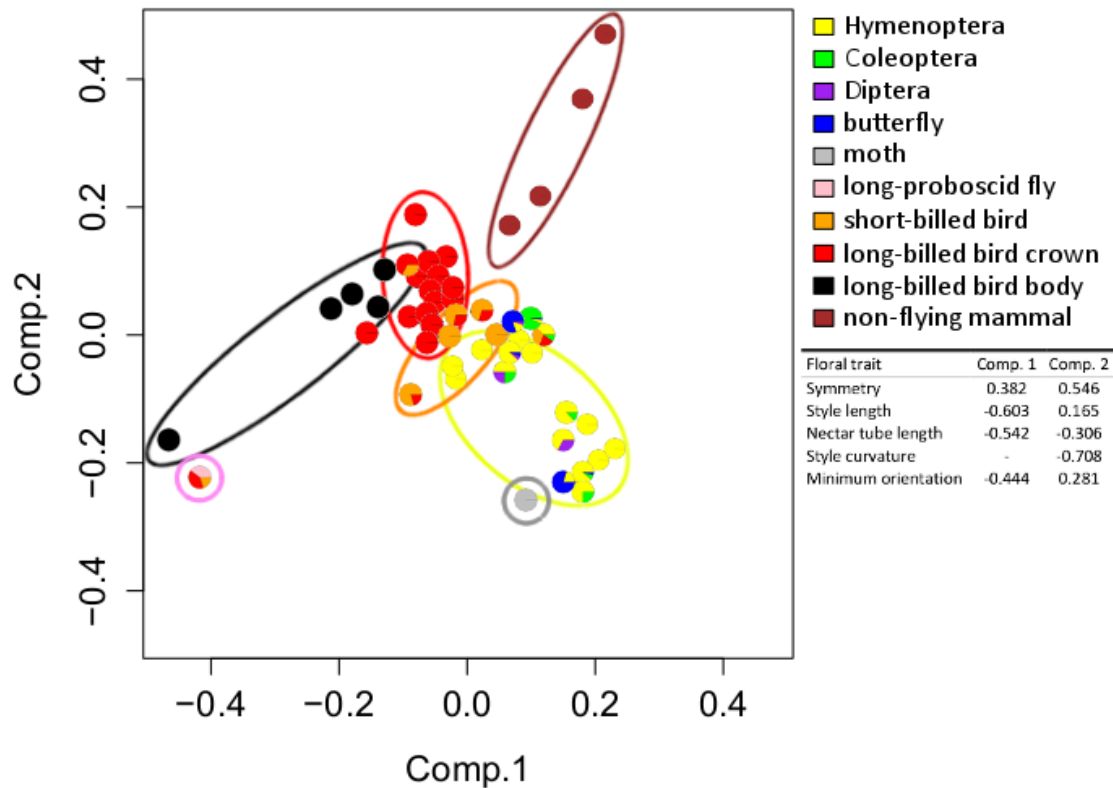


Figure 4.3. Principle component analysis of 49/52 *Leucospermum* taxa bases on 5 floral traits. Pie charts represent of the proportions of pollinator importance values for each taxa. Eigenvalues are given in the adjacent table.

Floral measurements, pollination mode, and autofertility are illustrated alongside the maximum credibility phylogeny (Fig 4.2). Flower symmetry was correlated with pollination by insect (actinomorphic) and the bird pollinator functional groups (zygomorphic)(Table 4.1). These correlations were not vulnerable to phylogenetic uncertainty (100% of  $P$ -values  $<0.05$ ).

Style length was negatively correlated with insect pollinator importance and positively correlated with bird pollinator importance (Table 4.1). Within bird pollinated taxa, style length was negatively correlated with short-billed bird pollinator importance and positively correlated with long-billed bird pollinator importance (Table 4.3). Within long-billed bird pollinated taxa, style length was positively correlated with taxa that utilize the body feather pollen transfer site (Table 4.4). None of these analyses were vulnerable to phylogenetic uncertainty (100% of  $P$ -values  $<0.05$ ).

Nectar tube length was negatively correlated with NFM pollinator importance and positively correlated with both bird and long-proboscid fly pollinator importance (Table 4.1). These correlation were not vulnerable to phylogenetic uncertainty (100% of  $P$ -values  $<0.05$ ).

In the analysis of broad scale functional groups, flower curvature was correlated with NFM pollinator importance (highly curved styles). Within the insect pollinated group, flower curvature was correlated with Hymenoptera pollinator importance (straight styles). Within bird pollinated taxa, long-billed bird pollinator importance was correlated with higher flower curvature (Table 4.2). These correlations were not vulnerable to phylogenetic uncertainty (100% of  $P$ -values $<0.05$ ).

Within insect pollinated taxa, moth pollination was negatively correlated with minimum flower orientation (Table 4.2). For taxa pollinated by long-billed birds, taxa that transfer pollen

on body feathers were negatively correlated with both minimum and maximum flower orientation (Table 4.4). These correlations were not vulnerable to phylogenetic uncertainty (98.7% of  $P$ -values  $< 0.05$ ) except for the negative correlation between taxa that utilize the long-billed bird body transfer site and minimum orientation (61.6% of  $P$ -values  $< 0.05$ ) so this result should be taken with caution.

Flower density was negatively correlated with insect pollinator importance and positively correlated with bird pollinator importance (Table 4.1). Neither correlation was vulnerable to phylogenetic uncertainty (100% of  $P$ -values  $< 0.05$ ).

Selfing was found to be negatively correlated with both insect and long-proboscid fly pollinator importance and positively correlated with bird pollinator importance (Table 4.1). Within bird pollinated taxa, selfing was positively correlated with long-billed bird pollinator importance (Table 4.3) and within long-billed bird pollinated taxa selfing was positively correlated with taxa that transfer pollen on body feathers (Table 4.4). Selfing was also positively correlated with style length (Table 4.1). These correlations were not vulnerable to phylogenetic uncertainty (100% of  $P$ -values  $< 0.05$ ).

Table 4.1. Correlations of univariate PGLS analyses between morphological traits and pollinator functional groups, selfing and pollinator functional groups, and selfing and morphological traits. Significant correlations are bolded. Maximum likelihood values of  $\lambda$  generated from the PGLS are given. 95% confidence intervals for the analyses on 1000 generated trees are provided in brackets as well as the total number of models whose p-values were significant.

| Functional group     | symmetry   | Style length  | Tube length  | Curvature  | Minimum orientation                                | Maximum orientation                               | Flower density   | selfing  |
|----------------------|--|---|--|--|--|---|--|--|
| Insects              | <b>-0.50</b><br>$\lambda=0$<br>[-0.50,-0.50]<br>1000 | <b>-24.23</b><br>$\lambda=0.974$<br>[-29.23,-17.52]<br>1000 | -2.72<br>$\lambda=0$<br>[-2.72,-2.72]<br>0               | <b>0.046</b><br>$\lambda=0.976$<br>[0.034,0.065]<br>1000 | 4.92<br>$\lambda=0.240$<br>[4.10,6.18]<br>0        | 10.53<br>$\lambda=0.289$<br>[9.70,11.64]<br>0     | <b>-17.97</b><br>$\lambda=0.928$<br>[-30.33,-12.15]<br>784 | <b>-0.093</b><br>$\lambda=0.758$<br>[-0.122,-0.081]<br>986 |
| Long-proboscid flies | 0.48<br>$\lambda=0$<br>[0.48,0.48]<br>0              | 26.26<br>$\lambda=0.895$<br>[22.65,30.39]<br>0              | <b>73.09</b><br>$\lambda=0.814$<br>[72.27,73.75]<br>1000 | 0.020<br>$\lambda=0.979$<br>[0.015,0.035]<br>0           | -78.090<br>$\lambda=0.297$<br>[-84.29,-74.40]<br>0 | -59.17<br>$\lambda=0.413$<br>[-66.32,-56.19]<br>0 | 50.67<br>$\lambda=0.986$<br>[35.22,54.95]<br>131           | <b>-0.60</b><br>$\lambda=0.970$<br>[-0.66,-0.56]<br>407    |
| Birds                | <b>0.41</b><br>$\lambda=0$<br>[0.41,0.41]<br>1000    | <b>32.55</b><br>$\lambda=0.271$<br>[31.96,33.00]<br>1000    | <b>4.71</b><br>$\lambda=0.002$<br>[4.70,4.71]<br>1000    | -0.021<br>$\lambda=1$<br>[-0.034,-0.004]<br>320          | -10.081<br>$\lambda=0.101$<br>[-10.65,-9.65]<br>0  | -10.013<br>$\lambda=0.272$<br>[-11.23,-9.36]<br>0 | <b>27.39</b><br>$\lambda=0.680$<br>[25.50,33.76]<br>1000   | <b>0.23</b><br>$\lambda=0.642$<br>[0.21,0.27]<br>1000      |
| Non-flying mammal    | 0.30<br>$\lambda=0.580$<br>[0.21,0.33]<br>0          | -11.67<br>$\lambda=0.853$<br>[-18.06,-6.68]<br>44           | <b>-9.62</b><br>$\lambda=0.166$<br>[-9.85,-9.51]<br>1000 | <b>-0.13</b><br>$\lambda=0$<br>[-0.13,-0.13]<br>1000     | 13.070<br>$\lambda=0.261$<br>[12.17,14.98]<br>0    | -0.287<br>$\lambda=0.445$<br>[-1.94,2.96]<br>0    | -18.400<br>$\lambda=0.843$<br>[-27.34,-12.09]<br>6         | -0.15<br>$\lambda=0.901$<br>[-0.27,-0.067]<br>32           |
| selfing              | 0.084<br>$\lambda=0.864$<br>[0.046,0.17]<br>47       | <b>0.008</b><br>$\lambda=0$<br>[0.008,0.008]<br>1000        | -0.003<br>$\lambda=0.945$<br>[-0.004,0.005]<br>0         | -0.58<br>$\lambda=1$<br>[-2.90,1.93]<br>255              | -0.000<br>$\lambda=0.904$<br>[-0.001,0.000]<br>0   | -0.001<br>$\lambda=0.886$<br>[-0.002-0.000]<br>0  | 0.001<br>$\lambda=0.790$<br>[0.001,0.002]<br>149           |  |

Table 4.2. Correlations of univariate PGLS analyses between morphological traits and insect pollinator functional groups and selfing and insect pollinator functional groups. Significant correlations are bolded. Maximum likelihood values of  $\lambda$  generated from the PGLS are given. 95% confidence intervals for the analyses on 1000 generated trees are provided in brackets as well as the total number of models whose p-values were significant.

| Functional group | symmetry                                      | Style length                               | Tube length                                      | Curvature   | Minimum orientation  | Maximum orientation                                | Flower density                                     | selfing                                    |
|------------------|---|--|--|---|--|--|--|--|
| Hymenoptera      | -0.022<br>$\lambda=0$<br>[-0.022,-0.022]<br>0 | -3.98<br>$\lambda=0$<br>[-3.98,-3.98]<br>0 | 1.092<br>$\lambda=0.803$<br>[0.97,2.19]<br>1     | -0.012<br>$\lambda=0.450$<br>[-0.013,-0.010]<br>0 | 1.69<br>$\lambda=0$<br>[1.69,1.69]<br>0                      | -2.09<br>$\lambda=0.586$<br>[-3.31,-0.47]<br>0     | 7.77<br>$\lambda=0.706$<br>[5.72,16.01]<br>0       | 0.014<br>$\lambda=0$<br>[0.014,0.014]<br>0 |
| Diptera          | -0.030<br>$\lambda=0$<br>[-0.030,-0.030]<br>0 | -7.78<br>$\lambda=0$<br>[-7.78,-7.78]<br>0 | -2.864<br>$\lambda=0.813$<br>[-9.09,-0.15]<br>45 | 0.038<br>$\lambda=1$<br>[-0.041,0.063]<br>106     | -40.48<br>$\lambda=0$<br>[-40.48,-40.48]<br>0                | -12.559<br>$\lambda=0.537$<br>[-17.29,-4.29]<br>0  | -4.772<br>$\lambda=0.766$<br>[-65.73,15.21]<br>0   | -0.12<br>$\lambda=0$<br>[-0.12,-0.12]<br>0 |
| Coleoptera       | 0.43<br>$\lambda=0$<br>[0.43,0.43]<br>0       | 9.48<br>$\lambda=0$<br>[9.48,9.48]<br>0    | -0.834<br>$\lambda=0.814$<br>[-1.94,-0.69]<br>0  | 0.0212<br>$\lambda=1$<br>[0.001,0.037]<br>90      | 32.723<br>$\lambda=0$<br>[32.72,32.72]<br>0                  | 19.957<br>$\lambda=0.583$<br>[18.54,21.03]<br>0    | -13.644<br>$\lambda=0.788$<br>[-23.46,-7.29]<br>0  | 0.038<br>$\lambda=0$<br>[0.038,0.038]<br>0 |
| Butterfly        | -0.021<br>$\lambda=0$<br>[-0.021,-0.021]<br>0 | -2.23<br>$\lambda=0$<br>[-2.23,-2.23]<br>0 | -0.44<br>$\lambda=0.857$<br>[-1.64,-0.004]<br>0  | -0.001<br>$\lambda=0.993$<br>[-0.014,0.017]<br>26 | 2.98<br>$\lambda=0$<br>[2.98,2.98]<br>0                      | -4.732<br>$\lambda=0.595$<br>[-6.23,3.33]<br>0     | -32.646<br>$\lambda=0.680$<br>[-41.23,-29.42]<br>1 | 0.005<br>$\lambda=0$<br>[0.005,0.005]<br>0 |
| Moth             | -0.556<br>$\lambda=0$<br>[-0.55,-0.55]<br>0   | 1.523<br>$\lambda=0$<br>[1.52,1.52]<br>0   | 0.0292<br>$\lambda=0.854$<br>[-0.24,0.24]<br>0   | 0.0047<br>$\lambda=0.990$<br>[0.0027,0.0071]<br>0 | <b>-44.058</b><br>$\lambda=0.208$<br>[-44.69,-43.13]<br>1000 | -12.617<br>$\lambda=0.588$<br>[-13.33,-11.61]<br>0 | 0.837<br>$\lambda=0.766$<br>[-4.16,6.22]<br>0      | -0.03<br>$\lambda=0$<br>[-0.03,-0.03]<br>0 |

Table 4.3. Correlations of univariate PGLS analyses between morphological traits and bird pollinator functional groups and selfing and bird pollinator functional groups. Significant correlations are bolded. Maximum likelihood values of  $\lambda$  generated from the PGLS are given. 95% confidence intervals for the analyses on 1000 generated trees are provided in brackets as well as the total number of models whose p-values were significant.

| Functional group  | Style length  | Tube length                                | Curvature   | Minimum orientation                             | Maximum orientation                             | Flower density                                      | selfing  |
|-------------------|---|--|---|---|---|---|--|
| Short-billed bird | <b>-20.0738</b><br>$\lambda=0.702$<br>[-21.76,-19.38]<br>1000 | -2.57<br>$\lambda=0$<br>[-2.57,-2.57]<br>0 | 0.034<br>$\lambda=0$<br>[0.034,0.034]<br>0              | 9.866<br>$\lambda=0.210$<br>[8.76,10.55]<br>0   | 5.070<br>$\lambda=0.259$<br>[4.13,5.94]<br>0    | -16.727<br>$\lambda=0.980$<br>[-23.53,-2.52]<br>297 | -0.20<br>$\lambda=0.638$<br>[-0.23,-0.18]<br>1       |
| Long-billed bird  | <b>21.867</b><br>$\lambda=0.580$<br>[20.98,23.27]<br>1000     | -0.13<br>$\lambda=0$<br>[-0.13,-0.13]<br>0 | <b>-0.038</b><br>$\lambda=0$<br>[-0.038,-0.038]<br>1000 | -8.939<br>$\lambda=0.199$<br>[-9.57,-7.96]<br>0 | -6.198<br>$\lambda=0.258$<br>[-6.97,-5.35]<br>0 | 14.42<br>$\lambda=0.910$<br>[7.40,20.00]<br>0       | <b>0.26</b><br>$\lambda=0.650$<br>[0.23,0.28]<br>932 |

Table 4.4. Correlations of univariate PGLS analyses between morphological traits and long-billed bird pollinator functional groups and selfing and long-billed birdpollinator functional groups. Significant correlations are bolded. Maximum likelihood values of  $\lambda$  generated from the PGLS are given. 95% confidence intervals for the analyses on 1000 generated trees are provided in brackets as well as the total number of models whose p-values were significant.

| Functional group       | Style length  | Tube length                                | Curvature                                     | Minimum orientation   | Maximum orientation   | Flower density                                 | selfing   |
|------------------------|---|--|---|---|---|--|---|
| Long-billed bird crown | -12.94<br>$\lambda=0.007$<br>[-12.97,-12.92]<br>0     | -1.70<br>$\lambda=1$<br>[-4.12,0.22]<br>14 | -0.018<br>$\lambda=0$<br>[-0.018,-0.018]<br>0 | 24.499<br>$\lambda=0.311$<br>[22.49,25.85]<br>0             | 22.689<br>$\lambda=0.278$<br>[21.17,23.79]<br>8             | 3.597<br>$\lambda=0.960$<br>[-5.51,10.61]<br>0 | -0.024<br>$\lambda=0.862$<br>[-0.10,0.008]<br>0   |
| Long-billed bird body  | <b>28.636</b><br>$\lambda=0$<br>[28.63,28.63]<br>1000 | 2.603<br>$\lambda=1$<br>[0.78,4.29]<br>4   | 0.00049<br>$\lambda=0$<br>[0.000,0.000]<br>0  | <b>-32.010</b><br>$\lambda=0.436$<br>[-34.70,-27.58]<br>616 | <b>-30.487</b><br>$\lambda=0.411$<br>[-32.62,-27.06]<br>987 | 12.942<br>$\lambda=0.974$<br>[2.35,23.52]<br>8 | <b>0.42</b><br>$\lambda=0$<br>[0.42,0.42]<br>1000 |

## Discussion

We can add *Leucospermum* to the list of CFR genera that radiated during the Miocene (Fig S1) (Linder 2005; Verboom *et al.* 2009), as well as to the short, but growing, list of phylogenetic studies that address correlated evolution between floral morphology, pollination mode, and/or breeding system. With regards to studies of correlated evolution, our study is valuable due to the large number of taxa studied in relation to genus diversity (49/52), the expansion of pollinator functional groups (10) and floral traits (7), and the integration of selfing data with pollination modes and floral morphology.

### *Pollinators and floral trait correlations*

*Flower symmetry*- We found that pollination by insect was significantly correlated with the possession of actinomorphic flowers, whereas pollination by birds was significantly correlated with the possession of zygomorphic flowers (Table 4.1). These interactions hold up to tests of phylogenetic uncertainty (100% of *P*-values < 0.05).

It is hypothesized that zygomorphy evolves from actinomorphy as a way to manipulate pollinator behavior (Neal *et al.* 1998). Hymenoptera and moths are dusted with pollen on both their anterior and ventral surfaces as they crawl and feed on *Leucospermum* inflorescences (pers. obs.). This imprecise mechanism of pollen transfer would not select for zygomorphic flowers, which explains the widespread actinomorphy within *Leucospermum* flowers visited by these functional groups.

Our data supports the long held association between zygomorphic flowers and ornithophily (Stiles 1978), as shown by the significant correlation between zygomorphic flowers and pollinator importance of birds. Aside from *L. reflexum* (Fig 4.1c), all nectar feeding birds

perch on the top of *Leucospermum* inflorescences as they forage and almost all of the bird pollinated taxa lack flowers at this location. The presence of this perch, combined with zygomorphic flowers, controls the positioning of bird visitors, which results in consistent and discrete pollen placement (Holm 1988; Neal *et al.* 1998).

*Style length*- Pollinator importance of insects was negatively correlated with style length and pollinator importance of birds was positively correlated with style length (Table 4.1). These correlations all hold up well to tests of phylogenetic uncertainty (100% of  $P$ -values $<0.05$ ).

The short styles of insect visited *Leucospermum* taxa match the length of pollinator mouthparts. Similarly, taxa pollinated by birds have the longest styles, which match their long mouthparts. Correlations between style length and bird mouthparts go even deeper, and results of our PGLS among bird pollinated taxa show that short-billed bird pollinator importance is negatively correlated with style length and long-billed bird pollinator importance is positively correlated with style length (Table 4.3). We also found style length varies among taxa pollinated by long-billed birds. Our results show that *Leucospermum* taxa that transfer pollen to the body feathers of long-billed birds are correlated with longer styles (Table 4.4). Displacements in exertion lengths have been shown to form discrete pollen attachment sites on a shared pollinator (Brown & Kodric-Brown 1979; Armbruster *et al.* 1994; Muchhala 2008) and our study supports that divergent use of long-billed birds has led in part to the floral diversification of *Leucospermum*. These correlations were not vulnerable to phylogenetic uncertainty (100% of  $P$ -values $<0.05$ ).

