2020

Conospermum undulatum: insights into population genetics and pollination ecology of a threatened species

Nicola Delnevo
Edith Cowan University

Edith Cowan University

Copyright Warning

USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

Conospermum undulatum: insights into population genetics and pollination ecology of a threatened species

Nicola Delnevo

Thesis submitted for the degree of Doctor of Philosophy in the School of Science

Edith Cowan University

November 2020

"…*but Nature more*"

–Lord Byron

Abstract

Fragmentation of natural vegetation is currently one of the largest threats to biodiversity. Within the southwest Australia global biodiversity hotspot, the Swan Coastal Plain was historically cleared for agriculture and forestry and is now experiencing extensive land clearing for urbanisation. The wavy-leaved smokebush *Conospermum undulatum* is a rare species endemic to the Swan Coastal Plain, and its future persistence is threatened by urban expansion.

Throughout this research, I investigated the pollination ecology of this species and found a specific association between *C. undulatum* and native bees for pollination. I also demonstrated that *C. undulatum* has evolved pollen with resistance to the usually negative effect of ant secretions on pollen grains, with ants providing effective pollination services to this threatened species. Native pollinators were drastically reduced in small populations, and urbanisation limited the movement of pollen across built-up areas surrounding remnant bushland. This lack of both pollinators and inter-population pollen flow is severely limiting the production of healthy seeds in smaller populations.

I then performed molecular investigations combined with an ecological characterisation of the recently fragmented distribution range of *C. undulatum* to quantify the genetic structure and levels of genetic diversity across the entire distribution of the species. Despite the current intense fragmentation, I found levels of genetic diversity similar across populations and a weak spatial genetic structure. Since habitat fragmentation is recent and many adult plants are likely to be several decades old, they mainly reflect pre-fragmentation conditions. Therefore, the detailed characterization of fragmentation over time has shown how the low levels of genetic fixation can be attributed to pervasive gene flow through the pre-fragmented landscape, which mostly influenced the current adult cohort.

Early signals of the negative effects of habitat fragmentation were found during my study of contemporary gene flow through the paternity assignment of seedlings sampled at the end of the 2017 flowering season. Although gametes of *C. undulatum* could flow unimpeded through large expanses of unfragmented bushland, inter-population pollen flow was non-existent between fragments surrounded by built-up areas. This study supports the need for an understanding of contemporary mating patterns to detect early signals of gene flow failure in fragmented remnants.

Lastly, I found evidence for hybridisation occurring at the edge of the distribution of *C. undulatum* between this rare and threatened plant and two other related species. This adds to the threats posed by habitat fragmentation to the conservation of *C. undulatum*.

My research highlighted the importance of native pollinators for plants that coevolved with them and adds to the limited research on the effect of habitat fragmentation on native plants that rely exclusively on native insects for pollination. Such pollinators appeared unable to maintain an adequate inter- population pollen flow in heavily fragmented landscapes. Therefore, the often negative effects of habitat fragmentation can be exacerbated in small and isolated populations of plants that rely on species-specific pollinators for sexual reproduction. Outcomes of my research will inform recovery plans to enhance the future persistence of *C. undulatum* over the long term.

Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

i. incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;

ii. contain any material previously published or written by another person except where due reference is made in the text of this thesis; or

iii. contain any defamatory material.

Nicola Delnevo

19-11-2020

Acknowledgments

Countless people provided valuable support throughout my PhD. I sincerely appreciate and would like to thank all those who have contributed directly or indirectly to completing this work.

To Doctor Eddie van Etten and Professor Will Stock, for your supervisory style throughout the PhD and your passion for all the aspects of ecology. Your guidance is helping me shape a career beyond this thesis.

To Doctor Margaret Byrne, for your commitment to being involved in all aspects of this research. Your advice and experience have been truly invaluable.

To Doctor David Field, despite being the latest to join this journey, your passion and guidance were exactly what I needed through the last steps of my research. Your advice and support have been irreplaceable.

To Bronwyn Macdonald and Shelley McArthur, for your much-needed help with molecular work and for providing such a great place to work. You made spending months in the lab one of the best parts of this journey.

To Doctors Alessandro Petraglia, Michele Carbognani, and Andrea Piotti, for always supporting and believing in me. Countless “5 minutes” online meetings have exceeded the 2 hours mark.

To my colleagues at the School of Science, with emphasis on the postgraduate students past and present. You have made this entire experience enjoyable.
I am grateful to Edith Cowan University and the Department of Biodiversity, Conservation, and Attractions of Western Australia for providing me with an Industry Engagement Scholarship and the School of Science and the Centre for Ecosystem Management for research and conference funds. This research was also supported by a grant from the Holsworth Wildlife Research Endowment.

To all my closest friends, thank you for continuously harassing me despite my persistent “I’m really busy this week” response over the past three years.

Specifically, I would like to acknowledge my Mum and Dad for always supporting me and being blindly proud of my work despite being on the other side of the world. Your interest in my research, your happiness and love have permeated my day-to-day life and have helped carry this thesis to its completion. My brother for always being proud of my work despite not remembering what it is I am doing. My wife Giada who has loved, encouraged, supported, and believed in me every step of the way. It has been quite a journey. Thank you for being part of my life. Fabrizio and Cattia for their incredible support through all these years. Connor Gorham, Connor Campbell, Oscar Serrano, Anna Lafratta, Viena Puigcorbé, Nicole Said, and Cristian Salinas for your support inside and outside this world of academia. Thank you for ensuring I adhere some balance between work and life.

Thank you all.

Statement of contribution of others

Research funding

Department of Biodiversity, Conservation and Attractions of Western Australia for top-up scholarship and operating funds

Holsworth Wildlife Research Endowment: *Conospermum undulatum: insights into genetics and ecology of an endangered species*

Graduate Research School, ECU (scholarship)

School of Science, Edith Cowan University

Supervision

Dr Eddie van Etten

Prof Will Stock

Dr Margaret Byrne

Dr David Field

Field assistance

Dr Eddie van Etten

Dr Alessandro Petraglia

Nicola Clemente

Luna Fogu

Evelina Pavarani

Giada Zanantoni

Cristian Salinas

Co-authors

Dr Eddie van Etten

Prof Will Stock

Dr Margaret Byrne

Dr Andrea Piotti

Dr Alessandro Petraglia

Dr Michele Carbognani

Nicola Clemente

Luna Fogu

Evelina Pavarani

The research described in this thesis was my original idea, I led the development of the questions and hypotheses at all stages, undertook the fieldwork and analyses, and completed the writing. The above list of co-authors contributed in one or more of the following ways to the thesis components published in journals: data collection, analyses, or development and

editing of manuscripts. I was the lead author in all cases and my contributions are detailed at the end of the thesis in the Co-author statements section.

Publications arising from this research

The thesis is presented in the 'thesis with publications' format. Chapters 2, 3, 4, and Appendices G and F are presented as reformatted copies of the published articles; hence there is some repetition of site descriptions and methodology throughout. Populations codes have been changed compared published articles to increase consistency throughout the thesis. The acknowledgements, but not the abstracts, have been reproduced for each paper, although I present a single reference list for the entire thesis. The original abstracts can be found in the '*Copies of original publications'* section. Chapters 2, 3, 4, and Appendices G and F contain material adapted from published papers of which I am the lead author. I warrant that I have obtained, where necessary, permission to use in this thesis any my own published work in which the copyright is held by another party.

Chapter 2:

Delnevo N, van Etten EJ, Byrne M, Stock WD. 2019. *Floral display and habitat fragmentation: effects on the reproductive success of the threatened mass-flowering Conospermum undulatum (Proteaceae)*. Ecology and Evolution, 9: 11494–11503.

Chapter 3:

Delnevo N, van Etten EJ, Byrne M, Petraglia A, Carbognani M, Stock WD. 2020. *Habitat fragmentation restricts insect pollinators and pollen quality in a threatened Proteaceae species*. Biological Conservation, 252.

Chapter 4:

Delnevo N, van Etten EJ, Clemente N, Fogu L, Pavarani E, Byrne M, Stock WD. 2020. *Pollen adaptation to ant pollination – a case study from the Proteaceae*. Annals of Botany, 126: 377–386.

Appendix G:

Delnevo N, Piotti A, van Etten EJ, Stock WD, Byrne M. 2019. *Isolation, characterization, and cross-amplification of 20 microsatellite markers for Conospermum undulatum (Proteaceae)*. Applications in Plant Sciences, 7: e11283.

Appendix F:

Delnevo N, van Etten EJ. 2019. *Tales of the unexpected – ant pollination mutualism*. Frontiers in Ecology and the Environment, 17(10): 558.

Table of Contents

List of Figures

List of Tables

Chapter 1 - General introduction and study site description

The anthropogenic loss and fragmentation of natural habitats are widespread phenomena in terrestrial ecosystems (Sanderson et al., 2002; Aguilar et al., 2006; Ellis et al., 2010). The rate at which natural ecosystems have been converted to urban and agricultural use has been increasing during the last 60 years and now is at unprecedented levels. As of today, 40% of Earth's ice-free land area is being directly exploited by humans, and an additional 37% is surrounded by human-modified areas (Ellis et al., 2010; Winfree et al., 2011), making land use change as one of the most important drivers affecting biodiversity (Haddad et al., 2015; Sala et al., 2000). It is widely known that reproduction by seeds (RbS) has a key role for fitness, migration, adaptation and, ultimately, population persistence of flowering plant species (Fenner & Thompson, 2005), which are crucial component of most terrestrial ecosystems. Indeed, the rich biodiversity of such systems relies on these plants and their interactions with pollinators (Ollerton et al, 2006). However, as a consequence of land use change, many plant and pollinator populations are declining (Sánchez-Bayo & Wyckhuys, 2019) and mutualistic plant-pollinator interactions are frequently disrupted (Thomann et al., 2013), which can have direct effects on plant population viability. Therefore, it is paramount to understand the RbS processes at individual plant and population levels. At the level of individual plants, key phases for RbS are pollination and germination, whereas at the population level, processes such as gene flow may affect genetic diversity within or between populations.

Fragmentation of natural habitats leads to a change in the sizes of remnant plant populations and their spatial arrangement (i.e. increased isolation) (Young et al., 1996; Aguilar et al., 2008). The resulting small and isolated plant populations often present reduced fitness compared to larger populations, and this could lead to reduced reproductive output and increased seedling mortality (Holmes et al., 2008). Several ecological and genetic aspects may be involved. These include altered pollinator presence and behaviour (Ågren, 1996; Aguilar et al., 2006), pollen limitation, especially in self-incompatible species (e.g. Wagenius & Lyon, 2010; Delmas et al., 2015;), and expression of inbreeding depression because of reduced gene flow between unrelated individuals (e.g. Fischer et al., 2003; Willi et al., 2005).

This worldwide fragmentation process has not spared the South-West Australian Floristic Region (SWAFR; Hopper & Gioia, 2004), which encompasses an exceptional concentration of endemic species undergoing major loss of habitat, and it is recognised as a global 'biodiversity hotspot' (Myers et al., 2000; Mittermeier et al., 2004). Indeed, hotspots identify priority areas to preserve our natural heritage and its evolutionary potential (Mittermeier et al., 2011). The SWAFR is particularly noted for its floristic diversity, especially among the medium-sized shrubs of the Proteaceae, Myrtaceae and Ericaceae (Hopper & Gioia, 2004; Phillips et al., 2010). The ecological integrity of this region has been compromised by land clearing and fragmentation, over-exploitation, and introduction of alien species. Within the SWAFR, at a finer spatial scale, the Swan Coastal Plain bio-region was historically cleared for agriculture and forestry (mostly pine plantations), and is now experiencing extensive land clearing for urbanisation (Wardell-Johnson et al., 2016). Urbanisation is centred around Perth, the capital city of Western Australia, which has more than doubled in area and population since the 1970s, with impacts readily visible on the biodiversity of the region (e.g. Davis et al., 2013; Heterick et al, 2013). Urbanisation has reduced natural and semi-natural vegetation on the Swan Coastal Plain to 34.7% of its original extent, of which only the 10% occurs in protected areas (Wardell-Johnson et al., 2016). What remains is usually degraded and under continuous pressures (e.g. inappropriate fire regimes, further urban development, weed invasion, feral animals). Moreover, the region presents a naturally fragmented flora due to both the effects of distinct soil and landform patterning as a result of an old and stable landscape, and oscillating historical climatic conditions (Hopper, 1979; Byrne et al., 2007). The combination of these factors has resulted in an increasingly high number of endemic flowering plant species listed as 'threatened' on both local and international conservation lists (https://www.iucnredlist.org/; https://www.environment.gov.au/biodiversity/threatened/species; W.A. Government Gazette, 2018).

Conospermum (Proteaceae) is an insect-pollinated plant genus endemic to Australia with its centre of distribution being the south-west corner of Western Australia. This genus includes 53 species (Bennett, 1995) and is of increasing conservation concern, with four taxa already listed among the threatened flora of Western Australia (W.A. Government Gazette, 2018). Plants of many *Conospermum* species have white woolly flowers, which looks like smoke when seen *en-masse*, therefore, they are often referred to as 'smokebush'. Like all Proteaceae, the perianth has four tepals, although in *Conospermum* the tepals are of unequal

size, with the upper one being much larger than the other three (Bennett, 1995; Douglas, 1997). The flowers of most *Conospermum* species are small and possess an active pollination mechanism. The style is bent, and the flower opens in a state of tension (Fig. 1.1) (Douglas, 1997; Stone et al., 2006). When a visiting insect applies pressure with its mouthparts to the base of the style, the style quickly flicks away from the fertile anthers to strike the visitor. The moist cup-shaped stigma is forced down onto the pollinator and thereby picks up pollen carried by the insect; at the same time the fertile anthers dehisce explosively, casting new pollen onto the visitor (Morrison et al., 1994; Stone et al., 2006). *Conospermum* flowers, therefore, need to be visited by insects carrying a suitable pollen load from previous floral visits in order for pollination to occur, leading to development of fruits.

In particular, *Conospermum undulatum* is a lignotuberous plant that grows as an erect, compact shrub up to 1.5 m tall with distinctive fibrous, longitudinally fissured stems. The glabrous leaves are to 12 cm long and 3.8 cm wide with a characteristic undulating margin. It is a long-lived resprouter species, which means that individual shrubs can resprout from semi-subterranean woody lignotubers after disturbance such as fire or mechanical damage. Although younger plants are single stemmed, they start to form multiple stems when regenerating after disturbance or responding to stress (e.g., drought); thus, the number of stems could be a useful proxy for plant age. This species relies on massive population-level floral display for attracting pollinators (i.e. mass-flowering species; Heinrich & Raven, 1972). The flowering period usually ranges from late August to late October and during this reproductive season its white inflorescences dominate the (non-fragmented) landscape. *Conospermum* flowers have a sessile ovary with a single pendulous ovule (Douglas, 1997) and, after pollination has occurred, they develop cone-shaped fruits, covered with tan orange hairs, containing only one seed (i.e. achenes). While there is no conclusive evidence in the field, where a remarkable low number of seedlings and juvenile plants was observed, *C. undulatum* germination response in the lab is known to be slow and variable (Crawford A., DBCA, personal communication, 2017) and can take several months.

Conospermum undulatum is a declared rare plant species endemic to the Swan Coastal Plain; it has a naturally restricted distribution in eastern Perth region of 55 km^2 but is now threatened due to clearing for urban expansion and fragmentation. It was declared as Rare Flora under the Western Australian *Wildlife Conservation Act 1950* (now *Biodiversity Conservation Act 2016*) in 1997 and is currently listed in the threatened flora of Western Australia (W.A Government Gazette, 2018). It has been assessed as "Vulnerable" using IUCN red list criteria under the Environment Protection and Biodiversity Conservation Act

1999 (EPBC Act). It was originally considered a variety of *C. triplinervium*, which also occurs in the region but has a different habit (i.e. *C. undulatum* never develops a thick trunk and is typically multi-stemmed) and leaf morphology (Bennett, 1995). Further, genetic evidence has established *C. undulatum* as a distinct species (Close et al., 2006).

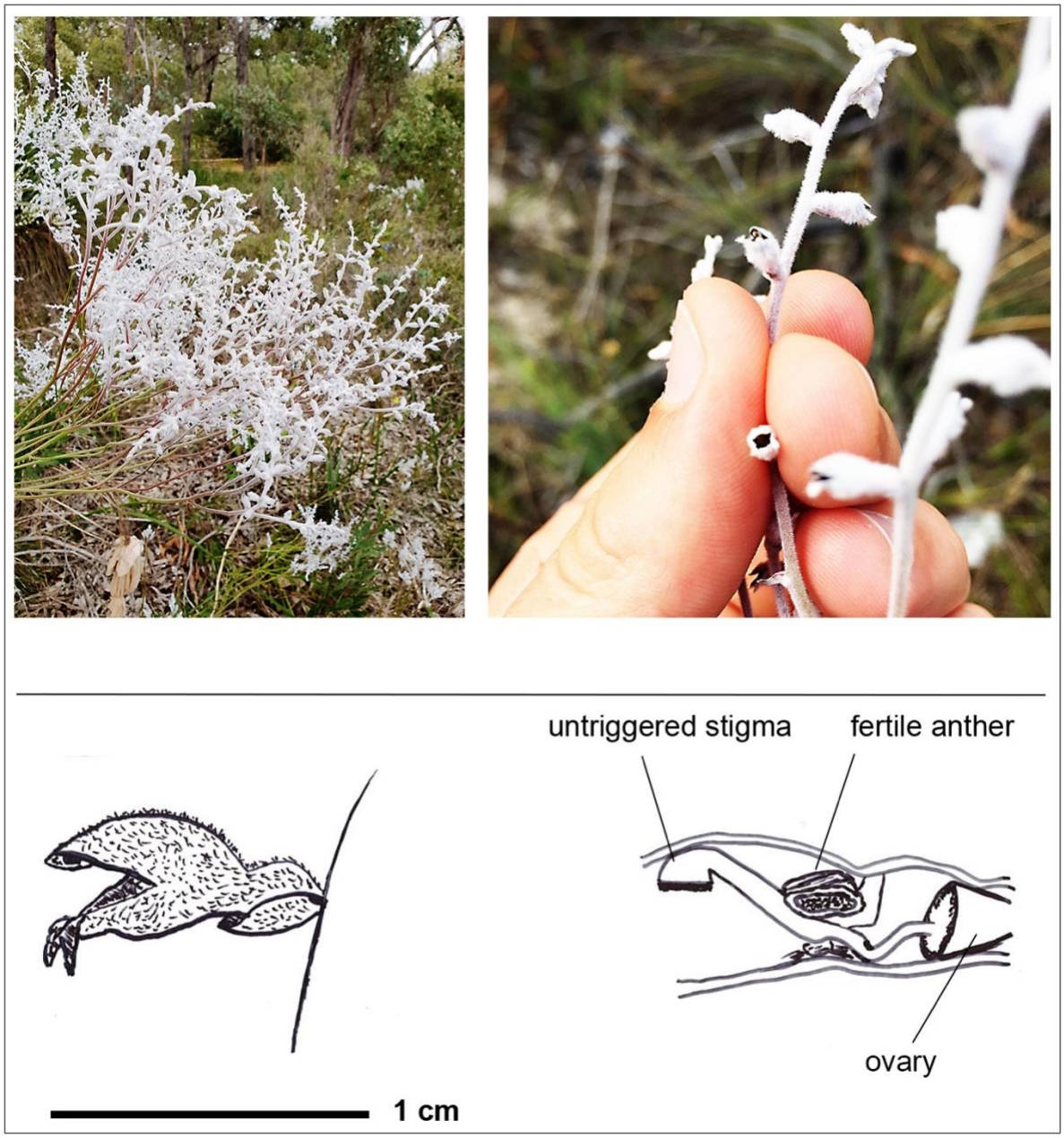


Figure 1.1. Above, *Conospermum undulatum* flowers. Below, mature flower drawing and drawing of longitudinal section of flower showing style and stigma in upper part of flower before being triggered.

In reviewing the existing literature, I found a lack of knowledge about the pollination and seed ecology of *C. undulatum*. Although Proteaceae are amongst the most widely studied Australian native plants, most research has focused on species of *Banksia*, *Grevillea* (Goldingay & Carthew, 1998), and more recently Persoonia (e.g. Rymer, Whelan, Ayre,

Weston, & Russell, 2005), with only a few studies specifically on *Conospermum* species (e.g. Stone et al., 2006; Sinclair et al., 2008). Moreover, most of these latter papers are focused on identifying the cues that stimulate seed germination (e.g. Roche et al., 1997; Tieu et al., 2007), without taking into account the pre-dispersal stages. Indeed, although germination of seeds is a crucial life-history event, it may not inform the conservation of the species if considered in isolation, because other important processes such as plant-pollinator interactions and gene flow are also likely to constrain reproduction. Therefore, a broader understanding of the 'reproduction by seed' process of *C. undulatum* was needed to fully appreciate the ecology and the genetic features of this rare species.

Pollinators and pollination

Mutualistic plant-animal interactions are a common ecological process with almost 90% of wild flowering plant species relying on animals for gamete dispersal and, ultimately, fruit and seed production (Ollerton, Winfree, & Tarrant, 2011). The majority of animals involved in such interactions are insects, accounting for the pollination of ~88% of all animal-pollinated plants (Potts et al., 2010; Thomann et al., 2013), with the remaining ~10% being vertebrate pollinators, mainly nectar-feeding birds and mammals (e.g. Letten & Midgley, 2009). Bird pollination, albeit present across the world, is particularly well represented among the south-western Australian flora (Orians & Milewski, 2007). In particular, within the south-western Australia biodiversity hotspot, Hopper and Gioia (2004) reported that 40% of threatened plants are vertebrate-pollinated, which has led researchers to focus on these unusual pollination mutualisms (e.g. Whelan & Burbidge, 1980; Paton, 2000; Van Der Kroft, Roberts, & Krauss, 2019). Despite this, the majority of south-western Australian plants, and indeed its threatened endemic species, are insect pollinated (Hopper & Gioia, 2004; Phillips et al., 2010). Australia's native bee fauna is large and diverse, and differs in major respects from bees of other continents (Houston, 2018). Many species are solitary and smaller in size compared to widespread boreal genera such as *Apis* and *Bombus*. Therefore, many of the ancient Gondwanan proteaceous plant species in south-western Australia coevolved over long periods with a range of native bees (many of them only recently described) and other invertebrates as pollen vectors. Such long periods allow for specialization to occur, resulting in the development of very specific flower morphologies and pollination systems (Phillips et al., 2010). Houston (1989) reported the identification of a south-western Australian species-group of native bees (*Leioproctus conospermi*), which

consists of three species oligolectic on flowers of *Conospermum* spp. which possess morphological adaptations to enable this remarkable pollination. He also reported other possible generalist pollinators, such as flies of the families Bombyliidae and Syrphidae, that were collected on smokebush flowers. However, at least some of the generalist flies who visit smokebush flowers may not produce any effective pollination due to the trigger mechanism of the flower. The stigma can strike with such a force that most dipterans visiting the flowers become fatally trapped, making these generalist pollinators ineffective. Very small insects can also reach the nectar without receiving or rendering pollen grains, as the anthers only release pollen when the mechanism is activated (N. Delnevo, personal observation). Further, the most abundant insect pollinator in the SWAFR, the introduced honeybee *Apis mellifera* (Apidae) (Phillips et al., 2010; Lambers, 2014), is probably not able to pollinate the small and characteristic flowers of smokebushes. Since a narrow specialisation between plants and pollinators could have important implications for conservation (Bond, 1994; Geerts & Pauw, 2012) my thesis investigated the assemblage of floral visitors associated with effective pollination in *C. undulatum* and the degree of specialisation of such pollinators within the plant community.

Besides bees, ants were observed visiting mature flowers *of C. undulatum* in the field (N. Delnevo, personal observation). Interestingly, although bees and other close relatives are recognised as important pollinators worldwide (Rico-Gray & Oliveira, 2007) pollination by ants appears to be poorly represented (de Vega & Gómez, 2014). This large difference between bees and ants as recognised effective pollinators has been attributed at least in part to the antimicrobial metapleural gland secretions of ants, which are toxic to pollen grains (i.e. the 'antibiotic hypothesis'; Beattie, Turnbull, Hough, Jobson, & Knox, 1985; Beattie, Turnbull, Knox, & Williams, 1984). The primary function of this cuticular secretion is very likely antiseptic (Poulsen et al., 2002; Stow & Beattie, 2008; Yek & Mueller, 2011), with ants spreading antibiotic secretions diffusely through the nest to prevent fungal growth and infections (Hölldobler & Wilson, 1990). However, bacteria and fungi are likely to impose stronger selection on ants for antimicrobial defences in warm, humid tropical rainforests than in deserts and Mediterranean-type habitats, such as the south-western Australia (Dutton & Frederickson, 2012). Despite many theories that have advanced the important role of ant in seed dispersal (Majer, 1982; Gove, Majer, & Dunn, 2007) little attention has been given to their possible role as pollinators in these region. Possible reduced selection for antimicrobial secretions in the dry Mediterranean climate of the Swan Coastal Plain and observations of

ants visiting flowers suggest ants may act as effective pollinators and a thorough investigation of this ant-plant association will significantly contribute to the scientific understanding of the ecological roles that ants might play in the region.

Visits by pollinators carrying an adequate amount of pollen grains are an essential requirement for the reproduction by seeds of most angiosperm species. In fact, a scarce *quantity* (i.e. amount of pollen) or *quality* (i.e. conspecific pollen with suitable viability and genetic makeup to result in viable seed) of pollen can reduce the quantity or quality of seeds; the common term used to describe this phenomenon is 'pollen limitation' (Ashman et al., 2004; Burd, 1994). Extensive reviews outline that pollen limitation is widespread among flowering plants (Ashman et al., 2004; Burd, 1994; Larson & Barrett, 2000), however, extensive habitat fragmentation may exacerbate this phenomenon and, consequently, decrease population viability (Ågren, 1996; Lennartsson, 2002; Aguilar et al., 2006; Christopher G. Eckert et al., 2010; Thomann et al., 2013). That is, the ecological impacts of pollination failure on plant populations depends on the availability of both pollinators and mates (i.e. number of genetically different conspecific individuals) (Aizen & Harder, 2007; Eckert et al., 2010; Delmas et al., 2016). Pollinator and mate limitation may occur independently (e.g. Campbell & Husband, 2007; Wagenius & Lyon, 2010), or together, for instance where habitat disturbance decreases both population size and pollinator availability (Delmas et al., 2016). As it can be easily understood, self-incompatible species are particularly susceptible to a reduction in reproductive output in response to limitation by pollen *quantity* and/or *quality* compared to self-compatible plants (Eckert et al., 2010).

Pollination ecology studies in the SWAFR are unevenly distributed within the Proteaceae (Goldingay & Carthew, 1998), with a pronounced bias towards bird-pollinated species (e.g. Yates et al., 2006; Llorens et al., 2012). Only a few studies have investigated the pre-dispersal stages of *Conospermum* seed ecology and its mating system. In line with the most common mating system in Proteaceae and, more generally, the strategy of most resprouter plants (Bond & Midgley, 2001), *C. undulatum* is generally considered a self-incompatible plant species that relies on insect-mediated outcrossing pollination to set seeds. However, no studies have been carried out on the reproductive biology of this species and only two studies have reported findings on the breeding and mating system of species within the *Conospermum* genus. Among these, one was on four Western-Australian blue-flowered species (*C. eatoniae, C. caeruleum, C. amoenum* and *C. brownii*; Stone et al., 2006), and the other was on four white-flowered species (*C. taxifolium, C. ericifolium, C. ellipticum* and *C.*

longifolium) growing in eastern Australia (Morrison et al., 1994). In particular, Stone et al. (2006) observed seeds set by plants pollinated with pollen originating from ramets (i.e. pollinated with self-pollen), leaving open questions about both the breeding and mating system of the genus. Hence, it was important to assess, firstly, the mating system of *C. undulatum* and, secondly, if this species suffers from pollen limitation. An understanding of the importance of size, connectivity and floral display of the populations in determining the reproductive output and the degree and type (i.e. quality *vs* quantity) of pollen limitation was also important for understanding the impacts of fragmentation on our target species and, consequently, for the development of management plans which effectively protect the species.

Reproductive output and seed germinability

Seeds generally are the main vector for plant regeneration in non-sprouter species (i.e. seeders), which are killed by fire and rely on seedling recruitment for population persistence (Clarke et al., 2013; Lambers, 2014). In resprouting plants, despite their ability to immediately recover after fire by regenerating from surviving buds, recruitment from seed is still vital. Indeed, over short- to medium-term their resprouting ability could buffer the effects of poor seed recruitment (Bond & Midgley, 2001), however, regeneration from seeds is crucial for long-term adaptation and population persistence. Fragmentation is also well known to have important consequences for pollen flow, seed production, seed germination and, consequently, population viability of resprouter, as well as seeder plant species (Whelan et al., 2000; Young et al., 2000; Hobbs & Yates, 2003; Aguilar et al., 2006). This is critical for outcrossing species, as plant populations become smaller and more isolated, mating between closely related individuals is more likely. This might lead to bi-parental inbreeding, which might reduce levels of seed production, seed viability and the number of germinants (Dudash & Fenster, 2000; Yates et al., 2007).

As a consequence of different life-history strategies (i.e. seeder *vs* resprouter), different patterns of historical fragmentation among SWAFR species (Hopper, 1979; 2009) and different pollen vectors (e.g. vertebrates or insects), the effect of recent human-induced fragmentation may lead to different responses among plant species. For instance, if pollen-mediated gene flow is extensive among fragments, small populations might avoid inbreeding and no correlation should be expected between population variables (such as population size and connectivity) and fecundity (fruit set, seed set, and seed germination). Alternatively, if

inbreeding is higher in small and isolated fragments, the number of seeds should decrease along with decreasing population size and connectivity due to higher probability of post-zygotic lethal alleles operating. Yates et al. (2007) observed a strong positive relation between number of seeds per fruit and increasing population size in the common bird-pollinated shrub *Calothamnus quadrifidus*. On the other hand, it is also possible that the unique history of speciation experienced by many SWAFR species might lead to a different result. This is true in the case of a highly restricted endemic species that might have been derived from founder populations, the small initial population size might have pushed the population to a severe genetic bottleneck, purging a high proportion of lethal alleles. This might allow products of bi-parental inbreeding to survive, and thus no correlation between population variable and reproductive output is expected in this case. Gibson et al. (2012) reached this conclusion in their work about the rare *C. quadrifidus* subsp. *teretifolius*.

The above discussion demonstrates it is currently difficult to generalise about the relationship between fruit output, seed viability, and number of germinants among SWAFR species. The combination of this reproductive output analysis and the genetic investigations may help to disentangle these different scenarios. For instance, if I find no correlation between population variables and reproductive output, I will be able to establish whether this lack of relationship is mainly due to either the extensive pollen-mediated gene flow or the possible reduction of deleterious alleles through genetic bottlenecks. This allows a deeper understanding of the effects of fragmentation on the rare shrub *C. undulatum*, and is critical in a conservation perspective that aims to maximise persistence of this threatened species in a fragmented urban landscape.

Reproductive phenology

Phenology was defined by Lieth (1974) as "the study of the timing of recurring biological events" and deals with classification and registration of relevant stages in an organism's development, principally for poikilothermic organisms such as plants and insects. Phenological patterns might not only differ between different species, but also between individuals of the same species in relation to different genetic provenance (Herrera, 2009; Morellato et al., 2016).

Flowering synchrony is important in relation to reproductive success in out-crossing entomophilous species, with populations reaching flowering peak too early or too late in the season missing the activity peak of pollinators (Ollerton et al., 2011). Moreover, flowering

time is highly relevant as it is the first mechanism of reproductive isolation (Hendry & Day, 2005). Synchronous flowering allows plant populations to produce a large floral display and promotes a high flower visitation rate by insects, which eventually cascades into high reproductive success provided the absence of resource limitation (Forsyth, 2003; Kudo & Harder, 2005; Rodrìguez-Pérez & Traveset, 2016). Understanding whether *C. undulatum* populations are synchronous in reaching their flowering peak or if populations differ in their flowering developmental time across the species range is crucial. For instance, if a population surrounded by others (such a population would have low spatial isolation) flowers later than its neighbours, then its effective reproductive isolation would be high. Therefore, investigating flowering phenology could complement the pollen limitation assessment of this study and give insights into population connectivity.

Genetic diversity

Genetic diversity within populations and the levels of genetic differentiation among populations are known to have fundamental effects on the evolutionary trajectories of species (Hughes et al., 2008; Broadhurst et al., 2016). Large and biogeographically central populations generally maintain more diversity than small and isolated ones (Hamrick & Godt, 1989), with marginal populations expected to be genetically diverged from central populations due to their isolation, divergent natural selection, and restricted gene flow (Lesica & Allendorf, 1995; Eckert, Samis, & Lougheed, 2008; Chhatre & Rajora, 2014). In a recent meta-analysis, Broadhurst et al. (2017) showed that the effect of range size, abundance, and range disjunction of Australian tree species was consistent with predictions based on population genetics theory. However, while Australian trees have been extensively studied, shrub species need more investigations to reach definitive conclusions due to a lower number of studies available (Broadhurst et al., 2017a). In this context, research about *Conospermum* is scarce. *Conospermum undulatum* was originally considered a variety of the more common *C. triplinervium*. A revision of the *Conospermum* genus by Bennett (1995) pointed out the morphological distinction between *C. undulatum* (multi-stemmed shrub) and *C. triplinervium* (single-stemmed tree). Only recently, Close et al. (2006) investigated the genetic differentiation between these smokebushes and found a clear genetic distinction between *C. undulatum* and *C. triplinervium* populations in bushland adjacent to Perth airport which underlined the recognition of these as distinct taxa at the species level. The authors also found that two of the four investigated *C. undulatum* populations only partially

overlapped in a PCA ordination of the genetic similarity (Close et al., 2006). This may indicate a low level of connectivity between at least some of the fragments.

The Close et al. (2006) study was confined to a restricted portion of the species distribution, in an area with relatively large and intact bushland. Thus, little is known about genetic variation across its entire range, much of which is highly fragmented due to intensive urbanisation. This leaves open questions about the effects of fragmentation on the distribution of genetic variation within *C. undulatum* remnant populations. As a direct effect of fragmentation, isolated populations are expected to exhibit reduced genetic connectivity and they might not be able to counteract genetic drift, leading to genetic impoverishment and strong genetic structure among populations. The genetic variability of populations might be even further diminished due to possible genetic bottlenecks and/or other processes leading to collapses in the effective population size.

Contemporary gene flow

Anthropogenic habitat fragmentation is now considered as one of the most important factors contributing to the loss of plant biodiversity worldwide as it can increase the physical isolation of populations (Sala et al., 2000; Heywood & Iriondo, 2003; Kramer et al., 2008). Changes in the landscape affect many plant and ecological processes, such as the dispersal of propagules and the rate of gene flow. Understanding the level of gene exchange between populations under current environmental conditions could be a key point for guide management decisions in a long-term conservation perspective. The pattern of gene flow that could emerge from indirect genetic methods (e.g. F_{ST}; Wright, 1951), however, might reflect population past histories, especially for long-lived resprouter plants, such as *C. undulatum*, which could be buffered from the effects of relatively recent processes (such as human-induced fragmentation). This makes it difficult to detect long-term genetic effects of anthropogenic changes (Fuchs & Hamrick, 2011). The use of a direct methods to analyse pollen dispersal, such as paternity analysis, therefore provides precise estimates of contemporary gene flow (Jones & Ardren, 2003). Pollen dispersal and the distance over which it occurs will affect the reproduction by seeds and, ultimately, the genetic layout of fragmented populations (Ellstrand & Elam, 1993; Yates et al., 2007). Consequently, gene flow patterns deserve a thorough evaluation (Ellstrand, 2014).

Population biology theory predicts that isolation will lead to decreased gene flow, increased mating among relatives and, consequently, reduced genetic diversity through a

magnified effect of genetic drift (Young et al., 1996; Hamrick, 2004). Empirical evidence, on the other hand, indicates that many species in which pollen dispersal has been investigated in disturbed landscape have shown extensive gene flow due to the high mobility of their pollen grains (i.e. wind dispersal or high motility of pollen vectors, such as birds and domestic honeybees) (Byrne et al., 2007; Byrne et al., 2008; Bezemer et al., 2016). In fragmented landscapes, an extensive pollen-mediated gene flow might buffer isolated plant populations against the loss of genetic variability. This research, however, has been limited mainly to plant species characterised by highly mobile pollen vectors such as birds and European honeybees (e.g. Byrne et al., 2007; 2008). Therefore, studies on SWAFR plants that rely on native insects for pollination are scarce. Since the impact of fragmentation is known to be greater on plant species with less motile pollinators (Breed et al., 2013; Lowe et al., 2015), we cannot generalise about the impact on SWARF plants because of different foraging behaviour of their pollination vectors (i.e. birds *vs.* native insects).

Hybrids

Since *C. undulatum*, *C. canaliculatum*, and *C. triplinervium* grows in close proximity in the Perth area, putative hybrid individuals have been reported between these congeneric species which bear leaves with unusual morphology somewhat intermediate between these species (i.e. narrower leaves with less undulate margins). Close et al. (2006) found that the observed variation in leaf morphology within the target *C. undulatum* population at Perth airport, compared with confirmed *C. undulatum* specimens, did not reflect hybridization between the target population and a nearby *C. triplinervium* population; although, the authors themselves stated that such genetic distinction might not be detected by the AFLP markers used in their work.

It is widely known that the consequences of hybridisation are relevant aspects in conservation biology. Indeed, the presence of hybrids is a pervasive threat for biodiversity worldwide, especially for rare species contacting more abundant ones (Rhymer & Simberloff, 1996; Prentis et al., 2007).

The risk of genetic assimilation and the eventual loss of the rare taxa is particularly true for small populations already threatened by abiotic stresses, such as fragmentation and disturbance (Ellstrand & Elam, 1993; Burgess et al., 2005; Zalapa et al., 2009). The use of microsatellites markers specifically developed for *Conospermum* should help shed light on

the presence of hybrids, and to evaluate the threat posed by hybridisation (if any) to the rare *C. undulatum*.

Study site description

The study was conducted within the Swan Coastal Plain bioregion (Fig. 1.2), a low-lying coastal plain that extends from Jurien Bay, north of Perth, to Cape Naturaliste in the south, and is part of the Southwest Australia global biodiversity hotspot (Mittermeier et al., 2004). The area experiences a dry, Mediterranean-type climate (Beard, 1984), with hot dry summers (December-March), and mild wet winters (June-August) with 600-1000 mm of rainfall on average across the region (762 mm year^{-1} on average at the Perth Airport meteorological station, the closest to study area, within a 10 km radius from the centre of the species distribution range; Bureau of Meteorology, http://www.bom.gov.au).

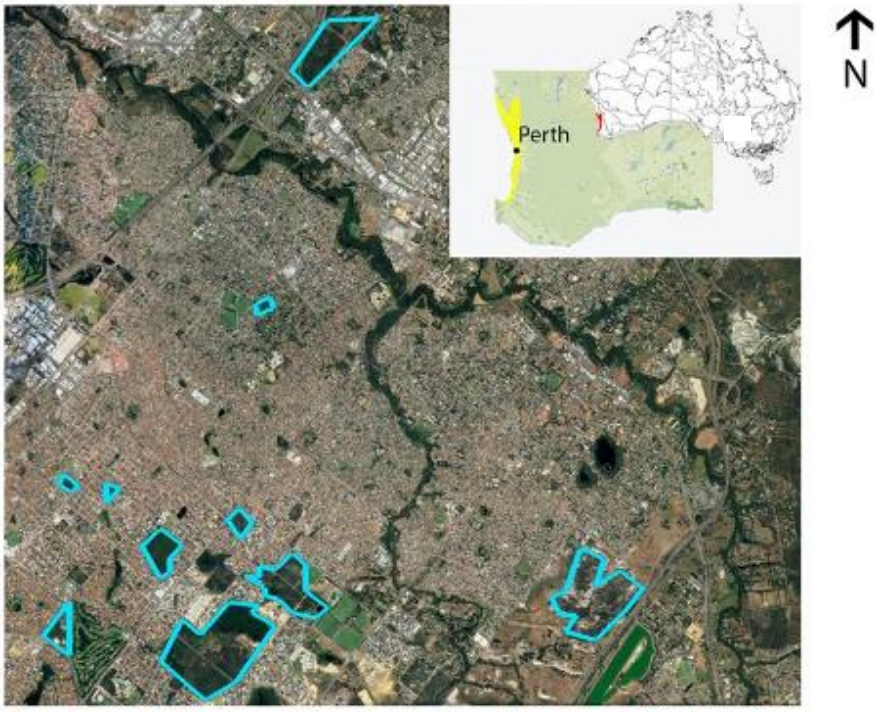


Figure 1.2. *E*xample of fragmented bushland in an urban matrix in the Swan Coastal Plain; blue polygons represent remnant vegetation patches such as the ones where *C. undulatum* is found.

Vegetation

The Swan Coastal Plain is dominated by *Banksia* woodlands. These woodlands are confined to the Mediterranean region of south-west Australia and are integral part of the rich

fabric of biodiversity in this world-class floristic region. They form the dominant structural vegetation on these deep, highly leached sand dune systems (McArthur & Bettenay, 1974).

The *Banksia* woodland is a low-stature woodland dominated by small trees 4-8 m tall. The woodlands associated with the Swan Coastal Plain typically consist of a prominent tree layer of *Banksia* species (mainly *B. attenuata*, *B. menziesii* and, less commonly, *B. ilicifolia*), together with scattered eucalypts and other tree species within or above the *Banksia* canopy (mainly *Eucalyptus marginata*, *Allocasuarina fraseriana* and the hemiparasite *Nuytsia floribunda*) (Beard, 1989). Surveys have recorded 1130 native taxa from 616 sampled points on the Swan Coastal Plain (Keighery & Keighery, 2016). Although the dominant trees comprise a relatively small number of species, the associated understorey is typically highly diverse. In particular, within the understorey, native sclerophyll shrubs are the most diverse, accounting for 42% of the species recorded. These communities not only are species rich, with an average of 50 taxa recorded in 100 m^2, but also have high floristic endemism, with 86% of *Banksia* woodland flora being endemic to Western Australia.

Moreover, *Banksia* woodlands provide vital habitat for over 20 nationally threatened species such as Carnaby's and forest red-tailed black cockatoos, the western quoll and western ringtail possum; as well as many wildflowers unique to the south-west and other animals that depend on them, such as the honey possum (*Tarsipes rostratus*) (Department of the Environment and Energy, 2016).

Banksia attenuata *woodland and Conospermum undulatum*

The richest of any *Banksia* community found on the Swan Coastal Plain is the *Banksia attenuata* woodlands over species rich dense shrublands, also known as Swan Coastal Plain community type 20a (SCP20a). The average number of species recorded in 100m^2 quadrats established for Gibson et al. (1994) was 67, with some sites having over 80 species per 100 m^2. These *B. attenuata* woodlands are regionally rare with the distribution limited to the sand ridges between Chittering (north of Perth) and the base of the Darling Scarp in an area heavily cleared for urbanisation and agriculture, with only around 600 ha remaining (Department of Parks and Wildlife, 2016). The community is listed as an endangered TEC (Threatened Ecological Community) by the WA State Government, with major threats being land clearing for urbanisation, too frequent fires, and weeds (Department of Parks and Wildlife, 2016). Among other threatened species (both flora and fauna), *Conospermum* species occur within this endangered community and *C. undulatum* is frequently recorded in southern occurrences. Of the known populations of *C. undulatum*,

47% are in areas currently identified as SCP20a (*sensu* Gibson et al. 1994; Table 1.1). The relationship between *C. undulatum* and the endangered community appears modest but not unimportant, especially considering that *Banksia* woodlands may provide important habitat for the poorly known native pollinators of *C. undulatum*.

Soils

The origin of coastal sandplains in south-west Australia is linked with sea-level fluctuations over the Neogene and the Quaternary, with a progressive lowering during the between *ca*. 23 and 2.6 million years ago. Subsequently, during the Quaternary, these fluctuations have been linked to Milankovitch cycles and subsequent glacial to inter-glacial cycles (Miller et al., 2011; Wyrwoll et al., 2014; Zachos et al., 2001). In particular, the Swan Coastal Plain is bounded by the Darling Scarp on the East and is a combination of sand deposited from the ocean during these sea-level variations and, secondarily, sediment washed down from the scarp.

The Swan Coastal Plain comprises four broad dune successions running roughly parallel to the existing coastline and their geological ages increase moving away from the coast. The distribution range of the target species of this study, *C. undulatum*, is found on the eastern part of the plain, towards the scarp, on the oldest succession, namely the older Bassendean dune system. This system is a series of shoreline deposits and coastal dunes that developed during several dune-building events in an interglacial period that began about 240,000 years ago. The dunes are made up of bleached white-grey sands and, because of their age, they present a low carbonate status and are now mostly pure silica sand (McArthur & Bettenay, 1974). To the west, the Bassendean system is bounded by the Pinjarra plain; this is an alluvial tract of unconsolidated clays and loams and consists of alluvial fans and floodplains along the rivers which have cut through the Darling Scarp and meander through the Swan Coastal Plain to the ocean.

Conospermum undulatum appears to grow on rapidly drained soils at the interface between the plain and the scarp (Fig. 1.3). The entire distribution range of the species is characterised by sandy soils, including deep sands of the Bassendean system, as well as areas of the Pinjarra and Forrestfield (foothills of Darling Scarp with raised very old dunes from when sea levels were at the base of scarp over 2 million years ago) systems where sand deposits occur, sometimes over heavier soils, such as clays. Details of soil types for each population are given in Table 1.1. Precise coordinates cannot be provided for Threatened Flora.

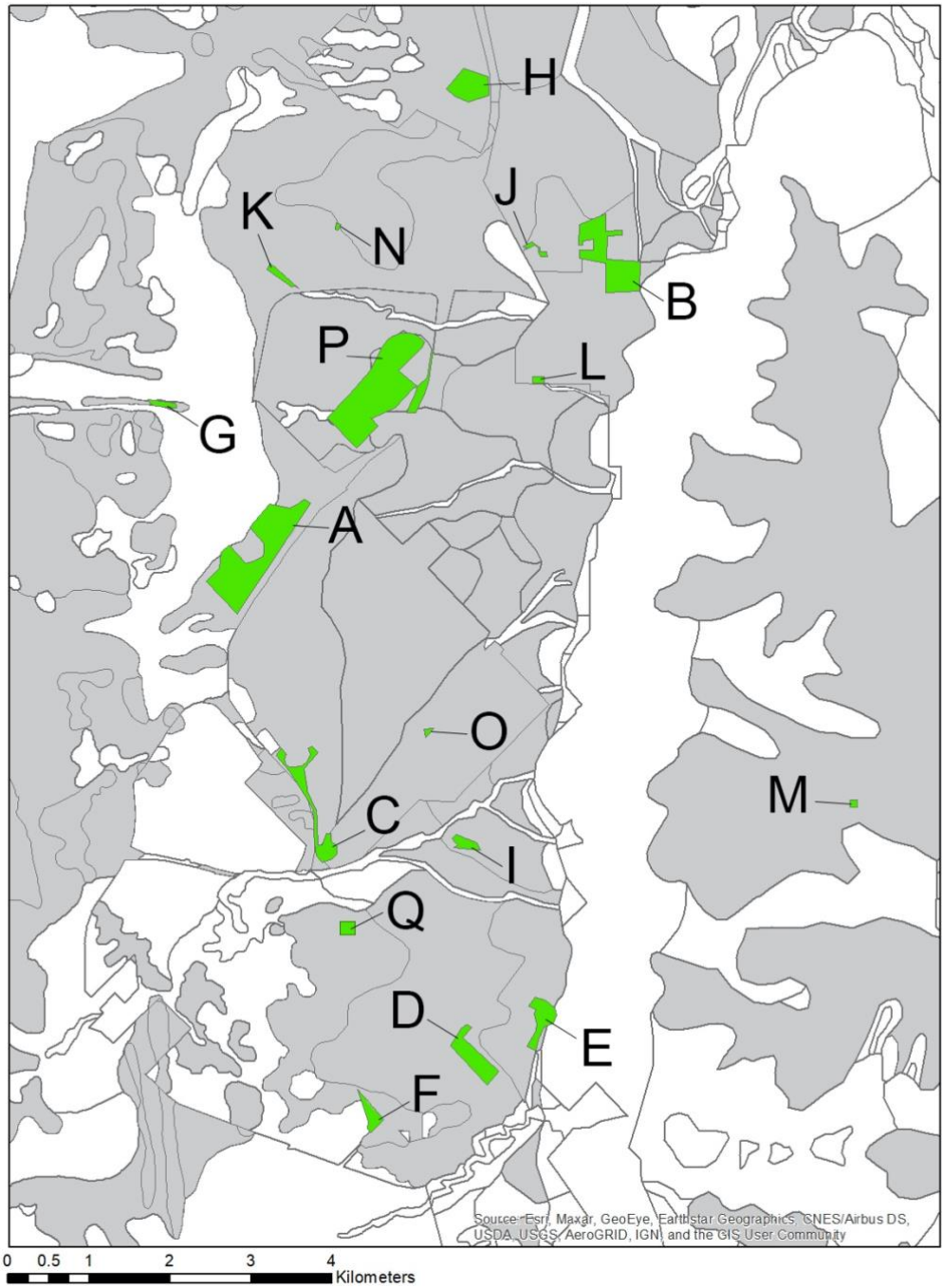


Figure 1.3. Map of the locations of bushland remnants with populations of *Conospermum undulatum* (in green). Grey shading represents suitable soil (sands), whereas white represents unsuitable soil for *C. undulatum* (clays and other heavy soils). Coordinates cannot be provided for specific locations of Threatened Flora.

Table 1.1. Soil system, type and relative description of *Conospermum undulatum* populations

Population	SCP20a[†]	Landform	Soil type	Soil description
A	Yes	Bassendean	S8 Phase	Sand - very light grey at surface, yellow at depth, fine to medium-grained, sub-rounded quartz, moderately well sorted of eolian origin
B	Yes*	Forrestfield	(D Range) F1 Phase	Foot and low slopes < 10% with deep rapidly drained siliceous yellow brown sands, and pale or bleached sands with yellow-brown subsoil.
C	Yes*	Forrestfield	Forrestfield System	Undulating foot slopes of the Darling and Whicher Scarps. Duplex sandy gravels, pale deep sands and grey deep sandy duplexes.
D	Yes	Forrestfield	(D Range) F1 Phase	Foot and low slopes < 10% with deep rapidly drained siliceous yellow brown sands, and pale or bleached sands with yellow-brown subsoil.
E	No	Forrestfield	(D Range) F2 Phase	Foot and low slopes < 10%. Well drained gravelly yellow or brown duplex soils with sandy topsoil.
F	No	Pinjarra	Phase Gf7	Minor rises with deep rapidly drained brownish, siliceous or bleached sands underlain by mottled yellow clay.
G	No	Bassendean	S8 Phase	Sand - very light grey at surface, yellow at depth, fine to medium-grained, sub-rounded quartz, moderately well sorted of eolian origin
H	Yes	Pinjarra	Gf7 Phase	Minor rises with deep rapidly drained brownish, siliceous or bleached sands underlain by mottled yellow clay.
I	Yes	Forrestfield	(D Range) F1 Phase	Foot and low slopes < 10% with deep rapidly drained siliceous yellow brown sands, and pale or bleached sands with yellow-brown subsoil.
J	Yes	Forrestfield	(D Range) F1 Phase	Foot and low slopes < 10% with deep rapidly drained siliceous yellow brown sands, and pale or bleached sands with yellow-brown subsoil.
K	No	Pinjarra	S10 Phase	Sand - as S8 as relatively thin veneer over sandy clay to clayey sand. Of eolian origin.
L	No	Forrestfield	Forrestfield System	Undulating foot slopes of the Darling and Whicher Scarps. Duplex sandy gravels, pale deep sands and grey deep sandy duplexes.
M	No	Dwellingup	2 Phase	Very gently to gently undulating terrain (<10%) with well drained, shallow to moderately deep gravelly brownish sands, pale brown sands and earthy sands overlying lateritic duricrust.
N	No	Pinjarra	S10 Phase	Sand - as S8 as relatively thin veneer over sandy clay to clayey sand. Of eolian origin.
O	No	Forrestfield	Forrestfield System	Undulating foot slopes of the Darling and Whicher Scarps. Duplex sandy gravels, pale deep sands and grey deep sandy duplexes.
P	Yes	Forrestfield	(D Range) F1 Phase	Foot and low slopes < 10% with deep rapidly drained siliceous yellow brown sands, and pale or bleached sands with yellow-brown subsoil.
Q	No	Pinjarra	Phase Gf7	Minor rises with deep rapidly drained brownish, siliceous or bleached sands underlain by mottled yellow clay.

[†] Endangered Floristic community type: '*Banksia attenuata* woodland over species rich dense shrublands'

* Part of the population falls within SCP20a plant community

Note. Soil descriptions retrieved from https://catalogue.data.wa.gov.au/dataset/soil-landscape-mapping-best-available

Study sites

At the beginning of this project, in 2017, I gathered information on populations of *C. undulatum* through a database of the Department of Biodiversity, Conservation and Attractions (DBCA) of Western Australia. All extant populations of this threatened species are well known thanks to the efforts of Conservation/Flora officers of different districts across the distribution range of *C. undulatum*. Prior the beginning of the 2017 flowering season, I visited all known remnant population to confirm the presence of my target species and record the GPS location of every individual *C. undulatum* plant. From the 25 populations recorded in various surveys starting from 1997, only 17 currently contain extant plants. By means of ArcGIS (ESRI, Redlands, USA), I used the GPS data to characterise each population by their size (i.e. number of *C. undulatum* plants), fragment area, and percentage of vegetated (native and not native) land around the population centroid. Subsequently, I calculated an isolation index based on a modified version of the incidence function model (Hanski, 1994). Details on the characterisation of population variables are given in the following chapters. Pairwise population distances in km are given in Table 1.2.

From a total of 17 remnant populations of *C. undulatum*, we selected a subset of populations encompassing the entire range of population sizes and levels of isolation. Population size ranged from 5 to 880 plants (mean = 243.4); fragment area ranged from 0.34 to 51.25 ha (mean = 22.06); isolation index ranged between -0.04 and -21.41 (mean = -7.65); and floral display ranged from 0.21 to 715.70 (mean = 288.02). The number of selected populations varied (from 14 to seven) in relation to the aim of the investigation and is reported in the relevant chapters.

Table 1.2. Pairwise population distance matrix (in km) of *Conospermum undulatum*.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
A	0																
B	4.85	0															
C	1.77	5.79	0														
D	5.06	7.93	2.35	0													
E	5.26	7.53	2.78	0.95	0												
F	5.53	9.07	2.82	1.21	2.05	0											
G	1.63	4.70	3.92	7.20	7.39	7.71	0										
H	5.48	1.86	6.99	9.74	9.53	10.68	4.55	0									
I	3.43	6.05	1.20	1.97	1.83	3.01	5.58	7.79	0								
J	4.48	0.62	5.67	8.16	7.88	9.20	4.13	1.63	6.24	0							
K	2.89	3.32	5.05	8.16	8.20	8.89	1.82	2.74	6.26	2.65	0						
L	3.57	1.31	4.49	6.86	6.52	7.87	3.86	3.06	4.87	1.39	2.99	0					
M	6.77	6.02	5.55	4.79	3.96	5.94	8.28	8.41	4.03	6.61	8.23	5.52	0				
N	3.67	2.74	5.47	8.34	8.19	9.22	2.54	1.94	6.55	1.89	0.79	2.61	8.09	0			
O	2.42	5.06	1.23	3.17	3.08	4.07	4.35	6.60	1.26	5.14	5.09	3.78	4.56	5.36	0		
P	2.02	2.18	4.32	7.15	7.10	8.06	2.18	2.66	5.26	1.62	1.48	1.50	6.56	1.38	3.78	0	
Q	3.64	7.22	0.80	1.48	1.92	1.93	5.89	8.76	1.22	7.31	6.94	5.97	5.23	7.31	2.13	5.61	0

Study rationale and aims

A detailed investigation of the genetics and ecology of *C. undulatum* will help develop and improve conservation plans for this threatened rare species. Current conservation actions (certainly required in the short term) are mainly focused on protecting remnant individuals from further decline caused by uncontrolled access to remnant populations, feral rabbits and the spreading of the pathogen *Phytophthora cinnamomi.* This has been mostly achieved by surveying, marking, and fencing extant populations (Commonwealth of Australia, 2000; Department of Environment and Conservation, 2009). The knowledge gained from my research will help to generate long-term plans needed to enhance the future persistence of *C. undulatum* by means of an improved awareness of factors that constrain both its reproduction and its capacity for adaptation over the long-term.

The thesis is structured around seven key objectives:

Objective 1: *Determine the effects of habitat fragmentation on the reproductive output and seed germination of this threatened shrub* (Chapter 2);

Objective 2: *Identify the pollinator assemblage of* C. undulatum *and quantify the effects of anthropogenic fragmentation on such pollinators*. (Chapter 3);

Objective 3: *Quantify the effects of fragmentation on the* quantity *and* quality *component of pollen limitation in* C. undulatum (Chapter 3);

Objective 4: *Define the ecological role of ants as floral visitors of* C. undulatum (Chapter 4);

Objective 5: *Quantify the intra-specific genetic diversity within and among populations, and the genetic structure across the entire distribution range of* C. undulatum (Chapter 5);

Objective 6: *Determine the contemporary gene flow among recently isolated populations of the threatened* C. undulatum (Chapter 6); and

Objective 7: *Clarify the threat posed by hybridisation with sympatric* Conospermum *species* (Chapter 7).

Studies on the pollination and seed ecology, population structure, and historical and current estimates of gene flow can complement each other, giving an unprecedented resolution on the processes that constrain the generation of vital offspring. In the final chapter (Chapter 8), I integrate the findings of the six experimental chapters into a broad understanding of the factors that impact on the persistence of the threatened *C. undulatum*

and how this information can support the development of management plans aimed to the long-term conservation of this endemic species.

Chapter 2 - Effects of habitat fragmentation on the reproductive output and seed germination of *Conospermum undulatum* (Proteaceae)

This chapter has been published as the following paper:

Delnevo N, van Etten EJ, Byrne M, Stock WD. 2019. Floral display and habitat fragmentation: effects on the reproductive success of the threatened mass-flowering *Conospermum undulatum* (Proteaceae). *Ecology and Evolution*, 9: 11494–11503.

Introduction

Pollinators visiting flowers with adequate amounts of pollen grains are an essential requirement for pollen dispersal and, ultimately, reproduction for *ca.* 87% of the world's flowering plant species (Ollerton, Winfree, & Tarrant, 2011; Winfree, Bartomeus, & Cariveau, 2011). Flowering plants are a crucial component of most terrestrial ecosystems (Ollerton Johnson, & Hingston, 2006) and the rich biodiversity of such systems relies on these plants and their interactions with pollinators. It is widely known that reproduction by seeds has a key role for fitness, migration, adaptation and ultimately population persistence of plant species (Fenner & Thompson, 2005). Yet, as a consequence of global change, many plant and pollinator populations are declining (Biesmeijer et al., 2006), with mutualistic plant-pollinator interactions frequently disrupted (Thomann, Imbert, Devaux, & Cheptou, 2013), which can have direct effects on plant population viability. Fragmentation of vegetation is one of the most pervasive changes in terrestrial ecosystems that affects plants and their pollinators. The rate at which natural habitats have been fragmented by clearing for urban and agricultural land uses has increased substantially during the last 60 years and now is at unprecedented levels (Ellis, Goldewijk, Lightman, & Ramankutty, 2010). Based on a meta-analysis of plant reproductive susceptibility to habitat fragmentation, Aguilar, Ashworth, Galetto, and Aizen (2006) suggested that a decrease in size and connectivity of plant populations resulting from habitat fragmentation could locally reduce the reproductive success of plants. Indeed, small and/or isolated fragments of plant populations may be less attractive for pollinators (Dauber et al., 2010; Delmas et al., 2014), leading to a reduction in both pollen *quantity* (i.e. decrease in pollination events) and pollen *quality* (i.e. less

deposition of conspecific and outcrossed pollen grains on stigmas) (Aizen & Harder, 2007; Eckert et al., 2010). Pollen quality is particularly important for self-incompatible species that lack the reproductive assurance that self-reproduction may provide (Morgan & Wilson, 2005). In addition, a factor that has rarely been considered, especially for conservation purposes, is how plant species that rely on massive population floral display for attracting pollinators (i.e. mass-flowering species; Heinrich & Raven, 1972) respond to habitat fragmentation.

