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Population genetics of South African *Protea* L. (Proteaceae) species associated with various pollinator guilds

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“If I had an hour to solve a problem and my life depended on it, I would spend the first 55 minutes determining the proper questions to ask”

– Albert Einstein

Contents

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Abstract

South Africa hosts plant species with a large variety of floral traits associated with different pollinator guilds. Suites of specialised floral traits associated with particular pollinators are known as pollination systems or syndromes. However, it is often uncertain how these pollination systems affect gene flow between plant populations, mating system outcome, and subsequent genetic health of plant species. Genetic variability is an important aspect in understanding the long-term survival of a species because excessive homozygosity, as a result of high amounts of inbreeding, may restrict a species' long-term local adaptive potential. The African genus, *Protea*, is an ideal study system for exploring the evolution and maintenance of mating systems, because the pollination system for many species have been confirmed by pollinator exclusion experiments. The genus has several pollination systems including birds, insects and non-flying mammals. Comparative estimates of pollinators' outcrossing abilities are rare and confined to bird and insect guilds. Furthermore, 10 microsatellite markers have been developed for the genus, but have not been used to assess the outcrossing abilities of various pollinators. This study thus had two aims: Firstly, to use microsattelite markers to estimate the outcrossing rates and subsequent genetic diversity of *Protea* species primarily pollinated by either birds (*P. laurifolia* and *P. roupelliae*), insects (*P. caffra* and *P. simplex*) or non-flying mammals (*P. amplexicaulis* and *P. humiflora*). Secondly, I aimed to understand how birds and non-flying mammal pollinators affect population connectivity and gene dispersal. This was done by comparing indirect estimates of gene flow in the therophilous, *P. amplexicaulis,* and ornithophilous*, P. laurifolia.* These estimates included population differentiation (G''_{st}) between subpopulations and spatial genetic structuring within metapopulations of each species. I expected that less vagile pollinators, such NMP's (non-flying mammal pollinators), would contribute the least to gene flow and cause high selfing rates and low genetic diversity within therophilous populations. Contrastingly, flying pollinators would be able to travel longer distances resulting in more genetic connectivity between plant populations. The high energetic demands and interplant movements of flying animals were predicted to result in high outcrossing rates in ornithophilous and entomophilous species.

Non-flying mammal-pollinated (NMP) *Protea* species had high (> 0.8) and non-significantly different ($p > 0.05$) outcrossing rates relative to species pollinated by flying animals. Similarly, hand pollen supplementation experiments also revealed that small mammals were effective pollen vectors since *P. amplexicualis* individuals were not pollen limited. High multilocus outcrossing rates (> 0.80) may have resulted in all sampled

Protea species exhibiting high levels of heterozygosity (> 0.7) and low levels of inbreeding. However, some of the outcrossing events were between kin (approximately 14 %), giving evidence for biparental inbreeding. Furthermore, the relationship between geographic distance and genetic distance was significant for sampled *P. amplexicaulis* individuals. In contrast, sampled *P. laurifolia* individuals were genetically similar across the landscape. This pattern was reflected in fine-scale (> 500 m) spatial genetic structuring in a sampled metapopulaton of *P. amplexicaulis* and a homogeneous distribution of *P. laurifolia* genotypes. Restricted gene dispersal recorded for *P. amplexicaulis* in this study may have been a result of the high levels of biparental inbreeding. It was not clear whether poor gene flow was primarily a result of restricted pollen dispersal created by NMP's or restricted seed dispersal. Nevertheless, continuous limited gene dispersal between NMP populations may result in allopatric speciation over time. This provides a hypothesized reason as to why many of the therophilous *Protea* species are located in small and isolated populations. Additionally, there is a concern that restricted population size in combination with poor gene flow may lead to increased levels of inbreeding over time. This study provided the first evidence for localised gene dispersal, but high outcrossing rates, in NMP proteas.

1. Introduction

Reproduction is essential for gene transfer through generations. Levels of genetic variability (variation in the DNA sequence of a genome) in an individual are highly influenced by the type of reproduction employed. Angiosperms have three main mechanisms for reproduction, namely outcrossing, selfing and asexual reproduction. Where at least two of the three strategies are employed, this results in a mixed mating system (Goodwille et al., 2005; Lewis, 1942). Richards (1996) realised that the specific mating system of a plant could control a population's genetic variability. The extent to which individuals are outcrossing or selfing (i.e. mating system) influences the amount of gene flow between populations, population size (a critical indicator of a population's vulnerability to allele loss via genetic drift) and the expression of inbreeding depression within a population (Barrett and Harder, 1996; Espeland and Rice, 2010; Rodger *et al*., 2013). Therefore, gene flow and genetic differentiation can be directly linked to a species' mating strategy (Hamrick *et al*., 1992).

High rates of gene flow can increase genetic variability while reducing population differentiation (Richards, 1996; Rodger *et al*., 2013). Differences in gene flow and genetic differentiation between populations is often a reflection of a species' mating system. It is a long and withstanding consensus that outcrossers show higher levels of genetic diversity (Darwin, 1862) rather than selfers and that such variability is found among populations in the former while remains within populations in the latter (Barrett, 1998). In a selfing species, the low rates of cross pollen dispersal decreases the size of the gene pool of a species and results in reduced genetic variation within a population (Ingvarsson, 2002; Schoen and Brown, 1991). Low levels of genetic diversity compromise the ability of populations to adapt to rapid environmental change and subsequently reduces the probability of long-term persistence (Franklin *et al*., 2002). The hypothesis that species that employ self-fertilisation rarely contribute to evolutionary trends (Stebbins, 1957) is still under debate (Wright *et al*., 2013). However, there are some aspects of the hypothesis that are supported by empirical evidence suggesting that this breeding mechanism may allow for the accumulation of deleterious mutations and lower diversification rates in lineages, and lower species richness in populations (Ferrer and Good, 2012) suggesting that self-fertilisation may be considered an evolutionary dead end (Brown, 1976).

Despite the mostly negative consequences of selfing, some hermaphroditic species make use of both self-and cross-pollen resulting in a mixed mating system (Goodwille et al., 2005; Lewis, 1942). Intermediate outcrossing (i.e. a mixed mating system), allows for a varying ability to reproduce through inbreeding (Goodwillie *et al*., 2005). In stressful environments, such as unproductive habitats, selfing or asexuality is often favoured as it serves as a reproductive insurance policy to the plant when the probability of outcrossing is uncertain (Barrett, 1998; Busch and Delph, 2012; Morgan and Wilson, 2005). It is beneficial when a plant radiates into a temporary, pioneer habitat and ecologically useful for colonisation and expansion of plant species (Zhang *et al*., 2014). In extreme circumstances, autonomous breeding can be used in order to decrease the reliance on available mates and pollinators (Busch, 2005; Larson and Barrett, 2000; Stiles, 1978) which is also known as Baker's rule of mating systems in colonising species (Baker, 1955). Although this can increase the per- flower seed production (reproductive assurance), it causes seed discounting by pre-empting ovules and resources that could be used to make outcrossed seeds (Eckert and Herlihy, 2004). This makes selfing costly since outcrossed progeny appear to have a higher rate of survival and fitness (i.e. lower inbreeding depression) (Barrett, 1998; Charlesworth and Charlesworth, 1987). In their review, Charlesworth and Willis (2009) state that the overall probability of reduced fitness and seed germination in usually hermaphroditic plant species, such as *Silene latifolia* (Caryophyllaceae) (Richards, 2000), is halved in those species that are predominantly outcrossing. For example, Dudash (1990) found significant (> 50 %) reduction in fitness (lower seed germination, survival to reproduction and flower production) in selfed progeny compared to outcrossed progeny in *Sabatia angularis* (Gentianaceae) individuals.

Outcrossing in approximately 90 % (250 000 species) of flowering plants is facilitated by animal pollen vectors (Buchmann and Nabhan, 1996; Ollerton *et al*., 2011). The maintenance of high outcrossing rates is thus a function of the plant's ability to attract a pollinator and the promotion of repeat visitation (Barrett and Harder, 1996; Richards, 1996). This requirement has resulted in the evolution of numerous floral adaptations to attract floral visitors, ensuring outcrossing (Barrett, 1998; Barrett and Harder, 1996). For instance, some plants allocate more resources to exhibiting larger displays of flowers to encourage pollinator visitation (Goodwillie *et al*., 2010). Despite a plant employing a pollen vector, inbreeding may still arise through geitonogamy (movement of self-pollen between florets) especially in hermaphroditic plants and close relatives (biparental inbreeding) (Goodwillie *et al*., 2005). Consequently, floral traits that can limit self-fertilisation (e.g. protrandry or heterostyly) and promote the attraction of a pollinator (e.g. scent, size, colour, morphology) will be selected for (Darwin, 1876; Proctor and Yeo, 1996). The latter will be moulded by the effectiveness and visitation rates of pollinators, as these will promote outcrossing (Mayfield *et al*., 2001). Consequently, high outcrossing rates can be a function of the floral morphology in relation to a specific pollinator (Barrett and Harder, 1996).

Many studies of mating systems often exclude the influence of a pollinator and the pollination process (e.g. Awadalla and Ritland 1997; Barret and Shore, 1987). Consequently, mating system mechanisms are often separated from mating system outcomes (Harder and Barrett, 1996). For instance, few studies link pollinator movement to the realized mating system of flowers visited by those specific pollinators (Brunet and Sweet, 2006; Karron *et al*., 2004). This has led to a research gap and failure to understand that mating systems are influenced by floral biology, which determines the genetic structure and evolutionary outcome of a population.

1.1 Pollinator-driven diversification in southern Africa taxa.

The south Western Cape Province of South Africa is a known global biodiversity hotspot. The province is home to the Cape Floristic Region (CFR), an area with exceptionally high species richness (approximately 9000 species) and endemism (60-69 %) (Goldblatt and Manning, 2002; Linder and Hardy, 2004). The region has, therefore, become a focal point for studying patterns of evolutionary diversification and speciation (Goldblatt, 1978; Johnson, 1996; Schnitzler *et al.*, 2011). The high species richness and rapid lineage diversification in the Cape were first hypothesised to be a result of geographic and parapatric radiation caused by a mosaic of localised habitats due to steep ecological gradients and extreme contrasts in seasonal climate (Goldblatt and Manning, 2002). However, quantitative analyses show that sister taxa in some of the South African lineages involved mainly floral rather than vegetative traits (van der Niet and Johnson, 2009). Lineages that show floral diversification are likely to have diversified through a selection of pollinators, rather than a selection from the abiotic factors or herbivores (Stebbins, 1970). Consequently, it also became apparent that reproductive isolation caused by pollinator shifts has also played a large role in speciation in this region (van der Niet *et al*., 2006).

The conceptual Grant-Stebbins model of pollinator-driven diversification recognises that pollinators are distributed in a geographic mosaic and that the selection of flowers would differ across a plant species' range (Grant, 1949; Stebbins, 1970). In some cases, these differences in floral morphology can lead to pollinator shifts which precipitate speciation. Stebbins (1970) outlined several key principles associated with pollinator-mediated speciation and diversification including the most effective pollinator principle, the evolution of character syndromes, selection along the lines of least resistance, transfer of function, and reversals of evolutionary trends. However, many lineage radiations also involve divergent use of the same pollinator rather than whole pollinator shifts. For example, some lineages of two of the largest South African plant families, Orchidaceae and Iridaceae, are highly morphologically diverse despite being pollinated by one animal species (Johnson and Steiner, 2003). Consequently, there are several proposed modes of pollinator-mediated diversification other than pollinator shifts.

Pollinator-driven diversification in the CFR is reflected in the number of highly diverse floral genera. These genera often have specialised pollination systems. This includes pollination by birds (e.g. sugarbirds and sunbirds), insects (e.g. butterflies, bees, monkey beetles, hawkmoths) and non-flying mammals (e.g. elephant shrews, rodents) (Alexandersson and Johnson, 2002; Johnson and Steiner, 1997; Johnson, 1996; Kehinde and Samways, 2012; Kleizen *et al*., 2008; Rebelo *et al*., 1984). Therefore, the high floral diversity in this region is associated with a large variety of pollinators found here. It is, however, unclear how these pollination systems affect mating systems and subsequent genetic diversity of plant populations

1.2 Pollinator behaviour and mating system

Since animal pollen vectors differ in morphological and behavioural traits, pollinators can affect a plant's mating system through poor pollen transfer (efficiency) or foraging habits (e.g. flying versus walking or crawling) (Devaux *et al*., 2014; Frankie and Baker, 1974). Two main aspects of foraging behavior are important for patterns of gene dispersal. First, flight distances between plants will determine the distance over which pollen is transferred (Schmitt, 1980). Secondly, in self-compatible plants the number of flowers visited will determine the ratio of selfed versus outcrossed seeds that are produced (Schmitt, 1980). For instance, the high mobility of birds (e.g. Breed *et al*., 2015) and insects are expected to result in long distance interplant movements (e.g. honeyeaters have been recorded to fly over 10 m between plants) (Vaughton, 1990) and limited flower visits on a single plant individual (e.g. Hopper and Moran, 1981; Phillips *et al*, 2010) promoting pollen carryover and high mate diversity in the plants they pollinated (Whelan *et al*., 2009; Krauss *et al*., 2017). For example, foraging by honeyeaters resulted in considerable pollen movement between florets of the Australian Proteaceae species, *Banksia prionotes* (Collins and Spice, 1998). Similarly, bees were estimated to visit *B. menziesii* inflorescences ten times as much as birds showcasing that insects are as capable of dispersing and removing pollen (Ramsey, 1988). Contrastingly, floral visitors that cannot fly (such as rodents or shrews) are expected to frequently sample from a smaller gene pool resulting in high levels of inbreeding in a population (Collins and Rebelo, 1987). A pollinator's small foraging range can result in plants becoming genetically similar because the introduction of new genes into a population is scarce. Further cross-pollination between genetically related relatives may increase the levels of biparental inbreeding in a population (Kelly and Willis, 2002). Biparental inbreeding is also seen in plants that employ highly territorial pollinators such as bats, despite these pollinators being highly mobile (Collevatti *et al*., 2001). In this instance, bat-pollinated plants have a high outcrossing potential, but by sampling from a small gene pool, mating between kin increases and thus, the overall levels of inbreeding increase within the plant population.

Pollinators can also influence outcrossing rates by visiting the same flowers in succession on a singular plant, and thus increasing levels of geitonogamous (between flowers) self-pollination, leading to inbreeding (Matsuki *et al*., 2008; Karron *et al*., 2009). Camera trap data illustrated that non-flying mammals often probe the inflorescences on the same individuals of geoflorous *Protea* species (Zoeller *et al*., 2017), increasing the chances of geitonogamous selfing. Karron *et al.* (2009) provided empirical evidence that large bumblebees probed several flowers consecutively on *Mimulus ringens* multiflower displays. This pattern of pollinator visitation increased selfing rates (to 78%) in *M. ringens* flowers. Contrastingly, pollinators with high energetic demands and those that rely on nectar as a primary food source, often visit many inflorescences across a landscape, promoting high outcrossing rates (e.g. honeybees were recorded visiting inflorescences of *Grevillea macleayana* two-six times per hour) (Collins and Rebelo, 1987; Whelan *et al*., 2009). These pollinators are often birds or insects because they generally do not accumulate fat reserves resulting rapid utilization of the sugars sourced from nectar (Heinrich, 1975).

Infrequent or low pollinator visitation may create a pollen limited environment (e.g. Larson and Barret, 2001), and in this case, self-compatibility can reduce mate limitation by allowing a plant to make use of its own pollen, whereby autonomous self-pollination can provide reproductive assurance (Eckert *et al*., 2006; Kalisz and Vogler, 2003; Kalisz *et al*., 2004; Willi, 2009). For example, significantly high amount of seeds were set in autonomously selfed *Ruellla nudiflora* (Acanthaceae) individuals (Abdala-Roberts *et al*., 2012). This may indicate an inefficient and unreliable pollinator because pollinator visitation has been estimated to be directly proportionate to pollen receipt (Cayenne and Irwin, 2003).

These variations in the outcrossing rates are often represented in the amount of genetic variation within a population (Brown, 1989) where high homozygosity is often a reflection of high selfing rates and short distance pollen dispersal (Enjalbert and David, 2000; Richards, 1996; Wright *et al*., 2013). Charlesworth and Charlesworth (1995) used various models to illustrate that highly self-fertilising plant populations exhibited reduced genetic variability in contrast to outbred populations. Understanding how pollinators affect the mating system of plant species is imperative to understanding their long-term survival.

1.3 Comparative outcrossing abilities of pollinators

Comparative estimates of pollinator effectiveness in pollination ecology studies are often done by using seed set as a proxy for reproduction following the exclusion of specific pollinators or pollen supplementation (e.g. Angoh *et al*., 2017; Fleming and Nicolson, 2002a; Hargreaves *et al*., 2004; Wiens *et al*., 1983; Wright and Giliomee, 1991). For example, Angoh *et al.* (2017) illustrated that six *Erica* species with insect and bird pollination syndromes produced significantly more viable seed when flowers were cross-pollinated in comparison flowers that were supplemented with self pollen. This suggested that these *Erica* species are self-incompatible and that both birds and insects were important for *Erica* reproduction. These experiments do not work well in species that are self-compatible or have low and variable seed set since significant differences between treatments are difficult to determine (Wright, 1994). Alternatively, flowers can be emasculated to prevent self-fertilisation (e.g. Rodger *et al*., 2013), but this approach can also be difficult to implement in some species (such as those belonging *Protea* and *Banksia* genera) where pollen is used as a reward for pollinators or pollen is presented on a specialised portion of the style (pollen presenter) and in the vicinity of the stigma (Ayre and Whelan, 1989). Recently, advancements in genetically based techniques has made it possible to overcome some of these challenges and enable researchers to estimate parentage and outcrossing rates of plants pollinated by different pollinator guilds. These measures can be used to quantify the outcrossing abilities of different pollinators at a plant population level and/or supplement seed set data (Matsuki *et al*., 2008).

Outcrossing rates are a major determinant of the role pollen dispersal plays in gene flow (Fenster, 1991) and can be efficiently calculated using co-dominant markers such as allozymes and microsatellites (e.g. Karron *et al*., 1995). Few studies have made attempts at partitioning the contributions of various animal pollinators to outcrossing rates. Where comparisons between outcrossing abilities of pollinators are made, they are confined to birds and insects (e.g. Ayre and Whelan, 2009; Ottewell *et al*., 2009; England *et al*., 2001; de Almeida Vieira and de Carvalho., 2008). For example, using allozye markers, Schmidt-Adam *et al.* (2009) estimated higher outcrossing rates in bird-pollinated individuals of *Metrosideros excelsa* (Myrtaceae) than individuals pollinated by native bees. Allozyme markers were also used to show that hawkmoth pollinators increased outcrossing rates by 87% in *Aquilegia coerulea* in comparison to other groups of floral visitors such as syrphid flies, solitary bees and bumblebees (Brunet and Sweet, 2006). Allozyme markers are based on the protein variants within enzymes and different sizes of these proteins can indicate relatedness between individuals, parentage assignment, overall heterozygosity and inbreeding in a population (Ellstrand, 1984; Franklin *et al*., 2002; Hamrick *et al*., 1992; Cunningham., 1991). However, heterozygosity can be overestimated through misdetection of null alleles and they generally lack the variability required for in-depth parentage analysis (Lian *et al*., 2001; Schlötterer, 2004). Consequently, and because many of these molecular techniques have become cheaper and more accessible, these markers have been largely replaced by co- dominant nuclear markers, such as microsatellites (Schlötterer, 2004).

Microsatellites are simple sequence repeats (SSR's) of DNA sequences that are only one- six basepairs long (Franklin *et al*., 2002). They are considered the marker of choice compared to conventional allozymes because they occur frequently and randomly in the genome, are codominant (homozygosity and heterozygosity can be distinguished), have high information content and can be hypervariable and polymorphic (Franklin *et al*., 2002). The hypervariability and Mendelian inheritance of microsatellite loci is beneficial to detecting differences in the genotype of closely related species and individuals, thus making them suitable in studies for mating systems, including parentage analysis (Reusch, 2000) and pollen dispersal (Dow and Ashley, 1996; Isagi *et al*., 2004), as well as genetic variability in a population (Mable and Adam, 2007; Muneer *et al*., 2009). Furthermore, microsatellites have been proven to be powerful markers in differentiating populations (Blambert *et al*., 2016; Chaix *et al*., 2003) as they are the most likely to conform to the assumption of neutrality (Zardoya *et al*., 1996).

Microsatellite markers have been used to calculate outcrossing rates particularly in fragmented and isolated plant populations (Barrell *et al*., 1997) to conserve a species' genetic resources (Bezemer *et al*., 2016; Byrne *et al*., 2007; Collevatti *et al*., 2001; Lian *et al*., 2001). For example, Ritchie and Krauss (2012) used microsatellite markers to show that there was extensive pollen dispersal within and between restored populations of *B. attenuata* which indicated successful genetic management. Similarly, Frick *et al* (2014) used microsatellites to illustrate that birds cause complete outcrossing as well as high expected heterozygosity (0.59) in fragmented populations of *B. menziesii*. However, studies comparing pollinator efficiencies using microsatellites are rare (e.g. England *et al*., 2001; Ottewell *et al*., 2009; Breed *et al*., 2015) and are often restricted to invertebrate pollinators. Kudo *et al*. (2011) utilised these markers to assess how pollinator activity affects the contributions of various bees to outcrossing rates in the alpine shrub *Rhododendron aureum*. Although outcrossing rates were high (~ 80 %) throughout seasons, they fluctuated since worker bees were responsible for geitonogamous pollination throughout winter (Kudo *et al*., 2011). A similar study used microsatellite analyses to supplement visitation rate data to show that beetles transport large amounts of genetically diverse pollen in *Magnolia obovata* (Magnoliaceae) in relation to bumble bees which were responsible for causing inbreeding depression through geitonogamy (Matsuki *et al*., 2008). Genetic markers such as microsatellites are proving to be a useful tool in assessing pollinator efficiencies but are rarely used.

Ten microsatellites markers have been developed for the "white protea" clade (Prunier and Latimer, 2010) that have the potential to show population differentiation within the *Protea* genus (section: Exsertae). These microsatellites have previously been used to estimate a lack of gene flow between populations of several, primarily bird-pollinated, *Protea* species including *P. punctata*, *P. aurea*, *P. lacticolor*, *P. mundii*, *P. venusta,* and *P. subvestita* indicating that geographic isolation played a role in the diversification of the lineage (Prunier *et al*., 2014; Prunier and Holsinger, 2010). Although these genetic markers were developed for white proteas, they are also variable in other *Protea* species that do not belong to clade including geoflorous *P. amplexicaulis* and *P. humiflora*; and grassland proteas *P. caffra* and *P. simplex* (R. Prunier, pers. comm.). This indicates that there is potential to use these developed markers to calculate outcrossing rates and relatedness of a variety of *Protea* species.

1.4 The study system: South African *Protea* (Family: Proteaceae)

The Proteaceae is an ancient Gondwanaland family that is represented approximately 130 million years ago (Johnson and Briggs, 1975). The entire family now consists of about 1400 species and is virtually restricted to the Southern Hemisphere with its two richest concentrations occurring in Australia and Africa (Johnson and Briggs, 1975). The African clade has 14 genera (10 endemic), whereby most species (approximately 360 species) are situated within South Africa (centre of endemism and diversity is within the Cape Floristic Region) and only 69 species occurring in the rest of Africa (Valente *et al*., 2010). Most African species, with the exception of *Brabejum*, are proteoid, woody shrubs employing life history strategies such as serotiny, reseeding and resprouting that are indicative of adaptations to regions with a Mediterranean-like climate, e.g. South Western Cape of South Africa (Le Maitre, 1992).

Species in the *Protea* genus are characterised by spectacular floral diversity. High floral diversity implies that the *Protea* clade has undergone numerous pollination shifts since the break-up of Gondwanaland (Valente *et al*., 2010). However, the role that these pollinator shifts have played in lineage diversification is still under debate (Johnson, 2010; van der Niet and Johnson, 2012). Floral adaptations to various pollinators were most likely a response to the selection for the increase in efficiency of female function in flowers in a pollen-limited region (Johnson, 1996). A shift to a new pollinator escapes pollinator competition whereby pollen dispersal and deposition can decrease when the pollinator is shared amongst species (Muchala *et al*., 2010). *Protea* species employing the same pollination system often do not co-occur geographically (Schurr *et al*., 2012) or often flower consecutively, potentially avoiding competition for pollinators (e.g. Kuhn *et al*., 2017).

Protea species are visited by a variety of animal pollinators, such as birds (e.g. Hargreaves *et al*., 2004; Rebelo *et al*., 1984), insects (Steenhuisen and Johnson, 2012b) and mammals (e.g. Biccard and Midgely, 2009; Wiens and Rourke, 1978b; Zoeller *et al.,* 2017). This is reflected in the high variation of morphological traits, such as floral morphology, relative to these pollinators (Rebelo, 1987). Species pollinated by birds (e.g. *P. laurifolia*) typically produce large, tall and narrow or bowl-shaped inflorescences that produce copious amounts of nectar (Hargreaves *et al*., 2004). Ornithophily is the most common pollination system in *Protea* where sugarbirds (e.g. *Promerops cafer*) and sunbirds (e.g. *Nectarinia famosa*) are regular floral visitors attracted to the brightly coloured inflorescences (Rebelo *et al*., 1984). Entomophilous species (e.g. *P. simplex*) produce sweet-smelling inflorescences that are usually cream or white in colour (Steenhuisen *et al*., 2012a; Steenhuisen and Johnson, 2012c). These species are most associated with fruit chafer beetles (Cetoniine beetles) because inflorescences often do not provide enough nectar resources to meet the energetic demands of birds (Collins and Rebelo, 1987; Steenhuisen and Johnson, 2012b). The shift to mammal pollination in *Protea* is associated with inflorescences that produce strong smelling, cryptic and bowel shaped geoflorous or pendulous inflorescences (e.g. *P. humiflora*) (Wiens *et al*.,1983; Rebelo, 1987). The "yeasty" odour produced by these species' inflorescences attracts a variety of rodents, such as *Aethomys namaquensis*, *Acomys subspinosus*, *Mus minutoides*, *Micaelamys namaquensis* and *Rhabdomys pumilio* (Fleming and Nicolson, 2002a; Kühn *et al*., 2017; Zoeller *et al*., 2017); elephant shrews; and occasionally, small carnivores such as genets and mongoose (Kühn *et al*., 2017; Steenhuisen *et al*., 2015).

1.4.1 The role of pollinators in Protea *reproduction*

Colins and Rebelo (1987) have suggested that there are large overlaps of pollination systems in Proteaceae, as species can be exposed to a variety of floral visitors. However, various plant characteristics, animal visitor size and behaviour indicate that there are primary pollinators that may be more important than others for reproductive success (Collins and Rebelo, 1987). For instance, nocturnal rodents may be more significant pollinators than birds for plants whose flowers open at night or in plants that have geoflorous or cryptic inflorescences (Wiens and Rourke, 1978). Poor pollen transfer among flowers creates conditions of pollen limitation and thus, autogamy could be selected for as reproductive assurance (Kalisz and Vogler, 2003). Different pollinators could affect levels of autogamy by causing variations of pollen limitation. Therefore, the extent to which a species requires a pollinator for reproductive assurance is indicative of not only a cause of pollination shifts in a lineage but also affects species survival if pollinator abundance changes (Eckert *et al*., 2010). Early evidence regarding pollinator efficiency in plants was based on opportunistic pollen found on the animals. Consequently, quantitative evidence relating to importance of these pollinators as outcrossers is still lacking especially in the *Protea* genus.