*Nectar tube length*- Nectar tube length is perhaps the most common morphological feature attributed to selection by pollinators (Schemske and Horvitz 1989; Hodges 1997; Johnson



1997; Johnson and Steiner 1997; Whittall and Hodges 2007; Anderson *et al.* 2010). We found that birds and long-proboscid flies were positively correlated the nectar tube length and NFM pollination was negatively correlated with nectar tube length (Table 4.1). These results were not vulnerable to phylogenetic uncertainty (100% of  $P$ -values $<0.05$ ). The positive correlation between bird pollinator importance and nectar tube length is likely driven by matching the longer mouthparts of these pollinators. Nectar tube length does not show the same divergence within taxa pollinated by birds with different bill lengths or pollen transfer sites, thus this fine scale adaptation occurs primarily through differences in the degree of style exertion rather than tube elongation. The strong positive correlation between long-proboscid fly pollination and long nectar tubes is a theme that has been repeatedly shown within the CFR (Manning and Goldblatt 1996, 1997; Goldblatt and Manning 2000; Pauw *et al.* 2009; Anderson *et al.* 2014) and our study can be added the wealth of data pointing towards a coevolved system .

Non-flying mammals are notoriously destructive foragers, and modifications to nectar storage and presentation that lessen these effects are imperative to specialize on this function group. We found modified nectar tubes in all four taxa pollinated by NFM. *L. hamatum* possess nectar tubes in the form of fused capsules that are ruptured by rodents to access nectar. *L. harpagonatum* nectar is stored in a similar fashion to *L. hamatum*, but the perianths are not fused and, instead of being ruptured, are peeled open while sipping nectar. *L. cordatum* nectar tubes are wide and untapered, allowing open access to pollinators with wide mouthparts. An extensive study was conducted on *L. arenarium* which showed how nectar was transported from deep within nectar tubes and stored in a nectar storing cup formed from fused perianth segments (Johnson and Pauw 2014). We scored all these nectar tube lengths as 0 in our PGLS analysis, as nectar storage in the form of traditional nectar “tubes” for these taxa has been abandoned.

In a similar study conducted on *Iochroma* (Smith *et al.* 2007), no correlations were found between corolla length and pollinators, leading to the conclusion that floral structure was not heavily influenced by pollinators. In *Leucospermum*, however, we found both style and nectar tube length to be strongly matched with varying pollinator mouthparts.

*Style curvature*- Whether less prevalent in nature or simply overlooked, style curvature is a morphological trait that has been largely absent from plant-pollinator studies. In *Leucospermum*, we found that insect pollinator importance was negatively correlated with flower curvature and NFM importance was positively correlated with flower curvature (Table 4.1). Within bird pollinated taxa, long-billed bird pollinator importance was positively correlated with curvature (Table 4.3). Correlations were not vulnerable to phylogenetic uncertainty (100% of  $P$ -values < 0.05).

In our discussion of insect pollinator importance with actinomorphic flowers, we interpreted the correlation as being advantageous for dusting pollinators with pollen on both their anterior and ventral surfaces as they crawl and feed on inflorescences. Similarly, we suggest this imprecise pollen attachment target does not favor selection for style curvature or for any form of elaborate inflorescence architecture in general.

Highly curved or “hooked” styles with a nectar stigma distance of 10 mm are a key trait in the rodent pollination syndrome (Rourke and Wiens 1977; Carpenter 1978; Weins *et al.* 1983; Johnson *et al.* 2001; Johnson and Pauw 2014), but selection for these traits has never been studied. The hooked styles found in NFM pollinated *Leucospermum* are the appropriate shape to actively force pollen onto the broad rostrum of a rodent as it backs out of the inflorescence (Carpenter 1978). Much more research needs to be conducted on the significance of curved

flowers in NFM pollinated Proteaceae taxa, as this relationship is repeatedly seen in both South African (Fleming and Nicolson 2003; Biccard and Midgley 2009; Johnson and Pauw 2014) and Australian (Carpenter 1978; Hackett and Goldingay 2001) genera.

*Flower orientation*- Similar to flower curvature, the role of pollinators in shaping flower orientation has received little attention (but see Fulton and Hodges 1999; Ushimaru and Hyodo 2005; Fenster *et al.* 2009b; Ushimaru *et al.* 2009). In our study, we found that pollinator importance of moths was negatively correlated with minimum flower orientation (Table 4.2) and long-billed birds that transfer pollen on body feathers were negatively correlated with both minimum and maximum flower orientation (Table 4.4). The negative correlation between minimum flower orientation and pollinator importance of long-billed birds that transfer pollen on body feathers was vulnerable to phylogenetic uncertainty (61.6% of  $P$ -values $<0.05$ ) so this result should be taken with caution.

Only one *Leucospermum* taxon, *L. heterophyllum*, is moth pollinated and it displays its flowers in a more horizontal fashion than its sister taxa, leading to the negative correlation between moth pollinator importance and minimum flower orientation. During our observations of *L. heterophyllum*, moth pollinators were seen landing on inflorescences to feed on flowers and exhibit the imprecise method of pollen transfer indicative of insect pollination in this genus. However, hawkmoths were also observed pollinating *L. heterophyllum* and these individuals instead hovered while accessing nectar. Therefore, selection for more horizontal flowers may have occurred in order to utilize hawkmoth pollinators.

The finding of negative correlations between pollinator importance of long-billed birds that transfer pollen on body feathers and minimum and maximum flower orientation provides

more evidence for floral modification of *Leucospermum* via divergent use of a pollinator. Along with increased style length, this decrease in flower orientation reflects a shift from the typical crown feather transfer site in favor of utilizing body feathers (Fig 4.1).

*Flower density*- Flower density was found to be negatively correlated with insect pollinator importance and positively correlated with bird pollinator importance (Table 4.1), but the correlation with insects was vulnerable to phylogenetic uncertainty (78.4% of  $P$ -values $<0.05$ ) so this finding should be taken with caution. Greater flower density likely translates into higher nectar rewards per inflorescence, which would support the high energy requirements of large bodied birds. Smith *et al.* (2007) found a similar correlation in *Iochroma*, where both reward and floral display were positively correlated with hummingbird importance. This also explains the negative correlation with insect pollinator importance, as these pollinators have low energy requirements.

*Selfing*- The question of how and why autonomous self-pollination evolves has garnered much attention from botanists (Stebbins 1957; Lande and Schemske 1985; Schemske and Lande 1985; de Vos *et al* 2014), yet few studies have attempted to utilize phylogenetic analyses to answer such questions (but see Schoen *et al.* 1997; Pérez *et al.* 2009; Martén-Rodríguez *et al.* 2010). We were interested in testing if autonomous selfing in *Leucospermum* was correlated with particular pollination modes and/or with floral morphology and found evidence for both. Selfing was negatively correlated with insect and long-proboscid fly pollinator importance but positively correlated with bird pollinator importance (Table 4.1). Within bird pollinated taxa, autonomous selfing was positively correlated with long-billed bird pollinator importance (Table 4.3) and within long-billed bird pollinated taxa there was a positive correlation between selfing and taxa that utilize the body feather pollen attachment site (Table 4.4). We also found that style length

was positively correlated with autonomous selfing (Table 4.1). None of these effects were vulnerable to phylogenetic uncertainty (93.2% of  $P$ -values $<0.05$ ), except for long-proboscid fly importance (40.7% of  $P$ -values $<0.05$ ), so this result should be taken with caution.

*L. tottum* var. *tottum*, which exhibits no autonomous selfing and is the only *Leucospermum* pollinated by long-proboscid flies, resides in a clade dominated by taxa that are pollinated by long-billed birds and have high selfing rates (Fig 4.2). This leads to the negative correlation between long-proboscid fly pollination and selfing. Waser *et al.* (1996) theorized that pollinator specialization leads to greater variance of reproductive success and failure, which would favor selfing in specialized taxa as a mode of reproductive assurance. *L. tottum* var. *tottum* is supplementally pollinated by short- and long-billed birds (Johnson *et al.* 2014). This functional generalization likely suppresses the need for reproductive assurance to emerge in the form of selfing. Similarly, many insect pollinated *Leucospermum* taxa are pollinated by a variety of functional groups and species specialized for one functional group, such as Hymenoptera, are ecologically generalized by the diversity of species available within this order (Ollerton *et al.* 2007).

Nectarivorous birds have large nectar requirements and track spatial and temporal variability in nectar availability, with the result that they are very heterogeneously distributed and may be absent from small populations and isolated individuals (Feinsinger 1976; Cotton 2007). In addition, regular fires in the CFR drive strong temporal fluctuations in the abundance of nectarivorous birds (Fraser and McMahon 1992; Fraser 1989; Geerts *et al.* 2012). We speculate that the unpredictability of bird visitors caused taxa specialized on these pollinators to evolve a capacity for autonomous self-pollination (Table 4.1). Large-bodied birds, such as Cape Sugarbirds and Malachite Sunbirds, show greater avoidance of small and isolated populations,

which explains the positive correlation between long-billed bird pollinator importance and autonomous selfing (Table 4.3). We also found that, among *Leucospermum* taxa pollinated by long-billed birds, taxa that utilize the body feather pollen transfer site are positively correlated with autonomous selfing. This may be explained by variation in fire regimens or community assemblages or mechanical variation promoting geitonogamy, as many of these styles are bent at the pollen presenter (Fig 4.2) but further research must be conducted in order to answer this question. Our study supports the assertions made by Martén-Rodríguez *et al.* (2010), who also found that selfing is associated with bird pollinated lineages and suggested this was due to their unpredictability as pollinators. Style length was also found to have a significant positive correlation with autonomous selfing; however, we feel this result is due to style length merely being correlated with bird pollinated taxa.

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# Chapter 5

Mutualism and coexistence between Proteaceae shrubs  
that share pollinating Sugarbirds and thieving Sunbirds

Christopher Michael Johnson and Anton Pauw

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## Abstract

The question of how ecologically similar species coexist has been one of the most useful pursuits in Ecology. Until recently, mutualism was seldom part of this debate because the best-known mutualisms occur between ecologically dissimilar species that seem unlikely to compete. However, diverse examples of mutualism among competitors are now coming to light, while new models demonstrate their stable coexistence. *Leucospermum conocarpodendron* and *Mimetes fimbriifolius* are two large shrubs in the Proteaceae that share many ecological features, but coexist in the Cape Floristic Region, one of South Africa's Biodiversity Hotspot. We studied their pollination biology and found that, although they share pollinating Cape Sugarbirds (*Promerops cafer*) and nectar thieving Sunbirds (Nectariniidae), this overlap resulted in mutualism rather than competition. The mutualism via pollinators occurs because Cape Sugarbirds concentrate on nectar-dense areas of the landscape and co-flowering enhances nectar density. The mutualism via nectar thieves occurs because Sunbirds are satiated at high floral densities with the result that their negative effect is diluted across plant species. The occurrence of mutualism, rather than the expected competition, helps to explain the frequent occurrence of pollinator sharing and convergent floral evolution.



## Introduction

On Red Hill, in South Africa's Cape Floristic Region, only two plant species attain a height of 2 m: *Leucospermum conocarpodendron* Rourke and *Mimetes fimbriifolius* Salisb. Ex Knight (Fig 5.1). Both co-occur on rocky outcrops that offer some protection against fires and, partly due to convergence, are so phenotypically similar that they are difficult to distinguish when not flowering. Both are in the Proteaceae, their leaves are broad, hairy and tipped with extra-floral nectaries, and their root systems combine surface foraging "proteoid" roots with deep, penetrating roots (Midgley *et al.* 1998). The species pair epitomizes the classic ecological question: "How do competing species coexist?" This question is particularly relevant to plants, because most species require the same resources: light, water, nitrogen, phosphorus and carbon dioxide. All else being equal, the species best at acquiring these resources should competitively exclude all others and diversity should be lost (Chesson 2000; Silvertown 2004).

Researchers studying this species pair hypothesized that coexistence was allowed by differences in regeneration niche (Grubb 1977; Midgley *et al.* 1998; Bond and Midgley 2001). In the Cape Floristic Region, decadal fires erase the above ground biomass. Most of the plant species are fire-killed and regenerate only from seed, but some resprout from buds protected underground. On Red Hill, only *L. conocarpodendron* and *M. fimbriifolius* sprout new growth from branches that remain standing above ground level, giving them the advantage in the race for height. Their above ground buds are protected by unusually thick bark. In addition, both species recruit via large ant-buried seeds that germinate after fires. Fires vary in intensity, frequency and season, but Midgley *et al.* 1998) found that recruitment and survival rates after fires were similar in the two species and thus ruled out the possibility that diversity was maintained by a diversity of fires, which sometimes favoured one species and at other times favoured the other (Midgley *et al.* 1998). Having found no evidence that fires provide temporal niche opportunities,

they suggested instead that fire allows coexistence because it keeps populations below their combined carrying capacity, such that resource competition does not occur (Midgley *et al.* 1998). This hypothesis has been offered before, but is inconsistent with the findings of Chesson and Huntly (1997), who used modelling to show that environmental harshness, such as frequent fires, does not make coexistence of competitors any more likely.

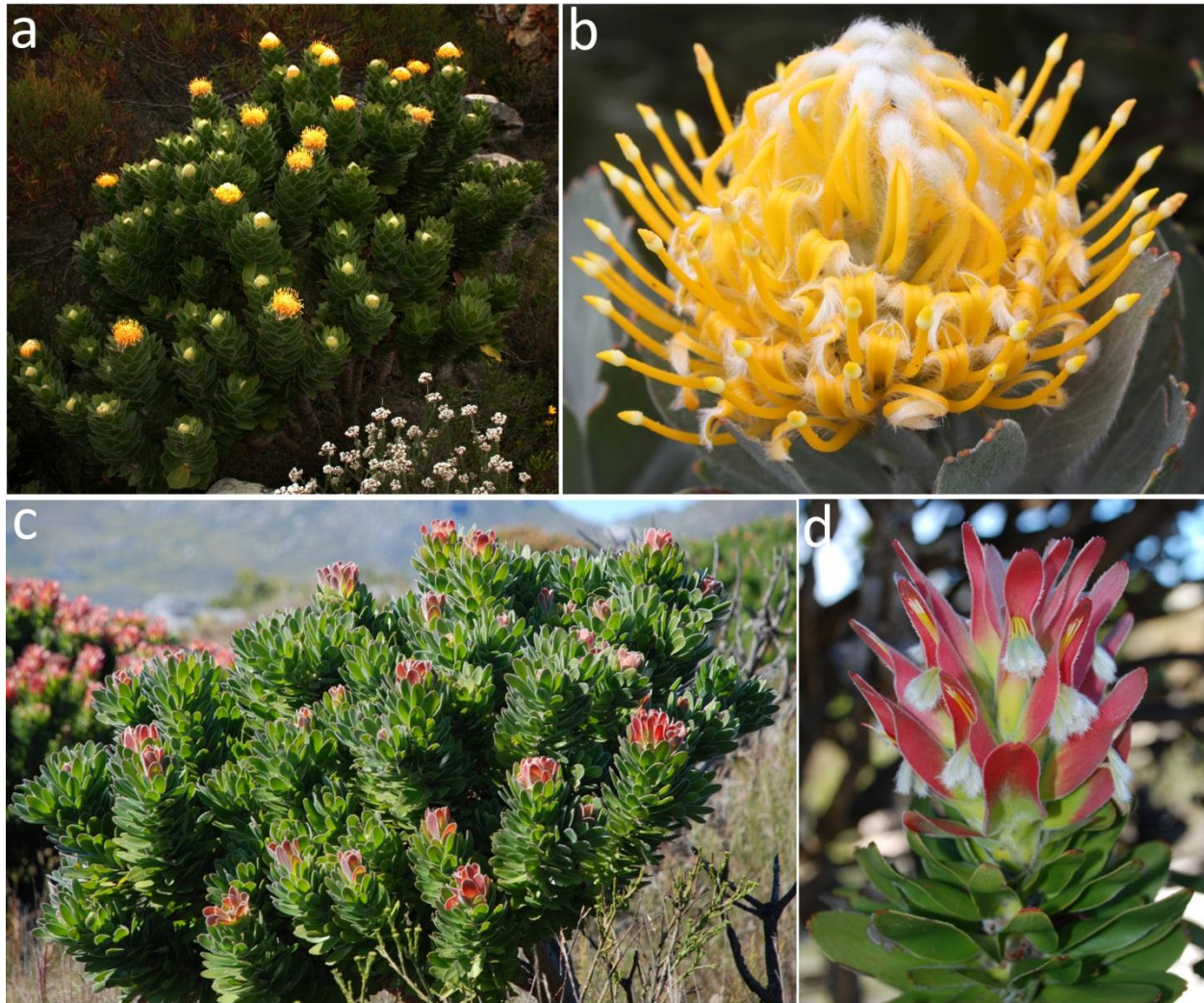


Figure 5.1. Ecologically equivalent co-flowering Proteaceae. a) *L. conocarpodendron* in the early flowering stage. b) *L. conocarpodendron* inflorescence with open and pre-anthesis flowers. c) *M. fimbriifolius* coming into flower. d) *M. fimbriifolius* inflorescence with open and pre-anthesis flowers.

With this background in mind we studied, for the first time, the pollination of *L. conocarpodendron* and *M. fimbriifolius*. It is now becoming clear that pollinators can determine patterns of coexistence (Sargent and Ackerly 2008). When plant species occupy different pollination niches, i.e. use different pollinators or flower at different times, coexistence may be possible even in the absence of other differences (Pauw 2013). When plant species share a pollination niche, much depends on how their shared pollinators respond to increases in floral density that results from co-flowering. If pollinators are satiated, co-flowering plants will compete for visits and this competition can lead to reduced seed set and displacement of the competitively inferior species. But if larger stands attract disproportionately more individuals or visits, co-flowering plants may enhance each other's pollination. In the latter case, pollinator-sharing plant species are engaged in a mutualism. Whereas hundreds of studies demonstrate competition for pollination (reviewed in: Rathcke 1983; Mitchell *et al.* 2009; Morales and Traveset 2009; Pauw 2013), relatively few have found mutualism between pollinator-sharing plant species (Thomson 1978; Waser and Real 1979; Thomson 1981; Gross *et al.* 2000; Moeller 2004; Ghazoul 2006; Molina-Montenegro *et al.* 2008; Liao *et al.* 2011).

Until recently, mutualism seldom entered the coexistence discussion, because the best-known mutualisms, such as pollination and seed dispersal, occur between rather than within trophic levels. However, examples of mutualism among competitors are now coming to light (Bruno *et al.* 2003; Crowley and Cox 2011) and include co-operative hunting among fish species (Bshary *et al.* 2006), mutualisms among plants via shared mycorrhizal fungi (Bever 2002), mutualism between ant species via shared ant-plants (Lee and Inouye 2010), mutualism between anemone fish species via shared anemones (Schmitt and Holbrook 2003), and the mutual amelioration of abiotic conditions in clump forming plants (Nunez *et al.* 1999). Encouraged by

these case studies, a flurry of new models have shown that mutualisms may facilitate coexistence of competitors (Bever 2003; Zhang 2003; Feldman *et al.* 2004; Butterfield 2009, Holland and DeAngelis 2010; Lee and Inouye 2010; Johnson and Amarasekare 2013). This belated interest is surprising, given that the first model of this sort was proposed 80 years ago by Gause and Witt (1935), who showed that stable coexistence between mutualists is obtained simply by changing the signs of the competition coefficients in the Lotka-Volterra equations from negative (competition) to positive (mutualisms). Some of the more complex new models suggest that mutualism may allow coexistence even in the absence of external density dependent factors (Johnson and Amarasekare 2013).

When evaluating the effects of co-flowering, it is important to consider both the quantity and quality of visits (Feinsinger *et al.* 1991). The benefits of co-flowering may be counteracted if pollinators transfer large amounts of pollen between species (Thomson 1982), resulting in pollen loss, stigma clogging or the formation of unfit hybrid seed (Waser 1978; Murcia and Feinsinger 1996; Bell *et al.* 2005; Morales and Traveset 2008; Aizen and Rovere 2010; Muchhala and Thomson 2012; Muchhala *et al.* 2014). Some pollinators exhibit constancy, meaning that they visit conspecifics in sequence; others are indiscriminate and move frequently between plant species (Waser 1986, Heystek *et al.* 2014). Even with indiscriminate pollinators, plants can reduce interspecific pollen transfer by attaching their pollen to different parts of the shared pollinator, and plant communities are often structured such that plant species that use the same pollen attachment site do not co-occur (Armbruster *et al.* 1994; Muchhala and Potts 2007; Waterman *et al.* 2011; Eaton *et al.* 2012). However, to the best of our knowledge, no empirical studies that have simultaneously considered the possible benefits of enhanced visitation rate via

co-flowering and the cost of interspecific pollen transfer, although Feldman *et al.* (2004) did include both factors in one model.