Conospermum (Proteaceae) is an endemic genus to Australia with its centre of distribution being South-west Western Australia (Bennett, 1995). The South-West Australian Floristic Region (SWAFR; Hopper & Gioia, 2004) encompasses an exceptional concentration of endemic flora and is recognised as a global biodiversity hotspot (Mittermeier et al., 2004; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000) and has been impacted by fragmentation because of urban and agricultural development. *Conospermum undulatum* is a mass-flowering species and during the reproductive season its white inflorescences dominate the (non-fragmented) landscape resembling drifting smoke, hence its common name: smokebush. This species is currently listed in the threatened flora of Western Australia (W.A. Government Gazette, 2018) and has been assessed as 'Vulnerable' using IUCN red list criteria (Department of Environment and Conservation, 2009).

In general, mass-flowering crops and native species have been shown to be attractive to a larger diversity of pollinators and may attract a higher abundance of floral visitors from surrounding flowers (Hegland & Totland, 2005; Westphal et al., 2003). Therefore, it may be expected that mass-flowering plants may not be impacted by the detrimental effects of fragmentation by remaining highly attractive to a large pool of pollinators due to their high flower abundance. However, this hypothesis has only been tested on crops and boreal plant species (e.g. Diekötter, Kadoya, Peter, Wolters, & Jauker, 2010; Mitchell, Karron, Holmquist, & Bell, 2004), that are pollinated by honeybees (*Apis mellifera*) and bumble bees (*Bombus* sp.), important pollinators in Europe. Results from these studies may not be transferable to plants in the SWAFR where plant-pollinator interactions have evolved over long timeframes. Plants within the SWAFR have coevolved with different pollen vectors such as birds, mammals, and small native bees, leading to the development of specific flower morphologies and pollination systems. *Conospermum undulatum* plants possess small and characteristic flowers with an active pollination mechanism described by Holm (1978) that involves a tactile stimulation within the calyx tube to trigger the stigma, so it makes contact with the visitor. Houston (1989) reported the identification of a south-western Australian

species-group of bees (*Leioproctus conospermi*), which consists of three species oligolectic on flowers of *Conospermum* that possess morphological adaptations to enable this remarkable pollination. He also reported that, besides these native bees, smokebush flowers are visited by argid sawflies (Argidae), and flies of families Bombyliidae and Syrphidae, although the true pollinators remain uncertain. The majority of other common generalist pollinators, such as dipterans, are unable to produce an effective pollination (i.e. untriggered style and non-dehisced anthers, or insect trapped fatally by the triggered style). Further, the most abundant insect pollinator in the SWAFR, the introduced European honeybee (Phillips, Hopper, & Dixon, 2010), is too large to pollinate the flowers of the smokebush (N. Delnevo, personal observation).

In addition, within small populations of mass-flowering species, pollinators tend to have higher numbers of within-plant floral visits compared to those in larger populations (Eckert, 2000), with a consequent increase in levels of geitonogamy (i.e. transfer of pollen between different flowers of the same plant). Such transfer of self-pollen may represent a reproductive assurance to compensate for the lack of outcross pollen, but this would depend on the strength of inbreeding depression (Campbell & Husband, 2007). However, many genera of Proteaceae exhibit self-incompatibility systems and evidence of selective fruit development (Goldingay & Carthew, 1998; Vaughton & Carthew, 1993). Accordingly, *C. undulatum* is considered to be a self-incompatible species (Goldingay & Carthew, 1998; Morrison, McDonald, Bankoff, & Quirico, 1994), and this would reduce the reproductive assurance of geitonogamy, especially in small fragments. However, the reproductive biology of this species has not been studied in detail as yet, and there is a need to understand the reproductive responses of this rare plant in a highly fragmented landscape to inform future conservation efforts. Here, we studied the effects of fragmentation on the reproductive biology of *C. undulatum* to inform conservation. Specifically, we asked: 1) were fruit production and seed production related to aspects of fragmentation?; 2) was seed germination following the same trends?; and 3) to what extent was geitonogamy evident in the mating system of *C. undulatum*? We expected small populations in isolated fragments with low floral display to produce fewer fruits and seeds compared to large, connected, highly visible populations due to a lack of pollen quantity and quality. Consequently, if pollen-mediated gene flow is not able to extend the mating pool beyond the single fragment providing genetic rescue in small and isolated population from the effects of inbreeding, then the number of germinants should also be related to our population descriptors. Finally, as for many proteaceous species, we expected our target species *C. undulatum* to be self-incompatible and

therefore reproductive assurance via autogamy would be zero to inconsequential. However, even if the trigger mechanism of the stigma is a physical barrier to self-pollination, geitonogamy may still occur, especially in small populations, making predictions less clear.

Materials and methods

Study site and species

The study was conducted in south-west Western Australia within the Swan Coastal Plain bioregion (Fig. 2.1). This region is a low lying coastal plain that extend from Jurien Bay, north of Perth, to Cape Naturaliste in the south, and it is part of the Southwest Australia biodiversity hotspot (Mittermeier et al., 2004). The Swan Coastal Plain was historically cleared for agriculture and forestry, and is now experiencing extensive land clearing for urbanisation. Urbanisation has more than doubled since the 1970s, and is centred around Perth, the capital city of Western Australia and has impacted biodiversity of the region (e.g. Davis, Gole, & Roberts, 2013). Urban expansion has reduced natural or semi-natural vegetation on the Swan Coastal Plain to 34.7%, with only 10% in protected areas (Wardell-Johnson et al., 2016). Our target species, *Conospermum undulatum*, is a threatened, naturally rare plant species with a range restricted to ca. 55 km^2 in an expanding urban zone.

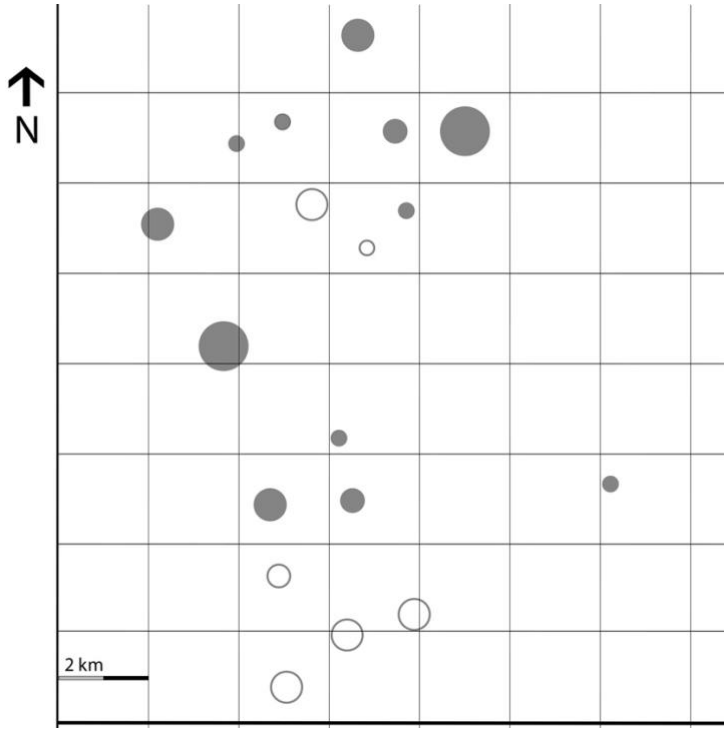


Figure 2.1. Spatial disposition of all extant *Conospermum undulatum* populations. Filled circles are populations selected for this study, empty circles are population not selected; the size of the circle reflect the population size. A precise map cannot be provided for Threatened flora.

Conospermum undulatum is a monoecious plant which grows as an erect, compact shrub up to 1.5 m tall with distinctive fibrous, longitudinally fissured stems and glabrous leaves to 12 cm long and 3.8 cm wide; leaves have characteristic undulating margins. It was originally considered to be a variety of *C. triplinervium*, which also occurs in the region but with different habit (i.e. *C. undulatum* never develops a thick trunk and is typically multi-stemmed) and leaf morphology (Bennett, 1995). Molecular evidence has established *C. undulatum* as a distinct species (Close et al., 2006), and recently developed genetic resources will further clarify its genetic relationships (Delnevo et al., 2019). Our target species is classified as resprouter; hence, it can survive fire by regenerating from rootstock. The hermaphroditic woolly flowers have long, white hairs, and are produced in inflorescences held well above the leaves. The flowering period usually ranges from late August to late October. Fruits are cone-shaped, covered with tan orange hairs and contain only one seed (Bennett, 1995). *Conospermum undulatum* is an entomophilous species and possesses an active pollination mechanism that involves a tactile stimulation within the calyx, which causes the style to flick down on the back of the insect, and simultaneously, causing the fertile anthers to dehisce explosively, casting pollen onto the visitor (Holm, 1978; see

Douglas (1997) for a morphological description). Thus, its flowers need to be visited by insects carrying a suitable pollen load for pollination to occur leading to fruit development.

Data collection

Prior the beginning of the flowering season, in August 2017, we recorded the GPS location of every individual plant in all the existing populations of *C. undulatum*. Then, by means of ArcGIS (ESRI, Redlands, USA) we characterised each population by their size (i.e. number of *C. undulatum* plants), fragment area, and percentage of vegetated (native and not native) land within a 500 m radius area around the population centroid. Since the foraging range of bees is related to body size (Greenleaf et al., 2007), a radius of 500 m was selected based on the fact that small native bees, with restricted ranges, were the most likely pollinators of our target species. Subsequently, we calculated an isolation index based on a modified version of the incidence function model (Hanski, 1994). This model accounts both for distances to all possible neighbouring populations, and the area of those populations, providing a better estimate in highly fragmented habitats and small data sets compared to either nearest neighbour or buffer measures (Moilanen & Nieminen, 2002). Population isolation was calculated as: $S_i = -\Sigma_{j \neq i} \exp(-\alpha d_{ij}) A_j^b$, where S_i is the isolation of the patch i; α is a scaling parameter for the effect of distance to migration ($1 / \alpha$ is the average migration distance); d_{ij} represent the distance between fragment i and j; A_j is the area of fragment j; b is a scaling parameter of immigration as a function of the area of fragment j. Again, since *C. undulatum* seeds are gravity dispersed (Close et al., 2006) and small native bees are the likely main pollen vector, we estimated an average migration distance up to 500 m (Campbell & Husband, 2007). However, isolation may be both spatial and temporal. Indeed, flowering time is highly relevant as it is the first mechanism of reproductive isolation. To account for possible effects of temporal isolation, we recorded the reproductive phenology of this species once a week for the entire flowering season, and we evaluated the flowering synchrony between populations using a modified version of the method proposed by Freitas & Bolmgren (2008), replacing individuals with populations. Overall, populations were synchronous with a score of 0.53 on a scale from 0 to 1, being 0 asynchrony, 0.25 low synchrony, 0.5 synchrony, and 1 perfect synchrony (Fig. A.1 in Appendix A). Thus, there was no temporal isolation between populations. Finally, during the flowering season we counted the total number of inflorescences of each individual in populations with less than 20 plants, and from 20-40 randomly selected individuals in larger populations. We then

estimated the floral display of each *C. undulatum* population as: FD = ($I \bullet A_C$) / 100, where FD is the floral display index of a population; I is the mean number of inflorescences per plant in the specific population; and A_C is the area (in m^2) covered by *C. undulatum* plants within the fragment (obtained through ArcGIS using the minimum convex polygon method). *Conospermum undulatum* seeds are gravity dispersed and plants appear in clumps of similar density across all populations. Therefore, due to the biology of the species, plant density was not informative and was not considered further in this study.

From a total of 17 remnant populations of *C. undulatum*, we selected 12 populations encompassing the entire range of population sizes and levels of isolation. Since *C. undulatum* is a threatened species, licence conditions restricted collections to 20% of fruits per plant from 20% of plants in a population. So, at the end of the flowering season when flowers began to senesce, we collected fruits (and seeds) from haphazardly placed bags over 5 inflorescences per plant in 20-40 randomly selected plants per population. In small populations with less than 20 plants we bagged all the individuals; however, only seeds from 20% of the plants were kept, the rest was returned to the population of origin after being recorded. In the lab, we counted the number of flowers, fruits, and seeds collected for each plant (total of 65,020 flowers and 2,505 seeds from 210 selected plants). The number of flowers was assessed by counting the scars left on the white, woolly inflorescence stalk.

Seed viability was assessed by carefully nicking off a small portion of the fruit wall under a dissecting microscope. Seeds with firm, white embryo were classified as viable, as opposed to seeds with rotting embryos. Also, nicking the fruit wall is part of the recommended method for germinating *C. undulatum* seeds (Cochrane, 2007). Nicked viable seeds were placed in a 10% plant preservative mix (Plant Cell Technology, Washington, USA) for 10 minutes to prevent the formation of mould on the exposed embryo. Then, following the best-known germination treatment (Crawford A., personal communication, 2017) we soaked the seeds in 10% Regen2000© smokewater (Grayson Australia, Victoria, Australia) for 24 h before sowing them on 75% agar with 100 mg L^{-1} of gibberellic acid solution, to aid germination. Seeds were placed in a germination chamber with 12 h of daily photoperiod at 15°C, and scored for radicle emergence every two weeks for nine months. All seeds from each mother plant were kept separate.

To experimentally assess the extent of self-compatibility in *C. undulatum* we performed three experimental treatments in the field: pollinator exclusion (PE), pollinator-excluded triggered flowers without pollen supplementation (PET), and hand geitonogamous

self-pollination on pollinator-excluded flowers (PES). In a medium-sized population of *C. undulatum* (216 plants), we randomly selected ten plants per treatment two weeks before anthesis, and we placed fine mesh bags around three inflorescences per plant. In this way, we prevented insects from visiting the flowers (i.e. PE treatment). During anthesis, we triggered the stigma of PET flowers, and we hand-pollinated flowers of PES treatment with pollen from different flowers on the same plant by means of a 1-mm flathead screwdriver as this enabled us to reach the stigma.

Data analysis

Following data exploration, we removed fragment area and percentage of vegetated land around the population centroid from the variables list because of high multicollinearity, with a variance inflation factor (VIF) of 44.35 and 31.43, respectively. The variables retained were population size, isolation, and floral display which had no collinearity, with a VIF below the selected cut-off value of 2.5 (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). Separate GLMs were fitted for the following response variables: 1) proportion of fruit production; 2) proportion of seed production; and 3) proportion of germinated seeds. To account for non-normal distribution of residuals, non-homogeneous variances, and moderate overdispersion, we used quasi-binomial error distributions (appropriate for proportional data) and checked that the assumptions were fulfilled by visual inspection of residual patterns (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). Full models for fruit and seed production contained all the retained population descriptors (i.e. population size, isolation, and floral display) as the explanatory variables. Some small populations produced no viable seeds to be tested for germination, and so we removed those populations from the dataset of the third model (i.e. proportion of germinated seeds) because this could not be determined. However, by doing so, floral display presented a collinearity issue, with VIF above the 2.5 cut-off value; thus, we removed this variable from the relative full model. Starting from each of the three full models, model selection was then performed by excluding non-significant terms. Furthermore, the absolute value of the standardised regression coefficient (ß) of each scaled explanatory variable can be a useful metric for determining the relative importance of the respective predictors (Murray & Conner, 2009). Each explanatory variable was scaled by subtracting its mean and dividing by its standard deviation. We used separated models to rank the predictors. All statistical analyses were performed with R version 3.5.2 (R Development Core Team, 2018).

Results

Self-pollination

The results of the self-compatibility experiments are outlined first as these provide an important basis for understanding the results of the reproductive success analyses. Total insect exclusion treatment (PE) yielded zero fruits (and zero seeds) in all the ten replicates (Table 2.1). Similarly, even if the stigma was triggered, PET treatment resulted in zero fruits (and zero seeds). Together, these two treatments (PE and PET) demonstrate *C. undulatum* flowers do not self-pollinate and develop fruit unless visited by insects carrying a suitable load of pollen from previous floral visits. The hand self-pollination treatment (PES) produced fruits among the ten replicates (Table 2.1), with an average proportion of success of 0.264 (± 0.105). However, all the fruits contained aborted embryos and no viable seeds developed.

Table 2.1. Reproductive output of *Conospermum undulatum* in term of fruit and seed production for pollinator exclusion (PE), exclusion and triggered flowers (PET), and exclusion and hand-self-pollination (PES) treatments.

PE				PET				PES			
Plant ID	Flowers	Fruits	Seeds	Plant ID	Flowers	Fruits	Seeds	Plant ID	Flowers	Fruits	Seeds
1	75	0	0	*11*	49	0	0	*21*	10	0	0
2	69	0	0	*12*	57	0	0	*22*	10	5	0
3	72	0	0	*13*	38	0	0	*23*	18	9	0
4	47	0	0	*14*	53	0	0	*24*	45	0	0
5	99	0	0	*15*	41	0	0	*25*	40	0	0
6	63	0	0	*16*	41	0	0	*26*	10	1	0
7	109	0	0	*17*	37	0	0	*27*	28	1	0
8	67	0	0	*18*	44	0	0	*28*	10	9	0
9	99	0	0	*19*	40	0	0	*29*	10	6	0
10	101	0	0	*20*	39	0	0	*30*	14	0	0

Fruit production and seed production

Population size ranged from 5 to 880 plants (mean = 243.4); fragment area ranged from 0.34 to 51.25 ha (mean = 22.06); isolation index ranged between -0.04 and -21.41 (mean = -7.65); and floral display ranged from 0.21 to 715.70 (mean = 288.02). Fruit production was significantly related to the variability in floral display ($F_{1,175} = 38.28$, $P < 0.001$), with populations with higher floral display having the largest fruit output, as opposed to less visible populations, where the probability that a flower will develop a fruit dropped by 15 percentage points (Fig. 2.2; Table 2.2). There was no significant effect of population size ($F_{1,173} = 0.04$, $P = 0.835$) and level of isolation ($F_{1,174} = 0.18$, $P = 0.667$), therefore they were removed from the final model (Table 2.2).

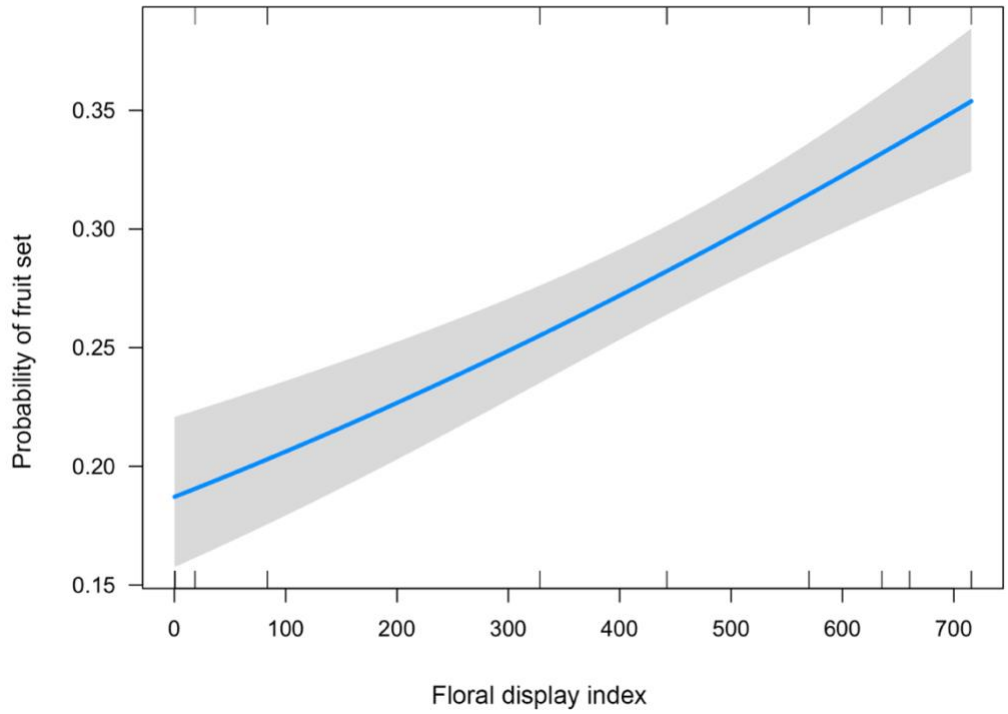


Figure 2.2. Effect of floral display index on the probability that a flower in *Conospermum undulatum* will develop into a fruit. Confidence intervals are in grey.

The probability that a flower will develop a seed showed a significant relationship with all the explanatory variables of population size, isolation, and floral display ($F_{1,173}$ = 29.80, $P < 0.001$; $F_{1,173}$ = 14.88, $P < 0.001$; $F_{1,173}$ = 16.80, $P < 0.001$, respectively). In particular, the response variable was positively related to population size (Table 2.2), with large populations having twice the probability of setting seeds than small populations (Fig. 2.3a). In contrast, the isolation effect was negative (Table 2.2), but of similar magnitude, with isolated patches having half the probability of setting seeds compared to more connected fragments (Fig. 2.3b). The effect of floral display was positive (Table 2.2), with the probability that a flower sets a seed increasing from 2.7% to 4.6% between the less visible and more visible populations (Fig. 2.3c). The three explanatory variables population size, isolation and floral display had a standardised ß coefficient of 0.239, -0.156, and 0.203, respectively (Table A.1 in Appendix A).

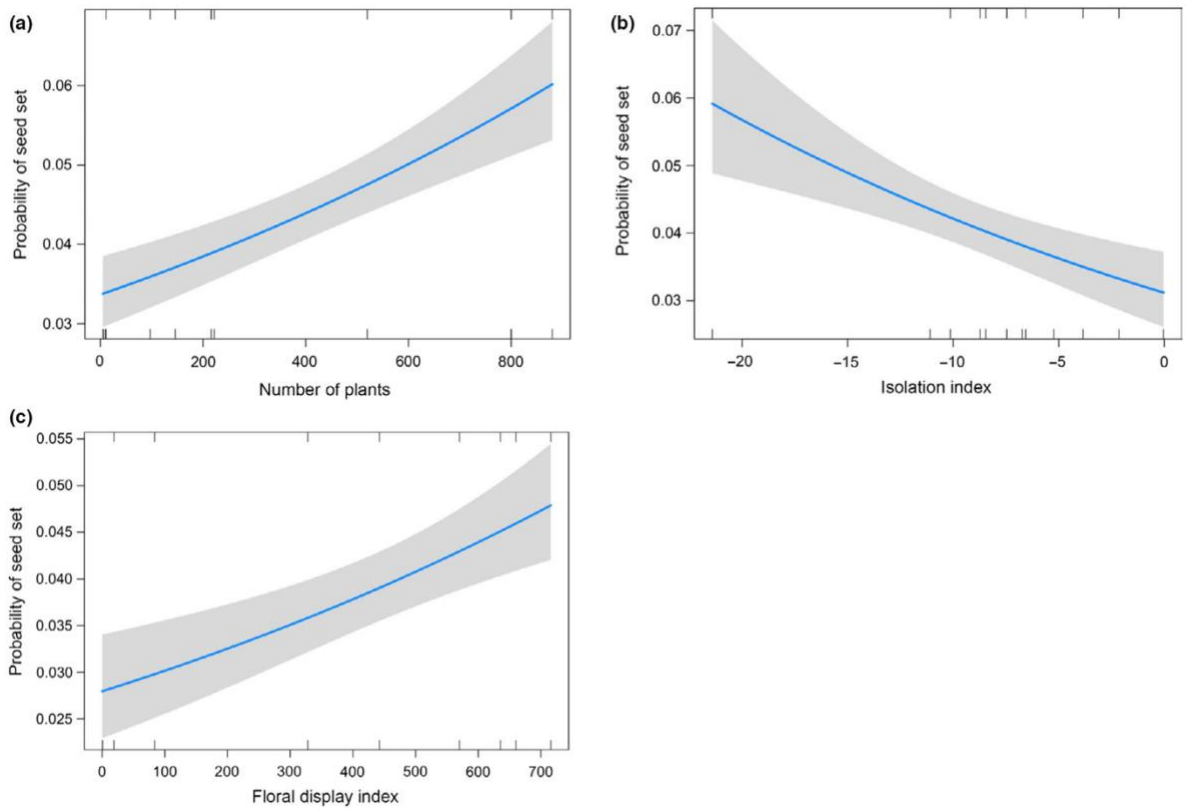


Figure 2.3. Effect of (a) population size, (b) isolation and (c) floral display on the probability of a flower in *Conospermum undulatum* to develop a seed. Confidence intervals are in grey.

Seed germination

Conospermum undulatum germination responses are known to be slow and highly variable (Crawford A., personal communication, 2017). From the 2,505 viable seeds obtained, we recorded 434 radicle emergences (17.33%) in the nine months germination period. There were significant effects of population size and isolation on the probability of a seed to germinate ($F_{1,160} = 11.01$, $P = 0.001$; $F_{1,160} = 10.90$, $P = 0.001$, respectively). The effect of population size was positive (Table 2.2), increasing from ~10% to ~20% probability of seed germination from small to large populations (Fig. 2.4a). A similar effect size, but negative, was found for the isolation variable (Fig. 2.4b; Table 2.2).

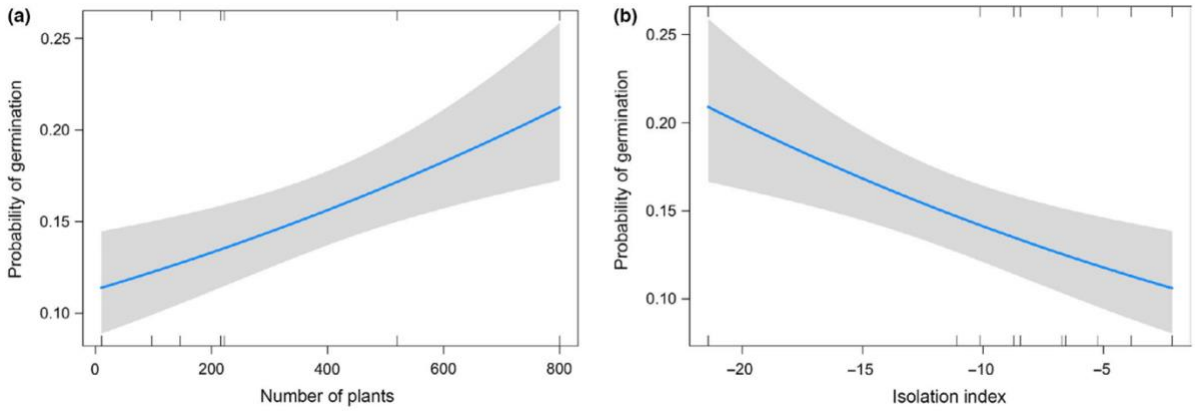


Figure 2.4. Effect of (a) population size and (b) isolation on the probability of a seed in *Conospermum undulatum* to germinate. Confidence intervals are in grey.

Table 2.2. Regression parameter estimates for fruit production, seed production, and germination models related to population size, isolation, and floral display variables in *Conospermum undulatum*.

Fruit production model			
	Estimate	Standard error	*P*
Intercept	-1.468943	0.105955	<0.001 ***
Floral display	0.001211	0.000201	<0.001 ***

Seed production model			
	Estimate	Standard error	*P*
Intercept	-3.935817	0.1508498	<0.001 ***
Population size	0.0006916	0.0001247	<0.001 ***
Isolation	-0.031324	0.0081266	<0.001 ***
Floral display	0.0007804	0.0001935	<0.001 ***

Germination model			
	Estimate	Standard error	*P*
Intercept	-2.423098	0.218199	<0.001 ***
Population size	0.0009374	0.000281	0.001 **
Isolation	-0.0415218	0.012637	0.001 **

Discussion

The mating system in *Conospermum undulatum* is consistent with those found in the majority of proteaceous species (Collins & Rebelo, 1987). Total exclusion of insects from flowers resulted in our target species not being able to produce fruits, which demonstrated the requirement of visitation by a pollinator. The stigma, once triggered, flicks away from the anthers towards the lower tepals; this mechanism can only be activated once, thus the exclusion plus triggered flowers treatment showed that pollen grains exploded from the anthers were unable to reach the downward-facing fertile part of the triggered style within the same flower, highlighting the efficacy of the trigger mechanism as a physical barrier to

autogamous self-pollination. This is similar to other observations on eastern Australian species in the genus, including *C. taxifolium*, *C. ericifolium*, *C. ellipticum* and *C. longifolium* where no autogamous self-pollination was found when pollinators were excluded (Morrison, McDonald, Bankoff, & Quirico, 1994). Results from the hand self-pollination treatment (i.e. geitonogamous selfing) suggests that self-incompatibility in *C. undulatum* was not only due to its specific flower morphology that prevents autogamy but was also a genetic response to prevent geitonogamy.

We have demonstrated that habitat fragmentation, when combined with the *C. undulatum* mating system, has far reaching effects on the reproductive potential of the species. Against our initial expectations, fruit production responded to only one population descriptor, that being floral display, suggesting the only variable that affected the production of fruits was the capacity of a population to attract pollinators. This result shows the importance that floral display may have from a conservation point of view, particularly for mass-flowering species that rely on huge floral displays to attract pollinators. Indeed, fragmentation of the habitat may result in patches that are not attractive for floral visitors due to a lack of resources. This result agrees with observations in other species where habitat fragmentation and its effect on floral display were the key determinant of pollinator abundance and, ultimately fruit production (Delmas et al., 2014; Goulson et al., 2008). This is particularly important considering that the native bee *L. conospermi* (Hymenoptera) is likely to be the main pollen vector of *C. undulatum*, since hymenopterans are found to be influenced to a greater extent by a reduction in floral display of mass-flowering plants compared to dipterans (Delmas et al., 2014). Moreover, since a fruit can only develop after an insect visit, this suggests that the populations of *C. undulatum* with lower floral display index may be limited by pollen quantity due to a lack of pollinators. This may have a cascading effect on reproductive success and is worthy of further investigation, especially considering that pollen deposition and fruit production are essential steps in plant sexual reproduction.

A second, no less important step leading to seed production is the development of a healthy embryo. *Conospermum undulatum* is a resprouting plant, with a life-history strategy adopted by 66-80% of the plant species in the SWAFR (Bell, 2001). These species are able to regenerate vegetatively after disturbance, such as fire or herbivory, and their seed set is generally low (Lamont et al., 2011). Nonetheless, although the seed production was expected to be low in *C. undulatum*, our results showed that the probability of setting seeds is doubled in large and connected populations with a high floral display index compared to small, isolated and less attractive ones. The effect of habitat fragmentation on seed production has

been investigated in numerous plant species with different compatibility systems and life-history strategies and found to be detrimental (Aguilar et al., 2006; Marcelo A. Aizen et al., 2002). In particular, these studies found that the reduction of the reproductive output was mainly due to disrupted interaction between plants and their pollinators following habitat fragmentation. The present study is consistent with these results and suggests that this negative effect can also be observed for mass-flowering resprouter species.

Moreover, if the lack of floral visitors was the only factor involved, it would have been reasonable to expect floral display to be the only significant factor for seed production, as it was for fruit production. However, in this case population size and isolation also became highly significant factors, consistent with our initial hypothesis. This suggests that besides the lack of floral visitors, genetic factors that prevent the development of the embryo and result in empty fruits may be present in small and isolated populations. Following our results of hand self-pollination, it is reasonable to conclude that the recorded discrepancy between fruit production and seed production may reflect late acting self-incompatibility, possibly due to an increased geitonogamy rate in small populations (Eckert, 2000), and/or inbreeding effects, resulting in a higher proportion of aborted seeds. Furthermore, the standardised regression coefficients demonstrate that population size was the most important variable in determining the production of seeds, with floral display and isolation also found to be important factors. These factors are essential considerations when planning conservation actions, such as translocations and reintroductions, in order to maintain adequate seed production in a population. In particular, the standardised ß coefficient of floral display was higher than that of isolation, underpinning the importance of floral display in the reproductive success of the mass-flowering *C. undulatum*.

The last step in the (sexual) reproductive cycle is seed germination. We found patterns of response variable for germination to be similar to those of seed production, and in line with our initial hypothesis, namely that seeds produced in small and isolated populations resulted in a lower probability of germination. Since a viable seed has been produced, self-incompatibility issues are drastically reduced at this point of the reproductive cycle of *C. undulatum*. Recent studies have found that in some cases increased isolation and a reduction of population size is not associated with an increase in biparental inbreeding (e.g. Byrne, Elliott, Yates, & Coates, 2007). This is due to an expansion of the usual foraging range of highly motile pollinators, such as birds or honeybees, in response to fragmentation. However, for plants pollinated by small, less-motile pollen vectors, this is unlikely to be the case. This hypothesis was tested by Breed et al. (2015) in a case study of three *Eucalyptus* tree species;

for the two small-insect-pollinated eucalypts, increased selfing and decreased pollen diversity was correlated with increased fragmentation, but no such relationship was evident for the bird-pollinated eucalypt species. Therefore, our result is consistent with the hypothesised lack of extended gene flow able to rescue small and/or isolated populations from the effects of inbreeding (Aguilar et al., 2008; Honnay & Jacquemyn, 2007).

Although the Proteaceae are amongst the most widely studied Australian native plants, most research has focused on species of *Banksia*, *Hakea* and *Grevillea*, with only a few studies specifically on *Conospermum* species. Moreover, most of the *Conospermum* research had different purposes being mainly focused on identifying the cues that stimulate seed germination (e.g. Tieu, Dixon, Meney, & Sivasithamparam, 2007), without taking into account other factors influencing seed production. Indeed, although germination of seeds is a crucial life-history event, it may not inform conservation planning if considered on its own, because other important processes such as plant-pollinator interactions and gene flow are also likely to constrain reproduction.

This study has identified several aspects of the reproductive biology of *Conospermum undulatum* that add to the growing base of knowledge of this genus, and Proteaceae in general. Habitat fragmentation appears to be a significant threat to the future persistence of *C. undulatum*, and its effects were readily visible in the results of this study. Every stage of sexual reproduction was directly and significantly affected by aspects of habitat fragmentation. Ultimately, urban expansion on the Swan Coastal Plain may result in patches of native vegetation that are unattractive for pollinators, and too small and isolated to ensure long-term population viability and adaptation ability based on reproduction by seeds. Future studies to help maximise the conservation effort should focus on clearly identifying the pollinator assemblage associated with successful pollination of this endemic species, as well as assessing the impact of habitat fragmentation on these essential floral visitors.

Supplementary material

Flowering phenology of different populations of *C. undulatum* (Fig A.1 in Appendix A), standardised ß coefficient of explanatory variables (Table A.1 in Appendix A).

Chapter 3 - Habitat fragmentation restricts insect pollinators and pollen quality in a threatened Proteaceae species

This chapter has been published as the following paper with only minor changes to population codes to ensure consistency throughout this thesis:

Delnevo N, van Etten EJ, Byrne M, Petraglia A, Carbognani M, Stock WD. 2020. Habitat fragmentation restricts insect pollinators and pollen quality in a threatened Proteaceae species. Biological Conservation, accepted.

Introduction

Anthropogenic loss and fragmentation of natural habitats are pervasive problems in terrestrial ecosystems (Haddad et al., 2015). The rate at which such natural systems have been converted to urban and agricultural use has been increasing during the last 60 years and now is at unprecedented levels (Ellis et al., 2010), with both demography and reproduction of wild plants being directly and negatively affected (Aguilar et al., 2006). It is widely known that reproduction by seeds has a key role for fitness, migration, adaptation and, ultimately, population persistence of plant species. Yet, as a consequence of land use change, many plant and pollinator populations are declining and mutualistic plant-pollinator interactions are frequently disrupted (Potts et al., 2016; Sánchez-Bayo & Wyckhuys, 2019). Consequently, since 90% of flowering plants depend on animal pollinators (Ollerton et al., 2011), it is important to understand how land use changes affect the pollinator fauna and the impact of this on plants.

Habitat fragmentation leads to a change in the sizes of remnant populations and their spatial arrangement (i.e. decreased connectivity). Such change may alter pollinator assemblages between fragments, with small and/or isolated populations expected to lose the most specialized taxa of floral visitors, with possible cascading effects on the visitation rate (Lázaro et al., 2020). This could lead to a phenomenon known as pollen limitation. Although extensive reviews outline that pollen limitation is widespread among flowering plants (Knight et al., 2005), habitat fragmentation may exacerbate this phenomenon and, consequently, decrease plant population viability (Newman et al., 2013; Wagenius & Lyon,

2010). A reduction in pollination events following fragmentation can reduce the number of pollen grains deposited on stigmas, leading to a decrease in pollen quantity. However, pollen limitation can also depend on the quality of pollen. Indeed, genetic drift in small populations of self-incompatible plants can cause a reduction of genetically unrelated mating partners (Les et al., 1991). Therefore, even if an adequate amount of pollen is transferred, increased self-pollen transfer (Eckert, 2000) or the presence of only a few unrelated mates render most pollination events unsuccessful (due to decrease in pollen quality; Campbell & Husband, 2007; Wagenius et al., 2007). All these factors combined with an increased exposure to demographic and environmental stochasticity, are likely to increase the risk of extinction of small, isolated populations of plants (Frankham, 2005). This makes understanding of plant-pollinator interactions vital for the conservation of threatened plant species that are experiencing habitat reduction.

Although several studies have assessed the effects of fragmentation on pollination and reproduction of species with massive floral displays (e.g. Aguilar et al., 2012; M. A. Aizen & Feinsinger, 1994; Herrerías-Diego et al., 2006), the impact of varying floral display on pollinators assemblages and flower visitation rate has received less attention. In general, small populations of mass-flowering species have been shown to remain highly attractive to pollinators and may not be affected by the detrimental effects of fragmentation (Westphal et al., 2003). However, this hypothesis has mainly been tested on plant species able to rely on European honeybee *Apis mellifera* and bumble bees (*Bombus* spp.) for pollination (e.g. Diekötter et al., 2010), important and widespread pollinators in Europe, but not native to many parts of the world. Consequently, results from these studies may not be transferable to plants that evolved species-specific pollination mutualisms with native pollinators, such as the ancient Gondwanan family of Proteaceae. Understanding the impact of habitat fragmentation on such highly specialized systems, therefore, is particularly relevant, especially when native plants are rare and threatened.

The native bee fauna of Australia is large and diverse, and differs from bees of other continents (Houston, 2018). In particular, the ground-nesting genus *Leioproctus*, one of the largest genera in the predominant family of Australian bees, Colletidae, is believed to have evolved before the break-up of Gondwana (Almeida et al., 2012), and is unlikely to have reached the Australian continent across expanses of ocean during more recent periods (Houston, 2018). Further, many flowering plants in Australia have evolved in isolation for at least 34 million years (Mcloughlin, 2001). Such a long period of isolation allowed for the development of very specific floral morphologies and pollination systems, making Australia

an ideal location for studying the effects of habitat fragmentation on specialized mutualisms between plants and their native pollinators.

Although many proteaceous species coevolved with birds for pollination, plant-insect mutualisms are also an important component of Australian ecosystems and a high number of species belonging to this ancient family rely exclusively on native insects for pollination (Hopper & Gioia, 2004). *Conospermum* (Proteaceae) is an insect-pollinated genus with its center of distribution being the south-west corner of Western Australia. This genus includes 53 species (Bennett, 1995) with four taxa already listed among the threatened flora of Western Australia (W.A. Government Gazette, 2018). The majority of species in this genus have characteristic flowers that appear to be too small to be effectively pollinated by introduced *A. mellifera* (Delnevo et al., 2020 a). In the southwest Western Australia, the native bee *Leioproctus conospermi* is the primary pollinator of several *Conospermum* species, with ants providing effective pollination service as secondary pollinators (Delnevo et al., 2020 a).

In this study, we combined an observational approach to clearly define the pool of floral visitors, their activity, and their flower visitation rate, with an experimental manipulation to assess the degree and type of pollen limitation (quantity vs quality) in the threatened *Conospermum undulatum*. Specifically, we ask: 1) Which species constitute the pollinator assemblage of *C. undulatum* and does the assemblage differ among fragments?; 2) is the insect visitation rate for *C. undulatum* similar among fragments characterized by different sizes and levels of connectivity?; 3) is *C. undulatum* limited by pollen quantity and/or quality?; and 4) does the type of pollen limitation differ between small and large fragments?

Materials and methods

Study area

The study was performed over two flowering seasons (2017 and 2018) in a total of eleven populations of *C. undulatum* (out of 17 known populations) encompassing the range of population sizes and levels of connectivity of the species (Table 3.1). Populations were separated by 0.9–9 km and embedded within an urban matrix in the Swan Coastal Plain bioregion. This region is a low-lying coastal plain that extends from Jurien Bay, north of Perth, to Cape Naturaliste in the south, and is part of the Southwest Australia global biodiversity hotspot (Mittermeier et al., 2004). The climate is typically Mediterranean and

was very similar in 2017 and 2018, with mean annual temperature of 18.8 and 18.9°C, and annual precipitation of 729 and 744 mm, respectively (which is just below the long-term average for the area). This region has been recently cleared for urbanization, which is centred around Perth, the capital city of Western Australia. Urbanization has more than doubled since the 1970s and has reduced natural and seminatural vegetation on the Swan coastal plain to 34.7%, of which only 10% occurs in protected areas (Wardell-Johnson et al., 2016). Our target species, *C. undulatum*, has a naturally restricted distribution in eastern Perth region of 55 km^2 but is now threatened due to clearing for urban expansion and associated fragmentation.

Table 3.1. The characteristics of the eleven populations of *Conospermum undulatum*.

Population	Population size	Connectivity index	Floral display index
A	880	7.46	328.3
B	800	8.72	635.4
C	520	10.13	715.7
E	310	18.60	674.3
H	216	2.15	660.2
J	139	21.41	83.7
K	12	6.57	18.8
L	10	11.09	0.6
M	7	0.04	0.3
N	7	5.24	0.21
O	4	6.73	0.6

Study species

Conospermum undulatum is a declared threatened plant species, and it is regarded as 'vulnerable' following IUCN red list criteria (Department of Environment and Conservation, 2009; W.A. Government Gazette, 2018). This species is a mass-flowering, lignotuberous plant that grows as an erect, compact shrub up to 1.5 m tall. The hermaphroditic flowers have long, white hairs and are produced in inflorescences held well above the leaves. During the flowering period (late August to late October), its white inflorescences dominate the (non-fragmented) landscape. The flowers of *Conospermum* possess an active pollination mechanism: the style is bent, and the flower opens in a state of tension (Douglas, 1997). When a visiting insect applies pressure to the base of the style with its mouthparts, the style

flicks away from the anthers and the downward-facing fertile stigma strikes the visitor and picks up any pollen carried by the insect. At the same time the fertile anthers dehisce explosively, casting new pollen onto the visitor. In a recent study, Delnevo et al. (2019 a) found that this pollination mechanism is an effective physical barrier against the pollination of a flower with its own pollen since the grains exploded by the anthers (positioned in the upper part or the flower) are unable to reach the downward-facing stigma (see Douglas (1997) for morphological description). Moreover, *C. undulatum* possesses a strongly developed self-incompatibility system that prevents the development of the embryo following self-pollination (Delnevo et al. 2019 a).

Conospermum have a sessile ovary with a single pendulous ovule (Douglas, 1997). Fruits are cone-shaped achenes (i.e. containing only one seed), covered with tan orange hairs. Seeds fall with the fruits and their dispersal, being gravity-driven, is mainly limited to a few meters from the mother plant (Close et al., 2006).

Population size, connectivity and floral display

In September 2017, population size was assessed in each fragment by direct count of plant individuals. The connectivity index was calculated based on a modified version of the incidence function model (Hanski, 1994), following the approach used by Delnevo et al. (2019 a). This model accounts both for distances to all neighbouring populations (including those not selected for this study) and the area of those populations, providing a better estimate in highly fragmented habitats and small datasets compared with either nearest neighbour or buffer measures (Moilanen & Nieminen, 2002). Temporal isolation of populations was investigated through observations of reproductive phenology and found to be negligible (Delnevo et al., 2019 a). Lastly, we counted the total number of inflorescences of each individual in populations with less than 20 plants, and from 20–40 randomly selected individuals in larger populations. We then calculated the floral display index (FD) of each population as: $FD = (I \bullet AC) / 100$, where I is the mean number of inflorescences per plant in a specific population; and AC is the area (in m^2) covered by *C. undulatum* plants within the fragment (minimum convex polygon method).

Since *C. undulatum* has a clumped distribution of individuals that is similar across all the populations (Close et al., 2006), plant density could be considered as non-informative and was not considered further in this study.

Surveys of flower visitors and flower visitation

Observations were performed on twelve randomly selected plants in each target population (or on all the plants in populations with less twelve individuals). Surveys were conducted during peak flowering on days with weather conditions favourable to pollinator activity (i.e. sunny days with slight to no wind). At the beginning of the census, the observer stood at 1.5 m from the selected individual for 2 minutes to allow insects to become accustomed to the observer's presence and then recorded insect activity for 10 minutes. We performed two rounds of observations on each plant each year in populations B, C, H, J, K, L and N, whereas populations A, M, and O were surveyed only in 2017 and population E only in 2018, for a total of 3100 minutes of observations. All the twelve selected plants in a population were observed during the same session (one after the other). Days and hours of observation were randomly applied across populations for the first round. In the following round, we performed the survey of a population in the afternoon if it had been done in the morning during the first round and vice versa. During each census, we recorded the number of available (untriggered) flowers, and a visit was recorded each time an insect probed an untriggered flower; visitation frequency was low enough to allow all visits to be recorded. For identification purposes, the insects were collected from inflorescences of *C. undulatum* using clear 50 mL tubes after recording whether there was stigmatic contact. We then induced cold anaesthesia by placing the tube containing the insect on ice. This allowed us to take macro photographs of the insect that were used for the identification with the help of professional entomologists (B. Heterick & T. Houston; Western Australian Museum). The captured insects were released as soon as the photographs had been taken. When a species level identification was unfeasible, we identified visitors to recognizable taxonomic unit (RTU; Oliver & Beattie, 1996).

Pollen limitation

To assess the amount of pollen quantity and/or quality limitation we performed three experimental treatments: cross-pollination with pollen from within the same population (+Within), cross-pollination with pollen from different populations (+Between), and open pollinated control. Since *C. undulatum* is a threatened species, license conditions restricted the availability of plants that could be manipulated within a population. This led to a reduction in the set of populations used for the pollination experiment. Small populations with seven (populations M and N) and four (population O) individuals were not included in the experiment due to a lack of available plants to perform the three treatments. In each

population, we randomly selected three to seven individuals per treatment (depending on population size). To prevent insects from visiting the flowers, we placed fine mesh bags around three inflorescences per plant one week prior to anthesis (except for control plants). Upon treatment, each flowering stalk was culled to an average of ten mature flowers; this was done to prevent accidental triggering of flowers during the manipulation. Flowers were then treated by means of inserting a 1-mm flathead screwdriver with pollen on its tip into the floral tube, as this enabled us to reach the stigma. The inflorescences were re-bagged immediately after manipulations. For the within-population crosses (+Within treatment), we collected pollen from mature untriggered flowers from random individuals at least 10 m away from the receiving plant to reduce the change of sampling genetically related individuals. This was not always possible in small populations due to the spatial occurrence of *C. undulatum* plants. For the between-population crosses (+Between treatment), pollen from mature flowers was collected from five random individuals in the source populations and used in the receiving population within one hour. The source populations were selected as the closest large population to the receiving fragment. Inflorescences of control plants were left unmanipulated and freely available to pollinators. For the controls, the bags on three random inflorescences were only placed at the end of the flowering period to collect the fruits.

In the laboratory, we counted the number of flowers (only for the control treatment), fruits, and seeds collected for each plant and we obtained a total of 4145 flowers.

Data analysis

Variation in insect visitor assemblage among populations was investigated by means of Principal Component Analysis (PCA). PCA was performed on the normalized average number of visits recorded in 2017 and 2018 (single year analyses in Fig B.3 of Appendix B). The broken stick criterion was used to select the number of axes retained in the PCA (Legendre & Legendre, 1998). To aid the interpretation of ordination axes, external variables (i.e., population size, connectivity, and floral display) scaled to zero-mean and unit-variance were projected onto PCA, and the significance of their correlations with ordination axes was assessed by means of permutation test. This analysis was carried out with the package "vegan" (Oksanen et al., 2019) of R v3.6.3 (R Development Core Team, 2020).

To analyse whether flower visitation was related to the population characteristics, we fitted a generalized linear mixed effect model (GLMM) with the proportion of visited flowers out of the total available flowers as the response variable. In this model, population size, connectivity, floral display (FD) and their two-way interaction were considered as fixed

effects. We used year and individual plant nested within population as random effects to account for population effect and repeated measurements in time on the same individual. Model selection was performed by excluding non-significant terms and multicollinearity among predictors was checked by calculating the variance inflation factor with a cut-off level of 3 (Zuur et al., 2009). The variables population size and FD were correlated (r = 0.67) but only FD was retained in the final model. The variance inflation factor of each explanatory variable in the final model was below the selected cut-off value.

Conospermum undulatum is a self-incompatible plant that shows a late acting self-incompatibility even after self-pollination between different flowers of the same plant; that is, the achene (i.e. single seeded fruit) starts to grow but the embryo never fully develops, ending up as an empty fruit (Delnevo et al., 2019 a). Therefore, to investigate the degree and type of pollen limitation we used both the proportion of fruits and the proportion of seeds out of the total number of flowers (i.e. ovules) for each individual plant as the response variables in two separate GLMMs. We ran these GLMMs with pollination treatment (3-level factor: +Within, +Between, and control), log-transformed number of plants, and their interaction as the explanatory variables, and individuals nested within populations as the random effect. In this analysis, we only used the number of plants as the population descriptor because we performed hand-pollination treatments, which meant the variables connectivity and floral display were no longer informative.

For GLMMs on both flower visitation and pollen limitation, transformations were applied to explanatory variables to improve their linear relationships with the response variables. In particular, we applied log-transformation to population size to reduce right skewness and square-root-transformation to FD, which depends on population area. Each explanatory variable in all the GLMMs was scaled by subtracting its mean and dividing by its standard deviation to allow comparison between predictors. All of our response variables were proportions, therefore we used binomial error distribution (appropriate for proportional data) to account for non-normal distribution of residuals in each model and checked that the model assumptions were fulfilled by visual inspection of residual patterns (Zuur et al., 2009). The effect of each fixed effect term in the GLMMs was tested by specifying type II Wald χ^2 tests via the "Anova" function in the R package 'car' (Fox & Weisberg, 2019). Analyses were performed using the function glmer in the R package lme4 (Bates et al., 2015).

Results

Pollinator assemblage and flower visitation

The flowers of *C. undulatum* were visited by eight insect RTUs from five families belonging to two different orders. Hymenopterans were the most frequent visitors; the native bee *Leioproctus conospermi* was the most active insect, followed by ants (Supplementary material, Appendix B). *Apis mellifera* was excluded from the pollinator assemblage as they appeared too large to pollinate the small flowers of *C. undulatum*. Indeed, honeybees trigger the stigma with only their proboscis while foraging for nectar and without inserting their head into the calyx (Supplementary material, Appendix B). The triggered stigma, therefore, is unable to reach the body of the visitor to collect the pollen deposited during previous floral visits, thereby depleting floral resources and impeding the flower to be pollinated in subsequent visits by more effective pollinators. The small populations showed a marked reduction of taxa in their pollinator assemblages and were missing the most important pollinator of our target species, the native bee *L. conospermi* (Fig. 3.1a). In particular, we only recorded a small number of syrphid flies in small patches of *C. undulatum*.

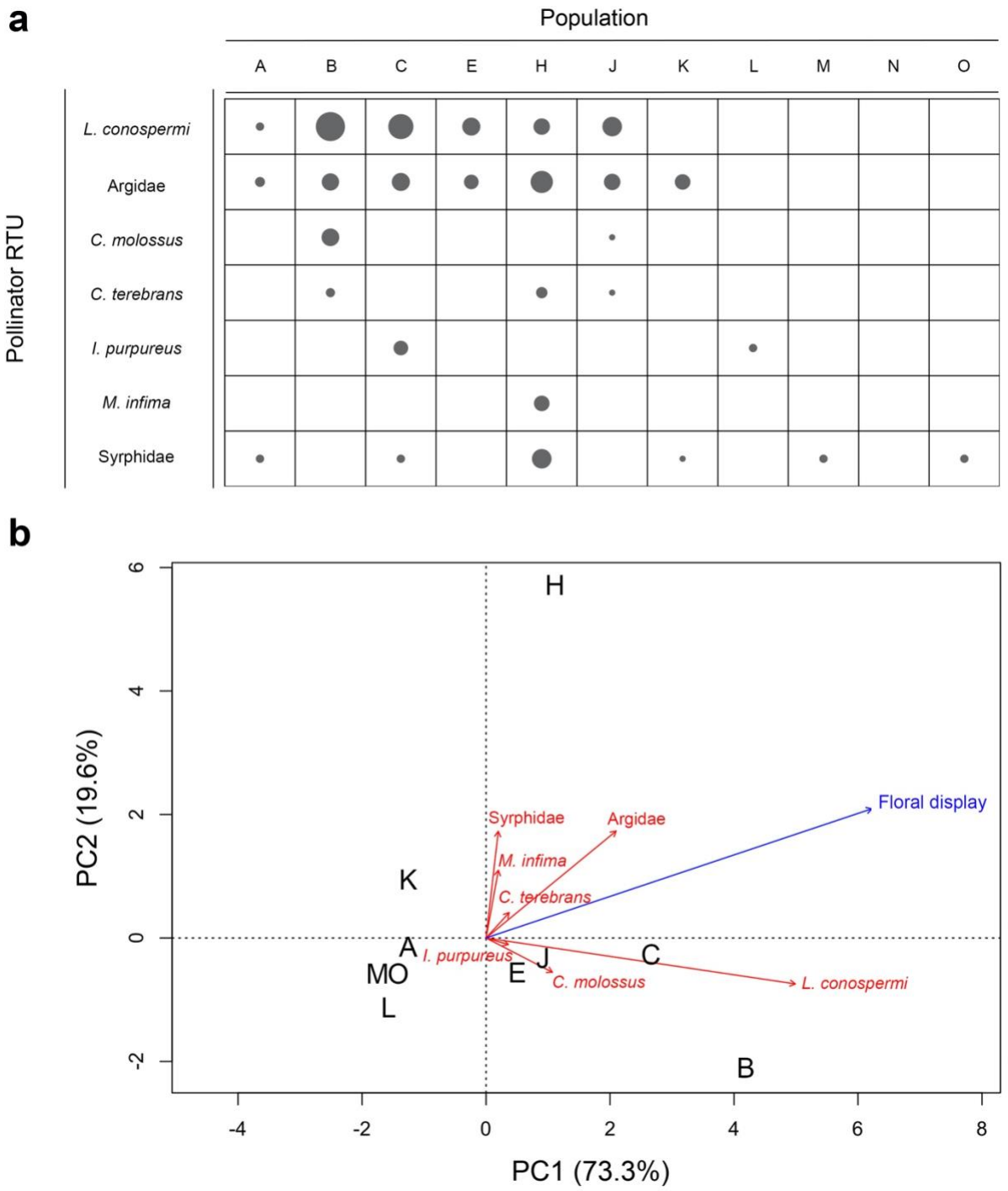


Figure 3.1. (a) Abundance of pollinator recognizable taxonomic units (RTUs) in different populations. Populations are ordered by decreasing population size and the size of circle is proportional to number of pollinator visits. (b) Principal Component Analysis (PCA) diagram (biplot type) with vector of floral display.

The populations investigated were separated in two groups along the first ordination axis, explaining 73.3% of the variation in visitor assemblages (Fig. 3.1b). In particular, the populations B, C, E, H and J, with positive values of PC1, were primarily characterized by a greater occurrence of the native bee *L. conospermi*, whereas the other populations showed a

reduced pool of pollinators. Along the second ordination axis, explaining 19.6% of the variation, the population H was markedly distinguished from the other ones by a higher occurrence of the ant *Myrmecia infima* and Syrphidae.

Among the three external variables tested, only the floral display was significantly correlated with the first two ordination axes ($R^2 = 0.70$, $p = 0.004$; Fig. 3.1b), showing the highest positive correlations with flying hymenopterans, such as *L. conospermi* and argid sawflies. Similar results were obtained when analysing the two years separately (Fig B.3 in Appendix B.

The probability that a flower was visited by an insect showed a significant relationship with the variables floral display (FD) and connectivity (Wald $\chi^2_1 = 70.63$, $p < 0.001$; Wald $\chi^2_1 = 10.19$, $p = 0.001$, respectively). In particular, the response variable was mainly related to FD, for which the probability of flower visitation increased from 1.5% to 74.3% between populations with lower and higher levels of floral display (Fig. 3.2a). A positive effect was also found in relation to connectivity, with flowers of plants in more isolated populations having four times less probability of being visited than those in connected populations (Fig. 3.2b). There was no significant effect of population size (Wald $\chi^2_1 = 0.47$, $p = 0.492$); therefore, this variable was removed from the final, parsimonious model.

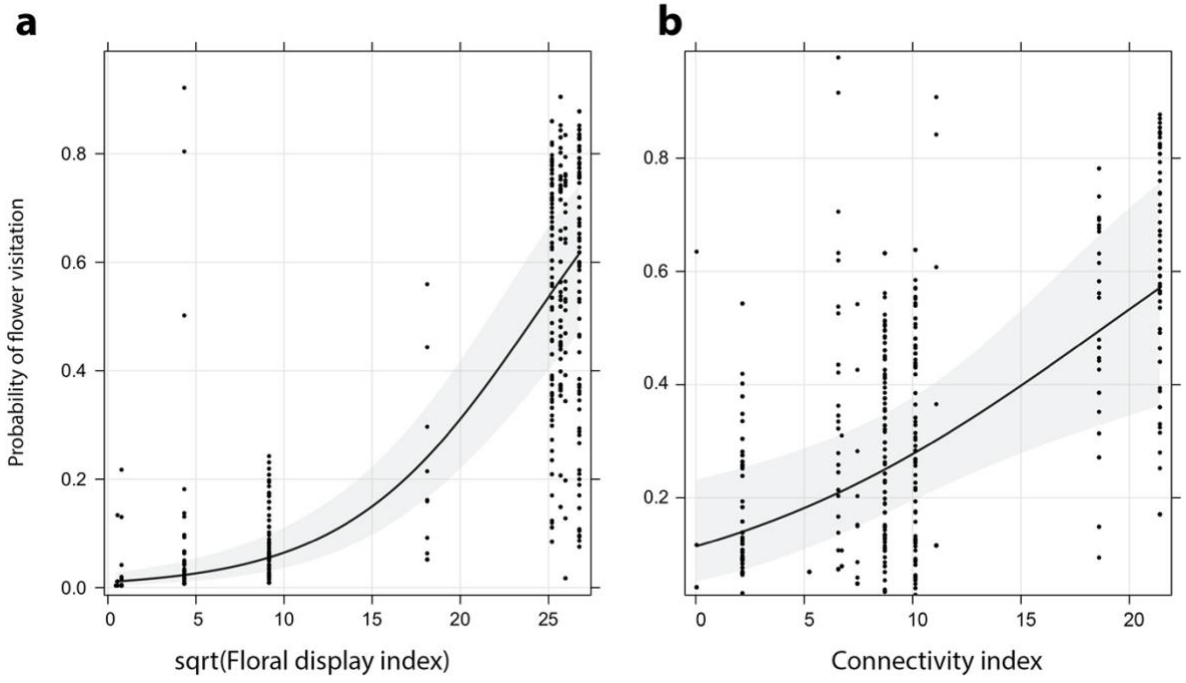


Figure 3.2. Effect of (a) floral display (square-root-transformed) and (b) connectivity on the probability of a flower of *Conospermum undulatum* to be visited by a pollinator. Grey bars indicate 95% CI.

