

**Ecology and conservation genetics of the rare and
threatened hammer orchid *Drakaea elastica***

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Abstract

Highly specific ecological interactions can pose significant conservation challenges, as practitioners require an intimate knowledge of all species involved. Orchids are frequently dependent on both specialised pollinator and mycorrhizal interactions. When combined with their tendency to exist in naturally patchy populations they may be particularly susceptible to disturbance. The rare and threatened orchid, *Drakaea elastica* is endemic to southwestern Australia and is the focus of ongoing conservation programs. As a consequence of extensive land clearing across its range the majority of known plants are restricted to two regions. *Drakaea elastica* is pollinated by male *Zaspilothynnus gilesi* thynnine wasps that it lures to the flower by sexual deception. The goal of this study was to investigate the habitat requirements, pollination ecology and population genetic structure of *D. elastica* to inform *in situ* conservation and guide future reintroduction and habitat restoration programs.

A major limitation to plant recruitment is the availability of suitable microsites for germination and growth. The structural microhabitat requirements of adult *D. elastica* plants were investigated by comparing occupied microsites between regions and occupied to random microsites within regions. Although there were significant regional differences in median values for some microhabitat variables, the differences between occupied and random microsites were similar for both regions. This analysis revealed that *D. elastica* requires open microsites with patches of bare sand, sparse understorey and moderate canopy cover. The observed regional differences in microhabitat variables may be a result of differing groundwater tables. The requirement for open sandy microsites with low litter and shrub cover may be linked to the competitive performance of the mycorrhizal endophyte, or due to the detrimental effects of shading of adult plants.

Pollinator experiments, floral odour analysis and gas chromatography coupled with electroantennographic detection (GC/MS–EAD), unexpectedly revealed evidence for the existence of two chemotypes, each attracting a specific variant of the pollinator. Microsatellite markers revealed low levels of population genetic differentiation (F_{ST}); and an absence of isolation-by-distance across the species range. However, low but significant regional genetic differentiation was detected, with evidence for two partially-overlapping genetic clusters, indicating some admixture. Spatial autocorrelation

analysis showed significant patterns of fine-scale positive spatial genetic structure. Additionally, *D. elastica* exhibits small scale clonality. These results indicate that, at least historically, populations of *D. elastica* were connected by gene flow, most likely through long distance seed dispersal events. Despite the low levels of population genetic differentiation, the significant regional differentiation tentatively supports the unexpected pollinator and chemistry findings.

The potential consequence of pollinator mate-searching behaviour for pollen movement in *Drakaea* was investigated using a capture-mark-recapture study the rare *Z. gilesi* and common *Z. nigripes* thynnine wasps. Median movement distances were not significantly different between the species but recapture rates were considerably lower in *Z. nigripes* compared to *Z. gilesi*. Both species utilised the same food plants, thus this factor is unlikely to be responsible for differences in abundance. Differences in recapture rate between the two species may arise due to variation in the spatial distribution of the respective wingless females. As the majority of movements for both species were less than 100 m, it is predicted that pollen movement will largely be restricted to within populations of *Drakaea*, with few movements between populations in continuous habitats.

The findings of this study raise some important conservation implications. For example, *D. elastica* has very specific microhabitat preferences for an open understory with patches of bare sand. This raises concern about its high vulnerability to weed invasion. Minimising weed invasion may therefore be one of the most important conservation management strategies for this species. The unexpected discovery of multiple entities within both *D. elastica*, and the pollinator *Z. gilesi* suggests that conservation programs designed to protect this plant-pollinator interaction could be dealing with up to four cryptic species. In all likelihood existing populations may contain mixtures of these taxa. As a consequence, given that so few populations remain, it will be critical to conserve all existing populations. This discovery of unexpected complexity is consistent with emerging evidence in other sexually deceptive orchid genera, perhaps indicating that cryptic entities will be far more common in other orchid groups than presently considered.

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List of Appendices

While not directly forming part of the thesis, the appended publications arose from collaborations during my candidature and relate to my general research themes.

Appendix 1. Menz, M.H.M. & Arlettaz, R. (2012) The precipitous decline of the ortolan bunting: time to build on scientific evidence to inform conservation management. *Oryx*, **46**, 122-129. doi:10.1017/S0030605311000032

Appendix 2. Menz, M.H.M., Dixon, K.W. & Hobbs, R.J. (2013) Hurdles and opportunities for landscape-scale restoration. *Science*, **339**, 526-527. doi: 10.1126/science.1228334

Appendix 3. Menz, M.H.M., Phillips, R.D., Winfree, R., Kremen, C., Aizen, M.A., Johnson, S.D. & Dixon, K.W. (2011) Reconnecting plants and pollinators: challenges in the restoration of pollination mutualisms. *Trends in Plant Science*, **16**, 4-12. doi:10.1016/j.tplants.2010.09.006

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Foreword

This thesis is presented as a series of stand-alone manuscripts prepared for publication, with the exception of Chapters 1 and 5. Chapter specific appendices are presented at the end of each chapter. A single reference list is provided at the end of the thesis.

Declaration of Candidate Contribution

Chapters 2 to 4 are co-authored manuscripts of which I was the primary author. These are presented in a format as prepared for publication, one of which has been published. The contributions of the co-authors to each chapter are outlined below.

Chapter 2. Menz, M.H.M., Peakall, R. & Dixon, K.W. Microhabitat modelling as a tool for identifying habitat requirements of rare orchids.

The design of this study was developed by MHMM in consultation with RP and KWD. Fieldwork and data analysis was carried out by MHMM. MHMM wrote the chapter with editorial comments provided by RP and KWD.

Chapter 3. Menz, M.H.M., Phillips, R.D., Peakall, R., Dixon, K.W. & Didham, R.K. (2013) Mate-searching behaviour of common and rare thynnine wasps and the implications for pollen movement of the sexually deceptive orchids they pollinate. *PLoS ONE*, **8(3)**, e59111. doi:10.1371/journal.pone.0059111.

The design of this study was developed by MHMM in consultation with RP, KWD and RKD. Fieldwork and data analysis was carried out by MHMM. MHMM wrote the chapter with editorial comments provided by RKD, RP, KWD and RDP.

Chapter 4. Menz, M.H.M., Anthony, J.M., Bohman, B., Dixon, K.W. & Peakall, R. Ecological, genetic and chemical analysis reveals hidden complexity in a rare plant with specific pollinators.

The design of this study was developed by MHMM in consultation with RP and KWD. Fieldwork and sample collection was carried out by MHMM. Genetics laboratory work was carried out by JMA and MHMM. The analytical chemistry laboratory work was carried out by BB and MHMM. Data analysis was carried out by MHMM with advice from RP. MHMM wrote the chapter with editorial comments provided by RP, KWD, BB and JMA.