Both *L. conocarpodendron* and *M. fimbriifolius* conform to the bird-pollination syndrome and their flowering peaks coincide. Substantial pollination niche overlap seemed likely, but it was not clear how important insects were as pollinators, whether the species were specialized for pollination by different bird species, as often occurs in the Cape Floristic Region (Geerts and Pauw 2009), or whether the two species utilize discrete pollen attachment sites on the same bird species. In addition, detailed study is often necessary to distinguish mutualistic from antagonistic birds that consume nectar without transferring pollen. Their activity may result in reduced seed set (Inouye 1980; Irwin *et al.* 2001; Zhang *et al.* 2014), and so may be relevant to the coexistence question.

Antagonistic interactions have a well-established place in our thinking about coexistence. If two species occupy different “predation niches” they can coexist in the absence of other differences (Janzen 1970; Connell 1971; Chase and Leibold 2003). If they share the same predator species, the outcome depends again on how the predators respond to the density of their prey. If predators are satiated at high combined prey density (perhaps because predator abundance is limited by other factors), the prey species “compete” for their attention and this “competition” results in mutualism (Holt and Lawton 1994). Alternatively, co-flowering plants may collectively attract disproportionately more predators with the result that the best-defended species will displace the other (“apparent competition”, Holt 1977). Flower visitors almost always include nectar thieving species among them, but with very few exceptions, the effect of co-flowering on nectar larceny has not been considered in studies of flower visitor mediated interactions between co-occurring plant species (Irwin *et al.* 2001; Heystek and Pauw 2014).



Here we combined nectar analysis, pollinator exclusion experiments, pollinator observations and pollen load data from captured birds to answer the following questions: 1) Do *L. conocarpodendron* and *M. fimbriifolius* offer similar rewards, 2) employ the same pollinator and, 3) is seed production pollen limited? 4) Do pollinators move indiscriminately between the species or do they exhibit floral constancy? 5) Do the species utilize discrete pollen attachment sites, and 6) what is the effect of conspecific and heterospecific density on pollinator and nectar thief visitation rates in mixed stands? We explore these questions at a scale of 628 m<sup>2</sup> plots, which are an order of magnitude larger than the plots used in previous studies of flower visitor mediated interactions between plant species. Mutualistic effects are thought to occur over larger spatial scales, especially when mutualism is mediated by a pollinator with a wide range.

## Materials and Methods

**Study site and species-** The study was conducted in Fynbos vegetation along the Kleinplaas Dam Trail on Red Hill on the Cape Peninsula, South Africa (34° 10' 32.4" S; 18° 23' 56.8" E). The vegetation has three strata: at about 0.5 m there is a diverse layer of small shrubs and monocots among which are bird-pollinated *Erica* (Ericaceae) and *Chasmanthe* (Iridaceae) species; at 1 m, bright yellow *Leucadendron* (Proteaceae) is dominant; at 2 m, the stratum is composed only of the two study species. The last large fire occurred in January 2008, giving sufficient time for birds to have returned by now (Geerts *et al.* 2012).

*L. conocarpodendron* subsp. *viridum* (hereafter *L. conocarpodendron*) and *M. fimbriifolius* are both local endemics, with *M. fimbriifolius* confined to the Cape Peninsula. Although flowering overlaps broadly, *M. fimbriifolius* peaks earlier (Sept) than *L. conocarpodendron* (Oct), and *M. fimbriifolius* has a broader flowering window (May-Feb) than

*L. conocarpodendron* (Jul-Jan)(A. G. Rebelo, unpublished data). *L. conocarpodendron* inflorescences possess ~60 flowers with bright yellow, incurved pollen presenters (Fig 5.1a,b). *M. fimbriifolius* inflorescences are composed of separate headlets in multiple whorls. Each headlet consists of 4-7 flowers with long, straight pollen presenters and is enclosed in a tube formed by bracts (Fig 5.1c,d). The adaxial bract is pink and typically extends beyond the pollen presenters to form a hood.

In contrast with the 400 species of bird-pollinated plants, there are few specialist, nectar-feeding bird species in the Cape Floristic Region. On the Cape Peninsula, these are: the Cape Sugarbird (*Promerops cafer* Linnaeus, 37 g), Malachite Sunbird (*Nectarinia famosa* Linnaeus, 18 g), Orange-breasted Sunbird (*Anthobaphes violacea* Linnaeus, 10 g) and Southern Double-collared Sunbird (*Cinnyris chalybea* Linnaeus, 8 g)(Geerts and Pauw 2009). Sugarbirds are members of the Promeropidae and are equipped with brush-tipped tongues. The sunbirds are in the Nectariniidae and have tubular tongues (Pauw 1998). Additional opportunistic nectar feeders include Cape White-eyes (*Zosterops virens* Sundevall, Zosteropidae, 11g).

**Nectar properties-** We measured nectar volume and concentration from newly opened flowers in the field using capillary tubes (Blaubrand, Wertheim, Germany) and a handheld refractometer (Eclipse, Bellingham & Stanley, Basingstoke, U.K.). Nectar was kept on filter paper and sugar composition was determined by gas chromatography. Samples (N=5) were reconstituted in 80ml of methoxyamine hydrochloride (30 mg/ml in pyridine) and incubated in an oven for 2 h at 30 °C. After 2 h samples were derivatized with 140 ml of MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) for 1 h at 37 °C. One microlitre of each of the derivatized samples was then injected onto a gas chromatography column. Analyses were done at the Central Analytical Facility, Stellenbosch University, South Africa.

**Pollination experiments-** Pollination rate, and hence competition for pollinator visits, can affect plant densities if seed set is pollen limited and population growth is seed limited (Pauw and Bond 2011). We attempted to test if plants are pollen limited by supplementing the pollination of open inflorescences and comparing seed production to inflorescences that were left un-manipulated. Fresh, outcrossed pollen was added with a paintbrush. Additionally, we tested the role of birds versus insects as pollinators by caging inflorescences to exclude bird visitors while still allowing access to insects. Each treatment, including an open control, was applied to the same ten plants. Once flowers began to wilt, inflorescences were enclosed in mesh bags for ~50 days to capture seed production. The effect of treatment on seed set was compared with a Wilcoxon signed rank tests in R (R Development Core Team 2009).

**Visitor observations-** We conducted field observations in mixed stands of *L. conocarpodendron* and *M. fimbriifolius* to identify pollinators and thieves and their response to conspecific and heterospecific inflorescence density. Field observations took place on 5 warm, clear days from Aug 2-Oct 12, 2011. Observations were recorded in 30 min. intervals between 6:30 am and 11:00 am to coincide with peak bird activity. Thirty-two semi-circular plots with a radius extending from the observer to a distance of 20 meters (i.e. 628 m<sup>2</sup>) were selected to span a range of plant densities. The number of open *L. conocarpodendron* and *M. fimbriifolius* inflorescences was estimated by counting the number of inflorescences on a subset of individuals and extrapolating this over the total vegetative cover. Additional recordings included floral visitor, the number of inflorescences each visitor probed, the visitor's orientation (from on the top, or below the inflorescence) and whether pollen presenter contact was made.

**Pollinator constancy & partitioning-** To test whether Cape Sugarbirds move randomly between species or exhibit floral constancy, we observed a stand consisting of three *L.*



*conocarpodendron* and three *M. fimbriifolius* plants in peak flower. A Sugarbird had a 40% random expectation of moving between conspecific individuals (because the plant from which the bird departs is excluded)(Heystek *et al.* 2014). We compared observed movements with expected using a Pearson's Chi-squared test.

Data from mist-netted birds provided evidence of the effectiveness of different visitors as well as where pollen was attached. Mist-netting took place over three days (10/10; 11/10; 18/10/2011). Using equal effort, samples were taken from the crown and throat region with fuchsin gel cubes of approximately the same size (Beattie 1971). Samples were melted onto slides and pollen grains were counted using a compound light microscopy. *L. conocarpodendron* pollen grains are larger isosceles triangles with concave sides in polar view; *M. fimbriifolius* pollen grains are smaller equilateral triangles with straight sides. Analysis of pollen loads (crown vs. throat) were conducted using a Wilcoxon signed rank test.

**Competition for visits-** Bird species were categorized into functional groups (pollinators or thieves) based on whether or not they made pollen presenter contact. We constructed generalized linear models with the number of probes per 30 minutes by each functional group as dependent variables and *L. conocarpodendron* and *M. fimbriifolius* inflorescence density and their interaction as predictor variables. The same model was run for visits to *L. conocarpodendron* and *M. fimbriifolius*. The model had a Poisson error structure and a log link function. All analyses were done in R (R\_Core\_Team 2013).

## Results

**Nectar properties-** *L. conocarpodendron* produced 9.7  $\mu$ l (s.d.= 1.72, N=5) of nectar per flower with a 18.2% sugar concentration (w/w, s.d.=1.67, N=5). The sugar consists almost

entirely of fructose (73.71%, s.d.=0.22, N=5) and glucose (26.14%, s.d.=0.29, N=5) with small traces of sucrose (0.14%, s.d.=0.071, N=5) and no xylose (N=5). An inflorescence in full flower contained 57 flowers (s.d.=1.41, N=5). *M. fimbriifolius* produced 28.7  $\mu\text{l}$  (s.d.=4.7, N=5) of nectar per headlet, which translates into 4.36  $\mu\text{l}$  (s.d.=0.70, N=5) per flower. Sugar concentration was 22.5% (w/w, N=5, s.d.= 0.49) and sugars consist almost entirely of fructose (74.73%, s.d.=1.05, N=5) and glucose (25.13%, s.d.=0.10, N=5) with small traces of sucrose (0.12%, s.d.=0.13, N=5) and no xylose (N=5). In full flower an inflorescence contains 121.6 flowers (s.d.=10.781, N=5).

***Manipulative pollination experiments-*** Unfortunately, we were only able to retrieve seed set data for *L. conocarpodendron*. We found no significant difference in seed production between open (mean=4.5, S.D.= 2.42) and pollen supplemented (mean=4.8, S.D.=1.83) inflorescences ( $p=0.719$ ,  $V=23.5$ ,  $N=10$ , Wilcoxon signed rank test). Seed production was significantly reduced when only insects were allowed access to flowers (mean=0.9, S.D.=0.94,  $p<0.001$ ,  $V=6$ ,  $N=10$ , Wilcoxon signed rank test). Autogamous (bagged) seed production was also significantly lower than open pollination (mean=0.6, S.D.=0.82,  $p<0.001$ ,  $V=0$ ,  $N=10$ , Wilcoxon signed rank test).

***Visitor observations-*** Among the visitors to *L. conocarpodendron*, Cape Sugarbirds and Malachite Sunbirds perch on the apex of the inflorescence and forage downwards, contacting pollen presenters on their crown. Orange-breasted Sunbirds, Southern double-collared Sunbirds and Cape White-eyes forage from underneath inflorescences and fail to contact pollen presenters (Table 5.1; 5.2). Cape Sugarbirds were the only effective pollinators observed at *M. fimbriifolius*. They sit on the bracts at the apex of the inflorescence and contact pollen presenters with their throats while foraging downwards. Orange-breasted Sunbirds, Southern double-collared

Sunbirds and Cape White-eyes forage from underneath inflorescences and fail to contact pollen presenters. Hymenopterans were occasionally seen foraging on *L. conocarpodendron* and *M. fimbriifolius*, but were never seen contacting pollen presenters or collecting pollen. Since Cape sugarbirds and Malachite sunbirds are the only visitors to contact pollen presenters we grouped the remaining bird species into one category (nectar thieves). Malachite Sunbirds were very infrequent; including them with Cape Sugarbirds (pollinators) did not make a qualitative difference to the analysis results, so we excluded them from the main analyses.

Table 5.1. Mean visitation rates of birds (probes.inflorescence<sup>-1</sup>.30min<sup>-1</sup>) to two Proteaceae species followed by the percentage of legitimate visits.

|                            | Cape Sugarbird | Malachite Sunbird | Orange-breasted Sunbird | Southern Double-collared Sunbird | Cape White-eye |
|----------------------------|----------------|-------------------|-------------------------|----------------------------------|----------------|
| <i>L. conocarpodendron</i> | 0.359 (98)     | 0.035 (100)       | 0.113 (0)               | 0.012 (0)                        | 0.003 (0)      |
| <i>M. fimbriifolius</i>    | 0.212 (100)    | 0                 | 0.182 (1)               | 0.005 (0)                        | 0.007 (0)      |

Table 5.2. Number of *L. conocarpodendron* and *M. fimbriifolius* pollen grains sampled from the crown and throat of captured birds (median, range).

| Visitor                 | N  | <i>L. conocarpodendron</i> pollen grains |              | <i>M. fimbriifolius</i> pollen grains |              |
|-------------------------|----|--|--------------|---------------------------------------|--------------|
|                         |    | Crown                                    | Throat       | Crown                                 | Throat       |
| Cape Sugarbird          | 6  | 761.5 (18-6038)                          | 264 (5-1912) | 0                                     | 27.5 (3-143) |
| Orange-breasted Sunbird | 10 | 1.5 (0-15)                               | 2 (0-7)      | 0 (0-17)                              | 0            |
| Malachite Sunbird       | 2  | 5 (4-6)                                  | 1 (0-2)      | 0                                     | 0            |
| Cape White-eye          | 1  | 2  | 0            | 0                                     | 0            |

**Pollinator constancy & partitioning-** We found that Cape sugarbirds do not show constancy and instead move randomly between species. Of 33 recorded transitions, 17 were conspecific compared to 13.2 predicted ( $\chi^2=36.167$ ,  $df=33$ ,  $p=0.333$ ).

*L. conocarpodendron* pollen is deposited more often on the Cape Sugarbird's crown than throat feathers ( $p=0.031$ ,  $V=0$ ,  $N=6$ , Wilcoxon signed rank test, Table 5.2). In contrast, *M. fimbriifolius* pollen was found only on the Cape Sugarbird's throat ( $p=0.036$ ,  $V=21$ ,  $N=6$ , Wilcoxon signed rank test, Table 5.2). *L. conocarpodendron* and *M. fimbriifolius* pollen on Orange-breasted sunbirds and Cape White-eyes (Table 5.2) is likely the result of being showered with pollen that is dislodged from pollen presenters in the process of stealing nectar from below the inflorescence and is therefore unlikely to be transferred to stigmas.

**Competition for visits-** The number of pollinating Cape Sugarbird visits to both Proteaceae species is highest when their collective density is highest (significant positive interaction terms, Table 5.3; Fig 5.2a,b). In contrast, in both Proteaceae species the number of visits by nectar thieving birds is negatively impacted by increasing density of the co-occurring species (negative interaction terms, Table 5.3; Fig 5.2c,d). This effect is significant for *M. fimbriifolius*, but not for *L. conocarpodendron*.

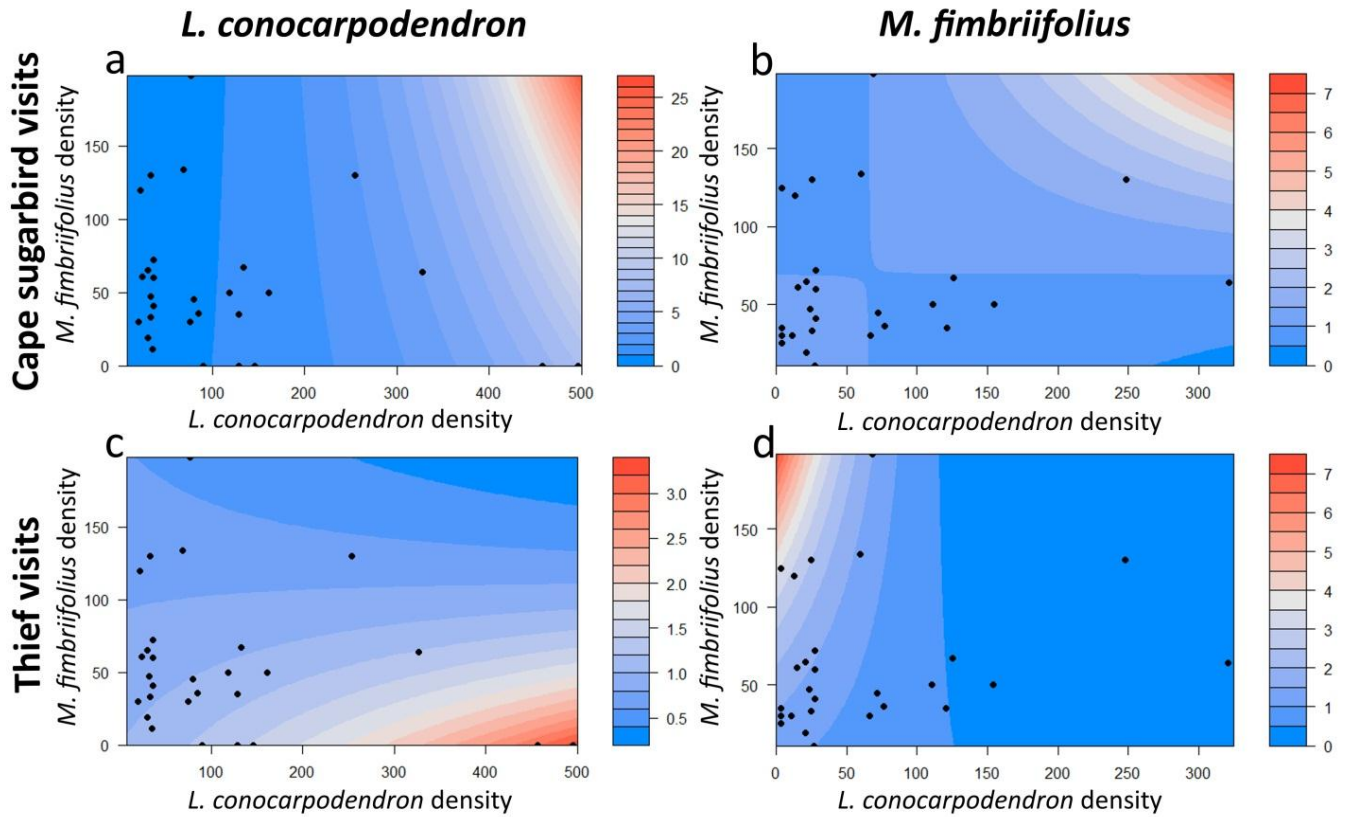


Figure 5.2. Predicted rate at which pollinating Cape Sugarbirds and nectar thieving birds visit *L. conocarpodendron* and *M. fimbriifolius* ( $\text{probes.inflorescence}^{-1} \cdot 30\text{min}^{-1}$ , red = high, blue = low, GLM, Table 5.3). Plots show the interactive effect of *L. conocarpodendron* and *M. fimbriifolius* density ( $\text{inflorescences} \cdot 628 \text{ m}^{-2}$ ). Dots indicate the spread of observed densities.