Early studies of the breeding systems of *Protea* inferred that self-incompatibility is ubiquitous in the genus (Horn, 1962). However, a recent study by Steenhuisen and Johnson (2012b) infers at least five shifts (including *P. caffra*, *P. dracomontana*, *P. simplex*, *P. welwitschii, P. decurrens,* and *P. roupelliae*) to self-compatibility in *Protea* (Steenhuisen and Johnson, 2012a; Zoeller *et al*., 2017; Hargreaves *et al*., 2004). Shifts to autonomous selfing appear to be mostly associated with a shift to beetle pollination (Steenhuisen and Johnson, 2012b). This suggests that the shift to self-compatibility could be linked to the pollen dispersal ability of certain pollinators.

The role of bird pollinators has been typically showcased through selective exclusion experiments (Coetzee and Giliomee, 1985; Hargreaves *et al*., 2004), pollen loads on pollinators (Hargreaves *et al*., 2004) and the estimation of how variation in characteristics of floral resources such as nectar sugar concentration can encourage visitation of local bird pollinators (Gideon *et al*., 1980; Schmid *et al*., 2016). Most *Protea* species require pollination by bird vectors to produce significant levels of viable seed (Botha, 2017; Gideon *et al*., 1980; Hargreaves *et al*., 2004). In cases where viable seeds are still produced in the absence of birds, it was thought that insects could contribute to pollination as they are also attracted to the high nectar rewards. Insects (e.g. beetles, long probiscus flies and butterflies) are often found on species that are thought to be entirely bird pollinated, including *Protea roupelliae*, *Protea repens, Protea punctata and Protea nitida* (Botha, 2017; Carlson and Holsinger, 2010; Coetzee and Giliomee, 1985; Hargreaves *et al*., 2004; Johnson *et al*., 2012)*.* Previous methods of seed set analysis have overestimated the value of insects to pollination success as autogamy has been overlooked (Coetzee and Giliomee, 1985). Apart from *Protea roupelliae*, it is unclear whether seed set in bird pollinated *Protea* species excluded from birds is associated with insect pollination or autogamy (Hargreaves *et al*., 2004).

Fewer studies have been conducted on the role that insects play in *Protea* reproduction in comparison to birds, except in terms of seed predation (e.g. Steenhuisen and Johnson, 2012a). It was thought that insect visitors promote inbreeding as they spend large amounts of time within *Protea* inflorescences, more frequently moving pollen between florets within inflorescences than between inflorescences, despite carrying large amounts of pollen (Matsuki *et al*., 2008). Therefore, birds are more likely to be efficient outcrossers as they move more frequently between plants (Hargreaves *et al*., 2004). Insect pollination may be of more importance in species that produce sweet smelling inflorescences with reduced amounts of low-quality nectar (Collins and Rebelo, 1987). In these cases, the nectar rewards would be too low to encourage repeat visitation by birds. However, some species primarily associated with pollination by cetoniine beetles (e.g. *P. caffra*), indicate levels of autonomous selfing and inbreeding depression (Steenhuisen *et al*., 2012b; Steenhuisen and Johnson, 2012b). This may suggest that although beetles are effective at pollen deposition, they may be facilitating self pollination and increasing levels of inbreeding within populations.

The relationship between small mammal and pollination in *Protea* species are difficult to record because many these pollinator species are nocturnal (Kleizen *et al*., 2008). High floral visitation (approximately 69 % of total visitation by vertebrates), recorded by camera trapping, could suggest that non-flying mammals are reliable pollinators in *Protea* (Kühn *et al*., 2017; Meek, 2011; Zoeller *et al*., 2017). Other methods have included live capturing of rodents which have estimated high amounts of pollen loads on their noses and in faeces (Kühn *et al*., 2017; Nicolson and van Wyk, 1998) as well as recording diel patterns in nectar production to assess the relationship between small mammals and *Protea* species (Kleizen *et al*., 2008; Wiens and Rourke, 1978). Studies have shown that excluding vertebrates (principally mammals) from geoflorous *Protea* species (e.g. *P. nana, P. humiflora*, and *P. foliosa*), reduces seed set, suggests that mammals could be effective pollinators (Biccard and Midgley, 2009; Fleming and Nicolson, 2002a; Melidonis and Peter, 2015). However, it is unclear whether seeds set in the absence of mammals (e.g. in *P. humiflora*) are thought to be a result of insect pollination or self compatibility (Fleming and Nicholson, 2002a; Zoeller *et al*., 2017). Therefore, the value of mammal pollination for these *Protea* species is unclear with just using seed set as a measure of reproductive success.

The extent to which different pollinators are contributing to reproductive success in *Protea* has been inferred, but is difficult to quantify because natural seed set in *Protea* is low (one to two seeds per inflorescence) (Ayre and Whelan, 1989) even in species that were thought to be self-compatible. Low seed set makes detecting significant differences between treatments difficult resulting in pollinator effectiveness and shifts to self-compatibility to be unclear in the genus (Hargreaves *et al*., 2004; Steenhuisen and Johnson, 2012a). Therefore, there is a need to estimate mating systems in *Protea* species using genetic techniques.

1.4.2 Genetic consequences of pollination systems in Protea

Studies exploring the genetic consequences of pollinators in Proteaceae are confined to bird-pollinated Australian species (Byrne *et al*., 2007). For example, Ritchie *et al.* (2012) showed that bird pollination in *B. hookeriana* results in extensive pollen dispersal and high multiple paternity. However, quantifying the evolutionary consequences of employing these pollination systems at a genetic level has not yet been fully explored in South African *Protea* species, despite the genus' high economic and ecological value. To date, there is only one study that has comparatively estimated the outcrossing abilities of pollinators in *Protea*. Steenhuisen *et al.* (2012b) used allozyme markers to estimate equal contributions of birds and insects (scarab beetles) to moderate (± 0.60) outcrossing rates in *P. caffra*. Similar conclusions were made by Whelan *et al.* (2009) when the outcrossing abilities of birds and introduced honeybees were compared in Australian *Grevillae* species (Proteaceae). So far, small mammals have yet to be included in comparative analyses of outcrossing rates. Virtually all non-flying mammal pollinated (NMP) *Protea* species are located in scattered and isolated pockets associated with specific soil types (Wiens *et al*., 1983). Species with restricted distributions may have formed a reliance on non-flying mammal pollinators over birds or insects (Ward and Johnson, 2005). The small and isolated populations of NMP *Protea* species are a concern for the survival for these species as they are potentially susceptible to inbreeding depression caused by a lack of gene flow (Franklin *et al*., 2002). However, research on the genetic consequences of mammalian pollinators is rare and restricted to bats (Collevatti *et al*., 2010; Lobo *et al*., 2005). The only study exploring the genetic consequences of non-flying pollinators is by Cunningham (1991). They used allozyme markers to estimate low (0.2) inbreeding in non-flying mammal pollinated *Banksia* species indicating that the limited mobility of these pollinators was not causing inbreeding. However, empirical estimates of the outcrossing rates of species pollinated by non-flying mammals are still unknown. To aid in conserving these species with often isolated ranges, it is imperative to understand the relative contribution by mammals to cross pollen dispersal and gene flow within and between populations, in comparison to other pollinator guilds.

1.5 Objectives of the thesis

While the importance of various pollinators has been estimated by pollinator exclusion experiments in the *Protea* genus, information on the relative outcrossing abilities of pollinators is superficial and confined to comparisons between birds and insects (Steenhuisen *et al*., 2012b). Therefore, in order to gain a deeper understanding of the evolution of pollination systems in the *Protea* genus, more information on how each of the main pollinator guild contributes to genetic diversity and gene flow in *Protea* species is needed.

This study focused on six species of *Protea* employing different pollination systems namely *Protea caffra* and *Protea simplex* (insect-pollinated), *Protea laurifolia* and *Protea roupelliae* (bird-pollinated), and *Protea amplexicaulis* and *Protea humiflora* (small mammal-pollinated). The species were chosen based on their adequate population size, polymorphism in microsatellite markers and conformity to the typical floral adaptations associated with the various pollinators (Steenhuisen and Johnson, 2012b; Wiens and Rourke, 1978a). Due to their lack of flight ability, small mammals are expected to contribute the least to population genetic diversity and gene flow (Wiens *et al*., 1983). However, empirical testing of outcrossing and pollen dispersal abilities is lacking. Since many mammal-pollinated *Protea* species occur as small and localised populations (Wiens *et al*., 1983), it is important to understand whether small mammals contribute to their evolutionary fitness as this will determine localised adaptability. *In situ* conservation management of these species may need to take place if they are susceptible to inbreeding depression and potential local extinction as a result of prolonged reproductive failure or nonviable genetic diversity.

Specifically, my objectives were to 1) determine whether the mammal-pollinated *Protea amplexicaulis* is self-compatible, capable of setting seed in the absence of pollinators, and pollen limited (Chapter 3). This information is lacking for *P. amplexicaulis* and is needed to fully interpret mating system results, 2) compare the outcrossing rates of *Protea* species with different pollination systems, namely bird, beetle and mammal pollination systems. The outcrossing rates and genetic diversity in *P. caffra*, *P. laurifolia*, *P. amplexicaulis, P. humiflora, P. simplex, and P. roupelliae* will be estimated using differences in allelic frequencies in developed microsatellites markers (Chapter 2), 3) investigate whether isolation-by-distance is taking place in NMP *Protea* species by comparing estimates in genetic distances and genetic diversity between populations of the mammal pollinated *P. amplexicaulis* and the bird-pollinated *P. laurifolia* (Chapter 3).

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2. Outcrossing abilities of bird, insect, and non-flying mammal pollinators and their effects on genetic diversity in *Protea* L.

Abstract

South Africa is home to plant species with a large variety of floral traits associated with different pollinator systems. It is often uncertain how these pollination systems affect gene flow between plant populations, mating system outcome, and subsequent genetic health of plant species. This study estimated the outcrossing rates and subsequent genetic diversity of *Protea* species primarily pollinated by birds (*P. laurifolia* and *P. roupelliae*), insects (*P. caffra* and *P. simplex*) and non-flying mammals (*P. amplexicaulis* and *P. humiflora*) using the developed microsatellite markers. Non-flying mammal pollinated (NMP) *Protea* species had high (> 0.8) and non-significantly different outcrossing rates relative to species pollinated by flying animals. High outcrossing rates may have resulted in all species exhibiting high levels of heterozygosity (> 0.7) and low levels of inbreeding. However, some (approximately 14 %) of the outcrossing events were between kin, evidenced by finding biparental inbreeding. It is not clear whether the biparental events are a result of limited seed or pollen dispersal. Nevertheless, long term biparental inbreeding can result in high population differentiation and isolation, precipitating speciation. Therefore, future studies should aim at determining the spatial genetic structuring and interpopulation gene flow in NMP proteas. This study was the first to illustrate that pollination by non-flying mammals can result in inbreeding in comparison to mobile pollinators such as birds or insects.

2.1 Introduction

The South Western Cape Province of South Africa is a known global biodiversity hotspot where exceptionally high species richness (approximately 9000 species) and endemism (60–69 %) occurring in only 90 000 km^2 (Goldblatt and Manning, 2002). The region has, therefore, become a focal point for studying patterns of evolutionary diversification and speciation (Goldblatt, 1978; Johnson, 1996). The high species richness and rapid lineage diversification in the Cape were first hypothesised to be a result of geographic and parapatric radiation caused by a pockets of localized habitats as a result of steep ecological gradients, spatial heterogeneity and extreme contrasts in seasonal climatic conditions (Goldblatt and Manning, 2002). In more recent literature, it has also become apparent that reproductive isolation caused by pollinator shifts has played a role in the radiation of plant lineages in this region (van der Niet *et al*., 2006). This is reflected in the number of highly diverse floral genera and specialisation of pollination systems in the Cape Floristic Region (CFR) such as pollination by birds (e.g. sugarbirds), insects (e.g. bees, monkey beetles, hawkmoths) and non-flying mammals (e.g. elephant shrews, mice) (Alexandersson and Johnson, 2002; Johnson and Steiner, 1997; Johnson, 1996; Kehinde and Samways, 2012; Kleizen *et al*., 2008; Rebelo *et al*., 1984; Biccard and Midgley, 2009).

It is unclear how various pollination systems affect mating systems (genetic relatedness and pairings between individuals e.g. levels of selfing or outcrossing) (Neal and Anderson, 2004) and subsequent genetic diversity of plant populations. Since animal pollen vectors differ in morphological and behavioural traits, pollinators can affect a plant's mating system through poor pollen transfer (efficiency) or foraging habits (e.g. flying versus walking or crawling) (Devaux *et al*., 2014; Frankie and Baker, 1974). For instance, small foraging ranges of pollinator species can increase the level of biparental inbreeding (mating between genetically similar relatives) in plant populations which have highly territorial floral visitors such as bats (Collevatti *et al*., 2001) or those with low energetic demands (e.g. Schmitt, 1980). Small foraging ranges of pollinators reduce the amount of novel genetic material that is introduced into a population resulting in relatives becoming genetically similar over time. If cross-pollination continues between these related individuals, overall inbreeding will increase in the plant population (Kelly and Willis, 2002). Pollinators can also influence outcrossing rates by visiting the same flowers in succession on a singular plant, and thus increasing levels of geitonogamous (between flowers) self-pollination, leading to inbreeding (Matsuki *et al*., 2008). Infrequent pollinator visitation may create a pollen limited environment, and in this case, self-compatibility can reduce mate limitation by allowing a plant to make use of its own pollen. In extremely pollen limited environments, autonomous self-pollination can provide reproductive assurance (Eckert *et al*., 2006; Kalisz and Vogler, 2003; Kalisz *et al*., 2004; Willi, 2009). These variations in the mating system are often represented in the amount of genetic variation within a population (Brown, 1989) where high homozygosity is often a reflection of high selfing rates and short distance pollen dispersal (Enjalbert and David, 2000; Richards, 1996; Wright *et al*., 2013). High selfing rates are considered an evolutionary 'dead end' whereby selfing taxa experience elevated extinction risk owing to a reduced potential for adaptation (Igic *et al*., 2008; Stebbins, 1957). Understanding how pollinators affect the mating system of plant species is imperative to understanding their long-term survival.

Empirical testing of how pollinators affect the mating or breeding system of a species is usually done through pollinator exclusion experiments (Angoh *et al*., 2017; Fleming and Nicolson, 2002a; Hargreaves *et al*., 2004; Wiens *et al*., 1983; Wright and Giliomee, 1991) and using seed set as a proxy for reproduction. However, this method does not work well in species that are self-compatible or have low and variable seed set since significant differences between treatments are difficult to determine (Wright, 1994). An alternative method is to estimate the relative contributions of each pollinator to outcrossing rates. Multi-locus outcrossing rates can be efficiently determined through the use of co-dominant markers such as allozymes or microsatellites (Franklin *et al*., 2002; Schmidt-Adam *et al*., 2000). Studies using these markers to assess the contribution of different pollinator guilds to outcrossing rates are rare and confined to comparisons between birds and insects (England *et al*., 2001; Shmidt-Adam *et al*., 2000; Steenhuisen *et al*., 2012b). For example, using allozymes Shmidt-Adam *et al.* (2000) found that bees and birds had similar contributions to outcrossing rates in the New Zealand shrub, *Metrosideros excelsa* (Myrtaceae). Another study used microsatellite markers to show that introduced honeybees could be as important to outcrossing as native birds in *Grevillea macleayana* (Proteaceae) (England *et al*., 2001). Similar outcrossing abilities could be a result of the flight ability of large flying insects and birds which allows them to move large distances between plants (Collins and Rebelo, 1987). These pollinators also generally have high nectar energy demands and require that they visit numerous inflorescences, and thus promote high levels of outcrossing (Collins, 1983; Schmid *et al*., 2016; Waser and Ollerton, 2006). Therefore, it is expected that the small foraging ranges of non-flying mammal pollinators (NMPs) will result in relatively high inbreeding in plant populations, and a decrease in intra-population genetic diversity in comparison to flying floral visitors (Faegri and Van der Pijl, 1979). However, evidence of the relative outcrossing rates of NMP's is lacking. Here, we aim to illustrate the extent at which less vagile floral visitors could be causing inbreeding relative to floral visitors that have flight ability.

The large (360 species) southern African genus, *Protea* L. (Proteaceae) exhibits pronounced variation in floral traits and breeding systems (Johnson and Briggs, 1975; Schurr et al., 2012). It forms an ideal study system since it employs specialised pollination systems primarily of birds (e.g. *Promerops cafer*), insects (e.g. Cetoniine beetles) and non-flying mammals (e.g. *Aethomys namaquensis*, *Rhabdomys pumilio*) (Biccard and Midgley, 2009; Fleming and Nicolson, 2002a; Hargreaves *et al*., 2004; Kühn *et al*., 2017; Meek, 2011; Steenhuisen and Johnson, 2012b; Wiens and Rourke, 1978; Zoeller *et al*., 2017). Ornithophily is the most common pollination system in *Protea.* Species primarily pollinated by birds often produce large, brightly coloured and terminal flower heads that secrete copious amounts of easily digested glucose-rich nectar (Hargreaves *et al*., 2004; Rebelo *et al*., 1984; Wright and Giliomee, 1991). Contrastingly, therophilous species generally exhibit cryptic inflorescences with brown or maroon involucral bracts (Collins and Rebelo, 1987). Non-flying mammals are attracted to the "yeasty" scent of these inflorescences which are annually produced from winter until spring (Collins and Rebelo, 1987; Fleming and Nicolson, 2002a, b; Zoeller *et al*., 2017). The least recorded pollination system is insect pollination because insects that were found in flower heads were identified as only attractants for birds (Gideon *et al*., 1980). However, several *Protea* species (e.g. *P. dracomontana*) are associated primarily with beetle pollination

(Steenhuisen and Johnson, 2012b). These species generally produce sweet, spicy or sour scented bowl-shaped inflorescences with pink, cream, white or yellow involucral bracts (Rebelo, 2000; Steenhuisen *et al*., 2012a).

The importance of bird, beetle and small mammal floral visitors for reproduction in *Protea* species is often supported by reduced seed set following exclusion experiments of pollinators (Biccard and Midgley, 2009; Wright and Giliomee, 1991; Zoeller *et al*., 2017). However, *Protea* species are characterised by low and variable seed set (Wright, 1994) and thus, using exclusion experiments may be an unreliable measure of the relative contribution by each pollinator to the species' seed set especially for self-compatible species (Steenhuisen *et al*., 2012b). In cases where species can produce seeds from self pollen, it is uncertain whether seed set is a result of an efficient pollinator. To date, there is only one study comparing the outcrossing rates of various floral visitors, where Steenhuisen *et al*. (2012) used allozymes to show similar, but moderate (0.6-0.65) outcrossing rates in *P. caffra* in inflorescences that were exposed to insect pollinators only and those exposed to all vertebrate and invertebrate floral visitors. However, allozymes generally lack the variability required for in-depth parentage analysis (Lian *et al*., 2001; Schlötterer, 2004) and banding patterns on starch gels were often very difficult to score (S-L. Steenhuisen, pers. comm.). Consequently, aided by dropping costs of genetic analyses, these markers have been largely replaced by nuclear markers, such as microsatellites (Cruzan, 1998; Schlötterer, 2004; Tautz, 1989).

Ten microsatellite markers have been developed for the "white protea" clade (Prunier and Latimer, 2010) that have the potential to show population differentiation within the *Protea* genus (section: *Exsertae*). However, these microsatellites have only been used to estimate a lack of gene flow between populations of several *Protea* species including *P. punctata*, *P. aurea*, *P. lacticolor*, *P. mundii* and *P. venusta* indicating that geographical isolation played a role in the diversification of the lineage. (Prunier *et al*., 2014; Prunier and Holsinger, 2010). This study is the first to use these microsatellite markers to understand the relative evolutionary consequences of various pollination systems in Cape flora taxa by estimating 1) the contribution of various pollinators to outcrossing rates, and 2) the resultant intrapopulation genetic diversity of non-flying mammal-, bird-and beetle-pollinated *Protea* species.

2.2 Methods

2.2.1 Study species and sampling sites

Six species belonging to the South African *Protea* genus (sugar bushes, family Proteaceae) were chosen for the study due to their known pollination systems and amplification of microsatellites in the desired marker region. These species include therophilous *P. amplexicaulis* (Salsib.) R.Br. (Fig 1-1) and *P. humiflora* Andrew (Fig 1-2); entomophilous *P. caffra* Meisn*.* (Fig 1-3) and *P. simplex* E. Phillips ex J.M. Wood. (Fig 1-4); and ornithophilous *P. laurifolia* Thunb (Fig 1-5) and *P. roupelliae* Meisn (Fig 1-6).

Figure 1: Inflorescences of six *Protea* species. The non-flying mammal-pollinated (NMP) proteas include *Protea amplexicaulis* (1) and *Protea humiflora* (2) (photo: Alex Connolly). Insect-pollinated proteas include *Protea caffra* (3) (photo: Sandy-Lynn Steenhuisen) and *Protea simplex* (4) (photo: Sandy-Lynn Steenhuisen). Bird-pollinated proteas include *Protea laurifolia* (5) and *Protea roupelliae* subs. *roupelliae* (6) (photo: Nigel Foreshaw).

P. amplexicaulis and *P. humiflora* are low sprawling shrubs (usually one to three meters wide) and endemic to the south Western Cape of South Africa (Collins and Rebelo, 1987; Rebelo, 2000). Populations of *P. amplexicaulis* are distributed on north-facing slopes of inland mountain ranges from Koue Bokkeveld to Langeberg and Caledon, and eastwards towards Worcester (Rebelo, 2000). Similarly, *P. humiflora* populations are located on dry slopes of inland mountain ranges in Dutoitskloof to the Langeberg and Wabooomsberg (Rebelo, 2000). The breeding systems of *P. humiflora* and *P. amplexicaulis* are uncertain but seed set in both species is significantly reduced in the absence of non-flying mammals (Fleming and Nicolson, 2002a; Wiens *et al*., 1983).

Grassland species, *P. caffra* and *P. simplex,* differ in their morphologies (Rebelo, 2000). *P. caffra* is an upright shrub that can grow up to eight meters tall and *P. simplex* is a low sprawling shrub up to 16 cm wide (Rebelo, 2000). However, both species are facultative resprouters that flower from December until March. There are overlaps in the distribution of both species where *P. caffra* populations are distributed on rocky outcrops or grassland areas of Gauteng, KwaZulu-Natal, Northern Province and as far south as the Katberg Mountains, and *P. simplex* is found in rocky grasslands of Mpumalanga and KwaZulu-Natal from Mariepskop to Vryheid (Rebelo, 2000). Both species are self-compatible and facultatively autogamous (Steenhuisen and Johnson, 2012a).

P. laurifolia and *P. roupelliae* are upright shrubs that grow between three and eight meters tall. *P. laurifolia* is self-incompatible while *P. roupelliae* can produce self-pollinated seeds, but cannot autonomously self (Collins and Rebelo, 1987; Hargreaves *et al*., 2004). Both species have wide geographic ranges where *P. laurifolia* populations are distributed on sandstone or granite soils on inland mountains ranges of the Western Cape including those in Nieuwoudtville to Franschhoek and Anysberg, whereas *P. roupelliae* populations are distributed in north eastern South Africa on quartzite ridges of Gauteng, Northern Province and Mpumalanga (Rebelo, 2000) including the Drakensberg mountains and surrounding foothills of Kwazulu-Natal.

Individuals of *P. laurifolia*, *P. amplexicaulis,* and *P. humiflora* were sampled on Jonaskop (33° 58′ 00 S 19° 30′ 00 E, altitude 1646 m) which is part of the Riviersonderend mountain range in the south Western Cape of South Africa (Fig 2). Samples of *P. amplexicaulis* and *P. laurifolia* were collected in February (summer) and June (winter) 2017 respectively whereas *P. humiflora* was collected in summer 2016. *P. humiflora* is found in dense stands in the driest fynbos of fynbos/succulent karoo ecotone on the lower slopes of the mountain, a habitat that is dominated by Asteraceae and Thymeleaceae where it usually is the only species of Proteaceae present (Fleming and Nicolson, 2002a). *P. laurifolia* and *P. amplexicaulis* were collected in proteoid fynbos on the upper slopes of Jonaskop. The south Western Cape has a seasonal climate of hot, dry summers and winter rainfall (Linder, 1991). Fires usually occur in summer, but no recent fires were recorded on Jonaskop during the time of sampling.

P. simplex, *P. caffra,* and *P. roupelliae* were sampled in July-August (winter) 2016 on Mount Gilboa (29° 17' 04.0" S 30° 17' 30.9" E, altitude 1750 m) which forms part of the Karkloof and Mt Gilboa Nature reserve in the Kwazulu-Natal Province of South Africa (Fig 2). The vegetation can be characterised as low, open and critically endangered grassland with rocky outcrops (Lechmere-Oertel, 2011). This site receives summer rainfall (approximately 900 mm) and cold, dry winters that are prone to frost. Fires usually occur during Autumn when wind speeds are high (Lechmere-Oertel, 2011).

2.2.2 Sampling protocol

2.2.2.1 Adult tissue

Leaves and seeds from one population of each of the six study species were sampled (Fig 1). Within each species' population, six mature and healthy (uneaten and no signs of disease) leaves and five seedheads (cohort one to two years) were collected from 20 adult individuals where plants were approximately 10 meters apart from one another. Three leaves from each individual plant were cut into half centimeter squared blocks and preserved in a concentrated Cetyl Trimethylammonium Bromide (CTAB) solution (see Appendix for protocol to make concentrated CTAB solution) in separate microcentrifuge tubes (Thomson, 2002) and then immediately stored at -20°C until DNA extraction. As an additional source of DNA, the remaining three leaves were placed in a coffee filter envelope and stored in an airtight container filled with silica gel. The silica gel was removed and re-dried at 60°C overnight every day for approximately three days to maximise leaf moisture absorption.

2.2.2.2 Offspring tissue

From each individual's infructescences, 20 plump and pliable seeds were selected by hand. Species in the *Protea* genus are known for low and variable seed set (Wright, 1994) and thus require hand separation of fertile and infertile seeds. Seeds thought to be fertile contained a white cotyledon, determined by cutting through the base of the seed. As a less destructive proxy for seed set, the plumpness of seeds was used to represent fertility. However, this can only be confirmed through successful germination.

The seeds of samples were germinated in order to harvest leaf material for further genetic analyses. Before sowing, the sorted seeds were soaked overnight (12 hours) in Kirstenbosch smoke primer discs (Super Smoke Plus, Kirstenbosch National Botanical Gardens, Cape Town, South Africa) prior to storing them dry at four degree celcius for another 12 hours (cold shocked) in order to encourage or induce germination (Rebelo and Rourke, 1985; Richards *et al.*, 1997). Each individual plant's 20 seeds were sown five centimeters apart in a 50 cm^2 seedling tray filled with a mixture of acidic soil (pH between five and six) and covered with a half a centimeter layer of sifted river sand (Richards *et al*., 1997). After sowing, the trays were placed underneath a shade cloth (Fig 3-1) at the Kirstenbosch National Botanical Garden's plant nursery (Cape Town, Western Cape). Watering procedures involved spraying municipal water over the trays until the soil was moist straight after sowing and then again, every one to two days until germination. Once germinated, all cotyledon leaves or two to three true leaves (Fig 3-2) were collected from six to eight individuals from each tray (representing the offspring of each maternal plant sampled), placed in a concentrated CTAB solution and stored at -20°C until DNA extraction (Thomson, 2002). Germination time varied between species but, all species showed signs of germination after a three month period. The germination trial was completed approximately four months after sowing when leaves from a sufficient number of offspring had been collected. Some seeds were predated upon by rodents or insects and this reduced the sample size of seedlings in some seed families.