Pollen limitation

Treatments significantly influenced the production of fruits (Wald $\chi^2_2 = 105.07$, $p < 0.001$), with an average proportion of flowers that produced fruits of 33.03% (SE 4.2) in control plants, whereas the +Within and +Between (within- and between-populations pollen addition) plants averaged 79.0% (SE 3.3) and 84.4% (SE 3.6), respectively. Fruit production was also significantly related to the population size (Wald $\chi^2_1 = 17.876$, $p < 0.001$). This population variable mostly affected control plants, which showed an increase from ~10% in small populations to ~50% in larger ones (Fig. 3.3a). The significant interaction between population size and treatment (Wald $\chi^2_2 = 7.074$, $p = 0.029$) indicated that pollen addition had different effects in small and large populations. Indeed, the increase produced by +Within and +Between compared with the control was greater for small populations in comparison with large ones (Fig. 3.3a; Table 3.2). In particular, the probability of fruit set in +Within treatment ranged between 75% and 85% from small to large populations, while the +Between treated plants had a similar probability of ~90% in all the populations (Fig. 3.3a).

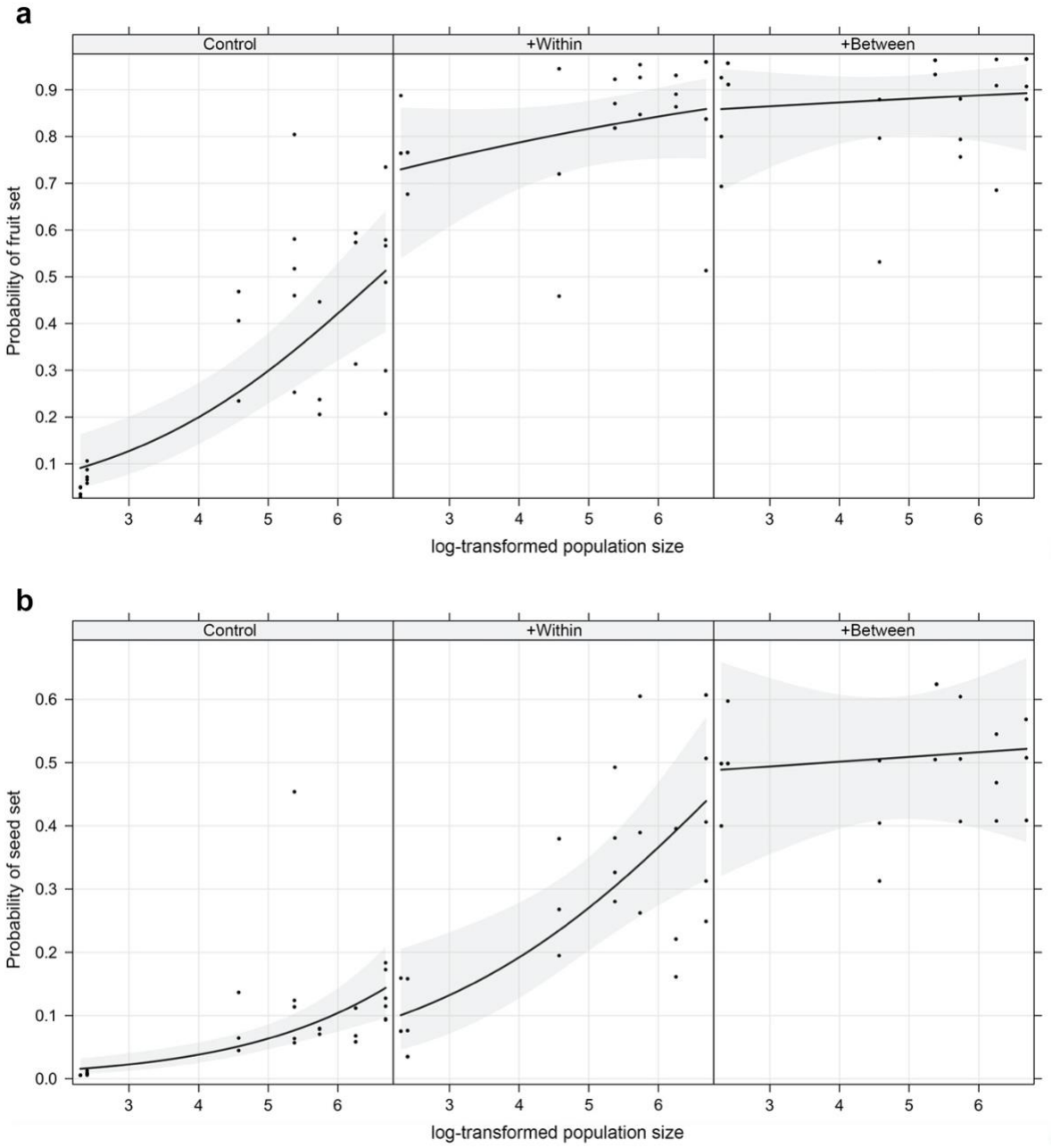


Figure 3.3. Effects of population size on (a) fruit and (b) seed production of *Conospermum undulatum* in relation to different pollination treatments. Bands indicate 95% CI. Control: unmanipulated flowers; +Within: within-population cross-pollination; +Between: between-populations cross-pollination.

Treatments significantly influenced the production of seeds (Wald $\chi^2_2 = 213.39$, $p < 0.001$); the probability that a flower will set a seed in naturally pollinated plants (controls) averaged 8.04% (SE 1.3), whereas the average for the +Within and +Between treatment were 31.6% (SE 3.4) and 50.5% (SE 2.4), respectively. The seed set per plant was positively correlated with increasing population size (Wald $\chi^2_1 = 12.33$, $p < 0.001$; Fig. 3.3b; Table 3.2). In particular, the probability that a naturally pollinated flower will develop a seed increased from 1.4% to ~15% with increasing population size (Fig. 3.3b). Similar to fruit production,

we found a significant interaction between the two explanatory variables (Wald $\chi^2_2 = 17.393$, $p < 0.001$). In this case, the increment in seed production with increasing population size was similar between control and +Within plants, which had a ten-fold increase in the probability of seed production in small populations (Fig. 3.3b). However, +Between-treated flowers were not affected by the variable population size (i.e. flat line response; Fig. 3.3b; Table 3.2), and their probability of seed set was five times higher than +Within treatment in small populations (~10% compared with ~50%), but the two treatments had similar outcomes in larger ones (Fig. 3.3b).

Table 3.2. Regression parameter estimates in *Conospermum undulatum* for fruit and seed production models related to treatments, population size, and their interaction.

	Estimate	SE	p value
Fruit set model			
Control (intercept)	-0.944	0.185	<0.001
+Within	2.406	0.289	<0.001
+Between	2.931	0.347	<0.001
Population size	0.901	0.184	<0.001
+Within × population size	-0.590	0.287	0.040
+Between × population size	-0.780	0.348	0.025
Seed set model			
Control (intercept)	-2.796	0.175	<0.001
+Within	1.733	0.199	<0.001
+Between	2.832	0.203	<0.001
Population size	0.920	0.194	<0.001
+Within × population size	-0.184	0.228	0.421
+Between × population size	-0.878	0.216	<0.001

Discussion

Pollination is a critical element of plant sexual reproduction and our study demonstrated that habitat fragmentation has far-reaching effects on a plant species that relies exclusively on native insects for pollen transfer. Fragmentation of remnant bushland affected the pool of pollinators, flower visitation, and both the quantity and quality of available pollen, which, in turn, is likely to affect the future persistence of the threatened *C. undulatum*.

Pollinator assemblage

This study has shown that the mutualistic relationship between the native bee *L. conospermi* and *C. undulatum* is consistent with the morphology of this bee species as described by Houston (1989). He reported the presence of morphological traits, such as the reduction of maxillary palpi, which might contribute to maximizing the pollination process in *Conospermum*. Ants were found to be potential cross-pollination agents for *C. undulatum* in a recent work aimed at clarifying the ecological role of ants in the region (Delnevo et al., 2020 a), and the present study confirmed their importance for this threatened species as ants were the second-most active floral visitors.

We found that populations with higher floral display have a more complex pollinator assemblage. In contrast, fragments with reduced floral display had an impoverished pool of pollinators, consisting only of generalist and poorly effective taxa (Delnevo et al., 2020 a). These results are consistent with other findings indicating that flying hymenopterans are more influenced by fragmentation compared with dipterans because the former are typically central place foragers fixed on a nest site (Winfree et al., 2009). Moreover, floral display had a substantial role in determining the number of recorded *L. conospermi* and argid sawflies (Hymenoptera). The influence of floral display became evident in population A which, despite being the largest population with a varied pollinator assemblage, presented a number of recorded pollinators lower than populations with higher floral display.

Honeybees were recorded in all the investigated fragments and, although are generally considered to be effective pollinators (Goulson, 2003), their robbing behaviour and the consequent depletion of floral resources for more effective pollinators is likely to limit the reproductive output of *C. undulatum*, as suggested by Delnevo et al. (2020 a). Moreover, the reduction of native bees recorded in small populations may be the result of the combined effect of both competition with honeybees and decreased attractiveness of small patches to specialized pollinators. Indeed, *A. mellifera* is not native to Australia and can occur at high densities due to the presence of domestic hives, with possible negative impacts on native wildlife (González-Varo & Vilà, 2017; Magrach et al., 2017). Although honeybees were present in all the populations, their possible impact on the native bee *L. conospermi* was only evident in the small fragments where the competition for floral resources may be stronger relative to large remnant bushlands. This suggests that, in the region, pollinator assemblages may be altered when habitat fragmentation occurs in combination with bee-keeping, as found by González-Varo et al. (2009) in southern Spain, and may have implications for the

conservation of specialized threatened plant species that rely on native pollinators for reproduction.

Flower visitation

Our result shows the importance that population floral display may have for mass-flowering species to attract pollinators, especially when combined with an increased isolation within an urban matrix. This is consistent with the theory of optimal foraging (Charnov, 1976) and with recent studies on *Conospermum* (Delnevo et al., 2019 a). On the other hand, our findings are opposite to results in other studies in the region on plants pollinated by birds and honeybees where more extensive pollen flow has been observed across remnants (Byrne et al., 2007; Byrne et al., 2008). Such different outcomes can be explained by the different foraging habits of small native bees and highly mobile flower visitors, and agrees with findings by Aguilar et al. (2019) showing that plant species pollinated by flying vertebrates can overcome the effects of fragmentation on the quality of progeny. Indeed, birds and honeybees are able to extend their foraging range in response to increased plant spacing (Morris, 1993; Saunders & De Rebeira, 1991). For most Australian bees, however, this is unlikely to be the case as they can only forage over short distances (Schwartz & Hurst, 1997). Since the results can differ greatly between these systems, generalizations on the effect of habitat fragmentation on plant-native insect mutualisms are inappropriate with greater research on this topic needed in the near future.

Pollen limitation

This study focused on the role that both pollen quantity and quality played in the limitation of the reproductive output in a rare plant species, threatened by habitat fragmentation. The fruit set of *C. undulatum* was significantly favoured by the quantity of pollen arrival, and the two types of hand pollination treatments had similar responses and were weakly related to the variable population size. In contrast to fruit set, the two hand pollination treatments had significantly different outcomes for seed set. The seed output of +Within-treated plants (within-population pollen transfer) increased relative to controls, indicating a pollen quantity limitation in both small and large populations of *C. undulatum*. Moreover, the seed set response was positively related to population size and this suggests the presence of a process that limits the development of the embryo in small populations compared with larger ones. The different outcomes between the +Within and +Between treatments reveals that this process is due to the reduced quality of available pollen in small

populations where an abnormally reduced availability of compatible mates is more likely to lead to the abortion of the embryo inside the fruit, just as following self-pollination. Hand pollination from different populations (+Between) allowed plants to reach their maximum seed production (possibly set by resources; Zimmerman & Pyke, 1988) equally across all the populations, regardless of their size, as pollen quality was no longer a constraint.

Similar fruit development in the two hand-pollination treatments but different seed set indicates the presence of a process that prevented the development of healthy embryos resulting in empty fruits in small patches of *C. undulatum* (compare Fig. 3.3a and Fig. 3.3b). Given the mating system and the poor seed dispersal of the species, it is reasonable to conclude that small remnant populations are likely to have a restricted availability of compatible, genetically unrelated mates that reduced the quality of pollen, even when this was outcrossed, suggesting the presence of early biparental inbreeding depression. This is also supported by the similarly high seed set for the two +Within and +Between treatments in large populations, where the effect of genetically unrelated mate availability is expected to be low.

Our results agreed with findings in other self-incompatible species, such as *Echinacea angustifolia* (Wagenius et al., 2007), and reinforce the importance of high inter-population pollinator activity in small fragments where unrelated mates may be limited. Manual pollination demonstrated that pollen limitation can be overcome by pollen availability in large populations; this is in line with recent studies about the effect of habitat fragmentation on pollen limitation (e.g. Chen et al., 2019). However, in small populations, this is unlikely to be sufficient to ensure an adequate reproductive output. Indeed, in such fragments, the pollen limitation is stronger than in large populations and is the result of both *quantity* and *quality* component.

Habitat fragmentation was found to be a serious threat to the future persistence of *C. undulatum* by altering the interactions with its pollinators. These mutualistic associations have evolved over long timeframes with native insects that appeared susceptible to the effect of land use change on plant populations and, possibly, to the increased competition with introduced bees, especially in small remnants. Habitat reduction may result in patches of native vegetation that are too unattractive and isolated to favour pollinator presence, limiting flower visitation and, consequently, pollen flow. This cross-population pollen flow is crucial for small populations where natural seed set is too low to ensure long-term population viability and adaptation based on reproduction by seeds. Future studies to help maximize the

conservation effort should focus on quantifying the amount of gene flow between different fragments, as well as identifying key populations that act as pollen sources.

Supplementary material

Flower visitors (Supplementary material B.1 in Appendix B), number of plant and flower visits grouped by family (Supplementary material B.2 in Appendix B), PCA of pollinator assemblage in 2017 and 2018 (Supplementary material B.3 in Appendix B).

Chapter 4 - Definition of the ecological role of ants as floral visitors of *Conospermum undulatum*

This chapter has been published as the following paper:

Delnevo N, van Etten EJ, Clemente N, Fogu L, Pavarani E, Byrne M, Stock WD. 2020. Pollen adaptation to ant pollination – a case study from the Proteaceae. Annals of Botany, 126: 377–386.

Introduction

Mutualistic plant-animal interactions are a common ecological process with almost 90% of wild flowering plant species relying on animals for gamete dispersal and, ultimately, fruit and seed production (Ollerton, Winfree, & Tarrant, 2011). Most animals involved in such interactions are insects, and they account for the pollination of ~88% of all animal-pollinated plants (Potts et al., 2010; Thomann et al., 2013). Among the insect-pollinated plants, pollination by ants appears to be poorly represented (de Vega & Gómez, 2014; Kuriakose, Sinu, & Shivanna, 2018; Rostás et al., 2018; Del-Claro et al., 2019), whereas bees and other close relatives are recognised as important pollinators worldwide (Potts et al., 2016). Moreover, interactions between ants and flowers are generally assumed to be antagonistic. This large discrepancy between the recognised roles of bees and ants has been attributed to peculiar characteristics of ants, such as their small size (being generally smaller than the reproductive structures of flowers), their aggressive behaviour that may deter other flower visitors, and their grooming, or self-cleaning, behaviour (Galen, 1983; Junker et al., 2007). Ants are also known to produce an antimicrobial secretion from their metapleural gland, which has been shown to have a negative effect on the viability of pollen (Beattie et al., 1985). This trait may have contributed to differences in pollination efficacy among the major hymenopteran lineages (i.e. the ‘antibiotic hypothesis’; Beattie et al., 1984; Beattie et al., 1985). The primary function of this cuticular secretion is very likely antiseptic (Poulsen et al., 2002; Stow & Beattie, 2008; Yek & Mueller, 2011), with ants spreading antibiotic secretions diffusely through the nest to prevent fungal growth and infections (Hölldobler & Wilson, 1990). Possibly, this is the reason why ant pollination appears to be mainly limited to dry, or sometimes cold, environments (Dutton & Frederickson, 2012); indeed, bacteria and fungi are likely to impose stronger selection on ants for antimicrobial defences in warm,

humid tropical rainforests than in deserts and Mediterranean-type habitats. Nonetheless, ant pollination may be an advantageous system with a low energetic cost, and could be favoured in habitats where ant frequency is high and plants produce small, open flowers with low amounts of pollen (i.e. the ant-pollination syndrome; Hickman, 1974). Reports of ants as effective pollinators are limited to a low number of convincing examples (46) (de Vega & Gómez, 2014) with the number of such studies increasing over recent years (Domingos-Melo, Nadia, & Machado, 2017; Del-Claro et al., 2019) suggesting that further studies are needed to evaluate some of the earlier generalizations about the negative role of ants as pollinators.

Ants are known to play an important role in seed dispersal in a number of regions and ecosystems (Lengyel et al., 2010; Suetsugu, Shitara, & Yamawo, 2017; Luna et al., 2018; Magalhães et al., 2018), including the sandplains of southwest Australia (also known as 'kwongan'). The region is noted for its rich floral diversity, especially among the medium-sized shrubs of the Proteaceae, Myrtaceae and Ericaceae families (Hopper & Gioia, 2004). It is characterised by an old, stable landscape and nutrient-poor soil (Hopper, 1979) with a climate that is typically Mediterranean with most rain concentrated in the winter months. Despite many theories that have advanced the importance of ant dispersal (Gove et al., 2007; Majer, 1982), little attention has been given to their possible role as pollinators in these regions. This became apparent during our recent studies on the pollination ecology of a threatened member of the Proteaceae (*Conospermum undulatum*) where we observed that ants were the second-most active floral visitors for this species (Delnevo et al., 2020). Thus, *C. undulatum* could represent a potential model species to test for ant pollination in a region where ants are abundant and diverse, and are already well known for their ecological role in dispersing seeds from many plant species, including members of the Proteaceae.

In this study, we evaluate the effectiveness of ants as pollinators and whether or not they negatively interfere with plant reproduction by rendering pollen grains unviable (and thus robbing nectar from the flowers) by assessing the effect of ant secretions on pollen germination. A lack of a negative response to ants could result from either the low production of secretions by local ants or because a plant species has adapted to potentially use ants as pollen vectors by producing pollen resistant to secretions. Therefore, to test for potential local adaptation we compared the response to ant secretions across several species of native ants and species of the Proteaceae. Possible reduced selection for antimicrobial secretions in this dry Mediterranean-climate region and observations of ants visiting flowers suggest ants may act as effective pollinators in the region. On the other hand, ants may still produce

antimicrobial secretions, but some plant species may have adapted to cope with such secretions, although this has never been tested before.

The effectiveness of a given pollinator not only depends on its floral visitation but also on the efficiency with which they deposit conspecific pollen (Herrera, 1987). Ants commonly are generalist floral visitors; however, short-term pollinator foraging specialisation on a particular plant species, known as floral fidelity, may occur (Brosi, 2016). For most plants, floral fidelity is critical because transfer of conspecific pollen must occur in order for fertilization to take place, so we investigated whether ants carry a suitable conspecific pollen load to enable successful pollination in *C. undulatum*. We also carried out an exclusion experiment to demonstrate if ants are effective pollinators in *C. undulatum* and to evaluate to what extent ants contributed to the reproductive output of this species. We hypothesised that because of the generally restricted foraging range of ants in comparison to winged hymenopterans and their possible antibiotic production, their contribution to seed set would be expected to be negligible (or negative) relative to naturally pollinated plants and those pollinated by flying insects, which we expected to be similarly high.

Materials and methods

Study area and species

The study was conducted in southwest Western Australia within the Swan Coastal Plain bioregion. This region is a low-lying coastal plain that extends from Jurien Bay, north of Perth, to Cape Naturaliste in the south, and it is part of the Southwest Australia global biodiversity hotspot (Mittermeier et al., 2004). The area experiences a dry, Mediterranean-type climate (Beard, 1984), with hot dry summers (December-March), and mild wet winters (June-August) with 600-1000 mm of rainfall on average across the region. The area is characterised by deep, highly leached sand dune systems (McArthur & Bettenay, 1974) with low woodland dominated by *Banksia* trees and highly diverse shrubby understorey.

Conospermum (Proteaceae) is an insect-pollinated genus endemic to Australia with its centre of distribution being the south-west corner of Western Australia. The genus includes 53 species (Bennett, 1995) and is of increasing conservation concern, with four taxa already listed among the threatened flora of Western Australia (W.A. Government Gazette, 2018). Like all Proteaceae, the perianth has four tepals, although in *Conospermum* the tepals are of unequal size, with the upper one being much larger than the other three. Zygomorphy is expressed in the bilabiate perianth, the upper tepal forming a broad hood over the other three

tepals, in each of which the distal-most portion flares and reflexes downward, allowing entry to the flower (Bennett, 1995; Douglas, 1997). The flowers of *Conospermum* possess an active pollination mechanism. The style is bent, and the flower opens in a state of tension (Stone et al., 2006; but see Douglas (1997) for morphological description). When a visiting insect applies pressure with its mouthparts at the base of the style it flicks away from the fertile anthers and strikes the visitor. The moist cup-shaped stigma is forced down onto the pollinator and thereby picks up pollen carried by the insect; at the same time the fertile anthers dehisce explosively, casting new pollen onto the visitor (Morrison et al., 1994; Stone et al., 2006). Thus, *Conospermum* flowers need to be visited by insects carrying a suitable pollen load from previous floral visits in order for pollination to occur, leading to development of fruits. These are cone-shaped, covered with tan orange hairs, and contain only one seed (i.e. achenes).

In particular, *Conospermum undulatum* is a monoecious plant that grows as an erect, compact shrub up to 1.5 m tall with distinctive fibrous, longitudinally fissured stems. The glabrous leaves are to 12 cm long and 3.8 cm wide with a characteristic undulating margin. This species is currently listed in the threatened flora of Western Australia (W.A Government Gazette, 2018) and has been assessed as "Vulnerable" using IUCN red list criteria (Department of Environment and Conservation, 2009). It was originally considered a variety of *C. triplinervium*, which also occurs in the region but with different habit and leaf morphology (Bennett, 1995). Molecular evidence has established *C. undulatum* as a distinct species (Close et al., 2006), and recently developed genetic resources are being used to further clarify genetic relationships among populations (Delnevo et al., 2019 b). The flowering period usually ranges from late August to late October. In a recent study Delnevo et al. (2019 a) found that the pollination mechanism in *C. undulatum* is an effective physical barrier against autogamous selfing, and also found that this species possesses a strongly developed self-incompatibility system that prevents the development of most embryos following geitonogamous selfing. The hermaphroditic flowers are small in size, measuring *ca*. 7 mm in length, with the tube being *ca*. 4 mm. They are covered in white hairs and are produced in inflorescences held well above the leaves. Flowers do not produce any obvious scent and offer a nectar reward located within the flower, at the base of the calyx tube. In this way an insect would trigger the mechanism by pushing on the trigger point near the anthers with its mouthparts whilst scavenging for nectar and/or pollen (Fig. 4.1).

Due to its characteristic floral morphology and pollination system, *C. undulatum* relies on a restricted group of pollinators, mainly hymenopterans. The native bee *Leioproctus*

conospermi (Colletidae) and native ants, including sugar ants, meat ants and bull ants, are the most active floral visitors of this species (Delnevo et al., chapter 3).

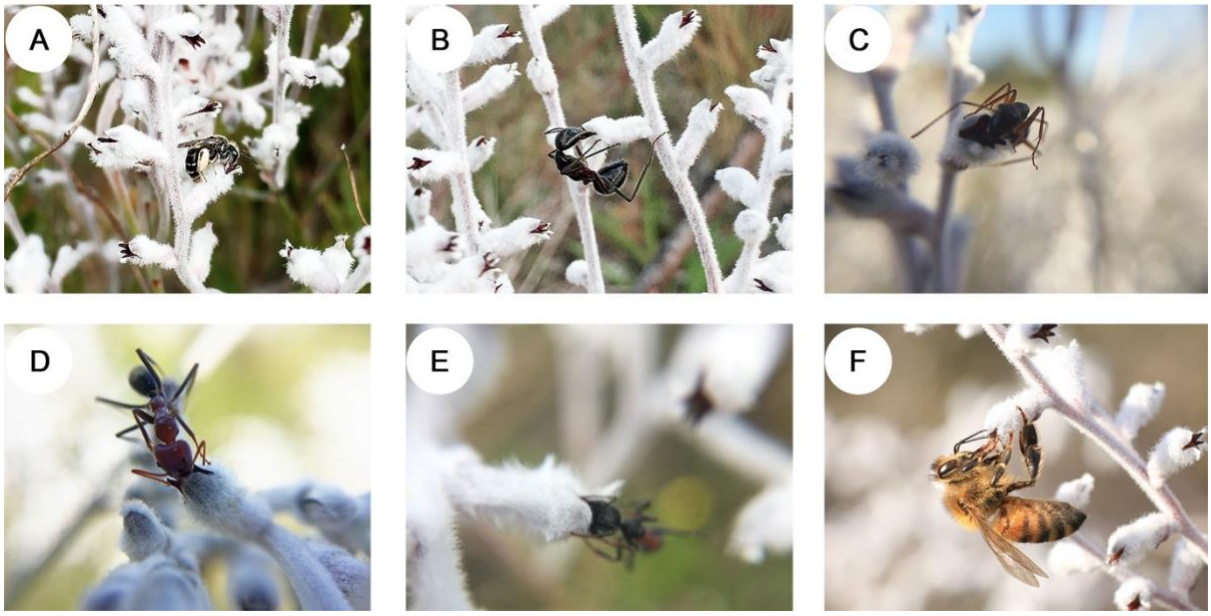


Figure 4.1. Insects visiting flowers of *C. undulatum*; (A) *Leioproctus conospermi*; (B) *Camponotus molossus*; (C) *Camponotus terebrans*; (D) *Iridomyrmex purpureus*; (E) *Myrmecia infima*; (F) *Apis mellifera*. Note that *A. mellifera* only insert its proboscis into the flower to steal nectar.

Pollen germination assays

To determine local adaptations of plants to cope with the detrimental effect of ant secretions on pollen viability we performed a pollen germination assay to compare the germination of pollen collected from *C. undulatum* to that of five other plant species after contact with three species of Australian ants, as well as honeybees and a control (no contact with insects). Specifically, we selected the ant species: *Iridomyrmex purpureus*, found throughout Australia, including our study region; *Camponotus terebrans*, mainly found in the southern part of Australia; and *Camponotus molossus*, native to the Swan Coastal Plain (Heterick, 2009). Following several field surveys we were unable to find any nests of the bull ant *Myrmecia infima*, therefore we were unable to test the response of pollen with this ant species even though it was observed visiting *Conospermum* flowers.

The plant species selected for this experiment were *Conospermum undulatum*, *Conospermum stoechadis*, *Conospermum canaliculatum*, *Grevillea eriostachya*, *Grevillea leucopteris*, and *Banksia nivea*. These species were selected as they are co-flowering shrub species that co-occur in the Swan Coastal Plain, and all three species of ant were recorded visiting flowers of these plants. They all belong to the Proteaceae family and were collected

within 20 km of the centre of the distribution of *C. undulatum*. For each species, freshly opened flowers within one day of anthesis were collected the same morning they were used. In the laboratory we pooled pollen from several flowers of the same species in Petri dishes. Subsequently, we gently picked up each ant or bee with tweezers, lightly dabbed it in the pollen grains and put the live insect in a clean 50 mL centrifuge tube for 30 minutes, a standard exposure time used in several similar studies (e.g. Peakall & Beattie, 1989; Dutton & Frederickson, 2012). For the control we left pollen grains in an empty tube for the same amount of time. Next, we transferred the pollen from ants, bees, or controls onto a microscope slide with a drop of pollen germination medium by gently dipping the insect into the drop and placed a coverslip to prevent desiccation. The pollen germination medium was prepared following a modified version of Brewbaker & Kwack (1963); briefly, the medium was made up of 100 mg L^{-1} of boric acid, 300 mg L^{-1} calcium nitrate, 200 mg L^{-1} magnesium sulphate, 100 mg L^{-1} potassium nitrate and 20% sucrose. The selected concentration of sucrose was found to be the one that maximised pollen tube growth for all the tested species following trials ranging from 10% to 60% of sucrose. After an incubation period of 48 h in the dark at room temperature (24 °C) we assessed the germination rate by counting the number of pollen grains with and without pollen tubes under a microscope. We tested pollen from each plant species against five individual workers of each ant species, five individual worker bees, and five controls (n = 150 germination assays).

Floral fidelity

In the field, we sampled the pollen load of 10 individuals of each species of floral visitor of *C. undulatum*. The insects were collected from inflorescences of *C. undulatum* using clear 50 mL centrifuge tubes after recording whether there was stigmatic contact. To avoid contamination of the pollen load a clean tube was used for every insect. We induced cold anaesthesia by placing the tube containing the insect on ice, and removed pollen non-destructively by dabbing the pollinator body in a standardised manner (i.e. two dabs on head and forehead) with a cube of fuchsin-stained gel (Kearns & Inouye, 1993; Brosi & Briggs, 2013). The captured insect was released as soon as the pollen had been sampled. We then mounted the pollen-containing gel on microscope slides and assessed floral fidelity in each pollen load by sorting pollen grains as either '*C. undulatum*' or 'other species' by means of a pollen reference slide of *C. undulatum*. To account for possible contamination in the field, we classified pollen loads as monospecific if >95% of pollen grains represented *C. undulatum*, and as heterospecific if otherwise, following the approach of Brosi and Briggs (2013).

Exclusion experiment

Autogamous selfing and anemophily have already been tested in a recent work by Delnevo et al. (2019 a) and no fruits were recorded in these total exclusion treatments demonstrating that *C. undulatum* completely relies on pollinators for pollen transfer. In this study, we aimed to experimentally assess the relative contribution of ants and flying visitors to the reproductive output of *C. undulatum*. We performed three experimental treatments in the field: flying insect exclusion (FLY_EXC), ant exclusion (ANT_EXC), and control (flowers freely exposed to all visitors). In three contiguous patches of *C. undulatum* characterised by similar population size (between 400 and 600 plants) we randomly selected a total of 27 plants. To implement the FLY_EXC treatment we covered the selected plants one week prior to anthesis with a net tent (0.25 mm^2 mesh) to 2 cm from the ground, so that only crawling insects could visit the inflorescences. Net tents were monitored for the presence of flying insects every week for the entire flowering period to ensure their efficacy, and no flying insects were recorded. The ANT_EXC treatment was performed by applying Tanglefoot® around the woody stems of selected *C. undulatum* plants one week prior to anthesis, to prevent crawling insects from reaching the opened flowers. At the end of the flowering period, when flowers began to senesce, we placed fine mesh bags around the inflorescences to collect the fruits. In the laboratory, we counted the number of flowers, fruits, and seeds collected for each plant. The number of flowers was assessed by counting the scars left on the white, woolly inflorescence stalk of *C. undulatum*, and we obtained a total of 3935 flowers.

Data analysis

Data from the germination assays were analysed using a generalised liner model (GLM) with the proportion of germinated pollen as the response variable and plant species, treatment, and their interaction, as the explanatory variables. We then compared all the combinations of levels of the explanatory variables with a Tukey's HSD test.

To analyse whether visitors showed floral fidelity, or they were generalists, we fitted a generalised linear mixed effect model (GLMM) with the proportion of *C. undulatum* pollen within the pollen load as the response variable, and the visitor taxon as the explanatory variable. Since individuals of the insect and plant species studied within a study site are likely to be closely related genetically, and environmental conditions are similar, data collected within a study site are not independent. To address this lack of independence and prevent

pseudoreplication, we used *Conospermum* population as a random effect. Again, we compared each level of the explanatory variable with a Tukey's HSD test. Syrphid flies (Syrphidae) were excluded from the analysis because of extremely small pollen load, whereas *Myrmecia infima* was excluded because we were unable to collect enough pollen load from this species in the field.

Finally, we used the proportion of seeds out of the total number of flowers as the response variable in a GLMM with the exclusion treatments as the explanatory variable and *Conospermum* patch as the random effect.

All of our response variables were proportions, therefore we used binomial error distribution (appropriate for proportional data) to account for non-normal distribution of residuals and non-homogeneous variances in each model, and checked that the assumptions were fulfilled by visual inspection of residual patterns (Zuur et al., 2009). All statistical analyses were performed with R version 3.5.2 (R Development Core Team, 2020).

Results

Pollen germination assays

The pollen germination response was different among treatments and the significant interaction term indicates different responses to the same treatment among species (Table 4.1). Pollen of *Conospermum* species subject to the control treatment had the highest germination response with *C. undulatum*, *C. stoechadis*, *C. canaliculatum* having 95.2%, 96.7%, and 96% of pollen grains germinated after the incubation period of 48 h, respectively (Fig. 4.2). The germination rates of pollen from the other plant species subject to the control treatment were all lower than that of *Conospermum*, and had similar germination rates of *ca*. 50%, with the least responsive species being *G. leucopteris* (41.8%).

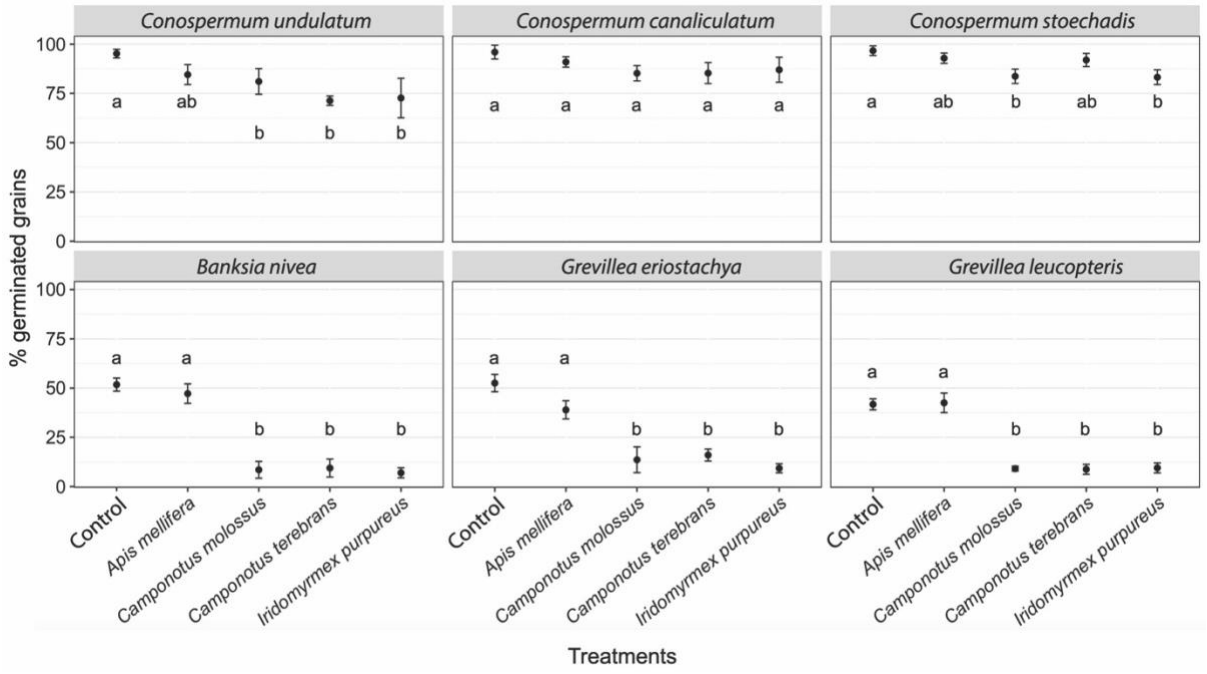


Figure 4.2. Pollen grain germination assays of six plant species. Mean (% ± SE) pollen germination after contact with the different treatments; treatments marked by different letters are significantly different at $\alpha = 0.05$ according to Tukey's HSD tests.

The treatment of the effect of honeybees showed there was no significant detrimental effect on pollen germination after contact with *A. mellifera* in any tested plant species compared to control treatments (Fig. 4.2). In contrast, contact with ants severely reduced the pollen germination to *ca*. 10% in *B. nivea*, *G. eriostachya*, and *G. leucopteris*, but not in *Conospermum* species. In particular, *C. undulatum* had a pollen germination after contact with the integument (outer covering) of *C. molossus*, *C. terebrans*, and *I. purpureus* of 81.1%, 71.3%, and 72.7%, respectively. The germination rate in *C. stoechadis* and *C. canaliculatum* was similar to *C. undulatum*, and did not statistically differ from the effect of bees (Fig. 4.2). For *B. nivea*, *G. eriostachya*, and *G. leucopteris*, contact with all the ant species led to significantly reduced pollen germination, being 38.9%, 26%, and 33.4% lower respectively, compared to bees ($P < 0.001$ in all cases). In contrast, pollen germination in *C. undulatum*, *C. stoechadis* and *C. canaliculatum* was reduced by only 9.3%, 6.6% and 5.1% with ant exposure, respectively, and did not differ from the effect of bees ($P = 0.532$, $P = 0.350$, $P = 0.702$; Fig. 4.3).

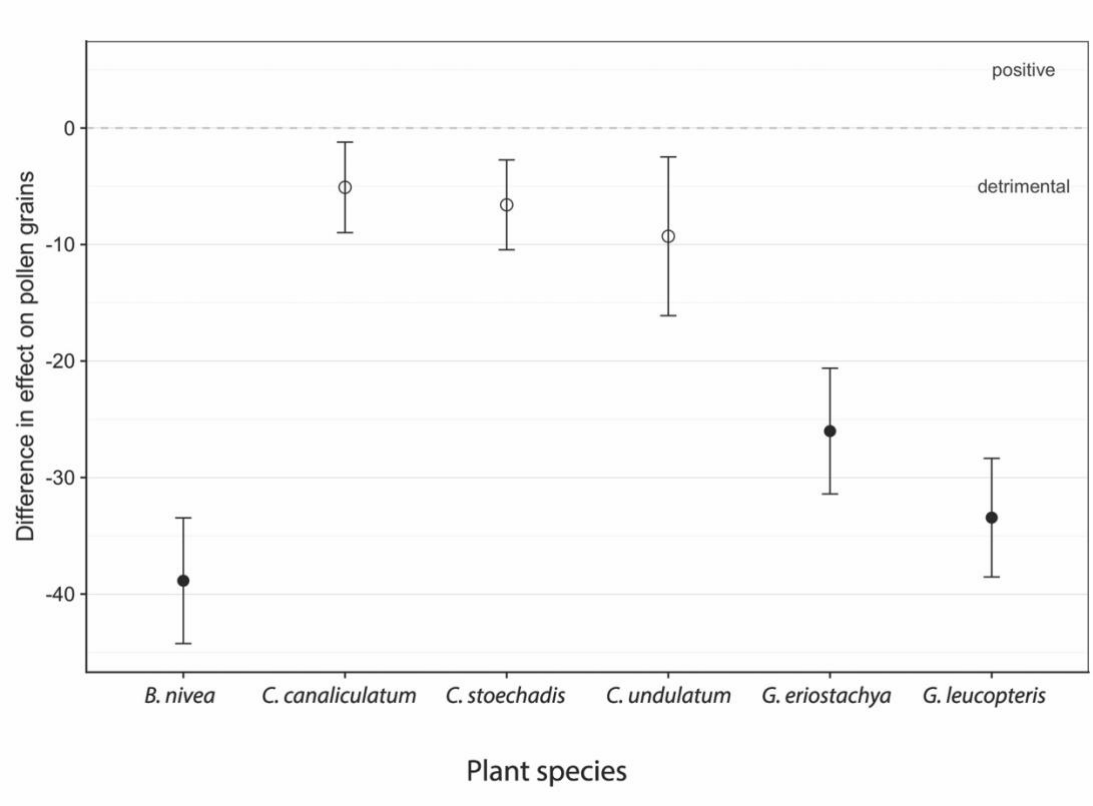


Figure 4.3. Difference between the effect of ants (pooled together) and the effect of *A. mellifera* on pollen germination; dots below the dashed line indicate a negative effect of ants. Closed dots indicate a statistically significant difference between *A. mellifera* and ants, open dots no significant difference.

Table 4.1. Analysis of variance table showing the effects of plant species, treatments, and their interactions on pollen germination response.

Variable	*df*	χ^2	*P*
Plant species	3	511.37	< 0.001
Treatment	4	360.03	< 0.001
Plant species x Treatment	12	21.35	0.04

Floral fidelity

The native bee *Leioproctus conospermi* was the only species that carried monospecific pollen (mean ± SE = 0.989 ± 0.006; Fig. 4.4), which was significantly different from all other species of pollinators, indicating highly specialised pollination of *C. undulatum* (Table 4.2). Argid sawflies, *Apis mellifera*, and *Iridomyrmex purpureus* were the most generalist pollinators, carrying a pollen load with average proportions of *C. undulatum* pollen being 0.57, 0.63, and 0.68 respectively. The two *Camponotus* species showed high proportion

of *C. undulatum* pollen grains within their pollen load, although not statistically different from the other generalist pollinators. In particular, *Camponotus terebrans* carried a pollen load with an average proportion of 0.82 of suitable grains, whereas *C. molossus* had 0.86 (Fig. 4.4).

Table 4.2. Tukey HSD pairwise comparison of the floral fidelity of the different recognisable taxonomic units of visitors of flowers of *Conospermum undulatum*. Estimate of contrasts, SE, and P-values are reported (significance codes: P-value < 0.001 '***'; <0.01 '**'; <0.05 '*'; >0.05 '').

Contrast	Estimate	SE	*P*
Argidae – *A. mellifera*	-0.238	0.6234	0.999
C. molossus – *A. mellifera*	1.318	0.6641	0.349
C. terebrans – *A. mellifera*	1.021	0.6096	0.545
I. purpureus – *A. mellifera*	0.238	0.6106	0.999
L. conospermi – *A. mellifera*	3.963	0.7212	< 0.001 ***
C. molossus – Argidae	1.556	0.6186	0.118
C. terebrans – Argidae	1.260	0.5597	0.212
I. purpureus – Argidae	0.477	0.5607	0.957
L. conospermi – Argidae	4.202	0.6795	< 0.001 ***
C. terebrans – *C. molossus*	-0.296	0.6047	0.997
I. purpureus – *C. molossus*	-1.079	0.6057	0.475
L. conospermi – *C. molossus*	2.645	0.717	0.003 **
I. purpureus – *C. terebrans*	-0.783	0.5454	0.703
L. conospermi – *C. terebrans*	2.942	0.6669	< 0.001 ***
L. conospermi – *I. purpureus*	3.725	0.6678	< 0.001 ***

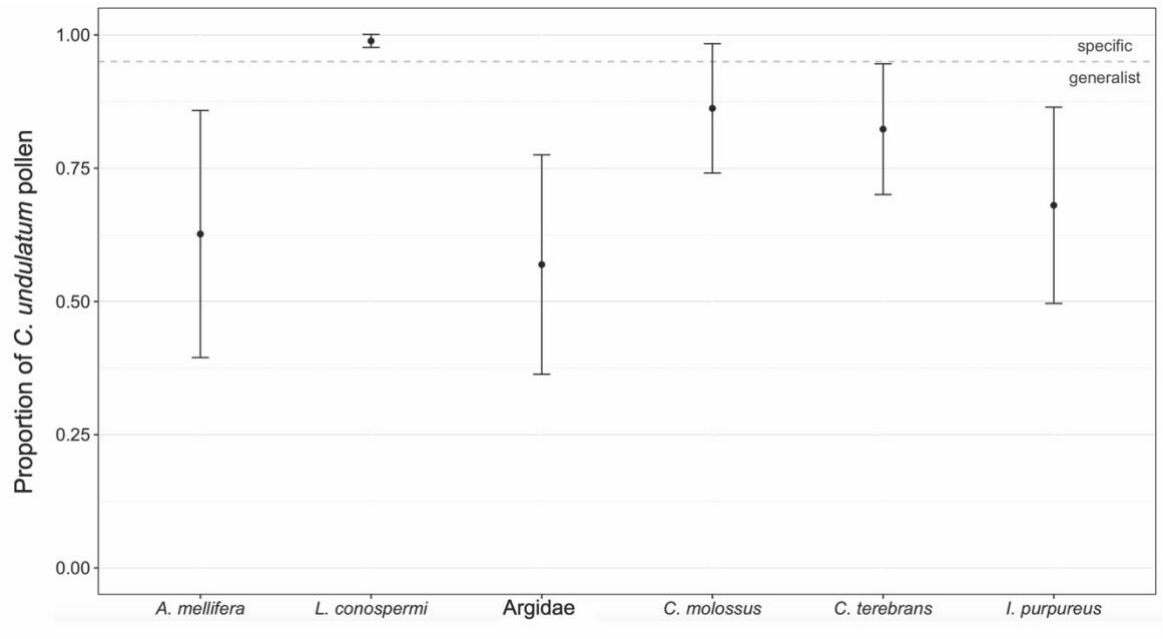


Figure 4.4. Proportion of *Conospermum undulatum* pollen grains (± SE) within the pollen load of insects recorded on *C. undulatum* plants. Dots above the dashed line represent insects that carried monospecific pollen load; dots below the line represent heterospecific pollen loads.

Field exclusion experiment

The probability that a flower developed a seed in freely exposed control plants was 10.5%, whereas flowers available only to flying visitors (ANT_EX treatment) resulted in a probability of 8.6% of seed set (Fig. 4.5A). Flying-visitor exclusion treatments (FLY_EX) showed that ants were effecting pollination resulting in a probability of setting seed of 6.7%. Using the controls as the reference for the maximum amount of seed that can be developed by freely exposed *C. undulatum* plants (Fig. 4.5B), the results showed that flying insects alone produced significantly less seeds than controls (84%; $P = 0.043$), and that ants alone contributed to 62.7% of the seed set of freely exposed control plants ($P = <0.001$). The results of the two treatments ANT_EX and FLY_EX were not significantly different from each other ($P = 0.096$).

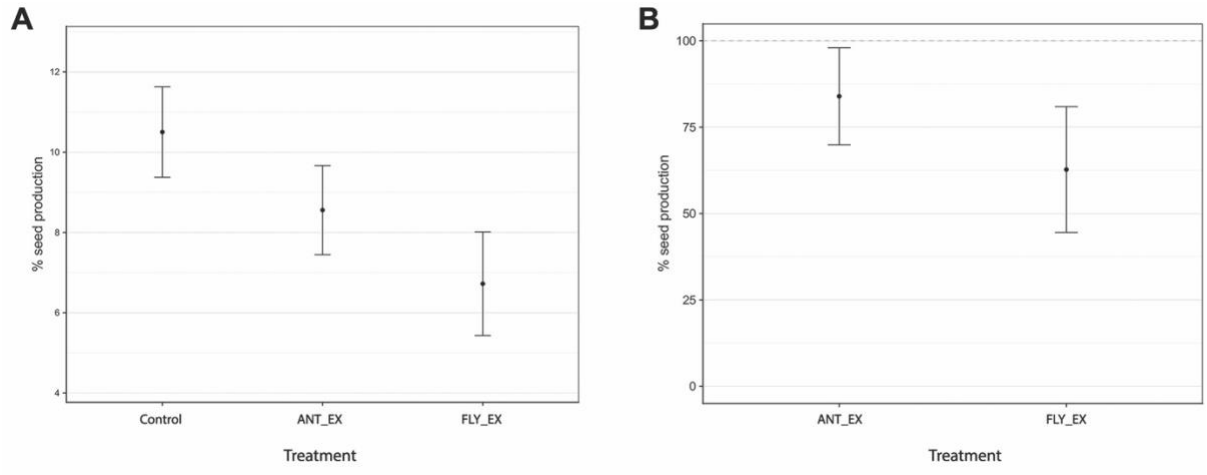


Figure 4.5. Seed production in *Conospermum undulatum* subject to experimental treatment. (A) Percentage of seeds produced by *C. undulatum* plants subject to treatments of natural pollination, ant exclusion, and flying-visitor exclusion. (B) Relative seed set of *C. undulatum* plants subject to ant exclusion and flying-visitors exclusion compared to freely exposed natural pollinated plants; the dashed line indicates controls' seed production.

Discussion

Pollination is a critical element of plant sexual reproduction and our study within a key genus of Proteaceae has revealed that ants are important secondary pollinators for *C. undulatum*, a threatened species in the Australian kwongan. We found evidence that within the genus *Conospermum* plants have adapted the biochemistry of their pollen grains to favour the action of these secondary pollinators. In addition, we demonstrated that *C. undulatum* has a highly specialised pollination mutualism with a native *Leioproctus* bee. Identification of such specific pollination associations are important for management of threatened species to ensure maintenance of effective pollination services to ensure long term population viability.

In contrast to the expectation under the antibiotic hypothesis where ant secretions mostly prevent the transfer of viable pollen (Beattie *et al*., 1984; Beattie *et al*., 1985; but see Peakall & Beattie, 1989; Gómez & Zamora, 1992; Gómez et al., 1996), we found that the germination of pollen grains was not inhibited in *C. undulatum*, as well as the other species of this genus studied. The germination of pollen grains in *B. nivea*, *G. eriostachya* and *G. leucopteris*, on the other hand, was drastically reduced after contact with the ant treatment and is consistent with the antibiotic hypothesis and with observations in other temperate and tropical plant species where the pollen germination rate decreased after contact with several different species of ants (Dutton & Frederickson, 2012). The opposite outcomes between

Conospermum and the other species strongly suggest that within the genus *Conospermum* plants have evolved to favour the action of ants as secondary pollinators by producing pollen with resistance to the negative effect of ant secretions on pollen grains that is common in the majority of plants. Moreover, the strong negative effect of ant secretions on pollen for all the analysed plant species except *Conospermum* species suggests that the investigated ants produce antimicrobial defences despite the dry summers that characterise the south-western Australia. It is noteworthy that although the sugar ants *C. molossus* and *C. terebrans* do not possess a metapleural gland (Heterick, 2009), the detrimental effect on pollen grains in these two species was comparable to that of the meat ant *I. purpureus*, which, as with most ant species, possesses this gland. Similar outcomes were found for the pollen of *Cytinus hypocistis* after contact with the ant *Camponotus pilicornis* by De Vega et al. (2009). This adds to the idea that antibiotic secretions may be secreted from different glands and distributed throughout the cuticle in at least some ant species (Hull & Beattie, 1988).
The lipoidal secretions of ants are able to penetrate the pollen grain via a hydrophobic pathway and render the plasma membrane and the organelle membranes ineffective (Beattie *et al*., 1985). The possible hydrophobic pathways are unknown, but it is plausible that *Conospermum* presents mechanism to mitigate the osmotic shock that leads to the lysis of the bilayer membrane of pollen. *Conospermum* pollen presents a remarkably fast tube growth, orders of magnitude faster than other plants (Stone et al., 2004). This may represent a possible difference in physiology that may be associated with its ability to cope with ant secretions. In fact, although pollen tube growth rate was not specifically investigated in this study, we noticed tube growth rates of the order of 50 µm s^{-1} which are in line with findings for other species in the genus, including *C. amoenum*, *C. spectabile*, *C. eatoniae*, *C. caeruleum*, *C. brownii* and *C. incurvum*, where pollen tubes emerged and grew at rates of up to 55 µm s^{-1} (Stone et al., 2004). These rates of pollen tube growth exceed some of the fastest recorded *in vivo* speeds, which were around 1.8 µm s^{-1} (evening primrose) to 2.7 µm s^{-1} (maize) (Stanley, 1971; Barnabas & Fridvalszky, 1984).

The effectiveness of a given pollinator not only depends on its abundance and floral visitation but also on the efficiency with which they collect and deposit pollen (De Vega et al., 2009; and see Herrera, 1987, 1989 for quantity and quality components of the plant-pollinator interaction, respectively). Ants are active floral visitors in the region and frequently visit our target species *C. undulatum*. Our results indicated that ants carried pollen of different plant species, but despite being generalist floral visitors, they presented a pollen load with a high proportion of *C. undulatum* grains. The characteristic pollination mechanism

of *Conospermum* makes pollination by small insects unlikely. Indeed, we recorded many dipterans and small ants fatally trapped by the triggered style of *Conospermum*. However, all the species of ants we studied have workers larger than 7 mm in length, which allows them to forage within the calyx of *Conospermum* flowers untroubled by the trigger mechanism of the stigma. The stigma, once triggered, can easily reach the ant visitor's body to collect the pollen deposited from previous floral visits to complete this characteristic pollination process. Plant adaptation to cope with ant secretions and evidence of suitable pollen load carried by ants suggests that *C. undulatum* likely relies on both ants and the native bee *L. conospermi* for pollination. The contribution of ants to the reproductive output of this species was tested by means of exclusion treatments, and, in contrast to our initial hypothesis, we found that pollination by ants only (FLY_EX treatment) produced an unexpected 62.7% of seeds compared to freely exposed controls; and ant-excluded plants resulted in significantly lower seed set than control plants available to both flying insects and ants. Thus, we demonstrated that pollination from winged visitors alone was not sufficient to allow *C. undulatum* to produce its maximum seed set in natural conditions, and therefore ants are likely playing an important role in filling this gap in the pollination of the species. Many plant-ant interaction systems studied observed an increased occurrence of geitonogamous selfing (i.e. transfer of pollen between different flowers of the same plant) following ant pollination due to the restricted foraging area exhibited by the investigated ants that led them to repeatedly visit individual flowers in close proximity (e.g. Peakall & Beattie, 1991; Gómez & Zamora, 1992; De Vega et al., 2009). However, in a recent study Delnevo *et al*. (2019 a) found that *C. undulatum* possesses a strongly developed self-incompatibility system that prevents the development of the embryo following both autogamous and geitonogamous selfing. This suggests that, although the species lacks the reproductive assurance of self-compatibility, ant pollination produced outcrossed progeny and did not contribute to the often-negative effects of selfing on plants (Herlihy & Eckert, 2002). Moreover, the discrepancy between the sum of ANT_EX and FLY_EX treatments and the controls (i.e. the sum of the exclusion treatments exceeds 100%) may be explained by the possible negative effect of introduced honeybees on the reproductive success of *C. undulatum*. Honeybees occur at high densities in the region due to the presence of domestic hives, and were recorded visiting *C. undulatum* flowers. However, *A. mellifera* is too big to pollinate the small flowers of *Conospermum*, and trigger the stigma with only their proboscis while foraging for nectar without inserting their head into the calyx; therefore, the stigma is unable to reach the body of the visitor to collect the pollen deposited during previous floral visits. Since the flowers of *Conospermum* can only be

triggered once, this behaviour possibly decreases the relative contribution of ants to the reproductive output of freely exposed plants by reducing the availability of flowers to true pollinators, and likely increases pollen limitation. The impact of *A. mellifera* robbing nectar and pollen, and, in the case of *C. undulatum*, triggering the stigma without pollinating the flower, may have cascading negative effects on the reproductive success of native plants that coevolved with native pollinators to develop characteristic flower morphologies over long timeframes. This may be particularly important for threatened species such as *C. undulatum* and is worthy of further investigation.

Ants have been traditionally considered nectar thieves, and some plants are known to produce volatiles that repel ants (Willmer et al., 2009). However, we have shown that mutualistic services by hymenopterans of the Formicidae family are important for maximising the seed output in *C. undulatum*, together with the native bee *L. conospermi*. This adds to the growing body of research highlighting the important role of ants in some plant-pollinator systems (De Vega et al., 2009; Del-Claro et al., 2019; Sugiura, Miyazaki, & Nagaishi, 2006). Nonetheless, there is a scarcity of experimental evidence on the adaptation of plant species to cope with the usually detrimental ant microbial secretions. In many ant pollination studies it is unclear whether the ants produced less harmful secretions or whether the plants were adapted to cope with such secretions. In a recent study, De Vega, Herrera, & Dötterl (2014) found evidence of adaptation by production of volatiles to attract ants in Mediterranean *Cytinus* species (Cytinaceae). However, pollen germination was negatively affected after contact with two species of ants (De Vega et al., 2009), suggesting possible adaptation of some ant species to the Mediterranean climate of south-west Spain rather than pollen resistance, which contrasts our finding for *Conospermum*. This highlights the complexity of ant-flower interactions and reinforces the fact that our understanding of these systems is still in its infancy.

Conospermum undulatum does not possess features of the proposed 'ant-pollination syndrome' (Hickman, 1974), such as small open flowers with a small amount of pollen and readily accessible nectaries, although this is also the case in a few other ant-pollinated plants (e.g. Peakall & Beattie, 1991; De Vega et al., 2009). Therefore, it seems that *C. undulatum* has coevolved to facilitate pollination by *L. conospermi*, although, coevolution also with native ants cannot be excluded.

Our study demonstrating the importance of ant pollination in this threatened species adds to the ecological roles that ants might play in the region, and the fact that ants produce antimicrobial secretions in this environment characterised by a Mediterranean climate do not

preclude ant pollination in the Australian kwongan. Instead, our results indicate that such mutualistic associations can happen in unexpected ways, and open the way for future studies to investigate flower-ant interactions in this global biodiversity hotspot. Studies on *Conospermum*, as well as phylogenetically related taxa, will provide an opportunity for understanding where and when this trait evolved and how common it is amongst the flora of south-western Australia.

Acknowledgements

We thank Dr A. Petraglia for his help with the field- and the laboratory-work. Special thanks to Dr B. Heterick for his help with the identification of ants.

ND led the writing of the manuscript, designed the experiment, collected and analysed the data; EJvE and WD contributed to design the experiment and to data collection and analysis; MB: helped improve the design of the experiment. NC, LF and EP contributed to collect and analyse the data. All authors contributed critically to the drafts and gave final approval for publication.

Chapter 5 - Genetic and ecological consequences of recent habitat fragmentation of a narrow endemic plant species

Introduction

Flowering plants are a crucial component of most terrestrial ecosystems (J Ollerton et al., 2006), providing food and shelter for vertebrate and invertebrate populations, which in turn are important for plant pollination and seed dispersal. Yet, as a consequence of human modification of landscapes, the extinction risk of many plant species is increasing worldwide (Haddad et al., 2015). Land use changes are predicted to drive the most significant effects on biodiversity throughout this century (Sala et al., 2000) and among the novel, human-built environments, urban areas are the most complex and fastest growing (Hobbs et al., 2006).

The structural changes imposed by anthropogenic habitat fragmentation can have a range of potentially deleterious genetic and demographic consequences for plants. Such consequences may result from the loss of pollinators and other alterations to plant-pollinator interactions, as well as from the reduction of reproductive output due to drastic changes in the size and isolation of populations (Aguilar et al., 2019; Lienert, 2004; Potts et al., 2010). Understanding the consequences of habitat fragmentation and the main drivers of declines in plant reproductive output is therefore critical in implementing effective restoration strategies.

Population genetics theory predicts that small and highly fragmentated populations will lose genetic variation due to genetic drift, leading to highly structured populations. Although some gene flow between remnant populations may be enough to maintain genetic diversity lost by genetic drift in small populations, increasing levels of fragmentation will eventually disrupt pollen and seed dispersal vectors from exchanging migrants between remnants. Under such conditions, extinction risk for plant species is expected to increase due to genetic erosion from increased inbreeding, reduced fitness due to expression inbreeding depression (Charlesworth & Willis, 2009), and/or a loss of mating types (e.g. self-incompatibility alleles; Pickup & Young, 2008). Empirical studies and meta-analyses, however, have found mixed support for these theoretical predictions (e.g. Aguilar et al., 2008; Kramer et al., 2008; Lowe et al., 2015) with often idiosyncratic species response to habitat fragmentation, highlighting the complexity of such systems. Indeed, for many plant

species, attempts to identify the effects of fragmentation on population genetics measures over ecological time scales are often confounded by life history and reproductive traits that buffer populations from expected loss of genetic variation and reproductive output (Broadhurst et al., 2017; Miles et al., 2019; Vranckx et al., 2012). These include overlapping generations, long generation times, seed banks, and diverse mating system and pollination dispersal mechanisms.