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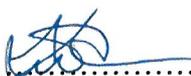
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Chapter 1 – General Introduction

Conservation of rare plants with specialised pollinator interactions

Highly specific plant-pollinator interactions can pose significant conservation and restoration challenges as practitioners need an intimate knowledge of all species involved to maximise conservation outcomes (Kearns *et al.* 1998; Menz *et al.* 2011). There are several key processes limiting the population size and distribution of rare plants. They include environmental factors such as limited availability of suitable microsites (Eriksson and Ehrlén 1992; Fiedler *et al.* 2007); demographic factors such as seed availability (Eriksson and Ehrlén 1992) and pollen limitation (Ackerman and Montalvo 1990); population genetic effects such as inbreeding depression (Ellstrand and Elan 1993); and reproductive limitation by associated organisms such as pollinators (Phillips 2010; Pauw and Bond 2011; Pauw and Hawkins 2011). Recently it has been shown that in species-specific pollination systems, the decline of a pollinator can lead to decreased seed production (Pauw 2007), altered plant density (Anderson *et al.* 2011) and population decline (Pauw and Hawkins 2011) of its dependent plant. Consequently, conservation programs involving specialised ecological interactions such as plant-pollinator relationships need to take a multi-faceted approach when investigating ecological limitations to rare plant populations.

The Orchidaceae is one of the most diverse plant families, estimated to contain at least 28,000 species worldwide (Joppa *et al.* 2011; WCSP 2013). However, globally the family is also characterized by more threatened species and narrow endemics than any other plant family (Cribb *et al.* 2003; Swarts and Dixon 2009). Two key anthropogenic threats to orchids include habitat destruction and over-collection (Cribb *et al.* 2003; Koopowitz *et al.* 2003; Swarts and Dixon 2009). Both pose major challenges for orchid conservation and restoration (Swarts and Dixon 2009; Wright *et al.* 2009; Menz *et al.* 2011). Three common features of orchid biology may also contribute to the high frequency of threatened orchid species worldwide: (1) specialised mycorrhizal interactions, (2) specific pollinator interactions and (3) specific habitat requirements. The combination of all three factors is thought to drive patterns of distribution, abundance and speciation in the Orchidaceae (Cribb *et al.* 2003; Gravendeel *et al.* 2004; Cozzolino and Widmer 2005; Tremblay *et al.* 2005; Schiestl and Schlüter 2009; Swarts and Dixon 2009; Phillips *et al.* 2011a; Phillips *et al.* 2011b). Furthermore, the

interaction between ecological specialisation and natural population patchiness (Phillips *et al.* 2011b) can lead to an increased susceptibility to anthropogenic threats and disturbance in orchids, however, there is evidence that pollinator specificity alone may not lead to extinction risk (Ackerman and Roubik 2012). Some orchids show features that may offer resilience in the event of pollinator decline. For example, Pauw and Bond (2011) and Pauw and Hawkins (2011) showed that high levels of clonality can reduce population decline in the event of the loss of a key pollinator species from remnant habitat. The high level of ecological specificity exhibited by orchids makes them excellent study systems for investigating the potential ecological limitations of specialised plant-pollinator interactions.

In this thesis, I use the rare, sexually deceptive *Drakaea elastica* (Orchidaceae) and its thynnine wasp pollinator *Zaspilothynnus gilesi* (Hymenoptera: Thynnidae) as a case-study for investigating key aspects of the ecology of rare plants with highly specific pollination interactions. While focusing on orchids, in this chapter I briefly review the concepts that underpin the subsequent chapters in the thesis, which includes the potential for suitable microsites as a limiting factor in plant populations, population genetic connectivity and conservation genetics of rare plants, and the implications for pollen movement in plants pollinated by sexual mimicry. Finally, I present the general aims of the thesis.

Microsite limitations

One major limitation to plant recruitment and population resilience is the availability of suitable microsites for germination, development and growth (Eriksson and Ehrlén 1992; Münzbergová and Herben 2005). Furthermore, the availability of suitable microsites can also strongly influence the persistence of plants to maturity. Consequently, understanding microhabitat requirements of a species can provide insight into the ecological factors that control species distributions (Münzbergová and Herben 2005; Moore and Elmendorf 2006). Similarly, the identification of microsite requirements is fundamental for determining the factors that limit populations of rare plants and hence for developing successful habitat conservation programs (Fiedler *et al.* 2007).

Plants may vary in their response to microhabitat characteristics at different stages of development (Facelli *et al.* 1999; Ibáñez and Schupp 2002). For example, most terrestrial orchids require the association of a mycorrhizal endophyte for germination and annual growth (Zettler *et al.* 2003; Rasmussen and Rasmussen 2009). Germination of orchid seed may be more prolific in high organic matter environments, such as areas with large quantities of leaf litter (Batty *et al.* 2001). However, while this may be the case when plants are at the achlorophyllous stage, once plants reach the photosynthetic stage, deprivation of sunlight by shading from heavy litter cover may be detrimental (Fowler 1988; Facelli and Pickett 1991; Willems *et al.* 2001).

Microhabitat characteristics may also affect the reproductive output of plants. Studies on European orchids have shown that increased shading can affect both floral characteristics, such as number of flowers (Shefferson *et al.* 2006) and reproductive output such as fruit or seed set (Jacquemyn *et al.* 2008; Jacquemyn *et al.* 2010a). For example, Jacquemyn *et al.* (2008) demonstrated that flowering and seed set was increased in *Orchis mascula* following the artificial opening of the canopy. Similarly, *O. purpurea* that grew naturally in sites with an open canopy had significantly higher fruit set than those at shaded sites (Jacquemyn *et al.* 2010a). By contrast, natural populations of *Spiranthes spiralis* exhibited increased seed production per fruit in partially-shaded plants compared to those in full sun (Willems *et al.* 2001). Further, microsite variation may affect pollination success. For example, pollination success may vary depending on vegetation structure, spatial overlap with pollinators, and the presence of co-occurring flowering plants (see Tremblay *et al.* 2005). Consequently, microsite characteristics may also affect recruitment and population persistence by affecting seed production.

Population genetic connectivity

The dispersal of pollen and seed enables both genetic and demographic connectivity between plant populations (Ghazoul 2005; Trapnell and Hamrick 2005). An insight into the relative contributions of pollen versus seed flow is best obtained by examining the patterns of population genetic structure across multiple scales (Loveless and Hamrick 1984; Vekemans and Hardy 2004). Consequently, the investigation of population genetic patterns at both the landscape (among populations) and local (within

populations) scale may provide important clues about the processes limiting population connectivity in rare plants.

In orchids, the long-distance dispersal capability of the dust-like seed (Arditti and Ghani 2000) is hypothesised to be the primary driver of the low levels of population genetic diversity typically reported (Phillips *et al.* 2012). However, despite the potential for long-distance seed dispersal (Arditti and Ghani 2000) at a local scale a number of studies have shown that the majority of orchid seed tends to fall within close proximity (<10 m) to the maternal plant (Murren and Ellison 1998; Jacquemyn *et al.* 2007; Jersáková and Malinová 2007). This can lead to strong patterns of local fine-scale positive genetic structure (the non-random distribution of genotypes in space) (Peakall and Beattie 1996; Smouse and Peakall 1999; Trapnell *et al.* 2004; Jacquemyn *et al.* 2009). Restricted pollen dispersal can also generate a similar spatial genetic pattern. Thus, ideally knowledge of pollen movements can greatly assist in disentangling the relative contributions of seed and pollen flow to population connectivity.