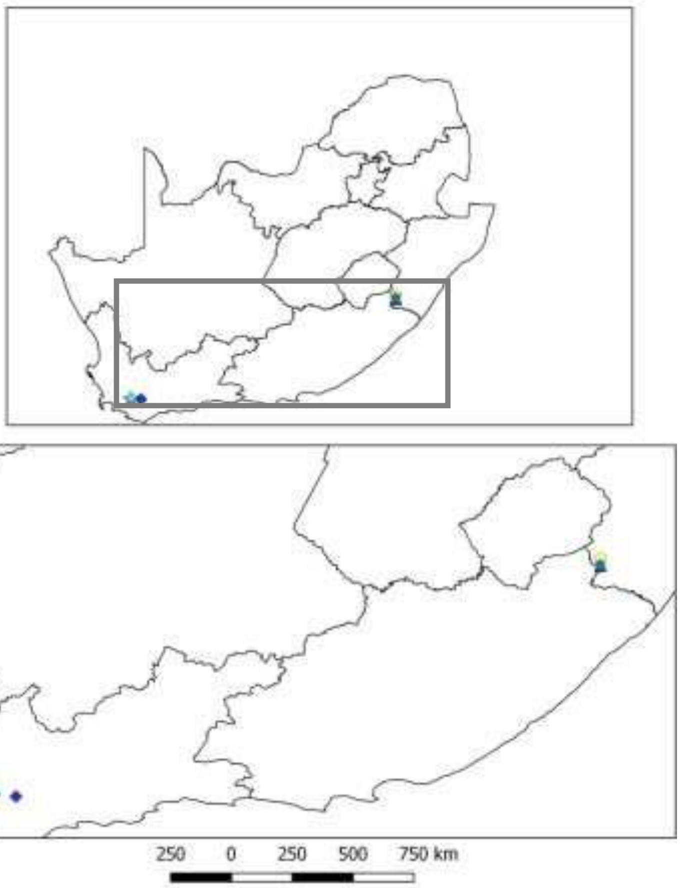

Figure 2: Sampling locations of all populations within South Africa is shown on the enlarged bottom map which is located in the region of the box demarcated in black. *Protea simplex* (⬠), *Protea roupelliae* (■) and *Protea caffra* (■) were sampled on the summit of Mount Gilboa (29° 17' 10.0" S 30° 17' 30.0" E, altitude 1750 m) in the Kwazulu-Natal Province. *Protea amplexicaulis* (★), *Protea humiflora* (●) and *Protea laurifolia* (◆) were collected on the slopes of Jonaskop (33°58′00 S 19°30′00 E, altitude 1646 m) within the Riviersonderend mountain range. Borderlines indicate demarcation of the various provinces in South Africa.

2.2.2.3 Genomic DNA extraction and amplification

Genomic DNA extraction was achieved using a modified CTAB procedure (Doyle and Doyle, 1987), quantified using a NanoDrop™ spectrophotometer (ThermoFischer Scientific, Applied Biosystems) and assayed by running four microlitres of each sample on a one percent agarose gel stain with ethidium bromide or SYBR Green gel stain (see Appendix for DNA extraction protocol using CTAB). Samples that had a DNA concentration above 20 ng/ul (R. Prunier, pers. comm) and were visible on an agarose gel were assumed to have a high enough DNA yield for further analysis. DNA was re-extracted from samples that did not meet these criteria.

Figure 3*:* Seedling tray layout (1) underneath a shade cloth at Kirstenbosch National Botanical Gardens. The offspring of different individuals of each species were sown into different trays. After seeds had germinated (2), the cotyledon leaves or true leaves were collected and stored in a concentrated CTAB solution at -20°C.

DNA was amplified using a QIAGEN multiplex PCR method using nine fluorescently tagged microsatellite primers (Table 1 in Appendix) (Prunier and Latimer, 2010) known to be polymorphic for these species (R. Prunier, pers. comm.). During analysis, an extra marker region was amplifying for primer 1.7.1 for all species, which we named 1.7.1 Long (Table 1 in Appendix). By finding this extra marker, it brought the number of loci used for the analysis to 10. The cycling conditions for amplifying microsatellites using the QIAGEN multiplex PCR method (see Appendix for reaction mixture) included an initial heat activation for 15 min at 95°C and a three-step cycling process involving denaturation for 30 s at 94°C, annealing for 90 s between 60°C, extension for 60 s at 72°C and then repeating the cycle 30 times after which a final extension of 30 min at 60°C occurs. PCR products were diluted in autoclaved distilled water (ddH$_2$0) in a one to nine ratio. Thereafter, GeneScan™ 600 LIZ size standard (Thermofischer Scientific, Applied Biosystems) and formamide were added to the diluted PCR products before fragment analysis. Fragment analysis for *P. simplex* (October, 2017), *P. caffra* (August, 2017)*,* and *P. humiflora* (December, 2018) was conducted at Cornell University, New York, USA and the remaining species' fragment analyses was completed at the Central Analysis Facility in Stellenbosch, South Africa (fragment analysis for *P. laurfolia* and *P. amplexicaulis* individuals was completed in November 2018 and December 2018 respectively). To keep PCR conditions constant, each individual belonging to a species was amplified on the same 2720 or Veriti thermal cycler (Applied Biosystems). GeneMarker software V2.2.0 (SoftGenetics, State College, PA, USA) was then used to score and visually verify all microsatellite scores. The fragments ranged from 100 to 500 base pairs.

2.2.3 Analyses

2.2.3.1 Genetic diversity

To compare the effects of pollination system on the population genetic variability in six *Protea* species (i.e. *Protea amplexicaulis*, *P. caffra*, *P. humiflora*, *P. laurifolia*, *P. roupelliae*, and *P. simplex*), six measures of genetic diversity were used: allelic richness (A_r) which is also interpreted as the mean number of alleles per locus; expected heterozygosity (H_e); percentage of polymorphic loci (%); gene diversity over loci (the probability that two alleles on a locus will be different in a population); effective allele number (A_e); and inbreeding co- efficient (F_{is}) calculated as 1-(H_o/H_e), where H_o represents observed heterozygosity. Effective allele number is used as a measurement of the number of equally frequent alleles a population would have to possess to achieve the same level of heterozygosity as with the observed number of alleles per locus (allelic richness). Therefore, the effective allele number can represent the number of alleles in the population with high frequencies. With the exception of gene diversity over loci and polymorphic loci (%), locus by locus values (Table 2 in Appendix) was estimated for each population in Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010) and GenAlex version 6.51b2 (Peakall and Smouse, 2012). Non-amplification of some individual's microsatellites resulted in a reduction in the number of loci used for each population. The number of missing loci varied between species (Table 2 in Appendix). Only loci with less than 10 % missing data and at least two allele variants were used for the analyses. This was to remove the biases that may arise in analysis as a result of missing data or the presence of null alleles. Because less than ten loci were used in the analysis and some loci had poor amplification success, the threshold of 10 % missing data (V. Naude, pers.comm) was chosen as a conservative measure of removing data from each loci (Table 2 in Appendix). Furthermore, the number of individuals used for the analysis in some population (i.e. species) reduced as result of sample degradation. Specifically, only 19 *P. laurifolia* individuals and 17 *P. roupelliae* individuals were used in the analysis. The statistical significance of any difference of each genetic diversity measurement between species was calculated by using a non-parametric Kruskal Wallis rank sum test and a pairwise Wilcox rank sum test when needed in R version 3.2.3 (RStudioTeam, 2015).

2.2.3.2 Outcrossing rates

To compare the outcrossing abilities of various pollinators in *Protea*, species-specific maximum likelihood estimates of single-locus (t_s) and multilocus (t_m) outcrossing rates were estimated for *P. amplexicaulis, P. caffra, P. laurifolia and P. simplex* using a multilocus mating system program, MLTR version 3.2 (Ritland, 2002). Ideally, the outcrossing rates of all the study species would be used for the analyses. However, collecting and genotyping *P. humiflora* and *P. roupelliae* offspring was beyond the financial and time constraints of the current study and outcrossing were not calculated for these species. Offspring leaf samples of *P. humiflora* were confiscated in September 2017 and time constraints did not allow for resampling. Standard deviations for estimates of t_s, t_m, and t_m–t_s were based on 1000 bootstraps with resampling within families. The difference t_m–t_s represents a test for biparental inbreeding where the difference will be zero in the absence of biparental inbreeding. The potential to detect outcrossing events increases with an increase in the number of sampled loci, and thus t_s will usually be lower than t_m in the presence of inbreeding. Differences between species' outcrossing rates were assessed using pairwise comparisons of bootstrap estimates. Two species' outcrossing rates were considered significantly different if 99.5 % of the differences between randomly paired bootstrap estimates (e.g. $t_{\text{P. laurifolia+k}}$ - $t_{\text{P. amplexicaulis+k}}$ for the k^{th} bootstrap estimate of t_m) were greater or less than zero (two-tailed test, $\alpha = 0.05$). The test percentage was calculated using the equation defined by Barrett and Eckert (1994) as 100*(1-a_{pc}/2) where a_{pc} is the type I error rate per contrast and calculated as 1- (1- a_{ew})$^{1/C}$. The experimentwise error rate (a_{ew}) was set at 0.05 and the number of species' contrasts (C) was 4. This method was also used to test whether all the estimates (t_s, t_m, and t_m–t_s) were significantly different from zero (one-tailed test, $\alpha = 0.05$). Discrepancies (39 discrepancies) between allele calls and maternal genotypes were excluded from analyses (Table 3 in Appendix). These discrepancies may have taken place as a result of mislabeling individuals. Furthermore, some individual's seed germination was poor, resulting in the number of offspring genotypes per maternal genotypes being low. The average number of offspring used per adult individual for each species was as follows: 5 offspring for each of 17 *P. amplexicaulis* adults, 4 offspring for 14 *P. caffra*, 2 offspring for *P.simplex*, and 4 offspring for 13 *P. laurifolia* individuals.

2.3 Results

2.3.1 Genetic diversity

Levels of allelic richness (A_r) were similar ($X^2_{(5)} = 3.5$, p = 0.6) and above 7 alleles per locus for all species, a trend that was also reflected in the number of polymorphic loci (> 70 %) across species (Table 1). The number of effective alleles (A_e) (4-7 alleles per locus) was similar to the respective values of A_r (8-11 alleles per locus) for all species, indicating an even spread of alleles across loci. Gene diversity over loci was high (> 0.50) across all species (Table 1). The lowest gene diversity over loci was recorded for both *P. roupelliae* (0.57 ± 0.32) and *P. simplex* (0.59 ± 0.36). Nevertheless, both species were also highly polymorphic (70 % for *P. roupelliae* and 40 % for *P. simplex*). Overall mean H_e was high across all species (0.6-0.8) and differences were non-significant ($X^2_{(5)} = 3.1$, p = 0.6). *P. simplex* and *P. amplexicaulis* exhibited the highest mean H_e values (0.75 ± 0.27 for *P. simplex* and 0.74 ± 0.2 for *P. amplexicaulis*, Table 1). Overall H_e for all species was interpreted as between 0.7 and 1 since the majority of H_e values were in this range (Table 2 in Appendix). Except for *P. simplex* and *P. caffra*, mean F_{is} was greater than 0 for all species, thus depicting that there was a deficiency of heterozygotes relative to Hardy-Weinberg expectations (Table 1). F_{is} values were the highest for non-flying mammal-pollinated species (0.34 ± 0.4 for *P. amplexicaulis* and 0.26 ± 0.35 for *P. humifora*). However, differences of F_{is} between species is not significant ($X^2(5) = 4.3$, p = 0.6).

Table 1: Genetic diversity estimates including mean ± SD expected heterozygosity (H_e), mean ± SD inbreeding co-efficient (F_{is}), mean ± SD allelic richness (A_r), mean ± SD effective allele number (A_e), percentage polymorphic loci and gene diversity over loci ± SD for six *Protea* species; *P. amplexicaulis* (20 individuals), *P. caffra* (20 individuals), *P. humiflora* (22 individuals), *P. laurifolia* (19 individuals), *P. roupelliae* (17 individuals), and *P. simplex* (20 individuals). Estimates were calculated using loci with less than 10 % missing data.

		Estimate					
Pollination system	Species	H_e ± SD	F_{is} ± SD	A_r ± SD	A_e ± SD	Polymorphic loci (%)	Genediversity over loci ± SD
Non-flying mammal-pollinated	*P. amplexicaulis*	0.74 ± 0.2	0.34 ± 0.4	11 ± 1.7	6 ± 0.8	50	0.64 ± 0.36
	P. humiflora	0.68 ± 0.17	0.26 ± 0.35	11 ± 7.5	6 ± 1.7	40	0.62 ± 0.34
Bird-pollinated	*P. laurifolia*	0.63 ± 0.23	0.17 ± 0.26	8 ± 1.3	4 ± 2.6	80	0.62± 0.34
	P. roupelliae	0.68 ± 0.31	0.18 ± 0.23	8 ± 5.7	4 ± 1.1	70	0.57± 0.32
Insect-pollinated	*P. caffra*	0.65 ± 0.3	0.05 ± 0.18	11 ± 7.5	6 ± 1.7	90	0.62 ± 0.34
	P. simplex	0.75 ± 0.27	0.06 ± 0.14	11 ± 6.3	7 ± 1.6	40	0.59 ± 0.36

2.3.2 Outcrossing rates

Overall outcrossing rates of species were high (> 0.85) and all measures were significantly different from zero (Table 2). Pairwise contrasts revealed that t_m multilocus outcrossing rates were similar for all species ($p > 0.05$) with the exception of the NMP *Protea*, *P. amplexicaulis,* which exhibited the lowest outcrossing rates of 88 %. In bird-and insect- pollinated species 94-95 % of mating events were a result of outcrossing. The mean ± SD difference t_m-t_s was the highest, but not significantly higher than other species', for *P. amplexicaulis* (0.13 ± 0.042) indicating that there is more biparental inbreeding occurring in this species' population. Multilocus outcrossing estimates were only marginally higher than single locus estimates for the bird- and insect-pollinated species representing little or no biparental inbreeding in these species.

Table 2: Maximum likelihood (MLTR) estimates (± SD) for multilocus outcrossing rates (*tm*), single locus (t_s) and biparental inbreeding (t_m-t_s) for four *Protea* species with various pollination syndromes, namely *Protea amplexicaulis* (NMP = non-flying mammal-pollinated)*, Protea caffra and Protea simplex* (insect-pollinated), and *Protea laurifolia* (bird-pollinated). Sample sizes are shown as the average number of progeny per locus and the number seeds with the seed families. * Denotes significant differences from zero (one tailed test, $p < 0.05$).

	NMP	Insect-pollinated		Bird-pollinated
MLTR estimate	*P. amplexicaulis*	*P. caffra*	*P. simplex*	*P. laurifolia*
t_m ± SD	0.88 ± 0.07*	0.96 ± 0.09*	0.94 ± 0.06*	0.94 ± 0.09*
t_s ± SD	0.78 ± 0.07*	0.92 ± 0.05*	0.92 ± 0.04*	0.86 ± 0.06*
t_m-t_s ± SD	0.14 ± 0.04*	0.04 ± 0.07	0.02 ± 0.04	0.08 ± 0.06*
Mean number of offspring per locus ± SD	66 ± 41	45 ± 5	43 ± 12	65 ± 23
Total number of seeds per population (total number of seed families)	82 (17)	49 (14)	30 (17)	42 (13)

2.4 Discussion

This is the first study to use microsatellite markers to understand the influences of pollination systems on outcrossing rates and genetic diversity of six African *Protea* species. Although inbreeding (F_{is}) and outcrossing rates (t_m) is no higher in non-flying mammal pollinated species in comparison to bird-and beetle pollinated species, both selfing and biparental inbreeding appear to be more common. Despite NMP proteas exhibiting high biparental inbreeding, population genetic diversity did not significantly decrease in this group. This suggests that inbreeding is higher in NMP *Protea* species (*Protea amplexicaulis* and *Protea humiflora*), but that this mating system difference hasn't led to noticeable differences in within population genetic diversity.

Similar outcrossing rates were estimated for both beetle- and bird-pollinated *Protea* species indicating that both pollinator guilds are effective cross-pollinators. This is supported by Steenhuisen *et al*. (2012b), the only other study to estimate the outcrossing abilities of various pollinators in *Protea*, which used allozymes to show that both birds and beetles are contributing to high (± 0.65) outcrossing rates in *P. caffra*. Centoniine beetles can spend long periods of time inside *Protea* inflorescences, spreading self-pollen from dehisced anthers from the inner florets to the receptive stigmas of the outer florets. Observations of scarab beetles found that they do not tend to visit several inflorescences on the same plant but, rather fly over several plants before settling again (Englund, 1993), making these beetles efficient at dispersing pollen over long distances since there was little grooming of pollen from their bodies during flights between plants.

Importantly, genetic analyses showed that inbreeding is not significantly higher in non-flying mammal-pollinated (NMP) *Protea* species than in *Protea* species pollinated by birds or insects. The low inbreeding values (mean F_{is} per locus = 0.2-0.34) found in NMP species (*P. amplexicaulis* and *P. humiflora*) are consistent with the low inbreeding values (F_{is} = 0.2) found in an Australian Proteaceae species, *Banksia intregifiolia,* which is pollinated by marsupials (Cunningham, 1991). Inbreeding co-efficient values above zero suggest that there is an excess of homozygotes, but the mean F_{is} values found in this study are still considered low (Cunningham, 1991). Low inbreeding values is reflected in the high multilocus outcrossing rates presented in this study for *P. amplexicaulis* (t_m = 0.88±0.07). High outcrossing rates were also found in *Banksia sphaerocarpa* (t_m = 0.86-0.99), a bird pollinated species (Llorens *et al*., 2012). Considerably high outcrossing rates and low inbreeding values found in *P. amplexicaulis* may be a result of the energy requirements of the small mammals that visit *Protea* inflorescences. These mammalian visitors are often medium-sized (> 100g) with metabolic requirements similar to that of birds or insects, demonstrating the need for these pollinators to visit large numbers of inflorescences in a foraging bout (Collins and Rebelo, 1987).

This study also found that all species had high outcrossing rates (tm = 0.8- 0.96) suggesting that all pollinators are effective at cross pollinating proteas. Contrary to expectations, non-flying mammal pollinated species, *P. amplexicaulis*, had high multilocus outcrossing rates (tm = 0.88 ± 0.07). Since these pollinators are considered to be less vagile than flying pollinators, it was expected that outcrossing rates of *P. amplexicaulis* would suggest high inbreeding (Rebelo, 1987). Carthew and Goldingay (1993) argued that high outcrossing rates for Proteaceae may be a reflection of postzygotic processes or preferential outcrossing (Heliyanto *et al*., 2005) rather than the outcrossing abilities of pollinators. This is supported by selective abortions of self- fertilized ovules in *Banksia spinulosa* after experimental self-pollen supplementation on inflorescences (Heliyanto *et al*., 2005; Vaughton and Carthew, 1993). Therefore, it is uncertain whether the high outcrossing rates amongst species is a reflection of a mobile visitor or the active selection against self-pollen (Goldingay and Carthew, 1998). There is little known about how intensively small mammals forage on a single plant and their interplant movements. Wiens *et al*. (1983) found that these animals can exhibit inter-plant movements as far as 15 m, similar to the distances that birds usually travel (20-30 m). However, the honey possum (*Tarsipes rostratus*), a main pollinator of *Banksia* species, have been recorded to travel less than 30 m even over several months (Garavanta *et al*., 2000), making it likely for these small mammals to pollinate near neighbours rather transfer pollen over long distance. Neverthless, Carthew (1994) found that 72 % of plant movements of NMP's employed by Australian Proteaceae were between plants rather than within plants where most (70 %) of these inter-plant movements were greater than five meters and could be as far as 59 m, indicating that non-flying mammals may be moving considerable distances between individuals.

Preferential outcrossing in Proteaceae may have resulted in the moderate outcrossing rates (± 0.65) of *P. caffra* estimated by Steenhuisen *et al.* (2012b) compared to those found in this study (± 0.9). Because *P. caffra* is self-compatible and lower outcrossing rates are expected for these species, it is surprising that the high outcrossing rate found in *P. caffra* (in the current study) is similar to species that are highly outcrossed (e.g. populations of *Grevillea barklyana* have been estimated to have outcrossing rates above 0.8) (Ayre *et al*., 1994). This may be explained by selective abortions of ovules fertilised by self-pollen (Wilcock and Neiland, 2002). Variations in outcrossing rates could also be a result of numerous other factors. For instance, the current study used a comparably low mean number of progeny (Table 3) per locus in *P. caffra* and *P. simplex* in relation to *P. amplexicaulis* and *P. laurifolia.* Therefore, only a major deviation from panmixis would have been detected in *P. caffra,* possibly resulting in an overestimation of outcrossing rates. Additionally, allozyme markers used by Steenhuisen *et al.* (2012b) may have underestimated relatedness between individuals because they can be difficult to visualise on starch gels (S-L. Steenhuisen, pers. comm.) and generally lack the variability required for in-depth parentage analysis (Lian *et al*., 2001; Schlötterer, 2004). Contrastingly, microsatellite loci are hypervariable making them beneficial for detecting differences in the genotypes of closely related species and individuals, making them suitable for estimating mating systems, multiple paternity (Reusch, 2000) and pollen dispersal (Dow and Ashley, 1996; Isagi *et al*., 2004). Nevertheless, allozymes are still considered a reliable measure to estimate population genetic parameters in comparison to microsatellites (Conte *et al*., 2008). As highlighted, there are various explanations that could provide reasons as to why estimated *P. caffra* outcrossing rates in this study were higher than those estimated by Steenhuisen *et al.* (2012b), making it difficult to determine the cause of this variation in outcrossing rates in populations of *P. caffra*.

Although outcrossing events are common in *P. amplexicaulis*, some of these events are occurring between relatives (t_m-t_s = 0.137 ± 0.04). This was almost double the amount of biparental inbreeding found in bird-pollinated congener, *P. laurifolia* (t_m-t_s = 0.08 ± 0.06). Since biparental inbreeding values significant from zero are considered high (Collevatti *et al*., 2001), the level of biparental inbreeding in *P. amplexicaulis* can affect the substructure of populations by restricting gene flow and causing isolation between populations. However, it is important to consider that seed dispersal can also play a large role in biparental inbreeding events since the distance between related individuals is a function of how far seeds are dispersed from one another and from the parent plant (Collevatti *et al*., 2001; Wright, 1969). Seed dispersal in members of the *Protea* genus is expected to be limited in space (Bond *et al*., 1984) since seeds are released *en masse* and most seedling recruitment was found to be closer than 10 m from the parent plant in, for example, *P. laurifolia* (Manders, 1986). Plant density estimates of *P. amplexicaulis* would indicate whether pollinator movements are the primary cause of biparental inbreeding events. Nevertheless, expected heterozygosity (0.6- 0.8) and allelic richness (8-11 alleles per locus) were not different amongst species, suggesting that this level of biparental inbreeding is not reflected in a decrease in population genetic diversity. Our levels of expected heterozygosity are consistent with estimates of *Banksia cuneata* (0.65-0.75) with considerably high gene flow between populations (Maguire and Sedgley, 1997). Therefore, this suggests that the level of biparental inbreeding taking place in *P. amplexicaulis* is not affecting intrapopulation genetic variability.

Short distance pollen dispersal, inferred from high levels of biparental inbreeding, may indicate that small mammals are ineffective at dispersing pollen over long distances. Long term biparental inbreeding can cause inbreeding depression especially in small populations where inbreeding events are common. This leads us to question why plant species have floral adaptations to non-flying mammals despite their suggested limited gene dispersal abilities. The shift to mammal pollination in the *Protea* genus has been debated in the literature and the most important cause of this shift is hypothesised to be associated with plant population size, structure, and locality (Wiens *et al*., 1983). Because NMP *Protea* species have restricted distributions, they may have formed a reliance on non-flying mammal pollinators over birds or insects (Ward and Johnson, 2005). A highly restricted and ephemeral resource (e.g. nectar and pollen from therophilous proteas) may attract non- hibernating, non-migratory and generalist feeders such as the small mammals associated with NMP species in South Africa rather than a specialist feeder such as a nectivorous bird (Wiens *et al*., 1983). Non-flying mammals may also provide a more reliable pollination service over insects whose activities are often affected by colder weather in winter months when most NMP *Protea* species flower (Kühn *et al*., 2017). For example, although *Aloe peglerae* exhibits floral traits that conform to a bird pollination syndrome, non-flying mammals are considered important contributors to reproductive success (Payne *et al*., 2016) especially in poor flowering seasons when birds are tracking more abundant resources elsewhere (Payne *et al*., 2019). In these cases, Payne *et al.* (2019) found that the small mammals that do not relocate, contributed to most of the pollination events in an *A. peglearea* population*,* compensating for the absence of birds. This suggests that while NMP's are not as mobile as birds, they can be reliable alternative pollinators. However, restricted gene dispersal has only been inferred in this study. Future research should aim to calculate whether isolation-by-distance is occurring in NMP proteas as a result of localised pollen movement (Chapter 3).

Outcrossing rates in the current study were also calculated with naturally pollinated plants, open to be visited by various other animals. This may have resulted in a variety of floral visitors contributing to the outcrossing rates estimated in this study. Although floral evolution in animal-pollinated plants typically results from the selection from their most effective and abundant pollinators, they can be visited by other pollinators that are of less importance to pollination (Johnson and Steiner, 2006). For example, birds (e.g. *Anthobaphes violacea)* often probe inflorescences of *Protea* species with typical therophilous (e.g. *P. decurrens* and *P. scabra*) and entomophilous (e.g. *P. caffra*) floral traits (Zoeller *et al*., 2017; Steenhuisen and Johnson, 2012b). Consequently, pollination systems should rather be viewed as a continuum where assemblages of animals will contribute to pollination in varying degrees (Botes *et al*., 2009; Steenhuisen *et al*., 2012). Future research, should aim to estimate outcrossing rates of offspring of adult *Protea* individuals pollinated by more specific pollinator guilds separated by exclusion experiments (e.g. Steenhuisen *et al*., 2012b). Peripheral floral visitors are expected to contribute less or not at all to pollination because they often lack the appropriate adaptations to effectively transfer pollen. For example, individuals of *A. violacea,* observed via camera traps, probing inflorescences of *P. decurrens* and *P. scabra* are considered ineffective pollinators because the beak length of these sunbirds exceeds the distance between nectar and pollen in these *Protea* species (Zoeller *et al*., 2017). This prohibits pollen from being deposited onto the body of individuals, preventing pollen transfer to a new inflorescence.

Although this study gives insight into the understanding of how population genetics of plant populations are affected by specific pollinators, only one population per species was used for these analyses. In a recent review (Whitehead et al., 2018), outcrossing rates and thus, genetic diversity has been recorded to vary among populations and estimates of outcrossing from a single population are often highly unreliable indicators of the mating system of an entire species. For example, estimates of outcrossing rates for six populations of *B. cuneata* ranged from $t_m = 0.67$ to $t_m = 0.95$ (Llorens *et al*., 2012; Maguire and Sedgley, 1997). Estimating a single population's outcrossing rates gives only a "snapshot" view of a species mating system. Therefore, estimating the mean outcrossing rate across several populations may be a more effective way of determining the mating system of a species (e.g. Ayre *et al*., 1994). Considering this, it would be imperative for future research to include more populations per species and especially populations that are sampled across a species geographic range

(Whitehead *et al*., 2018). Thus, despite some evidence that the outcrossing abilities of NFM's are weaker and lead to more biparental inbreeding than bird and insect pollinators of *Protea* species, more populations are needed to sampled in order to understand whether pollinator type has an effect on the mating systems of *Protea* species.

3. Preliminary evidence for isolation-by-distance and spatial genetic structuring in non-flying mammal-pollinated *Protea* L. species

Abstract

The link between pollinators and spatial genetic structuring (SGS), an effective tool to detect short distance pollen dispersal and gene flow, is often not made. Poor gene flow between populations affects the scale of local adaptation (i.e. whether populations act as evolutionary sub-units of a metapopulation) as well as the potential for allopatric speciation. Distance limited gene flow can cause reproductive isolation resulting in speciation. *Protea* is a large African genus with a large variation of floral adaptations to certain pollinator guilds including beetle, birds and small mammal pollinators. However, populations of non-flying mammal- pollinated proteas are often restricted spatially and in population size. This is, in contrast, bird-pollinated proteas which have wide and connected ranges. One hypothesis for this difference in geographic distribution is that birds can fly and thus can disperse genetic material (in the form of pollen) longer distances than non-flying mammals. Spatial autocorrelation revealed that a therophilous protea, *P. amplexicaulis*, has stronger spatial genetic structuring (maximum r = 0.25) than an ornithophilous congener, *P. laurifolia* (maximum r = 0.05). This was reflected in high population differentiation ($G''_{st} \geq 0.6$) between subpopulations of *P. amplexicaulis*. Non-flying mammals were also associated with biparental (mating between kin) inbreeding (t_m-t_s = 0.12-0.15). However, this can also be attributed to limited seed dispersal. High multilocus outcrossing rates (> 80) in *P. amplexicaulis i*ndicate that non-flying mammal pollinators (NMPs) are effective at transferring cross-pollen even if it is within a restricted range. Although there is a need to study realised pollen dispersal by NMP's, this study provides strong evidence that these pollinators may be causing reduced gene flow between therophilous *Protea* populations.