Table 5.3. Effect of conspecific and heterospecific inflorescence density on visitation rate (probes.inflorescence<sup>-1</sup>.30min<sup>-1</sup>) by pollinating Cape Sugarbirds and nectar thieving Sunbirds to *L. conocarpodendron* and *M. fimbriifolius* analyzed with a generalized linear model with a Poisson error structure and a logit link function (Fig. 2).

| Focal species              | Predictor variable   | Visitor    | Estimate | Std. Error | z-value | p-value        |
|----------------------------|--|------------|----------|------------|---------|----------------|
| <i>L. conocarpodendron</i> | <i>L. conocarpodendron</i> density                           | Sugarbirds | 0.00524  | 0.00026    | 20.56   | < <b>0.001</b> |
| <i>L. conocarpodendron</i> | <i>M. fimbriifolius</i> density                              | Sugarbirds | -0.00251 | 0.00150    | -1.504  | 0.133          |
| <i>L. conocarpodendron</i> | <i>L. conocarpodendron</i> x <i>M. fimbriifolius</i> density | Sugarbirds | 0.00002  | <0.00001   | 2.557   | <b>0.011</b>   |
| <i>L. conocarpodendron</i> | <i>L. conocarpodendron</i> density                           | Thieves    | 0.00239  | 0.00048    | 4.947   | < <b>0.001</b> |
| <i>L. conocarpodendron</i> | <i>M. fimbriifolius</i> density                              | Thieves    | -0.00243 | 0.00240    | -1.011  | 0.312          |
| <i>L. conocarpodendron</i> | <i>L. conocarpodendron</i> x <i>M. fimbriifolius</i> density | Thieves    | -0.00002 | 0.00001    | -1.400  | 0.161          |
| <i>M. fimbriifolius</i>    | <i>L. conocarpodendron</i> density                           | Sugarbirds | -0.00409 | 0.00182    | -2.249  | <b>0.025</b>   |
| <i>M. fimbriifolius</i>    | <i>M. fimbriifolius</i> density                              | Sugarbirds | -0.00391 | 0.00185    | -2.112  | <b>0.035</b>   |
| <i>M. fimbriifolius</i>    | <i>L. conocarpodendron</i> x <i>M. fimbriifolius</i> density | Sugarbirds | 0.00006  | 0.00002    | 2.994   | <b>0.003</b>   |
| <i>M. fimbriifolius</i>    | <i>L. conocarpodendron</i> density                           | Thieves    | -0.00608 | 0.00367    | -1.807  | 0.071          |
| <i>M. fimbriifolius</i>    | <i>M. fimbriifolius</i> density                              | Thieves    | 0.00969  | 0.00191    | 5.077   | < <b>0.001</b> |
| <i>M. fimbriifolius</i>    | <i>L. conocarpodendron</i> x <i>M. fimbriifolius</i> density | Thieves    | -0.00009 | 0.00004    | -2.155  | <b>0.031</b>   |

## Discussion

*L. conocarpodendron* and *M. fimbriifolius* flower together, secrete large volumes of dilute nectar, and employ the same pollinator, the Cape Sugarbird. The much smaller Sunbirds, Cape White-eyes and honeybees do not contact the pollen presenters of either species, but thieve nectar (Table 5.2). Seed set data for *L. conocarpodendron* extends the importance of Cape Sugarbirds. Seed set per inflorescence was near zero when only insects were allowed access to flowers and autogamous (bagged) seed set was negligible.

Sugarbirds move indiscriminately between the two plant species, but interspecific pollen transfer might be reduced by separation of the pollen loads on the body of the pollinator. *L. conocarpodendron* pollen is placed predominantly on the crown feathers whereas *M. fimbriifolius* pollen is placed on the throat feathers, although *M. fimbriifolius* is at a greater risk of stigma clogging due to the presence of *L. conocarpodendron* pollen on the neck feathers of

birds (Table 5.2). Further afield in the Cape Floristic Region, the study species are replaced by allopatric congeners, and since pollen attachment sites are mostly conserved within the genera, the mechanism of pollinator partitioning described here likely operates in many other communities where bird-pollinated *Mimetes* and *Leucospermum* species co-occur.

Our observations show that pollination biology can be added to the long list of ecological traits that *L. conocarpodendron* and *M. fimbriifolius* have in common (Midgley *et al.* 1998). In the case of pollination, however, similarity can lead to mutualism. The mutualism occurs via two different routes, one involving pollinators, the other nectar thieves. The mutualism via pollinators occurs because Cape Sugarbirds concentrate on nectar-dense areas of the landscape and co-flowering enhances nectar density (Table 5.3; Fig 5.2a,b). The mutualism via nectar thieves occurs because Sunbirds are satiated at high floral densities with the result that their negative effect is diluted across two plant species (Table 5.3; Fig 5.2c,d).

Why do the pollinating Cape Sugarbirds concentrate, whereas the nectar thieving birds do not? The heavy Cape Sugarbirds have large nectar requirements and it must be important for them to seek out dense nectar resources. In contrast, the nectar thieves are mainly small-bodied Orange-breasted Sunbirds that focus on the delicate, widely dispersed flowers of the genus *Erica*, not the Proteaceae (Skead 1967; Rebelo *et al.* 1984). It is also probable that the Sunbirds are unable to concentrate on dense patches of Proteaceae, because they are chased away by the larger Cape Sugarbirds (Wooller 1982; Geerts and Pauw 2009).

The demonstration of mutualism between plant species sharing a pollinator helps to explain the frequent occurrence of pollination guilds consisting of plants that share a pollinator species and a syndrome of floral traits. In the Cape Floristic Region, for example, there are about

80 bird-pollinated Proteaceae species, and plots with a diameter of 500 m contain up to 8 of these (Heystek and Pauw, unpublished data). Although it is difficult to prove that mutualism is the very mechanism of coexistence, at least the demonstration of positive rather than negative interactions makes it easier to understand floral trait convergence, especially when accompanied by divergence in morphological traits that determine pollen attachment site on the body of the pollinator (Brown and Kodric-Brown 1979; Pauw 2006).

Our analyses focussed on interspecific interactions and neglected detailed analyses of intraspecific interactions mainly because we obtained too few data points with which to confidently characterise the shape of the relationship between conspecific density and the number of pollinator visits to the patch. The shape of this relationship is critical: if the number of visits observed increases linearly with conspecific density, there is no per capita density dependent effect, but if the increase is accelerating or decelerating there is evidence for positive or negative density dependence, respectively (Feldman 2006). Statistically distinguishing between these possible functions, and others such as sigmoidal or hump-backed (Rathcke 1983), was not feasible in this study. A detailed study of intraspecific effects will be fascinating.

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# Chapter 6

## Conclusions

My research on the genus *Leucospermum* supports the common assertion that pollinators are key agents of selection on angiosperm morphology and diversity (Grant and Grant 1965; Stebbins 1970; Johnson; Hapeman and Inoue 1997; Johnson *et al.* 1998; Pérez *et al.* 2006). However, angiosperm diversification is not simply the result of a direct shift from one pollinator functional group to another. Instead, the process is gradual, and pollinator driven adaptive divergence experiences an intermediate stage of double function where both the ancestral and novel pollinators can provide a pollinator service (Stebbins 1970; Steiner 1998). Although pollinator selection can ultimately lead to morphological traits that appear specialized for a specific pollinator or functional group, the ancestral pollinator often remains as a supplemental pollinator that facilitates pollination without a fitness trade-off (Macior 1986; Sahley 1996; Aigner 2004; Devoto *et al.* 2006; Muchhala *et al.* 2008; Chalcoff *et al.* 2012). This appears to be the case with *Leucospermum tottum* var. *tottum*. We found this taxon to be highly adapted to accommodate long-proboscid flies along with being successfully pollinated by birds, which is the dominant pollination mode within its clade. Its localized sister taxon, *L. tottum* var. *glabrum*, specializes on bird pollinators and by doing so exhibits the ubiquitous bird-pollination syndrome. *L. tottum* var. *glabrum* has also developed the ability to autonomously self-pollinate, likely as a mode of reproductive assurance to supplement its specialization. These results, as presented in Chapter 2, provide valuable insights into the evolutionary process in the early stages of pollinator driven speciation.

There is a diverse pool of prospective pollinators within an ecosystem, each with the potential to elicit selection on angiosperms. Yet, certain pollination modes are studied more thoroughly than others. For example, studies of ornithophilous and entomophilous pollination modes have received considerable attention in both an evolutionary and ecological context, but

the few studies focused on non-flying mammal pollination are highly derivative and frequently end at validation of the pollination mode (Hackett and Goldingay 2001; Wooller and Wooller 2003; Kleizen *et al.* 2008; Letten and Midgley 2009; Wester *et al.* 2009; Turner *et al.* 2011; Wester 2011). We used our finding that *Leucospermum arenarium* is pollinated by mice and gerbils as an opportunity to explore features specific to this pollination mode and provide rare insights into this interaction. Specifically we wanted to show how nectar storage is modified to alleviate the destructive nature of these pollinators, test if geoflory, a common feature in non-flying mammal pollinated taxa, is essential for successful pollination, and study the rate of pollen loss due to pollinator grooming. The impact of pollinator grooming on pollen loss has time and time again been credited as the primary source of reduction to the male-phase fitness of angiosperms, yet empirical studies directly addressing pollinator grooming had been non-existent. Our study in Chapter 3 highlights a fascinating mode of nectar transport that allows non-flying mammals to sip nectar without damaging flowers. In addition, we provide evidence that non-flying mammals are adept climbers who can access inflorescences located above-ground. This result questions the assertion that geoflory is the result of selection by non-flying mammal pollinators (Rourke and Wiens, 1977). Lastly, we show that active grooming by non-flying mammals drastically reduces outcrossing. This raises the question of why angiosperms would adapt to such a wasteful, sedentary pollinator.

In order to test if pollinators select for *Leucospermum* morphology, we conducted a macroevolutionary study of floral divergence within *Leucospermum* as correlated with shifts in reproductive strategies. Along with producing the first *Leucospermum* phylogeny, we performed phylogenetic generalized least squares analyses and found that pollinator importance of insects, long-proboscid flies, non-flying mammals and birds are all correlated with specific floral traits.

Further demarcating bird pollinators into functional groups, we found that floral morphology is correlated with the utilization of birds with different bill lengths as well as divergent use of pollen transfer sites on long-billed birds. This highlights that a diverse array of pollinators are responsible, at least in part, for the diversification of *Leucospermum*, rather than strictly the result of abiotic factors. In addition, we found that autonomous self-pollination was positively correlated in taxa pollinated by long-billed birds. We suggest that the presence of these pollinators is erratic temporally, and autonomous selfing evolves in these taxa as a form of reproductive assurance.

Pollinators can not only drive diversification, but can also act to maintain it. Knowing this, we wanted to address the question of how ecological equivalent species that share pollination modes coexist, a question that has puzzled botanists and community ecologists alike. The results of this chapter show that our two “competing species,” *L. conocarpodendron* and *Mimetes fimbriifolius*, utilize discrete pollen attachment sites on a shared pollinator and that coexistence leads to a greater number of pollinator visits to each species, because co-flowering enhances nectar density. We also added a novel aspect to this study by incorporating nectar thieves, whose presence has an antagonistic effect (Inouye 1980, Irwin *et al.* 2001, Zhang *et al.* 2014). We found that nectar thieves were satiated at high floral densities and that co-flowering dilutes the thieving rate in communities. Overall we found that, instead of competition, the interaction between these two species represents a mutualism.

Our research, as a whole, highlights the importance of pollinators in floral evolution, lineage diversification and community composition. We provide valuable additions to the pollination literature by addressing overlooked plant-pollinator relationships and by broadening the scope of studies on floral adaptation. We also add to the literature by addressing the question

of community composition both from a pollinator and antagonist perspective. Overall, this research contributes to our understanding of angiosperm community interactions and the adaptive landscape, which helps to explain the origin and maintenance of angiosperm diversity.

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# Appendix A

Supplemental Figures & Tables

Table S2.1. GenBank accession numbers for all collected taken. An individual voucher specimen for each sample population is stored at the Stellenbosch University Herbarium.

| Samples ID | Species/taxa                                      | GenBank Accession Number |              |                        |  |  |                       |              |              |
|------------|---|--------------------------|--------------|------------------------|--|--|-----------------------|--------------|--------------|
|            |   | <i>rbcL</i>              | ITS          | <i>rpl16</i><br>intron | <i>trnL-trnF</i><br>Intergenic<br>spacer | <i>atpB-rbcL</i><br>Intergenic<br>spacer | <i>trnL</i><br>intron | <i>matK</i>  | <i>atpB</i>  |
| 10S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>287             | KJ481<br>322 | KJ481<br>391           | KJ4814<br>53                             | KJ4815<br>21                             | KJ481<br>589          | KJ481<br>699 | KJ481<br>738 |
| 11S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>288             | KJ481<br>323 | KJ481<br>392           | KJ4814<br>54                             | KJ4815<br>22                             | KJ481<br>590          | KJ481<br>700 | KJ481<br>739 |
| 12S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>289             | KJ481<br>324 | KJ481<br>393           | KJ4815<br>20                             | KJ4815<br>23                             | KJ481<br>591          | KJ481<br>657 | KJ481<br>740 |
| 13S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>290             | KJ481<br>325 | KJ481<br>394           | KJ4814<br>55                             | KJ4815<br>24                             | KJ481<br>592          | -            | KJ481<br>778 |
| 14S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>259             | KJ481<br>326 | KJ481<br>395           | KJ4814<br>56                             | KJ4815<br>25                             | KJ481<br>593          | KJ481<br>702 | KJ481<br>723 |
| 15S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>291             | KJ481<br>327 | KJ481<br>396           | KJ4814<br>57                             | KJ4815<br>26                             | KJ481<br>594          | KJ481<br>703 | KJ481<br>741 |
| 16S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>292             | KJ481<br>328 | KJ481<br>397           | KJ4814<br>58                             | KJ4815<br>27                             | KJ481<br>595          | KJ481<br>704 | KJ481<br>779 |
| 17S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>293             | KJ481<br>329 | KJ481<br>398           | KJ4814<br>59                             | KJ4815<br>28                             | KJ481<br>596          | KJ481<br>684 | KJ481<br>780 |
| 18S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | -                        | KJ481<br>330 | -                      | KJ4814<br>60                             | KJ4815<br>29                             | KJ481<br>597          | KJ481<br>705 | KJ481<br>742 |
| 19S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>294             | KJ481<br>331 | KJ481<br>399           | KJ4814<br>61                             | KJ4815<br>30                             | KJ481<br>598          | KJ481<br>658 | KJ481<br>743 |
| 20S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>260             | KJ481<br>332 | KJ481<br>400           | KJ4814<br>62                             | KJ4815<br>31                             | KJ481<br>599          | KJ481<br>706 | KJ481<br>744 |
| 21S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>295             | KJ481<br>333 | KJ481<br>401           | KJ4814<br>63                             | KJ4815<br>32                             | KJ481<br>600          | KJ481<br>707 | KJ481<br>781 |
| 22S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>296             | KJ481<br>334 | KJ481<br>402           | KJ4814<br>64                             | KJ4815<br>33                             | KJ481<br>601          | KJ481<br>659 | KJ481<br>724 |
| 23S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>297             | KJ481<br>335 | KJ481<br>403           | KJ4814<br>65                             | KJ4815<br>34                             | KJ481<br>602          | KJ481<br>708 | KJ481<br>745 |
| 24S        | <i>Leucospermum tottum</i><br><i>var. tottum</i>  | KJ481<br>279             | KJ481<br>336 | KJ481<br>404           | KJ4814<br>66                             | KJ4815<br>35                             | KJ481<br>603          | KJ481<br>709 | KJ481<br>746 |
| 25S        | <i>Leucospermum vestitum</i>                      | -                        | KJ481<br>337 | KJ481<br>405           | KJ4814<br>67                             | KJ4815<br>36                             | KJ481<br>604          | KJ481<br>660 | KJ481<br>782 |
| 27S        | <i>Leucospermum vestitum</i>                      | KJ481<br>298             | KJ481<br>338 | KJ481<br>406           | KJ4814<br>68                             | KJ4815<br>37                             | KJ481<br>605          | KJ481<br>661 | KJ481<br>747 |
| 28S        | <i>Leucospermum vestitum</i>                      | KJ481<br>299             | KJ481<br>339 | KJ481<br>407           | KJ4814<br>69                             | KJ4815<br>38                             | KJ481<br>606          | KJ481<br>662 | KJ481<br>748 |
| 29S        | <i>Leucospermum vestitum</i>                      | KJ481<br>300             | KJ481<br>340 | KJ481<br>408           | KJ4814<br>70                             | KJ4815<br>39                             | KJ481<br>607          | KJ481<br>685 | KJ481<br>725 |
| 31S        | <i>Leucospermum tottum</i><br><i>var. glabrum</i> | KJ481<br>261             | KJ481<br>341 | KJ481<br>409           | KJ4814<br>71                             | KJ4815<br>40                             | KJ481<br>608          | KJ481<br>686 | KJ481<br>749 |
| 32S        | <i>Leucospermum tottum</i><br><i>var. glabrum</i> | KJ481<br>262             | KJ481<br>342 | KJ481<br>410           | KJ4814<br>72                             | KJ4815<br>41                             | KJ481<br>609          | KJ481<br>710 | KJ481<br>726 |
| 33S        | <i>Leucospermum tottum</i><br><i>var. glabrum</i> | KJ481<br>263             | KJ481<br>343 | KJ481<br>411           | KJ4814<br>73                             | KJ4815<br>42                             | KJ481<br>610          | KJ481<br>711 | KJ481<br>750 |
| 34S        | <i>Leucospermum tottum</i><br><i>var. glabrum</i> | KJ481<br>301             | KJ481<br>344 | KJ481<br>412           | KJ4814<br>74                             | KJ4815<br>43                             | KJ481<br>611          | KJ481<br>687 | KJ481<br>751 |

|     |   |              |              |              |              |              |              |              |              |
|-----|---|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 35S | <i>Leucospermum tottum</i><br>var. <i>glabrum</i> | KJ481<br>264 | KJ481<br>345 | KJ481<br>413 | KJ4814<br>75 | KJ4815<br>44 | KJ481<br>612 | KJ481<br>676 | KJ481<br>783 |
| 36S | <i>Leucospermum tottum</i><br>var. <i>glabrum</i> | KJ481<br>302 | KJ481<br>346 | KJ481<br>414 | KJ4814<br>76 | KJ4815<br>45 | KJ481<br>613 | KJ481<br>677 | KJ481<br>752 |
| 37S | <i>Leucospermum tottum</i><br>var. <i>glabrum</i> | KJ481<br>303 | KJ481<br>347 | KJ481<br>415 | KJ4814<br>77 | KJ4815<br>46 | KJ481<br>614 | KJ481<br>678 | KJ481<br>753 |
| 38S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>304 | KJ481<br>348 | KJ481<br>416 | KJ4814<br>78 | KJ4815<br>47 | KJ481<br>615 | KJ481<br>712 | KJ481<br>754 |
| 39S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>305 | KJ481<br>349 | KJ481<br>417 | KJ4814<br>79 | KJ4815<br>48 | KJ481<br>616 | KJ481<br>679 | KJ481<br>755 |
| 40S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>265 | KJ481<br>350 | KJ481<br>418 | KJ4814<br>80 | KJ4815<br>49 | KJ481<br>617 | KJ481<br>680 | KJ481<br>727 |
| 41S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>306 | KJ481<br>351 | KJ481<br>419 | KJ4814<br>81 | KJ4815<br>50 | KJ481<br>618 | KJ481<br>681 | KJ481<br>756 |
| 42S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>266 | KJ481<br>352 | KJ481<br>420 | KJ4814<br>82 | KJ4815<br>51 | KJ481<br>619 | KJ481<br>663 | KJ481<br>757 |
| 43S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>307 | KJ481<br>353 | KJ481<br>421 | KJ4814<br>83 | KJ4815<br>52 | KJ481<br>620 | KJ481<br>688 | KJ481<br>758 |
| 44S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>308 | KJ481<br>354 | KJ481<br>422 | KJ4814<br>84 | KJ4815<br>53 | KJ481<br>621 | KJ481<br>682 | KJ481<br>759 |
| 45S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>309 | KJ481<br>355 | KJ481<br>423 | KJ4814<br>85 | KJ4815<br>54 | KJ481<br>622 | KJ481<br>713 | KJ481<br>760 |
| 46S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>280 | KJ481<br>356 | KJ481<br>424 | KJ4814<br>86 | KJ4815<br>55 | KJ481<br>623 | KJ481<br>674 | KJ481<br>761 |
| 47S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>267 | KJ481<br>357 | KJ481<br>425 | KJ4814<br>87 | KJ4815<br>56 | KJ481<br>624 | KJ481<br>714 | KJ481<br>762 |
| 48S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>268 | KJ481<br>358 | KJ481<br>426 | KJ4814<br>88 | KJ4815<br>57 | KJ481<br>625 | KJ481<br>683 | KJ481<br>763 |
| 49S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>310 | KJ481<br>359 | KJ481<br>427 | KJ4814<br>89 | KJ4815<br>58 | KJ481<br>626 | KJ481<br>675 | KJ481<br>728 |
| 50S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>269 | KJ481<br>360 | -            | KJ4814<br>90 | KJ4815<br>59 | KJ481<br>627 | KJ481<br>715 | KJ481<br>784 |
| 51S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>281 | KJ481<br>361 | KJ481<br>428 | KJ4814<br>91 | KJ4815<br>60 | KJ481<br>628 | KJ481<br>716 | KJ481<br>764 |
| 52S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>270 | KJ481<br>362 | -            | KJ4814<br>92 | KJ4815<br>61 | KJ481<br>629 | KJ481<br>664 | KJ481<br>729 |
| 53S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>311 | KJ481<br>363 | KJ481<br>429 | KJ4814<br>93 | KJ4815<br>62 | KJ481<br>630 | KJ481<br>665 | KJ481<br>788 |
| 54S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>312 | KJ481<br>364 | KJ481<br>430 | KJ4814<br>94 | KJ4815<br>63 | KJ481<br>631 | KJ481<br>689 | KJ481<br>777 |
| 55S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>313 | KJ481<br>365 | KJ481<br>431 | KJ4814<br>95 | KJ4815<br>64 | KJ481<br>632 | KJ481<br>690 | KJ481<br>765 |
| 56S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>271 | KJ481<br>366 | KJ481<br>432 | KJ4814<br>96 | KJ4815<br>65 | KJ481<br>633 | KJ481<br>666 | KJ481<br>766 |
| 57S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>282 | KJ481<br>367 | KJ481<br>433 | KJ4814<br>97 | KJ4815<br>66 | KJ481<br>634 | KJ481<br>691 | KJ481<br>730 |
| 58S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>314 | KJ481<br>368 | KJ481<br>434 | KJ4814<br>98 | KJ4815<br>67 | KJ481<br>635 | KJ481<br>717 | KJ481<br>731 |
| 59S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>272 | KJ481<br>369 | KJ481<br>435 | KJ4814<br>99 | KJ4815<br>68 | KJ481<br>636 | KJ481<br>718 | KJ481<br>767 |
| 60S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | -            | KJ481<br>370 | KJ481<br>436 | KJ4815<br>00 | KJ4815<br>69 | KJ481<br>637 | KJ481<br>719 | KJ481<br>768 |
| 61S | <i>Leucospermum tottum</i><br>var. <i>tottum</i>  | KJ481<br>273 | KJ481<br>371 | KJ481<br>437 | KJ4815<br>01 | KJ4815<br>70 | KJ481<br>638 | KJ481<br>692 | KJ481<br>732 |