In particular, adult cohorts of long-lived plant species may not show the expected population genetics consequences of fragmentation simply because insufficient time has elapsed since land use change for such signals to be detectable (Vranckx et al., 2012; Lowe et al., 2015). Accounting for the interaction between the time span of the fragmentation process and the life cycle of the focal species, therefore, appears to be particularly important when studying anthropogenic fragmentation effects on long-lived plants (Kramer et al., 2008; Lowe et al., 2015). Combining study of genetic information with current and past environmental characteristics of fragmented populations of plant species provides an integrated approach to understanding how present-day and historical environmental conditions may correlate with population genetics measures (Bacles & Jump, 2011). This approach, however, requires systems where quantitative indices of fragmentation can be placed into historical context together with genetic diversity. Nonetheless, a detailed characterisation of ecological indices (i.e. fragment size, isolation) through time is not always feasible since fragmentation can be a complex process over time, with insufficient historical records. As a consequence, relatively few studies have incorporated spatially explicit historical isolation measures and characteristics of the surrounding landscape as possible factors in explaining the genetic diversity of fragmented populations (e.g. Da Silva Carvalho et al., 2015; O. Honnay et al., 2007).

Although preventing the loss of genetic diversity is widely considered vital for maintaining the adaptive potential of populations in the face of environmental change (Booy et al., 2000; Reed & Frankham, 2003), we also need to whether current reproductive output in fragmented landscapes correlates with components of genetic variation and attributes of the fragmented populations. It has become increasingly accepted that population genetics and reproductive ecology are complementary approaches to address central questions about the conservation of plant biodiversity (Lowe et al., 2009; Real, 2017). However, interactions between the biotic and abiotic environment, genetics, and reproductive output of endangered flora remains poorly understood. It is known that genotype-environment interactions are crucial in explaining phenotypic variation, including reproductive traits (Via & Lande, 1985).

Therefore, looking at interactions between genetic and ecological variation and reproductive output is especially important in fragmented habitats where the ecological and environmental condition varies greatly and may be confounded.

Conospermum undulatum is a threatened insect-pollinated long-lived lignotuberous shrub with a narrow natural range embedded in the rapidly expanding metropolitan area of Perth (Department of Environment and Conservation, 2009). The area has been impacted by fragmentation because of relatively recent urban development (Wardell-Johnson et al., 2016). This species represents an ideal candidate to study the genetic consequences of recent fragmentation by urbanization on plants as it is feasible to quantify pre-fragmentation environmental characteristics across its entire distribution range. In this system, historical aerial photographs provide valuable information to distinguish historical from contemporary effects of environmental conditions on the present-day genetic diversity.

Delnevo et al. (2019 a) recently showed that habitat fragmentation negatively influenced the reproductive output of *C. undulatum* in terms of seed production and germination rate. However, it remains unclear to what extent the genetic diversity of this species has been affected by population size reductions and fragmentation or whether the genetic consequences of fragmentation are playing a role in changing the reproductive dynamics of the species. Moreover, the historical data enables a test of whether the genetic characterisation of remaining fragments pre-date recent population declines or reflects deeper timescales of population distributions.

To address these gaps, we performed a genetic characterisation of the remnant populations of *C. undulatum* and investigated how variation in genetic diversity is related to contemporary and historical environmental factors and to its current reproductive performance. The naturally restricted distribution of this species provides the opportunity to conduct a genetic survey at the whole species level to investigate its recent response to a large reduction in habitat. We quantified the extant genetic diversity of these populations embedded within a large urban area. This was used to address three questions: (1) Do recent decreases in population size and increased isolation explain current population genetic structure? (2) To what extent is the spatial distribution of genetic diversity associated with contemporary versus historical fragmentation and environmental factors? (3) Are genetic diversity and environmental factors correlated with reproductive output of this threatened species?

Material and methods

Study area

The study was conducted in southwest Western Australia within the Swan Coastal Plain bioregion (Fig. 5.1), a low-lying coastal plain that is part of the southwest Australia global biodiversity hotspot (Mittermeier et al., 2004). This region has experienced extensive land clearing for urbanisation, which is centred around the capital city of Perth. Approximately 35% of natural vegetation remains on the Swan Coastal Plain, although only the 10% occurs in protected areas (Wardell-Johnson et al., 2016). Our target species, *C. undulatum*, grows on rapidly drained soils on the eastern part of the Swan Coastal Plain adjacent to the Darling Scarp. The entire distribution range of the species is characterised by highly leached sandy soils of the Bassendean, the Pinjarra, and the Forrestfield systems (Fig. 5.1).

Biology of study species

Although occupying a narrow range, *Conospermum undulatum* (Proteaceae) is fairly widespread in remnant patches of native bushland within this range where compatible sandy soils occur. It is a long-lived non-clonal species that can survive at least several decades as individual shrubs can resprout from semi-subterranean woody lignotubers after disturbance such as fire or mechanical damage from strong winds or trampling by animals (Bennett, 1995). It is an entomophilous self-incompatible species, although it is unclear whether this is predominantly a genetic- or physiological-based incompatibility. The flowers possess an active pollination mechanism. Insect visitors apply pressure with their mouthparts at the base of the calyx which triggers the style to flick away from the fertile anthers and strike the insect. This remarkable pollination system is a physical barrier to autogamous selfing and is activated by a restricted group of native insect pollinators, with the small specialised native bee *Leioproctus conospermi* being the most effective pollinator (Delnevo et al., 2020 a). Highly mobile visitors, such as the introduced honeybee, are not able to pollinate the small and specialised flowers of *C. undulatum* (Delnevo et al., 2020. a).

Sample collection and genotyping

In 2018, we collected leaf samples from 24 individuals of *C. undulatum* from each of 14 of the 17 known populations (Fig. 5.1). Of the three unsampled populations, two were inaccessible, being located on private land, and one only contained three individuals, making

the sampling potentially detrimental to the population and the estimation of genetic indices unreliable. Census sizes of selected populations ranged from eight to approximately 880 plants. In populations with less than 24 plants, we collected leaf samples from all individuals. Populations with >24 plants were sampled using a stratified random approach, where sampling reflected the pattern of distribution of plants within a population and covered the full geographic extent of the population. We ensured each sampled plant was at least 10 m apart where possible.

A total of 293 samples were genotyped for the full set of 19 microsatellite loci developed for *Conospermum undulatum*; details of DNA extraction, polymerase chain reaction conditions and scoring of electropherogram profiles are provided in Delnevo et al. (2019 b).

Genetic diversity and population structure

To detect the possible presence of null alleles and genotyping failures in each population, we used the Bayesian approach implemented in INEST v2.2 (Chybicki & Burczyk, 2009). Each model was run for 5×10^5 cycles discarding the first 5×10^4 iterations and keeping results every 100 updates. After model selection based on the BIC criterion, we discarded loci for which null alleles were detected at >50% populations. Standard genetic diversity parameters (number of alleles, *A*; effective number of alleles, *Ae*; observed and expected heterozygosity, H_O and H_E, allelic richness, *Ar*; and private allelic richness, *PAr*) were then calculated using GenAlEx 6.5 (Peakall & Smouse, 2012) and HP-RARE (Kalinowski, 2005). Deviations of genotypic distributions from Hardy–Weinberg expectations (HWE) and genotypic linkage disequilibrium (LD) between all pairs of loci were assessed using GENEPOP 4.0 (Rousset, 2008). A measure of the contribution of each population to the total allelic diversity (A_T) and its within- (A_S) and between-population (D_A) components was estimated with METAPOP2 (López-Cortegano, Pérez-Figueroa, et al., 2019). The same program was used to estimate the percentage of individuals from each population contributing to a virtual pool of 1000 individuals created to maximise the total average number of alleles (maxK).

Parameters of genetic fixation (G_{ST}; Nei, 1977) and differentiation (Jost's *D*; Jost, 2008) among populations were estimated with GenAlEx 6.5, and their statistical significance were assessed with 999 permutations. The same software was used for calculating pairwise population genetic distance matrices used in both Principal Coordinate Analysis (PCoA) and Mantel test.

The Bayesian clustering algorithm implemented in STRUCTURE v. 2.3 (Pritchard et al., 2000) was used to assess the putative number of differentiated genetic clusters. The most-likely number of clusters (*K*) in which individuals can be divided was assessed using the empirical statistic ΔK calculated by STRUCTURE HARVESTER (Earl & Von Holdt, 2012; Evanno et al., 2005). The default parameter settings of the admixture model with correlated allele frequencies and no LOCPRIOR were used, with *K* varying from one to 15. Each run consisted of 1×10^5 burn-in iterations and 5×10^5 data collection iterations, and ten runs for each *K* were performed. Different runs for the same *K* were averaged using the software CLUMPP (Jakobsson & Rosenberg, 2007).

Effective population size

The effective population size (N_e) of the genetic clusters detected was assessed based on the linkage disequilibrium method implemented in NEESTIMATOR v2 (Do et al., 2014), discarding alleles with frequencies <0.02. The heterozygosity excess and M-ratio deficiency tests (Cornuet & Luikart, 1996; Garza & Williamson, 2001) as implemented in INEST v2.2 (Chybicki & Burczyk, 2009) were used to infer possible occurrence of a recent bottleneck. The two-phase-mutation model was used, with a proportion of multi-step mutations of 0.22. Statistical significance was assessed using the one-tailed Wilcoxon test after 1×10^6 permutations.

Fine-scale spatial genetic structure

Spatial autocorrelation analysis was used to assess the presence, intensity and extent of the spatial genetic structure possibly characterizing *C. undulatum* populations. It was performed by calculating the kinship coefficient F_{ij} (Loiselle et al., 1995) for each pair of individuals. For each distance class, tests for the statistical significance of average F_{ij} values were conducted by 5×10^3 permutations of individual geographical coordinates. Analyses were performed using 18 ad-hoc selected distance classes, from every 10 m at short spatial scale to every 1000 m at long spatial scale. The intensity of the spatial genetic structure (SGS) was measured by the *Sp* statistic (Vekemans & Hardy, 2004), computed as $Sp = b_F/(F_1–1)$, where b_F is the regression slope of the kinship estimator F_{ij} computed among all pairs of individuals against their geographical distances, and F_1 is the average kinship coefficient between individuals of the first distance class (0–10 m, for this calculation). An historical estimate of gene flow (gene dispersal distance parameter σ) was also obtained from the regression of pairwise kinship coefficients on the logarithm of the distance, computing b_F

within a restricted distance range (σ_e to $20\sigma_e$, Hardy et al., 2006) and using a range of effective density (d_e) estimates from 10 ind/ha to 100 ind/ha. All analyses were performed using SPAGeDi 1.5 (Olivier J Hardy & Vekemans, 2002).

Association between ecology and genetics

Redundancy analysis (RDA) was performed to assess the influence of environmental characteristics on the genetic indices of populations. In this analysis, the five genetic indices (Ar_{16}, H_E, F_{IS}, G_{ST} and Jost's D) were the response variables, whereas the following environmental variables were considered as predictors: number of plants, population area, fragment area, percentage of vegetated (both native and not native) land within a 500-m-radius area around the population centroid, current isolation index, historical isolation index, and historical percentage of vegetated land ('historical' refers to the 1950s before large-scale clearing for urbanisation occurred within the species range). Historical isolation index and historical percentage of vegetated land were quantified by means of historical aerial photographs sourced from https://map-viewer-plus.app.landgate.wa.gov.au/index.html. Prior to analysis, variables were scaled to zero-mean and unit-variance. Model selection was performed by assessing multicollinearity among and significance of predictors (respectively by means of the variance inflation factor, with a cut-off value of 3, and permutation tests).

Association between genetics and reproductive output was undertaken using extensive reproductive data collected in the 2017 flowering season (September-November 2017) by Delnevo et al. (2019. a). Briefly, they obtained a total of 64170 flowers and 3144 seeds from 198 selected plants (see Delnevo et al. (2019 a) for details on seed collection and germination methods).

To infer the relationship of genetic indices and current fragmentation conditions with plant reproductive variables, Principal Component Analysis (PCA) followed by variation partitioning (based on RDAs and partial-RDAs) were carried out. First, scaled genetic indices (i.e. Ar_{16}, H_E, F_{IS}, G_{ST} and Jost's D) and environmental variables (number of plants, population area, fragment area, percentage of vegetated land, floral display index, and current isolation index) were separately subjected to PCA in order to extract loading coefficients (i.e. scores of the populations in the multidimensional ordination space), providing a low-dimensional description of the datasets. Then, following the procedure suggested by Borcard et al. (2011), variation partitioning was performed on three variables related to plant reproduction (fruit production, seed production, and seed germination) constrained by the

genetic and environmental loadings. Multivariate analyses were run in R 3.6.1 (R Development Core Team, 2020) with the "vegan" package (Oksanen et al., 2019).

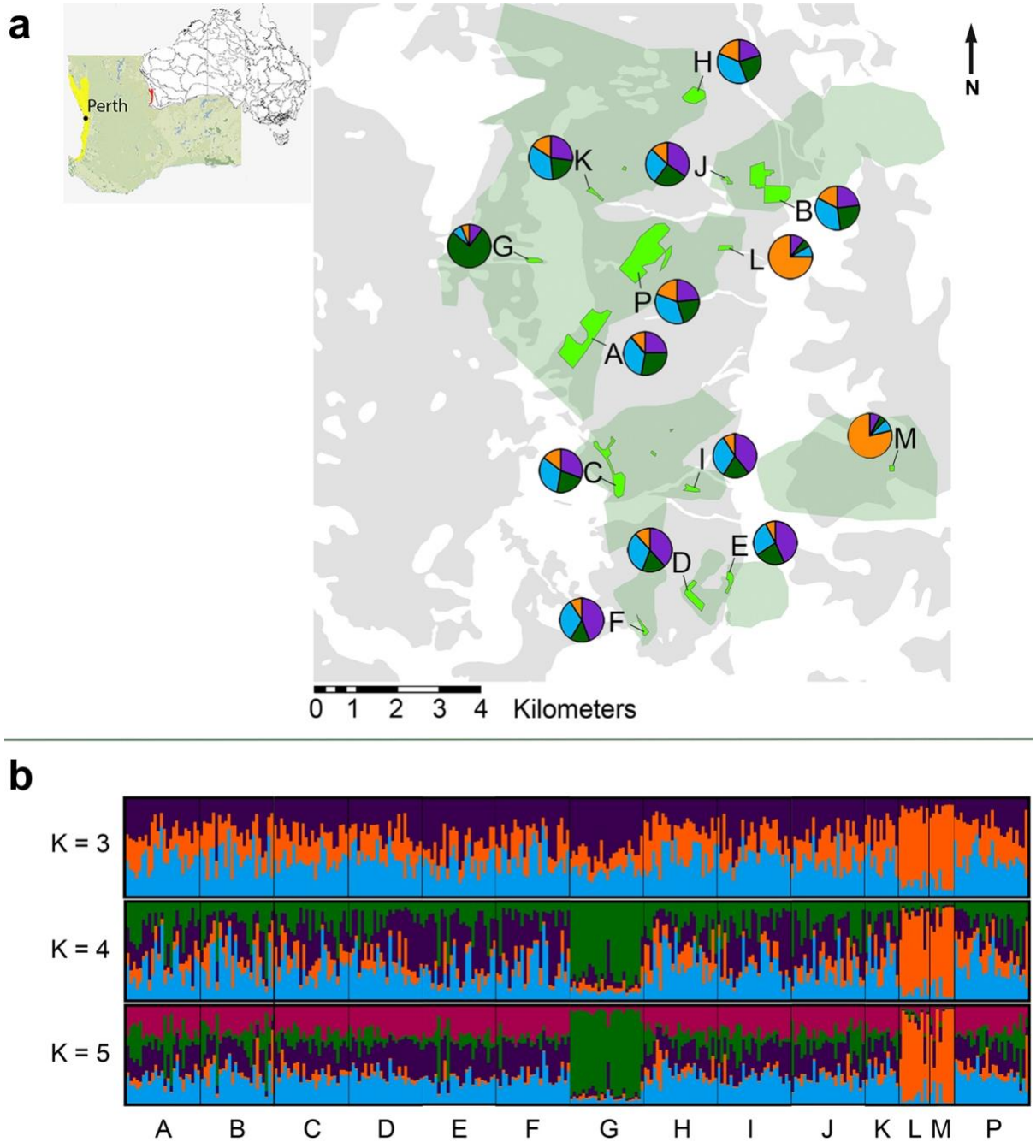


Figure 5.1. (a) Map of the location of *Conospermum undulatum* showing areas with suitable soil. Grey represents suitable soil (sands), whereas white represents unsuitable soil for *C. undulatum* (clays and other heavy soils). Darker green represents historical (1953) extent of native bushland (native bushland growing over incompatible soils unlikely to have contained *C. undulatum*). Bright green represents current extent of native bushland containing *C. undulatum*. Coordinates cannot be provided for specific locations of Threatened Flora. Pie charts shows the assignment probability of each population to genetic clusters inferred at K = 4 in Bayesian clustering. Inset map shows location of the Swan Coastal Plain in the

southwest Australia region. (b) Genetic ancestry of 293 individuals sampled from 14 populations, estimated using STRUCTURE analysis of microsatellite markers.

Results

Genetic diversity and structure

The final genetic dataset was assembled after discarding, as a precaution, 5 loci (Cu15, Cu17, Cu29, Cu41 and Cu45) that showed the possible presence of null alleles in the majority of populations at average frequencies ranging from 0.167 to 0.402. The levels of genetic diversity were mostly similar across populations, with the exception of populations E, G, L and M (Fig. 5.2; Table 5.1). These populations showed the lowest values of allelic richness and heterozygosity and, generally, the highest differentiation from the other populations (Table 5.1; Fig. 5.3; pairwise population matrices of G_{ST} and D in Table D.1 and Table D.2 in Appendix D). However, all populations carried at least one private allele, and private allelic richness varied between 0.10 in population G to 0.36 in population B. The generally even levels of genetic diversity and widespread presence of private genetic diversity reflects in the balanced percentage of individuals that should be collected from each population to maximise the allelic diversity (maxK; Table 5.1).

Table 5.1. Genetic diversity parameters of *Conospermum undulatum* populations.

Pop	Size	N	Na	PA	Ar_{16}	maxK %	PAr_{16}	H_O	H_E	F_{IS}	G_{ST}	Jost's D
A	880	24	9.36	1	6.24	7.4	0.17	0.675	0.709	0.050	0.015	0.063
B	800	24	9.21	5	6.24	7.2	0.36	0.667	0.725	0.080	0.018	0.083
C	520	24	8.86	5	6.00	7.3	0.28	0.612	0.708	0.134	0.019	0.086
D	780	24	9.00	3	6.17	7.1	0.33	0.694	0.719	0.061	0.023	0.106
E	230	24	8.21	1	5.70	8.1	0.17	0.658	0.684	0.047	0.021	0.091
F	675	24	9.21	4	6.16	8.4	0.27	0.698	0.725	0.030	0.019	0.085
G	236	24	7.21	1	5.50	6.4	0.10	0.586	0.699	0.195	0.033	0.152
H	216	24	10.07	3	6.34	8.2	0.25	0.711	0.718	0.024	0.017	0.076
I	183	24	9.50	3	6.31	9.2	0.32	0.652	0.720	0.128	0.019	0.085
J	139	24	8.93	2	6.15	4.6	0.16	0.679	0.720	0.062	0.018	0.081
K	11	11	7.29	2	6.34	5.3	0.18	0.649	0.680	0.020	0.019	0.085
L	10	10	4.00	1	3.81	7.1	0.11	0.550	0.569	0.081	0.054	0.218
M	8	8	4.00	1	4.00	6.0	0.16	0.563	0.471	-0.163	0.087	0.303
P	670	24	9.93	5	6.15	7.7	0.29	0.711	0.699	-0.023	0.017	0.075

Size: census size; N: sample size; Na: average number of alleles; PA: private alleles; Ar_{16}: allelic richness; *maxK*: distribution of alleles across populations of *Conospermum undulatum*; PAr_{16}: private allelic richness; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: fixation index; G_{ST}: mean G_{ST} value; Jost's D: mean D value.

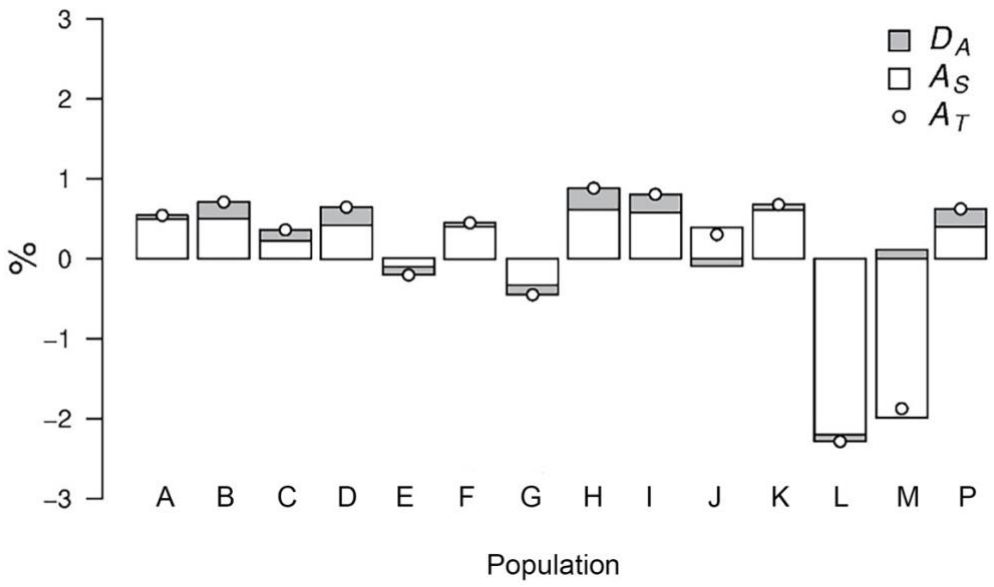


Figure 5.2. Percentage of loss (+) or gain (-) of total allelic diversity (A_T, dots) and its within-population (A_S, white bars) and between-population (D_A, grey bars) components after removal of each *Conospermum undulatum* population.

All analyses of genetic structure showed three populations (population G, and the small populations L and M) were markedly divergent from the rest of the populations that represented a genetically homogeneous group (Fig. 5.3). The latter group has an estimated *Ne* equal to 1589 [921-5082] and shows a typical isolation-by-distance (IBD) pattern where the pairwise genetic distance between populations is correlated with their geographical distance (Fig. 5.4a). This relationship becomes random when populations G, L, and M are included in the analysis (Fig. 5.4a). Populations L and M are the only two populations where the genetic signature of a recent bottleneck was detected according to the test for deficiency in M-Ratio (Wilcoxon signed-rank test: population L, Z score = -1.72. P = 0.041; population M, Z score = -2.20. P = 0.012).

At a finer spatial scale, a significant positive spatial autocorrelation of pair-wise kinship coefficients reflecting an average, non-random spatial distribution of genotypes up to 1000 m was found (Fig. 5.4b). Interestingly, a clear change in the slope of the relationship between kinship coefficients and spatial distance is recognizable between the 0-20 m and 20-1000 m intervals although the regression coefficient b_F is low overall (-0.00625). Such a slow decrease of the average kinship coefficient with distance indicates an overall low intensity of SGS (Sp = 0.007). Historical estimates of gene dispersal, calculated using the iterative procedure implemented in SPAGeDi, varied from 27.4 ± 3.29 m for d_e = 100 ind/ha to 116 ± 36.04 m for d_e = 10 ind/ha.

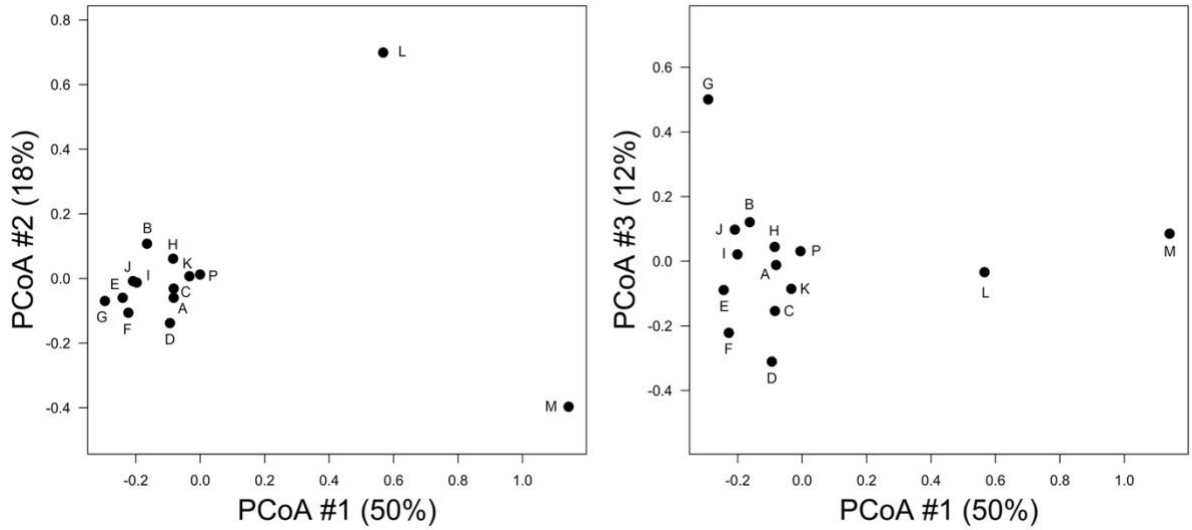


Figure 5.3. PCoA representing the genetic distance among *Conospermum undulatum* populations. The scores of populations are given in the space defined by the first and second (left panel) and first and third (right panel) principal coordinates.

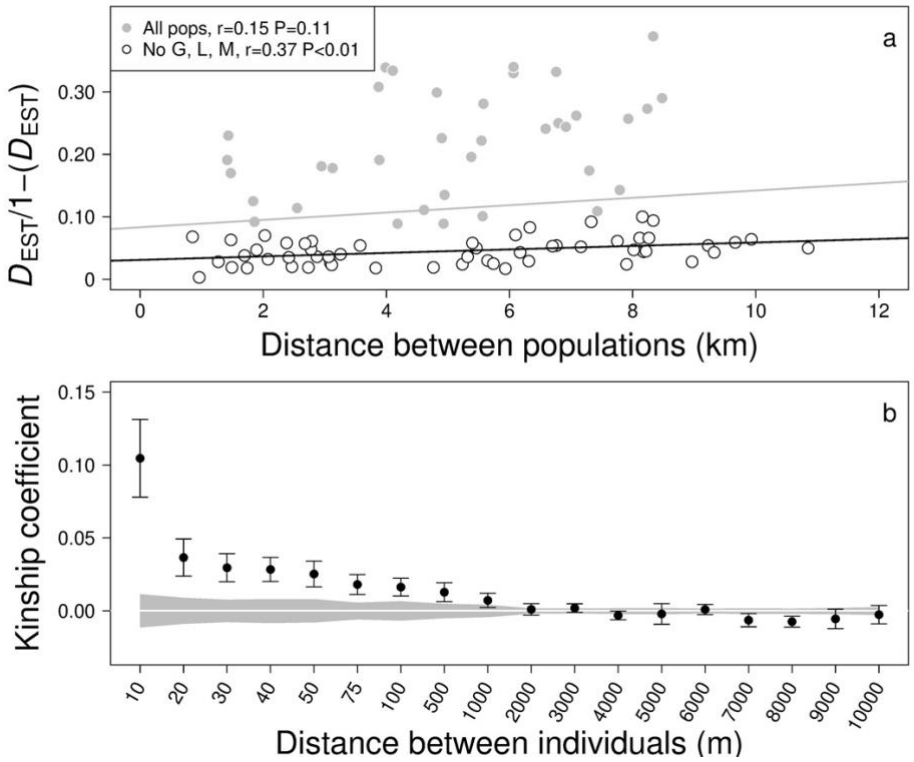


Figure 5.4. (a) Relationship between pairwise linearized Jost's D and geographic distance for pairs of populations. Pearson's correlation coefficients and P values after Mantel test with 1000 random cycles are reported. (b) Correlograms from spatial autocorrelation analysis using the correlation coefficient F_{ij} by Loiselle et al. (1995). The grey area represents the 95% CIs around the null hypothesis of absence of spatial genetic structuring, black lines around mean F_{ij} values represent their 95% confidence intervals generated by jackknifing loci.

Drivers of genetic diversity and reproductive performance

After excluding the variable number of plants from the model due to collinearity (VIF = 6.3), RDA showed that the variation in genetic indices of populations was significantly related to only one explanatory variable, namely the historical connectivity index (pseudo-$F_{1,12}$ = 7.46, P = 0.023). This parsimonious model, explaining 38.3% of the variation in genetic indices (based on the adjusted R^2), indicated that historical connectivity was negatively associated with among-population genetic differentiation and positively related to within-population genetic diversity (Fig. 5.5).

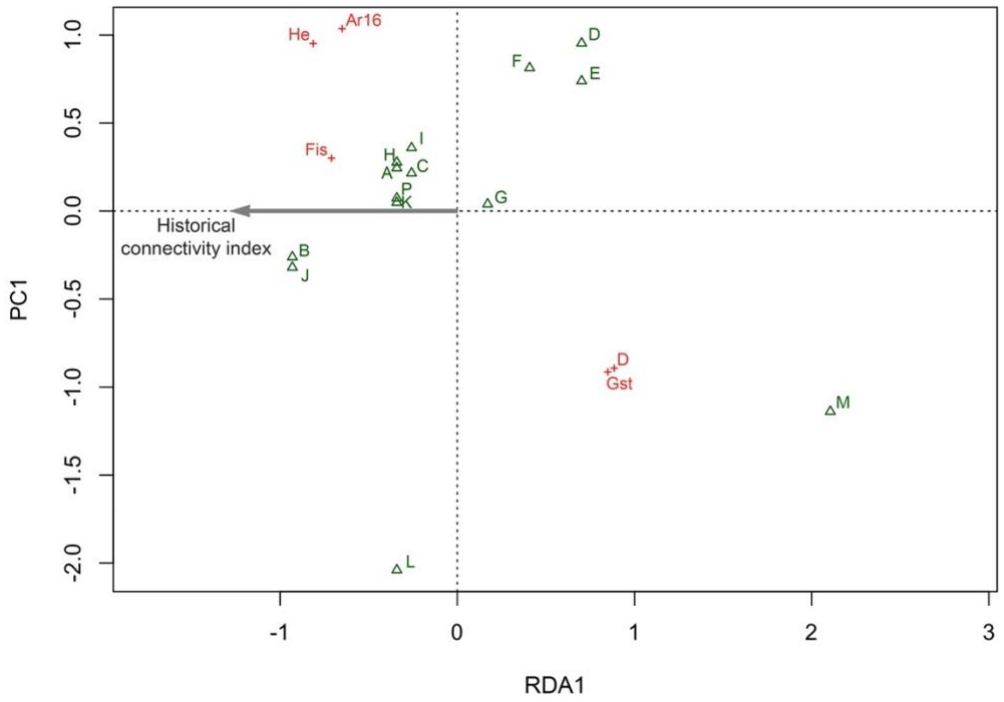


Figure 5.5. RDA triplot of the five genetic indices (crosses) of selected populations (triangles) constrained by the historical isolation index (arrow).

Model selection of RDAs performed on plant reproductive performance, constrained by the Principal Components (PCs) extracted by PCAs of genetic and environmental variables, retained the first genetic PC (pseudo-$F_{1,7}$ = 10.32, P = 0.009) and the first environmental PC (pseudo-$F_{1,6}$ = 12.66, P = 0.003). In particular, the first genetic PC had the highest correlation with genetic fixation index (G_{ST}), whereas the first environmental PC was correlated with natural vegetated land (fragment area) (Supplementary material Fig. C.2 in Appendix C). Variation partitioning of reproductive performances (Fig. 5.6) indicated significant influences for genetic, environmental and combined fractions (explaining *ca*. 65%

of the variance in the reproductive output; $F_{3,5} = 9.68$, $P = 0.001$), genetic and combined fractions ($F_{1,7} = 10.34$, $P = 0.004$), and environmental and combined fractions ($F_{2,6} = 12.66$, $P < 0.001$). However, whereas the proportion of variation in reproductive performance explained by the environmental fraction was significant ($F_{2,5} = 4.51$, $P = 0.020$), the one attributed to the genetic fraction alone was only marginally significant ($F_{1,5} = 3.21$, $P = 0.059$).

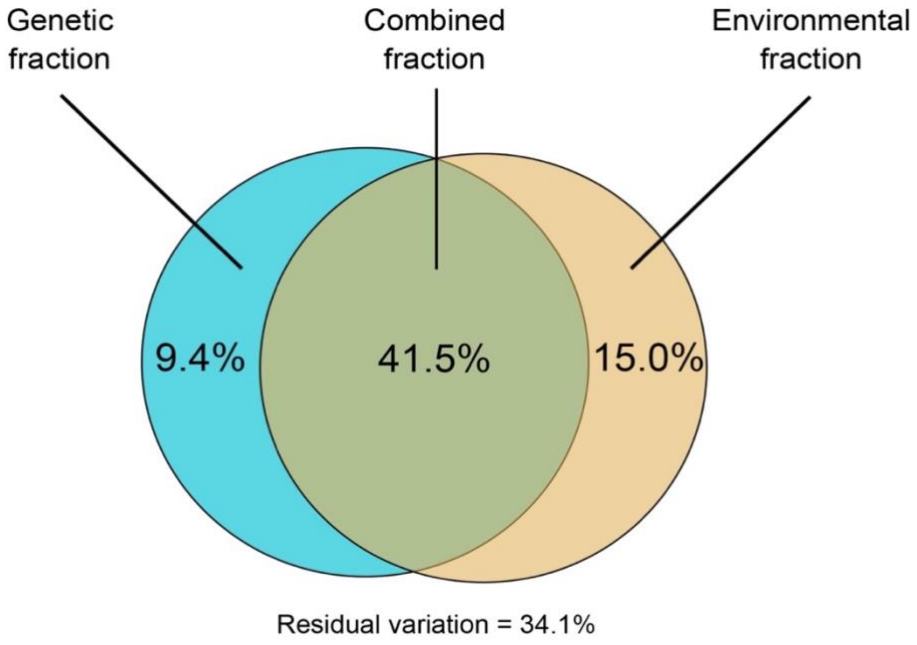


Figure 5.6. Variation partitioning analysis of the influences of genetic and environmental principal components on *Conospermum undulatum* reproductive performances. Venn diagram shows the percentage of variance explained by genetic (blue), environmental (orange), and combined (green) fractions.

Discussion

The relatively recent urban expansion of Perth allowed us to investigate the role of both current and historical environmental factors that have influenced the distribution of genetic diversity of *C. undulatum*. Genetic analysis of this threatened species revealed a relatively weak genetic structure and levels of genetic diversity and differentiation indices mainly influenced by historical levels of connectivity. We suggest that the low levels of genetic fixation and moderate differentiation in the core distribution area can be attributed to the combined effect of pervasive gene flow through the pre-fragmented landscape and the overall lack of detectable reduction in population size of this long-lived resprouting plant. Our study also demonstrates that the reproductive performance of *C. undulatum* were

correlated to a combination of current environmental conditions and level of genetic diversity, with important implications for its conservation.

Genetic diversity and structure

The patterns of genetic diversity and population structure found in our study were not consistent with theoretical expectations for highly fragmented populations. This result supports previous suggestions that the impact of fragmentation may depend on species-specific life-history traits and the timescale of the fragmentation process (Gibbs, 2001; Kramer et al., 2008). We found fixation index close to 0, moderate levels of genetic differentiation, and weak genetic structuring across the entire distribution range of *C. undulatum*. Although comparisons among studies involving different genetic markers have to be treated with caution, the only available study on the population genetics of *C. undulatum*, characterizing four populations using AFLP markers, showed similar weak genetic structuring (Close et al. 2006). The difference between fixation and differentiation indices (i.e. larger D than G_{ST}) might represent an early signal of failure of gene flow keeping populations genetically connected. The values of these indices depend on the accumulation of historic and recent migration and population sizes. Populations that are completely isolated from migration can still show low G_{ST} due to the maintenance of within-population diversity if deme size is still large, whereas D could be more influenced by past variation in migration rates (Jost et al., 2018; Whitlock, 2011). However, such indices should not be used to make inferences about current migration in populations which are likely not at equilibrium and, instead, should be interpreted at the empirical level to guide conservation decisions.

We also found a relatively even distribution of genetic diversity across the species' distribution. Ten out of 14 populations had similar levels of diversity and contributed a similar amount to the within-population component of total allelic diversity. Such a scenario, characterized by a rather genetically homogeneous group comprising the majority of populations, has been likely generated by extensive gene flow among populations over time. In fact, all the currently fragmented populations of *C. undulatum*, with the exception of two populations, were part of a continuous bushland 70 years ago, only punctuated by areas with incompatible soil. Peripheral populations that were historically isolated from the central distribution (i.e. populations G and M) showed a marked genetic divergence from the main group of populations. This may be explained by the presence of a large patch of incompatible soil that spatially separates these populations from the nearest conspecific individuals. Such barriers may have kept the populations separated well before the recent fragmentation event

that started in the late 1950s (Kelobonye et al., 2019), with levels of gene flow too low to avoid genetic drift. Indeed, populations G and M were historically separated from the main distribution by at least ~1500 m, well above the average distance that can be travelled by *C. undulatum* pollinators (based on the relation between body size and foraging distance in hymenopterans, Greenleaf et al., 2007).

The impact of recent fragmentation is also likely to be detected in the allelic diversity of populations that are now reduced to very small numbers of individuals (López-Cortegano et al., 2019). In our study, population L, which was part of the main continuous bushland in 1953 but is now reduced to 10 individuals, is one of the only two populations where the genetic signature of a recent bottleneck was detected and has the lowest allelic richness among all analysed populations (Ar_{16} = 3.810). Although geographically central, the genetic structure of population L differed from the main cluster of populations in the ordination space. This population also produced 20 fruits during the 2017 reproductive season, of which only two developed viable seeds (Chapter 2).

Gene dispersal is important to counteract the possible negative effects of genetic drift, and our investigation has also provided useful information about the dispersal capabilities of *C. undulatum*. Pre-fragmentation dispersal was not impeded, at least within large populations, with historical estimates of gene dispersal distance of up to 1000 m. Contemporary inter-population dispersal, on the other hand, is unlikely, given the current fragmentation and isolation of populations. The abrupt change in the slope of the kinship-distance relationship at ~20m may reflect the impact of different dispersal curves between pollen and seed dispersal on fine scale spatial genetic structure. The limited, gravity-driven seed dispersal of *C. undulatum* (to date only inferred by field observations; Close et al., 2006) is consistent with high average kinship coefficients observed over short distances. In contrast, pollen dispersal by the native bee *L. conospermi* is likely responsible for genetic connectivity until the upper limit of where fine-scale structure was significant (~1000 m). The genetic data is consistent with the inferred foraging range of *L. conospermi* (~500 m; Houston, 1989) and provides evidence for the spatial extent of pollen dispersal undertaken by this pollinator. The foraging range of *L. conospermi* is shorter compared with other common bird and insect pollinators encountered in similar habitats. Common pollinating birds such as honeyeaters have been tracked moving up to 12.5 km while foraging (Saunders & De Rebeira, 1991). European honeybees have been shown to maintain extensive pollen flow in fragmented landscapes (Byrne et al., 2007; Dick et al., 2003); indeed, *A. mellifera* is able to extend its foraging range up to 9.5 km from the hive (Beekman & Ratnieks, 2000). For the smaller native bee *L.*

conospermi, built-up urban areas may generate more significant barriers to inter-population movement (Andersson et al., 2017), limiting pollen-mediated gene flow only to within continuous populations of *C. undulatum*. These differences in dispersal highlight the great importance of considering species-specific pollinator groups when inferring the potential impact of habitat fragmentation on contemporary gene dispersal.

Historical connectivity

In many cases, failure to detect a genetic response to habitat fragmentation may be primarily due to insufficient time having elapsed since fragmentation. This may particularly be true for long-lived plant species, since they can carry a 'loss of genetic diversity' debt for many generations after a fragmentation event (Aguilar et al., 2008; Honnay & Jacquemyn, 2007; Vranckx et al., 2012). Our results for *C. undulatum* showed a geographical clustering of populations and a spatial distribution of genetic diversity not compatible with impeded gene flow. The genetic structure of these populations was related only to the connectivity among populations in 1953 inferred from historical records. Such effects of historical landscape structure on gene flow have also been reported for the shrub species *Grevillea caleyi*, another member of the Proteaceae (T. M. Llorens et al., 2004). Moreover, similar to our findings, Honnay et al. (2007) found that fragmented populations of the forb *Globularia bisnagarica*, historically belonging to the same grassland patch, still retained a spatial genetic structure mostly determined by pre-fragmentation processes. Our study indicated that the current spatial genetic structure of remnant *C. undulatum* populations reflects population distributions over deeper time scales. Population disturbance since 1953, has yet to impact patterns of spatial genetic structure in the contemporary adult cohort. Taken together, these results support hypotheses that plant longevity relative to time since disturbance and mean dispersal distances are key factors buffering populations from the effects of habitat fragmentation.

Reproduction

It is widely known that sexual reproduction plays a key role in migration, adaptation, and population persistence (e.g. Fenner & Thompson, 2005). Recent work in *C. undulatum* (Delnevo et al., 2019 a) found that reproductive output was significantly correlated with ecological consequences of habitat fragmentation, including reduction in population size, floral display and spatial connectivity among fragments. In line with these findings, in the current study we found a strong influence of fragmentation factors on the reproductive effort

of *C. undulatum*. The addition of the genetic information of populations to the analysis highlighted that most of the variation in key stages of sexual reproduction (i.e. pollination, fertilization and germination) was explained by the combined fraction of genetic and environmental characteristics of *C. undulatum* populations. Although the genetic fraction alone was only marginally significant, the environmental and the combined fractions explained 56.5% of the variation, indicating a joint influence of genetic diversity and the current environmental condition.

Changes in the spatial arrangement of remnant populations following anthropogenic fragmentation can lead to altered dynamics of gene flow, with small and/or isolated populations being less visited by pollinators (Dauber et al., 2010). In addition, reduced dispersal between populations may reduce the ability of small populations to recover lost genetic variation from larger remnants. In the short term, a loss of heterozygosity following population size reduction can reduce fitness due to inbreeding depression (Charlesworth & Willis, 2009). When this occurs in predominantly outcrossing plants, such as *C. undulatum*, the effects of inbreeding depression can impact early fitness traits such as the development of the embryo and seed germination (Aguilar et al., 2019; Vranckx et al., 2012). Our results highlight the current area of populations as an important variable influencing the reproduction of the species. A lack of contemporary inter-population pollen flow due to limited pollen dispersal may over time reduce the reproductive output in small, isolated populations. The weak but detectable effect of genetic factors on reproductive components may represent an early signal of the impact of increased inbreeding in small remnants. Given that inbreeding depression is often environmentally dependent (Yun & Agrawal, 2014), it is therefore not surprising that the interaction between genetics and environmental variables best explained reproductive components.

Conservation implications

This study highlights the importance of combining population genetics, reproductive data and ecological characteristics, together with the biogeographic history of populations, to obtain a more complete picture of the impact of habitat fragmentation. Many plants may tolerate habitat fragmentation due to naturally high levels of inbreeding and low genetic diversity due to self-compatible mating systems (e.g. Llorens et al., 2018). Other species may cope with habitat fragmentation due to highly mobile pollinators able to extend their foraging range to maintain pollen dispersal between remnant populations (e.g. Byrne et al., 2007). However, for plants such as *C. undulatum* which are self-incompatible species and rely on

small native bees for pollen flow (Delnevo et al., 2020 a) these population changes may have a stronger impact on reproductive output. Soil stored seed banks may provide some temporal buffer to these impacts, although seed viability in the related species *Conospermum triplinervium* have been shown to progressively decline in viability over 15 months (Tieu et al., 2007). For *C. undulatum*, the combination of life-history traits, mating system and pollinators in this system may therefore provide further challenges for its ability to regenerate after disturbance in fragmented landscapes.

Therefore, avoiding further decline in the size of medium and large populations is crucial to mitigating the effects of genetic drift and maintaining genetic diversity of *C. undulatum*. Nonetheless, populations of *C. undulatum*, like those of many other highly fragmented species, are more likely to face extinction from demographic factors (González-Varo et al., 2012) long before the lack of genetic variation impedes adaptation to changing environments. The reproductive performance of *C. undulatum* is already affected by current fragment size and the combination of environmental and genetic features of remnant populations. Our study did not identify any clear source or sink populations to be prioritised for conservation based on their contribution to within- and between-populations allelic diversity (López-Cortegano et al., 2019). This is particularly imporant for planning conservation actions such as translocations and reintroductions in order to maintain, or increase, adequate population size to sustain the genetic diversity within and among populations. Conservation efforts aimed at reconnecting remnant populations are predicted to be especially fruitful in species with limited pollen dispersal and should be combined with ongoing efforts to monitor reproductive output.

Supplementary material

Pairwise G_{ST} and D matrices (Table C.1, Table C.2 in Appendix C), STRUCTURE analysis (Fig C.1 in Appendix C), Principal Components of genetic and environmental variables (Fig. C.2 in Appendix C).

Funding sources

Funding: This project was supported by The Holsworth Wildlife Research Endowment & The Ecological Society of Australia [R12019/G1004377 to ND]

Chapter 6 – Contemporary pollen-mediated gene flow in a fragmented urban landscape

Introduction

The world's human population is rapidly moving to a high-density urban existence and, as a direct consequence, urban areas have become the most rapidly expanding habitat type worldwide (Grimm et al., 2008). Although cities and their associated sprawl now cover 3% of the world's land (Faeth et al., 2011), the associated anthropogenic fragmentation of natural habitats is one of the most important drivers affecting biodiversity loss (Haddad et al., 2015; Sala et al., 2000). Within urban environments, organisms experience unique pressures that can make long-term persistence of populations particularly difficult (Beninde et al., 2018; González et al., 2019).

In plant populations, land clearing and man-made infrastructure may act as barriers to gene flow, leading to genetic isolation and, consequently, greater fragmentation and reduction in size of originally continuous populations (Holderegger & Di Giulio, 2010; Johnson & Munshi-South, 2017). Habitat loss and fragmentation affect current plant–animal interactions and the associated ecological processes shaping patterns of mating and pollen dispersal (Aguilar et al., 2008, 2019; Brudvig et al., 2015; Gonzalez et al., 2011), and responses in different systems may differ based on the foraging behaviour of pollinators. Indeed, highly mobile pollinators, such as birds and European honeybees, are often able to extend their foraging range in response to increased plant spacing (Beekman & Ratnieks, 2000; Saunders & De Rebeira, 1991) and many plant species that rely on these vectors have shown extensive, or even increased, gene flow among populations (Bezemer et al., 2016; Byrne et al., 2007, 2008). Honeybees (*Apis mellifera*) have been introduced to many areas and are considered to be effective pollinators in disturbed landscapes because of their high density and social organization (Dick et al., 2003). However, they are not native to certain parts of the world, including Australia, where the native bee fauna is large and diverse, and differs from bees of other continents (Houston, 2018). Results from studies on the responses of honeybees to habitat fragmentation and their consequences on pollination dynamics are thus not transferable to plants of ancient Gondwanan families, such as the Proteaceae.

Many flowering plants in Australia have evolved in isolation for at least 34 million years (Exon et al., 2006; Mcloughlin, 2001); such a long period of isolation has facilitated

specialization, resulting in the development of specific floral morphologies and pollination systems. Although many proteaceous species coevolved with birds for pollination, plant-insect pollination mutualisms are also an important component of Australian ecosystems and a high number of species belonging to this ancient family rely exclusively on native insects for pollination (Hopper & Gioia, 2004). Such high specificity in plant-insect mutualistic associations, especially when native plants are rare and threatened, makes understanding the impact of habitat fragmentation on such systems particularly relevant.

Population genetic theory predicts that small and isolated plant populations can be prone to genetic impoverishment through the negative effects of genetic drift (Ellstrand & Elam, 1993), with important consequences for seed production and progeny fitness (Aguilar et al., 2019; González-Varo et al., 2010; Tamaki et al., 2009). However, there is mixed evidence emerging from empirical studies testing such predictions and recent syntheses have proposed a variety of factors related to this apparent lack of uniform genetic signals following habitat fragmentation (Aguilar et al., 2008; Kramer et al., 2008; Lowe et al., 2015). In addition, populations of long-lived plants have often experienced relatively recent fragmentation events, making it difficult to detect their genetic effects, in particular when the investigation is focused on adult trees (Lowe et al., 2015). In this regard, the amount of current (post-fragmentation) pollen dispersal and the distance over which it occurs have been proposed as early signals, more easily detectable in the short term, that will have significant impacts on shaping the future genetic layout and reproductive processes in fragmented populations (e.g. Cheptou et al., 2008; Lowe et al., 2015; Robledo-Arnuncio et al., 2014). For many flowering plants, the majority of which rely on pollination by biotic vectors (Ollerton et al., 2011), the long-term functioning of fragmented populations is dependent on the responses of pollinators to the changes in the spatial arrangement of remnant plants.

Conospermum (Proteaceae) is an insect-pollinated genus endemic to Australia with its centre of distribution being the south-west corner of Western Australia. It includes 53 species (Bennett, 1995), with four taxa listed among the threatened flora of Western Australia (W.A. Government Gazette, 2018). Of these, *Conospermum undulatum* is a long-lived lignotuberous shrub endemic to the southwestern Australia biodiversity hotspot (Mittermeier et al., 2004) and threatened by urban expansion, with its spatial distribution being now completely embedded within a large urban area. This species has coevolved to facilitate pollination by the small native bee *Leioproctus conospermi*, together with native ants as secondary pollinators (Delnevo et al., 2020 a; Delnevo & van Etten, 2019). Like most species within the genus, *C. undulatum* possesses characteristic flowers that appear too small to be effectively

pollinated by introduced European honeybees and therefore, represents an opportunity to study patterns of pollen flow in species that rely exclusively on native pollinators. Recent studies showed that habitat fragmentation negatively influenced the reproductive output of *C. undulatum* in terms of seed production and germination rate, and that most of the variation in key stages of sexual reproduction (i.e. fertilization and germination) was explained by the combined fraction of genetic and fragmentation characteristics of *C. undulatum* populations (Chapter 5). However, genetic characteristics alone showed a weak effect on the reproduction of the species, and this was attributed to the fact that population fragmentation was too recent to show the predicted effect of genetic drift on the adult cohorts of plants analysed.

Given the importance of identifying responses of plants to fragmentation, assessment of mating systems in fragmented landscapes provides a means of determining ongoing effects on maintenance of genetic diversity particularly in long lived plants where time since fragmentation has not been long enough for detection of genetic effects in current populations. In this study, we aimed to investigate the early signals of the effects of fragmentation on a threatened plant species that relies on small native pollinators for reproduction. In particular, we used paternity assignment to: (i) quantify the amount of pollen immigration into different populations, (ii) determine the distances over which local pollen dispersal occurred, and (iii) identify the effects of ecological and reproductive traits on the observed patterns of local pollen dispersal.

Material and methods

Study species and area

Conospermum undulatum (Proteaceae) is a long-lived species, with individual shrubs able to resprout from semi-subterranean woody lignotubers after disturbance such as fire or mechanical damage from strong winds. During the flowering season (late August- late October) it produces white inflorescences. *Conospermum undulatum* is an entomophilous species and has a specific suite of native insect pollinators, with the small specialised native bee *Leioproctus conospermi* being the most active floral visitor (Delnevo et al., 2020 b). Genetic connectivity via pollen is potentially maintained for up to 1000 m by this native pollinator through non-fragmented landscapes (Chapter 5). Flowers of *C. undulatum* have a characteristic pollination mechanism that involves a tactile stimulation within the calyx. When a visiting insect applies pressure with its mouthparts at the base of the style, it flicks away from the fertile anthers and strikes the visitor. The moist cup-shaped stigma is forced

down onto the pollinator and thereby picks up pollen carried by the insect; at the same time the fertile anthers dehisce explosively, casting new pollen onto the visitor (Douglas, 1997). This remarkable pollination system is an effective physical barrier that prevents autogamous selfing. Recent flower manipulation experiments suggest that self-incompatibility in *C. undulatum* was not only due to its specific flower morphology that prevents autogamy, but was also a genetic response to prevent self-pollination across different flowers of the same plant (Delnevo et al., 2019 a). *Conospermum undulatum* fruits are cone-shaped achenes (i.e. containing only one seed) that fall to the ground when mature and appear to have a spatially limited dispersal, mainly driven by gravity (Close et al., 2006). Low fecundity is common in resprouter species in the Proteaceae (Lamont et al., 2011) and, seed set in *C. undulatum* is low, especially when compared with the number of flowers produced.

Conospermum undulatum occurs in the Swan Coastal Plain bioregion, a low-lying coastal plain that extends from Jurien Bay, north of Perth, to Cape Naturaliste in the south, and it is part of the southwest Australia global biodiversity hotspot (Mittermeier et al., 2004). The region has experienced extensive land clearing for urbanisation, which is centred around the capital city of Perth, with impacts readily visible on its biodiversity (e.g. Davis et al., 2013; Heterick et al., 2013). Approximately 35% of native vegetation remains on the Swan Coastal Plain, and 10% occurs in protected areas (Wardell-Johnson et al., 2016). It is currently known from 17 populations ranging in size from a few individuals to several hundred plants. It has a naturally restricted distribution across 55 km^2 and has been impacted by land clearing for urban expansion. *Conospermum undulatum* is listed as 'vulnerable' among the threatened flora of Western Australia using IUCN red list criteria (Department of Environment and Conservation, 2009; W.A. Government Gazette, 2018).

All known populations of *C. undulatum* were intensively surveyed at the beginning of the flowering season 2017. The present study focused on eight populations (Table 6.1). Three of these were medium-sized populations, ranging from 139 to 236 adult plants (determined by direct counts); three were small populations, with eight, ten, and eleven plants; and two were large populations with an estimated size of <500 plants, one of which (population B) is part of an intact nature reserve. We restricted our sampling in the large populations to focus areas consisting of patches of *ca*. 70-100 m radius, similar to the fragment sizes of the other populations in order to compare patterns of pollen movement of smaller populations with those of large intact populations. All populations were discrete and distinctly separated by cleared land. All selected populations, except populations G and M, were within the 1000 m

distance that can potentially be covered by the main pollinator *L. conospermi* (see "Nearest unsampled plants" column in Table 6.2). To accurately assess pollen dispersal distances and spatial genetic structure, a BE-3300 GNSS Surveyor (Bad Elf, West Hartford, CT, USA) was used to obtain GPS coordinates of each plant to an accuracy of 1 m.

Table 6.1. Genetic diversity parameters of *Conospermum undulatum* populations.

Pop	Census size	*N*	*Na*	*PA*	Ar_{16}	PAr_{16}	H_O	H_E	F_{IS}	G_{ST}	Jost's *D*
B	800	72	11.29	7	6.11	0.50	0.645	0.736	0.115	0.030	0.133
C	520	47	10.50	5	6.17	0.57	0.658	0.715	0.075	0.031	0.133
G	236	236	10.64	3	5.69	0.26	0.604	0.723	0.167	0.041	0.185
I	183	183	14.29	25	6.30	0.68	0.674	0.739	0.096	0.028	0.124
J	139	139	13.00	14	6.01	0.41	0.631	0.726	0.134	0.027	0.117
K	11	11	7.29	1	6.34	0.48	0.649	0.680	0.020	0.029	0.122
L	10	10	4.00	0	3.81	0.12	0.550	0.569	0.081	0.058	0.228
M	8	8	4.00	1	4.00	0.22	0.563	0.471	-0.163	0.089	0.307

N: sample size; *Na*: average number of alleles; *PA*: private alleles; Ar_{16}: allelic richness; PAr_{16}: private allelic richness; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: fixation index; G_{ST}: mean G_{ST} value; Jost's *D*: mean *D* value.

Table 6.2. Mating system parameters calculated from seed crops sampled from six populations of *Conospermum undulatum*. Pollen immigration and selfing rates were estimated by modelling two different scenarios using NMπ and by direct estimate from paternity assignment data in *Cervus*.

Pop	Nearest unsampled plants (m)	Maternal plants	Progeny per mother	Mean pollen dispersal distance[†] (m)	Model	Pollen immigration (mp)	Selfing (s)	AIC[‡]
B[*]	–	7	3.50 (0.99)	25.12 (6.55)	mp + s	0.518 (0.124)	0.034 (0.055)	2105.5
					mp	0.516 (0.123)	–	2103.7
					Cervus	0.524	0.000	–
C[*]	–	14	2.57 (1.05)	27.14 (6.64)	mp + s	0.131 (0.083)	0.112 (0.074)	1851.2
					mp	0.238 (0.100)	–	1907.2
					Cervus	0.150	0.100	–
G	1680	11	2.18 (0.52)	35.18 (11.83)	mp + s	0.102 (0.102)	0.458 (0.109)	2220.6
					mp	0.494 (0.126)	–	2432.3
					Cervus	0.000	0.417	
I	650	8	1.75 (0.49)	35.28 (12.59)	mp + s	0.144 (0.095)	0.499 (0.134)	648.1
					mp	0.359 (0.129)	–	715.4
					Cervus	0.142	0.071	–
J	570	10	3.00 (0.77)	26.45 (5.10)	mp + s	0.000 (0.089)	0.101 (0.055)	3325.7
					mp	0.125 (0.084)	–	3364.9
					Cervus	0.000	0.100	–
K	600	2	1.50 (0.50)	30.98 (5.77)	mp + s	0.000 (NE)	0.000 (NE)	203.4
					mp	0.000 (0.285)	–	–
					Cervus	0.000	0	–
L	800	0	0		–	–	–	–
M	3900	0	0		–	–	–	–

Parameters are expressed as mean values per population. Standard errors are in parentheses
NE: not estimable.
[*] within focus area.
[†] for within population outcrosses.
[‡] Akaike information criterion.

Population variables

Population size was assessed in each fragment by direct count of plant individuals, except for the two large populations. A connectivity index was calculated based on a modified version of the incidence function model (Hanski, 1994), following the approach used by Delnevo et al. (2019 a). This model accounts both for distances to all neighboring populations (including those not selected for this study) and the area of those populations, and provides a better estimate in highly fragmented habitats and small datasets compared with either nearest neighbor or buffer measures (Moilanen & Nieminen, 2002). Temporal isolation of populations was investigated through observations of reproductive phenology and found to be negligible (Delnevo et al., 2019 *a*). The area covered by each population was obtained with ArcGIS (ESRI, Redlands, USA) using the minimum convex polygon method. We then calculated the plant density, and the floral display index (FD) of each population was calculated as: FD = (*I* • AC) / 100, where *I* is the mean number of inflorescences per plant in a specific population; and AC is the area (in m^2) covered by *C. undulatum* plants within the fragment.

Following disturbance, *C. undulatum* individuals regenerate through resprouting from the lignotuber to produce additional stems, and plants that go through more regeneration cycles have more stems than plants that have less regeneration cycles. Since fire in the area is fairly regular due to planned hazard reduction burning, the number of stems can be used as a proxy for age, that is plants with more stems are older than plants with fewer stems. Therefore, we counted the number of stems of each individual in all the investigated populations to calculate the average number of stems per population and obtain an overall estimate of population age.