3.1 Introduction

Gene flow or gene dispersal between populations is an important aspect of population genetics because it determines the scale of local adaptation as well as the role that population structure plays in evolutionary processes (Endler, 1977; Wright, 1977). Indirect estimations of gene flow focus on the impact that gene flow has on the population differentiation or by determining spatial genetic structure (SGS) (Sokal and Wartenberg, 1983; Vekemans and Hardy, 2004). According to models of isolation-by-distance, SGS arises from the interplay between gene flow and genetic drift whereby the strength of SGS is measured by the rate of increased genetic dissimilarity with distance (Hardy, 2003; Rousset, 2000). Understanding the biotic processes linked to the strength of SGS in a species is an essential question in population and conservation genetics (Vekemans and Hardy, 2004) because incorporating spatial data to infer genetic isolation between plant populations allow for less biased results (Merimans, 2012, Frantz *et al*., 2009). In plants, the extent of the non-random distribution of genotypes throughout a landscape is highly influenced by both pollen dispersal and seed dispersal (Howe and Smallwood, 1982; Loveless and Harmrick, 2004, Vekemans and Hardy, 2004). Since seed dispersal is often limited in plants (Loveless and Hamrick, 1984; Levin and Kerster, 1974), pollen dispersal is an important factor in determining the spatial distribution of genotypes. For example, species with more restricted pollen dispersal and poor gene flow between populations are expected to result in high genetic differentiation and strong fine-scale SGS (Hamrick and Godt, 1996; Ingvarsson, 2002, Vekemans and Hardy, 2004, Spoladore et al., 2017). Conversely, limited population differentiation causes subpopulations to act as a meta- population rather than singular evolutionary units (Endler, 1977) that are susceptible to the consequences of inbreeding (e.g. high homozygosity) and genetic drift (Levin and Kerster, 1974; Ellstrand, 1992). Therefore, SGS lends itself to understanding large-scale gene dispersal patterns which is necessary to inform conservation decisions (Ellstrand, 1992, Lee and Mitchell-Olds, 2011, Collevatti *et al*., 2014).

Almost three-quarters of angiosperms rely on animals for pollination (Ollerton *et al*., 2011). However, the pollen dispersal abilities of animal pollinators are often affected by various foraging behaviours including floral visitation rates, effective pollen transfer, distances travelled between plants, and whether pollen between near neighbor plants are frequently exchanged (Frankie and Baker, 1974; Frankie *et al*., 1976, Ollerton *et al*., 2011, Schmitt, 1980). For example, non-flying pollinators with restricted foraging ranges often increased mating events between kin, limiting pollen movement throughout a landscape. This results in inbreeding within populations and limited interpopulation gene flow (Collevatti *et al*., 2001). Similarly, opportunities for geitonogamous pollination can occur in self-compatible species through pollinators visiting the same flowers in succession on a singular plant (Matsuki *et al*., 2008). Infrequent pollinator visitation may also create a pollen limited environment. Consequently, an unreliable pollinator might result in a selection for self-compatibility to reduce mate limitation by allowing a plant to make use of its own pollen. In extremely pollen limited environments, facultative autonomous self-pollination can provide reproductive assurance (Karron *et al*., 2009). This short distance pollen dispersal reduces gene flow between populations and may strengthen SGS across a landscape. Therefore, determining how different pollinators affect gene dispersal is imperative to understanding the scale of local adaptation in species.

Protea L. (Proteaceae) is a large (360 species), hermaphroditic southern Africa genus with diverse and divergent floral characteristics and breeding systems (Collins and Rebelo, 1987).

Protea has its centre of endemism in the highly diverse Cape Floristic Region (Cowling *et al*., 2003). *Protea* species have a variety of specialised pollination systems (e.g. beetles, birds, and non-flying mammals) (Collins and Rebelo, 1987). However, the genus is dominated by bird (e.g. *Promerops cafer*) and non-flying mammal (e.g. *Aethomys namaquensis*, *Acomys subspinosus* and *Elephantulus edwardii*) pollinated species (Collins and Rebelo, 1987; Kuhn *et al*., 2017; Wiens *et al*., 1983; Zoeller *et al*., 2017). This is reflected in the diverse floral traits associated with these pollinators. Ornithophily is the most common of all pollination systems in *Protea*. Species pollinated by birds often produce brightly coloured and terminal flower heads that secrete copious amounts of easily digested glucose-rich nectar (Hargreaves *et al*., 2004; Rebelo *et al*., 1984; Wright and Giliomee, 1991). Contrastingly, therophilous species generally exhibit cryptic inflorescences with brown or maroon involucral bracts (Collins and Rebelo, 1987). Small mammals feed on the pollen and sucrose-rich nectar produced these inflorescences (Nicholson and van Wyk, 1998). Therophilous species are also characterised by the the "yeasty" scent produced by the inflorescences with flowers that are usually open from winter until spring (Collins and Rebelo, 1987; Fleming and Nicolson, 2002a, b; Zoeller *et al*., 2017).

Wiens *et al*. (1983) observed that therophilous South African and Australian Proteaceae tend to have small distributional ranges which comprise localised populations resulting in some species being categorised as endangered (e.g. *P. decurrens*) or near threatened (e.g. *P. scabra*) in the IUCN (2019) Red List of Threatened Species. This contrasts with the continuous and large distributional ranges of ornithophilous species (Colins and Rebelo, 1987). This difference in distributional ranges have been attributed to therophilous species having highly specialised ecological conditions, such as soil types, and non-flying mammals being attracted to the highly ephemeral and restricted resource. However, these reasons also apply to bird-pollinated species. It is hypothesised that because small mammals cannot fly, they are not able to move as far as birds during foraging bouts (Colins and Rebelo, 1987). Therefore, the distance of pollen transfer is reduced in non-flying mammal-pollinated (NMP) proteas in comparison to bird-pollinated species. Restricted pollen dispersal can cause reproductive isolation which is a precursor to speciation. Consequently, it is important to document whether populations of therophilous *Protea* species are experiencing poor gene flow since this will influence the amount of inbreeding and genetic drift occurring within these populations.

Empirical testing of the link between pollinator types and spatial genetic structure is rare. There is some evidence that primarily bird-pollinated species usually exhibit weak genetic structure within populations, high gene flow between populations and resultant high levels of genetic diversity (Coates and Sokolowski, 1992; England *et al*., 2003; Frick *et al*., 2014; Llorens *et al*., 2012; Ritchie and Krauss, 2012). For example, Frick *et al*. (2014) illustrated that birds cause complete outcrossing as well as high heterozygosity (0.59) and assumed resultant weak genetic structure (high gene flow between populations) in fragmented populations of Australian Proteaceae, *Banksia menziesii*. In support of this, birds have been documented to be able to fly considerable distances (20-30 m between plants) and display high amounts of interplant movements (Collins and Rebelo, 1987; Krauss *et al*., 2017). High floral visitation rates are most likely attributed to the elevated energetic requirement of birds. This is often reflected in the high outcrossing rates that bird-pollinated species typically have (Hoebee and Young, 2001; Krauss *et al*., 2017). Contrastingly, the link between non-flying mammals and the extent of their gene dispersal has not yet been made. Small mammals lack flight ability and are expected to forage from a smaller gene pool than birds (Collins and Rebelo, 1987). Biparental inbreeding (mating between kin) events documented in therophilous species, *P. amplexicaulis* (Chapter 1) suggest that foraging by NMP's could be limited to within a discrete area. Small foraging ranges may create fine-scale genetic clustering rather than dispersing genetic material homogenously across populations.

Estimating SGS within a population typically involves regressing a measure of pairwise genetic distance or relatedness with individual spatial distance (Volis *et al*., 2016). These measures of pairwise relatedness and genetic distance are often estimated through the use of co-dominant genetic markers (Volis *et al*., 2016). To obtain estimates of relatedness with enough precision, the number of polymorphic loci and the number of alleles per locus is a critical factor when deciding which type of marker to use. Microsatellite markers are often recognized as the marker of choice when considering estimations of the genetic structure of plants since they typically display many alleles (Estoup and Angers, 1998). Ten microsatellites markers have been developed for the "white protea" clade (Prunier and Latimer, 2010) that have the potential to show population differentiation within the *Protea* genus (section: *Exsertae*). These markers have been used to illustrate a lack of gene flow between populations of several primarily bird-pollinated *Protea* species, including *P. aurea, P. lacticolor*, *P. mundii*, *P. punctata,* and *P. venusta* showcasing that geographical isolation plays a role in the diversification of the lineage (Prunier *et al*., 2014; Prunier and Holsinger, 2010). The current study is the first to use these microsatellite markers to understand the relative indirect effects of non-flying mammal and bird pollinators on gene flow between *Protea* subpopulations and consequent intrapopulation genetic diversity by 1) estimating population differentiation between subpopulations of an ornithophilous and a therophilous *Protea* species, 2) determining the spatial genetic structuring within the meta-population of each *Protea* species including the subpopulations, and 3) estimating the genetic diversity within these subpopulations. We predict to detect more extensive genetic spatial structuring across the meta-population and population differentiation between subpopulations of the therophilous *Protea* species (*P. amplexicaulis*), but not in the ornithophilous *Protea* species (*P. laurifolia*) because NMP's are expected to be less vagile than bird pollinators. Along with poor gene flow, we expect intrapopulation genetic diversity to be lower within *P. amplexicaulis* than *P. laurifolia* as a result of short distance gene dispersal. Lastly, we also aim to understand whether expected poor gene flow is a result of pollen limitation or low amounts of outcrossing. This will be done by estimating seed set of inflorescences following pollen supplementation for *P. amplexicaulis* and measuring outcrossing rates in P. amplexicaulis populations that are increasing distances apart. We predict that the vagility of NMP's will be reflected in high pollen limitation in *P. amplexicaulis* and low outcrossing rates in populations that are the furthest distances apart from eachother.

3.2 Methods

3.2.1 Study site and species

To estimate population differentiation and spatial genetic structuring within therophilous *Protea* species, *Protea amplexicaulis* (Fig 1-1) was selected as a study species based on its known pollination system and polymorphism in the microsatellite marker region (R. Prunier, pers. comm.). *P. amplexicaulis* is a low, sprawling shrub (can grow up to one to three meters in diameter) and is classified as a "rodent sugarbush", possessing morphological traitsassociated with NMP (Collins and Rebelo, 1987). The primarily ornithophilous congener, *Protea laurifolia* (Fig 1-2) was used for comparison since the desired microsatellite regions were also polymorphic in this species. *P. laurifolia* is a large (three to five meters tall), self-incompatible (Wright *et al*., 1991), and upright shrub which produces bearded inflorescences with long and narrow, bright pink bracts. Both study species are endemic to the Western Cape region of South Africa with wide and overlapping distributional ranges (Rebelo, 2000). The ranges of both species occur on inland mountain ranges (e.g. the Bokkeveld escarpment near Nieuwoudtville and southwards along Gifberg, Cederberg and Franschhoek mountains to Villiersdorp, and then eastwards towards the Riversonderend Mountains). They are usually found in dense stands at altitudes between 400-600 m above sea level and occurring on mainly well-drained, sandstone-derived soils including Table Mountain Sandstone. However, *P. laurifolia* is able to also thrive on quartz-derived and shale soils (Rebelo, 2000).

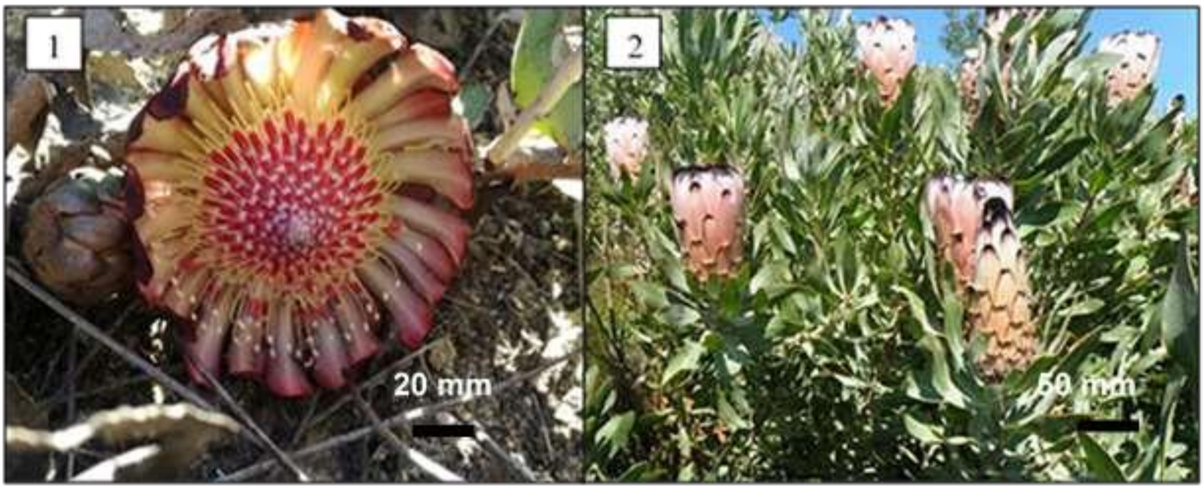

Figure 1: The inflorescences of *Protea amplexicaulis* (1) which are geoflorous, cryptic and generally have maroon involucral bracts; and *Protea laurifolia* (2) which are terminal and bearded with pink and white involucral bracts (photos: Megan Smith).

P. amplexicaulis and *P. laurifolia* populations were sampled in February (summer) and June (winter) 2017 respectively on Jonaskop (33° 58′ 00 S 19° 30′ 00 E, altitude 1646 m) which forms part of the Riviersonderend mountains in the Western Cape, South Africa. On the mountain, Succulent Karoo dominates in the lower slopes until approximately 600 m above sea level. Thereafter, there is a transition through a Renosterveld ecotone into Mountain Fynbos (Fleming and Nicolson, 2002a). Both species are found in large stands along the upper slopes of the mountain where proteoid fynbos dominates (Fleming and Nicolson, 2002a). The South Western Cape has a seasonal climate of hot, dry summers and cold, rainy winters (Linder, 1991). Fires usually occur in summer, but no recent fires had been recorded on Jonaskop during the time of sampling.

3.2.2 Genetic sampling procedure

3.2.2.1 Adult tissue

In order to detect small scale isolation-by-distance, three subpopulations of both *Protea laurifolia* and *P. amplexicaulis* were selected across Jonaskop. These subpopulations were sampled at increasing distances (Fig 2). Locality data (GPS coordinates) were recorded for each sampled individual plant of each species in all populations. Within each population, six mature and healthy (uneaten and no signs of disease) leaves and five seed heads (cohort one to two years) were collected from 20 adult individuals, each plant being approximately 10 m apart from each other. Three leaves from each individual plant were cut into half centimeter squared blocks and preserved in a concentrated Cetyl Trimethylammonium Bromide CTAB (see Appendix for protocol to make concentrated CTAB solution) solution

(Thomson, 2002) after which the leaves were immediately stored at -20°C until DNA extraction. As an additional source of DNA, the remaining three leaves were placed in a coffee filter envelope and stored in an airtight container filled with silica gel. The silica gel was re-dried at 60°C overnight every day for approximately three days to limit leaf moisture absorption

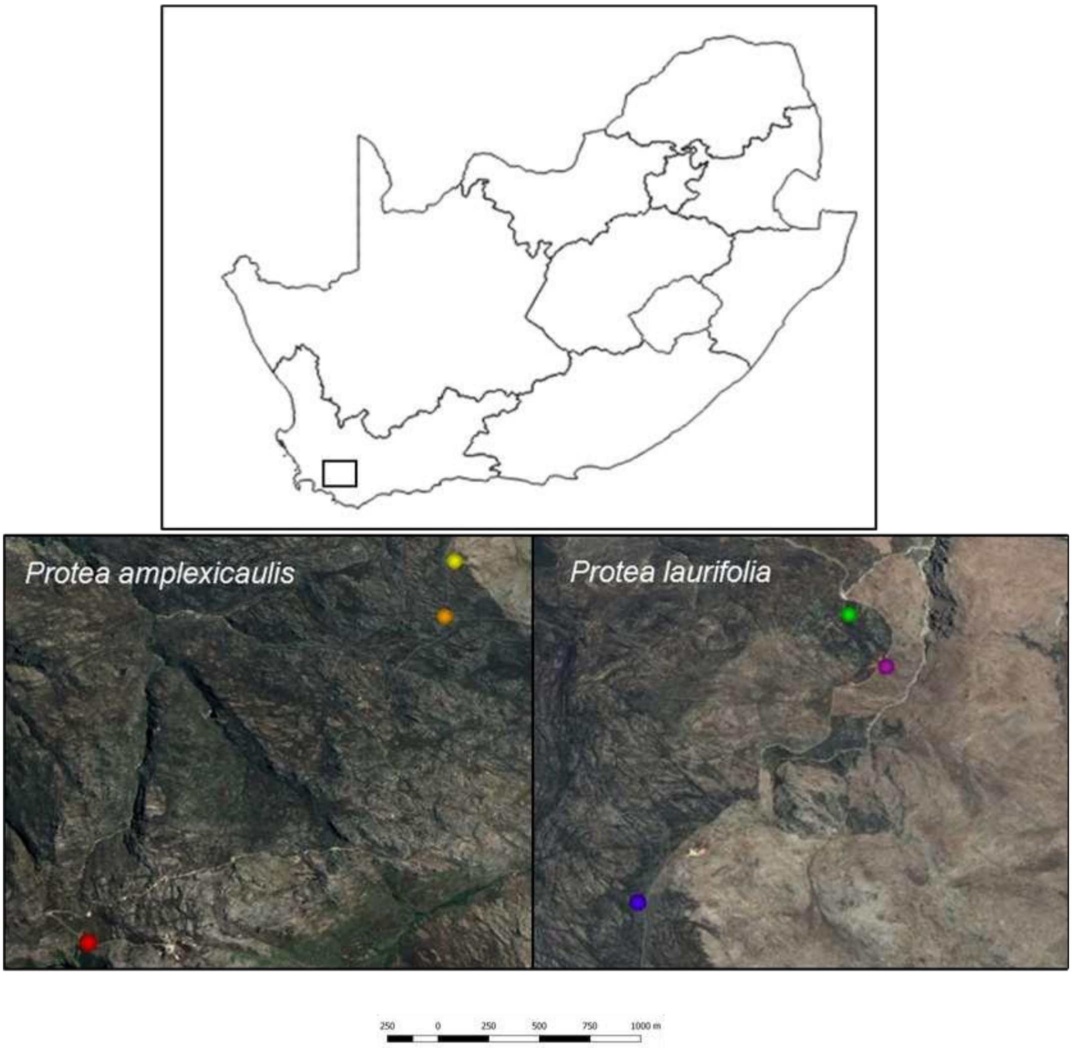

Figure 2: Sampling localities of *Protea amplexicaulis* and *Protea laurifolia* individuals on Jonaskop (33° 58′ 00 S 19° 30′ 00 E, altitude 1646 m) demarcated in the black box on the map of South Africa (top) with provincial border lines. Populations were sampled at varying distances apart. Distance between *P. amplexicaulis* populations 1 (●) and 2 (●) is 300 m; and population 2 and 3 (●) is 3500 m. Distance between *P. laurifolia* populations 1 (●) and 2 (●) is 300 m; and population 2 and 3 (●) is 1500 m. Inter-population distances were calculated using the centroids of each population. Distances are indicated by a scale bar below the map. Bottom map's source: Google earth.

3.2.2.2 Offspring tissue

From the sampled infructescences of each plant, 20 plump and pliable seeds were selected by hand. Species in the *Protea* genus are known for low and variable seed set (Wright, 1994) and therefore require hand separation of fertile and infertile seeds. Seeds were thought to be fertile when a white cotyledon is present which is typically determined by cutting through the base of the seed. As a less destructive proxy for cutting the seeds open, seeds that were plump and pliable were considered fertile (Steenhuisen and Johnson, 2012a). However, this could only be confirmed through successful germination.

The sorted seeds were germinated to harvest leaf material for further genetic analyses. Before sowing, the seeds were soaked overnight (12 hours) in Kirstenbosch smoke primer discs (Super Smoke Plus, Kirstenbosch National Botanical Gardens, Cape Town, South Africa) and then stored at four degrees celcius for another 12 hours (cold shocked) to encourage or induce germination (Rebelo and Rourke, 1985; Richards *et al*., 1997). Each individual plant's seeds were separately sown (March 2018) five centimeters apart in a 50 cm^2 seedling tray (Fig 3-1) filled with a mixture of acidic soil (pH between five and six) and covered with a half centimeter layer of sifted river sand (Fig 3-4). After sowing, the trays were placed beneath a shade cloth within the Kirstenbosch National Botanical Garden's plant nursery where the amount of water given to each tray was controlled. Watering procedures involved spraying municipal water over the trays until the soil was moist immediately after sowing and then every one-two days until germination. Once germinated, all cotyledon leaves or two to three true leaves (Fig 3-4) were collected from six to eight individuals from each tray (representing the offspring of each maternal plant) and placed in a concentrated CTAB solution and stored at -20°C until DNA extraction (Thomson, 2002). Germination time varied between species but both species showed signs of germination after a three-month period. The germination trial was completed approximately four months after sowing when leaves from a sufficient number of offspring had been collected. Some seeds were predated upon by rodents or insects (Fig 3-2) and this reduced the sample size of seedlings in some seed families.

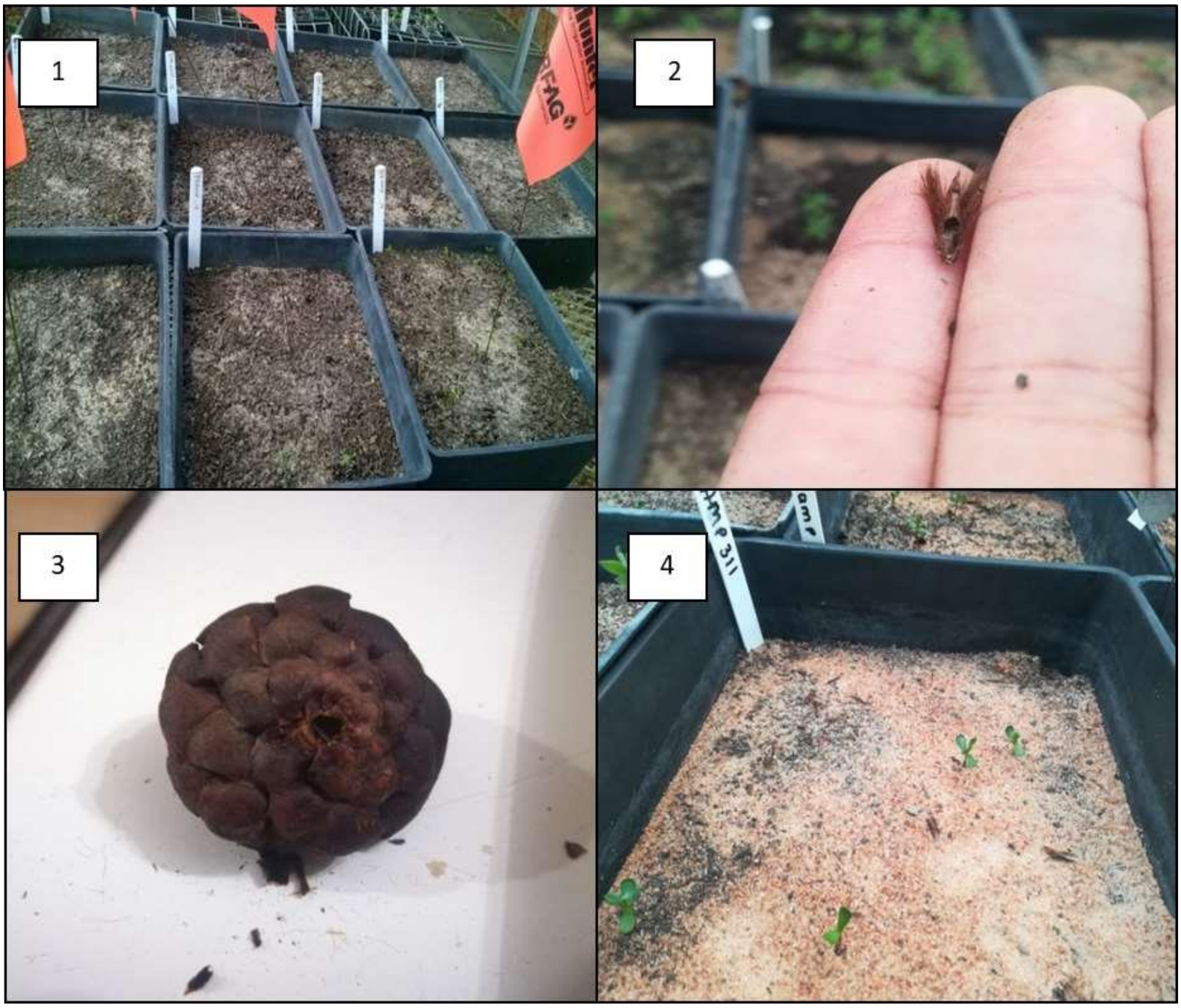

Figure 3: The layout of seedling trays (1) underneath shade cloth at Kirstenbosch Botanical Gardens. Offspring of each individual plant were sown into separate seedling trays. Seed predation, most likely from rodents which eat the cotyledons of the seeds (2) and insects which bore holes into the seed heads and predate on the seeds (3), was prevalent which reduced sample sizes in all experiments. After seeds were sown, the cotyledon or true leaves (4) were harvested from six to eight offspring per individual (photos: Megan Smith).

3.2.2.3 Genomic DNA extraction and amplification

To determine the outcrossing rates and genetic structuring for each population and across populations, the DNA of maternal leaves and corresponding offspring leaves was isolated for further amplification of specific microsatellite regions (Table 1 in Appendix). Genomic DNA was extracted (see Appendix for the DNA extraction protocol) using a modified Cetyl Trimethylammonium Bromide (CTAB) procedure (Doyle and Doyle, 1987). Extracted DNA was quantified using a NanoDrop spectrophotometer and assayed for degradation by running four microliters of each sample on a one percent agarose gel stained with ethidium bromide. Samples that had a DNA concentration above 20 ng/ul using a NanoDrop™

spectrophotometer (ThermoFischer Scientific, Applied Biosystems) and were visible on the agarose gel were assumed to have a high enough yield for further analysis. DNA was amplified using a QIAGEN multiplex PCR method using nine fluorescently tagged microsatellite primers (Table 1 in Appendix) known to be polymorphic for species in the *Protea* genus (Prunier and Latimer, 2010). The cycling conditions for amplifying microsatellites (see Appendix for reaction mixture) using the multiplex PCR method included an initial heat activation for 15 min at 95°C and a three-step cycling process involving denaturation for 30 s at 94°C, annealing for 90 s between 57-63°C, extension for 60 s at 72°C and then repeating the cycle 30 times after which a final extension of 30 min at 60°C occurred. PCR products were diluted in autoclaved distilled water (ddH$_2$0) in a one to nine ratio before fragment analysis. Fragment analysis was completed at the Central Analytical Facility in Stellenbosch, South Africa. GeneMarker software version 2.2.0 (SoftGenetics, State College, PA, USA) was then used to score and visually verify all microsatellite scores. The fragments ranged in sizes from 100 to 500 base pairs.