|      |  |              |              |              |              |              |              |              |              |
|------|--|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 62S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>274 | KJ481<br>372 | KJ481<br>438 | KJ4815<br>02 | KJ4815<br>71 | KJ481<br>639 | KJ481<br>667 | KJ481<br>787 |
| 63S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>283 | KJ481<br>373 | KJ481<br>439 | KJ4815<br>03 | KJ4815<br>72 | KJ481<br>640 | KJ481<br>668 | KJ481<br>769 |
| 64S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>315 | KJ481<br>374 | KJ481<br>440 | KJ4815<br>04 | KJ4815<br>73 | KJ481<br>641 | KJ481<br>669 | KJ481<br>770 |
| 65S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>316 | KJ481<br>375 | KJ481<br>441 | KJ4815<br>05 | KJ4815<br>74 | KJ481<br>642 | KJ481<br>693 | KJ481<br>785 |
| 66S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>275 | KJ481<br>376 | KJ481<br>442 | KJ4815<br>06 | KJ4815<br>75 | KJ481<br>643 | KJ481<br>670 | KJ481<br>771 |
| 67S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | -            | KJ481<br>377 | -            | KJ4815<br>07 | KJ4815<br>76 | KJ481<br>644 | -            | -            |
| 68S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | -            | KJ481<br>378 | -            | -            | -            | -            | -            | -            |
| 69S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>276 | KJ481<br>379 | -            | KJ4815<br>08 | KJ4815<br>77 | KJ481<br>645 | KJ481<br>720 | KJ481<br>772 |
| 70S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>284 | KJ481<br>380 | KJ481<br>443 | KJ4815<br>09 | KJ4815<br>78 | KJ481<br>646 | KJ481<br>694 | KJ481<br>733 |
| 71S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>277 | KJ481<br>381 | KJ481<br>444 | KJ4815<br>10 | KJ4815<br>79 | KJ481<br>647 | KJ481<br>671 | KJ481<br>734 |
| 72S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | -            | KJ481<br>382 | -            | KJ4815<br>11 | KJ4815<br>80 | KJ481<br>648 | KJ481<br>672 | -            |
| 73S  | <i>Leucospermum tottum</i><br><i>var. tottum</i>                         | KJ481<br>317 | KJ481<br>383 | KJ481<br>445 | KJ4815<br>12 | KJ4815<br>81 | KJ481<br>649 | KJ481<br>721 | KJ481<br>735 |
| C17S | <i>Leucospermum tottum</i><br><i>var. tuttum</i>                         | KJ481<br>285 | KJ481<br>384 | KJ481<br>446 | KJ4815<br>13 | KJ4815<br>82 | KJ481<br>650 | KJ481<br>673 | KJ481<br>786 |
| C2S  | <i>Leucospermum</i><br><i>conocarpodendron</i><br><i>subsp. viridium</i> | KJ481<br>318 | KJ481<br>385 | KJ481<br>447 | KJ4815<br>14 | KJ4815<br>83 | KJ481<br>651 | KJ481<br>722 | KJ481<br>736 |
| C9S  | <i>Leucospermum</i><br><i>grandiflorum</i>                               | KJ481<br>286 | KJ481<br>386 | KJ481<br>448 | KJ4815<br>15 | KJ4815<br>84 | KJ481<br>652 | KJ481<br>695 | KJ481<br>737 |
| C13S | <i>Leucospermum</i><br><i>gracile</i>                                    | KJ481<br>319 | KJ481<br>387 | KJ481<br>449 | KJ4815<br>16 | KJ4815<br>85 | KJ481<br>653 | KJ481<br>696 | KJ481<br>773 |
| C15S | <i>Leucospermum</i><br><i>guenzii</i>                                    | KJ481<br>320 | KJ481<br>388 | KJ481<br>450 | KJ4815<br>17 | KJ4815<br>86 | KJ481<br>654 | KJ481<br>697 | KJ481<br>774 |
| C19S | <i>Leucospermum</i><br><i>oleifolium</i>                                 | KJ481<br>321 | KJ481<br>389 | KJ481<br>451 | KJ4815<br>18 | KJ4815<br>87 | KJ481<br>655 | KJ481<br>701 | KJ481<br>775 |
| C20S | <i>Mimetes cucullatus</i>  | KJ481<br>278 | KJ481<br>390 | KJ481<br>452 | KJ4815<br>19 | KJ4815<br>88 | KJ481<br>656 | KJ481<br>698 | KJ481<br>776 |

Table S4.1. GenBank accession numbers for all collected taxa. An individual voucher specimen for each sample population is stored at either the Stellenbosch University or University of Curtin Herbarium.

| ID       | rbcL   | GenBank Accession Numbers |              |              |              |              |              |              |              |
|----------|--|---------------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
|          |  | rbcL                      | ITS          | matK         | rpl16        | trnL         | atpB_rbcL    | trnL_trnF    | atpB         |
| 14       | <i>Aulax umbellata</i>   | -                         | KM659<br>706 | KM659<br>534 | -            | -            | -            | -            | KM659<br>474 |
| 12       | <i>Adenanthos drummondii</i>   | KM659<br>414              | KM659<br>705 | KM659<br>537 | KM659<br>753 | KM659<br>591 | KM659<br>810 | KM659<br>649 | KM659<br>477 |
| 89       | <i>Adenanthos linearis</i>   | KM659<br>415              | KM659<br>701 | KM659<br>538 | KM659<br>754 | KM659<br>592 | KM659<br>811 | -            | KM659<br>478 |
| 187      | <i>Conospermum longifolium</i> subsp<br><i>longifolium</i>               | KM659<br>404              | KM659<br>699 | -            | -            | -            | KM659<br>808 | -            | KM659<br>467 |
| 19       | <i>Conospermum wycherleyi</i>  | KM659<br>405              | KM659<br>703 | -            | -            | KM659<br>590 | KM659<br>809 | KM659<br>648 | KM659<br>468 |
| C7<br>S  | <i>Diastella divaricata</i> subsp<br><i>divaricata</i>                   | KM659<br>463              | -            | KM659<br>578 | KM659<br>786 | KM659<br>628 | KM659<br>849 | KM659<br>686 | KM659<br>517 |
| C5<br>S  | <i>Diastella divaricata</i> subsp<br><i>montana</i>                      | KM659<br>464              | KM659<br>732 | KM659<br>579 | KM659<br>784 | KM659<br>626 | KM659<br>847 | KM659<br>684 | KM659<br>516 |
| 100      | <i>Diastella fraterna</i>  | -                         | -            | KM659<br>542 | KM659<br>745 | KM659<br>583 | KM659<br>799 | KM659<br>642 | KM659<br>496 |
| 99       | <i>Diastella thymelaeoides</i><br>subsp<br><i>thymelaeoides</i>          | KM659<br>422              | -            | KM659<br>541 | KM659<br>744 | KM659<br>582 | KM659<br>798 | KM659<br>641 | KM659<br>495 |
| 52       | <i>Isopogon formosus</i>   | KM659<br>410              | KM659<br>702 | KM659<br>536 | KM659<br>751 | KM659<br>588 | KM659<br>805 | KM659<br>646 | KM659<br>473 |
| 15       | <i>Isopogon gardneri</i>   | KM659<br>409              | KM659<br>704 | KM659<br>535 | KM659<br>750 | KM659<br>587 | KM659<br>804 | -            | KM659<br>472 |
| 108      | <i>Leucadendron platyspermum</i>   | KM659<br>416              | -            | KM659<br>539 | KM659<br>746 | KM659<br>584 | KM659<br>800 | KM659<br>643 | KM659<br>480 |
| 11       | <i>Leucadendron xanthoconus</i>  | KM659<br>418              | KM659<br>707 | KM659<br>540 | KM659<br>741 | -            | KM659<br>795 | KM659<br>639 | KM659<br>479 |
| C10<br>S | <i>Leucospermum arenarium</i>  | KM659<br>457              | KM659<br>735 | KM659<br>570 | KM659<br>788 | KM659<br>630 | KM659<br>851 | KM659<br>688 | KM659<br>521 |
| 142      | <i>Leucospermum bolusii</i>  | KM659<br>437              | -            | KM659<br>566 | KM659<br>780 | KM659<br>622 | KM659<br>842 | KM659<br>680 | KM659<br>515 |
| C6<br>S  | <i>Leucospermum calligerum</i>   | KM659<br>458              | KM659<br>733 | KM659<br>576 | KM659<br>785 | KM659<br>627 | KM659<br>848 | KM659<br>685 | KM659<br>526 |
| 134      | <i>Leucospermum catherinae</i>   | KM659<br>421              | KM659<br>719 | -            | KM659<br>765 | KM659<br>606 | KM659<br>825 | -            | -            |
| 122      | <i>Leucospermum cuneiforme</i>   | KM659<br>448              | -            | KM659<br>557 | KM659<br>759 | KM659<br>598 | KM659<br>817 | KM659<br>655 | KM659<br>483 |
| 124      | <i>Leucospermum conocarpodendron</i><br>subsp<br><i>conocarpodendron</i> | KM659<br>452              | KM659<br>725 | KM659<br>565 | KM659<br>760 | KM659<br>599 | KM659<br>818 | KM659<br>656 | KM659<br>512 |
| 121      | <i>Leucospermum cordatum</i>   | KM659<br>449              | KM659<br>726 | KM659<br>560 | KM659<br>758 | KM659<br>597 | KM659<br>816 | KM659<br>654 | KM659<br>511 |
| 125      | <i>Leucospermum cordifolium</i>  | KM659<br>423              | KM659<br>724 | KM659<br>550 | KM659<br>761 | KM659<br>600 | KM659<br>819 | KM659<br>657 | KM659<br>491 |

|          |   |              |              |              |              |              |              |              |              |
|----------|---|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 128      | <i>Leucospermum erubescens</i>  | KM659<br>429 | KM659<br>722 | KM659<br>553 | -            | KM659<br>603 | KM659<br>822 | KM659<br>660 | KM659<br>487 |
| 118      | <i>Leucospermum formosum</i>  | KM659<br>441 | KM659<br>709 | -            | KM659<br>774 | KM659<br>615 | KM659<br>835 | KM659<br>671 | KM659<br>508 |
| 120      | <i>Leucospermum fulgens</i>   | KM659<br>438 | -            | -            | KM659<br>775 | KM659<br>617 | KM659<br>836 | KM659<br>673 | KM659<br>488 |
| 338      | <i>Leucospermum gerrardii</i>   | KM659<br>425 | -            | KM659<br>546 | KM659<br>740 | KM659<br>580 | KM659<br>794 | KM659<br>638 | KM659<br>503 |
| 144      | <i>Leucospermum glabrum</i>   | KM659<br>443 | KM659<br>713 | KM659<br>547 | KM659<br>772 | KM659<br>612 | KM659<br>832 | KM659<br>668 | KM659<br>501 |
| 114      | <i>Leucospermum hamatum</i>   | KM659<br>436 | -            | KM659<br>551 | KM659<br>756 | KM659<br>594 | KM659<br>813 | KM659<br>651 | KM659<br>498 |
| 130      | <i>Leucospermum harpagonatum</i>  | KM659<br>447 | KM659<br>720 | -            | KM659<br>764 | KM659<br>605 | KM659<br>824 | KM659<br>662 | KM659<br>507 |
| 129      | <i>Leucospermum heterophyllum</i>   | KM659<br>424 | KM659<br>721 | -            | -            | KM659<br>604 | KM659<br>823 | KM659<br>661 | KM659<br>499 |
| C8<br>S  | <i>Leucospermum hypophyllocarpod endron</i> subsp<br><i>canaliculatum</i>           | KM659<br>461 | KM659<br>734 | KM659<br>572 | KM659<br>787 | KM659<br>629 | KM659<br>850 | KM659<br>687 | KM659<br>518 |
| C22<br>S | <i>Leucospermum hypophyllocarpod endron</i> subsp<br><i>hypophyllocarpod endron</i> | KM659<br>456 | KM659<br>739 | KM659<br>577 | KM659<br>792 | KM659<br>634 | KM659<br>855 | KM659<br>692 | KM659<br>524 |
| 126      | <i>Leucospermum innovans</i>  | KM659<br>426 | -            | KM659<br>552 | KM659<br>762 | KM659<br>601 | KM659<br>820 | KM659<br>658 | KM659<br>497 |
| C11<br>S | <i>Leucospermum lineare</i> subsp<br><i>lineare</i>                                 | KM659<br>455 | KM659<br>738 | KM659<br>571 | KM659<br>793 | KM659<br>635 | KM659<br>844 | KM659<br>677 | KM659<br>520 |
| 133      | <i>Leucospermum lineare</i> subsp<br><i>calocephalum</i>                            | -            | -            | -            | -            | -            | -            | KM659<br>637 | -            |
| 111      | <i>Leucospermum muirii</i>  | KM659<br>442 | KM659<br>711 | KM659<br>549 | KM659<br>773 | KM659<br>613 | KM659<br>833 | KM659<br>669 | KM659<br>500 |
| 137      | <i>Leucospermum mundii</i>  | KM659<br>431 | -            | KM659<br>555 | -            | KM659<br>621 | KM659<br>841 | KM659<br>679 | KM659<br>486 |
| 33       | <i>Leucospermum pedunculatum</i>  | KM659<br>428 | -            | KM659<br>567 | KM659<br>781 | KM659<br>623 | KM659<br>843 | KM659<br>681 | KM659<br>489 |
| C3<br>S  | <i>Leucospermum parile</i>  | KM659<br>459 | KM659<br>730 | KM659<br>568 | KM659<br>782 | KM659<br>624 | KM659<br>845 | KM659<br>682 | KM659<br>527 |
| 139      | <i>Leucospermum patersonii</i>  | KM659<br>420 | KM659<br>717 | -            | KM659<br>768 | KM659<br>609 | KM659<br>828 | KM659<br>665 | KM659<br>506 |
| 117      | <i>Leucospermum pluridens</i>   | KM659<br>450 | KM659<br>727 | KM659<br>548 | -            | KM659<br>596 | KM659<br>815 | KM659<br>653 | KM659<br>502 |
| 135      | <i>Leucospermum praecox</i>   | KM659<br>446 | KM659<br>718 | -            | KM659<br>766 | KM659<br>607 | KM659<br>826 | KM659<br>663 | KM659<br>504 |
| 112      | <i>Leucospermum praemorsum</i>  | KM659<br>433 | KM659<br>710 | KM659<br>562 | -            | KM659<br>614 | KM659<br>834 | KM659<br>670 | KM659<br>509 |
| 127      | <i>Leucospermum profugum</i>  | KM659<br>432 | KM659<br>723 | KM659<br>561 | KM659<br>763 | KM659<br>602 | KM659<br>821 | KM659<br>659 | KM659<br>510 |
| C14<br>S | <i>Leucospermum prostratum</i>  | KM659<br>453 | KM659<br>737 | KM659<br>573 | KM659<br>790 | KM659<br>632 | KM659<br>853 | KM659<br>690 | KM659<br>522 |
| 132      | <i>Leucospermum reflexum</i>  | KM659<br>434 | KM659<br>708 | KM659<br>563 | KM659<br>778 | KM659<br>619 | KM659<br>839 | KM659<br>676 | KM659<br>514 |



|          |  |              |              |              |              |              |              |              |              |
|----------|--|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| C21<br>S | <i>Leucospermum rodolentum</i>                               | KM659<br>462 | KM659<br>712 | KM659<br>574 | KM659<br>791 | KM659<br>633 | KM659<br>854 | KM659<br>691 | KM659<br>523 |
| 136      | <i>Leucospermum royenifolium</i>                             | KM659<br>439 | -            | KM659<br>545 | KM659<br>779 | KM659<br>620 | KM659<br>840 | KM659<br>678 | KM659<br>494 |
| 131      | <i>Leucospermum saxatile</i>                                 | KM659<br>430 | -            | KM659<br>554 | KM659<br>777 | -            | KM659<br>838 | KM659<br>675 | KM659<br>485 |
| 138      | <i>Leucospermum saxosum</i>                                  | KM659<br>427 | -            | -            | KM659<br>767 | KM659<br>608 | KM659<br>827 | KM659<br>664 | KM659<br>505 |
| 116      | <i>Leucospermum secundifolium</i>                            | KM659<br>419 | KM659<br>728 | KM659<br>543 | KM659<br>757 | KM659<br>595 | KM659<br>814 | KM659<br>652 | KM659<br>490 |
| 141      | <i>Leucospermum spathulatum</i>                              | KM659<br>444 | KM659<br>715 | KM659<br>559 | KM659<br>770 | -            | KM659<br>830 | -            | KM659<br>513 |
| C4<br>S  | <i>Leucospermum tomentosum</i>                               | KM659<br>460 | KM659<br>731 | KM659<br>575 | KM659<br>783 | KM659<br>625 | KM659<br>846 | KM659<br>683 | KM659<br>519 |
| C12<br>S | <i>Leucospermum truncatulum</i>                              | KM659<br>454 | KM659<br>736 | KM659<br>569 | KM659<br>789 | KM659<br>631 | KM659<br>852 | KM659<br>689 | KM659<br>525 |
| 123      | <i>Leucospermum truncatum</i>                                | KM659<br>440 | -            | KM659<br>558 | KM659<br>776 | KM659<br>618 | KM659<br>837 | KM659<br>674 | KM659<br>484 |
| 113      | <i>Leucospermum utriculosum</i>                              | KM659<br>451 | KM659<br>729 | KM659<br>556 | KM659<br>755 | KM659<br>593 | KM659<br>812 | KM659<br>650 | KM659<br>482 |
| 119      | <i>Leucospermum winteri</i>                                  | -            | -            | -            | -            | KM659<br>616 | -            | KM659<br>672 | -            |
| 140      | <i>Leucospermum wittebergense</i>                            | KM659<br>445 | KM659<br>716 | KM659<br>544 | KM659<br>769 | KM659<br>610 | KM659<br>829 | KM659<br>666 | KM659<br>493 |
| 18       | <i>Petrophile shuttleworthiana</i>                           | -            | -            | KM659<br>532 | KM659<br>749 | KM659<br>586 | KM659<br>803 | -            | KM659<br>476 |
| 28       | <i>Petrophile squamata</i>                                   | KM659<br>411 | KM659<br>696 | KM659<br>531 | KM659<br>748 | KM659<br>585 | KM659<br>802 | KM659<br>645 | KM659<br>475 |
| 85       | <i>Protea laevis</i>   | KM659<br>413 | -            | -            | KM659<br>743 | KM659<br>581 | KM659<br>797 | KM659<br>693 | KM659<br>471 |
| 82       | <i>Protea scolopendriifolia</i>                              | KM659<br>412 | -            | KM659<br>533 | KM659<br>742 | KM659<br>636 | KM659<br>796 | KM659<br>640 | KM659<br>470 |
| 124      | <i>Stirlingia anethifolia</i>                                | KM659<br>406 | KM659<br>700 | KM659<br>530 | KM659<br>752 | -            | KM659<br>807 | KM659<br>694 | KM659<br>469 |
| 114      | <i>Synaphea bifurata</i>                                     | KM659<br>408 | KM659<br>698 | KM659<br>529 | -            | -            | KM659<br>806 | KM659<br>647 | KM659<br>466 |
| 92       | <i>Synaphea canaliculata</i>                                 | KM659<br>407 | KM659<br>697 | KM659<br>528 | -            | KM659<br>589 | -            | -            | KM659<br>465 |
| 110      | <i>Mimetes hirtus</i>  | KM659<br>417 | KM659<br>695 | KM659<br>564 | KM659<br>747 | -            | KM659<br>801 | KM659<br>644 | KM659<br>492 |
| 143      | <i>Mimetes pauciflorus</i>                                   | KM659<br>435 | KM659<br>714 | -            | KM659<br>771 | KM659<br>611 | KM659<br>831 | KM659<br>667 | KM659<br>481 |
| C15<br>S | <i>Leucospermum gueinzii</i>                                 | KJ4813<br>20 | KJ4813<br>88 | KJ4816<br>97 | KJ4814<br>50 | KJ4816<br>54 | KJ4815<br>86 | KJ4815<br>17 | KJ4817<br>74 |
| C9<br>S  | <i>Leucospermum gradiflorum</i>                              | KJ4812<br>86 | KJ4813<br>86 | KJ4816<br>95 | KJ4814<br>48 | KJ4816<br>52 | KJ4815<br>84 | KJ4815<br>15 | KJ4817<br>37 |
| C19<br>S | <i>Leucospermum oleifolium</i>                               | KJ4813<br>21 | KJ4813<br>89 | KJ4817<br>01 | KJ4814<br>51 | KJ4816<br>55 | KJ4815<br>87 | KJ4815<br>18 | KJ4817<br>75 |
| C13<br>S | <i>Leucospermum gracile</i>                                  | KJ4813<br>19 | KJ4813<br>87 | KJ4816<br>97 | KJ4814<br>50 | KJ4816<br>54 | KJ4815<br>86 | KJ4815<br>17 | KJ4817<br>74 |
| C2<br>S  | <i>Leucospermum conocarpodendron</i><br>subsp <i>viridum</i> | KJ4813<br>18 | KJ4813<br>85 | KJ4817<br>22 | KJ4814<br>47 | KJ4816<br>51 | KJ4815<br>83 | KJ4815<br>14 | KJ4817<br>36 |
| 39S      | <i>Leucospermum tottum</i> var <i>tottum</i>                 | KJ4813<br>05 | KJ4813<br>49 | KJ4816<br>79 | KJ4814<br>17 | KJ4816<br>16 | KJ4815<br>48 | KJ4814<br>79 | KJ4817<br>55 |

|          |   |              |              |              |              |              |              |              |              |
|----------|---|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 31S      | <i>Leucospermum tottum</i> var <i>glabrum</i> | KJ4812<br>61 | KJ4813<br>41 | KJ4816<br>86 | KJ4814<br>09 | KJ4816<br>08 | KJ4815<br>40 | KJ4814<br>71 | KJ4817<br>49 |
| 25S      | <i>Leucospermum vestitum</i>                  | -            | KJ4813<br>37 | KJ4816<br>60 | KJ4814<br>05 | KJ4816<br>04 | KJ4815<br>36 | KJ4814<br>67 | KJ4817<br>82 |
| C20<br>S | <i>Mimetes cucullatus</i>                     | KJ4812<br>78 | KJ4813<br>90 | KJ4816<br>98 | KJ4814<br>52 | KJ4816<br>56 | KJ4815<br>88 | KJ4815<br>19 | KJ4817<br>76 |