Sampling and genotyping

Leaf samples were collected from each plant in all the eight populations in accordance with license restrictions to collect biological material from threatened species. Following intensive surveys, we sampled all individuals in small and medium sized populations to assign/exclude plants within populations as fathers. Similarly, all plants were sampled from within the selected focus areas. We obtained a total of 706 samples that were stored in silica gel in individual paper bags before DNA extraction. Due to the expected low seedling production in *C. undulatum* (Delnevo et al., 2019 a), we maximised fruit collection for inclusion in the study according to restrictions stipulated in the collection licence and a total of 1206 fruits were collected. Seed germination followed the best-known protocol as outlined in Delnevo et al. (2019 a). A total of 131 seedlings were obtained from 52 maternal plants representing the product of the 2017 flowering season. Two

of the small populations M and L produced zero and two viable seeds, respectively, of which none germinated.

Genomic DNA was extracted from adult leaf tissue and from entire seedlings, harvested 1–2 weeks after germination, and genotyped at 19 microsatellite loci that have been developed for *C. undulatum*. Details of DNA extraction, polymerase chain reaction conditions and scoring of electropherogram profiles are provided in Delnevo et al. (2019 b). All aspects of genotyping involved extensive manual checking to minimize errors. All allele bins were defined manually, and all allele assignments were manually checked and corrected where necessary. Parental samples that did not amplify clearly or presented very uncommon alleles were re-amplified, and DNA was re-extracted where necessary.

Deviations of genotypic distributions from Hardy–Weinberg expectations (HWE) and genotypic linkage disequilibrium (LD) between all pairs of loci were assessed using GENEPOP 4.0 (Rousset, 2008). Standard diversity indexes (Na, *PA*, H_O, H_E, F_{IS}; *Ar*; and *PAr*) were then calculated using GenAlEx 6.5 (Peakall & Smouse, 2012) and HP-RARE (Kalinowski, 2005). For this analysis we discarded, as a precaution, 5 loci (Cu15, Cu17, Cu29, Cu41 and Cu45) that showed the possible presence of null alleles according to the Bayesian approach implemented in INEST v2.2 (Chybicki & Burczyk, 2009). Parameters of genetic fixation (G_{ST}; Nei, 1977) and differentiation (Jost's *D*; Jost, 2008) among populations were estimated with GenAlEx 6.5, and their statistical significance was assessed with 999 permutations.

Contemporary pollen dispersal

Despite the power of recently developed mating models to estimate mating system and gene flow parameters, the use of different approaches is suggested in the literature (Bacles & Ennos, 2008; Jones et al., 2010). In all the populations that produced seedlings, paternity analysis was therefore performed on microsatellite data using two different approaches to control for the robustness of gene flow estimates. First, we used NMπ version 1.2 (Chybicki, 2018) to model paternity probabilities of progeny based on the spatially explicit neighbourhood model of Adams and Birkes (1991), which simultaneously produces maximum-likelihood estimates of several mating and dispersal parameters. Second, we used *Cervus* version 3.0.7 (Kalinowski et al., 2007; Marshall et al., 1998) to perform a maximum likelihood categorical paternity assignment, including the correction to accommodate genotyping inconsistencies.

An advantage of NMπ over traditional parentage methods is that seed and pollen dispersal parameters, seed- and pollen-mediated gene flow, and their confidence intervals, are jointly

estimated. Whereas, in the *Cervus* approach paternity is investigated in subsequent steps (see Jones et al. (2010) for a comprehensive discussion on this issue). Nonetheless, for datasets with a high exclusion probability and a good sample of the possible pollen donors, paternity assignment can elucidate the patterns of fine-scale pollen dispersal within populations.

We used NMπ to estimate the rates of selfing (s) and pollen immigration (m_p) (and their confidence intervals) using the default value for the neighbourhood size and simultaneously allowing the maximum-likelihood estimation of null allele frequencies. We ran two different models, one with m_p only and no selfing allowed; the other allowing for self-pollination ($m_p + s$). Model selection was performed using the Akaike Information Criterion (AIC) and the model with the lowest AIC value used.

Before performing the paternity analysis in *Cervus*, 100000 simulations were run to estimate the LOD thresholds above which paternity was assigned at the 95% (strict) and 80% (relaxed) confidence levels. The parameters for the simulations were: 0.005 as mistyping rate, the number of individuals in each population as probable candidate fathers in each respective population, 0.99 as proportion of sampled fathers, 10 as minimum number of typed loci, and self-fertilization allowed. Once paternity was assessed, we calculated selfing and pollen immigration rates directly from the paternity assignment data and compared them with the equivalent rates obtained through NMπ. Pollen immigration in the focus areas within large populations most likely represent immigration into the area rather than immigration into the population but can be useful for comparisons between intact natural bushland and fragmented populations (e.g. Llorens et al., 2012).

We then determined the distributions of pollen dispersal events for the six populations that produced seedlings. Pollination distances were calculated as the Euclidean distance between pollen donors and maternal trees, and selfed progeny were given a value of 0 m. Patterns of within-population pollen movement were explored by examining the distances over which successful pollen travelled between paternal and maternal plants.

Variation in selfing rates, pollen immigration, and proportion of successful sires within a population (directly estimated using paternity assignment data) was investigated by means of Principal Component Analysis (PCA). The broken stick criterion was used to select the number of axes retained in the PCA (Legendre & Legendre, 1998). To aid the interpretation of ordination axes, external variables (i.e., population size, area, connectivity, floral display, density, and mean number of stems per plant) scaled to zero-mean and unit-variance were projected onto PCA, and the significance of their correlations with ordination axes was assessed by means of permutation test. This analysis was carried out with the package "vegan" (Oksanen et al., 2019) of R v3.6.3 (R Core Team, 2020).

Results

Genetic diversity

The levels of genetic diversity were mostly similar across populations, with the exception of the small populations K, L, and M (Table 6.1). In particular, populations L and M showed the lowest values of allelic richness (Ar_{16}) and heterozygosity (H_O) and, generally, the highest differentiation from the other populations (Table 6.1; pairwise population matrices of G_{ST} and D in Table E.1 and Table E.2 in Appendix E). Moreover, population L was the only population without private alleles.

Selfing rates and pollen immigration

The NMπ model that included both m_p and s outperformed the model that only included m_p (Table 6.2). The presence of selfing was surprising given expectation of *C. undulatum* being self-incompatible, suggesting that transfer of self-pollen between different flowers of the same plant can, in some cases, lead to the production of viable seeds. The results from NMπ model were also supported by direct estimates from paternity assignment. The selfing rate in the largest populations B and C was low, as well as in population J with less than 150 plants, compared to the high rates detected in the medium sized populations G and I (Table 6.2). Selfing rate in the small population K was zero (Table 6.2). Interestingly, population C had a single plant that produced an unexpected high number of selfed progeny (13 selfed seeds out of a total of 16). This plant went through numerous regeneration cycles (11 stems) and was a clear outlier. Therefore, we ran the neighbourhood model both with and without this plant.

Pollen immigration rates were the highest in the two large populations (Table 6.2). Two medium populations showed differing responses with similar pollen immigration to larger populations detected in population I but no pollen immigration detected in population J. Population G, although being the third largest population with 236 plants, had a non-significant pollen immigration rate (being 0.000 following direct estimate after paternity assignment with *Cervus*; Table 6.2). Small populations did not produce any seeds with the exception of population K, that showed no pollen immigration (estimates for population K are generated from only three seedlings).

Direct estimates of pollen immigration and selfing rates from paternity assignment with *Cervus* were consistent with the ones obtained using the neighbourhood model (Table 6.2). The only exception was population I, for which NMπ gave a selfing rate of 0.499 (± 0.134 s.e.), whereas direct calculation from *Cervus* resulted in a selfing rate of 0.071. This discrepancy was due to the

fact that NMπ assigned three seeds of a mother plant as selfed progeny, while *Cervus* assigned the paternity of those seeds to a nearby plant (only 0.97 m away), with selfing being the second-most likely assignment. It is possible that these two plants in close proximity were closely related (i.e. mother and offspring), generating the different paternity assignment.

The overall dispersal distances for outcrossed pollination events detected by the paternity assignment had a median distance of 16.80 m, a mean of 28.96 m (30.24 s.d.) and ranged between 0.34 m and 125.04 m. The distributions of pollen dispersal distances were generally lower than the expected distributions considering the potential distances among all possible pollen donors and maternal plants (Fig. 6.1). This trend was less evident in the two large populations B and C (Fig. 6.1) that also had lower frequencies of local fathers within the first 10 m from the mother plants. In particular, population G had the most skewed distribution of dispersal distances compared to the other medium populations I and J, with only a few successful pollination events over 20 m from the mother plant.

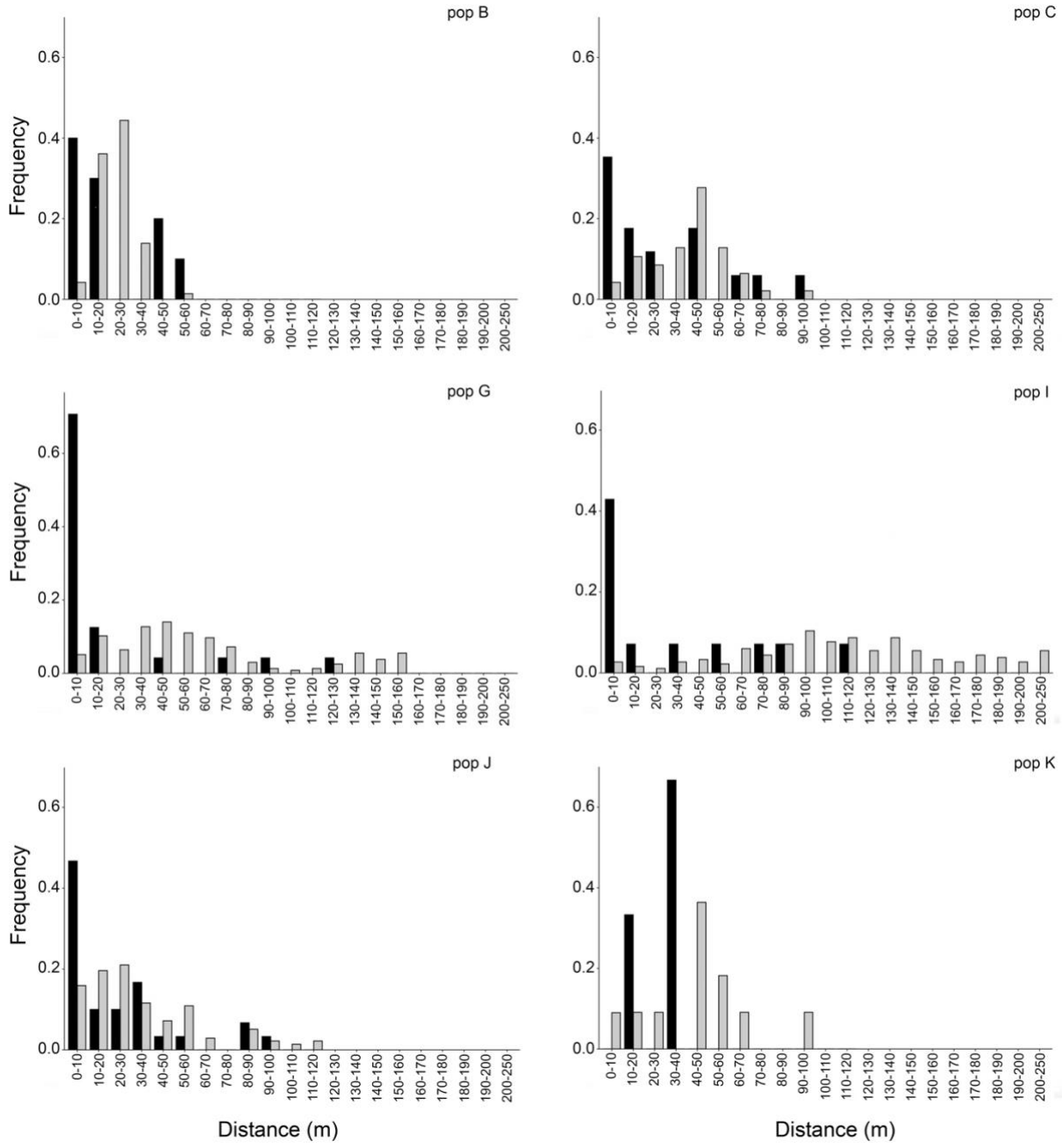


Figure 6.1. Frequency distribution of inferred pollination distances (black bars) and interplant distances of all plants relative to sampled seed mothers (grey bars).

Individual reproductive success

Individual siring success, measured as the proportion of gametes assigned to each pollen donor, ranged between 20% and 26.8% in populations G, I, and J; whereas, in the two large populations B and C, the most successful father sired 9.5% and 8.3% of the seedlings, respectively (Fig. 6.2). Population K was excluded from this analysis due to the low number of seeds produced.

Populations were mostly similar in terms of number of contributors to local offspring, showing a high number of reproductively inactive sires (Fig. 6.2). Except for population C, where

only 31 of the 47 sampled individuals (66%) had null reproductive success, the percentage of reproductively inactive local plants ranged between 81.8% and 95.1%.

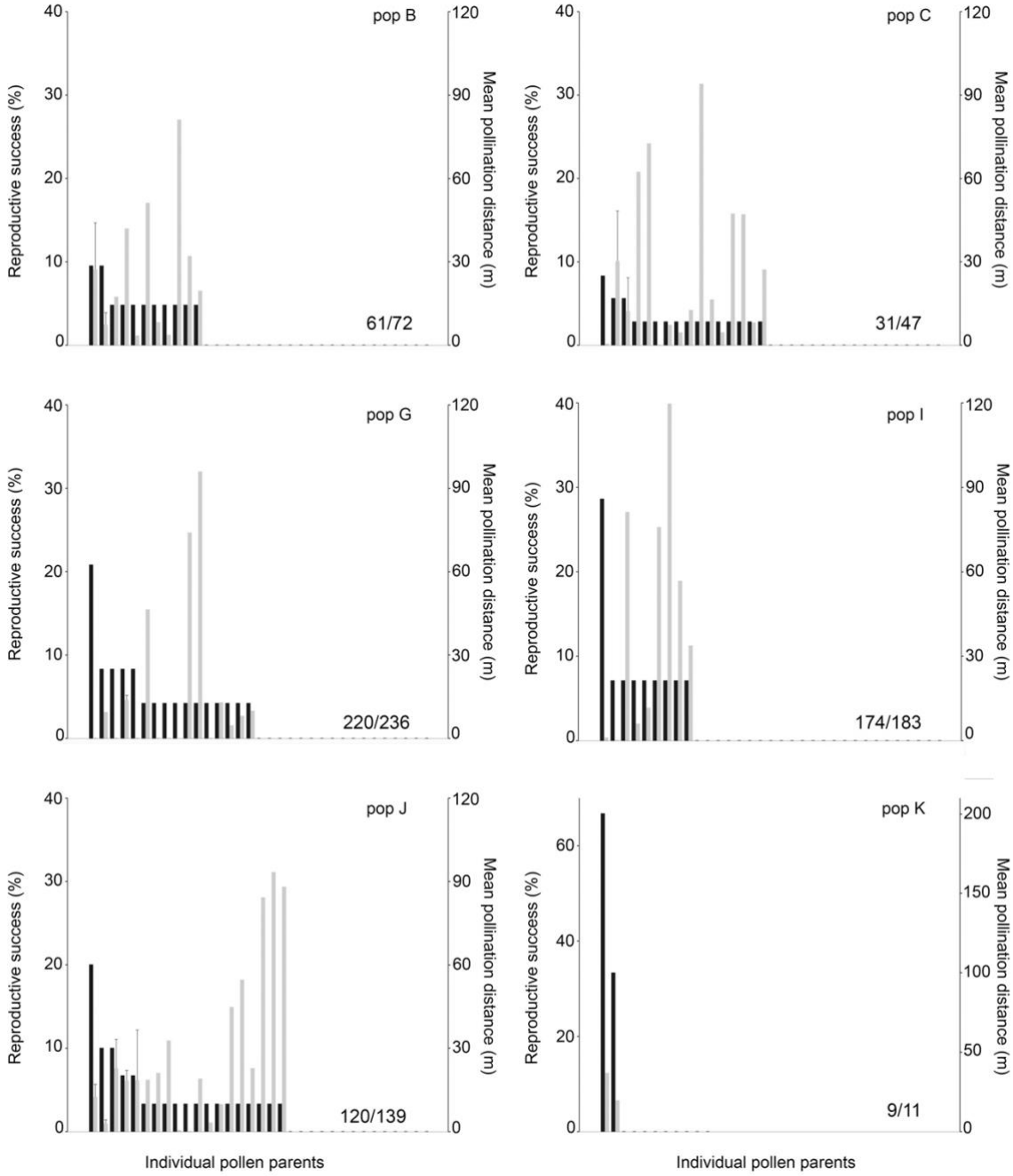


Figure 6.2. Individual siring success and pollination distances in study populations of *Conospermum undulatum*. For each individual pollen parent, the percentage of inferred pollinations (black bars) and mean distances of inferred pollinations (grey bars with standard errors). In the bottom left corner, the number of sampled pollen plants with null reproductive success.

Variation in selfing rates, pollen immigration, and proportion of active sires

The populations investigated were separated into two groups along the first ordination axis, explaining 45.5% of the variation in reproductive parameters (Fig. 6.3). In particular, populations G, I, and J, with positive values of PC1, were primarily characterized by a greater occurrence of selfing. The second ordination axis explained 33.5% of the variation, with population B distinguished from the others by a higher rate of pollen immigration.

Among the six external variables tested, only three were correlated with the ordination axes (Fig. 6.3). The external variable number of stems showed the highest positive correlation with selfing rate ($R^2 = 0.978$, $P = 0.038$); population area was positively correlated to pollen immigration rate ($R^2 = 0.976$, $P = 0.043$); and population size, although only marginally significant, was similarly related to immigration rate ($R^2 = 0.972$, $P = 0.057$).

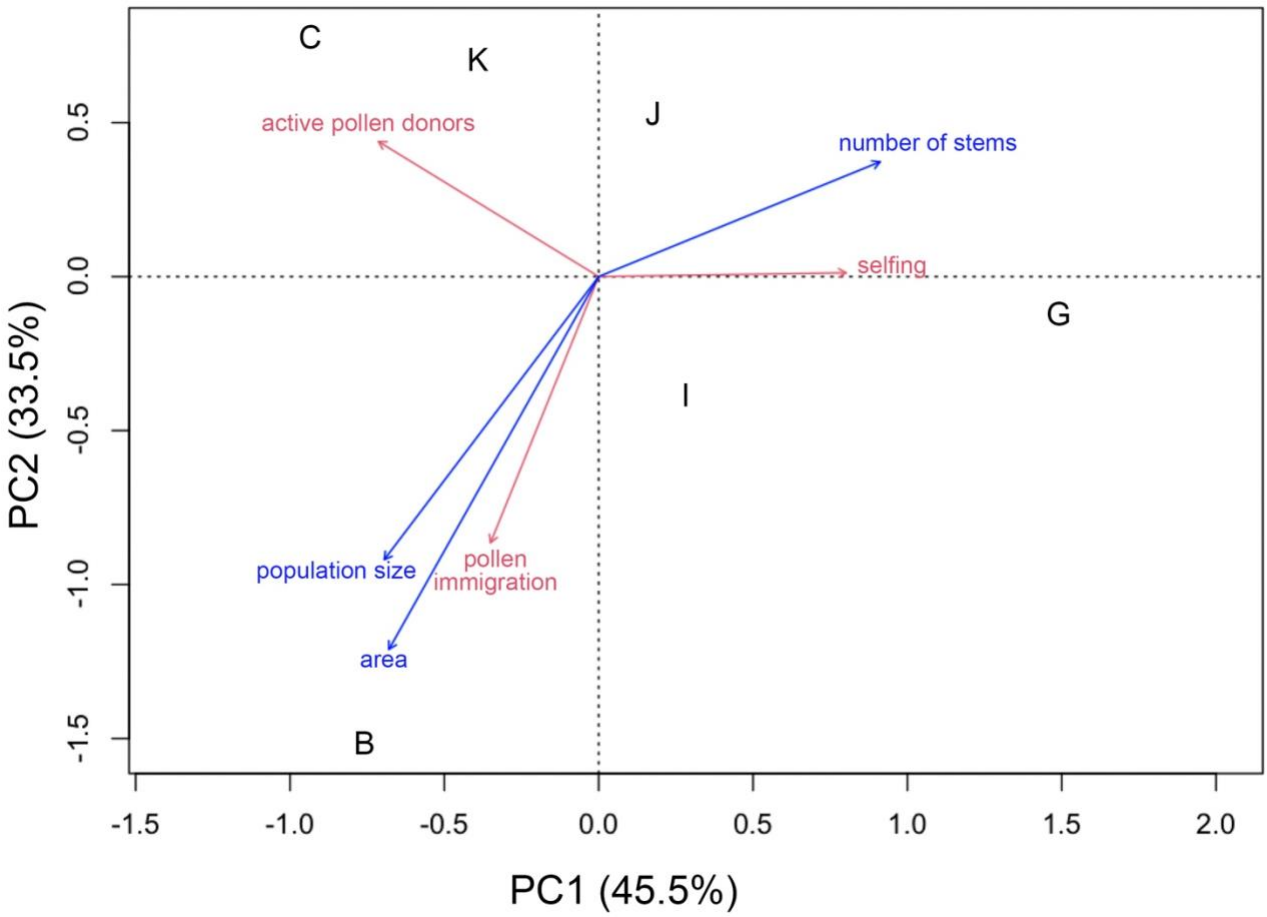


Figure 6.3. Principal Component Analysis (PCA) diagram (biplot type) with vector of population size, area, and number of stems.

Discussion

Gene flow is a fundamental force in determining how populations respond to anthropogenic habitat fragmentation. The recent urban expansion of Perth (Kelobonye et al., 2019) allowed us to investigate the short-term effects of fragmentation on the inter-population pollen flow of *C. undulatum*, a species coevolved with small native bees for gamete dispersal. Paternity analysis of this threatened species revealed almost no connection among urban remnant populations, with consequent implications for conservation. This fine-scale comprehensive investigation complements previous studies on *C. undulatum* (Chapter 5), and it provides more complete insight for understanding the responses of this threatened species to land use change.

Contemporary selfing and pollen immigration rates

Conospermum undulatum, as in the majority of proteaceous species (Collins & Rebelo, 1987; Goldingay & Carthew, 1998), has been considered as a self-incompatible plant. In a recent work by Delnevo et al. (2019 a), undertaking flower manipulation and hand pollination treatments, the authors found that although manual pollination with pollen from different flowers of the same plant produced an average of 26.4% fruits, all the embryos never fully developed, and the treatment yielded zero viable seeds. This suggests that self-incompatibility in *C. undulatum* was not only due to its specific flower morphology that prevents autogamy but was likely a late-acting self-incompatibility that prevented self-pollination across different flowers of the same plant (geitonogamy). However, our paternity investigation revealed an unexpected, more complex scenario. We found a total of 13 plants that produced selfed progeny. The occurrence of selfed seeds in self-incompatible plants has been reported in other systems that present late-acting self-incompatibility, such as *Ceiba pentandra* (Gribel, Gibbs & Queiroz, 1999) and *Akebia quinata* (Kawagoe & Suzuki, 2005). As reported by Gibbs (2014), in late-acting self-incompatible species it would appear that mixed pollen loads can reduce fecundity but also give rise to some mixed mating. Alternatively, the presence of selfed progeny may be due to the resprouting ability of *C. undulatum* combined with likely longevity of plants. Analysis of 11 microsatellite loci as described in He et al. (2007) on five branches of 54 older individuals of the resprouter *Banksia attenuata*, also Proteaceae, showed that 7.4% possessed non-identical heterozygous alleles in different branches on the same plant (Lamont et al., 2011). Indeed, since resprouter species can survive many regeneration cycles and the accumulation of mutant alleles is simply a time-dependent process, older individuals may be more likely to develop somatic mutations, allowing, in some cases, for self-fertilisation between different stems of the same plant.

While the latter scenario appears less likely given that is exceptionally uncommon that such mutation would be functional, it cannot be excluded by our results. We found plants that sired selfed progeny had an average of 7.54 (± 0.86) stems, whereas, in general, the average value across all population of *C. undulatum* was 4.49. Such plants with above average number of stems, having more inflorescences, offer more opportunity for natural mixed pollination to occur (i.e. geitonogamous pollen mixed with crossed pollen), potentially leading to successful selfing (Gibbs, 2014). Nonetheless, our PCA analysis showed that selfing rates were positively correlated with the average number of stems per plant, meaning that populations that went through many fire cycles are more likely to contain plants able to produce selfed offspring. It should also be noted that while the selfing rate was generally low across all the analysed populations, ranging between 0 and 0.112 (± 0.074), population G showed a high rate of 0.458 (±0.109). This unusually high selfing rate may be the result of the possible breakdown of self-incompatibility following the likely hybridisation happening in population G (see Chapter 7, below) and is worth of further investigation.

The impact of recent fragmentation can be clearly detected by the almost complete absence of pollen immigration towards remnant populations of *C. undulatum*. Although we were unable to accurately determine the rate of pollen immigration in the two large populations B and C, the predominance of short-distance mating and the hundreds of ungenotyped plants surrounding the focus areas suggest that most, if not all, of the unassigned progeny were sired from within the same population. However, the focus areas of non-fragmented populations were comparable in size with fragmented ones. Our findings, therefore, suggested that gametes could travel unimpeded through unfragmented bushland maintaining a high genetic connectivity over large distances within them. This pervasive pollen-mediated gene flow once characterised the entire distribution range in the pre-fragmented landscape, as shown by the weak genetic structure found in this study, and agrees with the high estimates of genetic connectivity found in Chapter 5. These results support the growing body of literature demonstrating that the effects of recent habitat fragmentation in plant populations are unlikely to be detected by looking at genetic indexes in adult cohorts of plants that pre-date recent population declines (Aguilar et al., 2008, 2019; Mimura et al., 2009; Vranckx et al., 2012). Instead, an understanding of contemporary mating patterns is needed to detect early signals of failure of gene flow in fragmented remnants.

It is also worth noting that the only other population that showed a significant estimate of pollen immigration (0.144 ± 0.095, population I) is separated from the nearest unsampled plants by ~650 m of cleared rural land, as opposed to built-up areas (satellite image in Fig. E.1 Appendix E). This distance is within the estimated limit of genetic connectivity possible for *C. undulatum* (~1000

m, Chapter 5) and is in line with the inferred foraging range of the main pollinator *L. conospermi* (~500m; based on the relation between body size and foraging distance in hymenopterans, Greenleaf et al., 2007). Pollen-mediated gene flow therefore appears to be possible between close fragments in a rural matrix. Whereas populations separated by built-up residential areas, even if closer than 650 m from each other, are now completely isolated as such urban areas may generate more significant barriers to small native bees compared to honeybees and birds (Andersson et al., 2017). This reduction in contemporary inter-population gene flow, as a consequence, may reduce the ability of small populations to recover lost genetic variation through connection with larger remnants, with consequent impacts on the future persistence of this threatened species.

Conservation implications

This study highlights the importance of combining population genetics with contemporary pollen movements, together with ecological and environmental characteristics to obtain a greater understanding of the impact of recent habitat fragmentation on long-lived plant species. A loss of heterozygosity following population size reduction, evident in populations L and M, can reduce fitness due to inbreeding depression (Charlesworth & Willis, 2009). When this occurs in predominantly outcrossing plants, such as *C. undulatum*, the effects of inbreeding depression can impact early fitness traits such as the development of the embryo and seed germination (Aguilar et al., 2019; Vranckx et al., 2012). The lack of contemporary inter-population gene flow likely due to anthropogenic barriers to pollen dispersal, as found in this study, may over time reduce reproductive output in isolated populations.

Moreover, changes in the spatial arrangement of remnant populations following land use change can lead to altered dynamics of pollen flow, with small and/or isolated populations being less visited by pollinators (Dauber et al., 2010). This general trend was also observed in *C. undulatum* (Delnevo et al., 2020 b). In the short term, a reduction in pollen movements following fragmentation can decrease the reproductive success of self-incompatible plants. In addition, within small fragments, pollinators tend to increase the number of within-individual floral visits (Eckert, 2000), with a consequent increase in the transfer of self-pollen between different flowers of the same individual. Despite our finding that geitonogamous selfing is possible in *C. undulatum*, it appears more likely in larger populations and cannot provide a reliable reproductive assurance in this threatened species. This decline in reproductive output appeared evident in all the three small populations selected for this study (K, L, and M), where a few fruits were produced but no viable seeds developed (except in population K where we obtained three seedlings). The relatively high heterozygosity and weak genetic structure observed in adult cohorts of *C. undulatum* suggests that

selfing was not common in the pre-fragmented landscape. However, it is unclear whether the selfing rate has increased due to fragmentation or whether selfed progeny is purged at early stages of development.

Many plant species can withstand impacts of habitat fragmentation due to highly mobile pollinators able to maintain high inter-population pollen dispersal (e.g. Byrne et al., 2007, 2008). However, for plants such as *C. undulatum*, which are mainly self-incompatible and rely on small native bees and ants for pollen flow (Delnevo et al., 2020 a, 2020 b), these changes may have a stronger impact on their future persistence. Understanding species-specific life history and ecological traits is particularly imporant for planning conservation actions such as translocations and reintroductions in order to maintain, or increase, adequate population size to sustain the genetic diversity within populations.

Supplementary material

Pairwise G_{ST} and *D* matrices (Table D.1, Table D.2 in Appendix D), satellite image of the rural matrix separating population I from the nearest unsampled *Conospermum undulatum* plants (Fig. D.1 in Appendix D).

Funding sources

Funding: This project was supported by The Holsworth Wildlife Research Endowment & The Ecological Society of Australia [R12019/G1004377 to ND].
This research was jointly supported by Edith Cowan University Industry Collaboration Grant and the Department of Biodiversity, Conservation and Attractions, Western Australia [G1002531].

Chapter 7 – Identification of potential hybrids of *C. undulatum*

Introduction

It has long been recognized that hybridisation can have constructive or destructive outcomes in the evolution and future persistence of taxa (Prentis et al., 2007b; Rhymer & Simberloff, 1996; Wolf et al., 2001). Indeed, although the presence of hybrids can in some cases maintain or increase diversity through the formation of stable hybrid zones or the reinforcement of reproductive isolation (Abbott et al., 2013; Mallet, 2007), it can also lead to a decreased diversity through the merger of previously distinctive evolutionary lineages and, ultimately, to extinction of populations or species (Allendorf et al., 2001; Levin et al., 1996; Rhymer & Simberloff, 1996; Vuillaume et al., 2015). Hybridisation can lead to extinction following two main mechanisms, namely demographic and genetic swamping (Todesco et al., 2016). The first is the result of outbreeding depression, where parent taxa may decline below replacement rates due to reproductive energy being wasted on infertile or unfit hybrid offspring. The second outcome, genetic swamping, is more frequent and occurs when hybrids replace one or both parents over time (Rhymer & Simberloff, 1996). This makes the presence of hybrids one of the most challenging problems for protection and management of endangered species.

The risk of swamping and the eventual loss of the parental lineages is increased in narrow range endemic taxa compared to common species (Wolf et al., 2001, Prentis et al., 2007), with the risk even more amplified in rare species already threatened by abiotic stresses, such as habitat fragmentation and degradation (Burgess et al., 2005; Ellstrand & Elam, 1993; Todesco et al., 2016; Zalapa et al., 2009). Global biodiversity hotspots are areas with an exceptional concentration of endemic species undergoing major loss of habitat, and therefore represent priorities for conservation management (Mittermeier et al., 2004, 2011). The very old and stable landscapes of the southwest Australia biodiversity hotspot provided the conditions for a strong speciation resulting in high rates of local plant endemism (Hopper, 2009; Mucina & Wardell-Johnson, 2011). This region records 7380 native vascular plants, of which 2500 of conservation concern (Hopper & Gioia, 2004). This flora includes many groups of closely related taxa with natural narrow distributions that are (or at least were prior anthropogenic fragmentation) geographically proximal. For these threatened taxa, understanding risks of hybridisation and its consequences is crucial for effective conservation planning.

Conospermum (Proteaceae) is an insect-pollinated genus endemic to Australia with its centre of distribution being the south-west corner of Western Australia. It includes 53 species (Bennett, 1995), with four taxa already listed among the threatened flora of Western Australia (W.A. Government Gazette, 2018). Of these, *Conospermum undulatum* is a threatened and rare shrub endemic to southwest Australia, with a naturally narrow distribution now completely embedded within a large and expanding urban area. Recent studies showed that habitat fragmentation negatively influenced the reproductive output of the species and the gene flow among populations now separated by built-up areas (Delnevo et al., 2019 a, 2020 b; Chapter 5).

Two other species of *Conospermum*, namely *C. triplinervium* and *C. canaliculatum* (both non-threatened) are present near the known range of *C. undulatum* and potential hybridisation has been hypothesised due to the occurrence of plants with unusual leaf morphology (Department of Environment and Conservation, 2009). Therefore, potential hybridisation may pose a further threat to the survival of the rare *C. undulatum*. In this study, we combined a morphological analysis of leaf characters with a genetic characterisations of supposed hybrid plants to (1) define whether the unusual leaf forms fall within the natural variability of *C. undulatum* or whether they are intermediate forms between *C. undulatum* and one of the two other sympatric species; (2) verify the presence of hybrids individuals using molecular markers; and (3) define the species (if any) involved in the hybridisation with the threatened *C. undulatum*.

Methods

Study species

The genus *Conospermum* (Proteaceae) arose in a fire-prone environment ~36 Mya and speciated strongly in the old stable landscape of the south-west corner of Western Australia (Groom & Lamont, 2015). It is an insect-pollinated genus and most *Conospermum* species have small white flowers with an active pollination mechanism that requires specific insect for pollination (Delnevo et al., 2020 a).

Although there has been some confusion in the taxonomy of *Conospermum* species, the descriptions of *C. undulatum*, *C. triplinervium*, and *C. canaliculatum* are quite clear in the most recent revision of the genus (Bennett 1995). In particular, *Conospermum undulatum* is a multi-stemmed plant that grows as an erect, compact shrub up to 1.5 m tall with distinctive fibrous, longitudinally fissured stems. The glabrous leaves are around 12 cm long and 3.8 cm wide with a characteristic undulating margin. This species can regenerate after fire by resprouting from semi-subterranean woody lignotubers (Bennett, 1995). This differ from *C. triplinervium*, a single

stemmed tree up to 4 m tall, which mostly lacks a lignotuber and is killed by fire, but readily regenerate from seeds (Bennet, 1995). The leaves of *C. triplinervium* are around 13 cm long and 1-3 cm wide with straight margins. Lastly, *C. canaliculatum* is a dense, multi-stemmed, lignotuberous shrub with linear leaves up to 20 cm long and around 2 mm wide which are distinctively canaliculate, having a thin longitudinal groove.

The flowering period of these species spans August to November and mostly overlaps. *Conospermum* have a sessile ovary with a single pendulous ovule (Douglas, 1997). Pollination is facilitated by native bees and ants (Delnevo et al., 2020b; Delnevo & van Etten, 2019) that fertilise flowers to produce cone-shaped achenes (i.e. dry, one-seeded indehiscent fruits), covered with tan orange hairs. Seeds fall with the fruits and their dispersal, being gravity-driven, is mainly limited to a few meters from the mother plant (Close et al., 2006).

Study area

The narrow distribution range of *C. undulatum* is part of the Swan Coastal Plain bioregion and is restricted to an area of 55 km^2 in eastern Perth region. The area has experienced extensive land clearing for urbanisation starting from the 1950s (Kelobonye et al., 2019; Wardell-Johnson et al., 2016). This species is currently known from 17 populations, with one population (population G) having a high number of plants with unusual leaf morphology.

The other two *Conospermum* species have much broader distribution ranges with *C. canaliculatum* found further north, up to Geraldton, and *C. triplinervium* extending from Geraldton to Albany, in the southern part of the biodiversity hotspot.

Specifically, within the study area, a population of *C. triplinervium* is present 3.9 km south of population G, while a few *C. canaliculatum* plants grows in close proximity to population G but this species was more frequent in the area in past, based on herbarium records (FloraBase, https://florabase.dpaw.wa.gov.au).

Sample collection

We conducted field surveys at the end of the flowering season 2019. In two large reference populations of *C. undulatum*, we sampled 47 and 72 in accordance with license restrictions to collect biological material from threatened species. These populations were central in the geographic distribution of the species. Reference *C. triplinervium* plants were collected from a large population located in Welshpool Road, ~3.9 km of population A, while the nearest large population of *C. canaliculatum* was located near Yanchep. Leaf samples for genetic analysis were collected from individuals located at least 15 m from each other. In addition, we sampled all the 236 plants

present in population G. A total of 412 samples, across all 3 species, were collected for the genetic analysis, stored in in silica gel after collection then freeze-dried (Table 7.1).

For morphological analysis, four populations of *C. undulatum* encompassing the entire distribution range were evenly sampled, for a total of 126 samples of *C. undulatum* (Table 7.1). Thirty samples from each of the reference populations of *C. canaliculatum* and *C. triplinervium* were also collected. Lastly, we sampled 65 leaves from plants in population G, encompassing the full spectrum of leaf morphologies. The largest healthy leaf from each selected plant was collected for morphological analysis and pressed in the field. A total of 251 leaves were sampled. Again, samples were a minimum of 15 m apart.

Morphological analysis

Three leaf dimensions were measured for each sample: length along midrib from base to tip; maximum width perpendicular to midrib; and leaf area, calculated by means of Adobe Photoshop (Adobe, San Jose, USA). In addition, a score for waviness of the margins was attributed to each leaf, ranging from 0 to 4. Plants were grouped *a priori* in four groups based on their morphology (i.e. undulatum, canaliculatum, triplinervium, putative hybrid). The variation in the leaf morphology among populations and species was investigated with non-metric multi-dimensional scaling (NMDS). We used the Euclidean coefficient to quantify the distance matrix by means of the R package vegan v.2.5-4 (Oksanen et al., 2019). NMDS aims to represent the distance matrix in the lowest possible dimensional space, with level of stress measuring the resultant distortion. Low levels of stress indicate a that the chosen dimensional representation (two in this case) reasonably represents the objects relative positions.

We used analysis of similarity (ANOSIM) to determine statistical significance for differences in leaf shape between these *a priori* groups using PAST3 software, which allows for pairwise comparisons of groups (Hammer et al., 2001). This function operates directly on the dissimilarity matrix using Euclidean distance and 9999 permutations were applied.

Genetic analysis

Genomic DNA was extracted from adult leaf tissue and genotyped at 14 microsatellite loci (Chapter 5) that have been developed for *C. undulatum* and cross-amplified in three other species of *Conospermum*, including *C. canaliculatum* and *C. triplinervium* (Delnevo et al., 2019 b). Details of DNA extraction, polymerase chain reaction conditions and scoring of electropherogram profiles are provided in Delnevo et al. (2019 b).

Standard genetic diversity parameters (number of alleles, *A*; effective number of alleles, *Ae*; observed and expected heterozygosity, H_O and H_E, were then calculated using GenAlEx 6.5 (Peakall & Smouse, 2012). Parameters of genetic fixation (G_{ST}; Nei, 1977) and differentiation (Jost's *D*; Jost, 2008) among populations were estimated with GenAlEx 6.5, and their statistical significance was assessed with 999 permutations.

We then performed a discriminant analysis of principal components (DAPC) using the R package ADEGENET (Jombart, 2008). This multivariate method does not focus only on the global diversity but maximises the variance between inferred groups while minimising within-group variation. DAPC also provides membership probabilities of each individual for the different groups that be interpreted as proximities of individuals to the different clusters. Different populations of *C. undulatum* were grouped together as *C. undulatum* species. This group and the other two species were used as *a priori* clusters and the optimal number of PCs to use in the DAPC was determined through cross-validation using the xvalDapc() function and 999 replicates. We generated a total of 200 hybrid individuals through simulated crosses between *C. undulatum* and the other two species. Reassignment of individuals from known groups was performed to check the validity of the model and 82.6% of individuals were reassigned to their actual cluster. Once the model was created, assignment of individuals from population G was performed to allocate putative hybrid individuals to the parental populations. Pure species were delineated by a threshold at 0.3, meaning individuals assigned a value > 0.70 to a species were categorised as pure.

Table 7.1. Genetic diversity parameters of different species of *Conospermum* and putative hybrids.

Species	Morpho samples	Genetic samples	*Na*	*PA*	H_O	H_E	F_{IS}
C. undulatum	126	119	13.286	40	0.650	0.741	0.115
C. canaliculatum	30	27	8.929	9	0.609	0.719	0.146
C. triplinervium	30	30	2.500	0	0.043	0.269	0.762
Putative hybrids	65	236	10.786	12	0.604	0.724	0.168

Morpho samples: number of samples for morphological analysis; Genetic samples: number of samples for genetic analysis; *Na*: average number of alleles; *PA*: private alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: fixation index

Results

Morphology

The three analysed species have long been recognised as separate based on morphology and the analysis of morphological data using NMDS showed a clear separation between them with no overlap in ordination plot space (Fig. 7.1). Selected plants in population G occupied the broad ordination space between the *C. undulatum* and the *C. canaliculatum* clusters. The variability in leaf morphology of these plants exceeded the naturally high intra-specific variability of *C. undulatum* (Fig. E.1 in Appendix E). These samples showed a reduction in waviness and width, and an increase in length, suggesting hybridisation with *C. canaliculatum* and the limited influence of *C. triplinervium* as potential source of hybridisation. Only four plants showed a marked reduction of leaf waviness with no major loss of leaf area, indicating potential hybridisation with *C. triplinervium*. All the four classes were significantly different from each other (ANOSIM overall R = 0.683, $p < 0.001$; pairwise comparisons in Fig. 7.1).

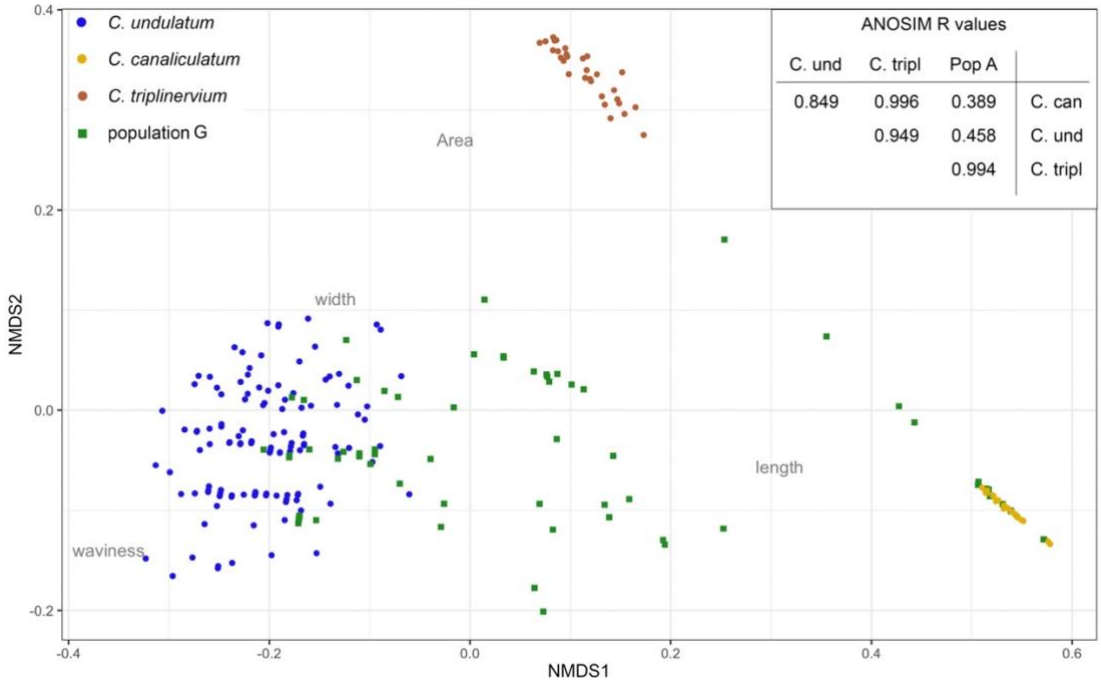


Figure 7.1. Ordination of morphological measurements of *Conospermum* leaves using nonmetric multidimensional scaling (NMDS). Different colours represent *a priori* groups of distinct species and population G. R values from ANOSIM are displayed with all groups being significantly dissimilar.

Genetic characterisation

Conospermum undulatum, *C. canaliculatum* and population G showed similar heterozygosity and number of alleles (Table 7.1), whereas *C. triplinervium* had much lower estimates of genetic diversity (Table 7.1). Pairwise genetic differentiation *D* was generally high

between the three groups, and the lowest differentiation value was recorded between *C. undulatum* and population G (Table 7.2). Interestingly, with the exception of *C. triplinervium*, pairwise G_{ST} values between populations were low (Table 7.3).

Table 7.2. Pairwise population matrix of *D* values.

	und	can	tripl	Pop G
und	0.000	0.001	0.001	0.001
can	0.161	0.000	0.001	0.001
tripl	0.632	0.433	0.000	0.001
Pop G	0.101	0.219	0.572	0.000

D values below the diagonal.
P-value based on 999 permutations above the diagonal.
und: *Conospermum undulatum*; can: *C. canaliculatum*; tripl: *C. triplinervium*.

Table 7.3. Pairwise population matrix of G_{ST} values.

	und	can	tripl	Pop G
und	0.000	0.001	0.001	0.001
can	0.042	0.000	0.001	0.001
tripl	0.253	0.174	0.000	0.001
Pop G	0.017	0.041	0.231	0.000

G_{ST} values below the diagonal.
P-value based on 999 permutations above the diagonal.
und: *Conospermum undulatum*; can: *C. canaliculatum*; tripl: *C. triplinervium*.

The full model comprising all three species showed possible hybridisation of *C. undulatum* mainly with *C. canaliculatum* and, to a lesser extent, with *C. triplinervium* (Fig 7.2). The optimal number of PCs retained for the analysis was 80 (Fig. E.2 in Appendix E). Assignment of individuals of population G showed the presence of hybridisation between the three species (Fig. 7.3). Of the 236 plants analysed for population G, 46.6% were assigned as pure *C. undulatum*, 28.4% resulted as F1 hybrids with *C. canaliculatum*, and 16.9% were assigned as backcrosses between these F1 hybrids and *C. undulatum* plants. One plant was identified as pure *C. canaliculatum* and only two plants backcrossed with this species. No pure *C. triplinervium* plants were found in population G, matching field observations, and eight were assigned as hybrids between *C. undulatum* and *C. triplinervium*.

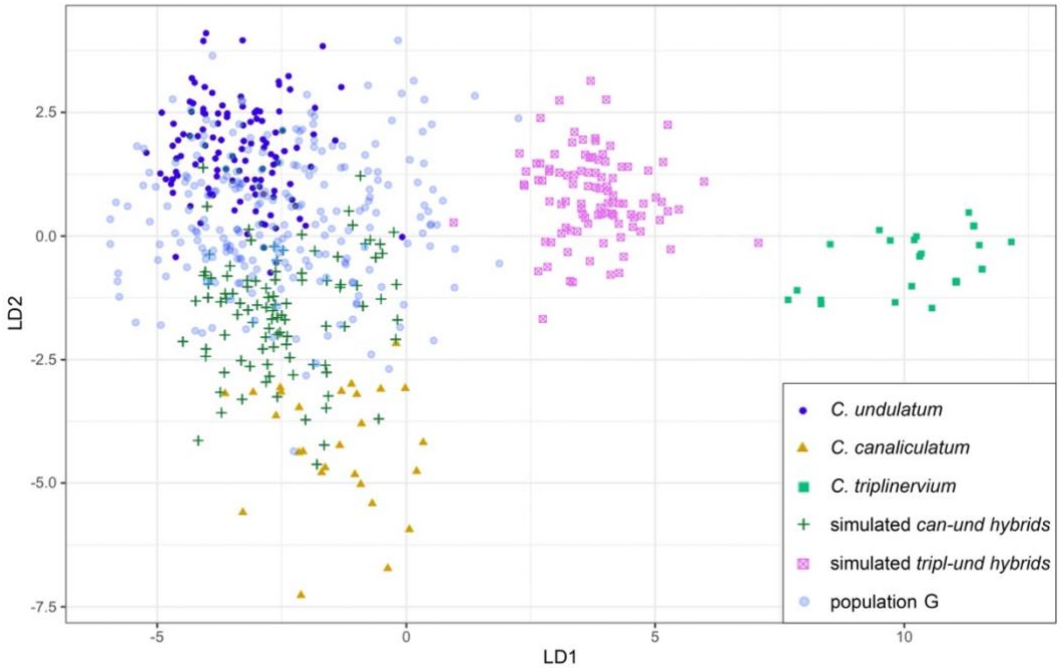


Figure 7.2. Two-dimensional ordination (Discriminant Analysis of Principal Components - DAPC) of 412 plants sampled from different groups and 200 simulated hybrids between *Conospermum undulatum* and the other two *Conospermum* species. Different colours and symbols represent different groups of distinct species, simulated hybrids, and population G.

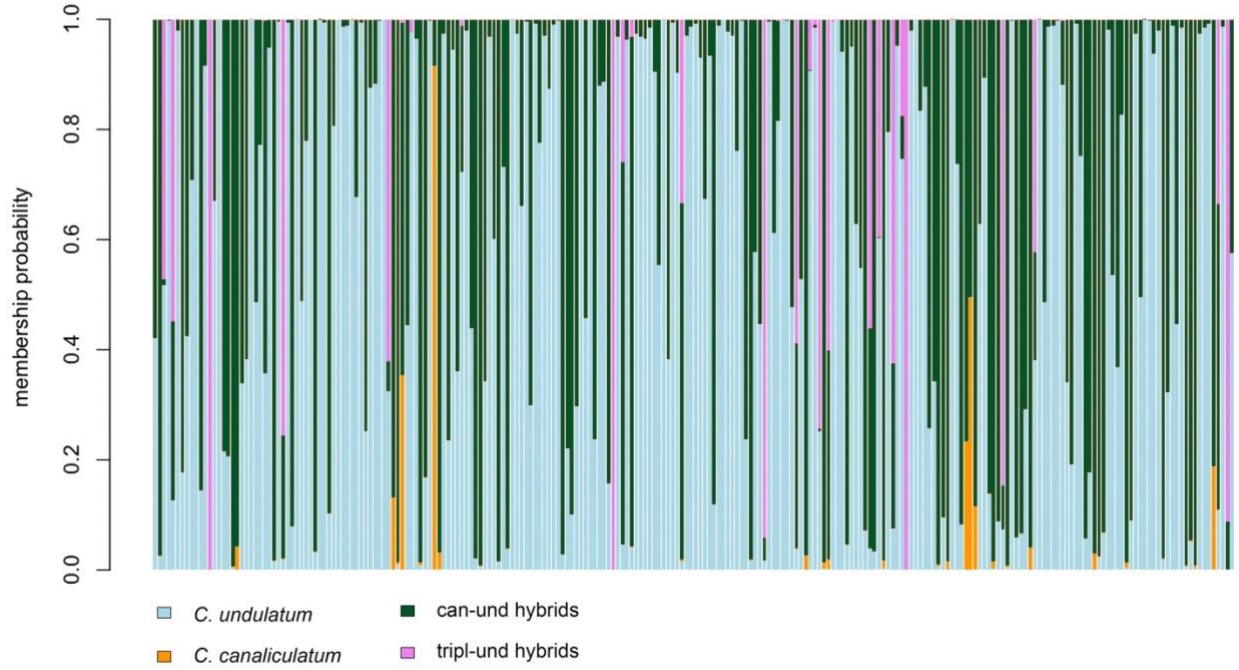


Figure 7.3. Genetic assignment probability of 236 putative hybrid plants from population G to different groups based on Discriminant Analysis of Principal Components. Different colours represent different groups of distinct species and simulated hybrids.

Discussion

Through the combination of morphological and genotyping approaches, this study provides evidence for the likely hybridisation between the threatened and rare *C. undulatum* with at least one more common species of *Conospermum*.

Morphological analysis highlighted the presence of phenotypic intermediates mainly between *C. undulatum* and *C. canaliculatum*, supporting previous claims of putative hybrids. Due to high intra-specific variability, however, leaf morphology alone is not able to provide conclusive evidence of hybridisation. The genetic characterisation performed in this study showed strong evidence for the presence of hybrids within population G. In a recent work performed using amplified fragment length polymorphism (AFLP) markers in the same population, Close et al. (2006) found no evidence of hybridisation between 20 selected plants in population G and 20 *C. triplinervium* plants sourced from the same population sampled for our study. The more thorough sampling conducted in our study and the inclusion of *C. canaliculatum* can explain the different outcomes. Indeed, it appeared from our analysis that hybridisation was polarised towards the latter species, with only a few plants having high probability of being *undulatum-triplinervium* hybrids. The limited sampling performed by Close et al. (2006) is likely to have missed those hybrids. We also found evidence of introgression and backcrossing, as hybrids exhibited a range of affinity to each species but particularly to *C. undulatum*. Without introgression, all hybrids would be expected to be F1 hybrids, and would display 100% affinity to their hybrid group.

The limited dispersal distance of propagule of *Conospermum* plants can be the reason for the low occurrence of *undulatum-triplinervium* hybrids. Indeed, despite a large population of *C. triplinervium* still present in the area, it is now separated from population G by almost 4 km of built-up industrial area able to stop contemporary inter-population gene flow (Chapter 6). Major land clearing of the area started in the 1950s (Kelobonye et al., 2019; Chapter 5), it is therefore likely that the hybrids between these two species found in this study are the result of historical admixture events occurred prior fragmentation of native bushland.

Conversely, as of today, there are no large populations of *C. canaliculatum* present in the area, but we found one plant assigned as pure *C. canaliculatum* within population G, suggesting a possible high historical proximity of the two species in the area. This was also supported by the numerous plants assigned with high probability to the F1 *undulatum-canaliculatum* group (28.4%) and by the presence of backcrosses between hybrids and pure *C. canaliculatum*.

Conservation implications

There are several potential positive and negative implications of hybridisation to the parent populations, but all are uncertain (Todesco et al., 2016). Hybridisation may have provided this population with new genetic variation, potentially maintain the high heterozygosity found in this study. It is possible that population G could have historically represented part of a stable hybrid zone able to isolate *C. undulatum* in the pre-fragmented bushland and therefore maintain or increase the diversity between the species. In the intact natural landscape, this barrier to inter-specific crossing was acting as a positive drive for maintaining the biodiversity in the area. This is also supported by the presence of a few other putative hybrid plants with intermediate morphology in the northern-most population of *C. undulatum* (population H; N. Delnevo, personal observation). This population represent the northern boundary of *C. undulatum* distribution and a few plants of *C. canaliculatum* are still present in close proximity to this remnant *C. undulatum* population.

Alternatively, hybridisation may lead to genetic swamping of the threatened *C. undulatum*, and, as we found evidence of introgression, population G could potentially be at risk. In today's landscape, land clearing and anthropogenic fragmentation are acting as a barrier to gene flow, reducing the threat posed by current hybridisation. Indeed, inter-specific crossing is unlikely between remnant *Conospermum* populations separated by built-up areas, as seen for the significantly hampered intra-specific gene flow (Chapter 6). Habitat fragmentation, however, is posing a serious threat to *C. undulatum* (Delnevo, et al., 2019 a, 2020 b; Chapter 5) and genetic swamping in population G would mean losing one of the few remnant populations of the rare *C. undulatum*. To clearly define the risk of genetic swamping, however, future studies should focus on the viability of pollen produced by hybrid plants, and on the viability and fitness of embryos and seedlings produced by the crossing of two hybrids and hybrid and *C. undulatum* plants. Accurate morphological analysis of seedling characters may also yield important extra information for early detection of hybrids in the field (e.g. Abasolo et al., 2012).

This study presents first evidence of hybridisation in *C. undulatum*. Two of the species involved, *C. undulatum* and. *C. canaliculatum,* despite having been sampled ~30 km apart, showed very low pairwise differentiation, suggesting a close relationship. Therefore, more in-depth investigations using more powerful tools, such as SNPs, are needed in the near future to provide guidance for both monitoring levels of hybridization and conservation planning.

Chapter 8 - Synthesis and conservation implications

In this thesis I examined the pollination ecology and the genetic relationships among populations of *Conospermum undulatum*. I first identified the mating system, the pollinator assemblage, and the intra-specific genetic diversity and structure across the entire distribution of *C. undulatum*. I then investigated the effects of recent anthropogenic fragmentation of habitat on these fundamental aspects to quantify the impact of land use change on pollination, reproduction, genetic diversity and gene flow, hybridisation, and, ultimately, future persistence of this threatened plant species. Although the work was focussed on *C. undulatum*, the results have broader implications for the management of other insect-pollinated native plants with specific mutualistic associations with native insects, in Australia or elsewhere. In addition, I combined field and laboratory experiments to examine the ecological role of ants as potential pollinators in the southwest Australia region.

In this final chapter, I aim to first summarise the key findings of this body of work in relation to the thesis objectives. I then provide a synthesis of the results and discuss how these findings can be applied to management plans and actions to enhance the conservation of *C. undulatum*.

Summary of major findings

Objective 1: Determine the effects of habitat fragmentation on the reproductive output and seed germination of this threatened shrub.

My investigation of the mating system of *C. undulatum* revealed this threatened species is incapable of autogamous self-pollination and requires a vector for moving the pollen grains across different flowers. Autogamous selfing cannot occur even in the case of the accidental triggering of the pollination mechanism, as demonstrated by the exclusion plus triggered flower manipulation (which resulted in no seed set). Pollen grains exploded from the anthers were unable to reach the downward-facing fertile part of the triggered style within the same flower, highlighting the efficacy of this mechanism as a physical barrier to self-pollination. *Conospermum undulatum* requires the visits of specific pollinators able to insert their head into the flower tube to reach the nectar reward at the base and thereby trigger the mechanism. In this way, the fertile face of the stigma can collect the pollen deposited on the back of the insect during previous flower visits. Simultaneously, the anthers can deposit more pollen on the insect, providing gametes for the next flower.

My flower manipulation experiments (Chapters 2) suggest that self-sterility in *C. undulatum* was not only due to its specific flower morphology that prevents autogamy but was also a genetic response to prevent self-pollination across different flowers of the same plant (geitonogamy). Although manual pollination with pollen from different flowers of the same plant produced an average of 26.4% fruits, all the embryos never fully developed, and the treatment yielded zero viable seeds. *Conospermum undulatum* appears, therefore, as a late-acting self-incompatible plant (Gibbs, 2014). The paternity assignment analysis, nonetheless, revealed a more complex scenario, where 13 individuals produced offspring following geitonogamous self-pollination. This may be due to the possible occurrence of mixed mating in some late-acting self-incompatible plants (Gribel, Gibbs & Queiroz, 1999; Kawagoe & Suzuki, 2005) where mixed pollen loads can reduce fecundity but also give rise to some selfed progeny. However, it cannot be conclusively defined whether *C. undulatum* is a late-acting self-incompatible or an early-acting inbreeding depression (Gibbs, 2014), and more studies are required to identify the process that cause self-sterility in this species. Alternatively, older plants that went through several regeneration cycles may be more likely to have non-identical heterozygous alleles in different stems on the same plant (Lamont et al., 2011), allowing, in some cases, for self-fertilisation between different stems of the same plant. Moreover, the higher selfing rate recorded in population G may also be due to a possible breakdown of self-incompatibility following likely hybridisation in that fragment.

Despite the ability of some older plants to produce viable offspring via geitonogamy, metrics of habitat fragmentation correlated with the key stages of sexual reproduction in *C. undulatum*:

1. Fruit production responded only to changes in floral display, suggesting the only variable that affected the production of fruits was the capacity of a population to attract pollinators.
2. Seed production was related to floral display, population size and isolation. The fact that population size and isolation also became highly significant factors suggests that besides the lack of floral visitors, genetic factors that prevent the development of the embryo and result in empty fruits may be present in small and isolated populations. It is reasonable to conclude from the results of the hand self-pollination experiment that the recorded discrepancy between fruit production and seed production may reflect late acting self-incompatibility.
3. Seed germination had patterns similar to those of seed production, where seeds produced in small and isolated populations had a lower probability of germination. This is

consistent with a possible lack of extended pollen flow able to rescue small and/or isolated populations from the effects of inbreeding.