3.2.3 Controlled pollination experiments

Two treatments were performed on 30 open-pollinated *P. amplexicaulis* individuals on Jonaskop during August and September 2018 to test whether small mammals were causing pollen limitation for *P. amplexicaulis*. These treatments included self-pollination and supplemented crossed pollination. Supplemented self-pollination treatments involved supplementing an individual's self-pollen onto each stigma in an inflorescence and leaving them open to pollinators. Since *Protea* flowers have a pollen presenter, a specialised section of the stigma that is coated with pollen once the anthers have dehisced, pollen was moved onto the stigma by rolling a toothpick over the presenters of all the stigmas in an inflorescence. This was to ensure that once the stigma became receptive, self-pollen was present. Supplemented cross pollination treatments were completed by supplementing cross pollen from individuals approximately ten meters away from the experimental individual onto each stigma in an inflorescence using a toothpick. A large amount of cross pollen was supplemented after the anthers had dehisced to insure that cross-pollination would occur. A third inflorescence on each shrub was left unmanipulated. Each inflorescence was tagged according to the treatment. *Protea* inflorescences consist of tightly packed flowers that open centripetally. Therefore, each inflorescence was pollinated between two to four times as the flowers sequentially opened and stigmas became receptive. Stigmas were thought to be receptive once pollen had been removed by pollinators. Seed heads were collected in January 2019 and plump and pliable seeds were hand sorted from each seed head. Nine infructescences showed damage by lepidopteron predators (as described by Steenhuisen and Johnson, 2012a) and were excluded from the analysis (Fig 3-3).

To test whether *P. amplexicaulis* is self-compatible, controlled pollination experiments were conducted in August 2017 by enclosing two inflorescences of 20 *P. amplexicaulis* individuals on Jonaskop with fine mesh nylon bags and 13 mm diameter chicken mesh wire cages from bud stage to exclude all pollinators. Plastic cages were placed inside each nylon bag to prevent the bag from touching pollen presenters and stigmas. The wire cages prevented rodents chewing through bags to reach the enclosed inflorescences. Two inflorescences were bagged from the bud stage and each inflorescence represented different treatments. These two treatments were autonomy and selfed. Autonomy treatments involved no pollen supplementation and selfed treatments involved supplementing self-pollen onto each stigma by rolling pollen over the stigmas of each flower in inflorescences. A third inflorescence was tagged by using robust colour tape and left open to natural pollinators. Selfed inflorescences were hand pollinated at least twice as the flowers within inflorescences sequentially opened. Seed heads were collected in November 2017 and plump and pliable seeds were hand sorted from each seed head. Infructescences (18 for the selfed treatment and 12 for the control and autonomy treatments) showed damage by lepidopteron predators (as described by Steenhuisen and Johnson, 2012a) were excluded from the analysis (Fig 3-3). Consequently, this reduced the sample size to two (selfed treatment) and eight (control and autonomy treatments) individuals per treatment.

3.2.3 Analyses

3.2.3.1 Genetic diversity and genetic structuring

To estimate genetic diversity within *P. ampexicaulis* and *P. laurifolia* populations, summary statistics of four measures of genetic diversity were calculated: number of alleles per locus or allelic richness (A_r); number of private alleles (A_p); expected heterozygosity (H_e); and percentage of polymorphic loci (%). Each measure, with the exception of A_p, was estimated per population in Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010). GenAlEx version 6.51b2 (Peakall and Smouse, 2012) was used to determine the number of private alleles for each population. Non-amplification of some individuals' microsatellites resulted in the reduction in the number of loci used for each population. Only loci and individuals with less than 10 % missing data were included in the analyses (Table 4 in Appendix). This was to remove the biases that may arise in analysis as a result of missing data or the presence of null alleles. Because less than ten loci were used in the analysis and some loci had poor amplification success, the threshold of 10 % missing data (V. Naude, pers. comm) was chosen as a conservative measure of removing data from each loci (Table 4 in Appendix). Furthermore, the number of individuals used for the analysis in some populations reduced as result of sample degradation. Specifically, only 18 and 19 *P. amplexicaulis* individuals were used in population two and three respectively for analysis. Similarly, 19 *P. laurifolia* individuals were used in population one for analysis. Since H_e and A_r were estimated locus by locus for each population, the statistical significance of any difference between species and populations was calculated in base R (RStudioTeam, 2015) by using a Kruskal-Wallis rank sum test and a pairwise Wilcox rank sum test when applicable. Differences genetic diversity estimates ($A_{r,}$ and H_e) between populations of *P. amplexicaulis* and *P. laurifolia* were tested using a Mann-Whitney U test for non-normal data calculated in the statistical in base R (RStudioTeam, 2015). Normality of the data for each population was tested using a Shapiro-Wilk's test for normality.

In order to detect population differentiation in each species, G''_{st} was calculated for each species using GenAlex version 6.51b. G''_{st} is an analog of F_{st} but is less sensitive to high population heterozygosity than F_{st}. The relationship between G''_{st} and geographic distance (m) can only be documented descriptively since a sample size of three populations is too low to detect a reliable significant relationship (M. Whitehead, pers. comm.). As an alternative to estimating population level genetic differentiation throughout space, individual pairwise genotypic distances (GD) were calculated using GenAlex version 6.51b and correlated with individual pairwise geographic distances (GGD) in meters. The relationship between pairwise GD and pairwise GGD was tested using a Mantel test with 999 permutations.

A classical spatial autocorrelation as defined by a multivariate approach (Smouse and Peakall, 1999) was used to determine spatial genetic structuring within each meta- population of *P. laurifolia* and *P. amplexicaulis*, This spatial autocorrelation analysis provides a multi-locus estimate of pairwise individual relatedness (r) which is correlated with pairwise individual geographic distance (m). Analyses were performed using variable distance classes set as 5 m, 10 m, 50 m, 250 m, 500 m, and 4000 m for *P. amplexicaulis and* 10 m, 50 m, 250 m, 1200 m, 1400 m for *P. laurifolia*. These distance classes were chosen in order to evenly distribute the sample sizes across the distance classes. Individuals and loci with no variability were excluded from the analysis. A statistical test for heterogeneity (Banks and Peakall, 2012) was used to test the significance of r in each correlogram. This was done by randomly resampling (1000 times) of each individuals geographic location to define an upper and lower limit of the 95 % confidence interval for each distance class and estimating 95 % confidence intervals around the mean r values by bootstrapping (1000 times) pairwise comparisons within each distance class. Homogeneity in the correlogram would indicate that r lies within 95% confidence of the upper and lower limit ($p > 0.05$) and thus heterogeneity would be represented as $p < 0.05$. If there is restricted gene flow in a species, it is expected that genetically similar individuals are physically clustered on a fine scale which will generally translate to $r > 0$ for smaller distance classes (or lags) and $r < 0$ for larger distance classes (Smouse *et al*., 2008). Subsequently, fine scale structuring would represent a short distance pollen movement. Since both correlograms were created using different distance classes, statistical significance between the two correlograms could not be determined. Consequently, it is not possible to determine if the relationship in each correlogram was significantly different to the eachother. Otherwise, this may have been done using the non-parametric test of heterogeneity as suggested by Banks and Peakall (2012).

3.2.3.2 Outcrossing rates.

To understand whether outcrossing rates in NMP proteas change with increasing distance between populations, species-specific maximum likelihood estimates of single-locus (t_s) and multilocus (t_m) outcrossing rates were estimated for two populations of *P. amplexicaulis* (population 1 and population 2) using a multilocus mating system program, MLTR version 3.2 (Ritland, 2002). The DNA of the third population of *P. amplexicualis* offspring degraded during a transit to South Africa and was excluded from analyses. Standard deviations for estimates of t_s, t_m, and t_m–t_s were based on 1000 bootstraps with resampling within families. The difference t_m– t_s represents a test for biparental inbreeding where the difference will be zero in the absence of biparental inbreeding. The potential to detect outcrossing events increases with an increase in the number of sampled loci and thus t_s will usually be lower than t_m in the presence of inbreeding. Differences between populations' outcrossing rates were assessed using pairwise comparisons of bootstrap estimates. Outcrossing rates of the two populations were considered significantly different if 99.5% of the differences between randomly paired bootstrap estimates (e.g., t_{PA1+K}-t_{PA2+K} for the kth bootstrap estimate of t_m) were greater or less than zero (two-tailed tests, α = 0.05). The test percentage was calculated using an equation defined by Barrett and Eckert (1994) as $100(1\text{-}a_{pc}/2)$ where a_{pc} is the Type I error rate per contrast and calculated as $1\text{-}(1\text{-}a_{ew})^{1/C}$. The experimentwise error rate (a_{ew}) was set at 0.05 and the number of contrasts (C) was 4. This method was also used to test whether all the estimates (t_s, t_m, and t_m–t_s) were significantly different from zero (one-tailed test, α = 0.05). Some seeds were predated upon by rodents or insects (Fig 3-2) and this reduced the sample size of seedlings in some seed families. The average number of offspring used per adult individual for each population of *P. amplexicaulis* was as follows: 5 offspring per 17 adult individuals for population one and 6 offspring per 19 adult individuals for population two.

3.2.3.3 Controlled pollination experiments

For both breeding system and pollen limitation experiments, statistical differences in seed set between treatments were tested using a generalized estimating equation model (GEE), using plant individual as a subject variable, with a logit link function for binomial data (events/trials) in IBM SPSS version 24 (IBMCorp, 2016). This analysis tests the effects of treatments on proportion seed set using Wald Chi-square statistics. Pairwise differences between treatments were estimated, if necessary (i.e. if significant differences were found between treatments), using a sidak sequential pairwise test

3.3 Results

3.3.1 Genetic diversity

Differences in expected heterozygosity (H_e) and allelic richness (A_r) between *P. amplexicaulis* populations were non-significant ($X^2_{(2)}$ = 0.14, p = 0.9 and $X^2_{(2)}$ =0.04, p = 0.9; Table 2). This trend was mirrored in *P. laurifolia* where H_e and A_r were significantly similar across populations ($X^2_{(2)}$ = 0.14, p =0.92 and $X^2_{(2)}$ = 0.03, p = 0.9 respectively, Fig 8) suggesting that levels of heterozygosity and allelic richness did not change with an increasing distance. Overall, H_e was higher for *P. amplexicaulis* (0.75 ± 0.2) than for *P. laurifolia* (0.63 ± 0.25), but the difference was non-significant (*U* = 411, p = 0.06; Fig 9). Differences in the overall measure of A_r also did not differ significantly (*U* = 379, p = 0.2) between *P. laurifolia* (mean = 8 ± 4.5) and *P. amplexicaulis* (mean = 8.7 ± 4.5; Table 2). The overall measure of A_p was less in *P. laurifolia* (1.3-1.7) populations than populations of *P. amplexicaulis* (4-5.8), which may indicate that there is isolation between *P. amplexicaulis* populations (Table 2). The percentage polymorphic loci was lowest for populations two and three of *P. amplexicaulis*. However, polymorphism was high (40-80 %) for all other populations of *P. amplexicaulis* and *P. laurifolia* (Table 2).

Table 2: Sample size (*N*), Percentage polymorphic loci, number of private alleles (A_p), expected heterozygosity (H_e) per locus and allelic richness (A_r), estimated for each subpopulation of *Protea amplexicaulis* and *Protea laurifolia*.Estimates were calculated using loci with less than 10 % missing data.

			Estimate			
Species	Population	*N*	Polymorphic loci (%)	A_p	H_e ± SD	A_r ± SD
P. laurifolia	1	19	80	1.7	0.63 ± 0.23	8 ± 4.2
	2	20	60	1.3	0.6 ± 0.28	8 ± 4.8
	3	20	40	1.7	0. 66 ± 0.2	8 ± 4.9
P. amplexicaulis	1	20	50	5.6	0.74 ±0.22	11 ± 1.7
	2	19	10	4	0.69 ± 0.21	6 ± 3
	3	18	10	5.8	0.84 ± 0.13	11 ± 4.6

3.3.2 Population differentiation and spatial genetic structuring

Pairwise population differentiation (G''_{st}) increased with distance in *P. amplexicaulis* (Fig 4) where the populations that were the furthest apart (3500 m and 4000 m) were the most genetically different (G''_{st} = 0.64 and 0.6 respectively). Contrastingly, *P. laurifolia* populations were genetically similar and thus little differentiation was found(G''_{st} = 0.1-0.5) amongst populations (Fig 4). Interpopulation distances varied (by 1500 m) between *P. laurifolia and P. amplexicaulis* making G”st comparisons between species difficult. Nevertheless, *P. laurifolia* had overall lower differentiation between populations (G''_{st} = 0.09-0.1) than *P. amplexicaulis* (G''_{st} = 0.3-0.63). The high population differentiation in P. amplexicaulis is supported by significantly increasing pairwise genetic distance (GD) across populations of *P. amplexicaulis* (n = 55, r^2 = 0.35, p = 0.001) with geographic distance (GGD) (Fig 5). However, the positive relationship between pairwise GD and pairwise GGD was not significant in *P. laurifolia* (n = 44, r^2 = 0.04, p = 0.2). The outcome of the spatial autocorrelation analysis of both *P. amplexicaulis* and *P. laurifolia* is illustrated in the correlograms (Fig 6). There was a positive ($r > 0$) and relationship (p = 0.001) between pairwise relatedness (*r*) and geographic distance (m) in *P. amplexicaulis* (Fig 6) for distances below 500 meters and a negative ($r < 0$) relationship for distances above 1400 m (p = 0.001).This provides strong evidence for spatial genetic structuring in *P. amplexicaulis*. There was a positive relationship between pairwise relatedness (*r*) and geographic distances below 50 m in *P. laurifolia* (Fig 6). Beyond 1200 meters, there was negative correlation between geographic distance (m) and individual pairwise relatedness. The relationship between geographic distance and relatedness was weaker in *P. laurifolia* (maximum *r* = 0.05) than in *P. amplexicaulis* (maximum *r* = 0.25) (Fig 6) which may provide evidence for weak spatial genetic structuring in *P. laurifolia*.

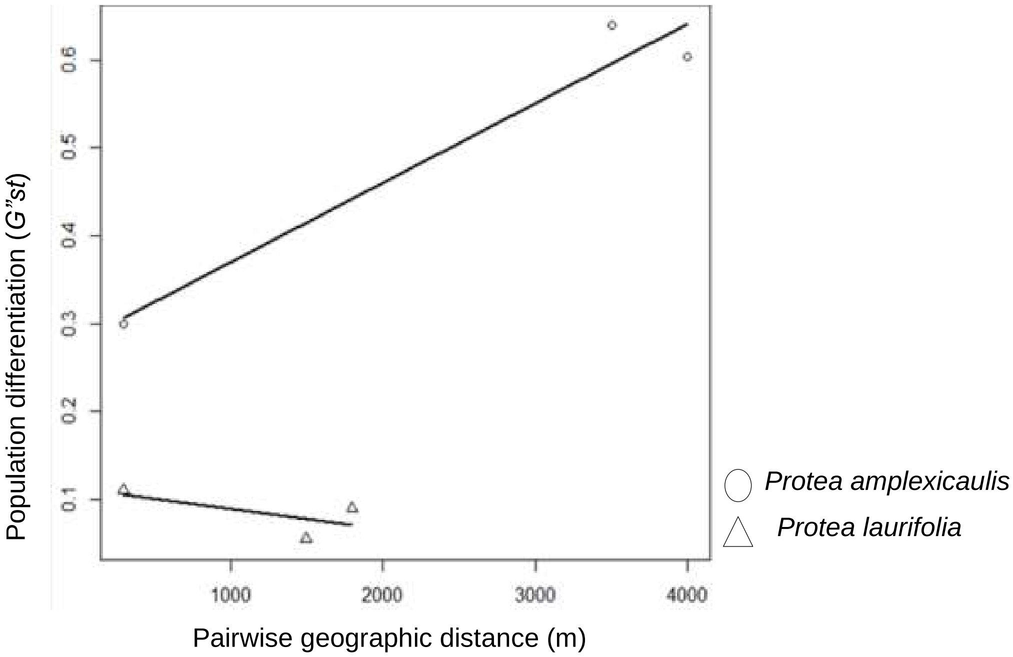

Figure 4: A scatter plot of the correlation between population differentiation (*G”st*) and geographic distance (m) across three subpopulations of *Protea amplexicaulis* and *Protea laurifolia*. Subpopulations were sampled at increasing distances apart. *P. amplexicaulis* populations were 300 m (population one and two) and 3500 m apart (Population two and three). *P. laurifolia* populations were 300 m (population one and two) and 1500 m (population two and three) apart. Expected trendlines for each species are drawn as solid black lines.

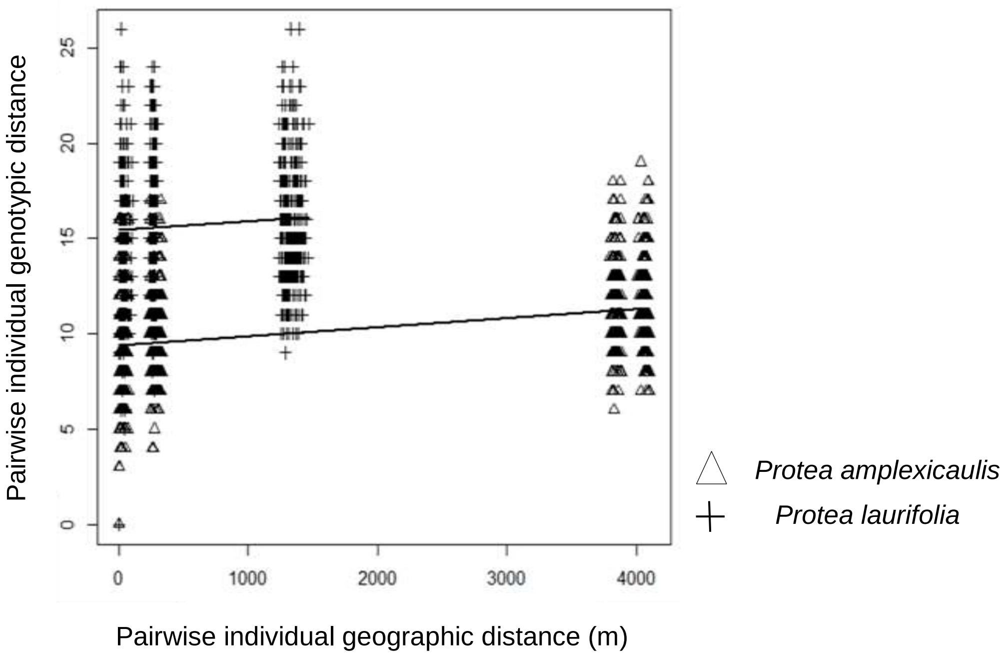

Figure 5: The scatter plot of the correlation between pairwise individual genotypic distance and pairwise individual geographic distance (m) of *Protea amplexicaulis* and *Protea laurifolia*. Each point on the scatterplot represents a single comparison between individual genotypes analysed across all loci. Expected trendlines for each species are drawn as solid black lines.

Table 3: Mantel's test results including sample size (*N*), Mantel's *r* and the p-value for the correlation between the pairwise individual genotypic distance and pairwise individual geographic distance (m) in *Protea amplexicaulis* and *Protea laurifolia*.

Species	*N*	Mantel's *r*	p-value
P. laurifolia	44	0.04	0.2
P. amplexicaulis	55	0.35	0.001

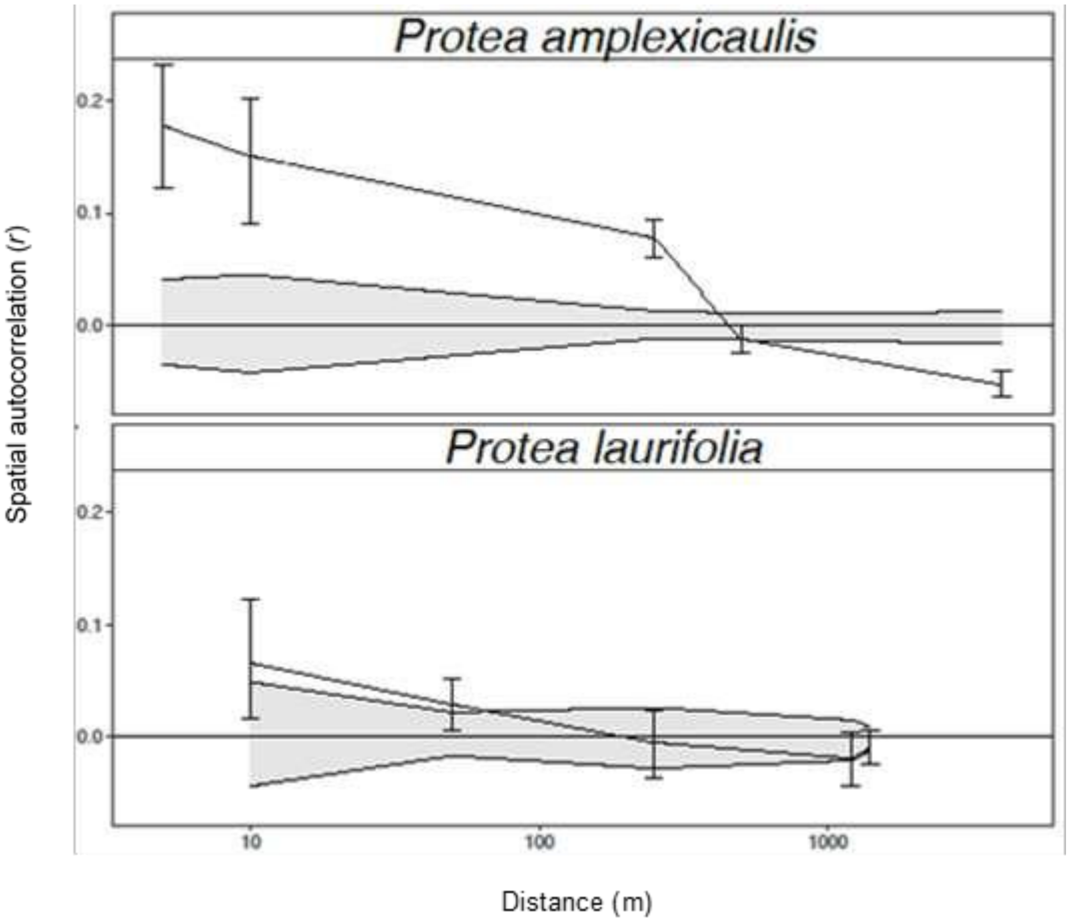

Figure 6: Correlogram generated from spatial autocorrelation analysis of *Protea amplexcaulis and Protea laurifolia* using the correlation co-efficient (*r*) described by Smouse and Peakall (1999), and variable distance classes. The upper and lower limits (indicated in the grey area) represent the 95 % confidence interval obtained by reshuffling (999 times) of individual geographic locations. Black lines around the mean *r* value (situated on the black solid line) represent 95 % confidence intervals around mean *r* generated by bootstrapping (1000 times) pair-wise comparisons of each individual in each distance class

3.3.3 Outcrossing rates

Both populations of *P. amplexicaulis* exhibited high t_m outcrossing rates (> 0.80) that were significantly different ($p < 0.05$) from zero (Table 4). The pairwise differences of t_m were similar between populations and thus, there is no evidence for differences in outcrossing rates over the spatial scale of this study (500 m). The difference, t_m-t_s, was significantly different from zero ($p < 0.05$) across populations illustrating that there was some biparental inbreeding taking place. However, this does not change with distance between populations.

Table 4 Maximum likelihood (MLTR) estimates (± SD) for multilocus outcrossing rates (t_m), single locus (t_s) and biparental inbreeding (t_m-t_s) for two populations of *Protea amplexicaulis*. Sample sizes are shown as the average number of progenies per locus and the number of seeds and seed families in parentheses. * Denotes a significant difference from zero; significance level: $p < 0.05$.

MLTR estimate	Population 1	Population 2
t_m ± SD	0.88 ± 0.07*	0.9 ± 0.04*
t_s ± SD	0.74 ± 0.08*	0.77 ± 0.06*
t_m-t_s ± SD	0.15 ± 0.04*	0.12 ± 0.03*
Mean number of progeny per locus	66 ± 41	85 ± 38
Number of seeds (seed families)	82 (17)	116 (19)

3.1.1 Pollen limitation and breeding system

Supplemental pollination did not increase seed set; the mean proportion of flowers that set seed seed was not-significantly different ($\chi^2_{(2)} = 3.01$, p = 0.2) between control ($N = 21$) (natural pollination) and selfed ($N = 21$) and crossed ($N = 21$) hand pollination treatments in the pollen limitation experiment (Fig 10). There is evidence that *P. amplexicaulis* relies on outcrossed pollen for seed set. Both selfed ($N = 3$) and autonomy ($N = 7$) bagged treatments yielded significantly lower seed set ($\chi^2_{(2)} = 80.1$, p = 0.001) than the control ($N = 10$) (open pollinated) (Fig 7). Since both autonomy and selfed treatments yielded zero or near-zero mean seed set, it is indicative that *P. amplexicaulis* is self-incompatible. Proportional seed set was low (adjusted mean proportion seed set = 0.2-0.25) across all treatments (Fig 7).

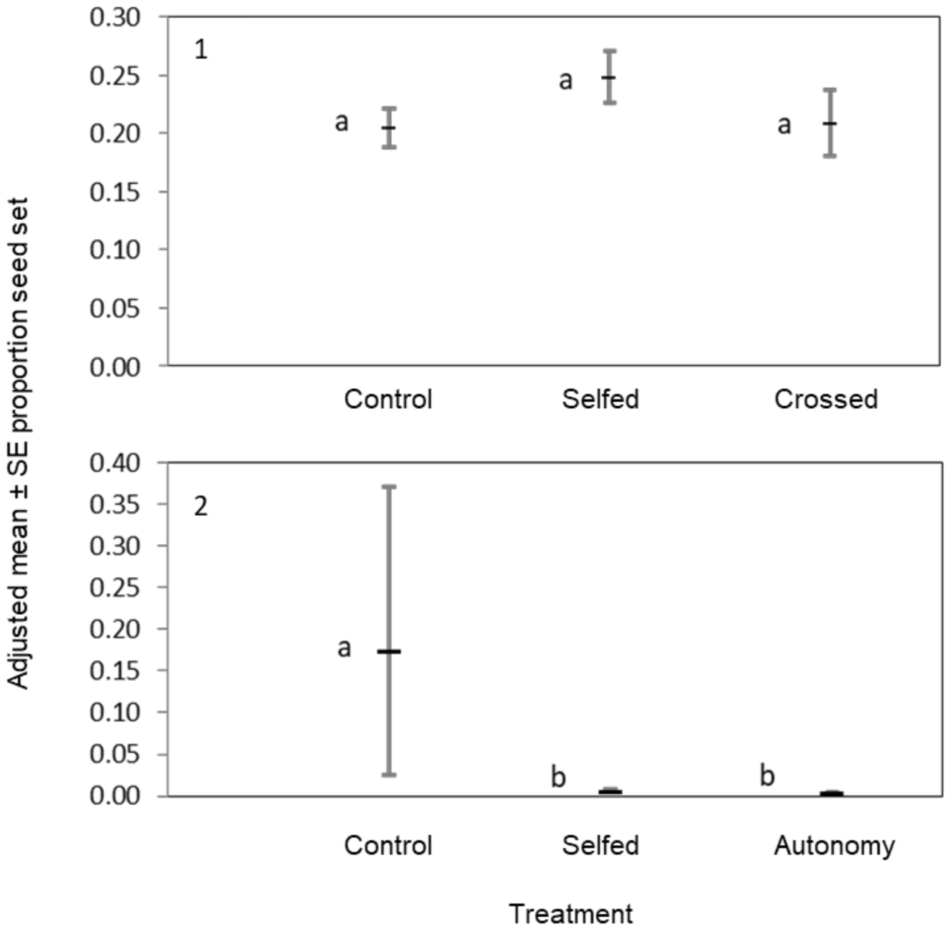

Figure 7: Adjusted mean ± SE seed set per inflorescence of *Protea amplexicaulis* exposed to 1) self-pollen (selfed) supplementation, cross-pollen (crossed) supplementation, and no pollen supplementation (control) as a test for pollen limitation, and 2) exclusion experiment treatments including bagged (autonomy), bagged and self-pollen supplementation (selfed), and inflorescences left open to natural pollinators (control). Significant differences between treatments are indicated with letters above each adjusted mean, $p < 0.05$. Different letters indicate that the means are significantly different from each other.