Pollinator behaviour directly influences patterns of pollen movement and the rate of outcrossing in plants, which are important for gene flow and seed fitness (Groom 1998; Ghazoul 2005; Trapnell and Hamrick 2005). Patterns of pollen movement will be driven primarily by pollinator behaviour (Ghazoul 2005; Trapnell and Hamrick 2005). Given the incredible diversity of orchid pollinators (Nilsson 1992; van der Cingel 2001; Gaskett 2011), it could be predicted that patterns of pollen movement will vary even within pollination strategies. The majority of flowering plants are pollinated by food-foraging insects, which typically display a pattern of optimal foraging that tends to result in small scale pollen movement and often within an inflorescence or plant (Pyke 1978; Pyke 1981). Dispersal or mate-searching behaviour by pollinators may lead to longer-distance pollen movement than optimal foraging behaviour. Pollination by sexual mimicry (sexual deception), where plants are pollinated by mate-searching male insects (Stoutamire 1975; Peakall 1990), provides an interesting example to test the consequences of mate-searching behaviour.

Pollination by sexual mimicry

Orchids are well known for their widespread exploitation of pollinators, with approximately one third of species being pollinated by deceptive strategies (Cozzolino

and Widmer 2005). Pollination by sexual mimicry of female insects (sexual deception) is one of the most specialised pollination systems known; whereby plants typically exploit only a single pollinator species (Paulus and Gack 1990; Bower 1996; Bower 2006; Bower and Brown 2009; Phillips *et al.* 2009; Peakall *et al.* 2010; Phillips 2010; Gaskett 2011). Sexual deception involves the mimicry of species-specific sex pheromones released by female insects, often coupled with visual and tactile cues, to attract mate-searching males (Schiestl *et al.* 1999; Schiestl *et al.* 2003; Jersáková *et al.* 2006; Peakall *et al.* 2010). Pollination is achieved when male insects are sexually attracted to the flower and often attempt copulation (pseudocopulation) thereby coming into contact with the pollinium and stigma (Stoutamire 1975; Peakall 1990).

Sexual deception is utilised almost exclusively by the Orchidaceae, aside from two recently discovered cases from a South African daisy (*Gorteria diffusa*) (Ellis and Johnson 2010) and a European *Oncocylus* irises (Vereecken *et al.* 2012). While Australia is recognised as one centre of diversity, sexual deception is known from other continents including Europe (*Ophrys*) (Paulus and Gack 1990), South America (*Geoblasta*) (Ciotek *et al.* 2006) and southern Africa (*Disa*) (Steiner *et al.* 1994). Globally, Hymenoptera are the most widely exploited group of pollinators (Gaskett 2011) with one known exception involving the exploitation of Diptera by *Lepanthes* (Blanco and Barboza 2005).

There are at least nine sexually-deceptive orchid genera recognised in Australia, most of which exploit thynnine wasps (Thynnidae) as pollinators (Stoutamire 1974; Stoutamire 1975; Stoutamire 1983; Peakall 1990; Phillips *et al.* 2009; Gaskett 2011; Griffiths *et al.* 2011). These orchids include *Caladenia* (Stoutamire 1975; Stoutamire 1983; Phillips *et al.* 2009), *Chiloglottis* (Bower 1996; Bower 2006; Bower and Brown 2009; Peakall *et al.* 2010), *Drakaea* (Peakall 1990; Phillips 2010), and *Spiculaea* (Alcock 2000). Within Australian orchids, other less widely utilised pollinator families include Ichneumonidae (Gaskett *et al.* 2008; Gaskett 2012), Scoliidae (Fordham 1946; Jones and Gray 1974; Gaskett 2011) and Formicidae (Peakall 1989b). Outside of Australia the most widely utilised group of Hymenoptera are bees (Andrenidae and Anthophoridae), which are used by the European orchid genus *Ophrys* (Paulus and Gack 1990; Peakall and Schiestl 2004).

Plants pollinated by sexual mimicry of female insects offer an interesting opportunity to test the consequences of mate-searching behaviour on pollen movement. Male insects are expected to search in such a way as to increase the chance of encountering unmated females (Alcock *et al.* 1978; Goh and Morse 2010). Given the taxonomic diversity of insects exploited in sexually-deceptive systems, considerable variation in the mate-searching behaviour of the associated insects and subsequent patterns of orchid pollen movement would be expected. For example, thynnine wasps have been reported moving 10's - 100's of metres in search of females (Peakall 1990; Peakall and Beattie 1996; Whitehead and Peakall 2012a; Whitehead and Peakall 2012b). In contrast, the bee *Colletes cunicularius*, which pollinates four species of *Ophrys* (Paulus and Gack 1990), moved on average only 5.2 m in search of mates (Peakall and Schiestl 2004). Similarly, a study on pollen movement in *Leporella fimbriata* showed the majority of pollen movements by the male winged ant *Myrmecia urens* (Formicidae) were less than 2 m, with an overall mean of 3 m (Peakall 1989b). While pollination by sexual mimicry is predicted to promote long-distance pollen movement and increased outcrossing, this may be dependent on the behaviour of the specific pollinator (Peakall and Beattie 1996).

Study system

Drakaea Lindl. (Hammer orchids) (Orchidaceae) is endemic to the South-west Australian Floristic Region (Hopper and Brown 2007), an area of approximately 302,627 km² characterised by a relatively flat landscape with few major topographic features (Hopper and Gioia 2004; Hopper 2009). The genus includes 10 species, with five species listed as Declared Rare Flora under the Western Australian Wildlife Conservation Act (1950), and one species presumed extinct (Hopper and Brown 2007; Phillips 2010).

Drakaea are herbaceous perennial geophytes, resprouting annually from a tuber (Hopper and Brown 2007). Plants produce a single small (approximately 1–3 cm diameter) flat, typically heart-shaped leaf in May (Hopper and Brown 2007) and may form small colonies (approximately 10 cm diameter) via clonal reproduction of daughter tubers (Peakall 1990). As with most terrestrial orchid genera, *Drakaea* are reliant on a mycorrhizal endophyte for germination and growth (Rasmussen and Rasmussen 2009; Phillips *et al.* 2011a). A recent study on *Drakaea* determined that across the five species studied, including those both common and rare, all use a single

monophyletic clade of *Tulasnella* mycorrhiza (Phillips *et al.* 2011a). This analysis concluded that mycorrhizal specificity does not contribute directly to the rarity of *Drakaea* within populations. However, fungal microhabitat specificity may limit *Drakaea* at the landscape scale (Phillips *et al.* 2011a).

Drakaea are single-flowered (Hopper and Brown 2007) and pollinated exclusively by the sexual deception of male thynnine wasps (Stoutamire 1974; Peakall 1990; Phillips 2010). Present evidence suggests that the pollination interaction is highly specific with each *Drakaea* species utilising a specific species of thynnine wasp (Hopper and Brown 2007; Phillips 2010).