Table S4.2. The 9 calibration point settings on the *Leucospermum* phylogeny analysis.

| Node | Priors | Minimum calibration<br>(mya) | Mean calibration<br>(mya) | Maximum calibration<br>(mya) |
|------|--------|------------------------------|---------------------------|------------------------------|
| 1    | Normal | 10.7                         | 12.3                      | 13.9                         |
| 2    | Normal | 28.7                         | 30.3                      | 31.9                         |
| 3    | Normal | 35.7                         | 37.3                      | 38.9                         |
| 4    | Normal | 42.9                         | 44.5                      | 46.1                         |
| 5    | Normal | 72                           | 73.6                      | 75.6                         |
| 6    | Normal | 74.1                         | 75.7                      | 77.3                         |
| 7    | Normal | 79.2                         | 80.8                      | 82.4                         |
| 8    | Normal | 54.9                         | 56.5                      | 58.1                         |
| 9    | Normal | 34.5                         | 36.1                      | 37.7                         |

Table S4.3. Mean morphological values and standard deviations for sampled specimens (N=5) and autofertility values (selfing).

| <i>Leucospermum</i> taxon  | Symmetry      | Style length (mm) | Tube length (mm) | Curvature    | Min orientation (degrees) | Max orientation (degrees) | Flower density | Selfing |
|--|---------------|-------------------|------------------|--------------|---------------------------|---------------------------|----------------|---------|
| <i>arenarium</i>   | Zygomorphic   | 28.2 (0.93)       | 9.1 (0.20)       | 0.890 (0.02) | 135 (13.40)               | 164.6 (7.12)              | 80.4 (4.92)    | 0       |
| <i>bolusii</i>   | Actinomorphic | 15.5 (0.45)       | 4.5 (0.77)       | 1 (0)        | 123 (4)                   | 155 (5.48)                | 42.2 (1.17)    | 0       |
| <i>calligerum</i>  | Actinomorphic | 24.5 (0.37)       | 7.8 (0.51)       | 0.957 (0.01) | 139.5 (3.51)              | 165 (2.76)                | 41 (4.24)      | 0       |
| <i>catherineae</i>   | Zygomorphic   | 73 (3.35)         | 12.5 (1.67)      | 0.948 (0.01) | 119 (4.90)                | 144 (5.83)                | 91.4 (2.50)    | 0.55    |
| <i>conocarpodendron</i> subsp. <i>conocarpodendron</i>             | Zygomorphic   | 46.6 (1.69)       | 10 (1.10)        | 0.936 (0.01) | 131 (2)                   | 153 (2.45)                | 70 (3.16)      | 0       |
| <i>conocarpodendron</i> subsp. <i>viridum</i>                      | Zygomorphic   | 51 (1.87)         | 12 (1.22)        | 0.971 (0.01) | 160 (0)                   | 170 (0)                   | 57.25 (1.48)   | 0.13    |
| <i>cordatum</i>  | Zygomorphic   | 24 (0.63)         | 0 (0)            | 0.889 (0.00) | 101 (2)                   | 119 (2)                   | 48 (2.10)      | -       |
| <i>cordifolium</i>   | Zygomorphic   | 49.1 (1.80)       | 7.1 (0.66)       | 0.930 (0.01) | 108 (4)                   | 153 (2.45)                | 167.8 (10.09)  | 0.36    |
| <i>cuneiforme</i>  | Zygomorphic   | 46.8 (1.33)       | 8.6 (0.49)       | 0.981 (0.01) | 127 (4)                   | 154 (2.74)                | 78.8 (6.65)    | 0.19    |
| <i>erubescence</i>   | Zygomorphic   | 53.4 (2.06)       | 11.2 (0.75)      | 0.966 (0.02) | 131 (13.9)                | 163 (2.45)                | 60.2 (5.64)    | 0       |
| <i>formosum</i>  | Zygomorphic   | 70.6 (1.36)       | 13.6 (1.2)       | 0.936 (0.02) | 102 (2.45)                | 134 (3.74)                | 72.2 (2.56)    | 0.62    |
| <i>fulgens</i>   | Zygomorphic   | 48.4 (1.85)       | 8.8 (0.24)       | 0.984 (0.01) | 125 (3.16)                | 152 (2.45)                | 62 (3.74)      | 0.22    |
| <i>glabrum</i>   | Zygomorphic   | 61.2 (1.03)       | 11.2 (0.81)      | 0.940 (0.01) | 145 (5.48)                | 160 (3.16)                | 42.4 (1.74)    | -       |
| <i>gracile</i>   | Zygomorphic   | 27.4 (1.36)       | 7.9 (1.11)       | 1 (0)        | 159 (6.63)                | 180 (0)                   | 57.2 (2.99)    | 0       |
| <i>grandiflorum</i>  | Zygomorphic   | 69.4 (1.98)       | 14 (3.03)        | 0.965 (0.01) | 142.6 (15.7)              | 164.2 (16.92)             | 89.4 (9.87)    | 0.50    |
| <i>gueinzii</i>  | Zygomorphic   | 72.7 (1.25)       | 10.1 (1.91)      | 0.942 (0.01) | 133 (2.45)                | 147 (2.45)                | 76 (1.90)      | 0.66    |
| <i>hamatum</i>   | Zygomorphic   | 15.2 (2.23)       | 0 (0)            | 0.768 (0.06) | 168 (2.45)                | 169 (2)                   | 5.8 (0.75)     | 0       |
| <i>harpogonatum</i>  | Zygomorphic   | 21 (1.10)         | 0 (0)            | 0.820 (0.03) | 158 (2.45)                | 170 (0)                   | 9.2 (0.75)     | -       |
| <i>heterophyllum</i>   | Actinomorphic | 20.9 (0.66)       | 6.4 (0.49)       | 1 (0)        | 93 (2.45)                 | 151 (4.90)                | 41.8 (3.19)    | 0       |
| <i>hypophyllocarpodendron</i> subsp. <i>canaliculatum</i>          | Zygomorphic   | 22.3 (0.748)      | 11.7 (1.08)      | 0.974 (0.02) | 114 (13.56)               | 147 (5.10)                | 111.8 (5.19)   | 0       |
| <i>hypophyllocarpodendron</i> subsp. <i>hypophyllocarpodendron</i> | Zygomorphic   | 21 (1.90)         | 11.9 (2.17)      | 0.981 (0.03) | 115.8 (9.33)              | 147.6 (8.43)              | 122.8 (18.74)  | 0.13    |
| <i>lineare</i> subsp. <i>calocephalum</i>                          | Zygomorphic   | 40.6 (2.15)       | 8.4 (0.37)       | 0.953 (0.02) | 127 (2.45)                | 152 (4)                   | 136 (12.08)    | 0.42    |
| <i>lineare</i> subs. <i>lineare</i>                                | Zygomorphic   | 49.3 (0.872)      | 8.3 (0.980)      | 0.944 (0.02) | 121 (5.83)                | 142 (9.27)                | 111.8 (19.26)  | 0.10    |
| <i>muirii</i>  | Zygomorphic   | 16.3 (0.40)       | 5 (1.26)         | 0.994 (0.01) | 133 (9.80)                | 164 (3.74)                | 52 (5.18)      | 0       |
| <i>mundii</i>  | Zygomorphic   | 29.6 (1.02)       | 7.4 (1.02)       | 1 (0)        | 153 (4)                   | 172 (2.45)                | 71 (7.32)      | 0       |
| <i>oleifolium</i>  | Zygomorphic   | 26.8 (0.51)       | 5.6 (0.58)       | 1 (0)        | 151 (2)                   | 180 (0)                   | 89.2 (2.32)    | 0       |
| <i>parile</i>  | Zygomorphic   | 19.4 (1.46)       | 5.5 (0.71)       | 0.990 (0.01) | 125 (9.63)                | 154.2 (2.23)              | 78.6 (6.44)    | 0       |
| <i>patersonii</i>  | Zygomorphic   | 42.4 (7.44)       | 11.2 (1.72)      | 0.858 (0.12) | 112 (12.88)               | 156 (4.90)                | 116.6 (7.79)   | 0.57    |
| <i>pedunculatum</i>  | Actinomorphic | 16.4 (0.80)       | 7.3 (0.4)        | 0.959 (0.2)  | 120 (7.07)                | 154.4 (2.33)              | 68 (4.94)      | 0       |

|                                   |               |             |                 |              |              |                 |               |      |
|-----------------------------------|---------------|-------------|-----------------|--------------|--------------|-----------------|---------------|------|
| <i>pluridens</i>                  | Zygomorphic   | 50.7 (2.79) | 11.6 (1.02)     | 0.977 (0.01) | 147 (2.45)   | 174 (2)         | 53.2 (2.79)   | 0.14 |
| <i>praecox</i>                    | Zygomorphic   | 41.8 (1.50) | 8.4 (0.37)      | 0.954 (0.02) | 132 (4)      | 148 (4)         | 56.8 (3.06)   | 0.06 |
| <i>praemorsum</i>                 | Zygomorphic   | 59.4 (2.65) | 9.2 (1.47)      | 0.943 (0.02) | 144 (5.83)   | 153 (4)         | 50.4 (3.67)   | 0.6  |
| <i>profugum</i>                   | Zygomorphic   | 48.7 (5.76) | 15.2 (1.72)     | 0.937 (0.01) | 101 (3.74)   | 141 (8)         | 103 (5.20)    | 0    |
| <i>prostratum</i>                 | Actinomorphic | 14.7 (0.60) | 8.6 (0.37)      | 0.961 (0.02) | 147 (4)      | 177 (2.45)      | 8.6 (0.37)    | 0    |
| <i>reflexum</i>                   | Zygomorphic   | 87.7 (0.75) | 16.7 (1.33)     | 0.978 (0.00) | 5 (0)        | 40.6 (2.33)     | 101.4 (13.09) | 0.56 |
| <i>rodolentum</i>                 | Zygomorphic   | 18.6 (1.62) | 6.5 (0.45)      | 1 (0)        | 123.8 (4.12) | 157.6(4.59)     | 65.8 (5.04)   | 0    |
| <i>royenifolium</i>               | Actinomorphic | 14.6 (0.50) | 6.8 (1.03)      | 1 (0)        | 150 (8.37)   | 167 (6)         | 21.6 (1.02)   | 0    |
| <i>saxatile</i>                   | Zygomorphic   | 29.1 (0.66) | 5.5 (0.77)      | 1 (0)        | 163 (2.45)   | 175 (0)         | 46.6 (2.15)   | 0    |
| <i>secundifolium</i>              | Actinomorphic | 15.6 (0.80) | 7.6(0.65)       | 1 (0)        | 133 (2.45)   | 162 (4)         | 8.2 (0.75)    | -    |
| <i>spathulatum</i>                | Zygomorphic   | 36.4 (1.02) | 12.4 (1.50)     | 0.997 (0.00) | 104 (4.90)   | 134 (3.74)      | 73.6 (2.87)   | 0    |
| <i>tomentosum</i>                 | Zygomorphic   | 20.1 (0.66) | 6.3 (0.98)      | 0.980 (0.02) | 135.2 (3.31) | 153.2<br>(8.23) | 53.4 (4.08)   | 0.04 |
| <i>tottum</i> var. <i>glabrum</i> | Zygomorphic   | 44.7 (2.89) | 8.35 (3.38)     | 0.971 (0.01) | 121.2 (5.90) | 138.9<br>(6.23) | 52.1 (9.58)   | 0.23 |
| <i>tottum</i> var. <i>tottum</i>  | Zygomorphic   | 55.5 (3.10) | 37.67<br>(4.25) | 0.976 (0.01) | 91.4 (5.30)  | 121.7<br>(7.49) | 75.5 (12.15)  | 0    |
| <i>truncatulum</i>                | Actinomorphic | 12.7 (0.75) | 7.4 (1.07)      | 1 (0)        | 128 (7.48)   | 180 (0)         | 38.6 (3.38)   | 0.33 |
| <i>truncatum</i>                  | Zygomorphic   | 27 (1.10)   | 7.2 (0.75)      | 0.951 (0.02) | 121 (2)      | 157 (4)         | 56.8 (3.54)   | 0    |
| <i>utriculosum</i>                | Zygomorphic   | 35.8 (1.60) | 8.4 (0.49)      | 0.952 (0.01) | 120 (0)      | 142 (2.45)      | 55.4 (3.88)   | 0    |
| <i>vestitum</i>                   | Zygomorphic   | 44.6 (2.33) | 7 (1.22)        | 0.916 (0.02) | 117 (6)      | 147 (10.30)     | 102 (11.38)   | 0.52 |
| <i>winteri</i>                    | Zygomorphic   | 19.5 (0.89) | 8.1 (0.66)      | 0.975 (0.00) | 118 (6)      | 148 (6.78)      | 89.8 (9.45)   | 0.03 |
| <i>wittebergense</i>              | actinomorphic | 14.2 (0.51) | 4.9 (.20)       | 1 (0)        | 135 (4.47)   | 160 (0)         | 24.2 (1.72)   | 0    |

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Table S4.4. Pollinator importance values for studied taxa. Values express the proportion of total visits belonging to each pollinator functional group.

| <i>Leucospermum</i> taxon  | Hymenoptera | Coleoptera | Diptera | Butterfly | Moth | Long-proboscid fly | Short-billed bird | Long-billed bird (crown) | Long-billed bird (body) | Non-flying mammal |
|--|-------------|------------|---------|-----------|------|--------------------|-------------------|--------------------------|-------------------------|-------------------|
| <i>arenarium</i>   | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 1                 |
| <i>bolusii</i>   | 0.89        | 0.07       | 0       | 0.04      | 0    | 0                  | 0                 | 0                        | 0                       | 0                 |
| <i>calligerum</i>  | 0.87        | 0.13       | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 0                 |
| <i>catherineae</i>   | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 1                       | 0                 |
| <i>conocarpodendron</i> subsp.                                     | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |
| <i>conocarpodendron</i> subsp. <i>viridum</i>                      | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |
| <i>cordatum</i>  | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 1                 |
| <i>cordifolium</i>   | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |
| <i>cuneiforme</i>  | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |
| <i>erubescence</i>   | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |
| <i>formosum</i>  | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 1                       | 0                 |
| <i>fulgens</i>   | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |
| <i>glabrum</i>   | 0           | 0          | 0       | 0         | 0    | 0                  | 0.33              | 0.67                     | 0                       | 0                 |
| <i>gracile</i>   | 1           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 0                 |
| <i>grandiflorum</i>  | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 1                       | 0                 |
| <i>gueinzii</i>  | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 1                       | 0                 |
| <i>hamatum</i>   | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 1                 |
| <i>harpagonatum</i>  | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 1                 |
| <i>heterophyllum</i>   | 0           | 0          | 0       | 0         | 1    | 0                  | 0                 | 0                        | 0                       | 0                 |
| <i>hypophyllocarpodendron</i> subsp. <i>canaliculatum</i>          | 1           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 0                 |
| <i>hypophyllocarpodendron</i> subsp. <i>hypophyllocarpodendron</i> | 1           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 0                 |
| <i>lineare</i> subsp. <i>calocephalum</i>                          | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |
| <i>lineare</i> subsp. <i>lineare</i>                               | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |
| <i>muirii</i>  | 1           | 0          | 0       | 0         | 0    | 0                  | 0                 | 0                        | 0                       | 0                 |
| <i>mundii</i>  | 0           | 0          | 0       | 0         | 0    | 0                  | 1                 | 0                        | 0                       | 0                 |
| <i>oleifolium</i>  | 0.38        | 0.1        | 0       | 0         | 0    | 0                  | 0.31              | 0.21                     | 0                       | 0                 |
| <i>parile</i>  | 0.88        | 0          | 0.06    | 0.06      | 0    | 0                  | 0                 | 0                        | 0                       | 0                 |
| <i>patersonii</i>  | 0           | 0          | 0       | 0         | 0    | 0                  | 0                 | 1                        | 0                       | 0                 |

|                                   |      |      |      |     |   |     |      |      |   |   |
|-----------------------------------|------|------|------|-----|---|-----|------|------|---|---|
| <i>pedunculatum</i>               | 0.67 | 0    | 0.33 | 0   | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>pluridens</i>                  | 0    | 0    | 0    | 0   | 0 | 0   | 0    | 1    | 0 | 0 |
| <i>praecox</i>                    | 0    | 0    | 0    | 0   | 0 | 0   | 0    | 1    | 0 | 0 |
| <i>praemorsum</i>                 | 0    | 0    | 0    | 0   | 0 | 0   | 0    | 1    | 0 | 0 |
| <i>profugum</i>                   | 0    | 0    | 0    | 0   | 0 | 0   | 0    | 1    | 0 | 0 |
| <i>prostratum</i>                 | 1    | 0    | 0    | 0   | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>reflexum</i>                   | 0    | 0    | 0    | 0   | 0 | 0   | 0    | 0    | 1 | 0 |
| <i>rodolentum</i>                 | 0.5  | 0.25 | 0.25 | 0   | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>royenifolium</i>               | 1    | 0    | 0    | 0   | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>saxatile</i>                   | 0.6  | 0.92 | 0    | 0.2 | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>secundifolium</i>              | 0.2  | 0    | 0    | 0.8 | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>spathulatum</i>                | 0    | 0    | 0    | 0   | 0 | 0   | 0.8  | 0.2  | 0 | 0 |
| <i>tomentosum</i>                 | 0.15 | 0    | 0.05 | 0.8 | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>tottum</i> var. <i>glabrum</i> | 0    | 0    | 0    | 0   | 0 | 0   | 1    | 0    | 0 | 0 |
| <i>tottum</i> var. <i>tottum</i>  | 0    | 0    | 0    | 0   | 0 | 0.4 | 0.2  | 0.4  | 0 | 0 |
| <i>truncatulum</i>                | 0.75 | 0.25 | 0    | 0   | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>truncatum</i>                  | 0    | 0    | 0    | 0   | 0 | 0   | 0.69 | 0.31 | 0 | 0 |
| <i>utriculosum</i>                | 0    | 0    | 0    | 0   | 0 | 0   | 0.71 | 0.29 | 0 | 0 |
| <i>vestitum</i>                   | 0    | 0    | 0    | 0   | 0 | 0   | 0    | 1    | 0 | 0 |
| <i>winteri</i>                    | 1    | 0    | 0    | 0   | 0 | 0   | 0    | 0    | 0 | 0 |
| <i>wittebergense</i>              | 1    | 0    | 0    | 0   | 0 | 0   | 0    | 0    | 0 | 0 |