3.4 Discussion

3.4.1 Spatial genetic structuring and genetic diversity

This study provides evidence that the mammal-pollinated *P. amplexicaulis* is genetically spatially structured (*r* is significantly larger than zero for distance classes smaller than 500 m and significantly smaller than zero for distance classes bigger than 500 m) and that genotypic distance is significantly correlated with geographic distance. This was supported by high genetic differentiation ($G''_{st} \geq 0.6$) between subpopulations of *P. amplexicaulis* that are 4000 m apart. This in strong contrast to its bird-pollinated congener, *P. laurifolia*, which had a homogenous distribution of genotypes even on both fine and distant spatial scales (maximum *r* = 0.05 and *r* values overlapped with confidence intervals). Subpopulations and individuals within the metapopulation of *P. laurifolia* were also genetically similar to each other (G''_{st} = 0.09-0.1) suggesting that gene flow is high in this species, and in fact might be panmicit. These findings indicate that NMP's could be poor facilitators of gene flow in *P. amplexicaulis* and cause genetic clustering on a small spatial scale. Localised gene dispersal is also reflected in the high proportion of biparental inbreeding events (0.12-0.14) estimated in *P. amplexicaulis* indicating that small-mammals are facilitating breeding between kin which are often found in close proximity to one another. This is the first study that has estimated the effects of NMP on associated plant population genetic structure and gene flow between populations. These findings are highly relevant given that many populations of NMP *Protea* species are localised and small (Collins and Rebelo, 1987). Over time, these species run a risk of becoming genetically depauperate, but additional sampling needs to take place to find a strong pattern of whether these populations are at risk of losing genetic diversity.

High levels of biparental inbreeding with associated strong spatial genetic structuring and low population genetic connectivity means that non-flying mammal floral visitors are not moving pollen considerable distances across the landscape. Although the NMP's associated with *Protea* species are capable of inter-plant movements similar to those of birds (Wiens *et al*., 1983), many of these pollinator species are highly territorial. In particular, *Aethomys namaquensis* and *Rhamdomys pumilio* (common NMP's associated with *Protea*) show a strong affinity for particular sites which can be small and discrete areas (e.g. 0.0035–0.0054 km^2) (Nicolson and Fleming, 2004; Schradin & Pillay, 2006). This is especially true for females during and between breeding seasons, shown by Nicolson and Fleming (2004) by the fact that they only captured certain *A. namaquensis* females within specific sites. Furthermore, *A. namaquensis* males are often highly aggressive and territorial, and leaving their specific territories puts them at risk of predation and fatality by intra-specific competition (Nicholson and Fleming, 2004). This suggests that although small mammals are mobile floral visitors, they might be foraging within a small area. There is increasing evidence that pollen movement is stochastic but, that routine short-distance pollen movement may affect limited population connectivity by decreasing neighbourhood area and effective population size (Ingvarsson, 2002).

Genetic clustering on a fine scale (> 50 m) is a common occurrence in plants and often a result of leptokurtic seed and pollen distributions (Beattie, 1978; Levin and Kerster, 1974). Therefore, it may be expected that *P. amplexicaulis* would exhibit strong genetic structuring. However, it is interesting that *P. laurifolia* exhibited a relatively homogenous distribution of genotypes throughout space. Weak genetic structure was also shown in populations of

Banksia attenuata (Proteaceae) (Ritchie and Krauss, 2012) and low population differentiation ($F_{st} < 0.01$) between populations of *B. menzeii* (Frick *et al*., 2014), both of which are pollinated by birds. As with NMP's, strong territoriality has also been recorded for *Promerops cafer*, the Cape Sugarbird, one of the most common bird pollinators associated with *Protea* (Calf *et al*., 2003a, b). However, territory ownership is maintained only until the end of their breeding system (Calf *et al*., 2003a). They may increase their foraging ranges outside of breeding seasons which allows these birds to move pollen beyond the borders of their territory. In addition to this, the potential mobility (birds have been regularly recorded to fly distances as far as one kilometer in one day) and high energy demands of birds (Collins, 1983) may promote the long-distance gene dispersal which results in limited differentiation between populations and a homogenous distribution of genotypes. High energy demands and large foraging ranges provide evidence that birds are dispersing pollen considerable distances and promoting gene flow between populations.

Spatial genetic structuring is also a function of seed dispersal such that limited seed dispersal would promote patterns of non-random spatial distribution of genotypes in populations (Loveless and Hamrick, 1984; Volis *et al*., 2016). Serotiny is ubiquitous in the *Protea* genus whereby seeds are released en masse prior to being wind dispersed (Bond, 1985). Seeds dispersed by wind and tumbling along the ground can result in neighbours of focal plants being relatives. *P. laurifolia* has been shown to have short distance seed dispersal (Hamrick and Loveless, 1986; Manders, 1986). Therefore, realised limited seed dispersal may be affecting the patterns of genetic structuring measured in this study. Measuring seed dispersal was beyond the scope of this study, but the relative contributions of pollen and seed dispersal to gene dispersal are important aspects to consider when determining the causes of limited gene flow. One way to disentangle the contributions to gene flow is to measure the relative pollen dispersal distances of each pollinator. For example, Krauss *et al*. (2009) estimated that birds created long-distance pollen dispersal events in *B. hookerinia*, but strong spatial genetic structuring up to approximately 30 m was detected. In this case, it is evident that seed dispersal is playing a large role in the species' strong genetic structuring. Since both species in this study represent similar seed dispersal mechanisms (i.e. wind dispersal after fire), it can be assumed that differences in spatial genetic structuring are likely to be caused by variations in pollen dispersal distances of pollinator movements.

Mating system is also a strong determinant of spatial genetic structure whereby high rates of selfing in populations promote strong genetic structuring (Vekemans and Hardy, 2004; Volis *et al*., 2016). Therefore, it is surprising that outcrossing rates are high (> 0.80) in *P. amplexicaulis* and consistent with the highly outcrossed Australian *B. sphaerocarpa* (tm =

0.86 - 0.99), a species primarily pollinated by birds (Llorens *et al*., 2012). Considerably high outcrossing rates found in *P. amplexicaulis* may be a result of high interplant movements caused by the energetic requirements of the small mammals that visit *Protea* inflorescences. These mammalian visitors are often medium sized (> 100 g) indicating that their metabolic requirements are similar to that of birds, which may demonstrate the need that these pollinators have to visit large numbers of inflorescences in a foraging bout (Collins and Rebelo, 1987). High outcrossing rates can also be a reflection of post-zygotic processes or preferential outcrossing often detected in Proteaceae (Goldingay and Carthew, 1998). High outcrossing rates could also be a result of inbred progeny not making it to maturity (inbreeding depression). For example, beetle-pollinated *P. caffra* individuals had low levels of inbreeding (–0.187 ± 0.065), but their offspring had increase measures of F_{is} (0.258 ± 0.002) (Steenhuisen *et al*., 2012b). This suggests that beetles could be facilitating inbreeding within this *P. caffra* population and that inbred offspring have a low survival rate. High heterozygosity (0.75 ± 0.2) found in adult individuals of the focal *P. amplexicaulis* metapopulation supplemented with evidence for sib-mating (0.12-0.15) suggests the presence of inbreeding depression within this population. Therefore, it is uncertain whether the high outcrossing rates amongst species is a reflection of a mobile visitor or the active selection against self-pollen or inbred progeny (Charlesworth and Willis, 2009; Goldingay and Carthew, 1998). Calculating inbreeding depression for *P. amplexicaulis* was beyond the scope of the current study, but future research should aim to compare values of offspring F_{is} with adult F_{is} to determine whether inbred progeny are reaching maturity. Nevertheless, high outcrossing rates are seemingly not a limiting factor in the high population differentiation and strong genetic structuring recorded in this study.

Since *P. amplexicaulis* shows evidence for limited pollen dispersal, it is expected that the inbreeding within populations of *P. amplexicaulis* would increase with distance (e.g. James and McDougall, 2014). Surprisingly, *P. amplexicaulis* genetic structure is similar to that found for a primarily bird-pollinated species, *P. punctata* (A_r = 8-9; H_e = 0.73-0.91), using the same ten microsatellite markers as in the current study (Prunier and Latimer, 2010). However, intra- population genetic diversity is not only dependent on gene dispersal via long distance pollen transfer but is also a function of a plant’s mating system, seed dispersal mechanism, and life-history traits and strategy (Hamrick *et al*., 1992; James and McDougall, 2014). Hamrick *et al*. (1992) reviewed various determinants of genetic structure and concluded that it is influenced mostly by life-history and mating systems where high H_e and A_r values are apparent for long-lived woody species that are highly outcrossed (Hamrick *et al*., 1992), which was found in *P. amplexicaulis* populations. In addition to this, proteas are found in fire- prone environments including the Cape Floristic Region (Cowling *et al*., 2003), and thus high values of genetic diversity indices may also be a reflection of strong natural selection for vigorous heterozygote genotypes that are adapted to a special post-fire regeneration niche (Gershberg *et al*., 2016). Serotinous species, such as *Protea* species (Bond, 1985), will release their seeds after a fire which exposes these seeds to a regeneration niche including thick ash beds and dead plant material (Gershberg *et al*., 2016). Therefore, intra- population genetic structure is a complex component of population health which is influenced by more factors than just the pollen dispersal mechanism of a species.

In order to fully understand the extent of SGS occurring in a species and whether isolation-by-distance is truly represented in the current dataset, the S_p statistic should be calculated for each metapopulation. However, this was beyond the scope of the current study because only preliminary data was recorded. Future studies should aim to calculate that statistic after increasing the number of species being analysed. This S_p statistic summarises the intensity of SGS, allowing for a quantitative comparison of SGS between species or populations. The statistic takes into account the sampling scheme and thus it is often used as a measurement to understand the biases in the SGS analysis (Fenster *et al*., 2003). For instance, the S_p statistic could indicate that the spatial scale used is inadequate or that the specific genetic marker is not yielding enough information to get sufficient reliable estimates of relatedness (Vekemans and Hardy, 2004). Therefore, SGS is often synthetically quantified by the S_p statistic. Additionally, since SGS is sensitive to sampling scheme, it may also be beneficial for future analyses to sample individuals continuously across the landscape rather than at several distances apart. Nevertheless, this study is a precursor to fully understanding SGS in *Protea* and provides some evidence that the genetic structuring shown within *P. amplexicaulis* is linked to pollinator type.

3.4.2 Controlled pollination experiments

Our study showed that pollen supplementation does not significantly increase natural seed set in *P. amplexicaulis.* A similar lack of response to pollen supplementation was also detected in the bird-pollinated *P. roupelliae* (Hargreaves *et al*., 2004), insect-pollinated *P. dracomontana*, *P. simplex* and *P. welwitschii* (Steenhuisen and Johnson, 2012), and small mammal-pollinated *P. decurrens* (Zoeller *et al*., 2017). Pollen availability in *P. amplexicaulis* is also reflected in strong self-incompatibility and inability to autonomously self-fertilise. Autonomous selfing is inversely related to the pollinator availability such that autogamy is selected as reproductive assurance when mates are limited (Brys *et al*., 2011). Because no seeds were set in the absence of all pollinators, it suggests that *P. amplexicaulis* requires facilitation for pollen movement by non-flying mammals. This is evidence that pollen transfer of non-flying mammals is efficient and mate limitation is not caused by infrequent visitation. High visitation rates (up to three visits per inflorescence in a 24 hour period) have been recorded by remote-camera analysis for several geoflorous *Protea* species (Zoeller *et al*., 2017). Therefore, this indicates that non-flying mammals are reliable vectors for pollen transfer in *P. amplexicaulis*.

Despite pollen availability not being a limiting factor for seed production in *P. amplexicaulis*, seed set is low (maximum adjusted mean proportion seed set = 0.25). Low and variable seed set is well documented in Proteaceae (Collins and Rebelo, 1987; Wright, 1994; Rebelo and Rourke, 1985) where the average seed set in the genus is 9.2 % (Collins and Rebelo, 1987). Previous studies documenting seed set in Proteaceae have suggested a few factors that may result in low seed set. In some species (e.g. *B. spinulosa* and *B. cunninghamii*), seed set increases with the addition of nutrients such as phosphorus (Dugal and O'Dowd, 1989; Vaughton and Ramsey, 2001). In other cases, seed mass has been noted to increase with redistribution of nutrients (Denton *et al*., 2007). However, there are many other hypotheses for low seed set in Proteaceae including high insect predation and limited space within the inflorescences (Coetzee and Giliomee, 1987). Investigating these hypotheses is beyond the scope of the study, but the low seed set detected also provides evidence that proportion fruit set can be an unreliable method when determining the breeding system of proteas. Significant differences are difficult to detect when seed set is low (Steenhuisen *et al*., 2012b) and thus, the use of alternative methods (e.g. using multilocus outcrossing rates) in conjunction with seed set data increases the accuracy of determining a species' mating system.

3.4.3 Conclusion

Strong fine-scale spatial genetic structuring within a metapopulation and high population differentiation between subpopulations of *P. amplexicaulis* provides preliminary evidence that isolation-by-distance is occurring in this therophilous species. Long-term limited gene flow can result in reproductive isolation between populations which precipitates speciation. The role that pollinator-mediated reproductive isolation plays in *Protea* diversification is still under debate (Johnson, 2010). It is hypothesised that reproductive isolation caused by limited gene flow between populations, has played a role in many therophilous *Protea* populations having localised distributions (Collins and Rebelo, 1987). A pattern of isolation-by-distance was estimated between populations of primarily bird-pollinated *P. repens* as a result of differences in rainfall seasonality and physical barriers to gene flow, placing populations in two or three groupings across the Cape Floristic Region (Prunier *et al*., 2017). However, this is the first study to provide evidence of isolation-by-distance and limited gene flow between small-mammal-pollinated *Protea* populations. Restricted gene dispersal in *P.*

amplexicaulis is hypothesised to be a result of the small home ranges (e.g. *Rhamdomys pumilio* in Schradin & Pillay, 2006) and territoriality (Nicolson and Fleming, 2004) of NMP's. This restricts the gene pool size that these pollinators are foraging from. Despite this study's *P. amplexicaulis* metapopulation showcasing spatial genetic structuring; small-mammals are considered effective cross-pollinators in *P. amplexicaulis*. This is evidenced by a significant reduction of seed set in the absence of these pollinators and high amounts of outcrossing events within populations. Although NMPs may be foraging between kin of individual plants in a population, they are also highly mobile (indicated by high outcrossing rates) and important for reproduction (indicated by low seed in the absence of non-flying mammal pollinators) in *P. amplexicaulis*.

4. Synthesis

4.1 Conclusion

Research on the outcrossing and gene dispersal abilities of various pollinators is important for understanding the diversification of a plant lineage as well as the maintenance of mating systems. In South Africa, the mating system outcome and subsequent genetic diversity of plant species that have specialised pollination syndromes are unclear. In this thesis, I conducted a comprehensive mating system and genetic structuring study on six *Protea* species pollinated by various animal guilds: *P. amplexicaulis* and *P. humiflora* (non-flying mammal-pollinated), *P. caffra* and *P. simplex* (insect-pollinated), *P. laurifolia* and *P. roupelliae* (bird-pollinated). This involved estimating multilocus outcrossing rates, spatial genetic structuring and genetic diversity using ten microsatellites developed for the *Protea* genus. In this concluding chapter, I will summarise the findings discussed in the previous chapters and examine how comparing population genetics of non-flying mammal- pollinated (NMP) *Protea* species to species associated with bird or beetle pollinators can further our understanding in the role that small mammals play in pollinator-mediated speciation and conservation genetics of proteas.

The role of pollinators in plant reproduction is often quantified using pollinator exclusion experiments where seed set is used as a proxy for reproductive output. However, *Protea* species have low and variable seed set and thus finding significant differences between treatments is difficult (Ayre and Whelan, 1989; Steenhuisen and Johnson, 2012a). This was evidenced in my breeding experiment (Chapter 3) of *P. amplexicaulis* where seed set was low (< 0.25) even in treatments supplemented with cross-pollen. Steenhuisen *et al.* (2012b) is the only study to date showing that molecular markers can be an alternative method to determine breeding or mating systems in African *Protea* species. However, Steenhuisen *et al.* (2012b) estimated outcrossing rates using allozymes markers which often lack variability for in-depth parentage and relatedness analyses (Franklin *et al*., 2002). Molecular data have been effectively used to determine the mating system in several other genera. For example, Frick et al (2014) recorded complete outcrossing in *Banksia meziesii* (Proteaceae) using *microsatellites*. Similarly, inbreeding depression was detected in *Magnolia obovata* (Magnoliaceae) using molecular data. Microsatellite markers developed for the "white protea clade" have the potential to determine relatedness between individuals belonging to the same *Protea* species (Prunier and Laitmer, 2010). Here I have given evidence that these markers are effective at estimating high multilocus outcrossing rates (> 0.80) and genetic diversity (H_e > 0.7) in *Protea* species (Chapter 2). Furthermore, I illustrated that these markers can be used for species that are not considered white proteas (e.g. *P. amplexicaulis* and *humiflora*). Therefore, breeding or mating system analysis of Protea are not restricted to pollinator exclusion experiments or analysis using allozymes markers.

Pollination systems are identified as a mode of plant lineage diversification where pollinator shifts are associated with floral trait specialisation (Johnson, 2010; Armbruster *et al*., 2014). Pollinator shifts are often precipitated when a plant encounters a novel pollinator on its range margin and maximises outcrossing by filtering out less effective pollinators (Grant, 1949; Ashworth *et al*., 2015). Improving reproductive success is tied into attracting the most efficient pollinator by reward, the timing of flowering, scent or colour (Kay and Sergent, 2009). Because *Protea* species have a diverse array of floral traits associated with various pollinator guilds, it is hypothesised that pollinator shifts may have played a large role in the diversification of the lineage. However, this is still debated (Valente *et al*., 2010; Schnitzler *et al*., 2011). I have shown that all focal *Protea* species used in this study had high outcrossing rates which illustrates that the insect, non-flying mammal and bird pollinators associated with these species are equally effective at transferring cross-pollen (Chapter 2). This suggests that specialisation to the most effective pollinator (i.e. pollinator shifts) may have played a large role in the diversification of lineage.

A pollinator's mobility is an important aspect of its contribution to a plant's outcrossing because it affects the distance of interplant pollen movements, the number of plants that are visited, and whether visitation is limited to inflorescences on the same plant (Devaux *et al*., 2014; Frankie and Baker, 1974). I have shown here that although small mammals cannot fly, they are still effective outcrossers relative to flying pollinators (Chapter 2). High outcrossing rates found in the NMP protea, *P. amplexicaulis*, also suggests that NMPs could be more reliant on the nectar resources of proteas than previously thought. Because nectar and pollen are regarded as an alternative food source for small mammals, these floral visitors were classified as unreliable pollen vectors (Fleming and Nicolson, 2002a; Wiens *et al*., 1983). Consequently, floral specialisation associated with non-flying mammals has become a topic of interest amongst evolutionary and pollination biologists (Wiens *et al*., 1983). However, I have shown here that non-flying pollinators are as effective at cross-pollinating plant individuals as flying pollinators. High energetic demands of non-flying mammal pollinators may result in these pollinators visiting many inflorescences in one foraging bout resulting in high levels of pollen carryover amongst plant individuals. Large numbers of inflorescences that are probed by a pollinator often lead to increased levels of outcrossing rates in a population if these plant individuals are genetically unrelated or dissimilar.

The effectiveness of non-flying mammal pollinators is also evidenced by strong self-incompatibility in *P. amplexicaulis*, shown with pollinator exclusion experiments (Chapter 3), which suggests that small mammals are required for pollination. Pollinator specialisaton of plant species is often a method to insure and increase fecundity (Aigner, 2004). In cases where pollinators are poor outcrossers or have infrequent flower visits, self-compatibility can be seen as a method to insure fertilization. In these cases, lower outcrossing rates could be a result of an ineffective pollinator (Shuttleworth and Johnson, 2006; Wilcock and Nieland, 2002). Previous research on the breeding system of NMP *Protea* species also showed a significant reduction in seed set of *P. decurrens*, *P. scabra*, *P. humiflora*, *P. cordata*. *P. nana*, and *P. foliosa* when NMP's were excluded from pollination (Biccard and Midgely, 2009; Zoeller *et al*., 2017; Wiens *et al*., 1983). However, I have given the first evidence that *P. amplexicaulis* is self-incompatible using pollinator exclusion experiments (Chapter 3). This information is imperative to understanding the importance of non-flying mammals in therophilous *Protea* species, supporting the results from the genetic analysis (Chapter 3).

Our results also revealed that some of the outcrossing events in *P. amplexicaulis* were between kin (Chapter 3). Over time, high levels of biparental inbreeding can result in isolation-by-distance between populations (Yamaguchi and Iwasa, 2013). A significant pattern of population differentiation was illustrated between *P. amplexicaulis* subpopulations supported by strong fine-scale spatial genetic structuring (Chapter 3). Because complete isolation of populations can cause allopatric speciation (Yamaguchi and Iwasa, 2013), short distance gene dispersal associated with NMPs has the potential to facilitate pollinator-mediated speciation resulting in localised therophilous *Protea* populations. Contrastingly, bird-pollinated *Protea* species are found in large and continuous populations (Collins and Rebelo, 1987). Our results illustrate that birds are capable of long-distance gene dispersal which resulted in population connectivity and weak spatial genetic structuring within a *P. laurifolia* metapopulation (Chapter 3). Therefore, gene dispersal differences in ornithophilous and therophilous *Protea* species may be contributing to the contrast in current population distributional ranges.

High outcrossing rates (Chapter 2) in NMP proteas show that non-flying mammals are highly vagile, but the fine scale spatial genetic structuring (Chapter 3) associated with NMP proteas also indicates that these pollinators are sampling from a small gene pool. Localised pollination is most likely a result of non-flying mammal pollinators being territorial. Many of the small mammals that pollinate *Protea* species protect discrete areas, preventing males from crossing territory boundaries (Nicolson and Fleming, 2004). The high outcrossing rates (> 0.80) found in *P. amplexicaulis* (Chapter 2) illustrates that NMP's have high interplant movements, but these movements are localised. Therefore, territoriality is hypothesised to play a role in gene dispersal in NMP *Protea* species.

4.2 Research gaps

Several areas of concern were raised during this study. While the focus of this study was primary pollinators, there are various other floral visitors that may have pollinated individuals of the focal species (Bawa 1990, Olesen and Jordano, 2002). The study species are specialised in their floral morphology to be pollinated by certain pollinator guilds, but other floral visitors may have contributed to pollination. For example, *P. caffra* floral traits are associated with specialisation to beetle pollination but are often pollinated by birds (Steenhuisen *et al*., 2012b). To gain a better insight into the outcrossing abilities of each pollinator, exclusion experiments should be done by preventing pollination by certain animals prior to estimating outcrossing rates of individuals (e.g. Steenhuisen *et al*., 2012b) This would ensure that outcrossing rates are determined for individuals visited by one pollinator guild. Preventing specific animals from pollination would be imperative to understand the actual contribution each pollinator has to outcrossing rates of the focal species.

Future studies would benefit from using a variety of populations per species to increase replication. Outcrossing rates and genetic diversity can vary between populations of species (Whitehead *et al*., 2018). For example, estimates of outcrossing rates for six populations of *B. cuneata* ranged from a mixed mating system (t_m = 0.67) to complete outcrossing (t_m = 0.95) (Llorens *et al*., 2012; Maguire and Sedgley, 1997). Variations of mating systems can be a result of several ecological factors. Fluctuations in pollinator abundances often affect selfing rates in self-compatible species such as *Hypochaeris salzmanniana* (Asteraceae) (Arista *et al*., 2017). Nevertheless, this study provides preliminary evidence for the outcrossing abilities of pollinators. However, an increase in the number of populations per species would be imperative to understanding whether mating system outcomes are a result of pollinator efficiency or ecological differences between species. This can also be supplemented by the addition of species because outcrossing can also differ between species (Whitehead *et al*., 2018). This would allow for a reliable estimate of the outcrossing abilities of pollinators rather than a “snapshot” view. Along with the pollination syndrome, it is recommended that species used in future research should be selected based on the species’ breeding system. Ideally, species should be facultatively self-compatible to ensure that estimated high outcrossing rates are not a result of preventative measures against self- fertilisation (e.g. selective abortions of self-fertilised ovules in *Banksia* species) which are often found in obligate outcrossers (Silva and Goring, 2001).

In some cases, inbred offspring do not make it maturity (also known as inbreeding depression) because increased homozygosity can result in lowered fitness these progenies (e.g. Richards, 2000). Inbreeding depression is predominantly caused by the expression of deleterious alleles

(Charlesworth and Willis, 2009). Subsequently, the presence of inbreeding depression within a population indicates genetic variation in fitness traits exists in the natural population (Charlesworth and Willis, 2009). Calculating inbreeding depression can give an indication as to whether high levels of genetic diversity and outcrossing rates within populations are a result of selection against inbred progeny or an efficient cross- pollinator. For example, beetle-pollinated *P. caffra* individuals had high levels of heterozygosity, but their offspring had increase measures of F_{is} (Steenhuisen *et al*., 2012b). This suggests that beetles could be facilitating inbreeding within this *P. caffra* population and that inbred offspring have a low survival rate. High heterozygosity within *P. amplexicaulis* populations and evidence for sib-mating (Chapter 2 and 3) suggests that progeny do not make it maturity. Calculating inbreeding depression was beyond the scope of the current study. Future research should aim to estimate measures of *P. amplexicaulis* progeny F_{is} in comparison to adult values to determine the level of inbreeding depression that is occurring within the population. This will indicate if high outcrossing rates in *P. amplexicaulis* populations are a result of a selection against inbred individuals.

The results of the breeding system experiment were highly affected by seed predation which reduced the sample size to only 8 focal individuals. Most of the individuals could only be tested for autonomous self-pollination which resulted in only two plants that were supplemented with self-pollen. Seed predation is a common occurrence in *Protea* species and is often results in low seed set in the genus (Ayre and Whelan, 1989; Coetzee and Giliomee, 1987; Steenhuisen and Johnson, 2012a; Rebelo and Rourke, 1985). *P. amplexicaulis* exhibited strong self-incompatibility (Chapter 3). Increasing the sample size of the number of self-pollinated supplemented individuals would not only strengthen statistical analyses, but also the reliability of the results.

While biparental inbreeding was prevalent in *P. amplexicaulis*, it is uncertain whether this is a result of restricted pollen or seed dispersal. Seed dispersal is often limited in wind- dispersed seeds which results in related individuals recruiting in close proximity to one another (Manders, 1986). Therefore, future research should focus on determining realised seed dispersal in *Protea* in order to estimate the relative contributions of seed and pollen dispersal to gene flow. However, this could also be achieved by estimating pollen movement across a landscape (Scheepens *et al*., 2012). This would also indicate the distance pollinators are moving between plants and whether pollination is restricted within a discrete or small area (Scheepens *et al*., 2012). Subsequently, determining pollen movements would result in a direct estimation of how pollinators contribute to pollinator-mediated gene flow between populations.