Drakaea elastica

Drakaea elastica Lindl. is a rare, winter-active terrestrial orchid, endemic to the Swan Coastal Plain of southwest Western Australia and occur over an area of approximately 300 km between Cataby and Busselton (Figures 1.1, 1.2A) (Brown *et al.* 1998; Hopper and Brown 2007; Department of Environment and Conservation 2009, 2011). *Drakaea elastica* is listed as critically endangered under the Australian Federal Environmental Protection and Biodiversity Conservation Act (EPBC). The species is confined to a small number of remnant populations within a highly fragmented environment with much of the species' habitat being cleared for agriculture and housing (Government of Western Australia 2000; Department of Environment and Conservation 2009). The total population is estimated at less than 5,000 plants (Department of Environment and Conservation 2011). Approximately sixty percent of the locations from where *D. elastica* is known harbour less than 50 leaves (Department of Environment and Conservation 2011). Currently, the majority of remaining plants occur in two regional strongholds approximately 110 km apart, near the towns of Mandurah and Capel (Figure 1.2). Continued land clearing is further threatening the species by reducing and fragmenting suitable habitat (Brown *et al.* 1998; Department of Environment and Conservation 2008). Other threats include grazing of leaves and flowers, competition with weeds, increasing density of ground-level vegetation and inappropriate fire regimes (Department of Environment and Conservation 2008).

Within its remaining strongholds, *D. elastica* occurs on patches of grey sand usually in association with *Kunzea glabrescens* thickets within *Banksia* woodland (*B. menziesii*, *B.*

attenuata, and *B. ilicifolia*) and often in low-lying areas of the landscape near to wetlands and depressions (Hopper and Brown 2007; Department of Environment and Conservation 2009). Population size, as measured by number of leaves, may fluctuate annually and has been correlated to autumn (April-May) rainfall, with greater autumn rainfall associated with higher population counts (Carstairs and Coates 1994).

Biology of thynnine wasps

Drakaea elastica is pollinated via sexual deception of male *Zaspilothynnus gilesi* Turner thynnine wasps (Figure 1.1) (Phillips 2010). Thynnine wasps (Hymenoptera, Thynnidae) represent a diverse, predominantly Australian insect group with an estimated 1500–2000 species; many of which are undescribed (Brown 2009; Griffiths *et al.* 2011). Thynnine wasps are parasites on scarab beetle larvae (Coleoptera: Scarabeidae), which the females locate and parasitise below ground (Burrell 1935; Given 1953; Ridsdill Smith 1970b; Ridsdill Smith 1971). Little is known about the host specificity of these wasps, although studies conducted in eastern Australia have shown that multiple thynnine species may utilise the same host species of scarab beetle (Ridsdill Smith 1970b).

Mating occurs when the apterous females emerge from the ground and are picked up by the alate males. The males then fly with them to a food source and feed *in copula* (Ridsdill Smith 1970a; Alcock 1981). It is proposed the males then drop the females back near the collection site, where they burrow in search of a host (Ridsdill Smith 1970b). Given that females spend most of the time underground, or *in copula*, competition is intense for the few receptive females. This results in a scramble mating system (Alcock *et al.* 1978; Alcock 1981; Alcock 2000).

Thynnine wasps are known to utilise a number of food sources, particularly nectar and other sugar producing sources such as lerps (Phillips *et al.* 2009). Aside from casual field observations, little is known of the foraging preference of these wasps. However, it has been noted that nectar-feeding species in southwestern Australia can be found in large numbers on flowering plants from the families Myrtaceae, Proteaceae and Xanthorrhoeaceae (Phillips *et al.* 2009).

Zaspilothynnus gilesi

Our current knowledge on the biology of *Zaspilothynnus gilesi* is scant and little more than anecdotal field observations exist. The species was described by (Turner 1910) who determined the existence of two additional variants based on morphology, in particular femoral colour and the size and colouration of abdominal spots. *Zaspilothynnus gilesi* is approximately 20–30 mm long. *Zaspilothynnus gilesi* is widespread but patchily distributed from Kalbarri to Busselton in southwestern Australia and is known to occur in a variety of habitats across its range (Figure 1.2B) (M.H.M. Menz & R.D. Phillips unpublished data).

There is no information on the host specificity of *Z. gilesi*. There are few observations of *Z. gilesi* foraging on nectar plants with solitary males and pairs *in copula* observed mainly on open-flowered Myrtaceae, specifically *Astartea fascicularis* (Labill.) DC. and *Chamelaucium uncinatum* Schauer (M.H.M. Menz, R.D. Phillips & J. Hardwick unpublished data) and *Xanthorrhoea* Sm. sp. (Xanthorrhoeaceae) (S.M. Tomlinson pers. comm.).

Thesis objectives

The goal of this thesis was to investigate the structural microhabitat requirements, pollination ecology and population genetic structure of *D. elastica* to inform *in situ* conservation and guide reintroduction and habitat restoration programs.

Firstly, the structural microhabitat requirements of adult *D. elastica* were investigated by comparing microhabitat characteristics between occupied and random microsites across the species range. This helped to identify characteristics that best explained the occurrence of *D. elastica*.

Secondly, the mate-searching movement patterns of *Z. gilesi* were compared with the common sympatric *Z. nigripes* using a capture-mark-recapture (CMR) study. This study allowed the first investigation of interspecific differences in mate-searching behaviour between two of thynnine wasp species. Further, this study also offered insights into the possible patterns of pollinator mediated pollen movement within and among populations of *Drakaea*.

Thirdly, a regional-scale pollinator-baiting experiment was conducted. This was coupled with laboratory-based gas chromatography/mass spectrometry–electroantennographic detection (GC/MS–EAD) to test for differences in the pollinator attracted by different *D. elastica* flowers. This was combined with a population genetic study to investigate the patterns of population genetic structuring of *D. elastica* across multiple scales, spanning the landscape (km), within populations (m) and within colonies (cm).

Finally, these ecological and genetic findings were placed in the context of the conservation of *D. elastica*.