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Table S4.5. Number of probes by bird species for bird pollinated *Leucospermum* taxa. Detailed pollinator data for both *L. tottum* varieties and for *L. conocarpodendron* subsp. *viridum* are provided in chapters 2 and 5.

| <i>Leucospermum</i> taxon                              | <i>Promerops cafer</i> | <i>Nectarinia famosa</i> | <i>Anthobaphes violacea</i> | <i>Cinnyris chalybeus</i> |
|--|------------------------|--------------------------|-----------------------------|---------------------------|
| <i>catherineae</i>                                     | 139                    | 0                        | 0                           | 0                         |
| <i>conocarpodendron</i> subsp. <i>conocarpodendron</i> | 407                    | 0                        | 0                           | 0                         |
| <i>cordifolium</i>                                     | 573                    | 0                        | 0                           | 0                         |
| <i>cuneiforme</i>                                      | 150                    | 0                        | 0                           | 0                         |
| <i>erubescence</i>                                     | 237                    | 0                        | 0                           | 0                         |
| <i>formosum</i>  | 43                     | 0                        | 0                           | 0                         |
| <i>fulgens</i>   | 29                     | 0                        | 0                           | 0                         |
| <i>glabrum</i>   | 48                     | 0                        | 21                          | 0                         |
| <i>grandiflorum</i>                                    | 36                     | 0                        | 0                           | 0                         |
| <i>gueinzii</i>  | 214                    | 0                        | 0                           | 0                         |
| <i>lineare</i> subsp. <i>calocephalum</i>              | 80                     | 0                        | 0                           | 0                         |
| <i>lineare</i> subsp. <i>lineare</i>                   | 79                     | 0                        | 0                           | 0                         |
| <i>mundii</i>  | 0                      | 0                        | 89                          | 0                         |
| <i>oleifolium</i>                                      | 101                    | 0                        | 119                         | 0                         |
| <i>patersonii</i>                                      | 45                     | 0                        | 0                           | 0                         |
| <i>pluridens</i>                                       | 543                    | 0                        | 0                           | 0                         |
| <i>praecox</i>   | 110                    | 0                        | 0                           | 0                         |
| <i>praemorsum</i>                                      | 14                     | 55                       | 0                           | 0                         |
| <i>profugum</i>  | 0                      | 15                       | 0                           | 0                         |
| <i>reflexum</i>  | 0                      | 56                       | 0                           | 0                         |
| <i>spathulatum</i>                                     | 6                      | 0                        | 101                         | 0                         |
| <i>truncatum</i>                                       | 44                     | 0                        | 148                         | 0                         |
| <i>utriculosum</i>                                     | 13                     | 0                        | 0                           | 36                        |
| <i>vestitum</i>  | 127                    | 0                        | 0                           | 0                         |

Table S6: Number of captured or observed rodent species on non-flying mammal pollinated *Leucospermum* taxa. Pollen on fur was measured by taking a swab of the entire forehead region using a fuchsin gel cube. Pollen in feces was measured by taking a sample from three haphazardly chosen fecal pellets. Pollinators on *L. cordatum* were identified using camera traps, so no pollen samples were recorded.

| <i>Leucospermum</i> taxon | Rodent individual | Species                     | Pollen grains (fur) | Pollen grains (feces) |
|---------------------------|-------------------|-----------------------------|---------------------|-----------------------|
| <i>arenarium</i>          | 1                 | <i>Gerbillurus paeba</i>    | 18                  | 94                    |
|                           | 2                 | <i>Gerbillurus paeba</i>    | 13                  | 17                    |
|                           | 3                 | <i>Gerbillurus paeba</i>    | 24                  | 143                   |
|                           | 4                 | <i>Gerbillurus paeba</i>    | 13                  | 85                    |
|                           | 5                 | <i>Gerbillurus paeba</i>    | 7                   | 23                    |
|                           | 6                 | <i>Gerbillurus paeba</i>    | 8                   | 54                    |
|                           | 7                 | <i>Rhabdomys pumilio</i>    | 3                   | 28                    |
|                           | 8                 | <i>Rhabdomys pumilio</i>    | 11                  | 36                    |
|                           | 9                 | unknown                     | 6                   | 44                    |
| <i>cordatum</i>           | 1                 | <i>Acomys subspinosus</i>   | -                   | -                     |
|                           | 2                 | <i>Acomys subspinosus</i>   | -                   | -                     |
|                           | 3                 | <i>Aethomys namaquensis</i> | -                   | -                     |
|                           | 4                 | <i>Aethomys namaquensis</i> | -                   | -                     |
| <i>hamatum</i>            | 1                 | <i>Rhabdomys pumilio</i>    | 46                  | 87                    |
|                           | 2                 | <i>Rhabdomys pumilio</i>    | 63                  | 98                    |
|                           | 3                 | <i>Acomys subspinosus</i>   | 32                  | 62                    |
|                           | 4                 | <i>Acomys subspinosus</i>   | 53                  | 93                    |
|                           | 5                 | <i>Acomys subspinosus</i>   | 18                  | 42                    |
| <i>harpagonatum</i>       | 1                 | <i>Acomys subspinosus</i>   | 63                  | 106                   |
|                           | 2                 | <i>Acomys subspinosus</i>   | 28                  | 37                    |
|                           | 3                 | <i>Acomys subspinosus</i>   | 43                  | 68                    |



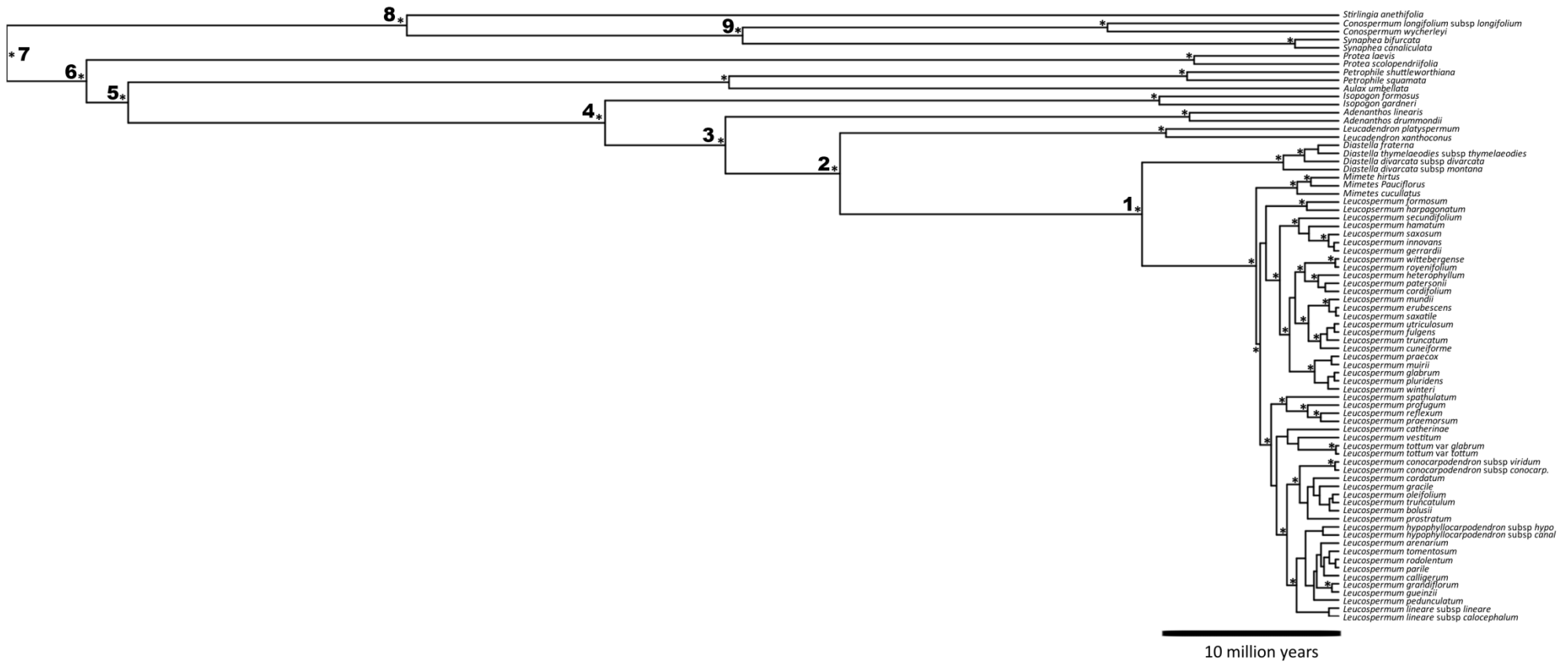


Figure S4.1. Maximum credibility phylogeny of *Leucospermum* and out groups from a Bayesian analysis of 8 genes. An asterisk denotes branches with >80% posterior probability.

# Appendix B

All photographs were taken by CM Johnson and A Pauw



B.1: *L. arenarium*. Population found near Redelinghuys -32.62490, 18.59182 on September 17, 2012. Pollinator shown is the hairy-footed gerbil *Gerbillurus paeba*. Scale bar= 1cm.





B.2: *L. bolusii*: Population found in Gordon's Bay -34.17429, 18.83605 on October 8, 2013. Pollinator shown is a member of the genus *Coelioxys*. Scale bar= 1cm.





B.3: *L. calligerum*: Found near Abbotsdale -33.4889, 18.6709 on August 18, 2012. Honeybee pollinators show are members of the genus *Apis* and monkey beetle is a member of the genus *Peritichia*. Scale bar= 1cm.





B.4: *L. catherinae*. Population found in the Cederberg Wilderness Area -32.43964, 19.1917 on October 2, 2013. Pollinator shown is the *Cape Sugarbird Promerops cafer*. Scale bar= 1cm.





B.5: *L. conocarpodendron* subspecies *conocarpodendron*. Population found above Camp's Bay - 33.96341, 18.38532 on November 26, 2013. Scale bar= 1cm.





B.6: *L. conocarpodendron* subspecies *viridum*. Found north of Simon's Town -34.17303, 18.39905 on August 17, 2012. Pollinator shown is the Cape Sugarbird *Promerops cafer*. Scale bar= 1cm.





B.7: *L. cordatum*. Population found near Rooi-Els -34.27712, 18.83743 on September 11, 2014.  
Scale bar= 1cm.





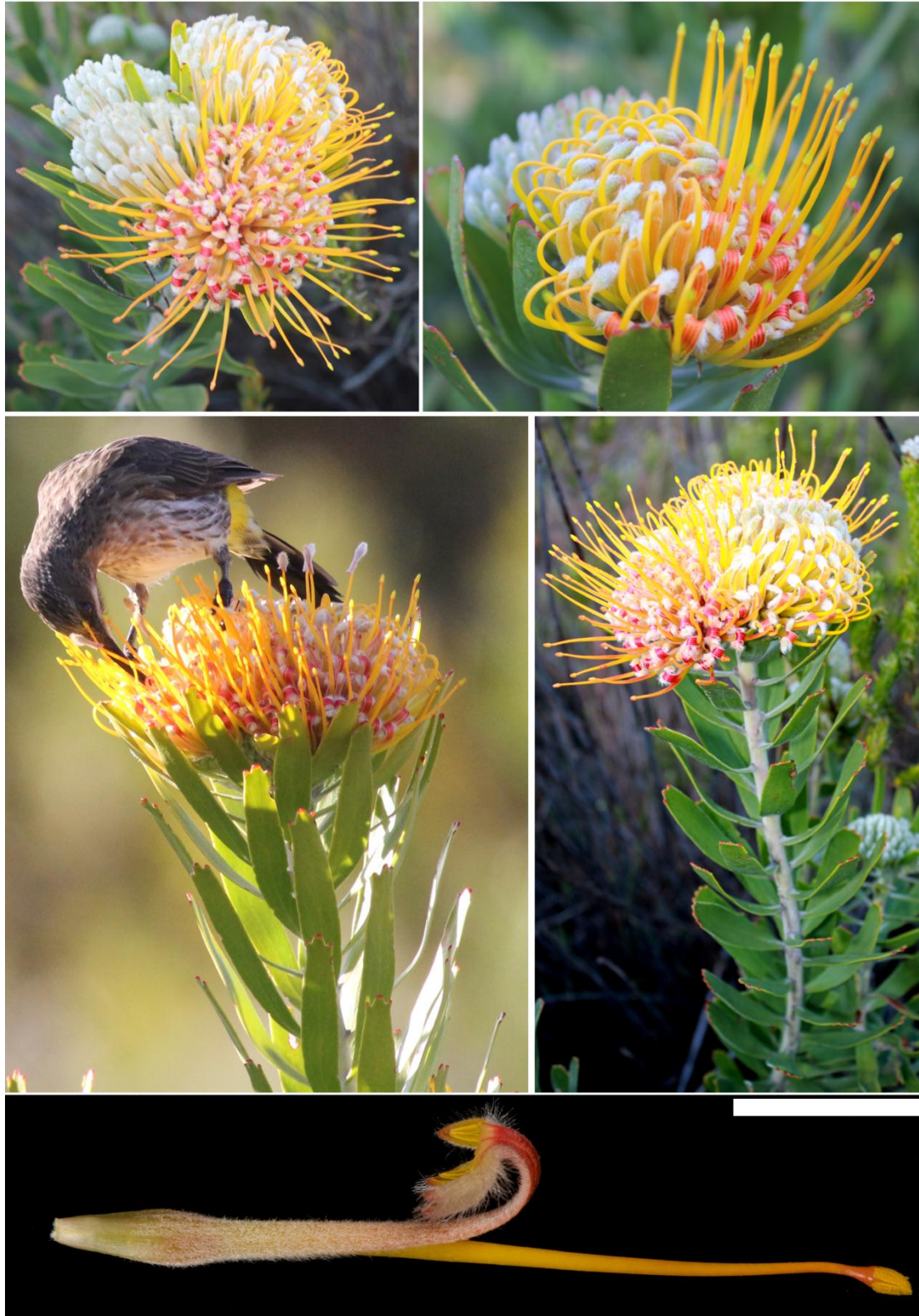
B.8: *L. cordifolium*. Population found near Bot River -34.21016, 19.16218 on November 4, 2013. Scale bar= 1cm.





B.9: *L. cuneiforme*. Population found in De Hoop Nature Reserve -34.4276, 20.65794 on November 6, 2013. Scale bar= 1cm.





B.10: *L. erubescens*. Population found near Garcia Pass -33.93198, 21.2041 on September 19, 2013. Pollinator shown is the Cape Sugarbird *Promerops cafer*. Scale bar= 1cm.





B.11: *L. formosum*. Population found in Dassieshoek Local Nature Reserve -33.74525, 19.85041 on October 15, 2013. Scale bar= 1cm.





B.12: *L. fulgens*. Found in De Hoop Nature Reserve -34.42746, 20.63924 on November 6, 2013.  
Scale bar= 1cm.





B.13: *L. glabrum*. Found near George -33.91834, 22.5074 on September 16, 2013. Pollinator shown is the Orange-breasted Sunbird *Anthobaphes violacea*. Scale bar= 1cm.





B.14: *L. gracile*. Found in Fernkloof Nature Reserve -34.39658, 19.26559 on September 11, 2012. Scale bar= 1cm.





B.15: *L. grandiflorum*. Found in Paarl Mountain Local Nature Reserve -33.731, 18.9235 on September 2, 2012. Scale bar= 1cm.





B.16: *L. gueinzii*. Population found in Jonkershoek Nature Reserve -33.99197, 18.98256 on December 6, 2012. Pollinator shown is the Cape Sugarbird *Promerops cafer*. Scale bar= 1cm.





B. 17: *L. hamatum*. Population found in Doringrivier Wilderness Area -33.86409, 22.17541 on September 14, 2013. Scale bar= 1cm.





B. 18: *L. harpagonatum*. Population found south of McGergor -34.03866, 19.9237 on October 17, 2013. Pollinator shown is the cape spiny mouse *Acomys subspinosus*. Scale bar= 1cm.





B.19: *L. heterophyllum*. Population found near Bredasdorp -34.60952, 19.9068 on October 29, 2013. Scale bar= 1cm.





B.20: *L. hypophyllocarpodendron* subspecies *canaliculatum*. Found in Mamre Nature Reserve - 33.5236, 18.4886 on August 18, 2012. Scale bar= 1cm.





B.21: *L. hypophyllocarpodendron* subspecies *hypophyllocarpodendron*. Population found in Kleyn Kloof Private Nature Reserve -34.65876, 19.56591 on September 26, 2013. Scale bar= 1cm.





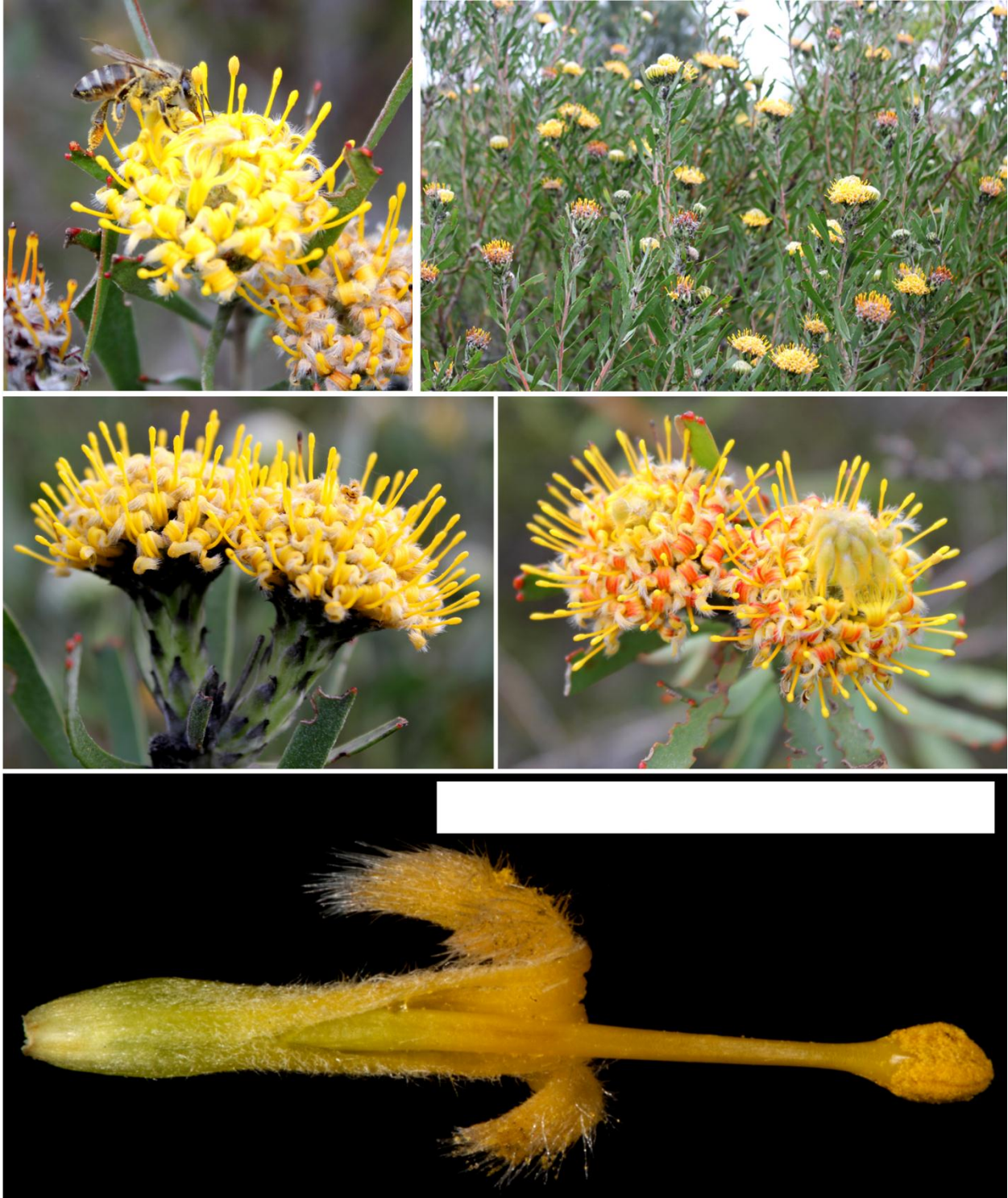
B.22: *L. lineare* subspecies *lineare*. Found in Jonkershoek Nature Reserve -33.946, 18.972 on November 2, 2012. Scale bar= 1cm.