This thesis has provided preliminary evidence for the role that pollinators play in gene dispersal and the effect they have on plant population genetics. In order to fully understand the role that pollinators play in the maintenance of plant mating systems; more studies need to be conducted on the comparative pollen dispersal abilities of pollinators using genetic analyses. For Proteaceae in particular, much of the current research is centred on the gene flow between fragmented or rare *Banksia* and *Grevillea* populations primarily pollinated by birds (England *et al*., 2002; Llorens *et al*., 2011; Maquire and Sedgely, 1997). Therefore, there is a need to compare the mating systems and interpopulation gene flow of populations primarily pollinated by small-mammals and insects. This is the first study to comparatively understand how pollinator specialisation can affect plant population genetics of South African taxa. Specifically, this is the first study to estimate the outcrossing abilities of small- mammals and subsequent effects on gene flow. In order to gain a better understanding of pollinator-driven speciation in *Protea* and other southern Africa taxa, more research is required to compare the pollen dispersal abilities of different pollinator guilds. This is especially necessary for small mammals and insects where information is lacking.

References

Abdala-Roberts, L., Marrufo-Zapata, D., Arceo-Gómez, G. and Parra-Tabla, V., 2014. Pollen limitation, fruit abortion, and autonomous selfing in three populations of the perennial herb *Ruellia nudiflora*. *Plant Species Biology* 29(1), 25-33.

Aigner, P.A., 2004. Ecological and genetic effects on demographic processes: pollination, clonality and seed production in *Dithyrea maritima*. *Biological conservation* 116(1):27-34.

Alexandersson, R., Johnson, S.D., 2002. Pollinator-mediated selection on flower–tube length in a hawkmoth–pollinated *Gladiolus* (Iridaceae). *Proceedings of the Royal Society of London. Series B: Biological Sciences* 269, 631-636.

Angoh, S., Brown, M., Midgley, J., 2017. Selfing versus out-crossing in six *Erica* species of the southern Cape. *South African Journal of Botany* 111, 387-391.

Ashworth, L., Aguilar, R., Martén-Rodríguez, S., Lopezaraiza-Mikel, M., Avila-Sakar, G., Rosas-Guerrero, V. and Quesada, M., 2015. Pollination syndromes: a global pattern of convergent evolution driven by the most effective pollinator. In *Evolutionary biology: biodiversification from genotype to phenotype* (pp. 203-224). Springer, Cham.

Awadalla, P., Ritland, K., 1997. Microsatellite variation and evolution in the Mimulus guttatus species complex with contrasting mating systems. *Molecular Biology and Evolution* 14(10), 1023-1034.

Ayre, D.J., Whelan, R.J., 1989. Factors controlling fruit set in hermaphroditic plants: Studies with the Australian proteaceae. *Trends in Ecology and Evolution* 4, 267-272.

Ayre, D.J., Whelan, R.J., Reid, A., 1994. Unexpectedly high levels of selfing in the Australian shrub *Grevillea barklyana* (Proteaceae). *Heredity* 72, 168.

Banks, S.C., Peakall, R., 2012. Genetic spatial autocorrelation can readily detect sex-biased dispersal. *Molecular Ecology* 21, 2092-2105.

Barrell, P., Richardson, T., Gardner, R., 1997. Molecular markers and experimental pollinations reveal self-fertility and high levels of natural inbreeding in the New Zealand endemic tree *Vitex lucens* (puriri). *New Zealand Journal of Botany* 35, 535-543.

Barrett, S.C., Shore, J.S., 1987. Variation and evolution of breeding systems in the *Turnera ulmifolia* L. *complex* (Turneraceae). *Evolution* 41(2), 340-354.

Barrett, S.C., 1998. The evolution of mating strategies in flowering plants. *Trends in plant science* 3, 335-341.

Barrett, S.C., 2003. Mating strategies in flowering plants: the outcrossing–selfing paradigm and beyond. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 358, 991-1004.

Barrett, S.C., Harder, L.D., 1996. Ecology and evolution of plant mating. *Trends in Ecology and Evolution* 11, 73-79.

Bawa, K.S., Beach, J.H., 1981. Evolution of sexual systems in flowering plants. *Annals of the Missouri Botanical Garden* 68, 254-274.

Beattie, A., 1978. Plant-animal interactions affecting gene flow in *Viola*. *The pollination of Flowers by Insects*, 151-164.

Bezemer, N., Krauss, S.L., Phillips, R.D., Roberts, D.G. and Hopper, S.D., 2016. Paternity analysis reveals wide pollen dispersal and high multiple paternity in a small isolated population of the bird-pollinated *Eucalyptus caesia* (Myrtaceae). *Heredity* 117, 460.

Biccard, A., Midgley, J., 2009. Rodent pollination in *Protea nana*. *South African Journal of Botany* 75, 720-725.

Blambert, L., Mallet, B., Humeau, L., Pailler, T., 2016. Reproductive patterns, genetic diversity and inbreeding depression in two closely related *Jumellea* species with contrasting patterns of commonness and distribution. *Annals of Botany* 118, 93-103.

Bond, W., Volk, J., Viviers, M., 1984. Variation in seedling recruitment of Cape Proteaceae after fire. *Journal of Ecology* 72, 209-221.

Botha, P.W., 2017. The world without birds: an experimental test of the ecological significance of pollinating birds for plant communities. MSc thesis, Stellenbosch University.

Breed, M.F., Ottewell, K.M., Gardner, M.G., Marklund, M.H., Dormontt, E.E. and Lowe, A.J., 2015. Mating patterns and pollinator mobility are critical traits in forest fragmentation genetics. *Heredity* 115(2), 108.

Brown, A.H.D., 1989. Plant population genetics, breeding, and genetic resources, in: International Symposium on Population Genetics and Germplasm Resources in Crop Improvement. Sinauer Associates, California.

Brunet, J., Sweet, H.R., 2006. Impact of insect pollinator group and floral display size on outcrossing rate. *Evolution* 60, 234-246.

Brys, R., De Crop, E., Hoffmann, M. and Jacquemyn, H., 2011. Importance of autonomous selfing is inversely related to population size and pollinator availability in a monocarpic plant. *American Journal of Botany* 98(11), 1834-1840.

Buchmann, S.L. and Nabhan, G.P., 1996. The forgotten pollinators. Island Press, Washington.

Busch, J.W., 2005. The evolution of self-compatibility in geographically peripheral populations of *Leavenworthia alabamica* (Brassicaceae). *American Journal of Botany* 92, 9

Busch, J.W., Delph, L.F., 2012. The relative importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. *Annals of Botany* 109, 553-562

Byrne, M., Elliott, C., Yates, C., Coates, D., 2007. Extensive pollen dispersal in a bird-pollinated shrub, *Calothamnus quadrifidus,* in a fragmented landscape. *Molecular Ecology* 16, 1303-1314.

Calf, K.M., Downs, C.T., Cherry, M.I., 2003a. Territoriality of Cape Sugarbirds (*Promerops cafer*) between and within breeding seasons. *Ostrich-Journal of African Ornithology* 74, 125 -128

Calf, K.M., Downs, C.T., Cherry, M.I., 2003b. Territoriality and breeding success in Cape Sugarbirds (*Promerops cafer*). *Emu* 103, 29-35

Carlson, J.E., Holsinger, K.E., 2010. Natural selection on inflorescence colour polymorphisms in wild *Protea* populations: the role of pollinators, seed predators, and intertrait correlations. *American Journal of Botany* 97, 934-944.

Carthew, S.M., 1994. Foraging behaviour of marsupial pollinators in a population of *Banksia spinulosa*. *Oikos*, 133-139.

Chaix, G., Gerber, S., Razafimaharo, V., Vigneron, P., Verhaegen, D., Hamon, S., 2003. Gene flow estimation with microsatellites in a Malagasy seed orchard of *Eucalyptus grandis*. *Theoretical and Applied Genetics* 107, 705-712.

Charlesworth, D. and Charlesworth, B., 1995. Quantitative genetics in plants: the effect of the breeding system on genetic variability. *Evolution* 49(5), 911-920.

Charlesworth, D. and Willis, J.H., 2009. The genetics of inbreeding depression. *Nature reviews* *genetics* 10(11), 783.

Coates, D.J., Sokolowski, R.E., 1992. The mating system and patterns of genetic variation in *Banksia cuneata* AS George (Proteaceae). *Heredity* 69, 11.

Coetzee, J., Giliomee, J., 1985. Insects in association with the inflorescence of *Protea repens* (L.) (Proteaceae) and their role in pollination. *Journal of the Entomological Society of southern Africa*.

Coetzee, J., Giliomee, J., 1987. Seed predation and survival in the infructescences of *Protea repens* (Proteaceae). *South African Journal of Botany* 53, 61-64.

Collevatti, R.G., Estolano, R., Garcia, S.F. and Hay, J.D., 2010. Short-distance pollen dispersal and high self-pollination in a bat-pollinated neotropical tree. *Tree Genetics & Genomes* 6(4), 555-564.

Collevatti, R.G., Grattapaglia, D., Hay, J.D., 2001. High resolution microsatellite based analysis of the mating system allows the detection of significant biparental inbreeding in *Caryocar brasiliense*, an endangered tropical tree species. *Heredity* 86, 60.

Collevatti, R.G., Telles, M.P., Lima, J.S., Gouveia, F.O., Soares, T.N., 2014. Contrasting spatial genetic structure in *Annona crassiflora* populations from fragmented and pristine savannas. *Plant Systematics and Evolution* 300:1719-1727.

Collins, B., 1983. A first approximation of the energetics of Cape sugarbirds (*Promerops cafer*) and orange-breasted sunbirds (*Nectarinia violacea*). *African Zoology* 18, 363-389.

Collins, B.G and Spice, J., 1986. Honeyeaters and the Pollination Biology of *Banksia prionotes* (Proteaceae). *Australian Journal of Botany* 34, 175 – 185.

Collins, B.G., Rebelo, T., 1987. Pollination biology of the Proteaceae in Australia and southern Africa. *Australian Journal of Ecology* 12, 387-421.

Conte, R., Sedrez dos Reis, M., Mantovani, A. and Vencovsky, R., 2008. Genetic structure and mating system of *Euterpe edulis* Mart. populations: a comparative analysis using microsatellite and allozyme markers. *Journal of Heredity* 99(5), 476-482.

Cowling, R.M., Pressey, R.L., Rouget, M. and Lombard, A.T., 2003. A conservation plan for a global biodiversity hotspot—the Cape Floristic Region, South Africa. *Biological conservation* 112(1-2), 191-216.

Cruzan, M.B., 1998. Genetic markers in plant evolutionary ecology. *Ecology* 79, 400-412.

Cunningham, S.A., 1991. Experimental evidence for pollination of *Banksia* spp. by non-flying mammals. *Oecologia* 87, 86-90.

Darwin, C., 1862. On the various contrivances by which British and foreign orchids are fertilised by insects and on the good effects of intercrossing. J. Murray, London.

Darwin, C., 1876. The effects of cross and self-fertilisation in the vegetable kingdom. J. Murray, London.

Devaux, C., Lepers, C., Porcher, E., 2014. Constraints imposed by pollinator behaviour on the ecology and evolution of plant mating systems. *Journal of evolutionary biology* 27, 1413- 1430.

Dow, B., Ashley, M., 1996. Microsatellite analysis of seed dispersal and parentage of saplings in bur oak, *Quercus macrocarpa*. *Molecular Ecology* 5, 615-627.

Doyle, J., Doyle, J., 1987. A rapid procedure for DNA purification from small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19, 11-15.

Dudash, M.R., 1990. Relative fitness of selfed and outcrossed progeny in a self-compatible, protandrous species, *Sabatia angularis* L. (Gentianaceae): a comparison in three environments. *Evolution* 44, 1129-1139.

Eckert, C.G., Herlihy, C.R., 2004. Using a cost–benefit approach to understand the evolution of self-fertilization in plants: the perplexing case of *Aquilegia canadensis* (Ranunculaceae). *Plant Species Biology* 19, 159-173.

Eckert, C.G., Kalisz, S., Geber, M.A., Sargent, R., Elle, E., Cheptou, P.-O., Goodwillie, C., Johnston, M.O., Kelly, J.K., Moeller, D.A., 2010. Plant mating systems in a changing world. *Trends in Ecology and Evolution* 25, 35-43.

Eckert, C.G., Samis, K.E., Dart, S., 2006. Reproductive assurance and the evolution of uniparental reproduction in flowering plants. *Ecology and evolution of flowers* 183, 203.

Ellstrand, N.C., 1984. Multiple paternity within the fruits of the wild radish, *Raphanus sativus*. *The American Naturalist* 123, 819-828.

Ellstrand, N.C., 1992. Gene flow by pollen: implications for plant conservation genetics. *Oikos*, pp.77-86.

England, P., Whelan, R., Ayre, D., 2003. Effects of seed bank disturbance on the fine-scale genetic structure of populations of the rare shrub *Grevillea macleayana*. *Heredity* 91, 475.

England, P.R., Beynon, F., Ayre, D.J., Whelan, R.J., 2001. A molecular genetic assessment of mating-system variation in a naturally bird-pollinated shrub: contributions from birds and introduced honeybees. *Conservation Biology* 15, 1645-1655.

Englund, R., 1993. Movement patterns of Cetonia beetles (Scarabaeidae) among flowering *Viburnum opulus* (Caprifoliaceae). *Oecologia* 94, 295-302.

Enjalbert, J., David, J.L., 2000. Inferring recent outcrossing rates using multilocus individual heterozygosity: application to evolving wheat populations. *Genetics* 156, 1973-1982.

Espeland, E., Rice, K., 2010. Ecological effects on estimates of effective population size in an annual plant. *Biological Conservation* 143, 946-951.

Estoup, A., Angers, B., 1998. Microsatellites and minisatellites for molecular ecology: theoretical and empirical considerations, in: Advances in Molecular Ecology. IOS Press, Amsterdam.

Excoffier, L., Lischer, H.E., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular ecology resources* 10, 564-567.

Faegri, K., Van der Pijl, L., 1979. The Principles of Pollination Ecology, third ed. Pergamom, United Kingdom.

Ferrer, M. M., Good S. V., 2012 Self-sterility in flowering plants: preventing self-fertilization increases family diversification rates. *Annals of Botany* 110: 535 – 553.

Fleming, P., Nicolson, S., 2002a. How important is the relationship between *Protea humiflora* (Proteaceae) and its non-flying mammal pollinators? *Oecologia* 132, 361-368.

Fleming, P., Nicolson, S., 2002b. Opportunistic breeding in the Cape spiny mouse (*Acomys subspinosus*). *African Zoology* 37, 101-105.

Frankie, G. W., Baker, H. G. 1974. The importance of pollinator behavior in the reproductive biology of tropical trees. *Anales del Instituto de Biologia, Universidad Nacional Autonoma de Mexico, Serie Botanica* 45, 1-10

Frankie, G. W., Opler, P. A., Bawa, K. S. 1976. Foraging behaviour of solitary bees: Implications for outcrossing of a neotropical forest tree species. *Journal of Ecology*. 64:1049-

Franklin, R., Ballou, J.D., Briscoe, D.A., 2002. Introduction to conservation genetics. Cambridge University Press, United Kingdom.

Frantz, A. C., Cellina, S., Krier, A., Schley, L., & Burke, T., 2009. Using spatial Bayesian methods to determine the genetic structure of a continuously distributed population: clusters or isolation by distance?. *Journal of Applied Ecology* 46(2): 493-505.

Frick, K.M., Ritchie, A.L., Krauss, S.L., 2014. Field of dreams: restitution of pollinator services in restored bird-pollinated plant populations. *Restoration ecology* 22, 832-840.

Garavanta, C.A.M, Wooller, R.D, Richardson, K.C., 2000. Movement patterns of honey possums, *Tarsipes rostratus*, in the Fitzgerald River National Park, Western Australia.*Wildlife Research* 27,179-183.

Gershberg, A., Ne'eman, G; Ben-Shlomo, R., 2016. Genetic structure of a naturally regenerating post-fire seedling population: *Pinus halepensis* as a case study. *Frontiers of Plant Science* 7, 549

Gideon, N., Mostert, D., Siegfried, W., 1980. *Protea* nectar and satellite fauna in relation to the food requirements and pollinating role of the Cape Sugarbird. *South African Journal of Science* 76, 409-412.

Goldblatt, P., 1978. An analysis of the flora of southern Africa: its characteristics, relationships, and origins. *Annals of the Missouri Botanical Garden* 65, 369-436.

Goldblatt, P., Manning, J.C., 2002. Plant diversity of the Cape region of southern Africa. *Annals of the Missouri Botanical Garden* 89, 281-302.

Goldingay, R.L., Carthew, S.M., 1998. Breeding and mating systems of Australian Proteaceae. *Australian Journal of Botany* 46, 421-437.

Goodwillie, C., Kalisz, S., Eckert, C.G., 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution and Systematics* 36, 47-79.

Goodwillie, C., Sargent, R.D., Eckert, C.G., Elle, E., Geber, M.A., Johnston, M.O., Kalisz, S., Moeller, D.A., Ree, R.H., Vallejo-Marin, M., 2010. Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. *New Phytologist* 185, 311-321.

Grant, V., 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* 3, 82-97.

Hamrick, J., Godt, M., 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 351, 1291-1298.

Hamrick, J., Loveless, M., 1986. The influence of seed dispersal mechanisms on the genetic structure of plant populations, *Vegetatio* 107(1), 281-297

Hamrick, J.L., Godt, J.W., Sherman-Broyles, S., 1992. Factors influencing levels of genetic diversity in woody plant species. *New Forests* 6. 95-124

Hardy, O.J., 2003. Estimation of pairwise relatedness between individuals and characterization of isolation-by-distance processes using dominant genetic markers. *Molecular Ecology* 12, 1577-1588

Hargreaves, A.L., Johnson, S.D., Nol, E., 2004. Do floral syndromes predict specialization in plant pollination systems? An experimental test in an “ornithophilous” African *Protea*. *Oecologia* 140, 295-301.

Heliyanto, B., Veneklaas, E.J., Lambers, H., Krauss, S.L., 2005. Preferential outcrossing in *Banksia ilicifolia* (Proteaceae). *Australian Journal of Botany* 53, 163-170.

Hoebee, S.E., Young, A.G., 2001. Low neighbourhood size and high interpopulation differentiation in the endangered shrub *Grevillea iaspicula* McGill (Proteaceae). *Heredity* 86, 489.

Holsinger, K., 1996. Pollination biology and the evolution of mating systems in flowering plants. *Evolutionary biology* 29, 107-149.

Horn, W., 1962. Breeding research on South African plants: II. fertility of Proteaceae. *Journal of South African Botany* 28, 259-268.

Howe, H. F., Smallwood, J. 1982. Ecology of seed dispersal. *Annual Review of Ecology and Systematics* 13:201-28.

IBMCorp., 2016. IBM SPSS Statistics for Windows. Version 24.0.

Igic, B., Lande, R., Kohn, J.R., 2008. Loss of self-incompatibility and its evolutionary consequences. *International Journal of Plant Sciences* 169, 93-104.

Ingvarsson, P., 2002. A metapopulation perspective on genetic diversity and differentiation in partially self-fertilizing plants. *Evolution* 56, 2368-2373.

Isagi, Y., Kanazashi, T., Suzuki, W., Tanaka, H., Abe, T., 2004. Highly variable pollination patterns in *Magnolia obovata* revealed by microsatellite paternity analysis. *International Journal of Plant Sciences* 165, 1047-1053.

IUCN 2019. The IUCN Red List of Threatened Species. Version 2019. http://www.iucnredlist.org.

James, E.A., McDougall, K.L., 2014. Spatial genetic structure reflects extensive clonality, low genotypic diversity and habitat fragmentation in *Grevillea renwickiana* (Proteaceae), a rare, sterile shrub from south-eastern Australia. *Annals of Botany* 114, 413-423.

Johnson, L., Briggs, B.G., 1975. On the Proteaceae—the evolution and classification of a southern family. *Botanical Journal of the Linnean Society* 70, 83-182.

Johnson, S., Newman, E., Anderson, B., 2012. Preliminary observations of insect pollination in *Protea punctata* (Proteaceae). *South African Journal of Botany* 83, 63-67.

Johnson, S., Steiner, K., 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* 51, 45-53.

Johnson, S.A., van Tets, I.G., Nicolson, S.W., 1999. Sugar preferences and xylose metabolism of a mammal pollinator, the Namaqua rock mouse (*Aethomys namaquensis*). *Physiological and Biochemical Zoology* 72, 438-444.

Johnson, S.D and Steiner, K.E., 2003. Specialized pollination systems in southern Africa. *South African Journal of Science* 99, 345-348.

Johnson, S.D., 1996. Pollination, adaptation and speciation models in the Cape flora of South Africa. *Taxon*, 59-66.

Johnson, S.D., 2010. The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 365, 499-516.

Kalisz, S., Vogler, D.W., 2003. Benefits of autonomous selfing under unpredictable pollinator environments. *Ecology* 84, 2928-2942.

Kalisz, S., Vogler, D.W., Hanley, K.M., 2004. Context-dependent autonomous self- fertilization yields reproductive assurance and mixed mating. *Nature* 430, 884.

Karron, J., Mitchell, R., Holmquist, K., Bell, J., Funk, B., 2004. The influence of floral display size on selfing rates in *Mimulus ringens*. *Heredity* 92, 242.

Karron, J.D., Holmquist, K.G., Flanagan, R.J. and Mitchell, R.J., 2009. Pollinator visitation patterns strongly influence among-flower variation in selfing rate. *Annals of Botany 103*, 1379-1383.

Karron, J.D., Thumser, N.N., Tucker, R. and Hessenauer, A.J., 1995. The influence of population density on outcrossing rates in *Mimulus ringens*. *Heredity 75*, p.175.

Kehinde, T. and Samways, M.J., 2012. Endemic pollinator response to organic vs. conventional farming and landscape context in the Cape Floristic Region biodiversity hotspot. *Agriculture, ecosystems & environment* 146(1), 162-167.

Kleizen, C., Midgley, J., Johnson, S.D., 2008. Pollination systems of *Colchicum* (Colchicaceae) in southern Africa: evidence for rodent pollination. *Annals of Botany* 102, 747-755.

Koenig, W.D., Ashley, M.V., 2003. Is pollen limited? The answer is blowin' in the wind. *Trends in ecology & evolution* 18, 157-159.

Krauss, S.L., Phillips, R.D., Karron, J.D., Johnson, S.D., Roberts, D.G., Hopper, S.D., 2017. Novel consequences of bird pollination for plant mating. *Trends in plant science* 22, 395-410

Kudo, G., Hirao, A.S., Kawai, Y., 2011. Pollination efficiency of bumblebee queens and workers in the alpine shrub *Rhododendron aureum*. *International Journal of Plant Sciences* 172, 70-77.

Kühn, N., Midgley, J. and Steenhuisen, S.L., 2017. Reproductive biology of three co-occurring, primarily small-mammal pollinated *Protea* species (Proteaceae). *South African Journal of Botany* 113, 337-345.

Kühn, N., Midgley, J., Steenhuisen, S.-L., 2017. Reproductive biology of three co-occurring, primarily small-mammal pollinated *Protea* species (Proteaceae). *South African Journal of Botany* 113, 337-345.

Lande, R., Barrowclough, G.F., 1987. Effective population size, genetic variation, and their use in population management. *Viable populations for conservation* 87, 124.

Larson, B.M., Barrett, S.C., 2000. A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnean Society* 69, 503-520.

Le Maitre, D., 1992. The relative advantages of seeding and sprouting in fire-prone environments: a comparison of life histories of *Protea neriifolia* and *Protea nitida,* in: Fire in South African mountain fynbos. Springer, pp. 123-144.

Lee, C., Mitchell-Olds, T., 2011. Quantifying effects of environmental and geographical factors on patterns of genetic differentiation. *Molecular ecology* 20 22: 4631-42.

Lechmere-Oertel, R., 2011. Karkloof Nature Reserve: Management Plan. Version 1.

Levin, D. A., Kerster, H. W., 1974. Gene flow in seed plants, in: Dobzhansky. T., Hecht, M.K., Steere, W.D (eds.), Evolutionary Biology. Plenum, New York, pp. 139-220

Lewis, D., 1942. The evolution of sex in flowering plants. *Biological Reviews* 17, 46-67.

Lian, C., Miwa, M., Hogetsu, T., 2001. Outcrossing and paternity analysis of *Pinus densiflora* (Japanese red pine) by microsatellite polymorphism. *Heredity* 87, 88.

Linder, H., 1991. Environmental correlates of patterns of species richness in the south-western Cape Province of South Africa. *Journal of Biogeography*, 509-518.

Linder, H.P. and Hardy, C.R., 2004. Evolution of the species–rich Cape flora. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 359(1450), 1623-1632.

Llorens, T., Byrne, M., Yates, C., Nistelberger, H., Coates, D., 2012. Evaluating the influence of different aspects of habitat fragmentation on mating patterns and pollen dispersal in the bird-pollinated *Banksia sphaerocarpa* var. *caesia*. *Molecular Ecology* 21, 314-328.

Lobo, J.A., Quesada, M. and Stoner, K.E., 2005. Effects of pollination by bats on the mating system of *Ceiba pentandra* (Bombacaceae) populations in two tropical life zones in Costa Rica. *American Journal of Botany* 92(2), 370-376.

Loveless, M.D., Hamrick, J.L., 1984. Ecological determinants of genetic structure in plant populations. *Annual review of ecology and systematics*, 65-95.

Mable, B.K., Adam, A., 2007. Patterns of genetic diversity in outcrossing and selfing populations of *Arabidopsis lyrata*. *Molecular Ecology* 16, 3565-3580.

Maguire, T.L., Sedgley, M., 1997. Genetic diversity in *Banksia* and *Dryandra* (Proteaceae) with emphasis on *Banksia cuneata*, a rare and endangered species. *Heredity* 79, 394.

Manders, P., 1986. Seed dispersal and seedling recruitment in *Protea laurifolia. South African Journal of Botany* 52, 421-424.

Matsuki, Y., Tateno, R., Shibata, M., Isagi, Y., 2008. Pollination efficiencies of flower-visiting insects as determined by direct genetic analysis of pollen origin. *American Journal of Botany* 95, 925-930.

Mayfield, M.M., Waser, N.M., Price, M.V., 2001. Exploring the 'most effective pollinator principle' with complex flowers: bumblebees and *Ipomopsis aggregata*. *Annals of Botany* 88, 591-596.

Meek, S., 2011. Small mammal pollination in *Protea witzenbergiana*. University of Cape Town.

Melidonis, C.A., Peter, C.I., 2015. Diurnal pollination, primarily by a single species of rodent, documented in *Protea foliosa* using modified camera traps. *South African Journal of Botany* 97, 9-15.

Meirmans, P.G., 2012. The trouble with isolation by distance. *Molecular ecology* 21(12): 2839-2846.

Morgan, M.T., Wilson, W.G., 2005. Self-fertilization and the escape from pollen limitation in variable pollination environments. *Evolution* 59, 1143-1148.

Muchhala, N., Brown, Z., Armbruster, W.S. and Potts, M.D., 2010. Competition drives specialization in pollination systems through costs to male fitness. *The American Naturalist* 176(6), 732-743.

Muchhala, N., Brown, Z., Armbruster, W.S., Potts, M.D., 2010. Competition drives specialization in pollination systems through costs to male fitness. *The American Naturalist* 176, 732-743.

Muneer, P.A., Gopalakrishnan, A., Musammilu, K., Mohindra, V., Lal, K.K., Basheer, V., Lakra, W., 2009. Genetic variation and population structure of endemic yellow catfish, *Horabagrus brachysoma* (Bagridae) among three populations of Western Ghat region using RAPD and microsatellite markers. *Molecular biology reports* 36, 1779-1791.

Nicolson, S., Fleming, P., 2004. Sex differences in space use, body condition and survivorship during the breeding season in the Namaqua rock mouse, *Aethomys namaquensis*. *African Zoology* 39, 123-132.

Nicolson SW, Van Wyk, B.-E., 1998. Nectar sugars in Proteaceae: patterns and processes.

Australian Journal of Botany 46, 489–504

Ollerton, J., Alarcón, R., Waser, N.M., Price, M.V., Watts, S., Cranmer, L., Hingston, A., Peter, C.I., Rotenberry, J., 2009. A global test of the pollination syndrome hypothesis. *Annals of Botany* 103, 1471-1480.