Objective 2: Identify the pollinator assemblage of C. undulatum and quantify the effects of anthropogenic fragmentation on such pollinators.

By means of direct observations, I recorded the flower visitors of *C. undulatum* over two flowering seasons (2017 and 2018; Chapter 3). While many plant species have generalised invertebrate pollinators, results showed that *C. undulatum* has a highly specialised pollination mutualism with the native bee *L. conospermi*, by far the most prevalent flower visitor, suggesting coevolution of this plant and bee species. Dipterans can be important generalist pollinators for wild plant species worldwide, but they provide a negligible contribution to the pollen flow of my target species due to both low number of visits and scarce activity. We also recorded several flies that were fatally trapped by the triggered style of *C. undulatum*. Ants were the second-most frequent visitors, and our field observations suggest that hymenopterans of the Formicidae family are important visitors in *C. undulatum*, together with *L. conospermi*. Since ant pollination is remarkably rare, I examined this association in detail to provide evidence for the role of ants as pollinators of *C. undulatum* and, more broadly, in the region (*Objective 4*).

Fragmentation of natural vegetation affected the pool of pollinators available for *C. undulatum*. Larger populations can rely on a more complex assemblage comprising all key pollinator species; in contrast, small fragments had an impoverished pool of pollinators, lacking *L. conospermi* and consisting only of generalist, poorly effective taxa. Domestic honeybees were present in all the populations and, although the interaction between honeybees and native bees was not directly investigated, the reduction of native bees observed in small populations may have resulted from the combined effect of both competition with honeybees and decreased attractiveness of small patches to specialised pollinators. This suggests that, in the region, pollinator assemblages may be altered when habitat fragmentation occurs in combination with bee-keeping or the presence of feral honeybee colonies (now common throughout Australia) and may have implications for the conservation of specialised threatened plant species that rely on native pollinators for reproduction.

Objective 3: Quantify the effects of fragmentation on the quantity *and* quality *component of pollen limitation in C. undulatum.*

I examined the variation of pollen quantity and quality limitation along a fragmentation gradient (Chapter 3). The reproductive output of the threatened *C. undulatum* was positively related to the size of the population and was limited by both pollen quantity and pollen quality, with the

type of limitation being different between large and small populations. There was no quality limitation in larger populations, whereas the reduction in flower visitation rate, the increase of geitonogamous selfing, and the limited compatible mate availability in small remnants resulted in a significant impact of both pollen quantity and quality on the reproductive output. Small populations likely lacked inter-population pollen flow and the maintenance of such cross-population gamete exchange is crucial for these populations where natural seed set is too low to ensure long-term population viability and adaptation based on sexual reproduction.

Objective 4: Definition of the ecological role of ants as floral visitors of C. undulatum.

I performed germination assays of pollen from several species of plants (including *C. undulatum* and two other *Conospermum* species) after contact with three species of ants and bees (Chapter 4). In contrast with the expectation under the 'antibiotic hypothesis', where ant secretions mostly prevent the transfer of viable pollen (Beattie *et al*., 1984), pollen of all the *Conospermum* species had a germination rate after contact with ants of ~80% which did not differ from the effect of bees. In contrast with *Conospermum* and in line with the antibiotic hypothesis, the other plant species tested showed a drop in the germination rate to ~10% following ant treatments.

The potential effectiveness of ants as pollinators for *Conospermum* was also tested in the field by means of exclusion treatments and by investigating the floral fidelity of *C. undulatum* flower visitors. Although ants were generalist pollinators, they showed a high proportion of conspecific pollen grains and their pollination services led to the production of a significant portion of the total seed set of the threatened *C. undulatum*.

Conospermum has likely adapted the biochemistry of their pollen grains to favour the action of ants as secondary pollinators. This result highlighted the complexity of ant-flower interactions and suggested that generalizations neglecting the importance of ants as pollinators cannot be made and adds to the ecological roles that ants might play in the region. This study also showed that such mutualistic associations can happen in unexpected ways and open the way for future studies to investigate flower-ant interactions in this global biodiversity hotspot.

Objective 5: Quantify the intra-specific genetic diversity within and among populations, and the genetic structure across the entire distribution range of C. undulatum.

I developed and used genetic markers to assess the genetic diversity and structure across 14 populations of *C. undulatum* (Chapter 5). The levels of genetic diversity were mostly similar across populations. Populations G, L, and M showed the lowest values of allelic richness and heterozygosity, and the genetic structure analysis showed they were markedly divergent from the

rest of the populations that represented a genetically homogeneous group. Of these three populations, populations L and M showed genetic signature of a relatively recent bottleneck. Fine-scale spatial genetic structure revealed a seed dispersal limited to ~20 m and a pollen dispersal able to maintain a significant relationship between kinship coefficients and spatial distance of up to 1000 m.

The variation in genetic indices of populations was significantly related to only one explanatory variable, namely the historical connectivity index in 1953. This variable was negatively associated with among-population genetic differentiation and positively related to within-population genetic diversity.

The analysis I performed to investigate the relationship between *C. undulatum* reproductive performance and genetic and environmental variables revealed that influences of genetic, environmental, and combined fractions explained 65% of the variance in reproductive output. Although the genetic fraction alone was only marginally significant, the environmental and the combined fractions explained 56.5% of the variation, indicating a joint influence of genetic diversity and the current environmental condition.

Objective 6: Determine the contemporary gene flow among recently isolated populations of the threatened C. undulatum.

To address this objective, I collected and germinated seeds from eight populations of *C. undulatum* to obtain the multilocus genotype of seedlings representing the reproductive output of the 2017 flowering season (Chapter 6). After thorough surveys and in accordance with license restrictions to collect biological material from threatened species, we sampled a single leaf from every plant in the selected populations, for a total of 706 plants. Despite the increased sample size, we found levels of genetic diversity mostly similar across populations, as it was in Chapter 5. Population L and M showed the lowest heterozygosity and, generally, the highest differentiation from the other populations.

The selfing rate was generally low and, except for population G (0.458 ± 0.109), ranged between 0 and 0.112 (± 0.074). Although, the unusually high selfing rate recorded in population G may be linked to the likely presence of hybrids (see Chapter 7) and the possible breakdown in self-incompatibility following hybridisation and is worth of further investigation. Contemporary pollen immigration rates were zero in the smaller populations (J, K, L, M) and varied between 0.131 (± 0.083) and 0.518 (± 0.124) in larger populations B and C. Interestingly, population G, despite being

the third largest population investigated, with 236 plants, had a non-significant pollen immigration rate of 0.102 (± 0.102) (0.000 from direct estimate after paternity assignment).

The number of reproductively active sires within the analysed populations was generally low. Population C had the highest number of contributors to local offspring (16 different fathers), meaning that the remaining 66% of pollen donors in the population had null reproductive success. In the other populations the percentage of inactive pollen donors ranged between 81.8% and 95.1%.

The external variable number of stems showed the highest positive correlation with selfing rate ($R^2 = 0.978$, $P = 0.038$), supporting the idea that plants that survive many regeneration cycles may be more likely to develop somatic mutations, which, in some cases, allow geitonogamous self-fertilisation between different stems of the same plant.

Objective 7: Clarify the threat posed by hybridisation of C. undulatum with sympatric Conospermum species.

Representatives of two other *Conospermum* species were sampled from pure large populations to provide a reference to facilitate identification of hybrids individuals. In addition, measurements of leaf samples were carried to compare the morphology of putative hybrid plants in population G with the pure species *C. undulatum*, *C. canaliculatum*, and *C. triplinervium* (Chapter 7).

The three pure species separated well in the ordination space based on morphology, whereas the unusual leaf shape of plants in population G was mostly intermediate between *C. undulatum* and *C. canaliculatum*, with leaves of these intermediate plants showing a reduction in leaf area and waviness combined with increased elongation.

Genetic characterisation of the plants in population G showed the presence of hybridisation between *C. undulatum* and *C. canaliculatum*, and to a lesser extent, with *C. triplinervium*. Nearly half of the population was assigned as pure *C. undulatum* plants (46.6%), and the other half was assigned as either hybrid (28.4%) or *C. undulatum* backcross (16.9%). No pure *C. triplinervium* plants were found, matching field observations, and eight were assigned as hybrids between *C. undulatum* and this species. Since no close large population of *C. canaliculatum* is now present in the area, possibly due to land clearing, population G may represent historical hybridisation events that may have been important to maintain genetic differentiation among the species prior anthropogenic fragmentation events.

Synthesis

The naturally restricted distribution range of *Conospermum undulatum* has experienced recent reduction and fragmentation, mainly due to urban expansion.

At the beginning of the thesis, I tested the breeding and mating system of *C. undulatum* to determine whether this species is capable of autogamous self-pollination or if it requires pollinator-mediated transfer of crossed pollen to allow fertilisation. Autogamous selfing was not possible for *C. undulatum*, and due to the development of fruits following geitonogamous selfing, the results of this work excluded 'conventional homomorphic self-incompatibility' (*sensu* Gibbs, 2014). Indeed, unlike species with conventional self-incompatibility in which self pollen is inhibited at the stigma surface or whilst pollen tubes are growing in the style, *C. undulatum* failed to set seed after selfing even though the self-pollen tube has growth to the ovary and penetrated the ovule, starting the development of the embryo. This result strongly suggests the presence of late-acting self-incompatibility (Gibbs, 2014). However, it also raises the alternative possible explanation that the rejection of self-fertilized embryos is due to the consequences of early-acting inbreeding depression due to deleterious recessive alleles. This early acting inbreeding depression has been recorded in many *Stylidium* species, which lack self-incompatibility mechanisms and experience fruiting failure because of high loads of deleterious recessives which act soon after fertilization to prevent seed set from selfed ovules (Burbidge & James, 1991). Future studies with targeted full diallel crosses are required to conclusively identify the cause of the self-sterility recorded in *C. undulatum*.

Throughout the rest of this thesis, I have examined plant-pollinator interactions and population genetics of this threatened plant species and have investigated how anthropogenic habitat reduction is impacting on these important factors. *Conospermum undulatum*, as for many Australian plants, coevolved in isolation with native pollinators over long timeframes, allowing for the development of very specific floral morphologies and pollination associations. In particular, the main pollinator of *C. undulatum* identified during this work was the native bee *Leioproctus conospermi*. This ground-nesting genus is believed to have evolved before the break-up of Gondwana (Almeida et al., 2012). As opposed to some wood-nesting bees, such as Megachilidae, that could potentially have reached Australia more recently by "rafting" in driftwood, *Leioproctus* is unlikely to have reached the Australian continent across expanses of ocean during more recent periods (Houston, 2018). Although a detailed phylogeny of the species *C. undulatum* is not available, it is known that within the old Proteaceae family, the subfamily Proteoideae arose in a fire-prone environment *ca*. 90 million years ago (Mya) and speciated strongly, giving rise to major shrub genera now accounting for 250 species in the southwest Australian flora (Groom & Lamont,

2015). Of these, the genus *Conospermum* appeared ~36 Mya (Groom & Lamont, 2015), emphasizing again the long timeframes that allowed coevolution between these native pollinators and *Conospermum* spp., providing a solid base to understand the high specificity of this pollination mutualism. *Conospermum undulatum*, as well as the other *Conospermum* species investigated in Chapter 4, also appeared to have coevolved with native ants for pollination. Ants were found visiting *C. undulatum* flowers foraging for nectar and the high proportion of *C. undulatum* pollen found within their pollen loads suggests that ants were actively selecting for *C. undulatum*. These non-flying hymenopterans were able to carry viable outcrossed pollen to different plants thanks to the resistance of *Conospermum* pollen grains to the usually harmful secretions present on ants' bodies. Since *C. undulatum* does not possess features of the proposed 'ant-pollination syndrome' (Hickman, 1974), it seems to have coevolved to facilitate pollination by *L. conospermi*. However, coevolution also with native ants cannot be excluded. The uncommon resistance of *C. undulatum* pollen grains allows ants to increase the reproductive output of this threatened plant, making them an important secondary pollinator for the species.

Hopper (2009) introduced landscape age, climate buffering, and soil nutrient status as descriptors to develop an integrated series of hypotheses explaining the evolution and ecology of biota on very old, climatically buffered, infertile landscapes (OCBIL theory). This conceptual framework has been expanded by Mucina & Wardell-Johnson (2011) where they redefined Hopper's climate buffering as a dimension of climate stability, identified soil impoverishment as a function of landscape age, and recognised fire regime predictability as another important dimension. One of the first predictions of the OCBIL theory is the increase in local endemism and common rarity of plant species in these old landscapes. Although *C. undulatum* distribution range does not fall within the definition of OCBIL *sensu stricto* (Hopper, 2009), it can still be characterised as an intermediate-age stable landscape (*sensu* Mucina & Wardell-Johnson, 2011; Gosper et al., 2020) as it has experienced prolonged tectonic quiescence, older than the Late Cenozoic, and has not experienced large scale glacial scouring during the Plio-Pleistocene (McArthur & Bettenay, 1974; Playford et al., 1976), and is characterized by nutrient-poor sandy soils (Table 1.1). It is therefore likely that *C. undulatum* was a naturally rare species with a naturally narrow distribution range before European settlement. Nonetheless, land clearing and fragmentation of the habitat are now exserting unprecedented pressure on this system.

Being a mass-flowering plant, *C. undulatum* relies on its massive population-level floral display to attract flower visitors. The pool of potential pollinators of *C. undulatum* recorded during this work was extremely narrow, drastically reducing the number of species able to pollinate the smokebushes. Other studies have shown that many generalist mass-flowering plants may not be

affected by the detrimental effects of fragmentation by remaining attractive to a higher diversity of floral visitors from surrounding flowers (Hegland & Totland, 2005; Westphal et al., 2003). However, this has mainly been tested on plant species able to rely on widespread and generalist pollinators and is not transferable to highly specialized systems. Overall, I found anthropogenic fragmentation of natural habitat to have readily visible effects on the essential interactions between *C. undulatum* and its pollinators. Such effects are not limited only to reduced pollination events and, therefore, reduced pollen quantity, but extended to direct and indirect negative consequences, both in the short/medium term (i.e. pollen quality and reproduction) and in the long term (i.e. reduced pollen flow leading to genetic erosion and reduced fitness due to expression of inbreeding depression). A thread that connects the different parts of this thesis is the importance of maintaining high population floral displays to avoid creating small patches not attractive to pollinators due to a lack of resources. This was also found in other studies where a reduction in floral display was the key determinant of the decrease in pollinator abundance (Delmas et al., 2014; Goulson et al., 2008). Flying hymenopterans, in particular, have been found to be more influenced by a reduction in floral display of mass-flowering plants compared to dipterans (Delmas et al., 2014). This trend remained true also for the main pollinator of *C. undulatum*, namely *L. conospermi* (Hymenoptera).

Very few studies simultaneously assessed how metrics of fragmentation may affect pollinator assemblage, their visitation frequency, and the pollination process in terms of the amount and quality of pollen delivered. This is especially true for plant species with very specific pollination associations, such as *C. undulatum*. Through manual cross-pollination experiments, I showed that pollen limitation can be overcome by pollen availability in large populations. In small remnant populations, however, an increase in pollination events is unlikely to be sufficient to ensure an adequate reproductive output because such small populations are more likely to have a restricted availability of genetically unrelated mates. This lack of compatible mates reduced the quality of pollen, even when this was outcrossed, suggesting the presence of early biparental inbreeding depression acting during the development of the embryo (Aguilar et al., 2019; Vranckx et al., 2012). Maintaining a consistent inter-population pollen flow, therefore, appeared crucial for small populations where natural seed set is too low to ensure long-term population persistence.

The genetic investigation performed in this work showed pre-fragmentation estimates of gene dispersal distance of up to 100 m. This is the result of the combination of a restricted seed dispersal of up to 20 m and longer pollen dispersal distances facilitated by *L. conospermi* of up to ~1000 m, with the majority of pollination events occurring within 120 m from the mother plants. Fruits (and seeds) of *C. undulatum* lack any special dispersal mechanism and they simply fall off

the plants when ripe. This very restricted seed dispersal is compatible with the limitation imposed by the mosaic of soils that characterize southwest Australian old stable landscapes (Mucina & Wardell-Johnson, 2011). Local dispersal allows the offspring to retain the benefits of the parent's environment, which is suitable by definition. Indeed, for many species in the region, the "struggles for existence" are generally waged against the environment, rather than competitors, and the major source of early seedling mortality are largely density-independent (e.g. being mostly the result of moisture stress; Ellner & Shmida, 1981; Fenner & Thompson, 2005). Unfortunately, in the human modified landscape of the Swan Coastal Plain, these gene dispersal distances suggest that the recent fragmentation and isolation of remnant populations, which started in the late 1950s (Kelobonye et al., 2019), may act as a significant barrier to inter-population gene flow, given that the pairwise distance between most of the populations is now well above 1000 m (Table 1.2). The difficulty for propagules of *C. undulatum* to cross such distances was confirmed by the population genetic investigation carried during this project (Chapter 5), where population G and M – populations historically separated from the main distribution by patches of unsuitable soils– showed a marked genetic divergence from the main group of populations.

The investigation of population genetics (Chapter 5) also suggests population disturbance since the 1950s has yet to impact patterns of spatial genetic structure in the contemporary adult cohort. This is consistent with findings in many synthesis and meta-analyses, where emerged that long-lived plants can carry a 'loss of genetic diversity' debt for many generations after a fragmentation event (Aguilar et al., 2008; Honnay & Jacquemyn, 2007; Vranckx et al., 2012). The exact life span of *C. undulatum* is not known (and it is worth of future investigations), but it can survive at least several decades, as individual shrubs can resprout from semi-subterranean woody lignotubers after disturbance such as fire or mechanical damage from strong winds or trampling by animals (Bennet, 1995). The current spatial genetic structure of remnant *C. undulatum* populations, therefore, reflects population distributions over deeper time scales, and the lack of significant genetic responses to habitat fragmentation should not encourage complacency in management as negative effects may yet develop in the longer term.

Early signals of the negative impact of anthropogenic habitat fragmentation on *C. undulatum* populations emerged from the paternity investigations performed in this work, and in particular, from the lack of pollen immigration detected in most of the populations. Results from this analysis suggested that although gametes of *C. undulatum* can flow unimpeded through large expanses of unfragmented bushland, as showed by the pollen immigration rates found in the focus areas selected within the two large populations B and C, inter-population gene flow is non-existent between urban

fragments. The two focus areas were comparable in size with medium fragments and represented non-fragmented patches embedded within large native bushland, similar to the pre-fragmented range of *C. undulatum*. Although most, if not all, of the unassigned progeny in these focus areas were likely sired from within the same population, this result showed that pollinators are able to move freely in undisturbed areas, maintaining a high genetic connectivity over larger distances. This pervasive pollen-mediated gene flow once characterised the entire distribution range in the pre-fragmented landscape, as showed by the genetic diversity and differentiation indexes found in this study (Chapters 5 and 6). This supports the need for an understanding of contemporary mating patterns to detect early signals of gene flow failure in fragmented remnants (Aguilar et al., 2008, 2019; Mimura et al., 2009; Vranckx et al., 2012).

The fact that urban areas may represent a significant barrier to small native bees compared to honeybees and birds is highlighted by the pollen immigration estimate found for population I. This population was the only fragment that showed a significant pollen immigration rate and is separated from the nearest unsampled plants by ~650 m of cleared rural land, as opposed to built-up areas. This distance is within the estimated range of genetic connectivity possible for *C. undulatum* (~1000 m as found in this work) and in line with the inferred foraging range of the main pollinator *L. conospermi* (~500m; based on the relation between body size and foraging distance in hymenopterans, Greenleaf et al., 2007). Pollen-mediated gene flow appeared therefore to be possible between close fragments in a rural matrix. On the other hand, populations separated by built-up residential areas, even if closer than 650 m from each other, are now completely isolated and, consequently, more exposed to long term threats, such as a reduction in fitness due to loss of genetic diversity (Charlesworth & Willis, 2009), and a reduction in reproductive output in the short term.

Many OCBIL species, in their pursuit of heterozygosity, have coevolved pollination mutualisms with native birds able to maintain genetic connectivity across their naturally fragmented landscape (e.g. Bezemer et al., 2016). These pollination systems are able to enhance the resilience of such plants to the increased habitat fragmentation caused by human activities (e.g. Ritchie et al., 2019). The outcomes of this work offer a point of reflection as numerous southwest Australian plants, developed pollination interactions with native insects that are likely to be negatively affected by land clearing and urbanisation. As a consequence, the resilience of such plant species, similar to *C. undulatum*, may not be as high as expected by the OCBIL theory and more studies on plants with specific native insect pollination mutualisms are needed to increase our understanding of the impact of anthropogenic fragmentation in the region.

Given the current absence of large populations of *C. canaliculatum* in the area and the relative isolation of the only remnant population of *C. triplinervium* in the area, the evidence for hybridisation of *C. undulatum* found in this study (Chapter 7) suggests historical inter-specific breeding between the analysed species. Hybridisation events were more common between *C. undulatum* and *C. canaliculatum* and population G may represent an historical stable hybrid zone that was able to maintain the diversity between the species in the natural pre-urbanisation landscape. However, given the current lack of proximity between these species, hybridisation has shifted from being a positive drive for maintaining biodiversity, to a threat for the rare *C. undulatum*, which is now threatened by urban expansion and fragmentation, with important implications for conservation.

Conservation implications

This body of work highlights the importance of combining ecological and genetic studies (both current and historical) to fully understand the impacts of habitat fragmentation on native species that coevolved with poorly known native pollinators. Many Australian native bee species are yet to be described and the conservation status of very few of the ~1500 (at the time of writing) described species has been assessed. This reflects the generally poor state of knowledge of Australia's bees, their geographic ranges and habitat requirements (Houston, 2018). The only bees with officially assessed conservation status are three species in southwest Australia, one of which is presumed to be extinct. Due to the specificity of the mutualism between *C. undulatum* and *L. conospermi* emerged in this study (Chapter 4), clearly appears the need for expanding our understanding on the ecology of this native pollinator. Since this native bee is the main pollen vector for the threatened *C. undulatum*, the conservation of the shrub is closely interlinked with the conservation of the bee. Understanding the environmental requirements of *L. conospermi* can also be useful in order to successfully reintroduce this vital pollinator in populations where its presence was no longer recorded.

Despite the unexpected ability of *C. undulatum* to produce selfed offspring via geitonogamy, this appeared as a random and uncommon process, more likely in multi-stemmed plants generally occurring in larger and older populations (Chapter 6). Therefore, the increased geitonogamy rate usually experienced in small, fragmented populations (Eckert, 2000) did not lead to the production of selfed progeny in small populations of *C. undulatum*, but, on the contrary, it led to the production of empty fruits with aborted embryos. Both the ecological and the genetic approaches concurred in identifying the lack of pollen flow across different populations due to habitat fragmentation as a

threat to the persistence of *C. undulatum*. In particular, a decrease in the population level floral display was significantly correlated with an overall decrease in fruit production due to a decreased complexity in the pollinator assemblage and the absence of the specialised pollinator *L. conospermi*. The decreased probability of obtaining a healthy embryo despite outcrossed pollination events in small populations compared to manual cross-pollination with pollen sourced from other populations reinforced the idea that an increased connectivity among populations would be ideal for increasing the reproductive output of the species. Conservation efforts aimed at reconnecting remnant populations are predicted to be especially fruitful in *C. undulatum*. Increasing the connectivity level of populations by means of bushland corridors, however, appears unfeasible due to the high level of urbanisation already present in the area. Stepping-stones corridors may be ineffective, too. Indeed, the built-up urban matrix that surrounds the remnant patches of native bushland appeared as a significant barrier to pollinator-mediated gene flow, even across distances well below the possible foraging distance that can be travelled by *L. conospermi*. Since no clear source or sink populations to be proritised for conservation based on their contribution to within- and between-populations allelic diversity were identified in this study and demographic stochasticity is likely to play an important role in the survival or the extinction of extremely fragmented populations (González-Varo et al., 2012), the short-term goal of conservation planning should focus on avoiding further decline in the size of medium and large populations of *C. undulatum* and to increase the number of individuals in small populations. The latter could be achieved, at least in the short term, by manual outcross pollination in small remnants using pollen sourced from nearby larger populations to increase the seed output in small patches that are currently reproductively inactive. This approach increased the probability that a flower will develop a viable seed from 1.4% to ~50% in my flower manipulation experiment (Chapter 3); however, due to the small size of the flowers and their trigger mechanism, it may not be easily applied and would require significant time in the field to be accurately performed.

An alternative of easier applicability is represented by the translocation of viable seeds from larger populations. My genetic investigations showed an overall similarity across the entire range of *C. undulatum* (Chapter 5 & 6). Therefore, although the seed dispersal is naturally very limited in *C. undulatum*, this weak genetic structure suggests that seeds could be sourced from any (but possibly large) populations and translocated to very small populations in order to increase the population size over time. This approach requires the least effort as seeds can be sourced from different mother plants by placing bags around the inflorescences at the end of the flowering season. Since the germinability of seeds of *C. undulatum* is generally low (17.33% of the 2505 seeds collected for this work germinated under optimal conditions), translocation of seeds can be programmed prior to

prescribed burning to enhance germination, because *Conospermum* seeds, as many southwest Australian species, may benefit from smoke stimulation to enhance germination (Close et al., 2006).

Land clearing is not only isolating *C. undulatum* populations but is also separating *C. undulatum* from other potentially sympatric species. The hybrids in population G have therefore lost their potential positive effects of maintaining genetic differentiation between the species, but now pose a potential risk of genetic swamping in one of the few remnant populations of the rare *C. undulatum*.

In this work, I identified the basal requirements needed for the persistence of *C. undulatum* in the long term. Given that fire represent a significant disturbance in the region (Burrows & McCaw, 2013), future studies should focus on understanding the responses of this species to different fire regimes. Indeed, the season and/or intensity of prescribed burns can affect the rate of recovery of the species and also affect the phenological development of *C. undulatum*, with possible mismatch between the flowering peak and the presence of pollinators. Moreover, the frequency of prescribed fire may affect the regeneration of *C. undulatum* both via resprouting and seedling establishment. Such future research has the opportunity to improve management of fire in the area and help conservation practitioners in maximising the conservation effort for the threatened wavy-leaved smokebush *C. undulatum*. Research on habitat requirements for the main pollinator of *C. undulatum*, namely *L. conospermi*, and an understanding of the effects of fire on this native bee is also required for maintaining self-sustainable smokebush populations.

Future studies on the hybridisation of *C. undulatum* should focus on assessing the viability of pollen grains produced by hybrid individuals and on the reproductive success and fitness of seedlings produced by experimental crossings between different species, between hybrids, and between hybrids and *C. undulatum* plants (taking care of avoiding spreading hybrids in the field). Given the close relationship between the species involved in the hybridisation process, more informative markers, such as SNPs, should be used to provide a higher resolution to provide accurate guidance for conservation planning.

Appendix A - Supplementary material for Chapter 2

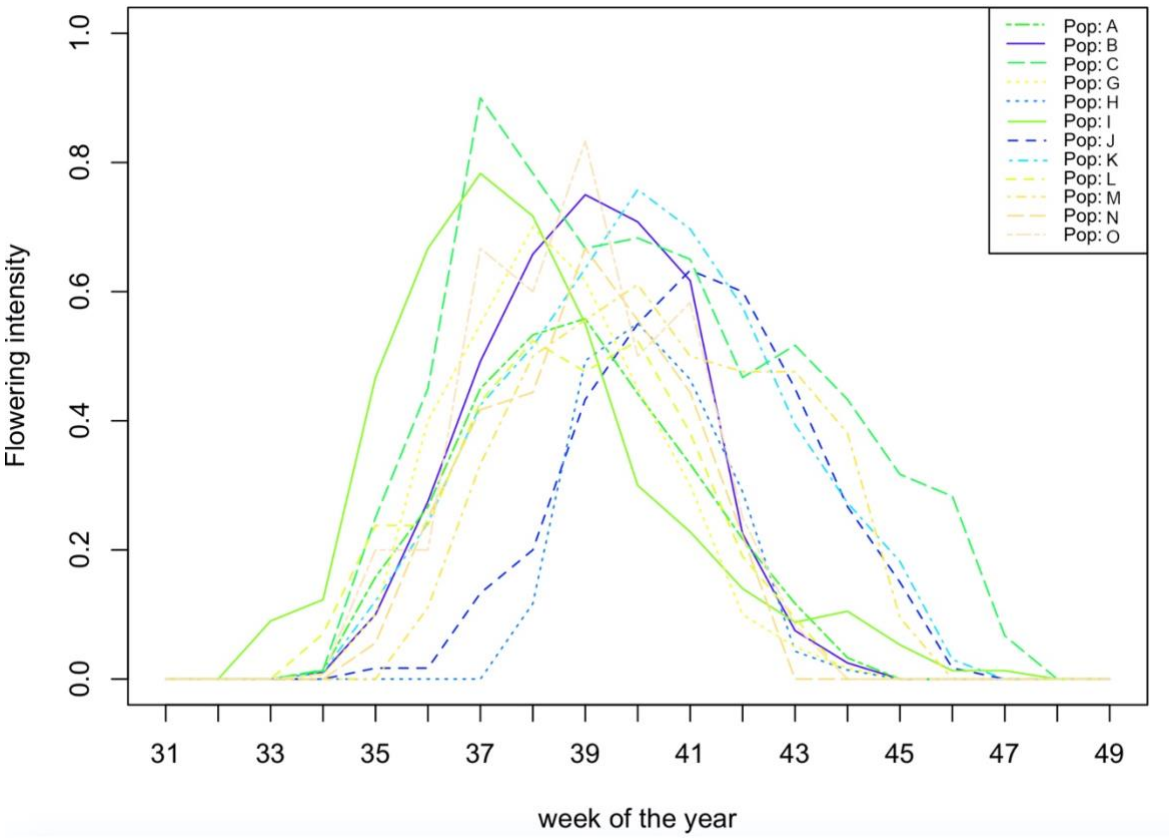


Figure A.1. Flowering phenology of studied populations of *C. undulatum* across the entire flowering season.

Table A.1. Standardised regression coefficient ß of predictors utilised in each model for variable comparison; standard error in parenthesis.

Predictor	Fruit production model	Seed production model	Germination model
Population size	/	0.240 (0.043)	0.247 (0.074)
Isolation index	/	-0.156 (0.041)	-0.256 (0.078)
Floral display index	0.314 (0.052)	0.203 (0.050)	/

Appendix B - Supplementary material for Chapter 3

Supplementary material

Pollinator assemblage

The flowers of *C. undulatum* were visited by eight insect RTUs from five families belonging to two different orders (Fig. B.2). From a total of 447 landings recorded in the two years, hymenopterans were the most frequent visitors, with the native bee *Leioproctus conospermi* (Fig. B.1a) being the most recorded insect, followed by the argid sawfly (Argidae), ants and honeybees (Fig. B.2a). Dipterans were represented only by syrphid flies and poorly contributed to the total records. In terms of visitor activity, the native bee *L. conospermi* was again the most active among all the 4962 recorded visits (Fig. B.2b). Argid sawflies, despite being the second most frequently recorded insect, was relatively inactive, visiting less flowers than ants and honeybees, respectively.

Within the Formicidae family, the ant *Camponotus molossus* was the most abundant and the most active, whereas the other ant species *Camponotus terebrans*, *Iridomyrmex purpureus* and *Myrmecia infima* showed similar abundances and activities (Fig. B.2).

Figure B.1. Flowers and visitors of *Conospermum undulatum*: (a) *Leioproctus conospermi*, (b) Argid sawfly, (c) *Myrmecia infima*, and (d) *Apis mellifera*. Note that *A. mellifera* only insert its proboscis into the flower to steal nectar.

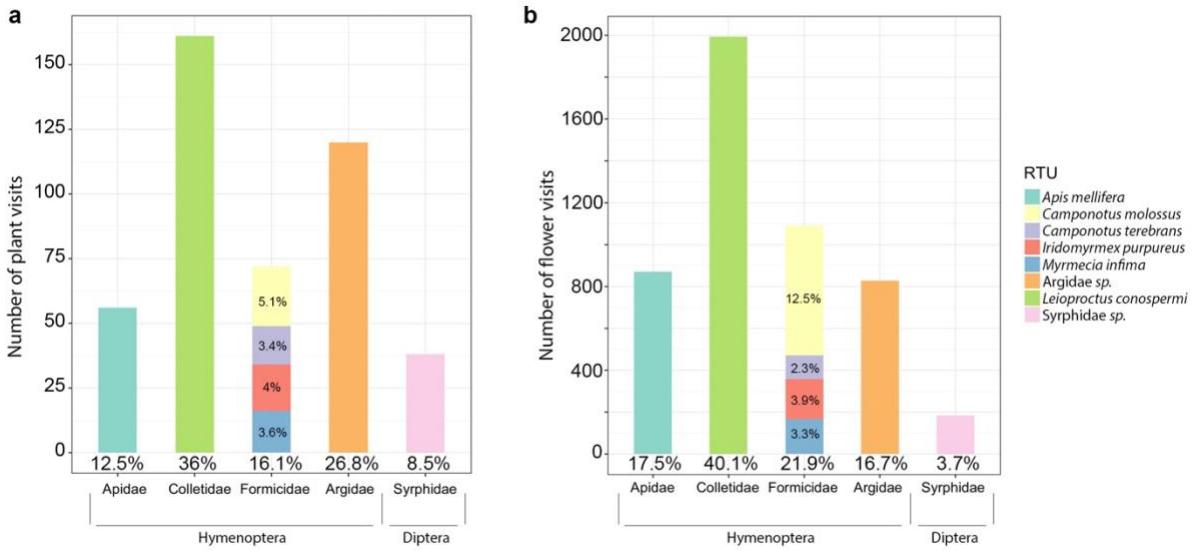


Figure B.2. Observations of flower visits to *Conospermum undulatum* showing (a) number of plant visits and (b) number of floral visits recorded for the different invertebrates grouped by family and order. Relative percentage reported at the base of each bar and within each sub-bar.

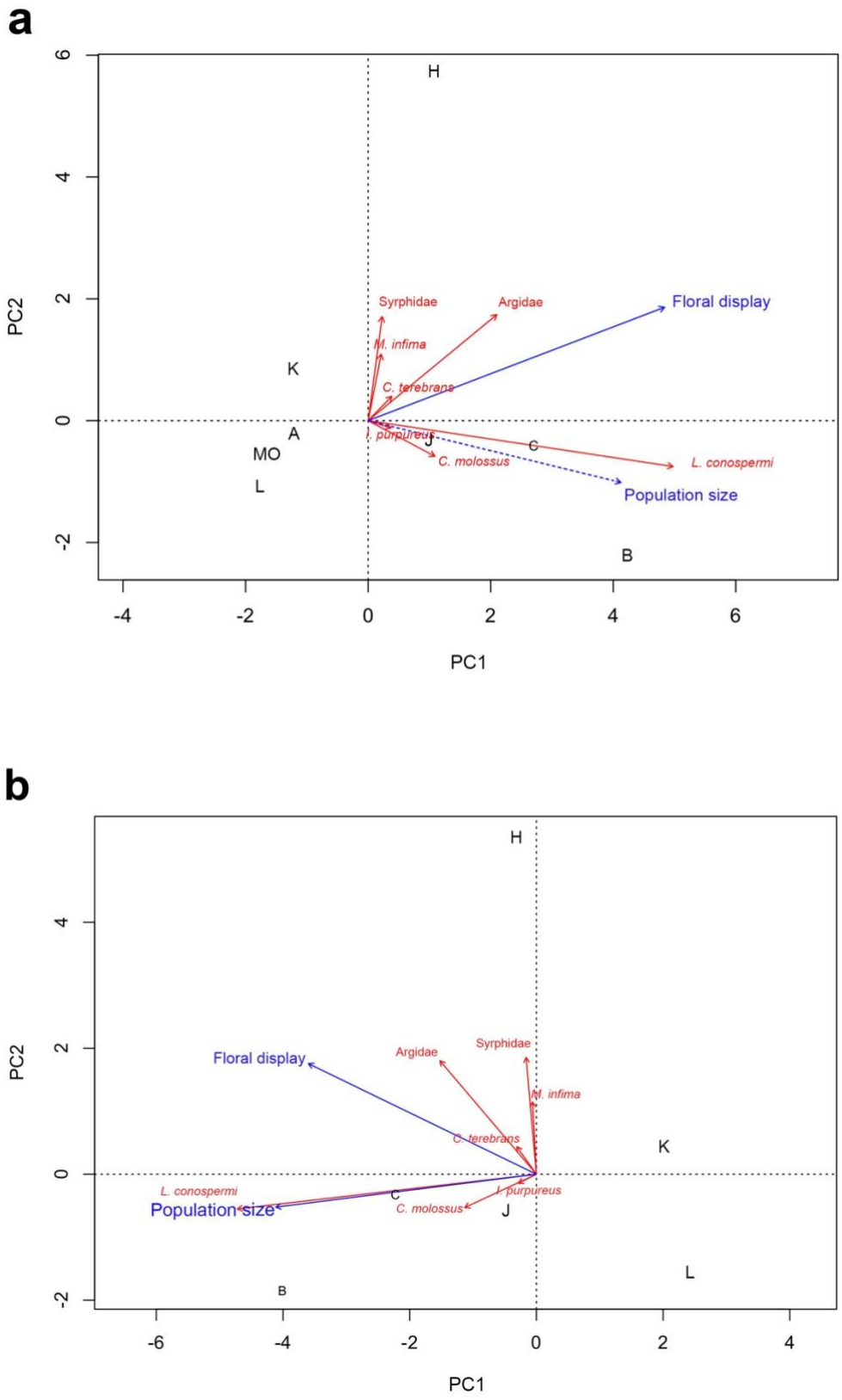


Figure B.3. Principal Component Analysis (PCA) diagrams (biplot type) in (a) 2017 and (b) 2018 with vectors of floral display and population size.

Appendix C - Supplementary material for Chapter 5

Supplementary material

Table C.1. Pairwise population matrix of G_{ST} values.

A	B	C	D	E	F	G	H	I	J	K	L	M	P	
0.000	0.031	0.025	0.002	0.008	0.084	0.001	0.024	0.059	0.059	0.019	0.001	0.001	0.021	A
0.004	0.000	0.002	0.001	0.001	0.003	0.001	0.049	0.003	0.372	0.004	0.001	0.001	0.001	B
0.005	0.010	0.000	0.006	0.003	0.006	0.001	0.001	0.002	0.001	0.004	0.001	0.001	0.011	C
0.009	0.017	0.014	0.000	0.001	0.023	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	D
0.006	0.012	0.010	0.013	0.000	0.006	0.001	0.001	0.003	0.003	0.014	0.001	0.001	0.011	E
0.003	0.009	0.009	0.005	0.019	0.000	0.001	0.002	0.010	0.002	0.072	0.001	0.001	0.003	F
0.018	0.016	0.025	0.032	0.022	0.026	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.022	G
0.005	0.004	0.012	0.011	0.012	0.009	0.021	0.000	0.018	0.071	0.002	0.001	0.001	0.004	H
0.003	0.008	0.011	0.012	0.009	0.006	0.019	0.004	0.000	0.016	0.001	0.001	0.001	0.007	I
0.003	0.001	0.010	0.017	0.009	0.008	0.017	0.003	0.005	0.000	0.004	0.001	0.001	0.007	J
0.008	0.010	0.014	0.012	0.009	0.005	0.024	0.011	0.015	0.011	0.000	0.001	0.001	0.004	K
0.046	0.044	0.047	0.057	0.061	0.059	0.073	0.042	0.052	0.054	0.045	0.000	0.001	0.042	L
0.076	0.090	0.080	0.084	0.101	0.093	0.110	0.082	0.092	0.092	0.081	0.083	0.000	0.074	M
0.004	0.011	0.002	0.017	0.002	0.008	0.001	0.038	0.006	0.002	0.105	0.001	0.001	0.000	P

G_{ST} values below the diagonal.
P-value based on 999 permutations above diagonal.

Table C.2. Pairwise population matrix of *D* values.

A	B	C	D	E	F	G	H	I	J	K	L	M	P	
0.000	0.031	0.026	0.002	0.008	0.084	0.001	0.024	0.059	0.059	0.019	0.001	0.001	0.021	A
0.024	0.000	0.002	0.001	0.001	0.003	0.001	0.049	0.003	0.372	0.004	0.001	0.001	0.001	B
0.026	0.054	0.000	0.035	0.003	0.036	0.001	0.001	0.002	0.001	0.004	0.001	0.001	0.058	C
0.050	0.100	0.014	0.000	0.001	0.023	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	D
0.030	0.061	0.048	0.068	0.000	0.032	0.001	0.001	0.003	0.003	0.013	0.001	0.001	0.052	E
0.017	0.054	0.009	0.028	0.019	0.000	0.001	0.002	0.010	0.003	0.078	0.001	0.001	0.003	F
0.092	0.089	0.135	0.174	0.109	0.143	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.114	G
0.025	0.020	0.066	0.064	0.059	0.050	0.111	0.000	0.018	0.072	0.002	0.001	0.001	0.019	H
0.018	0.043	0.063	0.070	0.047	0.036	0.101	0.024	0.000	0.016	0.001	0.001	0.001	0.036	I
0.019	0.003	0.053	0.094	0.047	0.043	0.089	0.018	0.029	0.000	0.004	0.001	0.001	0.038	J
0.040	0.054	0.071	0.066	0.045	0.028	0.125	0.061	0.083	0.057	0.000	0.001	0.001	0.019	K
0.191	0.191	0.196	0.244	0.241	0.257	0.308	0.178	0.226	0.230	0.181	0.000	0.001	0.170	L
0.262	0.330	0.281	0.299	0.339	0.340	0.389	0.290	0.334	0.332	0.273	0.222	0.000	0.250	M
0.023	0.058	0.002	0.092	0.002	0.044	0.001	0.038	0.006	0.002	0.102	0.001	0.001	0.000	P

D values below the diagonal.
P-value based on 999 permutations above diagonal.

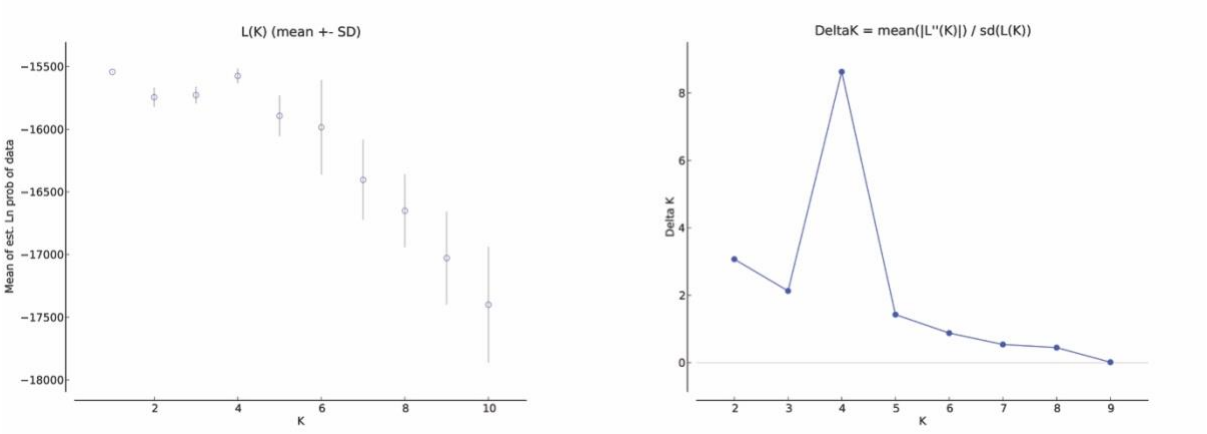


Figure C.1. Top left: The most-likely number of clusters (K); top right: ΔK calculated by STRUCTURE HARVESTER; bottom: genetic ancestry of 293 individuals sampled from 14 populations, estimated using STRUCTURE analysis of microsatellite markers.

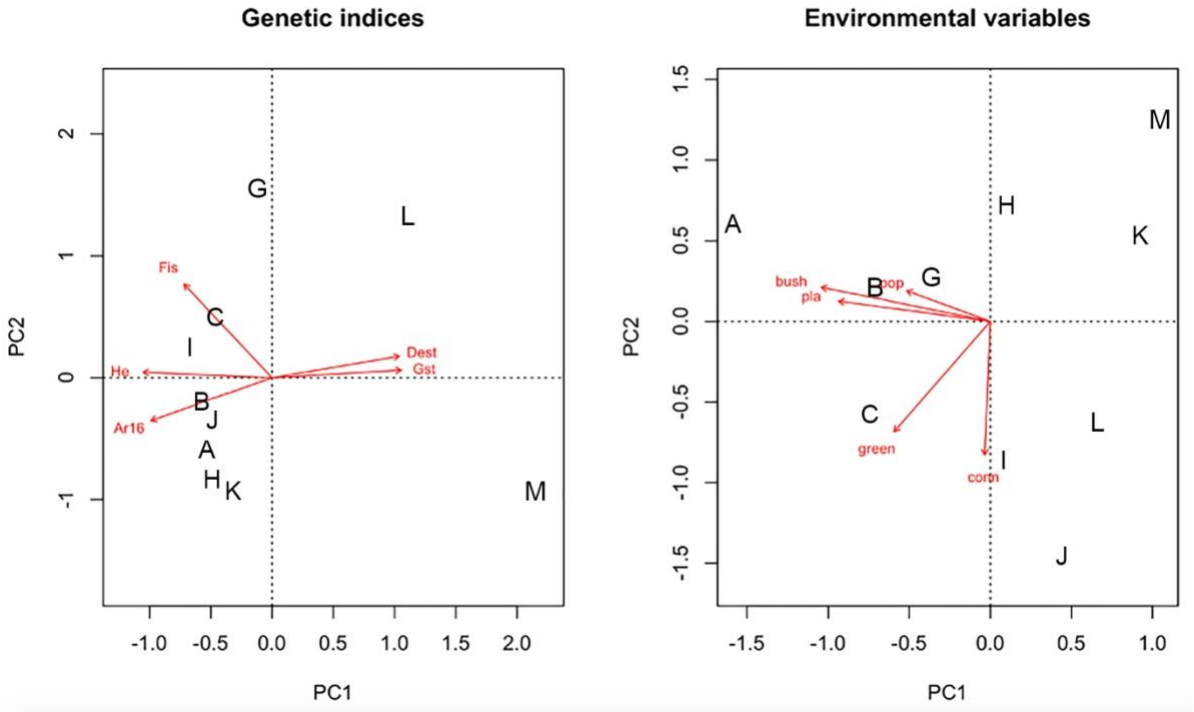


Figure C.2. Principal Component Analysis (PCA) diagram (biplot type).

Appendix D – Supplementary material for Chapter 6

Supplementary material

Table D.1. Pairwise population matrix of G_{ST} values.

	B	C	G	I	J	K	L	M
B	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001
C	0.013	0.000	0.001	0.001	0.001	0.001	0.001	0.001
G	0.023	0.018	0.000	0.001	0.001	0.001	0.001	0.001
I	0.012	0.008	0.019	0.000	0.001	0.001	0.001	0.001
J	0.007	0.012	0.022	0.010	0.000	0.008	0.001	0.001
K	0.018	0.013	0.021	0.014	0.009	0.000	0.001	0.001
L	0.051	0.055	0.076	0.048	0.049	0.045	0.000	0.001
M	0.083	0.099	0.111	0.083	0.081	0.081	0.083	0.000

G_{ST} values below the diagonal.
P-value based on 999 permutations above diagonal.

Table D.2. Pairwise population matrix of *D* values

	B	C	G	I	J	K	L	M
B	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001
C	0.075	0.000	0.001	0.001	0.001	0.002	0.001	0.001
G	0.131	0.097	0.000	0.001	0.001	0.001	0.001	0.001
I	0.067	0.046	0.107	0.000	0.001	0.001	0.001	0.001
J	0.040	0.063	0.119	0.055	0.000	0.006	0.001	0.001
K	0.097	0.068	0.109	0.079	0.046	0.000	0.001	0.001
L	0.222	0.230	0.327	0.209	0.206	0.181	0.000	0.001
M	0.301	0.354	0.404	0.305	0.288	0.273	0.222	0.000

D values below the diagonal.
P-value based on 999 permutations above diagonal.

Figure D.1. Map showing the clear distinction between built-up areas (north) and cleared rural land (centre and south).

Appendix E – Supplementary material for Chapter 7

Figure E.1. Above - Photos of leaf samples of different *Conospermum* species. Below – selection of leaf images from population G showing the range of intermediate morphologies from *C. undulatum*-like to *C. canaliculatum*-like.

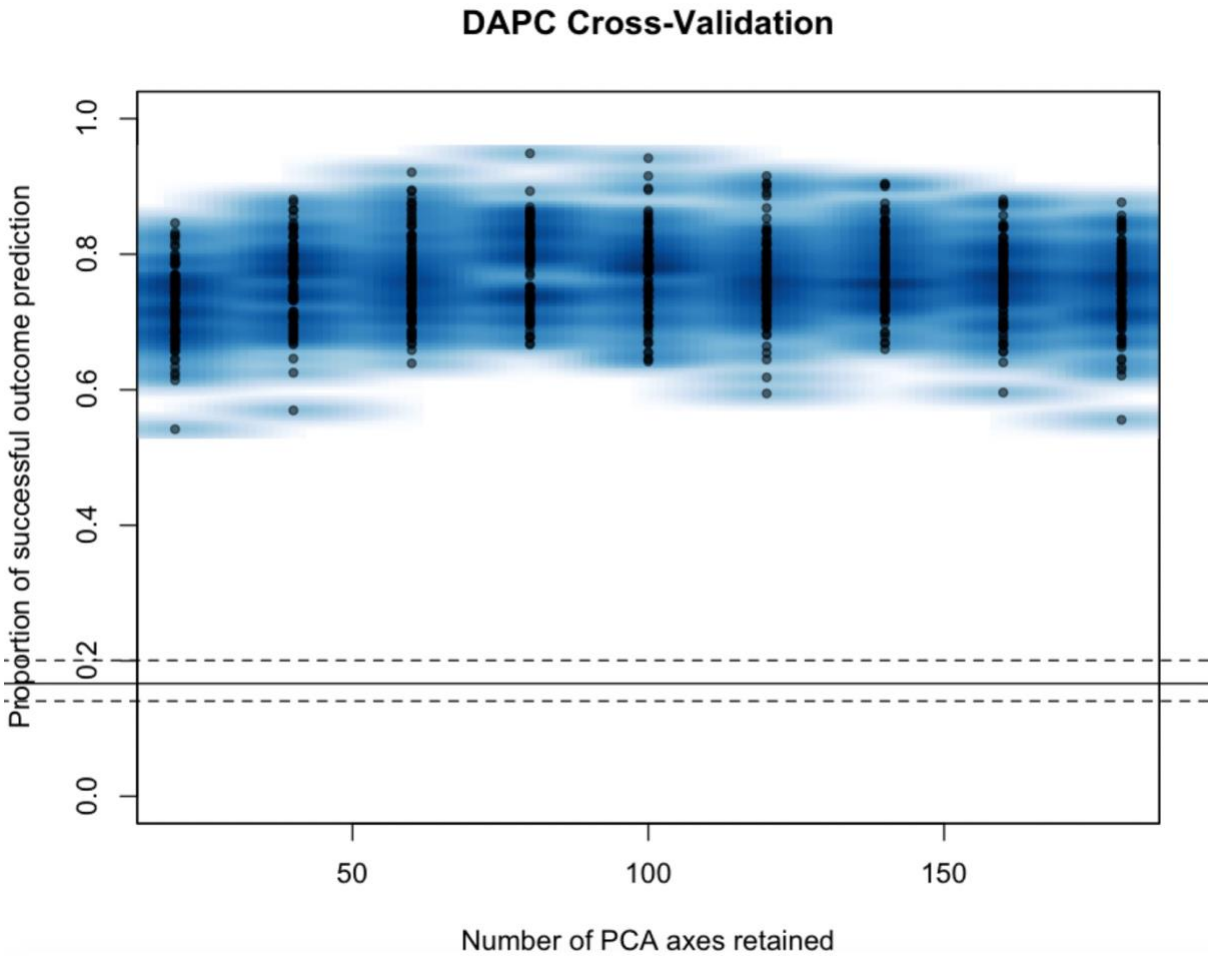


Figure E.2. Results from cross-validation function xvalDapc() showing 80 Principal Components as the best number of PCs to retain for the analysis.

Appendix F - Isolation, characterization, and cross-amplification of 20 microsatellite markers for *Conospermum undulatum* (Proteaceae)

This chapter has been published as the following paper:

Delnevo N, Piotti A, van Etten EJ, Stock WD, Byrne M. 2019. Isolation, characterization, and cross-amplification of 20 microsatellite markers for *Conospermum undulatum* (Proteaceae). Applications in Plant Sciences, 7: e11283.

Introduction

The genus *Conospermum* Sm. (Proteaceae) represents an important component of the heathlands and woodlands of Western Australian sandplains. The genus has 53 species endemic to Australia, with its centre of distribution in south-western Western Australia (Bennett, 1995). Within the South West Australian Floristic Region, a global biodiversity hotspot (Myers et al., 2000; Hopper and Gioia, 2004), many *Conospermum* species are of increasing conservation concern, with four taxa already declared rare by the Western Australia government (W.A. Gazette, 2018). Moreover, as for many Proteaceous species, various *Conospermum* species are widely utilised in floriculture (Bennett, 1995; Stone et al., 2006). *Conospermum undulatum* is a diploid shrub with its range restricted to *ca*. 55 km^2 in a rapidly expanding urban zone in the metropolitan area of Perth (Close et al., 2006; Wardell-Johnson et al., 2016). This species is listed as Vulnerable under the Environment Protection and Biodiversity Conservation Act 1999. Habitat fragmentation and hybridization with sympatric *Conospermum* species are likely to pose a risk to the future persistence of *C. undulatum*.

In *Conospermum*, studies of population genetics and reproductive biology have been undertaken using AFLP and RAPD markers for only a few species (Stone et al., 2006; Sinclair et al., 2008). To our knowledge, no microsatellite resources have been developed for this genus to date. Considering the growing concern about this endemic genus and the number of species within it, we expect that microsatellite markers will have broad applicability for conservation and population genetic analyses. Here, we report the development and characterization of 20 microsatellite markers for *C. undulatum* that will be useful for the study of its genetic structure, spatial patterns of genetic diversity and dispersal dynamics. Additionally, we tested for cross-amplification of these loci in three related

Conospermum species to evaluate the utility of the marker set more broadly and specifically to allow assessment of hybridization between *C. undulatum* and neighbouring species.

Methods and results

Genomic DNA was extracted from freeze-dried leaf material (approx. 50 mg) using a modified 2% CTAB method, with 1% polyvinylpyrrolidone and 0.1% sodium sulphite added to the extraction buffer (Byrne et al., 2001). High quality DNA extracted from a single *C. undulatum* individual was used by the Monash University Malaysia genomics facility (Petaling Jaya, Selangor, Malaysia) for microsatellite development. Briefly, the extracted DNA was sheared to 500 bp using a Covaris M220 Focused-Ultrasonicator (Covaris, Woburn, Massachusetts, USA), and a NEBNext Ultra DNA preparation kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) was used for library preparation after sequencing on the Illumina MiSeq desktop sequencer (Illumina, San Diego, California, USA). Sequencing resulted in a total of 313174 reads and a total data output of 78 Mb (data available from the Dryad Repository: 10.5061/dryad.f81k3q7). The obtained reads were searched for microsatellite loci having a minimum of five repeats using the QDDv3.1 pipeline (Meglécz et al., 2014).

The resulting 9848 loci were sorted based on PCR product size, repeat class, repeat length, and multiplexing potential. Of these, forty-eight candidate loci characterized by perfect repeat motifs and different expected product sizes within the 90-300 bp interval were tested for amplification on a total of six individuals from different populations. Initial screening was performed with Eppendorf Mastercycler ep (Eppendorf, Hamburg, Germany) using 15 µL reaction volumes containing 10 ng of genomic DNA, 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 0.2 mM each dNTP, 2 µM of forward and reverse primers, 2.75 µM $MgCl_2$, and 0.1 µL Taq DNA polymerase. PCR reactions were performed with the following conditions: initial denaturation at 96°C for 2 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 5 min. The PCR products were checked on 8% polyacrylamide gels to assess for successful amplification across all tested individuals. Of the markers that amplified successfully based on their multiplexing potential and consistent amplification within the expected size range, 22 were selected for initial test for polymorphism and labelled with fluorescent dyes (VIC, PET, NED, 6-FAM; Applied Biosystems, Foster City, California, USA). In this step, we used 3.75 µL 2x Master Mix (QIAGEN, Hilden, Germany), 0.75 µL of 2µM primer mix, 1 µL of 5-20 ng genomic DNA and 2 µL sterile RNase-free water (QIAGEN) in a 7.5 µL reaction with the following PCR conditions: initial denaturation at

95°C for 15 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, and extension at 72°C for 60 s; and a final extension at 60°C for 30 min. The PCR products were diluted 10× and 1.0 μL of the dilution was added to a mix of 12.0 μL Hi-Di Formamide (Applied Biosystems) and 0.1 μL GeneScan 500 LIZ Size Standard (Applied Biosystems) for sequencing on a 3730xl DNA analyzer (Applied Biosystems). Following testing, 20 markers (17 containing di-nucleotide and three containing tri-nucleotide microsatellites) that consistently amplified with easily scorable peaks were selected and combined into five multiplexes (Table B.1).

Table F.1. Characteristics of 20 microsatellite loci in *Conospermum undulatum*.