B.23: *L. lineare* subspecies *calocephalum*. Population found near the Berg River Dam, Franschhoek -33.95122, 19.076 on October 9, 2013. Pollinator shown is the Cape Sugarbird *Promerops cafer*. Scale bar= 1cm.





B.24: *L. muirii*. Population found near Albertinia -34.2129, 21.5859 on September 18, 2013. Pollinator shown is of the genus *Apis*. Scale bar= 1cm.





B.25: *L. mundii*. Population found near Garcia Pass -33.96027, 21.2367 on September 18, 2013. Pollinator shown is the Orange-breasted Sunbird *Anthobaphes violacea*. Scale bar= 1cm.





B.26: *L. oleifolium*. Population found in Paul Cluver Nature Reserve -34.14308, 19.10681 on October 24, 2012. Carpenter bee pollinator shown is a member of the genus *Coelioxys* and bird pollinator is the Orange-breasted Sunbird *Anthobaphes violacea*. Scale bar= 1cm.





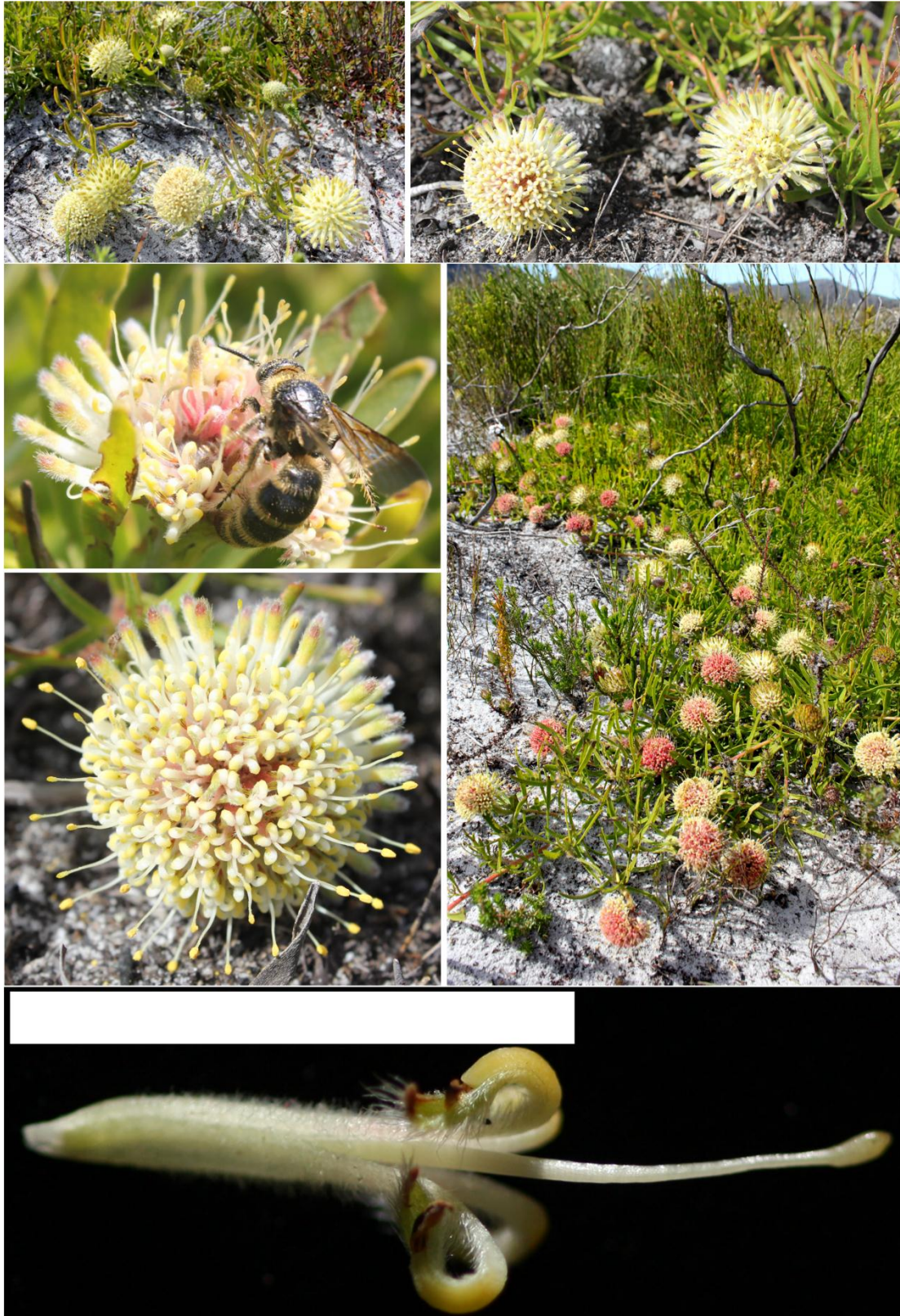
B.27: *L. parile*. Population found in Riverlands Nature Reserve -33.4905, 18.5989 on August 18, 2012. Pollinator shown is a member of the genus *Coelioxys*. Scale bar= 1cm.





B.28: *L. patersonii*. Found in Grootbos Private Nature Reserve -34.52797, 19.46865 on September 7, 2013. Scale bar= 1cm.





B.29: *L. pedunculatum*. Population found in Kleyn Kloof Private Nature Reserve -34.66201, 19.56385 on September 26, 2013. Pollinator shown is of the genus *Apis*. Scale bar= 1cm.





B.30: *L. pluridens*. Population found in Gamkaberg Nature Reserve -33.68883, 21.59108 on September 10, 2013. Pollinator shown is the Cape Sugarbird *Promerops cafer*. Scale bar= 1cm.





B.31: *L. praecox*. Population found north of Mossel Bay -34.06646, 22.06646 on September 18, 2013. Scale bar= 1cm.





B.32: *L. praemorsum*. Population found north of Clanwilliam -32.00109, 18.83877 on October 1, 2013. Scale bar= 1cm.



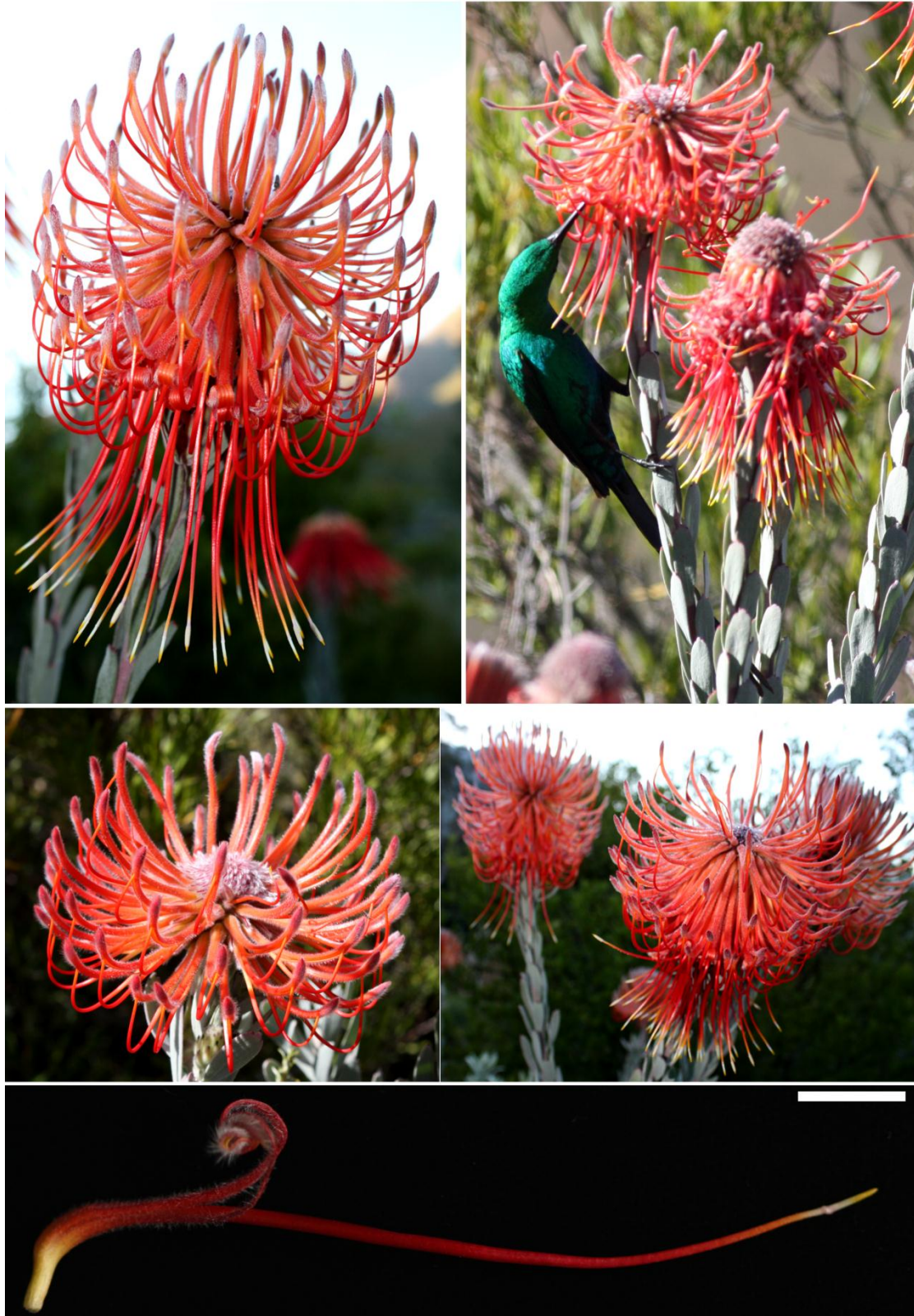
B.33: *L. profugum*. Population found in Piketberg -32.87747, 18.73123 on October 14, 2013.  
Scale bar= 1cm.





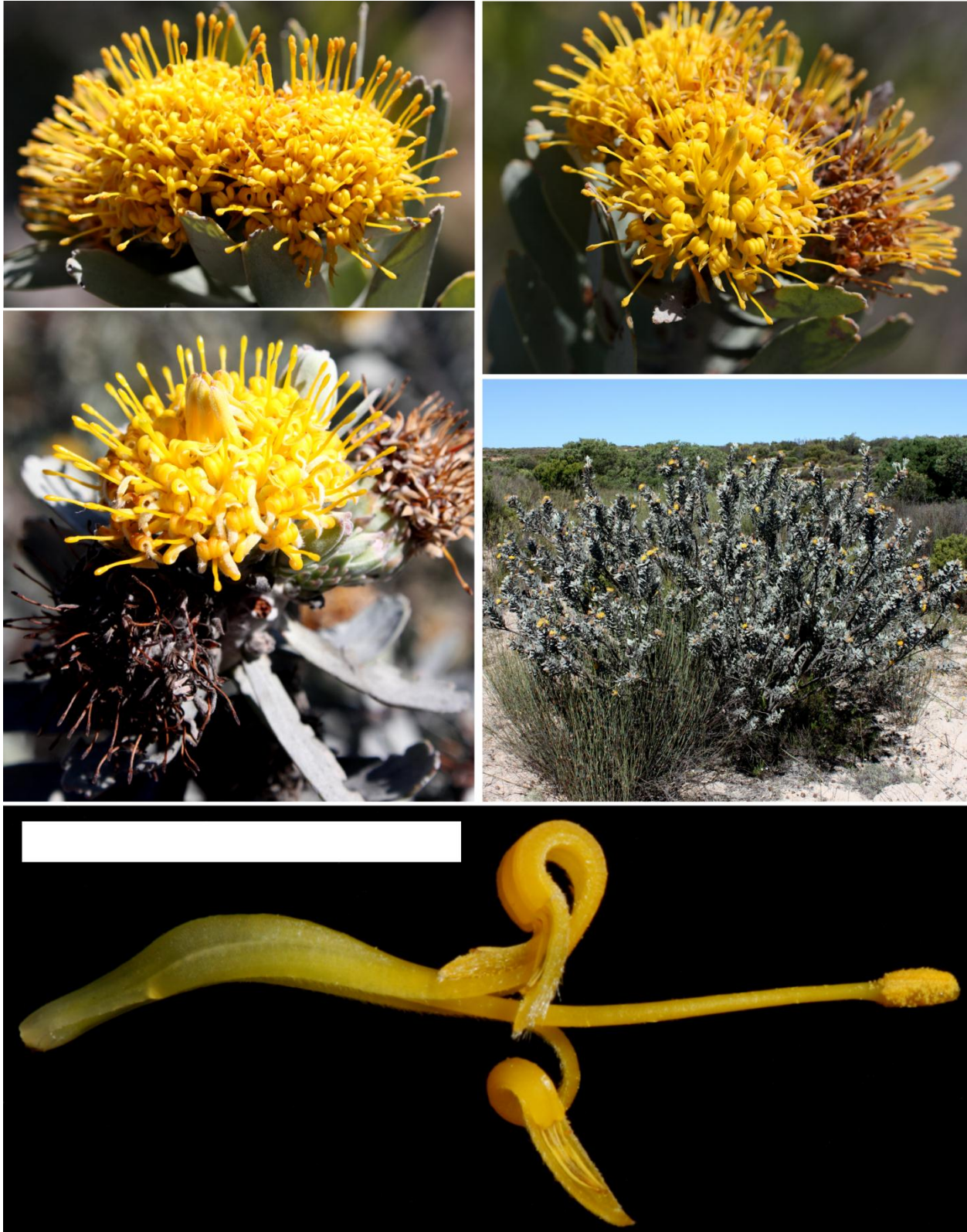
B.34: *L. prostratum*. Found in Fernkloof Nature Reserve -34.39658, 19.26559 on September 11, 2012. Scale bar= 1cm.





B.35: *L. reflexum*. Population found in the Cederberg Wilderness Area -32.54145, 19.27884 on October 3, 2013. Pollinator shown is the Malachite Sunbird *Nectarinia famosa*. Scale bar= 1cm.





B.36: *L. rodolentum*. Population found near Redelinghuys -32.631972, 18.781169 on September 17, 2012. Scale bar= 1cm.





B.37: *L. royenifolium*. Population found in Kammanassie Nature Reserve -33.62817, 22.58667 on September 17, 2013. Pollinator shows is of the genus *Apis*. Scale bar= 1cm.





B.38: *L. saxatile*. Population found east of Garcia Pass -33.92746, 21.34497 on September 19, 2013. Pollinator shows is of the family Scarabaeidae. Scale bar= 1cm.





B.39: *L. secundifolium*. Population found near Ladismith -33.45137, 21.20174 on December 4, 2013. Scale bar= 1cm.





B.40: *L. spathulatum*. Population found near Wolfberg Arch in the Cederberg Wilderness Area GPS -32.4515, 19.25544 on November 28, 2013. Pollinator shown is the Orange-breasted Sunbird *Anthobaphes violacea*. Scale bar= 1cm.





B.41: *L. tomentosum*. Population found in Jakkalsfontein Private Nature Reserve -33.40325, 18.2738 on August 18, 2012. Scale bar= 1cm.





B.42: *L. tottum* var. *glabrum*. Found northwest of Worcester -33.53888, 19.32832 on December 12, 2012. Pollinator shown is the Orange-breasted Sunbird *Anthobaphes violacea*. Scale bar= 1cm.



B.43: *L. tottum* var. *tottum*. Population found on du Toit's Kloof Pass -33.7167, 19.0693 on December 12, 2012. Long-tongued fly pollinator shown in *Philoliche rostrata* and bird pollinator is the Cape Sugarbird *Promerops cafer*. Scale bar= 1cm.





B.44: *L. truncatulum*. Found in Fernkloof Nature Reserve -34.39658, 19.26559 on September 11, 2012. Scale bar= 1cm.





B.45: *L. truncatum*. Population found in De Hoop Nature Reserve -34.39979, 20.41428 on November 5, 2013. Scale bar= 1cm.





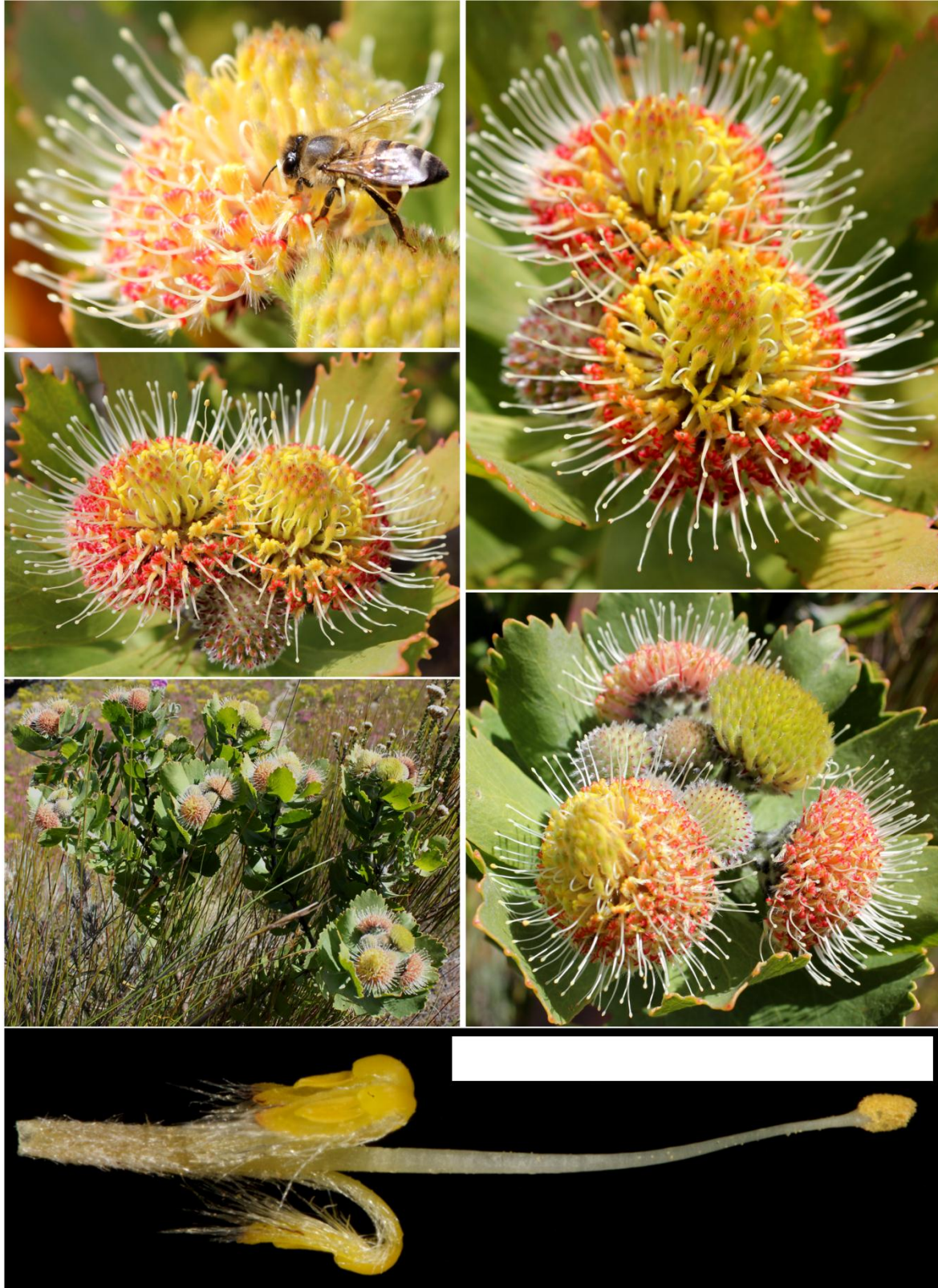
B.46: *L. utriculosum*. Population found north of De Hoop Nature Reserve -34.37199, 20.64839 on November 11, 2013. Scale bar= 1cm.





B.47: *L. vestitum*. Population found near Wolseley -33.4639, 19.224 on December 8, 2012. Scale bar= 1cm.





B.48: *L. winteri*. Population found near Garcia Pass -33.97277, 21.30182 on September 19, 2013. Pollinator shown is of the genus *Apis*. Scale bar= 1cm.





B.49: *L. wittebergense*. Population found north of George -33.79098, 22.43019 on September 20, 2013. Scale bar= 1cm.