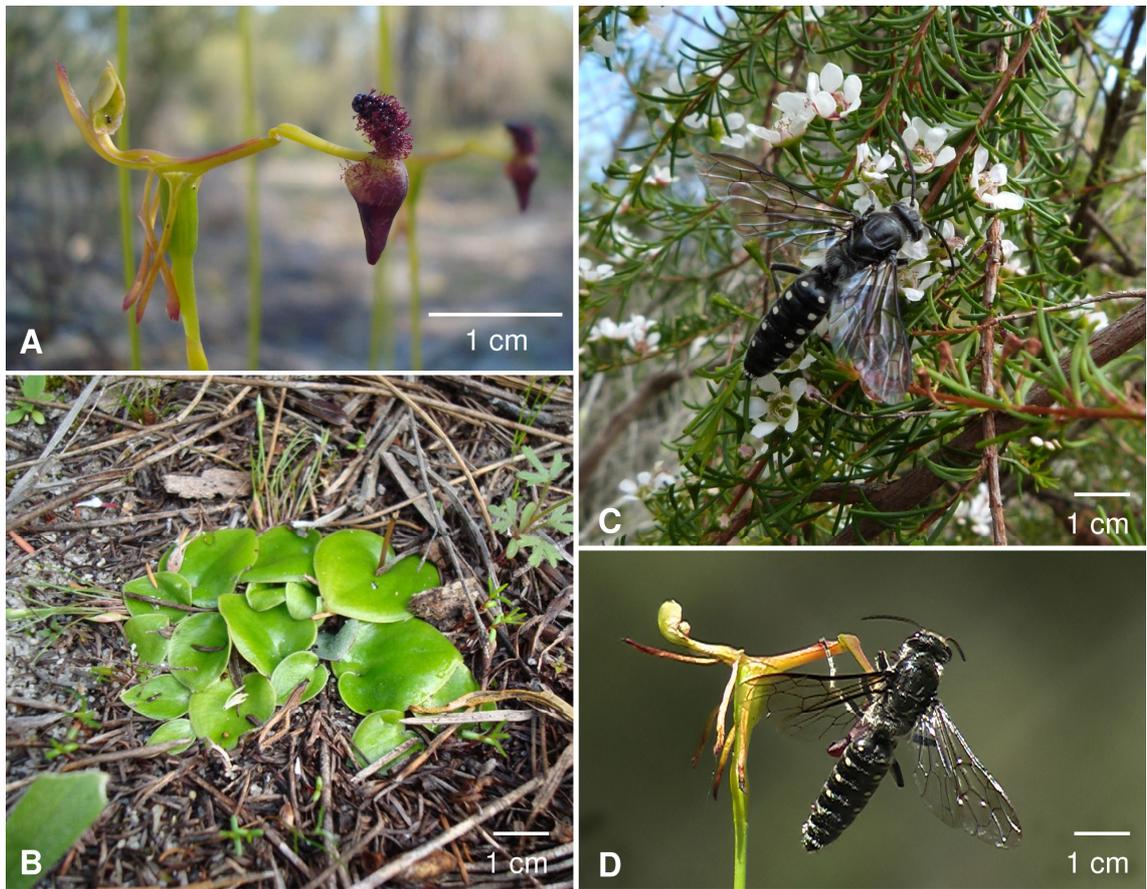


Figure 1.1. *Drakaea elastica* flower (A) and leaves (B) clustered in a small colony. The grey-green leaf in the centre of the colony is *D. glyptodon*. Male *Zaspilothynnus gilesi* feeding on *Astartea fascicularis* (C) and attempting to copulate with a *D. elastica* flower (D). Photographs: (A) and (B) M.H.M. Menz; (C) J. Hardwick; (D) R. Peakall.

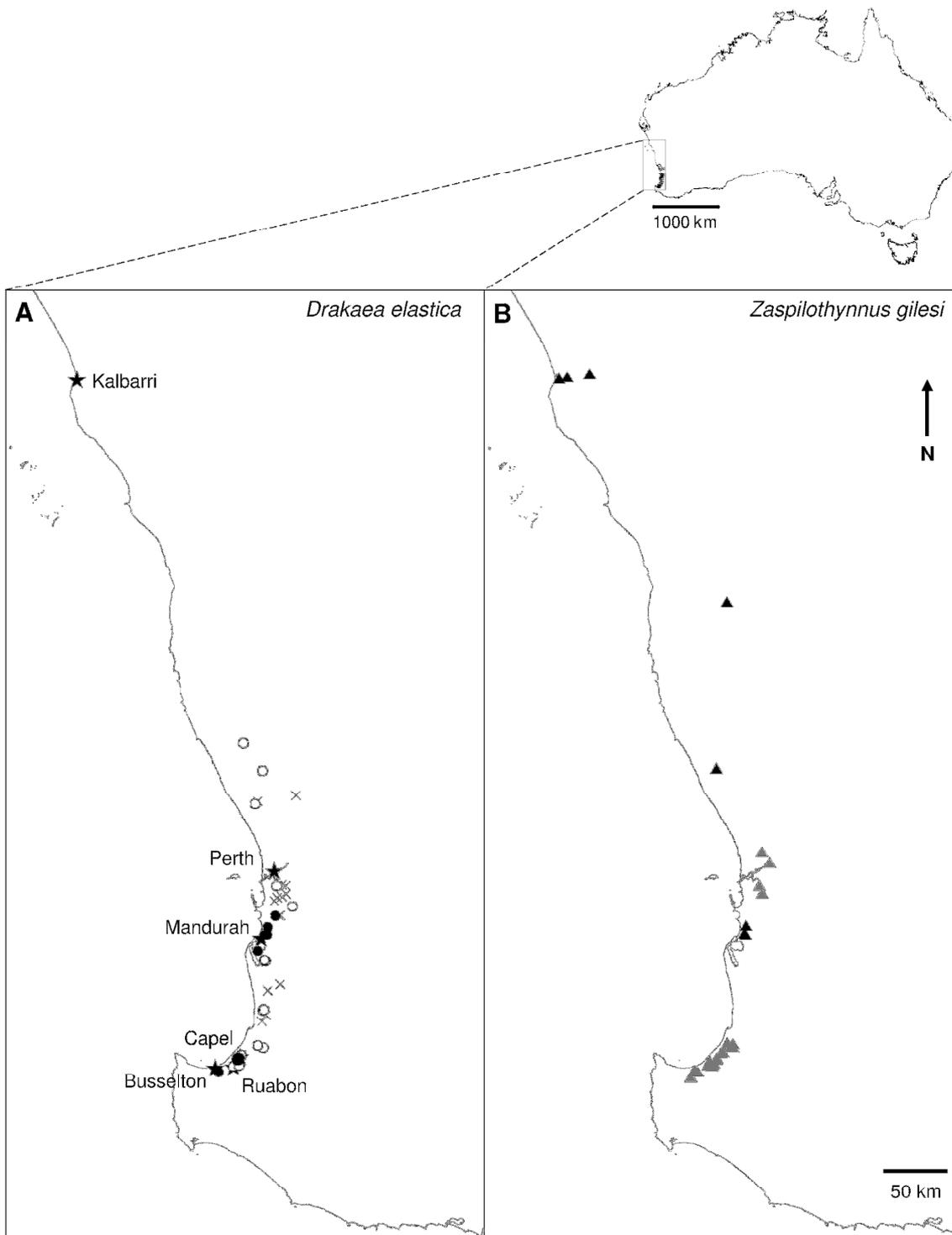


Figure 1.2. Map including place names referred to in the text (stars) and (A) location of all known populations of *Drakaea elastica* (source: Western Australian Department of Environment and Conservation Rare Flora Database, 2011). Solid circles indicate populations of less than 50 leaves; open circles indicate populations of greater than 50 leaves and crosses indicate extinct populations. (B) Records of *Zaspilothynnus gilesi* [grey triangles; variant *b* (black triangles), as *per* Turner (1910)] collected by M.H.M. Menz, R.D. Phillips and from specimens in the Western Australian Museum.