Locus	Primer sequences (5'–3')	Repeat motifs	Allele size range (bp)	T_a (°C)	Fluorescent label[a]	Primer mix (µL)[b]	GenBank accession no.
Multiplex 1							
Cu4	F: GGAGACGGGAGAACTGTGGT	$(AG)_{10}$	88 – 112	60	VIC	3	MH917262
	R: TACTAAACCCTACCACCCTACCC						
Cu16	F: AGGATCCATATAGCCGACCC	$(AAC)_7$	100 – 127	60	PET	3	MH917265
	R: AGCAGTTGCAGTTTCTGTGG						
Cu31	F: GCAAGACAGACGCCCTAAGT	$(AG)_{18}$	131 – 207	60	6-FAM	3	MH917272
	R: GGCATTGTGGGTCATCTCCA						
Cu41	F: ATGTCCCACCGGTATTCAGA	$(AG)_6$	242 – 276	60	VIC	3	MH917277
	R: CTGAAGAGGAAGCAGGCCTT						
Multiplex 2							
Cu8	F: GCATATGGCCCTCATTGTCT	$(AG)_6$	95	60	VIC	3	MH917263
	R: GACCTCCCAAAGATATGAGGCT						
Cu17	F: AGCACTCACAAGTCTGACCC	$(AG)_6$	103 – 111	60	PET	6	MH917266
	R: GCTCAGTAGCTGCCTTTGTC						
Cu24	F: TGAGACCACAAACCAGACCC	$(AAG)_7$	140 – 146	60	NED	5	MH917269
	R: TGTGTCTTCTGTGGCAGTAGT						
Cu32	F: ACACAACAAGCCCTCATCAGT	$(AG)_9$	159 – 189	60	6-FAM	4	MH917273
	R: GGTCTGGCAAGTCCACTCTT						
Multiplex 3							
Cu9	F: GACTCTACAGAAGTCTCGCCC	$(AG)_{12}$	86 – 132	60	VIC	3	MH917264
	R: GGCAAAGCAAGAGCATGGTT						
Cu18	F: AACCCGCCAACAGAATCGAT	$(AG)_8$	105 – 111	60	PET	6	MH917267
	R: TGATCACATGAGGGTAGTAAGC						
Cu28	F: GTTCCTCCATTTCGAAACCCT	$(AG)_{17}$	124 – 160	60	NED	4	MH917270
	R: ACCGTTTCGTTTCGTCTCAGT						
Cu33	F: AAGAAATGAAGCAAGGCGTG	$(AG)_{22}$	144 – 198	60	6-FAM	3	MH917274
	R: GTAGGAGTCCAAGACCCGTTG						
Multiplex 4							
Cu20	F: TCTCCATCAGTACCGCTACCT	$(AG)_7$	122 – 128	60	VIC	2	MH917268
	R: GCTTCCAGTTCCCACCAAAC						
Cu29	F: GACCAGTGAACTCTCAAGGACT	$(AG)_7$	142 – 186	60	PET	6	MH917271
	R: CGCAGCTAGCCGACTTAGAA						
Cu36	F: TGCTTCCTTTCAACGCTTGG	$(AGC)_8$	186 – 201	60	NED	4	MH917275
	R: TGTAAGTGTACAAGGGTCGCC						
Cu39	F: ACACCAAAGCAAGGCATGAA	$(AG)_{10}$	201 – 255	60	6-FAM	4	MH917276
	R: TGCAAACAAGTGGCCTACCA						
Multiplex 5							
Cu15	F: TCGTGATTTCAACCTTGACCA	$(AG)_9$	93 – 131	60	VIC	3	MK570861
	R: TGGAACTGGTCATCCCTCCA						
Cu22	F: TGCACAAAGAAGATGGAAGCTG	$(AG)_{11}$	122 – 158	60	NED	1	MK570862
	R: CCGTCCACGTATTGCAGAGA						
Cu38	F: AGTTCATATGCCAGCGTAATCG	$(AG)_9$	196 – 232	60	6-FAM	3	MK570863
	R: AACGTCCCAGACCAACGATC						
Cu45	F: CTCCAATGGCTACCGTCGAG	$(AG)_{10}$	249 – 281	60	PET	5	MK570864
	R: TGACAATTCACATGCATGATGC						

Note: T_a = annealing temperature.

[a] Florescent label refers to Applied Biosystems fluorescent dyes used in sequencing reactions;

[b] µL of primer working solution (2 µM) in 100 µL

Subsequently, those multiplexes were tested in 72 *C. undulatum* individuals from three populations (Table B.2) using the same PCR conditions as described above. Plants were selected as evenly spaced as possible throughout the populations by using a grid of 15x15 m quadrats where the closest plant to each corner of each quadrat was sampled. Leaves were kept separated per source plant and stored in silica gel after collection then freeze-dried. DNA was extracted from 50 mg freeze-dried leaf material using a modified 2% CTAB method, as outlined above. Multiple runs were performed to ensure both the consistency of scoring and the accuracy of the final data set. In addition, we tested cross-amplification with a total of 45 samples of congeners, i.e. *C. stoechadis*, *C. canaliculatum* and *C. triplinervium*, sampled within a 30 km radius from *C. undulatum* populations (Table B.2).

Allele size was determined using GeneMapper Software v5 (Applied Biosystems). GenAlEx v6.51 (Peakall and Smouse, 2012) was used to calculate number of alleles per locus and expected and observed heterozygosity for loci in three populations (Table B.3). Evidence of linkage disequilibrium were assessed by GENEPOP (Rousset, 2008) based on 10,000 permutations. The frequency of possible null alleles, genotyping failure and inbreeding was estimated using INEST 2.2 (Chybicki and Burczyk, 2009) after 500000 Markov Chain iterations.

Table F.2. Locality information for *Conospermum* species used in this study.

Species	Voucher[a]	Collection locality	Geographic coordinates
Conospermum undulatum	PERTH 06797083	Maida vale	Rare flora
	PERTH 09006176	Orange grove	Rare flora
	PERTH 09006192	Orange grove	Rare flora
Conospermum stoechadis	PERTH 07795092	Koondoola regional bushland	-31.846111, 115.874722
Conospermum canaliculatum	PERTH 06256430	7 km NE of Yanchep	-31.489641, 115.668538
Conospermum triplinervium	PERTH 07800754	Kings Park, Perth	-31.966827, 115.836388

[a] Vouchers are stored in the Western Australian Herbarium (PERTH), Perth, Western Australia, Australia.

Evaluation of loci showed no indication of linkage disequilibrium for any pairwise combination of loci, nor was there significant genotyping failure. Loci Cu15, Cu17, Cu29 and Cu41 showed evidence of null alleles in two out of three populations and should be used with caution in analyses whose results may be inflated by the occurrence of null alleles. One locus was monomorphic in the three *C. undulatum* populations investigated in this study (i.e. Cu8; Table B.3). However, this marker was polymorphic in two out of the three other *Conospermum* species considered. Overall, we observed 229 alleles at the 20 microsatellite

loci, with an average of 11.45 alleles per locus. Observed and expected heterozygosity per locus ranged from 0.000 to 1.000 and from 0.117 to 0.919, respectively (Table B.3). Inbreeding was never comprised in the most likely INEST model to explain excess of homozygosity and, therefore, average within-population inbreeding was not statistically different from zero in any of the three analysed populations.

Table F.3. Genetic characterization of 20 newly developed microsatellite loci across three populations of *Conospermum undulatum*[a].

		Population 1 (n=24)				Population 2 (n=24)				Population 3 (n=24)			
Locus	A_T	*A*	H_o	H_e	Null	*A*	H_o	H_e	Null	*A*	H_o	H_e	Null
Multiplex 1													
Cu4	11	9	0.667	0.811		10	0.750	0.822		9	0.833	0.824	
Cu16	8	5	0.667	0.745		8	0.625	0.694		6	0.333	0.681	0.173
Cu31	21	16	0.833	0.894		17	0.750	0.884		15	1.000	0.891	
Cu41	3	3	0.083	0.424	0.239	2	0.167	0.500		2	0.000	0.486	0.303
Multiplex 2													
Cu8	1	1	ND	ND		1	ND	ND		1	ND	ND	
Cu17	5	5	0.444	0.656		2	0.000	0.413	0.280	2	0.087	0.499	0.311
Cu24	3	3	0.391	0.322		2	0.333	0.278		2	0.125	0.117	
Cu32	9	8	0.667	0.637		5	0.625	0.648		5	0.542	0.582	
Multiplex 3													
Cu9	21	14	0.875	0.904		13	0.750	0.882		16	0.958	0.919	
Cu18	4	4	0.542	0.674		4	0.667	0.702		4	0.500	0.672	
Cu28	18	15	1.000	0.914		14	0.917	0.888		11	0.875	0.846	
Cu33	21	14	0.792	0.878		15	0.958	0.886		11	0.833	0.861	
Multiplex 4													
Cu20	4	4	0.250	0.463		2	0.435	0.499	0.155	2	0.250	0.413	
Cu29	16	10	0.417	0.800	0.176	9	0.174	0.696	0.297	8	0.375	0.643	
Cu36	6	5	0.375	0.419		5	0.417	0.436		4	0.417	0.355	
Cu39	17	12	0.652	0.863	0.138	14	0.875	0.888		10	0.917	0.852	
Multiplex 5													
Cu15	15	11	0.417	0.792	0.177	9	0.500	0.485		13	0.583	0.828	0.108
Cu22	15	10	0.708	0.822		10	0.833	0.841		12	0.792	0.823	
Cu38	15	10	0.917	0.800		10	0.833	0.808		8	0.833	0.742	
Cu45	16	14	0.708	0.839		11	0.625	0.870		12	0.750	0.877	
Mean	11.45	8.650	0.570	0.683		8.150	0.562	0.656		7.650	0.550	0.646	
S.E.	1.521	1.037	0.060	0.054		1.120	0.067	0.055		1.062	0.075	0.059	

Note: A_T = Overall number of alleles; *A* = number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; n = number of individuals sampled; Null = estimated frequency of null alleles where different from zero; ND = not determined.

[a] Localities are provided in Table A.2

All microsatellite loci showed successful cross-amplification in *C. stoechadis*, *C. canaliculatum* and *C. triplinervium* (Table B.4) using the same extraction method and amplification conditions outlined above. Analysis of amplification showed similar number of alleles amplified in *C. stoechadis* and *C. canaliculatum* as in *C. undulatum*, whereas fewer alleles were detected in *C. triplinervium*.

Table F.4. Cross-amplification of 20 microsatellite loci developed for *Conospermum undulatum* in three related species[a].

Locus	*C. stoechadis* (n=15)			*C. canaliculatum* (n=15)			*C. triplinervium* (n=15)		
	Amplification	A	Allele size (bp)	Amplification	A	Allele size (bp)	Amplification	A	Allele size (bp)
Multiplex 1									
Cu4	15	7	86 – 104	14	4	84 – 92	15	1	92
Cu16	15	5	100 – 112	15	5	100 – 112	15	2	103
Cu31	15	12	131 – 167	15	13	133 – 165	15	2	139 – 141
Cu41	15	3	242 – 246	15	4	242 – 248	15	2	246 – 250
Multiplex 2									
Cu8	15	1	95	15	2	75 – 95	15	2	75 – 95
Cu17	15	4	107 – 113	15	4	101 – 111	15	2	100 – 111
Cu24	15	3	140 – 146	15	3	140 – 146	15	1	140
Cu32	15	4	161 – 187	15	6	161 – 175	15	1	163
Multiplex 3									
Cu9	15	8	88 – 122	15	10	82 – 120	14	1	102
Cu18	15	4	105 – 111	15	4	105 – 111	15	1	109
Cu28	15	10	124 – 154	15	13	124 – 168	15	3	136 – 148
Cu33	15	11	146 – 178	15	12	152 – 188	15	4	164 – 168
Multiplex 4									
Cu20	15	2	124 – 126	15	4	122 – 128	15	2	128 – 132
Cu29	15	6	150 – 178	14	9	148 – 172	15	2	150 – 152
Cu36	15	3	189 – 201	15	6	186 – 201	15	2	186 – 198
Cu39	15	9	201 – 237	15	10	213 – 249	15	3	215 – 223
Multiplex 5									
Cu15	15	10	93 – 123	14	10	91 – 129	15	3	105 – 111
Cu22	14	6	126 – 152	13	11	116 – 152	14	4	128 – 148
Cu38	15	6	196 – 220	15	7	200 – 232	15	2	202 – 204
Cu45	15	11	247 – 275	15	12	247 – 281	14	3	259 – 269

Note: Amplification = number of individuals successfully amplified; A = number of alleles; n = number of individuals used.

[a] Localities are provided in Table A.2.

Conclusions

Twenty microsatellite markers were developed for *Conospermum undulatum*. These markers will be used for investigating population genetic structure, dispersal dynamics and possible hybridization events of this rare species to underpin its management and conservation. These newly developed markers are likely to be useful for genetic studies on phylogenetically related species given the successful cross-amplification for three different *Conospermum* species.

Acknowledgments

This research was jointly supported by Edith Cowan University Industry Collaboration Grant and the Department of Biodiversity, Conservation and Attractions, Western Australia [G1002531]. The authors thank B. Macdonald and S. McArthur for their help in the laboratory.

Appendix G – Tales of the unexpected – ant pollination interactions

This chapter has been published as the following paper:

Delnevo N, van Etten EJ. 2019. Tales of the unexpected – ant pollination mutualism. *Frontiers in Ecology and the Environment*, 17(10): 558.

South-western Australia constitutes a biodiversity hotspot where plants and animals have coevolved over long timeframes, resulting in exceptionally specialized interactions. The shrub Conospermum undulatum, also known as smokebush because it presents white, woolly flowers covered in white hairs that resemble drifting smoke when seen en masse, has developed a pollination mutualism with the native ant *Camponotus molossus*. Pollination by ants is extremely rare, with only about 40 convincing examples observed to date. Insects visiting smokebush flowers for nectar trigger the stigma, which flicks away from the anthers toward the lower tepals (outer part of flowers with no differentiation between petals and sepals) to make contact with the insect; simultaneously, the fertile anthers cast new pollen

onto the visitor. The stigma strikes with such a force that most dipterans visiting the flowers remain fatally trapped, making these generalist pollinators ineffective.

The hymenopteran *C. molossus* is an exception; this ant is large enough to escape such a fate and also facilitates pollination by feeding on smokebush. While the cuticular secretions of many ants are known to drastically reduce the pollen viability of most plants, laboratory experiments assessing the effect of ant secretions on pollen viability showed that smokebush pollen is resistant to these secretions. Moreover, mutualistic services by ants are important for maximizing the seed output of this endangered plant, which is threatened by urbanization across its limited distribution. Is the pollen tolerance to ant secretions present in other phylogenetically related taxa? Where and when did this trait evolve? Thousands of plant and animal species and their interactions have been described in this global biodiversity hotspot, but how many more are still to be discovered?

References

Abasolo, M., Lee, D. J., & Shepherd, M. (2012). Identification of intersectional *Corymbia* hybrids based on seedling morphology improves with parental divergence. *Forest Ecology and Management*, *279*, 189–202. https://doi.org/10.1016/j.foreco.2012.05.014

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C. A., Buggs, R., Butlin, R. K., Dieckmann, U., Eroukhmanoff, F., Grill, A., Cahan, S. H., Hermansen, J. S., Hewitt, G., Hudson, A. G., Jiggins, C., … Zinner, D. (2013). Hybridization and speciation. *Journal of Evolutionary Biology*, *26*(2), 229–246. https://doi.org/10.1111/j.1420-9101.2012.02599.x

Adams, W. T., & Birkes, D. S. (1991). Estimating mating patterns in forest tree population. In F. S. et Al (Ed.), *Biochemical Markers in the Population Genetics of Forest Trees* (pp. 157–172). SPB Academic Publishing: The Hague.

Ågren, J. (1996). Population size, pollinator limitation, and seed set in the self-incompatible herb *Lythrum salicaria*. *Ecology*, *77*(6), 1779–1790. http://www.jstor.org/stable/2265783

Aguilar, R., Ashworth, L., Calviño, A., & Quesada, M. (2012). What is left after sex in fragmented habitats? Assessing the quantity and quality of progeny in the endemic tree *Prosopis caldenia* (Fabaceae). *Biological Conservation*, *152*, 81–89. https://doi.org/10.1016/j.biocon.2012.03.021

Aguilar, R., Ashworth, L., Galetto, L., & Aizen, M. A. (2006). Plant reproductive susceptibility to habitat fragmentation: Review and synthesis through a meta-analysis. *Ecology Letters*, *9*(8), 968–980. https://doi.org/10.1111/j.1461-0248.2006.00927.x

Aguilar, R., Cristóbal-Pérez, E. J., Balvino-Olvera, F. J., de Jesús Aguilar-Aguilar, M., Aguirre-Acosta, N., Ashworth, L., Lobo, J. A., Martén-Rodríguez, S., Fuchs, E. J., Sanchez-Montoya, G., Bernardello, G., & Quesada, M. (2019). Habitat fragmentation reduces plant progeny quality: a global synthesis. *Ecology Letters*, *22*(7), 1163–1173. https://doi.org/10.1111/ele.13272

Aguilar, R., Quesada, M., Ashworth, L., Herrerias-Diego, Y., & Lobo, J. (2008). Genetic consequences of habitat fragmentation in plant populations: Susceptible signals in plant traits and methodological approaches. *Molecular Ecology*, *17*(24), 5177–5188. https://doi.org/10.1111/j.1365-294X.2008.03971.x

Aizen, M. A., & Feinsinger, P. (1994). Forest fragmentation, pollination, and plant reproduction in a Chaco dry forest, Argentina. *Ecology*, *75*(2), 330–351.

https://doi.org/10.1111/j.1600-0706.2010.18376.x

Aizen, M. A., Ashworth, L., & Galetto, L. (2002). Reproductive success in fragmented habitats: Do compatibility systems and pollination specialization matter? *Journal of Vegetation Science*, *13*(6), 885–892. https://doi.org/10.1111/j.1654-1103.2002.tb02118.x

Aizen, M. A., & Harder, L. D. (2007). Expanding the limits of the pollen-limitation concept: effects of pollen quantity and quality. *Ecology*, *88*(2), 271–281. https://doi.org/10.1890/07-1861.1

Allendorf, F. W., Leary, R. F., Spruell, P., & Wenburg, J. K. (2001). The problems with hybrids: setting conservation guidelines. *Trends in Ecology & Evolution*, *16*(11), 613–622. https://doi.org/https://doi.org/10.1016/S0169-5347(01)02290-X

Almeida, E. A. B., Pie, M. R., Brady, S. G., & Danforth, B. N. (2012). Biogeography and diversification of colletid bees (Hymenoptera: Colletidae): Emerging patterns from the southern end of the world. *Journal of Biogeography*, *39*(3), 526–544. https://doi.org/10.1111/j.1365-2699.2011.02624.x

Andersson, P., Koffman, A., Sjödin, N. E., & Johansson, V. (2017). Roads may act as barriers to flying insects: Species composition of bees and wasps differs on two sides of a large highway. *Nature Conservation*, *18*, 47–59. https://doi.org/10.3897/natureconservation.18.12314

Ashman, T. L., Knight, T. M., Steets, J. A., Amarasekare, P., Burd, M., Campbell, D. R., Dudash, M. R., Johnston, M. O., Mazer, S. J., Mitchell, R. J., Morgan, M. T., & Wilson, W. G. (2004). Pollen limitation of plant reproduction: Ecological and evolutionary causes and consequences. *Ecology*, *85*(9), 2408–2421. https://doi.org/10.1890/03-8024

Bacles, C. F. E., & Jump, A. S. (2011). Taking a tree's perspective on forest fragmentation genetics. *Trends in Plant Science*, *16*(1), 13–18. https://doi.org/https://doi.org/10.1016/j.tplants.2010.10.002

Barnabas, B., & Fridvalszky, L. (1984). Adhesion and germination of differently treated maize pollen grains on the stigma. In *Acta botanica Hungarica* (Vol. 30, pp. 329–332).

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/https://doi.org/10.18637/jss.v067.i01

Beard, J. S. (1984). Biogeography of the kwongan. In J. S. Pate & J. S. Beard (Eds.), *Kwongan, plant life of the sandplain* (pp. 1–26). University of Western Australia Press.

Beard, J. S. (1989). Definition and location of the *Banksia* woodlands. *Journal of the Royal Society of Western Australia*, *71*(4), 85–86.

Beattie, A. J., Turnbull, C., Hough, T., Jobson, S., & Knox, R. B. (1985). The vulnerability of pollen and fungal spores to ant secretions: evidence and some evolutionary implications. *American Journal of Botany*, *72*(4), 606–614. https://doi.org/10.2307/2443594

Beattie, A. J., Turnbull, C., Knox, R. B., & Williams, E. G. (1984). Ant inhibition of pollen function: A possible reason why ant pollination is rare. *American Journal of Botany*, *71*(3), 421–426. https://doi.org/10.2307/2443499

Beekman, M., & Ratnieks, F. L. W. (2000). Long-range foraging by the honey-bee, *Apis mellifera* L. *Functional Ecology*, *14*(4), 490–496. https://doi.org/10.1046/j.1365-2435.2000.00443.x

Bell, D. T. (2001). Ecological response syndromes in the flora of southwestern Western Australia: Fire resprouters versus reseeders. *The Botanical Review*, *67*(4), 417–440. https://doi.org/10.1007/BF02857891

Beninde, J., Feldmeier, S., Veith, M., & Hochkirch, A. (2018). Admixture of hybrid swarms of native and introduced lizards in cities is determined by the cityscape structure and invasion history. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1883). https://doi.org/10.1098/rspb.2018.0143

Bennett E. M. (1995). Flora of Australia. *Melbourne: CSIRO Australia*, *16*.

Bezemer, N., Krauss, S. L., Phillips, R. D., Roberts, D. G., & 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*(February), 1–12. https://doi.org/10.1038/hdy.2016.61

Biesmeijer, J. C. (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*, *313*(5785), 351–354. https://doi.org/10.1126/science.1127863

Bond, W J. (1994). Do mutualisms matter - Assessing the impact of pollinator and disperser disruption on plant extinction. *Philosophical Transactions of the Royal Society B-Biological Sciences*, *344*(1307), 83–90. https://doi.org/10.1098/rstb.1994.0055

Bond, W. J., & Midgley, J. J. (2001). Ecology of sprouting in woody plants: The persistence niche. *Trends in Ecology and Evolution*, *16*(1), 45–51. https://doi.org/10.1016/S0169-5347(00)02033-4

Booy, G., Hendriks, R. J. J., Smulders, M. J. M., Van Groenendael, J. M., & Vosman, B. (2000). Genetic diversity and the survival of populations. *Plant Biology*, *2*(4), 379–395. https://doi.org/10.1055/s-2000-5958

Borcard, D., Gillet, F., & Legendre, P. (2011). Numerical Ecology with R. In *Numerical Ecology with R*. Ney York: Springer. https://doi.org/10.1007/978-1-4419-7976-6

Breed, M. F., Ottewell, K. M., Gardner, M. G., Marklund, M. H. K., Dormontt, E. E., & Lowe, A. J. (2015). Mating patterns and pollinator mobility are critical traits in forest fragmentation genetics. *Heredity*, *115*, 1–7. https://doi.org/10.1038/hdy.2013.48

Brewbaker, J. L., & Kwack, B. H. (1963). The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany*, *50*, 859–865. https://doi.org/10.2307/2439772

Broadhurst, L., Breed, M., Lowe, A., Bragg, J., Catullo, R., Coates, D., Encinas-Viso, F., Gellie, N., James, E., Krauss, S., Potts, B., Rossetto, M., Shepherd, M., & Byrne, M. (2017). Genetic diversity and structure of the Australian flora. *Diversity and Distributions*, 41–52. https://doi.org/10.1111/ddi.12505

Brosi, B. J., & Briggs, H. M. (2013). Single pollinator species losses reduce floral fidelity and plant reproductive function. *Proceedings of the National Academy of Sciences*, *110*(32), 13044–13048. https://doi.org/10.1073/pnas.1307438110

Brosi, Berry J. (2016). Pollinator specialization: from the individual to the community. *New Phytologist*, *210*, 1190–1194. https://doi.org/10.1111/nph.13951

Brudvig, L. A., Damschen, E. I., Haddad, N. M., Levey, D. J., & Tewksbury, J. J. (2015). The influence of habitat fragmentation on multiple plant-animal interactions and plant reproduction. *Ecology*, *96*, 2669–2678. https://doi.org/10.1890/14-2275.1

Burbidge, A. H., James, S. H. (1991). Post-zygotic seed abortion in the genetic system of *Stylidium* (Angiospermae: Stylidiaceae). *Journal of Heredity*, *82*(4), 319–328. https://doi.org/10.1093/oxfordjournals.jhered.a111092

Burd, M. (1994). Bateman principle and plant reproduction - the role of pollen limitation in fruit and seed set. *Botanical Review*, *60*(1), 83–139. https://doi.org/10.1007/bf02856594

Burgess, K. S., Morgan, M., Deverno, L., & Husband, B. C. (2005). Asymmetrical introgression between two *Morus* species (*M. alba, M. rubra*) that differ in abundance. *Molecular Ecology*, *14*(11), 3471–3483. https://doi.org/10.1111/j.1365-294X.2005.02670.x

Burrows, N., & McCaw, L. (2013). Prescribed burning in southwestern Australian forests. *Frontiers in Ecology and the Environment*, *11*, 25–34. https://doi.org/10.1890/120356

Byrne, M., Macdonald, B., & Francki, M. (2001). Incorporation of sodium sulfite into extraction protocol minimizes degradation of *Acacia* DNA. *BioTechniques*, *30*(4), 742–748. https://doi.org/10.2144/01304bm06

Byrne, M., Elliott, C. P., Yates, C., & Coates, D. J. (2007). Extensive pollen dispersal in a bird-pollinated shrub, *Calothamnus quadrifidus*, in a fragmented landscape. *Molecular Ecology*, *16*(6), 1303–1314. https://doi.org/10.1111/j.1365-294X.2006.03204.x

Byrne, M., Elliott, C. P., Yates, C. J., & Coates, D. J. (2008). Maintenance of high pollen dispersal in *Eucalyptus wandoo*, a dominant tree of the fragmented agricultural region in Western Australia. *Conservation Genetics*, *9*(1), 97–105. https://doi.org/10.1007/s10592-007-9311-5

Campbell, L. G., & Husband, B. C. (2007). Small populations are mate-poor but pollinator-rich in a rare, self-incompatible plant, *Hymenoxys herbacea* (Asteraceae). *New Phytologist*, *174*(4), 915–925. https://doi.org/10.1111/j.1469-8137.2007.02045.x

Charlesworth, D., & Willis, J. H. (2009). The genetics of inbreeding depression. *Nature Reviews Genetics*, *10*(11), 783–796. https://doi.org/10.1038/nrg2664

Charnov, E. L. (1976). Optimal foraging, the marginal value theorem. *Theoretical Population Biology*, *9*, 129–136. https://doi.org/10.1016/0040-5809(76)90040-X

Chen, M., Zhao, X. Y., & Zuo, X. A. (2019). Comparative pollen limitation and pollinator activity of *Caragana korshinskii* Kom in natural and fragmented habitats. *Science of the Total Environment*, *654*, 1056–1063. https://doi.org/10.1016/j.scitotenv.2018.11.148

Cheptou, P. O., Carrue, O., Rouifed, S., & Cantarel, A. (2008). Rapid evolution of seed dispersal in an urban environment in the weed *Crepis sancta*. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(10), 3796–3799. https://doi.org/10.1073/pnas.0708446105

Chhatre, V. E., & Rajora, O. P. (2014). Genetic divergence and signatures of natural selection in marginal populations of a keystone, long-lived conifer, eastern white pine (*Pinus strobus*) from Northern Ontario. *PLoS ONE*, *9*(5), 1–13. https://doi.org/10.1371/journal.pone.0097291

Chybicki, I. J. (2018). NMπ—improved re-implementation of NM+, a software for estimating gene dispersal and mating patterns. *Molecular Ecology Resources*, *18*(1), 159–168. https://doi.org/10.1111/1755-0998.12710

Chybicki, I. J., & Burczyk, J. (2009). Simultaneous estimation of null alleles and inbreeding coefficients. *Journal of Heredity*, *100*(1), 106–113. https://doi.org/10.1093/jhered/esn088

Clarke, P. J., Lawes, M. J., Midgley, J. J., Lamont, B. B., Ojeda, F., Burrows, G. E., Enright, N. J., & Knox, K. J. E. (2013). Resprouting as a key functional trait: How buds, protection and resources drive persistence after fire. *New Phytologist*, *197*(1), 19–35. https://doi.org/10.1111/nph.12001

Close, D. C., Messina, G., Krauss, S. L., Rokich, D. P., Stritzke, J., & Dixon, K. W. (2006). Conservation biology of the rare species *Conospermum undulatum* and *Macarthuria keigheryi* in an urban bushland remnant. *Australian Journal of Botany*, *54*(6), 583–593.

https://doi.org/10.1071/BT05205

Cochrane, A. (2007). *Conospermum*. *Seed Notes for Western Australia*, *12*, 1–4. https://doi.org/10.1017/S0033291708004790

Collins, B., & Rebelo, T. (1987). Pollination biology of the Proteaceae in Australia and southern Africa. *Australian Journal of Ecology*, *12*(4), 387–421. https://doi.org/10.1111/j.1442-9993.1987.tb00958.x

Commonwealth of Australia. (2000). Declaration under s178, s181, and s183 of the Environment Protection and Biodiversity Conservation Act 1999 - List of threatened species, List of threatened ecological communities and List of threatening processes. F2005B02653. In *Federal Register of Legislative Instruments*.

Cornuet, J. M., & Luikart, G. (1996). Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, *144*(4), 2001–2014. https://doi.org/10.1093/oxfordjournals.jhered.a111627

Da Silva Carvalho, C., Ribeiro, M. C., Côrtes, M. C., Galetti, M., & Collevatti, R. G. (2015). Contemporary and historic factors influence differently genetic differentiation and diversity in a tropical palm. *Heredity*, *115*(3), 216–224. https://doi.org/10.1038/hdy.2015.30

Dauber, J., Biesmeijer, J. C., Gabriel, D., Kunin, W. E., Lamborn, E., Meyer, B., Nielsen, A., Potts, S. G., Roberts, S. P. M., Sõber, V., Settele, J., Steffan-Dewenter, I., Stout, J. C., Teder, T., Tscheulin, T., Vivarelli, D., & Petanidou, T. (2010). Effects of patch size and density on flower visitation and seed set of wild plants: A pan-European approach. *Journal of Ecology*, *98*(1), 188–196. https://doi.org/10.1111/j.1365-2745.2009.01590.x

Davis, R. A., Gole, C., & Roberts, J. D. (2013). Impacts of urbanisation on the native avifauna of Perth, Western Australia. *Urban Ecosystems*, *16*(3), 427–452. https://doi.org/10.1007/s11252-012-0275-y

De Vega, C., Arista, M., Ortiz, P. L., Herrera, C. M., & Talavera, S. (2009). The ant-pollination system of *Cytinus hypocistis* (Cytinaceae), a Mediterranean root holoparasite. *Annals of Botany*, *103*(7), 1065–1075. https://doi.org/10.1093/aob/mcp049

De Vega, C., & Gómez, J. (2014). Polinización por hormigas: conceptos, evidencias y futuras direcciones. *Ecosistemas: Revista Científica y Técnica de Ecología y Medio Ambiente*, *23*(3), 48–57. https://doi.org/10.7818/re.2014.23-3.00

De Vega, C., Herrera, C. M., & Dötterl, S. (2014). Floral volatiles play a key role in specialized ant pollination. *Perspectives in Plant Ecology, Evolution and Systematics*, *16*(1), 32–42. https://doi.org/10.1016/j.ppees.2013.11.002

Del-Claro, K., Rodriguez-Morales, D., Calixto, E. S., Martins, A. S., & Torezan-Silingardi,

H. M. (2019). Ant pollination of *Paepalanthus lundii* (Eriocaulaceae) in Brazilian savanna. *Annals of Botany*, *123*(7), 1159–1165. https://doi.org/10.1093/aob/mcz021

Delmas, C. E. L., Escaravage, N., Cheptou, P. O., Charrier, O., Ruzafa, S., Winterton, P., & Pornon, A. (2015). Relative impact of mate versus pollinator availability on pollen limitation and outcrossing rates in a mass-flowering species. *Plant Biology*, *17*(1), 209–218. https://doi.org/10.1111/plb.12200

Delmas, C. E. L., Escaravage, N., & Pornon, A. (2014). Massive floral display affects insect visits but not pollinator-mediated pollen transfer in *Rhododendron ferrugineum*. *Plant Biology*, *16*(1), 234–243. https://doi.org/10.1111/plb.12039

Delmas, C. E. L., Fort, T. L. C., Escaravage, N., & Pornon, A. (2016). Pollen transfer in fragmented plant populations: insight from the pollen loads of pollinators and stigmas in a mass-flowering species. *Ecology and Evolution*, *6*(16), 5663–5673. https://doi.org/10.1002/ece3.2280

Delnevo, N., Etten, E. J. Van, Byrne, M., Petraglia, A., Carbognani, M., & Stock, W. D. (2020 b). Habitat fragmentation restricts insect pollinators and pollen quality in a threatened Proteaceae species. *Biological Conservation*, *252*. https://doi.org/10.1016/j.biocon.2020.108824

Delnevo, N., Piotti, A., van Etten, E. J., Stock, W. D., & Byrne, M. (2019 b). Isolation, characterization, and cross-amplification of 20 microsatellite markers for *Conospermum undulatum* (Proteaceae). *Applications in Plant Sciences*, *7*(8), e11283. https://doi.org/10.1002/aps3.11283

Delnevo, N., & van Etten, E. J. (2019). Tales of the unexpected – ant pollination mutualism. *Frontiers in Ecology and the Environment*, 2135. https://doi.org/10.1002/fee.2135

Delnevo, N., van Etten, E. J., Byrne, M., & Stock, W. D. (2019 a). Floral display and habitat fragmentation: Effects on the reproductive success of the threatened mass-flowering *Conospermum undulatum* (Proteaceae). *Ecology and Evolution*, 1–10. https://doi.org/10.1002/ece3.5653

Delnevo, N., van Etten, E. J., Clemente, N., Fogu, L., Pavarani, E., Byrne, M., & Stock, W. D. (2020 a). Pollen adaptation to ant pollination – a case study from the Proteaceae. *Annals of Botany*, 1–10. https://doi.org/10.1093/aob/mcaa058

Department of Environment and Conservation. (2009). Wavy-leaved smokebush (*Conospermum undulatum*) Recovery Plan. *Commonwealth Department of the Environment, Water, Heritage and the Arts, Canberra.*

Department of the Environment and Energy. (2016). *Banksia* woodlands of the Swan Coastal Plain: a nationally protected ecological community.

Department of Parks and Wildlife. (2016). *Banksia attenuata* woodlands over species rich dense shrublands (Swan Coastal Plain community type 20a – Gibson et al. 1994). *Interim Recovery Plan No. 359.* 1–31.

Dick, C. W., Etchelecu, G., & Austerlitz, F. (2003). Pollen dispersal of tropical trees (*Dinizia excelsa*: Fabaceae) by native insects and African honeybees in pristine and fragmented Amazonian rainforest. *Molecular Ecology*, *12*(3), 753–764. https://doi.org/10.1046/j.1365-294X.2003.01760.x

Diekötter, T., Kadoya, T., Peter, F., Wolters, V., & Jauker, F. (2010). Oilseed rape crops distort plant-pollinator interactions. *Journal of Applied Ecology*. https://doi.org/10.1111/j.1365-2664.2009.01759.x

Do, C., Waples, R. S., Peel, D., Macbeth, G. M., Tillett, B. J., & Ovenden, J. R. (2014). NeEstimator v2: re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. *Molecular Ecology Resources*, *14*(1), 209–214. https://doi.org/10.1111/1755-0998.12157

Domingos-Melo, A., Nadia, T. L., & Machado, I. C. (2017). Complex flowers and rare pollinators: Does ant pollination in *Ditassa* show a stable system in Asclepiadoideae (Apocynaceae)? *Arthropod-Plant Interactions*, *11*, 339–349. https://doi.org/10.1007/s11829-017-9499-3

Douglas, A. W. (1997). The developmental basis of morphological diversification and synorganization in flowers of Conospermeae (Stirlingia and Conosperminae: Proteaceae). *International Journal of Plant Sciences*, *158*(6), 13–48. https://doi.org/10.1086/297505

Dudash, M., & Fenster, C. (2000). Inbreeding and outbreeding depression in fragmented populations. In A. G. Young & G. M. Clarke (Eds.), *Genetics, Demography and Viability of Fragmented Populations* (pp. 35–53). Cambridge University Press. https://doi.org/Genetic Considerations in Ecological Restoration

Dutton, E. M., & Frederickson, M. E. (2012). Why ant pollination is rare: New evidence and implications of the antibiotic hypothesis. *Arthropod-Plant Interactions*, *6*(4), 561–569. https://doi.org/10.1007/s11829-012-9201-8

Earl, D. A., & Von Holdt, B. M. (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, *4*(2), 359–361. https://doi.org/10.1007/s12686-011-9548-7

Eckert, C. G., Samis, K. E., & Lougheed, S. C. (2008). Genetic variation across species' geographical ranges: The central-marginal hypothesis and beyond. *Molecular Ecology*,

17(5), 1170–1188. https://doi.org/10.1111/j.1365-294X.2007.03659.x

Eckert, C. G. (2000). Contributions of autogamy and geitonogamy to self-fertilization in a mass-flowering, clonal plant. *Ecology*. https://doi.org/10.1890/0012-9658(2000)081[0532:COAAGT]2.0.CO;2

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., Porcher, E., Ree, R. H., Vallejo-Marín, M., & Winn, A. A. (2010). Plant mating systems in a changing world. *Trends in Ecology and Evolution*, *25*(1), 35–43. https://doi.org/10.1016/j.tree.2009.06.013

Ellis, E. C., Goldewijk, K. K., Siebert, S., Lightman, D., & Ramankutty, N. (2010). Anthropogenic transformation of the biomes, 1700 to 2000. *Global Ecology and Biogeography*, *19*(5), 589–606. https://doi.org/10.1111/j.1466-8238.2010.00540.x

Ellis, E. C., & Ramankutty, N. (2008). Putting people in the map: Anthropogenic biomes of the world. *Frontiers in Ecology and the Environment*, *6*(8), 439–447. https://doi.org/10.1890/070062

Ellner, S., & Shmida, A. (1981). Why are adaptations for long-range seed dispersal rare in desert plants? *Oecologia*, *51*(1), 133–144. https://doi.org/10.1007/BF00344663

Ellstrand, N. C. (2014). Is gene flow the most important evolutionary force in plants? *American Journal of Botany*, *101*(5), 737–753. https://doi.org/10.3732/ajb.1400024

Ellstrand, N. C., & Elam, D. R. (1993). Population genetic conseqences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics*, *24*, 217–242. https://doi.org/10.1146/annurev.es.24.110193.001245

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology*, *14*(8), 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Exon, N. F., Hill, P. J., Lafoy, Y., Heine, C., & Bernardel, G. (2006). Kenn plateau off northeast Australia: A continental fragment in the southwest pacific jigsaw. *Australian Journal of Earth Sciences*, *53*(4), 541–564. https://doi.org/10.1080/08120090600632300

Faeth, S. H., Bang, C., & Saari, S. (2011). Urban biodiversity: Patterns and mechanisms. *Annals of the New York Academy of Sciences*, *1223*(1), 69–81. https://doi.org/10.1111/j.1749-6632.2010.05925.x

Fenner, M., & Thompson, K. (2005). *The Ecology of Seeds*. Cambridge University Press. https://doi.org/https://doi:10.1017/CBO9780511614101

Fischer, M., Hock, M., & Paschke, M. (2003). Low genetic variation reduces cross-compatibility and offspring fitness in populations of a narrow endemic plant with a self-incompatibility system. *Conservation Genetics*, *4*(3), 325–336.

https://doi.org/10.1023/A:1024051129024

Forsyth, S. A. (2003). Density-dependent seed set in the Haleakala silversword: Evidence for an allee effect. *Oecologia*, *136*, 551–557. https://doi.org/10.1007/s00442-003-1295-3

Fox, J., & Weisberg, S. (2019). *An R Companion to Applied Regression* (3rd ed.).

Frankham, R. (2005). Genetics and extinction. In *Biological Conservation*. https://doi.org/10.1016/j.biocon.2005.05.002

Freitas, L., & Bolmgren, K. (2008). Synchrony is more than overlap: measuring phenological synchronization considering time length and intensity. *Revista Brasileira de Botânica*, *31*(4), 721–724. https://doi.org/10.1590/S0100-84042008000400017

Fuchs, E. J., & Hamrick, J. L. (2011). Mating system and pollen flow between remnant populations of the endangered tropical tree, *Guaiacum sanctum* (Zygophyllaceae). *Conservation Genetics*, *12*(1), 175–185. https://doi.org/10.1007/s10592-010-0130-8

Galen, C. (1983). The effects of nectar thieving ants on seed set in floral scent morphs of *Polemonium viscosum*. *Oikos*, *41*(2), 245–249. https://doi.org/10.2307/3544271

Garza, J. C., & Williamson, E. G. (2001). Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology*, *10*(2), 305–318. https://doi.org/10.1046/j.1365-294X.2001.01190.x

Government Gazette. (2018). *Wildlife Conservation (Rare Flora) Notice 2018*. *September*, 3226–3232.

Geerts, S., & Pauw, A. (2012). The cost of being specialized: Pollinator limitation in the endangered geophyte *Brunsvigia litoralis* (Amaryllidaceae) in the Cape Floristic Region of South Africa. *South African Journal of Botany*, *78*, 159–164. https://doi.org/10.1016/j.sajb.2011.06.007

Gibbs, J. P. (2001). Demography versus habitat fragmentation as determinants of genetic variation in wild populations. *Biological Conservation*, *100*(1), 15–20. https://doi.org/https://doi.org/10.1016/S0006-3207(00)00203-2

Gibbs, P. E. (2014). Late-acting self-incompatibility – the pariah breeding system in flowering plants. *New Phytologist, 203*, 717–734. https://doi.org/10.1111/nph.12874

Gibson, N., Keighery, B. J., Keighery, G. J., Burbidge, A. H., & Lyons, M. (1994). A floristic survey of the Southern Swan Coastal Plain. *Unpublished Report for the Australian Heritage Commission Prepared by the Department of Conservation and Land Management and the Conservation Council of Western Australia (Inc.)*.

Gibson, N., Yates, C., Byrne, M., Langley, M., & Thavornkanlapachai, R. (2012). The importance of recruitment patterns versus reproductive output in the persistence of a short-range endemic shrub in a highly fragmented landscape of south-western Australia.

Australian Journal of Botany, *60*(7), 643–649. https://doi.org/10.1071/BT12194

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

Gómez, J. M., & Zamora, R. (1992). Pollination by ants: consequences of the quantitative effects on a mutualistic system. *Oecologia*, *91*(3), 410–418. https://doi.org/10.1007/BF00317631

Gómez, J. M., Zamora, R., Hódar, J. A., & García, D. (1996). Experimental study of pollination by ants in Mediterranean high mountain and arid habitats. *Oecologia*, *105*(2), 236–242. https://doi.org/10.1007/BF00328552

González-Varo, J. P., Albaladejo, R. G., Aparicio, A., & Arroyo, J. (2010). Linking genetic diversity, mating patterns and progeny performance in fragmented populations of a Mediterranean shrub. *Journal of Applied Ecology*, *47*(6), 1242–1252. https://doi.org/10.1111/j.1365-2664.2010.01879.x

González-Varo, J. P., Arroyo, J., & Aparicio, A. (2009). Effects of fragmentation on pollinator assemblage, pollen limitation and seed production of Mediterranean myrtle (*Myrtus communis*). *Biological Conservation*, *142*(5), 1058–1065. https://doi.org/10.1016/j.biocon.2009.01.017

González-Varo, J. P., Nora, S., & Aparicio, A. (2012). Bottlenecks for plant recruitment in woodland remnants: An ornithochorous shrub in a Mediterranean “relictual” landscape. *Perspectives in Plant Ecology, Evolution and Systematics*, *14*(2), 111–122. https://doi.org/10.1016/j.ppees.2011.11.002

González-Varo, J. P., & Vilà, M. (2017). Spillover of managed honeybees from mass-flowering crops into natural habitats. *Biological Conservation*, *212*(June), 376–382. https://doi.org/10.1016/j.biocon.2017.06.018

Gonzalez, A., Rayfield, B., & Lindo, Z. (2011). The disentangled bank: How loss of habitat fragments and disassembles ecological networks. *American Journal of Botany*, *98*, 503–516. https://doi.org/10.3732/ajb.1000424

González, A. V., Gómez-Silva, V., Ramírez, M. J., & Fontúrbel, F. E. (2019). Meta-analysis of the differential effects of habitat fragmentation and degradation on plant genetic diversity. *Conservation Biology*, *00*(0), 1–9. https://doi.org/10.1111/cobi.13422

Gosper, C. R., Coates, D. J., Hopper, S. D., Byrne, M., & Yates, C. J. (2020). The role of landscape history in the distribution and conservation of threatened flora in the Southwest Australian Floristic Region. *Biological Journal of the Linnean Society*. https://doi.org/10.1093/biolinnean/blaa141

Goulson, D. (2003). Effects of introduced bees on native ecosystems. *Annual Review of*

Ecology, Evolution, and Systematics, *34*(1), 1–26. https://doi.org/10.1146/132355

Goulson, D., Lye, G. C., & Darvill, B. (2008). Decline and conservation of bumble bees. *Annual Review of Entomology*, *53*, 191–208. https://doi.org/10.1146/annurev.ento.53.103106.093454

Gove, A. D., Majer, J. D., & Dunn, R. R. (2007). A keystone ant species promotes seed dispersal in a "diffuse" mutualism. *Oecologia*, *153*(3), 687–697. https://doi.org/10.1007/s00442-007-0756-5

Greenleaf, S. S., Williams, N. M., Winfree, R., & Kremen, C. (2007). Bee foraging ranges and their relationship to body size. *Oecologia*, *153*(3), 589–596. https://doi.org/10.1007/s00442-007-0752-9

Gribel, R., Gibbs, P. E., Queiroz, A. L. (1999). Flowering phenology and pollination biology of *Ceiba pentandra* (Bombacaceae) in Central Amazonia. *Journal of Tropical Ecology 15*(3), 247–263. https://doi.org/10.1017/S0266467499000796

Grimm, N. B., Faeth, S. H., Golubiewski, N. E., Redman, C. L., Wu, J., Bai, X., & Briggs, J. M. (2008). Global change and the ecology of cities. *Science*, *319*(5864), 756–760. https://doi.org/10.1126/science.1150195

Groom, P. K., & Lamont, B. B. (2015). Evolution and diversity of the southwestern Australian flora. In *Plant life of southwestern Australia* (Issue May, pp. 6–29). DeGruyter Open.

Haddad, N. M., Brudvig, L. A., Clobert, J., Davies, K. F., Gonzalez, A., Holt, R. D., Lovejoy, T. E., Sexton, J. O., Austin, M. P., Collins, C. D., Cook, W. M., Damschen, E. I., Ewers, R. M., Foster, B. L., Jenkins, C. N., King, A. J., Laurance, W. F., Levey, D. J., Margules, C. R., … Townshend, J. R. (2015). Habitat fragmentation and its lasting impact on Earth's ecosystems. *Science Advances*, *1*(2), e1500052. https://doi.org/10.1126/sciadv.1500052

Hammer, Ø., Harper, D., & Ryan, P. (2001). PAST: Paleontological statistics software package for education and data analysis. *Paleontologia Electronica*, *22*, 1–9.

Hamrick, J. (2004). Response of forest trees to global environmental changes. *Forest Ecology and Management*, *197*(1–3), 323–335. https://doi.org/10.1016/j.foreco.2004.05.023

Hamrick, J. L., & Godt, M. J. W. (1989). Allozyme diversity in plant species. *Plant Population Genetics, Breeding and Genetic Resources*, 43–64.

Hanski, I. (1994). A practical model of metapopulation dynamics. *Journal of Animal Ecology*, *63*, 151–162. https://doi.org/https://doi.org/10.2307/5591

Hardy, O J, Maggia, L., Bandou, E., Breynne, P., Caron, H., Chevallier, M., Doligez, A., Dutech, C., Kramer, A., Latouche-Hallé, C., Troispoux, V., Veron, V., & Degen, B.

(2006). Fine-scale genetic structure and gene dispersal inferences in 10 Neotropical tree species. *Molecular Ecology*, *15*(2), 559–571. https://doi.org/https://doi.org/10.1111/j.1365-294X.2005.02785.x

Hardy, Olivier J, & Vekemans, X. (2002). SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*, *2*(4), 618–620. https://doi.org/10.1046/j.1471-8286.2002.00305.x

He, T., Krauss, S., & Lamont, B. B. (2007). Polymorphic microsatellite DNA markers for *Banksia attenuata* (Proteaceae). *Molecular Ecology Notes*, *6*, 1329–1331. https://doi.org/10.1111/j.1471-8286.2007.01871.x

Hegland, S. J., & Totland, Ø. (2005). Relationships between species' floral traits and pollinator visitation in a temperate grassland. *Oecologia*. https://doi.org/10.1007/s00442-005-0165-6

Heinrich, B., & Raven, P. H. (1972). Energetics and pollination ecology. *Science*, *176*(4035), 597–602. https://doi.org/10.1126/science.176.4035.597

Hendry, A. P., & Day, T. (2005). Population structure attributable to reproductive time: Isolation by time and adaptation by time. *Molecular Ecology*, *14*, 901–916. https://doi.org/10.1111/j.1365-294X.2005.02480.x

Herlihy, C. R., & Eckert, C. G. (2002). Genetic cost of reproductive assurance in a self-fertilizing plant. *Nature*, *416*(6878), 320–323. https://doi.org/10.1038/416320a

Herrera, C. M. (1987). Components of pollinator "quality": Comparative analysis of a diverse insect assemblage. *Oikos*, *50*, 79–90. https://doi.org/10.2307/3565403

Herrera, C. M. (1989). Pollinator abundance, morphology, and flower visitation rate: analysis of the "quantity" component in a plant-pollinator system. *Oecologia*, *80*, 241–248. https://doi.org/10.1007/BF00380158

Herrera, C. M. (2009). *Multiplicity in unity : plant subindividual variation and interactions with animals*. University of Chicago Press.

Herrerías-Diego, Y., Quesada, M., Stoner, K. E., & Lobo, J. A. (2006). Effects of forest fragmentation on phenological patterns and reproductive success of the tropical dry forest tree *Ceiba aesculifolia*. *Conservation Biology*, *20*(4), 1111–1120. https://doi.org/10.1111/j.1523-1739.2006.00370.x

Heterick, B. E. (2009). A guide to the ants of south-western Australia. Records of the Western Australian Museum Supplement 76. In M. Harvey & P. Doughty (Eds.), *Records of the Western Australian Museum, Supplement* (Vol. 76, Issue 1). Western Australian Museum. https://doi.org/10.18195/issn.0313-122x.76.2009.007-206

Heterick, B. E., Lythe, M., & Smithyman, C. (2013). Urbanisation factors impacting on ant

(Hymenoptera: Formicidae) biodiversity in the Perth metropolitan area, Western Australia: Two case studies. *Urban Ecosystems*, *16*(2), 145–173. https://doi.org/10.1007/s11252-012-0257-0

Heywood, V. H., & Iriondo, J. M. (2003). Plant conservation: old problems, new perspectives. *Biological Conservation*, *113*, 321–335. https://doi.org/10.1016/S0006-3207(03)00121-6

Hickman, J. C. (1974). Pollination by ants: A low-energy system. *Science*, *184*(4143), 1290–1292. https://doi.org/10.1126/science.184.4143.1290

Hobbs, R. J., Arico, S., Aronson, J., Baron, J. S., Bridgewater, P., Cramer, V. A., Epstein, P. R., Ewel, J. J., Klink, C. A., Lugo, A. E., Norton, D., Ojima, D., Richardson, D. M., Sanderson, E. W., Valladares, F., Vilà, M., Zamora, R., & Zobel, M. (2006). Novel ecosystems: theoretical and management aspects of the new ecological world order. *Global Ecology and Biogeography*, *15*(1), 1–7. https://doi.org/10.1111/j.1466-822X.2006.00212.x

Hobbs, R. J., & Yates, C. J. (2003). Impacts of ecosystem fragmentation on plant populations: Generalising the idiosyncratic. *Australian Journal of Botany*, *51*(5), 471–488. https://doi.org/10.1071/BT03037

Holderegger, R., & Di Giulio, M. (2010). The genetic effects of roads: A review of empirical evidence. *Basic and Applied Ecology*, *11*, 522–531. https://doi.org/10.1016/j.baae.2010.06.006

Hölldobler, B., & Wilson, E. O. (1990). The ants. *Cambridge: Belknap Press of Harvard University*.

Holm, E. (1978). Some unusual pollination mechanisms in Western Australian wildflowers. *Western Australian Naturalist*, *14*(3), 60–62.

Holmes, G. D., James, E. A., & Hoffmann, A. A. (2008). Limitations to reproductive output and genetic rescue in populations of the rare shrub *Grevillea repens* (Proteaceae). *Annals of Botany*, *102*(6), 1031–1041. https://doi.org/10.1093/aob/mcn195

Honnay, O., Adriaens, D., Coart, E., Jacquemyn, H., & Roldan-Ruiz, I. (2007). Genetic diversity within and between remnant populations of the endangered calcareous grassland plant *Globularia bisnagarica* L. *Conservation Genetics*, *8*(2), 293–303. https://doi.org/10.1007/s10592-006-9169-y

Honnay, O., & Jacquemyn, H. (2007). Susceptibility of common and rare plant species to the genetic consequences of habitat fragmentation. *Conservation Biology*, *21*(3), 823–831. https://doi.org/10.1111/j.1523-1739.2006.00646.x

Hopper, S. D. (1979). Biogeographical aspects of speciation in the southwest Australian

Flora. *Annual Review of Ecology and Systematics*, *10*, 399–422. https://doi.org/10.1146/annurev.es.10.110179.002151

Hopper, S. D. (2009). OCBIL theory: Towards an integrated understanding of the evolution, ecology and conservation of biodiversity on old, climatically buffered, infertile landscapes. *Plant and Soil*, *322*(1), 49–86. https://doi.org/10.1007/s11104-009-0068-0

Hopper, S. D., & Gioia, P. (2004). The Southwest Australian Floristic Region : Evolution and conservation of a global hot spot of biodiversity. *Annual Review of Ecology, Evolution, and Systematics*, *35*, 623–650.

Houston, T. F. (1989). *Leioproctus* bees associated with Western Australian smoke bushes (*Conospermum* spp.) and their adaptations for foraging and concealment (Hymenoptera: Colletidae: Paracolletini.). *Records of the Western Australian Museum*, *14*(3), 275–292.

Houston, T. F. (2018). *A guide to native bees of Australia* (J. Window (ed.)). CSIRO Publishing.

Hughes, A. R., Inouye, B. D., Johnson, M. T. J., Underwood, N., & Vellend, M. (2008). Ecological consequences of genetic diversity. *Ecology Letters*, *11*(6), 609–623. https://doi.org/10.1111/j.1461-0248.2008.01179.x

Hull, D. A., & Beattie, A. J. (1988). Adverse effects on pollen exposed to *Atta texana* and other North American ants: implications for ant pollination. *Oecologia*, *75*(1), 153–155. https://doi.org/10.1007/BF00378829

Jakobsson, M., & Rosenberg, N. A. (2007). CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics*, *23*(14), 1801–1806. https://doi.org/10.1093/bioinformatics/btm233

Johnson, M. T. J., & Munshi-South, J. (2017). Evolution of life in urban environments. *Science*, *358*(6363). https://doi.org/10.1126/science.aam8327

Jombart, T. (2008). *adegenet*: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, *24*(11), 1403–1405. https://doi.org/10.1093/bioinformatics/btn129

Jones, A. G., & Ardren, W. R. (2003). Methods of parentage analysis in natural populations. *Molecular Ecology*, *12*(10), 2511–2523. https://doi.org/10.1046/j.1365-294X.2003.01928.x

Jones, A. G., Small, C. M., Paczolt, K. A., & Ratterman, N. L. (2010). A practical guide to methods of parentage analysis. *Molecular Ecology Resources*, *10*, 6–30. https://doi.org/10.1111/j.1755-0998.2009.02778.x

Jost, L. (2008). G_{ST} and its relatives do not measure differentiation. *Molecular Ecology*, *17*(18), 4015–4026. https://doi.org/10.1111/j.1365-294X.2008.03887.x

Jost, L., Archer, F., Flanagan, S., Gaggiotti, O., Hoban, S., & Latch, E. (2018). Differentiation measures for conservation genetics. *Evolutionary Applications*, *11*(7), 1139–1148. https://doi.org/10.1111/eva.12590

Junker, R., Chung, A. Y. C., & Blüthgen, N. (2007). Interaction between flowers, ants and pollinators: additional evidence for floral repellence against ants. *Ecological Research*, *22*(4), 665–670. https://doi.org/10.1007/s11284-006-0306-3

Kalinowski, S. T. (2005). HP-RARE 1.0: A computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes*, *5*(1), 187–189. https://doi.org/10.1111/j.1471-8286.2004.00845.x

Kalinowski, S. T., Taper, M. L., & Marshall, T. C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, *16*(5), 1099–1106. https://doi.org/10.1111/j.1365-294X.2007.03089.x

Kawagoe, T., Suzuki, N. (2005). Self-pollen on a stigma interferes with outcrossed seed production in a self-incompatible monoecious plant, *Akebia quinata* (Lardizabalaceae). *Functional Ecology, 19*(1), 49–54. https://doi.org/10.1111/j.0269-8463.2005.00950.x

Kearns, C. A., & Inouye, D. W. (1993). *Techniques for pollination biologists*. University Press of Colorado.

Keighery, G. J., & Keighery, B. J. (2016). *Banksia* woodlands: A restoration guide for the Swan Coastal Plain. In J. C. Stevens, D. P. Rokich, V. J. Newton, R. L. Barrett, & K. W. Dixon (Eds.), *Banksia woodlands: A restoration guide for the Swan Coastal Plain* (pp. 7–30). UWA Publishing.

Kelobonye, K., Xia, J. C., Swapan, M. S. H., McCarney, G., & Zhou, H. (2019). Drivers of change in urban growth patterns: A transport perspective from Perth, Western Australia. *Urban Science*, *3*(2), 1–16. https://doi.org/10.3390/urbansci3020040

Knight, T. M., Steets, J. A., Vamosi, J. C., Mazer, S. J., Burd, M., Campbell, D. R., Dudash, M. R., Johnston, M. O., Mitchell, R. J., & Ashman, T. L. (2005). Pollen limitation of plant reproduction: Pattern and process. *Annual Review of Ecology, Evolution, and Systematics*, *36*, 467–497. https://doi.org/10.1146/annurev.ecolsys.36.102403.115320

Kramer, A. T., Ison, J. L., Ashley, M. V, & Howe, H. F. (2008). The paradox of forest fragmentation genetics. *Conservation Biology*, *22*(4), 878–885. https://doi.org/10.1111/j.1523-1739.2008.00944.x

Kudo, G., & Harder, L. D. (2005). Floral and inflorescence effects on variation in pollen removal and seed production among six legume species. *Functional Ecology*, *19*, 245–254. https://doi.org/10.1111/j.1365-2435.2005.00961.x

Kuriakose, G., Sinu, P. A., & Shivanna, K. R. (2018). Ant pollination of *Syzygium occidentale*, an endemic tree species of tropical rain forests of the Western Ghats, India. *Arthropod-Plant Interactions*, *12*, 647–655. https://doi.org/10.1007/s11829-018-9613-1

Lambers, H. (2014). *Plant life on the sandplains in Southwest Australia: a global biodiversity hotspot*. UWA Publishing. https://uwap.uwa.edu.au/products/plant-life-on-the-sandplains-in-southwest-australia-a-global-biodiversity-hotspot

Lamont, B. B., Enright, N. J., & He, T. (2011). Fitness and evolution of resprouters in relation to fire. *Plant Ecology*, *212*(12), 1945–1957. https://doi.org/10.1007/s11258-011-9982-3

Larson, B. M. H., & Barrett, S. C. H. (2000). A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnean Society*, *69*(4), 503–520. https://doi.org/10.1111/j.1095-8312.2000.tb01221.x

Lázaro, A., Fuster, F., Alomar, D., & Totland, Ø. (2020). Disentangling direct and indirect effects of habitat fragmentation on wild plants' pollinator visits and seed production. *Ecological Applications*, eap.2099. https://doi.org/10.1002/eap.2099

Legendre, P., & Legendre, L. (1998). *Numerical Ecology* (3rd ed.). Elsevier.

Lengyel, S., Gove, A. D., Latimer, A. M., Majer, J. D., & Dunn, R. R. (2010). Convergent evolution of seed dispersal by ants, and phylogeny and biogeography in flowering plants: A global survey. *Perspectives in Plant Ecology, Evolution and Systematics*, *12*(1), 43–55. https://doi.org/10.1016/j.ppees.2009.08.001

Lennartsson, T. (2002). Extinction thresholds and disrupted plant-pollinator interactions in fragmented plant populations. *Ecology*, *83*(11), 3060–3072.