Ollerton, J., Winfree, R., Tarrant, S., 2011. How many flowering plants are pollinated by animals? *Oikos* 120, 321-326.

Ottewell, K.M., Donnellan, S.C., Lowe, A.J. and Paton, D.C., 2009. Predicting reproductive success of insect-versus bird-pollinated scattered trees in agricultural landscapes. *Biological Conservation 142*, 888-898.

Payne, S. and Witkowski, C.S., Symes, C. T. 2019. Good times, bad times: Inter-annual reproductive output in a montane endemic succulent (*Aloe peglerae*; Asphodelaceae) driven by contrasting visitor responses of small mammals and birds. *Australian Journal of Botany* 67(2), 116-127.

Peakall, R., Smouse, P., 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28, 2537e2539.

Proctor, M., Yeo, P., 1996. The Natural History of Pollination. Timber Press Inc, Portland.

Prunier, R., Akman, M., Kremer, C.T., Aitken, N., Chuah, A., Borevitz, J. and Holsinger, K.E., 2017. Isolation by distance and isolation by environment contribute to population differentiation in *Protea repens* (Proteaceae L.), a widespread South African species. *American journal of botany* 104, 674-684.

Prunier, R., Holsinger, K., Carlson, J., 2014. Reconsidering a disjunct distribution: Molecular and morphological evidence for two evolutionarily distinct lineages in *Protea mundii* Klotsch. *South African Journal of Botany* 95, 64-69.

Prunier, R., Holsinger, K.E., 2010. Was it an explosion? Using population genetics to explore the dynamics of a recent radiation within *Protea* (Proteaceae L.). *Molecular Ecology* 19, 3968-3980.

Prunier, R., Latimer, A., 2010. Microsatellite primers in the white proteas (Protea section Exsertae, Proteaceae), a rapidly radiating lineage. *American Journal of Botany* 97, e1-e3.

Ramsey, M.W., 1988. Differences in pollinator effectiveness of birds and insects visiting *Banksia menziesii* (Proteaceae). *Oecologia* 76, 119-124

Rebelo, A., 1987. Preliminary synthesis of pollination biology in the Cape flora. National Scientific Programmes Unit: CSIR.

Rebelo, A., 2000. Protea Atlas Project: Protea biology. National Botanical Institute, Kirstenbosch, Cape Town, South Africa. protea.worldonline.co.za/home.htm.

Rebelo, A., Rourke, J., 1985. Seed germination and seed set in southern African Proteaceae: ecological determinants and horticultural problems. Acta Hortic 185, 75-88.

Rebelo, A., Siegfried, W., Crowe, A., 1984. Avian pollinators and the pollination syndromes of selected mountain fynbos plants. *South African Journal of Botany* 3, 285-296.

Reusch, T.B., 2000. Pollination in the marine realm: microsatellites reveal high outcrossing rates and multiple paternity in eelgrass *Zostera marina*. *Heredity* 85, 459.

Richards, A.J., 1996. Breeding systems in flowering plants and the control of variability. *Folia Geobotanica* 31, 283-293.

Richards, C.M., 2000. Genetic and demographic influences on population persistence: gene flow and genetic rescue in *Silene alba*. *Genetics, demography and viability of fragmented populations*, pp.271-291.

Richards, M., Cowling, R., Stock, W., 1997. Soil factors and competition as determinants of the distribution of six fynbos Proteaceae species. *Oikos*, 394-406.

Ritchie, A.L., Krauss, S.L., 2012. A genetic assessment of ecological restoration success in *Banksia attenuata*. *Restoration ecology* 20, 441-449.

Ritland, K., 2002. Extensions of models for the estimation of mating systems using n independent loci. *Heredity*, 221-228.

Rodger, J., van Kleunen, M., Johnson, S.D., 2013. Pollinators, mates and Allee effects: the importance of self-pollination for fecundity in an invasive lily. *Functional Ecology* 27, 1023-1033

Rousset, F., 2000. Genetic differentiation between individuals. *Journal of Evolutionary Biology* 13, 58-62

RStudioTeam, 2015. RStudio: integrated development for R. RStudio, Inc., Boston, MA URL http://www. rstudio. com.

Schlötterer, C., 2004. The evolution of molecular markers—just a matter of fashion? *Nature reviews genetics* 5, 63.

Schmid, B., Nottebrock, H., Esler, K., Pagel, J., Pauw, A., Böhning-Gaese, K., Schurr, F., Schleuning, M., 2016. Responses of nectar-feeding birds to floral resources at multiple spatial scales. *Ecography* 39, 619-629.

Schmidt-Adam, G., Murray, B.G. and Young, A.G., 2009. The relative importance of birds and bees in the pollination of *Metrosideros excelsa* (Myrtaceae). *Austral Ecology 34*, 490- 498.

Schmidt-Adam, G., Murray, B.G., Young, A.G., 2009. The relative importance of birds and bees in the pollination of *Metrosideros excelsa* (Myrtaceae). *Austral Ecology* 34(5), 490-498

Schmidt-Adam, G., Young, A.G., Murray, B.G., 2000. Low outcrossing rates and shift in pollinators in New Zealand pohutukawa *(Metrosideros excelsa;* Myrtaceae*). American Journal of Botany* 87, 1265-1271.

Schnitzler, J., Barraclough, T.G., Boatwright, J.S., Goldblatt, P., Manning, J.C., Powell, M.P., Rebelo, T. and Savolainen, V., 2011. Causes of plant diversification in the Cape biodiversity hotspot of South Africa. *Systematic Biology* 60(3), 343-357.

Schoen, D.J., Brown, A., 1991. Intraspecific variation in population gene diversity and effective population size correlates with the mating system in plants. *Proceedings of the National Academy of Sciences* 88, 4494-4497.

Schradin, C. and Pillay, N., 2006. Female striped mice (Rhabdomys pumilio) change their home ranges in response to seasonal variation in food availability. *Behavioral Ecology* 17(3), 452-458.

Schurr, F.M., Esler, K.J., Slingsby, J.A., Allsopp, N., 2012. Fynbos Proteaceae as model organisms for biodiversity research and conservation. *South African Journal of Science* 108, 12-16.

Shuttleworth, A. and Johnson, S.D., 2006. Specialized pollination by large spider-hunting wasps and self-incompatibility in the African milkweed *Pachycarpus asperifolius*. *International Journal of Plant Sciences* 167(6): 1177-1186.

Silva, N.F. and Goring, D.R., 2001. Mechanisms of self-incompatibility in flowering plants. *Cellular and Molecular Life Sciences* 58(14), 1988-2007.

Slatkin, M., 1987. Gene flow and the geographic structure of natural populations. *Science* 236, 787-792.

Smouse, P.E., Peakall, R., 1999. Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. *Heredity* 82, 561-573.

Smouse, P.E., Peakall, R., Gonzales, E., 2008. A heterogeneity test for fine-scale genetic structure. *Molecular Ecology* 17, 3389-3400.

Sokal, R.R. and Wartenberg, D.E., 1983. A test of spatial autocorrelation analysis using an isolation-by-distance model. *Genetics* 105(1), 219-237.

Spoladore, J., Mansano, V.D., Lemes, M.R., Freitas, L.C., & Sebbenn, A.M., 2017. Genetic conservation of small populations of the endemic tree *Swartzia glazioviana* (Taub.) Glaz. (Leguminosae) in the Atlantic Forest. *Conservation Genetics* 18:1105-1117.

Stebbins, G.L., 1957. Self-fertilization and population variability in the higher plants. *The American Naturalist* 91, 337-354.

Stebbins, G.L., 1970. Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. *Annual review of Ecology and Systematics* 1, 307-326.

Steenhuisen, S.-L., Balmer, A., Zoeller, K., Kuhn, N., Midgley, J., Hansen, D. and Johnson, S.D., 2015. Carnivorous mammals feed on nectar of *Protea* species (Proteaceae) in South Africa and likely contribute to their pollination. *African Journal of Ecology 53*,602-605.

Steenhuisen, S.-L., Johnson, S.D., 2012a. The influence of pollinators and seed predation on seed production in dwarf grassland *Protea* “sugarbushes” (Proteaceae). *South African Journal of Botany* 79, 77-83.

Steenhuisen, S.-L., Johnson, S.D., 2012b. Evidence for autonomous selfing in grassland *Protea* species (Proteaceae). *Botanical Journal of the Linnean Society* 169, 433-446.

Steenhuisen, S.-L., Johnson, S.D., 2012c. Evidence for beetle pollination in the African grassland sugarbushes (*Protea*: Proteaceae). *Plant Systematics and Evolution* 298, 857- 869.

Steenhuisen, S.-L., Raguso, R., Johnson, S., 2012a. Floral scent in bird-and beetle- pollinated Protea species (Proteaceae): Chemistry, emission rates and function. *Phytochemistry* 84, 78-87.

Steenhuisen, S.-L., van der Bank, H., D Johnson, S., 2012b. The relative contributions of insect and bird pollinators to outcrossing in an African *Protea* (Proteaceae). *American Journal of Botany* 99, 1104-1111.

Stiles, F.G., 1978. Ecological and evolutionary implications of bird pollination. *American Zoologist* 18, 715-727.

Tautz, D., 1989. Hypervariabflity of simple sequences as a general source for polymorphic DNA markers. *Nucleic acids research* 17, 6463-6471.

Thomson, J.A., 2002. An improved non-cryogenic transport and storage preservative facilitating DNA extraction from ‘difficult’ plants collected at remote sites. *Telopea* 9, 755- 760.

Valente, L.M., Reeves, G., Schnitzler, J., Mason, I.P., Fay, M.F., Rebelo, T.G., Chase, M.W., Barraclough, T.G., 2010. Diversification of the African genus *Protea* (Proteaceae) in the Cape biodiversity hotspot and beyond: equal rates in different biomes. *Evolution* 64, 745- 760.

van der Niet, T and Johnson, S.D., 2009. Patterns of plant speciation in the Cape floristic region. *Molecular Phylogenetic Evolution* 51, 85-93.

van der Niet, T., Johnson, S.D., 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution* 27, 353-361.

Vaughton, G., 1990. Seasonal variation in honeyeater foraging behaviour, infloresence abundance and fruit set in *Banksia spinulosa* (Proteaceae). *Australian Journal of Ecology* 15, 109-116.

Vaughton, G., Carthew, S.M., 1993. Evidence for selective fruit abortion in *Banksia spinulosa* (Proteaceae). *Biological Journal of the Linnean Society* 50, 35-46.

Vekemans, X., Hardy, O.J., 2004. New insights from fine-scale spatial genetic structure analyses in plant populations. *Molecular Ecology* 13, 921-935.

Volis, S., Ormanbekova, D., Shulgina, I., 2016. Fine-scale spatial genetic structure in predominantly selfing plants with limited seed dispersal: A rule or exception? *Plant diversity* 38, 59-64.

Wallace, D.D., O'Dowd, D.J., 1989. The effect of nutrients and inflorescence damage by insects on fruit-set by *Banksia spinulosa*. *Oecologia* 79, 482-488.

Ward, M., Johnson, S.D., 2005. Pollen limitation and demographic structure in small fragmented populations of *Brunsvigia radulosa* (Amaryllidaceae). *Oikos* 108, 253-262.

Waser, N.M., Ollerton, J., 2006. Plant-pollinator interactions: from specialization to generalization. University of Chicago Press.

Whelan, R.J., Ayre, D.J., Beynon, F.M., 2009. The birds and the bees: pollinator behaviour and variation in the mating system of the rare shrub *Grevillea macleayana*. *Annals of Botany* 103, 1395-1401.

Whitehead, M.R., Lanfear, R., Mitchell, R.J., Karron, J.D., 2018. Plant mating systems often vary widely among populations. *Frontiers in Ecology and Evolution* 6, 38.

Wiens, D., Rourke, J.P., 1978. Rodent pollination in southern African *Protea* spp. *Nature* 276, 71.

Wiens, D., Rourke, J.P., Casper, B.B., Rickart, E.A., LaPine, T.R., Peterson, C.J., Channing, A., 1983. Nonflying mammal pollination of southern African proteas: a non-coevolved system. *Annals of the Missouri Botanical Garden* 70(1), 1-31.

Wilcock, C., Neiland, R., 2002. Pollination failure in plants: why it happens and when it matters. *Trends in plant science* 7, 270-277.

Willi, Y., 2009. Evolution towards self-compatibility when mates are limited. *Journal of Evolutionary biology* 22, 1967-1973.

Wright, M., 1994. Unpredictable seed-set: a defence mechanism against seed-eating insects in *Protea* species (Proteaceae). *Oecologia* 99, 397-400.

Wright, M., Giliomee, J., 1991. Insect and bird pollination of *Protea* species in the Western Cape-further data. *South African Journal of Science* 87, 214-215.

Wright, S., 1969. Evolution and the genetics of populations, volume 2: The theory of gene frequencies. University of Chicago Press, Chicago.

Wright, S.I., Kalisz, S., Slotte, T., 2013. Evolutionary consequences of self-fertilization in plants. *Proceedings of the Royal Society. Series B: Biological Sciences* 280.

Zardoya, R., Vollmer, D.M., Craddock, C., Streelman, J.T., Karl, S., Meyer, A., 1996. Evolutionary conservation of microsatellite flanking regions and their use in resolving the phylogeny of cichlid fishes (Pisces: Perciformes). *Proceedings of the Royal Society of London. Series B: Biological Sciences* 263(1376), 1589-1598.

Zhang, A.Q., Xiong, Y.Z. and Huang, S.Q., 2014. Maintenance of self-incompatibility in peripheral populations of a circumboreal woodland subshrub. *AoB Plants* 6.

Zoeller, K., Midgley, J., Johnson, S., Steenhuisen, S.-L., 2017. Floral biology and breeding systems of geoflorous *Protea* species (Proteaceae). *South African Journal of Botany* 112, 452-45

Appendix

Table 1: Locus names, primer sequences, labelling method, primer mix number and base pair (bp) range for microsattelites markers used in this study. These markers were developed by Prunier and Latimer (2010) for white proteas (*Protea* section: *Exsertae*, Proteceae).

Locus	Forward Primer	Reverse primer	Labelling method	Primer mix	Bp range
1.1.3	CCATGTCCCCTCCTCAGTATC	ATGCTCTGATGCCATGTAACTG	label	1	151-245
1.1.8	ATTGCACGGAACCTTATTT	AGGAAGCAGCACATCTTTTA	label	2	122-192
1.12.7	TGGTTCTAGGGTCAAGTTATGG	TTCAAAGGAGGTGTGGTTGC	tagged	1	194-249
1.4.2	CAAAATCCAGTTCTTTCCAC	TAGCATAAATCTGACGGTGA	label	2	117-194
1.7.1	TGTTGTGTTGCCACTGATAG	ATTCAAAGCAGGTGCATGT	label	1	85-165
2.13.3	AAGATGAAGGTCGAAGAAGTGC	TATGTTTGTCCACAGCTCCAAG	label	2	168-278
2.13.7	ACTCACCGTCACTTTGGACAG	GTTCTATTTTCCCCTCCCTTTC	label	1	199-281
2.19.7	CTTTTCATCGTGGAGACCAGAG	CGCTTGTGGGAATCTTCTAGG	tagged	2	142-183
2.22.1	GAGAACTCCTCTCCTCGGTAG	GTCCTGCTTATGGGGTCAAATC	label	2	370-455

Protocol 1: Modified genomic DNA extraction protocol using Cetyl Trimethylammonium Bromide (CTAB) buffer from Doyle and Doyle (1987) used to extract genomic DNA from *Protea* leaves (R. Prunier, pers. comm.).

One half centimeter squared (0.5 cm^2) leaf of each sample was defrosted and the CTAB was rinsed off with distillied water. Under a fume hood, each leaf sample was placed into a mortor afterwhich 800 µl pre-prepared 2 x CTAB and three microlitres (3 µl) β- mercaptoethanol were added. These leaf sections were then individually grinded until a uniform consistency. This solution was incubated at 65°C in a water bath for one hour. Five microlitres (5 µl) RNAse A (10 mg/ul) was added to each sample and then immediately incubated at 37°C for 15 min. The samples were then placed in a -20°C freezer for 10 min. Once cooled, 600 µl chloroform was added to each sample and spun in a centrifuge at maximum speed for five min. The top 500 µl aqueous solution of each sampled was then removed and placed in an uncontaminated 1.5 µl microcentrifuge tube. This process is repeated once more. To each sample, 300 µl isopropanol, 36 µl 7.5M Ammonium Acetate and 300 µl 99 % Ethanol (EtOh) was added. To precipitate the DNA, the samples were stored at -20°C for atleast 12 hours. After 12 hours, the samples were centrifuged for 3 min at maximum speed and the supernantant for each sampled was drained. One mililitre (1 ml) 70 % ethanol was then added to each drained tube. To wash the DNA, the white DNA pellet was resuspended by gently flicking the side of each tube. The samples were then centrifuged for 3 min at maximum speed. The 70 % ethanol was then drained for each tube again and the process was repeated once more. Once the ethanol was drained a second time, the sample tubes were left open to dry for atleast 12 hours. When a tube was completey dry, 60 µl pre-prepared 1 x TE buffer was added to resuspend the DNA pellet.

Protocol 2: Contentrated Cetyl Trimethylammonium Bromide (CTAB) solution for the preservation of plant leaf DNA (Thomson, 2002).

To make one one litre CTAB, place one litre of distilled water into an autoclaved beaker and heat on a hot plate until approximately 80-90°C. A magnetic stirrer should also be placed and actively stirring inside the beaker. While the water was heating, 30 g of CTAB was slowly added to prevent aggregation. Once the CTAB was completely dissolved, 400 g of sodium chloride was added to the CTAB solution by dissolving 50-100 g at a time.

Protocol 3: Reaction mixture protocol to amplify DNA using the multiplex PCR method as stipulated in the QIAGEN multiplex PCR kit.

Three microlitres (3 µl) of RNAse free water was placed into a half microlitre (0.5 µl) reaction tube. Five microlitres of the 2 x QIAGEN mastermix (containing $MgCl_2$, dNTPs and HotStarTaq DNA polymerase) was added to the water inside the reaction tube. To add the primers, one microlitre of a specific primer mix (one or two) was added to the solution. Primer mixes were created by adding four microlitres from forward and reverse primers of each specified marker (Table 1, Appendix) add supplementing it with molecular grade water (H_2O) to make 200 µl of primer mix. To make the solution of each reaction 10 µl, one microliter of genomic DNA was added to the solution inside the reaction tube.

Table 2: The genetic diversity indices for each locus in six *Protea* species generated in GenAlex version 6.5.1b2 and Arlequin where *N* is the number of samples, A_r is the allelic richness, A_e is the number of effective alleles, H_o is the observed heterozygosity, H_e is the expected heterozygosity and F_{is} is the inbreeding co-efficient for each locus. Loci that are in bold represent those with more than 10 % missing data and were excluded from the analyses.

Species	Locus	*N*	A_r	A_e	H_o	H_e	F_{is}
P. roupelliae	1.7.1_short	19	1	1	0.00	0.000	0.00
	1.7.1_long	19	2	1	0.05	0.053	-0.03
	1.12.7	19	3	1	0.26	0.457	0.41
	1.4.2	19	11	7	0.63	0.90	0.28
	1.1.3	0	0	0	0.00	0.00	0.00
	2.13.7	0	0	0	0.00	0.00	0.00
	2.22.1	20	13	8	0.90	0.90	-0.03
	1.1.8	20	5	2	0.55	0.66	0.14
	2.13.3	20	16	1	0.75	0.92	0.17
	2.19.7	20	12	5	0.85	0.84	-0.03
P. simplex	1.7.1_short	20	6	2	0.70	0.68	-0.06
	1.7.1_long	15	19	13	0.70	0.95	0.06
	1.12.7	17	7	4	0.82	0.74	-0.15
	1.4.2.	0	0	0	0.00	0.00	0.00
	1.1.3	18	16	10	0.67	0.92	0.26
	2.13.7	19	2	1	0.05	0.05	-0.03
	2.22.1	18	15	10	0.67	0.93	0.26

	1.1.8	18	9	2	0.67	0.59	-0.17
	2.13.3	19	20	17	0.84	0.97	0.11
	2.19.7	20	8	4	0.80	0.80	-0.03
P. humiflora	1.7.1_short	22	4	2	0.36	0.51	0.27
	1.7.1_long	0	0	0	0.00	0.00	0.00
	1.12.7	18	8	3	0.67	0.72	0.044
	1.4.2	19	9	5	0.84	0.82	-0.052
	1.1.3	20	1	1	0.00	0.00	0.00
	2.13.7	21	13	8	0.91	0.90	-0.04
	2.22.1	0	0	0	0.00	0.00	0.00
	1.1.8	13	10	7	0.23	0.89	0.73
	2.13.3	18	5	2	0.44	0.61	0.25
	2.19.7	20	3	2	0.45	0.40	-0.17
P. caffra	1.7.1_short	19	5	2	0.42	0.41	-0.06
	1.7.1_long	19	17	8	0.95	0.90	-0.08
	1.12.7	19	4	2	0.42	0.55	0.22
	1.4.2	14	14	8	0.71	0.91	0.18
	1.1.3	19	15	7	0.79	0.88	0.08
	2.13.7	19	2	1	0.11	0.10	-0.06
	2.22.1	19	22	16	0.95	0.96	-0.01
	1.1.8	19	4	1	0.16	0.15	-0.06
	2.13.3	19	20	16	0.95	0.96	-0.01

	2.19.7	19	5	3	0.90	0.72	-0.28
P. laurifolia	1.7.1_short	17	5	3	0.88	0.72	-0.26
	1.7.1_long	17	3	2	0.18	0.35	0.48
	1.12.7	18	3	1	0.33	0.30	-0.14
	1.4.2	15	9	5	0.67	0.83	0.17
	1.1.3	18	5	2	0.39	0.35	-0.16
	2.13.7	18	8	3	0.56	0.69	0.18
	2.22.1	18	15	9	0.94	0.92	-0.06
	1.1.8	10	4	2	0.20	0.50	0.58
	2.13.3	18	13	6	0.50	0.87	0.41
	2.19.7	18	10	5	0.78	0.80	0.00
P. amplexicaulis	1.7.1_short	20	13	8	0.70	0.89	0.20
	1.7.1_long	0	0	0	0.00	0.00	0.00
	1.12.7	20	8	4	0.95	0.77	-0.28
	1.4.2	20	13	5	0.80	0.833	0.02
	1.1.3	5	2	1	0.00	0.36	1.00
	2.13.7	20	5	2	0.45	0.57	0.18
	2.22.1	14	11	9	0.21	0.92	0.76
	1.1.8	0	0	0	0.00	0.00	0.00
	2.13.3	20	16	11	0.75	0.93	0.17
	2.19.7	20	1	1	0.00	0.00	0.00

Table 3: The loci that illustrated different offspring alleles than the alleles of the adult individuals of four *Protea* species calculated with a multilocus mating system program, MLTR version 3.2 (Ritland, 2002). The alleles were entirely novel and were excluded from estimating outcrossing rates of these species.

Species	Adult	Offspring	Locus
P. amplexicaulis	18	1	2.19.7
	2	4	2.19.7
	4	1	2.13.3
	4	1	2.19.7
	4	2	2.13.3
	4	2	2.19.7
	4	3	2.13.3
	4	3	2.19.7
	6	1	2.19.7
	6	2	2.13.3
	6	2	2.19.7
	6	3	2.13.3
	6	1	2.19.7
	7	1	2.13.3
	7	1	2.19.7
	5	1	2.13.3
	5	1	2.19.7
	5	2	2.13.3

	5	2	2.19.7
	5	3	2.13.3
	5	3	2.19.7
	11	1	2.13.3
	11	1	2.19.7
	11	2	2.13.3
	11	2	2.19.7
	8	1	2.13.3
	8	1	2.19.7
	9	1	2.13.3
	9	1	2.19.7
P. caffra	3	4	2.13.3
	6	1	2.13.7
	6	1	1.1.8
	3	5	2.13.3
P.laurifolia	5	6	2.13.7
P. simplex	13	All	1.7.1_long
	19	All	1.4.2
	3	All	2.13.7

Table 4: The genetic diversity indices for each locus in three subpopulations of two *Protea* species generated in GenAlex version 6.5.1b2 and Arlequin where *N* is the number of samples, A_r is the allelic richness, H_o is the observed heterozygosity, H_e is the expected heterozygosity and F_{is} is the inbreeding co-efficient for each locus. Loci that are in bold represent those with more than 10 % missing data and were excluded from the analyses.

Species	Population	Locus	*N*	A_r	H_o	H_e	F_{is}
P. laurifolia	One	1.7.1_short	17	5	0.88	0.72	-0.26
		1.7.1_long	17	3	0.18	0.35	0.48
		1.12.7	18	3	0.33	0.30	-0.14
		1.1.3	18	5	0.39	0.35	-0.16
		2.13.7	18	8	0.56	0.69	0.18
		1.4.2	15	9	0.67	0.83	0.17
		2.22.1	18	15	0.94	0.92	-0.06
		1.1.8	10	4	0.20	0.50	0.58
		2.13.3	18	13	0.50	0.87	0.41
		2.19.7	18	10	0.78	0.80	0.00
	Two	1.7.1_short	20	4	0.45	0.42	-0.09
		1.7.1_long	20	2	0.00	0.19	1.00
		1.12.7	20	4	0.20	0.28	0.26
		1.1.3	20	4	0.55	0.48	-0.17
		2.13.7	19	4	0.32	0.76	0.57
		1.4.2	12	11	0.83	0.91	0.05
		2.22.1	18	13	0.83	0.91	0.06
		1.1.8	14	6	0.36	0.44	0.15

		2.13.3	19	15	0.58	0.94	0.36
		2.19.7	18	11	0.61	0.83	0.24
	Three	1.7.1_short	19	4	0.42	0.65	0.34
		1.7.1_long	19	3	0.16	0.24	0.33
		1.12.7	19	4	0.37	0.33	-0.14
		1.1.3	19	2	0.37	0.31	-0.23
		2.13.7	15	7	0.60	0.84	0.26
		1.4.2	16	14	0.50	0.94	0.45
		2.22.1	14	14	0.93	0.93	-0.04
		1.1.8	8	6	0.75	0.68	-0.19
		2.13.3	17	15	0.82	0.92	0.08
		2.19.7	17	10	0.71	0.83	0.13
P. amplexicaulis	One	1.7.1_short	20	13	0.70	0.89	0.20
		1.7.1_long	0	0	0.00	0.00	0.00
		1.12.7	20	8	0.95	0.77	-0.27
		1.1.3	5	2	0.00	0.36	1.00
		2.13.7	20	5	0.45	0.57	0.18
		1.4.2	20	13	0.80	0.83	0.02
		2.22.1	14	11	0.21	0.92	0.76
		1.1.8	0	0	0.00	0.00	0.00
		2.13.3	20	16	0.75	0.93	0.17
		2.19.7	20	1	0.00	0.00	0.00

	Two	1.7.1_short	19	17	0.90	0.94	0.02
		1.7.1_long	0	0	0.00	0.00	0.00
		1.12.7	19	9.	0.63	0.85	0.23
		1.1.3	0	0	0.00	0	0.00
		2.13.7	19	5	0.47	0.58	0.16
		1.4.2	17	14	1.00	0.91	-0.14
		2.22.1	9	10	0.56	0.88	0.33
		1.1.8	0	0	0.00	0.00	0.00
		2.13.3	18	16	0.89	0.94	0.02
		2.19.7	18	1	0.00	0.00	0.00
	Three	1.7.1_short	15	11	0.87	0.86	-0.05
		1.7.1_long	6	4	0.17	0.80	0.77
		1.12.7	17	7	0.82	0.83	-0.02
		1.1.3	16	3	0.75	0.55	-0.41
		2.13.7	13	8	1.00	0.80	-0.30
		1.4.2	11	9	0.82	0.90	0.05
		2.22.1	3	2	0.33	0.33	-0.20
		1.1.8	13	5	0.32	0.41	0.22
		2.13.3	15	10	0.93	0.87	-0.11
		2.19.7	17	1	0	0	0