Chapter 2 – Microhabitat modelling as a tool for identifying habitat requirements of rare orchids

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ABSTRACT

A major limitation to plant recruitment is the availability of suitable microsites for germination, development and growth. Therefore, the identification of microhabitat requirements is of key importance for developing effective conservation programs for rare plants. Using generalised linear mixed models, we defined the microhabitat requirements for a model rare species, *Drakaea elastica*, a threatened terrestrial orchid, and investigated regional patterns of microhabitat use. The majority of extant individuals are associated with two regional strongholds approximately 100 km apart. The structural microhabitat requirements of adult plants were investigated by comparing occupied and random microsites across two regions. There were significant regional differences in median values for microhabitat variables at occupied microsites. However, the pattern between occupied and random microsites was similar between regions. Across its range, *D. elastica* requires open microsites with patches of bare sand, sparse understorey and low canopy cover. The observed regional differences in microhabitat variables may result from differing groundwater levels. The requirement for open sandy microsites with low litter and shrub cover may be linked to the competitive performance of the mycorrhizal endophyte, or due to the detrimental effects of shading of adult plants. Our identification of the key microhabitat requirements of *D. elastica* inform present habitat management, and future reintroduction programs. The expanded use of microhabitat modelling to identify the microhabitat requirements of rare plants is recommended for conservation programs operating worldwide.

INTRODUCTION

Human induced habitat destruction, habitat fragmentation and habitat degradation is impacting many plant populations worldwide. As a consequence there is a growing number of plant species requiring conservation attention. The Orchidaceae, in particular, appear to be overrepresented in lists of rare and endangered plants (Cribb *et al.* 2003; Koopowitz *et al.* 2003; Swarts and Dixon 2009). This likely reflects the combination of their sheer diversity, estimated at over 26,000 species (Joppa *et al.* 2011; WCSP 2013), and the possibility they are more extinction prone due to peculiarities of their biology.

As the subject of growing conservation concern worldwide, an increasing number of studies are investigating a range of factors potentially limiting the distribution and recruitment of orchid populations. Factors investigated to date include pollinators (Phillips 2010), seed dispersal (Murren and Ellison 1998; Jacquemyn *et al.* 2007; Jersáková and Malinová 2007), the presence of mycorrhizal endophytes (Brundrett *et al.* 2003; Swarts *et al.* 2010; Phillips *et al.* 2011a; McCormick *et al.* 2012) and the effect of factors such as shading on reproductive output, dormancy and survival (Willems *et al.* 2001; Shefferson *et al.* 2005; Shefferson *et al.* 2006; Jacquemyn *et al.* 2008; Jacquemyn *et al.* 2010a; Jacquemyn *et al.* 2010b).

Surprisingly, there appear to have been few studies investigating the structural habitat requirements of adult orchid plants (but see Calder *et al.* 1989; Ackerman *et al.* 1996; Kull 1998; Bowles *et al.* 2005; Bougoure *et al.* 2008). An understanding of the environmental and habitat factors influencing the distribution and persistence of adult populations is crucial for the *in situ* conservation and management of rare orchids. Additionally, this knowledge will also assist in identification of suitable sites for reintroduction programs (Brundrett 2007; Swarts and Dixon 2009; Wright *et al.* 2009).

In the absence of pollen limitation (Ackerman and Montalvo 1990), orchids can produce abundant seed per capsule (Arditti and Ghani 2000). Consequently, in common species germination may be limited by the availability of appropriate mycorrhizal endophytes (Jacquemyn *et al.* 2007; Jacquemyn *et al.* 2010b). However, in some terrestrial orchid habitats, it has been shown that the mycorrhizal endophyte occurs at microsites that are unoccupied by adult plants (Rasmussen and Wigham 1993; Batty *et al.* 2001; Diez

2007; Phillips *et al.* 2011a). This suggests that other factors such as seed arrival, climatic events at establishment, or microsite variation (litter, plant cover, soil organic matter) may interact to lead to ensure a successful recruitment event (Ackerman *et al.* 1996; Kull 1998; Batty *et al.* 2001; Diez 2007; McCormick *et al.* 2012). For example, increased germination success has been found in close proximity to adult plants and is also known to vary with levels of soil potassium, moisture, organic matter, pH and leaf litter (Batty *et al.* 2001; Diez 2007).

Drakaea elastica is a rare and threatened terrestrial orchid, which is endemic to the Swan Coastal Plain, in the southwestern Australian biodiversity hotspot (Brown *et al.* 1998; Myers *et al.* 2000; Hopper and Brown 2007). It is the focus of ongoing conservation programs as the species is under threat from development, with more than 80% of the habitat for *D. elastica* already cleared for housing and agriculture (Department of Environment and Conservation 2009). This has resulted in the remaining populations existing in isolated habitat fragments with the majority of individuals associated with two regional strongholds approximately 100 km apart. *Drakaea elastica* is pollinated by male *Zaspilothynnus gilesi* (Hymenoptera: Thynnidae) wasps (Hopper and Brown 2007; Phillips 2010), which it lures to the flowers by mimicking the sex pheromones of the female wasps (Schiestl *et al.* 1999; Schiestl *et al.* 2003). While orchids may often be pollination-limited (Ackerman *et al.* 1996), fruit set in *D. elastica* is relatively high (55.6%) (Phillips 2010), indicating populations are likely to be limited by the availability of suitable microsites. Consequently, pollinator-limitation is more likely to affect the distribution of *D. elastica* at a broader scale.

Similarly, the distribution of *D. elastica* at the landscape scale will also be limited by the availability of suitable habitat, and the distribution of the associated mycorrhizal endophyte. Within habitat patches, the distribution of the orchid will be determined by both the presence of the endophyte, and suitable conditions for the orchid-mycorrhizal symbiosis to form. The mycorrhiza associated with *D. elastica* is known to occur at sites unoccupied by adult orchids (Phillips *et al.* 2011a), hence, microsites suitable for adult plant survival may be limiting populations of *D. elastica*.

Within habitats occupied by the orchid, the goal of this study was to investigate the structural microhabitat requirements of adult *D. elastica* plants compared to random microsites. Three specific questions were investigated: (1) Are there regional

differences in microhabitat structural characteristics at microsites occupied by adult *D. elastica*? (2) What are the specific microhabitat structural requirements of adult *D. elastica*? (3) Which microhabitat variables best predict the occurrence of *D. elastica*? We conclude by considering the conservation implications of our findings.

METHODS

Study species

Drakaea elastica is primarily threatened by habitat destruction, fragmentation and degradation (Brown *et al.* 1998; Department of Environment and Conservation 2009). Based on comprehensive survey efforts it is estimated that the total population of *Drakaea elastica* Lindl. now consists of less than 5,000 plants (ramets) (Department of Environment and Conservation 2011). The extant distribution of *D. elastica* is highly fragmented by intervening urban and agricultural land and composed of seven major populations; located primarily in the Mandurah (northern) and Capel (southern) regions (Figure 2.1). While additional sites outside of these strongholds are known, they typically support populations of less than 50 plants (Conservation 2011) (Figure 2.1).

Within those sites still containing the orchids, additional threats to the long-term persistence of these populations are evident. The grazing of leaves and flowers by native and introduced animals, competition with weeds, increasing density of ground-level vegetation, increase in soil organics and inappropriate fire regimes are recognised as likely threatening processes that will exacerbate the risk of further decline within available habitats (Department of Environment and Conservation 2009).

Drakaea elastica grows in low-lying patches of open grey or white sand within mixed stands of *Kunzea glabrescens* Toelken and/or *Banksia* woodland (Figure 2.2) (Brown *et al.* 1998; Department of Environment and Conservation 2009). This preference for sandy patches leads to naturally scattered populations (Phillips 2010). Plants produce a single small leaf (approximately 1–3 cm diameter), which is readily identifiable from other *Drakaea* by its glossy green colour (Figure 2.2) (Hopper and Brown 2007).