Les, D. H., Reinartz, J. A., & Esselman, E. J. (1991). Genetic consequences of rarity in *Aster furcatus* (Asteraceae), a threatened, self-incompatible plant. *Evolution*, *45*(7), 1641–1650. https://doi.org/10.2307/2409785

Lesica, P., & Allendorf, F. W. (1995). When are peripheral valuable populations for conservation? *Conservation Biology*, *9*(4), 753–760. https://doi.org/10.1046/j.1523-1739.1995.09040753.x

Letten, A. D., & Midgley, J. J. (2009). Rodent pollination in the Cape legume *Liparia parva*. *Austral Ecology*, *34*, 233–236. https://doi.org/10.1111/j.1442-9993.2008.01925.x

Levin, D. A., Francisco-Ortega, J., & Jansen, R. K. (1996). Hybridization and the extinction of rare plantspecies. *Conservation Biology*, *10*(1), 10–16. http://www.jstor.org/stable/2386938

Lienert, J. (2004). Habitat fragmentation effects of fitness of plant populations - A review. *Journal for Nature Conservation*, *12*(1), 53–72.

https://doi.org/10.1016/j.jnc.2003.07.002

Lieth, H. (1974). *Phenology and Seasonality Modeling* (Vol. 8). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-51863-8

Llorens, T. M., Ayre, D. J., & Whelan, R. J. (2004). Evidence for ancient genetic subdivision among recently fragmented populations of the endangered shrub *Grevillea caleyi* (Proteaceae). *Heredity*, *92*(6), 519–526. https://doi.org/10.1038/sj.hdy.6800444

Llorens, T. M., Byrne, M., Yates, C. J., Nistelberger, H. M., & Coates, D. J. (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*(2), 314–328. https://doi.org/10.1111/j.1365-294X.2011.05396.x

Llorens, T. M., Ayre, D. J., & Whelan, R. J. (2018). Anthropogenic fragmentation may not alter pre-existing patterns of genetic diversity and differentiation in perennial shrubs. *Molecular Ecology*, *27*(7), 1541–1555. https://doi.org/10.1111/mec.14552

Loiselle, B. A., Sork, V. L., Nason, J., & Graham, C. (1995). Spatial genetic structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). *American Journal of Botany*, *82*(11), 1420–1425. https://doi.org/https://doi.org/10.1002/j.1537-2197.1995.tb12679.x

López-Cortegano, E., Pérez-Figueroa, A., & Caballero, A. (2019). metapop2: Re-implementation of software for the analysis and management of subdivided populations using gene and allelic diversity. *Molecular Ecology Resources*, *19*(4), 1095–1100. https://doi.org/10.1111/1755-0998.13015

López-Cortegano, E., Pouso, R., Labrador, A., Pérez-Figueroa, A., Fernández, J., & Caballero, A. (2019). Optimal management of genetic diversity in subdivided populations. *Frontiers in Genetics*, *10*(September), 1–10. https://doi.org/10.3389/fgene.2019.00843

Lowe, A. J., Cavers, S., Boshier, D., Breed, M. F., & Hollingsworth, P. M. (2015). The resilience of forest fragmentation genetics—no longer a paradox—we were just looking in the wrong place. *Heredity*, *115*(2), 97–99. https://doi.org/10.1038/hdy.2015.40

Lowe, A. J., Harris, S., & Ashton, P. (2009). *Ecological genetics: Design, analysis, and application*. Wiley-Blackwell.

Luna, P., Anjos, D., García-Chávez, J. H., & Dáttilo, W. (2018). Exploring the vegetation: Seed harvester ants climb and remove seeds from a giant cactus in a semiarid environment. *Journal of Arid Environments*, *156*(April), 106–109. https://doi.org/10.1016/j.jaridenv.2018.05.002

Magalhães, V. B., Espírito Santo, N. B., Salles, L. F. P., Soares, H., & Oliveira, P. S. (2018).

Secondary seed dispersal by ants in Neotropical cerrado savanna: species-specific effects on seeds and seedlings of *Siparuna guianensis* (Siparunaceae). *Ecological Entomology*, *43*(5), 665–674. https://doi.org/10.1111/een.12640

Magrach, A., González-Varo, J. P., Boiffier, M., Vilà, M., & Bartomeus, I. (2017). Honeybee spillover reshuffles pollinator diets and affects plant reproductive success. *Nature Ecology & Evolution*, *1*(9), 1299–1307. https://doi.org/10.1038/s41559-017-0249-9

Mallet, J. (2007). Hybrid speciation. *Nature*, *446*(7133), 279–283. https://doi.org/10.1038/nature05706

Marshall, T. C., Slate, J., Kruuk, L. E. B., & Pemberton, J. M. (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, *7*, 639–655.

McArthur, W. M., & Bettenay, E. (1974). The development and distribution of the soils of the Swan Coastal Plain, Western Australia. In *Soil Publication*. CSIRO Publishing.

Mcloughlin, S. (2001). The breakup history of Gondwana and its impact on pre-Cenozoic floristic provincialism. *Australian Journal of Botany*, *49*(3), 271–300.

Meglécz, E., Pech, N., Gilles, A., Dubut, V., Hingamp, P., Trilles, A., Grenier, R., & Martin, J. F. (2014). QDD version 3.1: A user-friendly computer program for microsatellite selection and primer design revisited: Experimental validation of variables determining genotyping success rate. *Molecular Ecology Resources*, *14*(6), 1302–1313. https://doi.org/10.1111/1755-0998.12271

Miles, L. S., Rivkin, L. R., Johnson, M. T. J., Munshi-South, J., & Verrelli, B. C. (2019). Gene flow and genetic drift in urban environments. *Molecular Ecology*, *28*(18), 4138–4151. https://doi.org/10.1111/mec.15221

Miller, K. G., Mountain, G. S., Wright, J. D., & Browning, J. V. (2011). Sea level and ice volume variations from continental margin and deep-sea isotopic records. *Oceanography*, *24*(2), 40–53. https://doi.org/10.5670/oceanog.2011.26

Mimura, M., Barbour, R. C., Potts, B. M., Vaillancourt, R. E., & Watanabe, K. N. (2009). Comparison of contemporary mating patterns in continuous and fragmented *Eucalyptus globulus* native forests. *Molecular Ecology*, *18*, 4180–4192. https://doi.org/10.1111/j.1365-294X.2009.04350.x

Mitchell, R. J., Karron, J. D., Holmquist, K. G., & Bell, J. M. (2004). The influence of *Mimulus ringens* floral display size on pollinator visitation patterns. *Functional Ecology*, *18*(1), 116–124. https://doi.org/10.1111/j.1365-2435.2004.00812.x

Mittermeier, R. A., Larsen, F. W., Turner, W. R., & Larsen, F. W. (2011). Global biodiversity conservation: the critical role of hotspots. In F. E. Zachos & J. C. Habel

(Eds.), *Biodiversity Hotspots* (pp. 3–22). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-20992-5

Mittermeier, R. A., Robles-Gil, P., Hoffmann, M., Pilgrim, J., Brooks, T., Mittermeier, C. G., Lamoreux, J., & da Fonseca, G. A. B. (2004). Hotspots revisited: Earth's biologically richest and most endangered ecoregions. *CEMEX*, *14*(1), 2–10. https://doi.org/10.2744/ccab-14-01-2-10.1

Moilanen, A., & Nieminen, M. (2002). Simple connectivity measure in spatial ecology. *Ecology*, *83*(4), 1131–1145. https://doi.org/10.1890/0012-9658(2002)083[1131:SCMISE]2.0.CO;2

Morellato, L. P. C., Alberton, B., Alvarado, S. T., Borges, B., Buisson, E., Camargo, M. G. G., Cancian, L. F., Carstensen, D. W., Escobar, D. F. E., Leite, P. T. P., Mendoza, I., Rocha, N. M. W. B., Soares, N. C., Silva, T. S. F., Staggemeier, V. G., Streher, A. S., Vargas, B. C., & Peres, C. A. (2016). Linking plant phenology to conservation biology. *Biological Conservation*, *195*, 60–72. https://doi.org/10.1016/j.biocon.2015.12.033

Morgan, M. T., & Wilson, W. G. (2005). Self-fertilization and the escape from pollen limitation in variable pollination environments. *Evolution*, *59*(5), 1143–1148. https://doi.org/10.1111/j.0014-3820.2005.tb01050.x

Morris, W. F. (1993). Predicting the consequences of plant spacing and biased movement for pollen dispersal by honey bees. *Ecology*, *74*, 493–500. https://doi.org/10.2307/1939310

Morrison, D. A., McDonald, M., Bankoff, P., & Quirico, P. (1994). Reproductive isolation mechanism among four closely-related species of *Conospermum* (Proteaceae). *Botanical Journal of the Linnean Society*, *116*, 13–31. https://doi.org/https://doi.org/10.1006/bojl.1994.1050

Mucina, L., & Wardell-Johnson, G. W. (2011). Landscape age and soil fertility, climatic stability, and fire regime predictability: Beyond the OCBIL framework. *Plant and Soil*, *341*(1–2), 1–23. https://doi.org/10.1007/s11104-011-0734-x

Murray, K., & Conner, M. M. (2009). Methods to quantify variable importance: Implications for the analysis of noisy ecological data. *Ecology*, *90*(2), 348–355. https://doi.org/10.1890/07-1929.1

Myers, N., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. B., & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, *403*(6772), 853–858. https://doi.org/10.1038/35002501

Nei, M. (1977). *F*-statistics and analysis of gene diversity in subdivided populations. *Annals of Human Genetics*, *41*, 225–233. https://doi.org/10.1111/j.1469-1809.1977.tb01918.x

Newman, B. J., Ladd, P., Brundrett, M., & Dixon, K. W. (2013). Effects of habitat

fragmentation on plant reproductive success and population viability at the landscape and habitat scale. *Biological Conservation*, *159*, 16–23. https://doi.org/10.1016/j.biocon.2012.10.009

Oksanen, A. J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., Hara, R. B. O., Simpson, G. L., Solymos, P., Stevens, M. H. H., & Wagner, H. (2019). 'vegan' Package. In *Https://Cran.R-Project.Org*.

Oliver, I., & Beattie, A. J. (1996). Designing a cost-effective invertebrate survey: a test of methods for rapid assessment of biodiversity. *Ecological Applications*, *6*(2), 594–607.

Ollerton, J, Johnson, S. D., & Hingston, A. B. (2006). Geographical variation in diversity and specificity of pollination. In *Plant-pollinator interactions: from specialization to Generalizationeneralization*. https://doi.org/10.1016/j.ophtha.2006.06.003

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, *120*(3), 321–326. https://doi.org/10.1111/j.1600-0706.2010.18644.x

Orians, G. H., & Milewski, A. V. (2007). Ecology of Australia: The effects of nutrient-poor soils and intense fires. *Biological Reviews*, *82*(3), 393–423. https://doi.org/10.1111/j.1469-185X.2007.00017.x

Paton, D. C. (2000). Disruption of bird-plant pollination systems in southern Australia. *Conservation Biology*, *14*(5), 1232–1234.

Peakall, R., & Beattie, A. J. (1989). Pollination of the orchid *Microtis parviflora* R. Br. by flightless worker ants. *British Ecological Society*, *3*(5), 515–522. https://doi.org/10.2307/2389565

Peakall, R., & Beattie, A. J. (1991). The genetic consequences of worker ant pollination in a self-compatible, clonal orchid. *Evolution*, *45*(8), 1837–1848. https://doi.org/10.2307/2409835

Peakall, R., & Smouse, P. E. (2012). GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, *28*(19), 2537–2539. https://doi.org/10.1093/bioinformatics/bts460

Phillips, R. D., Hopper, S. D., & Dixon, K. W. (2010). Pollination ecology and the possible impacts of environmental change in the Southwest Australian Biodiversity Hotspot. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1539), 517–528. https://doi.org/10.1098/rstb.2009.0238

Pickup, M., & Young, A. G. (2008). Population size, self-incompatibility and genetic rescue in diploid and tetraploid races of *Rutidosis leptorrhynchoides* (Asteraceae). *Heredity*, *100*(3), 268–274. https://doi.org/10.1038/sj.hdy.6801070

Playford, P. E., Cockbain, A. E., & Low, G. H. (1976). Geology of the Perth basin Western

Australia. *Geological Survey of Western Australia Bulletin*, *124*.

Pontin, J., & Buckley, R. C. (1982). Ant-plant interactions in Australia. In *The Journal of Ecology* (Vol. 71, Issue 3). https://doi.org/10.2307/2259608

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology and Evolution*, *25*(6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007

Potts, S. G., Imperatriz-Fonseca, V., Ngo, H. T., Aizen, M. A., Biesmeijer, J. C., Breeze, T. D., Dicks, L. V., Garibaldi, L. A., Hill, R., Settele, J., & Vanbergen, A. J. (2016). Safeguarding pollinators and their values to human well-being. *Nature*, *540*(7632), 220–229. https://doi.org/10.1038/nature20588

Poulsen, M., Bot, A. N. M., Nielsen, M. G., & Boomsma, J. J. (2002). Experimental evidence for the costs and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants. *Behavioral Ecology and Sociobiology*, *52*(2), 151–157. https://doi.org/10.1007/s00265-002-0489-8

Prentis, P. J., White, E. M., Radford, I. J., Lowe, A. J., & Clarke, A. R. (2007). Can hybridization cause local extinction: A case for demographic swamping of the Australian native *Senecio pinnatifolius* by the invasive *Senecio madagascariensis*? *New Phytologist*, *176*(4), 902–912. https://doi.org/10.1111/j.1469-8137.2007.02217.x

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945–959. https://doi.org/10.1111/j.1471-8286.2007.01758.x

R Development Core Team. (2018). R Software. In *R: A Language and Environment for Statistical Computing* (3.5.2). R Foundation for Statistical Computing. https://www.r-project.org/

R Development Core Team. (2020). R Software. In *R: A Language and Environment for Statistical Computing* (4.0.3). R Foundation for Statistical Computing. https://www.r-project.org/

Real, L. (2017). *Ecological genetics*. Princeton University Press.

Reed, D. H., & Frankham, R. (2003). Correlation between fitness and genetic diversity. *Conservation Biology*, *17*(1), 230–237. https://doi.org/10.1046/j.1523-1739.2003.01236.x

Rhymer, J. M. ., & Simberloff, D. (1996). Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics*, *27*, 83–109.

Rico-Gray, V., & Oliveira, P. S. (2007). The ecology and evolution of ant-plant interactions. *Chicago: The University of Chicago Press.*

https://doi.org/10.7208/chicago/9780226713540.001.0001

Ritchie, A. L., Dyer, R. J., Nevill, P. G., Sinclair, E. A., & Krauss, S. L. (2019). Wide outcrossing provides functional connectivity for new and old *Banksia* populations within a fragmented landscape. *Oecologia*. https://doi.org/10.1007/s00442-019-04387-z

Robledo-Arnuncio, J. J., Klein, E. K., Muller-Landau, H. C., & Santamaría, L. (2014). Space, time and complexity in plant dispersal ecology. *Movement Ecology*, *2*(1), 1–17. https://doi.org/10.1186/s40462-014-0016-3

Roche, S., Dixon, K. W., & Pate, J. S. (1997). Seed ageing and smoke: Partner cues in the amelioration of seed dormancy in selected Australian native species. *Australian Journal of Botany*, *45*, 783–815. https://doi.org/10.1071/BT96099

Rodrìguez-Pérez, J., & Traveset, A. (2016). Effects of flowering phenology and synchrony on the reproductive success of a long-flowering shrub. *AoB Plants*, *8*, 1–15. https://doi.org/10.1093/aobpla/plw007

Rostás, M., Bollmann, F., Saville, D., & Riedel, M. (2018). Ants contribute to pollination but not to reproduction in a rare calcareous grassland forb. *PeerJ*, *6*, e4369. https://doi.org/10.7717/peerj.4369

Rousset, F. (2008). GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, *8*(1), 103–106. https://doi.org/10.1111/j.1471-8286.2007.01931.x

Sala, O. E., Stuart Chapin III, F., Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L. F., Jackson, R. B., Kinzig, A., Leemans, R., Lodge, D. M., Mooney, H. A., Oesterheld, M., Poff, L. N., Sykes, M. T., Walker, B. H., Walker, M., & Wall, D. H. (2000). Global biodiversity scenarios for the year 2100. *Science*, *287*, 1770–1774. https://doi.org/10.1126/science.287.5459.1770

Sánchez-Bayo, F., & Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, *232*(September 2018), 8–27. https://doi.org/10.1016/j.biocon.2019.01.020

Sanderson, E. W., Jaiteh, M., Levy, M. a., Redford, K. H., Wannebo, A. V., & Woolmer, G. (2002). The human footprint and the last of the wild. *BioScience*, *52*(10), 891–904. https://doi.org/10.1641/0006-3568(2002)052[0891:THFATL]2.0.CO;2

Saunders, D. A., & De Rebeira, C. P. (1991). Values of corridors to avian populations in a fragmented landscape. In D. A. Sounders & R. J. Hobbs (Eds.), *Nature conservation 2: the role of corridors* (pp. 221–240). Surrey Beatty & Sons, Chipping Norton.

Schwartz, M. W., & Hurst, P. S. (1997). Effects of introduced honey bees on Australia's native bee fauna. *The Victorian Naturalist*, *114*, 7–12.

Sinclair, E., Cheetham, B., Krauss, S., & Hobbs, R. (2008). Morphological and molecular variation in *Conospermum triplinervium* (Proteaceae), the tree smokebush: Implications for bushland restoration. *Australian Journal of Botany*, *56*(5), 451–460. https://doi.org/10.1071/BT07137

Stanley, R. G. (1971). Pollen chemistry and tube Growth. In *Pollen - Development and physiology.* Heslop-Harrison, J. (ed.). Butterworth-Heinemann. https://doi.org/10.1016/b978-0-408-70149-5.50019-1

Stone, L. M., Seaton, K. A., Kuo, J., & McComb, J. A. (2004). Fast pollen tube growth in *Conospermum* species. *Annals of Botany*, *93*(4), 369–378. https://doi.org/10.1093/aob/mch050

Stone, L. M., Seaton, K. A., Byrne, M., & Mccomb, J. A. (2006). A study of the reproductive biology of blue-flowered *Conospermum* species (Proteaceae). *Australian Journal of Botany*, *54*(6), 543–551. https://doi.org/10.1071/BT05125

Stow, A., & Beattie, A. (2008). Chemical and genetic defenses against disease in insect societies. *Brain, Behavior, and Immunity*, *22*(7), 1009–1013. https://doi.org/10.1016/j.bbi.2008.03.008

Suetsugu, K., Shitara, T., & Yamawo, A. (2017). Seed dispersal by ants in the fully mycoheterotrophic plant *Sciaphila secundiflora* (Triuridaceae). *Journal of Asia-Pacific Entomology*, *20*(3), 914–917. https://doi.org/10.1016/j.aspen.2017.06.011

Sugiura, N., Miyazaki, S., & Nagaishi, S. (2006). A supplementary contribution of ants in the pollination of an orchid, *Epipactis thunbergii*, usually pollinated by hover flies. *Plant Systematics and Evolution*, *258*, 17–26. https://doi.org/10.1007/s00606-005-0391-8

Tamaki, I., Ishida, K., Setsuko, S., & Tomaru, N. (2009). Interpopulation variation in mating system and late-stage inbreeding depression in *Magnolia stellata*. *Molecular Ecology*, *18*, 2365–2374. https://doi.org/10.1111/j.1365-294X.2009.04195.x

Thomann, M., Imbert, E., Devaux, C., & Cheptou, P. O. (2013). Flowering plants under global pollinator decline. *Trends in Plant Science*, *18*(7), 353–359. https://doi.org/10.1016/j.tplants.2013.04.002

Tieu, A., Dixon, K. W., Meney, K. A., & Sivasithamparam, K. (2007). Interaction of soil burial and smoke on germination patterns in seeds of selected Australian native plants. *Seed Science Research*, *11*(01), 69–76. https://doi.org/10.1079/SSR200061

Todesco, M., Pascual, M. A., Owens, G. L., Ostevik, K. L., Moyers, B. T., Hübner, S., Heredia, S. M., Hahn, M. A., Caseys, C., Bock, D. G., & Rieseberg, L. H. (2016). Hybridization and extinction. *Evolutionary Applications*, *9*(7), 892–908. https://doi.org/10.1111/eva.12367

Van Der Kroft, T., Roberts, D. G., & Krauss, S. L. (2019). The critical role of honeyeaters in the pollination of the catspaw *Anigozanthos humilis* (Haemodoraceae). *Australian Journal of Botany*, *67*(4), 281–289. https://doi.org/10.1071/BT18209

Vaughton, G., & Carthew, S. M. (1993). Evidence for selective fruit abortion in *Banksia spinulosa* (Proteaceae). *Biological Journal of the Linnean Society*. https://doi.org/10.1111/j.1095-8312.1993.tb00917.x

Vekemans, X., & Hardy, O. J. (2004). New insights from fine-scale spatial genetic structure analyses in plant populations. *Molecular Ecology*, *13*(4), 921–935. https://doi.org/10.1046/j.1365-294X.2004.02076.x

Via, S., & Lande, R. (1985). Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution*, *39*(3), 505–522. https://doi.org/10.1111/j.1558-5646.1985.tb00391.x

Vranckx, G., Jacquemyn, H., Muys, B., & Honnay, O. (2012). Meta-Analysis of susceptibility of woody plants to loss of genetic diversity through habitat fragmentation. *Conservation Biology*, *26*(2), 228–237. https://doi.org/10.1111/j.1523-1739.2011.01778.x

Vuillaume, B., Valette, V., Lepais, O., Grandjean, F., & Breuil, M. (2015). Genetic evidence of hybridization between the endangered native species *Iguana delicatissima* and the invasive *Iguana iguana* (Reptilia, Iguanidae) in the Lesser Antilles: Management implications. *PLOS ONE*, *10*(6), e0127575. https://doi.org/10.1371/journal.pone.0127575

Wagenius, S., Lonsdorf, E., & Neuhauser, C. (2007). Patch aging and the S-Allee effect: Breeding system effects on the demographic response of plants to habitat fragmentation. *American Naturalist*, *169*(3), 383–397. https://doi.org/10.1086/511313

Wagenius, S., & Lyon, S. P. (2010). Reproduction of *Echinacea angustifolia* in fragmented prairie is pollen-limited but not pollinator-limited. *Ecology*, *91*(3), 733–742. https://doi.org/10.1890/08-1375.1

Wardell-Johnson, G., Wardell-Johnson, A., Bradby, K., Robinson, T., Bateman, P. W., Williams, K., Keesing, A., Braun, K., Beckerling, J., & Burbridge, M. (2016). Application of a Gondwanan perspective to restore ecological integrity in the south-western Australian global biodiversity hotspot. *Restoration Ecology*, *24*(6), 805–815. https://doi.org/10.1111/rec.12372

Westphal, C., Steffan-Dewenter, I., & Tscharntke, T. (2003). Mass flowering crops enhance pollinator densities at a landscape scale. *Ecology Letters*. https://doi.org/10.1046/j.1461-0248.2003.00523.x

Whelan, R J, Ayre, D. J., England, P. R., Llorens, T., & Beynon, F. (2000). Ecology and genetics of *Grevillea* (Proteaceae): implications for conservation of fragmented populations. In A. G. Young & G. M. Clarke (Eds.), *Genetics, Demography and Viability of Fragmented Populations* (pp. 253–270). Cambridge University Press. https://doi.org/10.1017/CBO9780511623448.019

Whelan, Robert J., & Burbidge, A. H. (1980). Flowering phenology, seed set and bird pollination of five Western Australian *Banksia* species. *Australian Journal of Ecology*, *5*(1), 1–7. https://doi.org/10.1111/j.1442-9993.1980.tb01225.x

Whitlock, M. C. (2011). G'_{ST} and *D* do not replace F_{ST}. *Molecular Ecology*, *20*(6), 1083–1091. https://doi.org/10.1111/j.1365-294X.2010.04996.x

Willi, Y., Buskirk, J. Van, & Fischer, M. (2005). A threefold genetic allee effect: Population size affects cross-compatibility, inbreeding depression and drift load in the self-incompatible *Ranunculus reptans*. *Genetics*, *169*, 2255–2265. https://doi.org/10.1534/genetics.104.034553

Willmer, P. G., Nuttman, C. V., Raine, N. E., Stone, G. N., Pattrick, J. G., Henson, K., Stillman, P., McIlroy, L., Potts, S. G., & Knudsen, J. T. (2009). Floral volatiles controlling ant behaviour. *Functional Ecology*, *23*(5), 888–900. https://doi.org/10.1111/j.1365-2435.2009.01632.x

Winfree, R., Aguilar, R., Vazquez, D., LeBuhn, G., & Aizen, M. A. (2009). A meta-analysis of bees' responses to anthropogenic disturbance. *Ecology*, *90*(8), 2068–2076. https://doi.org/10.1890/14-0661.1

Winfree, R., Bartomeus, I., & Cariveau, D. P. (2011). Native pollinators in anthropogenic habitats. *Annual Review of Ecology, Evolution, and Systematics*, *42*, 1–22. https://doi.org/10.1146/annurev-ecolsys-102710-145042

Wolf, D. E., Takebayashi, N., & Rieseberg, L. H. (2001). Predicting the risk of extinction through hybridization. *Conservation Biology*, *15*(4), 1039–1053. https://doi.org/10.1046/j.1523-1739.2001.0150041039.x

Wright, S. (1951). The genetical structure of populations. *Annals of Eugenics*, *15*, 322–354. https://doi.org/10.1017/CBO9781107415324.004

Wyrwoll, K., Turner, B. L., & Findlater, P. (2014). On the origins , geomorphology and soils of the sandplains of south-western Australia. In H. Lambers (Ed.), *Plant life on the sandplains in southwest Australia, a global biodiversity hotspot* (pp. 3–22). UWA Publishing.

Yates, C. J., Coates, D. J., Elliott, C., & Byrne, M. (2006). Composition of the pollinator community, pollination and the mating system for a shrub in fragments of species rich

kwongan in south-west Western Australia. *Biodiversity and Conservation*, *16*(5), 1379–1395. https://doi.org/10.1007/s10531-006-6736-y

Yates, C. J., Elliott, C., Byrne, M., Coates, D. J., & Fairman, R. (2007). Seed production, germinability and seedling growth for a bird-pollinated shrub in fragments of kwongan in south-west Australia. *Biological Conservation*, *136*(2), 306–314. https://doi.org/10.1016/j.biocon.2006.12.003

Yek, S. H., & Mueller, U. G. (2011). The metapleural gland of ants. *Biological Reviews*, *86*(4), 774–791. https://doi.org/10.1111/j.1469-185X.2010.00170.x

Young, A., Brown, A. H. D., Murphy, B. G., Thrall, P. H., Miller, C. H., & Gosling, M. (2000). Genetic erosion, restricted mating and reduced viability in fragmented populations of the endangered grassland herb *Rutidopsis leptorrhynchoides*. In A. G. Young & G. M. Clarke (Eds.), *Genetics, demography and viability of fragmented populations* (Issue 4, pp. 335–359). Cambridge University Press.

Young, Andrew, Boyle, T., & Brown, T. (1996). The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology & Evolution*, *11*(10), 413–418. https://doi.org/10.1016/0169-5347(96)10045-8

Yun, L., & Agrawal, A. F. (2014). Variation in the strength of inbreeding depression across environments: Effects of stress and density dependence. *Evolution*, *68*(12), 3599–3606. https://doi.org/10.1111/evo.12527

Zachos, J., Pagani, M., Sloan, L., Thomas, E., & Billups, K. (2001). Trends, rhythms, and aberrations in global climate 65 Ma to Present. *Science*, *292*(5517), 686–693. https://doi.org/10.1126/science.1059412

Zalapa, J. E., Brunet, J., & Guries, R. P. (2009). Patterns of hybridization and introgression between invasive *Ulmus pumila* (Ulmaceae) and native *U. Rubra*. *American Journal of Botany*, *96*(6), 1116–1128. https://doi.org/10.3732/ajb.0800334

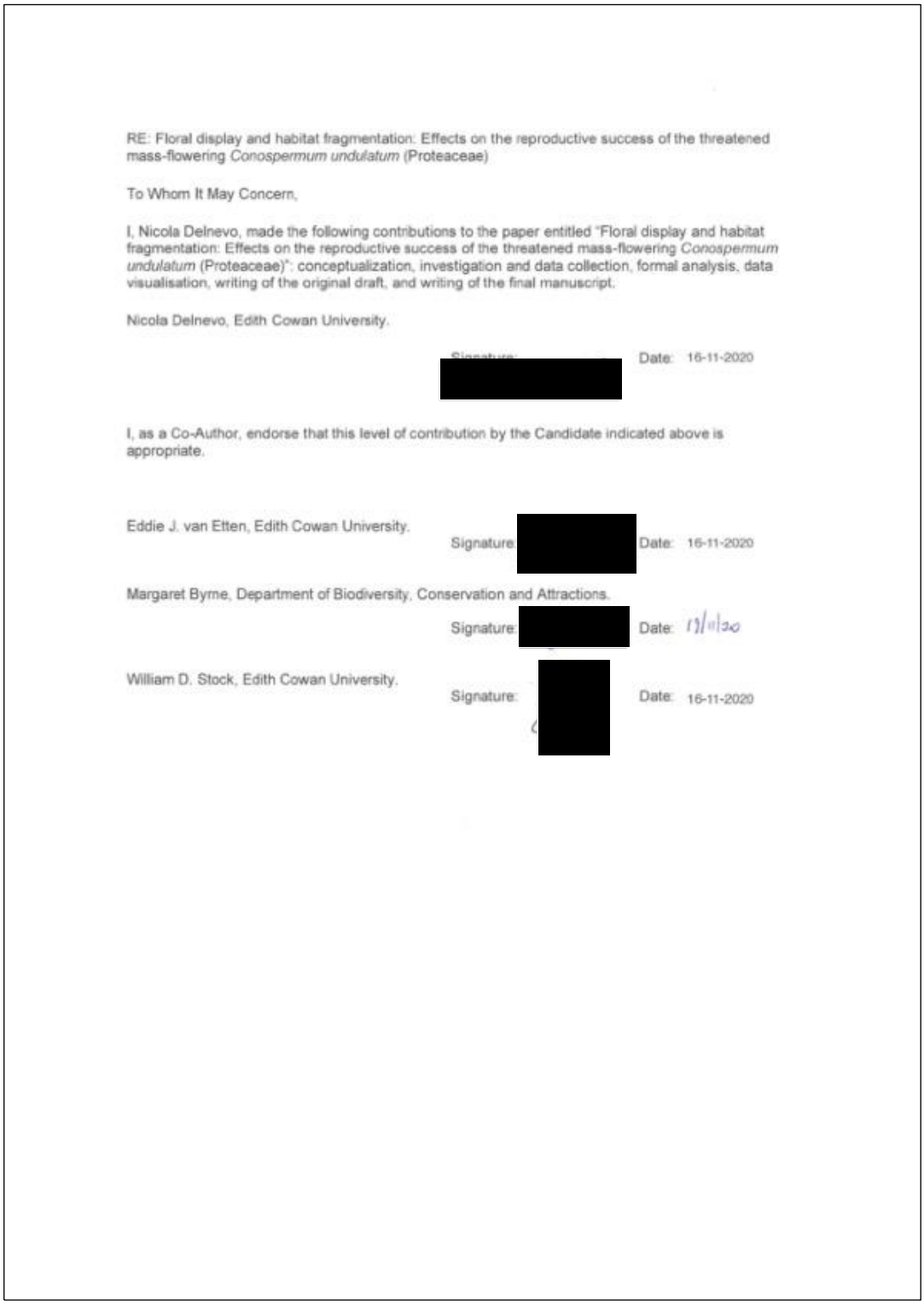
Zimmerman, M., & Pyke, G. H. (1988). Reproduction of *Polemonium*: assessing the factors limiting seed set. *American Naturalist*, *131*, 723–738. https://doi.org/10.1086/284815

Zuur, A. F., Ieno, E. N., Walker, N. J., Savaliev, A. A., & Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R*. Springer US.

Co-author statements

This section contains signed statements from my co-authors attesting to my level of contribution to the published papers in Chapters 2, 3, 4, and Appendices F and G.

Chapter 2.



RE: Floral display and habitat fragmentation: Effects on the reproductive success of the threatened mass-flowering *Conospermum undulatum* (Proteaceae)

To Whom It May Concern,

I, Nicola Delnevo, made the following contributions to the paper entitled "Floral display and habitat fragmentation: Effects on the reproductive success of the threatened mass-flowering *Conospermum undulatum* (Proteaceae)": conceptualization, investigation and data collection, formal analysis, data visualisation, writing of the original draft, and writing of the final manuscript.

Nicola Delnevo, Edith Cowan University.

Signature: [redacted] Date: 16-11-2020

I, as a Co-Author, endorse that this level of contribution by the Candidate indicated above is appropriate.

Eddie J. van Etten, Edith Cowan University. Signature: [redacted] Date: 16-11-2020

Margaret Byrne, Department of Biodiversity, Conservation and Attractions. Signature: [redacted] Date: 17/11/20

William D. Stock, Edith Cowan University. Signature: [redacted] Date: 16-11-2020

Chapter 3.

RE: Habitat fragmentation restricts insect pollinators and pollen quality in a threatened Proteaceae species

To Whom It May Concern,

I, Nicola Delnevo, made the following contributions to the paper entitled "Habitat fragmentation restricts insect pollinators and pollen quality in a threatened Proteaceae species": conceptualization, investigation and data collection, formal analysis, data visualisation, writing of the original draft, and writing of the final manuscript.

Nicola Delnevo, Edith Cowan University.

Signature: [redacted] Date: 16-11-2020

I, as a Co-Author, endorse that this level of contribution by the Candidate indicated above is appropriate.

Eddie J. van Etten, Edith Cowan University. Signature: [redacted] Date: 16-11-2020

Margaret Byrne, Department of Biodiversity, Conservation and Attractions. Signature: [redacted] Date: 17/11/20

Alessandro Petraglia, University of Parma. Signature: [redacted] Date: 16-11-2020

Michele Carbognani, University of Parma. Signature: [redacted] Date: 16-11-2020

William D. Stock, Edith Cowan University. Signature: [redacted] Date: 16-11-2020

Chapter 4.

RE: Pollen adaptation to ant pollination – a case study from the Proteaceae

To Whom It May Concern,

I, Nicola Delnevo, made the following contributions to the paper entitled "Pollen adaptation to ant pollination – a case study from the Proteaceae": conceptualization, investigation and data collection, formal analysis, data visualisation, writing of the original draft, and writing of the final manuscript.

Nicola Delnevo, Edith Cowan University.

Signature: [redacted] Date: 16-11-2020

I, as a Co-Author, endorse that this level of contribution by the Candidate indicated above is appropriate.

Eddie J. van Etten, Edith Cowan University. Signature: [redacted] Date: 16-11-2020

Nicola Clemente, University of Parma. Signature: [redacted] Date: 16-11-2020

Luna Fogu, University of Parma. Signature: [redacted] Date: 16-11-2020

Evelina Pavarani, University of Parma. Signature: [redacted] Date: 16-11-2020

Margaret Byrne, Department of Biodiversity, Conservation and Attractions. Signature: [redacted] Date: 19/11/20

William D. Stock, Edith Cowan University. Signature: [redacted] Date: 16-11-2020

Appendix F.

RE: Isolation, characterization, and cross-amplification of 20 microsatellite markers for *Conospermum undulatum* (Proteaceae)

To Whom It May Concern,

I, Nicola Delnevo, made the following contributions to the paper entitled "Isolation, characterization, and cross-amplification of 20 microsatellite markers for *Conospermum undulatum* (Proteaceae)": conceptualization, investigation and data collection, formal analysis, data visualisation, writing of the original draft, and writing of the final manuscript.

Nicola Delnevo, Edith Cowan University.

Signature: [redacted] Date: 16-11-2020

I, as a Co-Author, endorse that this level of contribution by the Candidate indicated above is appropriate.

Andrea Piotti, Institute of Biosciences and BioResources.

Signature: [redacted] Date: 16-11-2020

Eddie J. van Etten, Edith Cowan University.

Signature: [redacted] Date: 16-11-2020

William D. Stock, Edith Cowan University.

Signature: [redacted] Date: 16-11-2020

Margaret Byrne, Department of Biodiversity, Conservation and Attractions.

Signature: [redacted] Date: 11/11/20

Appendix G.

RE: Tales of the unexpected – ant pollination mutualism

To Whom It May Concern,

I, Nicola Delnevo, made the following contributions to the paper entitled "Tales of the unexpected – ant pollination mutualism": investigation, writing of the original draft, and writing of the final manuscript.

Nicola Delnevo, Edith Cowan University.

Signature: [redacted] Date: 16-11-2020

I, as a Co-Author, endorse that this level of contribution by the Candidate indicated above is appropriate.

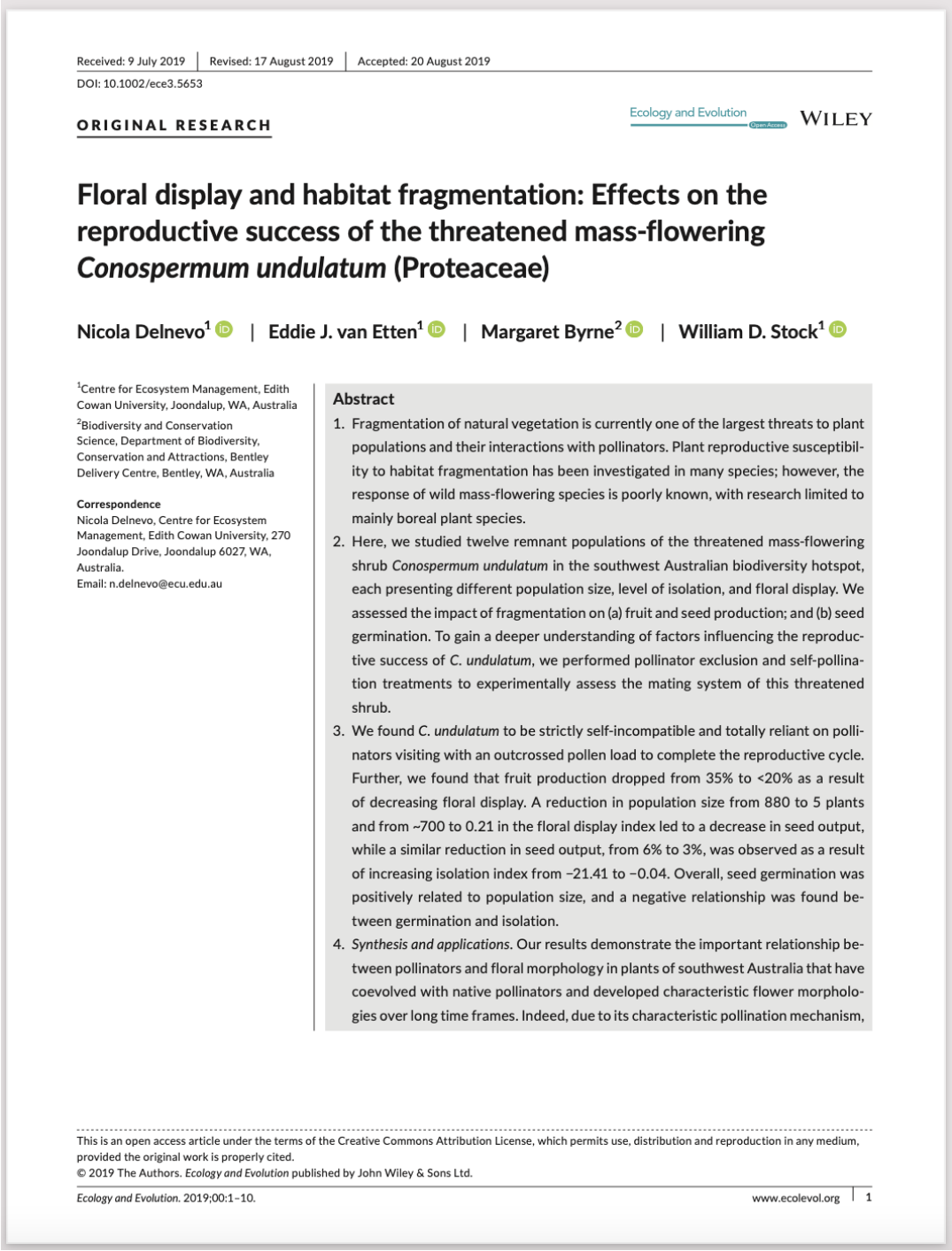
Eddie J. van Etten, Edith Cowan University.

Signature: [redacted] Date: 16-11-2020

Copies of original publications

This section contains the first page of each thesis component that has been published in the peer-reviewed literature and, where necessary, a copy of the licence agreement. Required for that publication to be reused in the thesis.

Chapter 2.



Received: 9 July 2019 | Revised: 17 August 2019 | Accepted: 20 August 2019

DOI: 10.1002/ece3.5653

ORIGINAL RESEARCH

Ecology and Evolution Open Access WILEY

Floral display and habitat fragmentation: Effects on the reproductive success of the threatened mass-flowering *Conospermum undulatum* (Proteaceae)

Nicola Delnevo[1] | Eddie J. van Etten[1] | Margaret Byrne[2] | William D. Stock[1]

[1]Centre for Ecosystem Management, Edith Cowan University, Joondalup, WA, Australia

[2]Biodiversity and Conservation Science, Department of Biodiversity, Conservation and Attractions, Bentley Delivery Centre, Bentley, WA, Australia

Correspondence
Nicola Delnevo, Centre for Ecosystem Management, Edith Cowan University, 270 Joondalup Drive, Joondalup 6027, WA, Australia.
Email: n.delnevo@ecu.edu.au

Abstract

1. Fragmentation of natural vegetation is currently one of the largest threats to plant populations and their interactions with pollinators. Plant reproductive susceptibility to habitat fragmentation has been investigated in many species; however, the response of wild mass-flowering species is poorly known, with research limited to mainly boreal plant species.
2. Here, we studied twelve remnant populations of the threatened mass-flowering shrub *Conospermum undulatum* in the southwest Australian biodiversity hotspot, each presenting different population size, level of isolation, and floral display. We assessed the impact of fragmentation on (a) fruit and seed production; and (b) seed germination. To gain a deeper understanding of factors influencing the reproductive success of *C. undulatum*, we performed pollinator exclusion and self-pollination treatments to experimentally assess the mating system of this threatened shrub.
3. We found *C. undulatum* to be strictly self-incompatible and totally reliant on pollinators visiting with an outcrossed pollen load to complete the reproductive cycle. Further, we found that fruit production dropped from 35% to <20% as a result of decreasing floral display. A reduction in population size from 880 to 5 plants and from ~700 to 0.21 in the floral display index led to a decrease in seed output, while a similar reduction in seed output, from 6% to 3%, was observed as a result of increasing isolation index from −21.41 to −0.04. Overall, seed germination was positively related to population size, and a negative relationship was found between germination and isolation.
4. *Synthesis and applications.* Our results demonstrate the important relationship between pollinators and floral morphology in plants of southwest Australia that have coevolved with native pollinators and developed characteristic flower morphologies over long time frames. Indeed, due to its characteristic pollination mechanism,

No license is required to reproduce this content.

Chapter 3.

Biological Conservation 252 (2020) 108824

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

Policy analysis

Habitat fragmentation restricts insect pollinators and pollen quality in a threatened Proteaceae species

Nicola Delnevo [a,*], Eddie J. van Etten [a], Margaret Byrne [b], Alessandro Petraglia [c], Michele Carbognani [c], William D. Stock [a]

[a] Centre for Ecosystem Management, Edith Cowan University, 270 Joondalup Drive, Joondalup 6027, WA, Australia
[b] Biodiversity and Conservation Science, Department of Biodiversity, Conservation, and Attractions, Bentley Delivery Centre, Locked Bag 104, Bentley, WA 6983, Australia
[c] Department of Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy

ARTICLE INFO

Keywords:
Australia
Biodiversity hotspot
Conospermum
Land use change
Native bee
Pollen limitation
Proteaceae

ABSTRACT

- Disruption of pollination services has been investigated in many species; however, fragmentation-induced effects on endemic plants that rely on species-specific invertebrate pollinators are still unclear. While honeybees can forage over long distances, many native bees with small body size are unable to extend their foraging range, making habitat fragmentation the largest threat to such systems.
- Here, we defined the pollinator assemblage of our target species, *Conospermum undulatum* (Proteaceae), among remnant populations characterized by different degrees of fragmentation. We explored the impact of fragmentation on flower visitation in eleven populations of *C. undulatum*. Lastly, we took advantage of the fragmentation gradient to tease apart the influences of pollen quantity and quality on pollen limitation.
- Small populations showed an impoverished pool of weakly effective pollinators. Flower visitation increased from 1.5% to 74.3% with increasing floral display index, while it increased from 1.3% to 5.6% with increasing connectivity levels of populations. We found evidence of pollen *quantity* limitation across all the populations; however, the overall pollen limitation was greater in small fragments where genetic factors limited the *quality* of pollen.
- Native bees were absent in small isolated remnants. Our study highlights the importance of such pollinators for plants that coevolved with them. The availability of compatible pollen is limited in small remnant populations and native pollinators appeared unable to maintain an adequate inter-population pollen flow in heavily fragmented landscapes. The effects of habitat fragmentation can be exacerbated in small and isolated populations of plants that rely on species-specific pollinators for sexual reproduction.

Chapter 4.

Annals of Botany **126**: 377–386, 2020
doi: 10.1093/aob/mcaa058, available online at www.academic.oup.com/aob

Pollen adaptation to ant pollination: a case study from the Proteaceae

Nicola Delnevo[1,*], Eddie J van Etten[1], Nicola Clemente[2], Luna Fogu[2], Evelina Pavarani[2], Margaret Byrne[3] and William D Stock[1]

[1]Centre for Ecosystem Management, Edith Cowan University, 270 Joondalup Drive, Joondalup 6027, WA, Australia, [2]Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy and [3]Biodiversity and Conservation Science, Department of Biodiversity, Conservation and Attractions, Bentley Delivery Centre, Locked Bag 104, Bentley, WA 6983, Australia
**For correspondence. E-mail n.delnevo@ecu.edu.au*

Received: 20 January 2020 Returned for revision: 6 March 2020 Editorial decision: 25 March 2020 Accepted: 27 March 2020

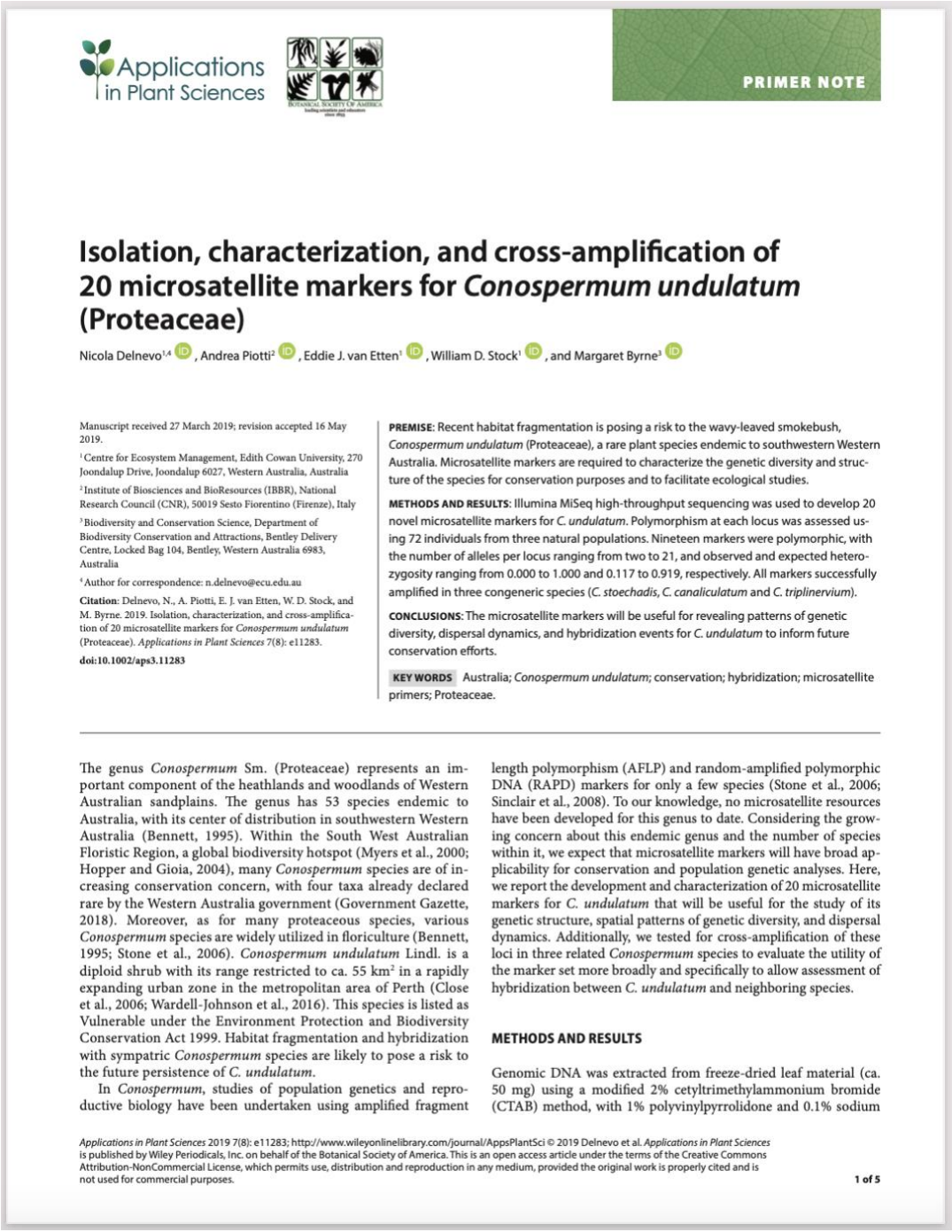
• **Background and Aims** Ant–plant associations are widely diverse and distributed throughout the world, leading to antagonistic and/or mutualistic interactions. Ant pollination is a rare mutualistic association and reports of ants as effective pollinators are limited to a few studies. *Conospermum* (Proteaceae) is an insect-pollinated genus well represented in the south-western Australia biodiversity hotspot, and here we aimed to evaluate the role of ants as pollinators of *C. undulatum*.
• **Methods** Pollen germination after contact with several species of ants and bees was tested for *C. undulatum* and five co-flowering species for comparison. We then sampled the pollen load of floral visitors of *C. undulatum* to assess whether ants carried a pollen load sufficient to enable pollination. Lastly, we performed exclusion treatments to assess the relative effect of flying- and non-flying-invertebrate floral visitors on the reproduction of *C. undulatum*. For this, we measured the seed set under different conditions: ants exclusion, flying-insects exclusion and control.
• **Key Results** Pollen of *C. undulatum*, along with the other *Conospermum* species, had a germination rate after contact with ants of ~80 % which did not differ from the effect of bees; in contrast, the other plant species tested showed a drop in the germination rate to ~10 % following ant treatments. Although ants were generalist visitors, they carried a pollen load with 68–86 % of suitable grains. Moreover, ants significantly contributed to the seed set of *C. undulatum*.
• **Conclusions** Our study highlights the complexity of ant–flower interactions and suggests that generalizations neglecting the importance of ants as pollinators cannot be made. *Conospermum undulatum* has evolved pollen with resistance to the negative effect of ant secretions on pollen grains, with ants providing effective pollination services to this threatened species.

Key words: Australia, ant–plant interaction, biodiversity hotspot, *Conospermum undulatum*, cuticular antimicrobial secretions, entomophily, floral fidelity, Hymenoptera, myrmecophily, mutualism, pollen germination.

INTRODUCTION

Mutualistic plant–animal interactions are a common ecological process with almost 90 % of wild flowering plant species relying on animals for gamete dispersal and, ultimately, fruit and seed production (Ollerton *et al.*, 2011). Most animals involved in such interactions are insects, and they account for the pollination of ~88 % of all animal-pollinated plants (Potts *et al.*, 2010; Thomann *et al.*, 2013). Among the insect-pollinated plants, pollination by ants appears to be poorly represented (de Vega and Gómez, 2014; Kuriakose *et al.*, 2018; Rostás *et al.*, 2018; Del-Claro *et al.*, 2019), whereas bees and other close relatives are recognized as important pollinators worldwide (Potts *et al.*, 2016). Moreover, interactions between ants and flowers are generally assumed to be antagonistic. This large discrepancy between the recognized roles of bees and ants has been attributed to peculiar characteristics of ants, such as their small size (being generally smaller than the reproductive structures of flowers), their aggressive behaviour that may deter other flower visitors, and their grooming, or self-cleaning, behaviour (Galen, 1983; Junker *et al.*, 2007). Ants are also known to produce an antimicrobial secretion from their metapleural gland, which has been shown to have a negative effect on the viability of pollen (Beattie *et al.*, 1985). This trait may have contributed to differences in pollination efficacy among the major hymenopteran lineages (i.e. the 'antibiotic hypothesis'; Beattie *et al.*, 1984, 1985). The primary function of this cuticular secretion is very likely antiseptic (Poulsen *et al.*, 2002; Stow and Beattie, 2008; Yek and Mueller, 2011), with ants spreading antibiotic secretions diffusely through the nest to prevent fungal growth and infections (Hölldobler and Wilson, 1990). Possibly, this is the reason why ant pollination appears to be mainly limited to dry, or sometimes cold, environments (Dutton and Frederickson, 2012); indeed, bacteria and fungi are likely to impose stronger selection on ants for antimicrobial defences in warm, humid tropical rainforests than in deserts and Mediterranean-type habitats. Nonetheless, ant pollination may be an advantageous system with a low energetic cost, and could

Appendix F.



Applications in Plant Sciences

PRIMER NOTE

Isolation, characterization, and cross-amplification of 20 microsatellite markers for *Conospermum undulatum* (Proteaceae)

Nicola Delnevo[1,4], Andrea Piotti[2], Eddie J. van Etten[1], William D. Stock[1], and Margaret Byrne[3]

Manuscript received 27 March 2019; revision accepted 16 May 2019.

[1] Centre for Ecosystem Management, Edith Cowan University, 270 Joondalup Drive, Joondalup 6027, Western Australia, Australia

[2] Institute of Biosciences and BioResources (IBBR), National Research Council (CNR), 50019 Sesto Fiorentino (Firenze), Italy

[3] Biodiversity and Conservation Science, Department of Biodiversity Conservation and Attractions, Bentley Delivery Centre, Locked Bag 104, Bentley, Western Australia 6983, Australia

[4] Author for correspondence: n.delnevo@ecu.edu.au

Citation: Delnevo, N., A. Piotti, E. J. van Etten, W. D. Stock, and M. Byrne. 2019. Isolation, characterization, and cross-amplification of 20 microsatellite markers for *Conospermum undulatum* (Proteaceae). *Applications in Plant Sciences* 7(8): e11283.

doi:10.1002/aps3.11283

PREMISE: Recent habitat fragmentation is posing a risk to the wavy-leaved smokebush, *Conospermum undulatum* (Proteaceae), a rare plant species endemic to southwestern Western Australia. Microsatellite markers are required to characterize the genetic diversity and structure of the species for conservation purposes and to facilitate ecological studies.

METHODS AND RESULTS: Illumina MiSeq high-throughput sequencing was used to develop 20 novel microsatellite markers for *C. undulatum*. Polymorphism at each locus was assessed using 72 individuals from three natural populations. Nineteen markers were polymorphic, with the number of alleles per locus ranging from two to 21, and observed and expected heterozygosity ranging from 0.000 to 1.000 and 0.117 to 0.919, respectively. All markers successfully amplified in three congeneric species (*C. stoechadis*, *C. canaliculatum* and *C. triplinervium*).

CONCLUSIONS: The microsatellite markers will be useful for revealing patterns of genetic diversity, dispersal dynamics, and hybridization events for *C. undulatum* to inform future conservation efforts.

KEY WORDS Australia; *Conospermum undulatum*; conservation; hybridization; microsatellite primers; Proteaceae.

The genus *Conospermum* Sm. (Proteaceae) represents an important component of the heathlands and woodlands of Western Australian sandplains. The genus has 53 species endemic to Australia, with its center of distribution in southwestern Western Australia (Bennett, 1995). Within the South West Australian Floristic Region, a global biodiversity hotspot (Myers et al., 2000; Hopper and Gioia, 2004), many *Conospermum* species are of increasing conservation concern, with four taxa already declared rare by the Western Australia government (Government Gazette, 2018). Moreover, as for many proteaceous species, various *Conospermum* species are widely utilized in floriculture (Bennett, 1995; Stone et al., 2006). *Conospermum undulatum* Lindl. is a diploid shrub with its range restricted to ca. 55 km^2 in a rapidly expanding urban zone in the metropolitan area of Perth (Close et al., 2006; Wardell-Johnson et al., 2016). This species is listed as Vulnerable under the Environment Protection and Biodiversity Conservation Act 1999. Habitat fragmentation and hybridization with sympatric *Conospermum* species are likely to pose a risk to the future persistence of *C. undulatum*.

In *Conospermum*, studies of population genetics and reproductive biology have been undertaken using amplified fragment length polymorphism (AFLP) and random-amplified polymorphic DNA (RAPD) markers for only a few species (Stone et al., 2006; Sinclair et al., 2008). To our knowledge, no microsatellite resources have been developed for this genus to date. Considering the growing concern about this endemic genus and the number of species within it, we expect that microsatellite markers will have broad applicability for conservation and population genetic analyses. Here, we report the development and characterization of 20 microsatellite markers for *C. undulatum* that will be useful for the study of its genetic structure, spatial patterns of genetic diversity, and dispersal dynamics. Additionally, we tested for cross-amplification of these loci in three related *Conospermum* species to evaluate the utility of the marker set more broadly and specifically to allow assessment of hybridization between *C. undulatum* and neighboring species.

METHODS AND RESULTS

Genomic DNA was extracted from freeze-dried leaf material (ca. 50 mg) using a modified 2% cetyltrimethylammonium bromide (CTAB) method, with 1% polyvinylpyrrolidone and 0.1% sodium

No license is required to reproduce this content.

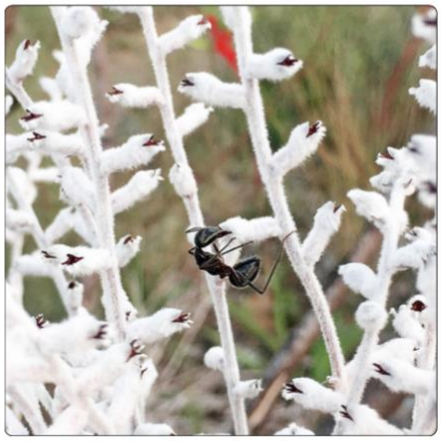
Appendix G.

FrontiersEcoPics

Tales of the unexpected – ant pollination mutualism

South-western Australia constitutes a biodiversity hotspot where plants and animals have coevolved over long time frames, resulting in exceptionally specialized interactions. The shrub *Conospermum undulatum*, also known as smokebush because it presents white, woolly flowers covered in white hairs that resemble drifting smoke when seen en masse, has developed a pollination mutualism with the native ant *Camponotus molossus*. Pollination by ants is extremely rare, with only about 40 convincing examples observed to date. Insects visiting smokebush flowers for nectar trigger the stigma, which flicks away from the anthers toward the lower tepals (outer part of flowers with no differentiation between petals and sepals) to make contact with the insect; simultaneously, the fertile anthers cast new pollen onto the visitor. The stigma strikes with such a force that most dipterans visiting the flowers remain fatally trapped, making these generalist pollinators ineffective.

The hymenopteran *C molossus* is an exception; this ant is large enough to escape such a fate and also facilitates pollination by feeding on smokebush. While the cuticular secretions of many ants are known to drastically reduce the pollen viability of most plants, laboratory experiments assessing the effect of ant secretions on pollen viability showed that smokebush pollen is resistant to these secretions. Moreover, mutualistic services by ants are important for maximizing the seed output of this endangered plant, which is threatened by urbanization across its limited distribution. Is the pollen tolerance to ant secretions present in other phylogenetically related taxa? Where and when did this trait evolve? Thousands of plant and animal species and their interactions have been described in this global biodiversity hotspot, but how many more are still to be discovered?



Nicola Delnevo and Eddie J van Etten
Centre for Ecosystem Management, Edith Cowan University, Joondalup, Australia
doi:10.1002/fee.2135