Microhabitat sampling

Sampling for microhabitat analysis was implemented as a two-stage process. The first stage involved the mapping of populations of *D. elastica*. The second stage involved the assignment of random quadrats within the bounds of the orchid populations. This was an important process for ensuring the randomly chosen quadrats used were ecologically relevant. The location of individual leaves/colonies was marked in the field using flagging tape and a hand-held GPS (Garmin eTrex). These locations were used to calculate a minimum convex polygon (MCP) (Mohr 1947; White and Garrott 1990) with a 5 m buffer around each population at each site using the Animal Movement extension (Hooge *et al.* 1999) in ArcView v3.3 (Environmental Systems Research Institute, Inc.). Minimum Convex Polygons are created by joining the outermost locations at each site to form a boundary polygon (White and Garrott 1990). Within each MCP an equal number of random locations to occupied locations were generated using ArcView v3.3. When recording microhabitat variables at minimum a 0.5 m buffer was employed between any surveyed quadrats (both random and occupied) to avoid fine-scale spatial autocorrelation.

Microhabitat requirements of *D. elastica* were investigated by comparing 30 x 30 cm quadrats occupied by the orchid with random quadrats. These surveys were conducted across 12 sites (four northern, eight southern) that supported populations of 30 or more plants (Table 2.1, Figure 2.1). Occupied quadrats were selected based on the presence of *D. elastica* irrespective of abundance given that it is weakly clonal (Chapter 4).

We recorded the following variables: elevation (m a.s.l.), percentage cover of bare sand, percentage cover of *Kunzea glabrescens* litter, percentage cover of other litter, percentage cover of herbs and grasses, percentage cover of low shrubs less than 0.5 m, and percentage cover of medium shrubs 0.5–1 m (Table 2.4). The estimated cover of the variables percentage of bare sand, percentage cover of *K. glabrescens* litter, percentage cover of other litter, percentage cover of herbs and grasses, and percentage cover of low shrubs less than 0.5 m, summed to 100%.

At nine of the 12 sites (four northern, five southern) we also recorded percentage canopy / tall shrub cover greater than or equal to 2 m, and distance to nearest *K. glabrescens* plant (up to a distance of 2 m). *Kunzea glabrescens* leaf litter was

distinguished from that of other species, due to its small size (3–4 mm) and different structure. The other litter types included *Banksia* (*B. menziesii* R.Br., *B. attenuata* R.Br. and *B. ilicifolia* R.Br.), *Allocasuarina* species and eucalypt (*Corymbia calophylla* (Lindl.) K.D.Hill & L.A.S.Johnson and *Eucalyptus marginata* Sm.). All variables were recorded as estimated percentage covers to the nearest 5% (Table 2.2). In the case where a microhabitat variable was present within a site, but cover was less than 5%, this was given an arbitrary value of 1%.

Data analysis

Structural microhabitat requirements of D. elastica

To test for significant regional differences in microhabitat requirements, microhabitat variables were first compared between northern and southern sites at microsites occupied by *D. elastica* (Table 2.1, Figure 2.2), using Mann-Whitney *U*-tests performed in GenAlEx 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012). Microhabitat variables were pooled across all sites within each region.

Multivariate comparison between microhabitat variables at occupied and random microsites within regions were made using a generalised linear mixed model (GLMMs) (Burnham and Anderson 2002; Bolker *et al.* 2009) with a binomial error distribution and logit link function, including all microhabitat variables. This was conducted to test for significant differences between microhabitat variables at occupied and random microsites. GLMMs provide a useful framework for analysis of non-normal data that includes random effects, such as blocks, sites, or individuals (Bolker *et al.* 2009). Site was included as a random effect in the models.

Modeling microhabitat requirements of D. elastica

Secondly, using a set of candidate models, GLMMs were used to determine which microhabitat variables best explain the presence of *D. elastica* by comparing occupied to random microsites within regions. Modelling was conducted separately for each region. Given the large number of potential models arising from all possible combinations of the six predictor variables, a reduced set of 31 *a priori* defined models were selected that were deemed ecologically plausible (Tables 2.3 and 2.4). Site was

included as a random factor in the models. All microhabitat variables were assessed for pair-wise correlation and multicollinearity prior to analysis, using Spearman's Correlation Coefficient (r_s) and variance inflation factors (VIF) (Fox and Monette 1992; Faraway 2005) respectively. Variables with a value of $r_s \geq |0.7|$ were considered highly correlated and the most biologically meaningful variable was retained for further analysis. The VIF represents the increase in the variance resulting from correlation among the regressors in a linear model (Fox and Monette 1992).

Model selection in ecology and evolution has received much attention in recent literature (e.g. (Burnham and Anderson 2002; Johnson and Omland 2004; Zuur *et al.* 2009). Model selection operates under likelihood theory and provides a framework whereby competing models, treated like independent hypotheses are ranked based on how appropriately they fit the data. Akaike's Information Criterion (AIC) (Akaike 1973) is a widely used method for ranking models (Burnham and Anderson 2002; Johnson and Omland 2004) and calculates the goodness-of-fit of the model while accounting for model complexity (Burnham and Anderson 2002; Johnson and Omland 2004; Zuur *et al.* 2009).

Model selection was based on Akaike's Information Criterion corrected for small samples (AIC_C) (Hurvich and Tsai 1989; Burnham and Anderson 2002; Johnson and Omland 2004). The use of AIC_C in place of AIC is suggested when the ratio of sample size (N) to the number of parameters (K) in the model with the most parameters is small (<40) (Burnham and Anderson 2002). When the ratio of N/K is large AIC and AIC_C tend to be similar (Burnham and Anderson 2002). Models were ranked based on Akaike weight (w_i), which is the probability that a particular model is the 'best' model, given the set of models tested (Burnham and Anderson 2002). Akaike weights for a given set of models are normalised to sum to one. The model with the highest w_i was considered to be the best model and was used to make predictions regarding the occurrence of *D. elastica*. The 95% confidence intervals for predictions were generated by bootstrapping with 1000 iterations.

All modelling was carried out in R v2.14.0 (R Development Core Team 2011) using the libraries glmmML (Broström and Holmberg 2011b; Broström and Holmberg 2011a) for mixed model analysis, and car (Fox and Weisberg 2011) to calculate VIF (libraries available from <http://cran.r-project.org>).

RESULTS

Structural microhabitat requirements of *D. elastica*

A total of 750 quadrats (374 occupied, 376 random) were sampled across the 12 sites (four northern, eight southern) supporting populations of *D. elastica* (Table 2.1). In both regions *D. elastica* tended to occur in open habitats with moderate, bare sand (median 30-40%), low litter cover (median <10%), low herb and grass cover (median <5%), low shrub cover (median 0%), and low cover of tall shrubs (median <5%) (Figure 2.3). There were significant differences in median cover of a number of microhabitat variables at microsites occupied by *D. elastica*, between the two geographic regions (Figure 2.3). Consequently, subsequent analysis was undertaken separately for each region. Significant differences were identified for elevation (median northern sites = 5 m, median southern sites = 27 m; $Z = -16.28$, $P < 0.001$), percentage of bare sand (median northern sites = 30%, median southern sites = 40%; $Z = -2.61$, $P = 0.009$) and litter cover (median northern sites = 10%, median southern sites = 5%; $Z = 2.46$, $P = 0.014$). Cover of *K. glabrescens* litter was high at both northern and southern sites (median northern sites = 45%, median southern sites 40%, ns) (Figure 2.3).

At the northern sites *D. elastica* occupied microsites with a significantly higher amount of bare sand ($P < 0.001$), and lower leaf litter cover ($P < 0.001$), low shrub ($P < 0.001$), medium shrub ($P = 0.05$) and canopy cover ($P < 0.001$) compared to random microsites (Figure 2.4). The pattern was similar at the southern sites where *D. elastica* occupied microsites with significantly higher amount of bare sand ($P < 0.001$), and lower leaf litter cover ($P < 0.001$), *K. glabrescens* litter cover ($P = 0.03$), herb cover ($P < 0.006$), low shrub cover ($P < 0.001$) and canopy cover ($P = 0.003$) compared to random microsites (Figure 2.5). All other variable comparisons were not significant (Figure 2.4 and 2.5).

Predictors of *D. elastica* occurrence

The microhabitat variable medium shrub cover was excluded from the modelling for both northern and southern *D. elastica* populations due to less than 10% of records being greater than zero. There were no significant pairwise correlations between the microhabitat variables (northern populations: $r_s \leq -0.48$; southern populations: $r_s \leq -$

0.52). Initial multicollinearity was high in both regions (northern populations, $VIF \leq 7.04$; southern populations, $VIF \leq 6.86$), however in both cases, this was reduced to an acceptable level (Zuur et al., 2009) ($VIF \leq 1.82$ and $VIF \leq 1.52$ respectively) following removal of the variable percent cover of *K. glabrescens* litter from the models. All other microhabitat variables were retained for the modelling.

The best microhabitat model for both regions contained the variables percentage of bare sand, canopy cover, and distance to the nearest *K. glabrescens* plant (Tables 2.2 and 2.3). For the southern sites, this model included the quadratic function for percentage of bare sand and clearly performed better than the other tested models ($w_i = 0.996$, Tables 2.2 and 2.5). For the northern sites, the inclusion of the quadratic term for bare sand generated a model that performed equally as well as the model with the linear term for percentage of bare sand ($w_i = 0.473$ and 0.527 respectively, Tables 2.3 and 2.6). For comparative purposes we retained the model that included the quadratic function when making predictions.

There was a clear quadratic relationship between predicted occurrence of *D. elastica* and the percentage of bare sand for both northern and southern sites; with an optimum cover of between 60–80% (Figure 2.6A, D). The relationship was less clear with percent canopy cover; however the predicted probability of occurrence decreased with increasing canopy (Figure 2.6B, E). The probability of occurrence for *D. elastica* increased with proximity to *K. glabrescens* (Figure 2.6C, F).

DISCUSSION

Understanding habitat requirements of rare plants is a key aspect for developing effective conservation programs (e.g. Wiser *et al.* 1998; Maliakal-Witt *et al.* 2005; Fiedler *et al.* 2007; Sommers *et al.* 2011). The benefits of microhabitat analysis may be particularly valuable for orchids, which are often characterised by patchy distributions at both the population and landscape levels (Phillips *et al.* 2011a). Such distributions suggest specific microhabitat features could be particularly critical for orchids. Furthermore, orchids often exhibit specialised above and below ground interactions, which may also be associated with specific microhabitats (Schiestl and Schlüter 2009; Phillips *et al.* 2011a; Waterman *et al.* 2012).

Here we present one of the few studies to investigate the microhabitat requirements of adult orchid plants (but see Calder *et al.* 1989; Bowles *et al.* 2005). To our knowledge, this is also the first study to model key microhabitat requirements predicting orchid species occurrence. Microhabitat characteristics between microsites occupied by *D. elastica* and random microsites were similar across the species range. Both univariate non-parametric analysis of the variables by regional groupings (Figure 2.3) and generalised linear mixed modelling (Tables 2.2 and 2.3, Figure 2.6) indicated that *D. elastica* requires open microsites with patches of bare sand, sparse understorey and low canopy cover.

The observed regional differences in microhabitat variables, in particular elevation, may be a result of differing ground water levels, which may restrict *D. elastica* to sites at lower elevations in some areas. How a superficially rooted species like *D. elastica* interacts with the water table, given the plant rarely roots deeper than 10 cm, may be related to the deep-rooted attendant vegetation most commonly associated with *D. elastica*, such as *K. glabrescens*. Some phreatophytes (deep-rooted plants capable of reaching the water table) are known to provide additional soil moisture to shallow-rooted plants through hydraulic lift (Caldwell *et al.* 1993; Horton and Hart 1998); however this has not been established for *K. glabrescens* and may be a useful area for future research. A dependence on adequate soil moisture is a likely explanation for the restriction of *Drakaea* to slight depressions in semi-arid regions (Hopper and Brown 2007; Phillips 2010; Phillips *et al.* 2011a).

The microhabitat preferences of *D. elastica* may reflect two different processes: (1) the mycorrhizal competency, and/or (2) the competitive ability of adult plants. The strong preference of *Drakaea* orchids for open sandy patches has been hypothesised to arise from the competitive ability of their associated obligate mycorrhizal endophyte. An investigation into the distribution of the mycorrhizal endophyte (*Tulasnella* sp.) of *Drakaea* species using *in situ* seed-baiting across their range has revealed a mycorrhizal preference for open sandy sites (Phillips *et al.* 2011a). This fungus is known to be very slow growing, at least on artificial media and may therefore be outcompeted by other fungi in high organic matter environments (Phillips *et al.* 2011a). If so, the fungal dependence on open sites may directly constrain the microhabitat preference of *D. elastica*, which as previously noted typically tends to occupy microsites with very little surface leaf litter (median northern sites = 10%, median southern sites = 5%).

Despite a strong preference for bare sand, *D. elastica* also may also occur at microsites with a high proportion of *K. glabrescens* litter (median northern sites = 45%, median southern sites 40%). Aside from affecting levels of organic matter in the soil, leaf litter can hamper germination and growth of seedlings (Fowler 1988) through deprivation of sunlight by shading (Facelli and Pickett 1991). Most terrestrial orchids in southwest Australia are herbaceous perennials, senescing to a below-ground tuber outside the growing season (Pate and Dixon 1982). Thus they rely on the accumulated carbohydrate reserves from the previous year for vegetative growth and flowering (Willems *et al.* 2001). Shading directly affects the amount of available sunlight, thus effecting photosynthesis and carbohydrate accumulation, as well as the capacity to provide photosynthate to the mycorrhizal partner (McKendrick *et al.* 2000; Cameron *et al.* 2006).

Since *Drakaea* leaves are small (approximately 1–3 cm diameter) and sit flat on the soil surface (Hopper and Brown 2007), it is likely that even as adult plants they can be easily covered by litter and so deprived of sunlight, which will affect survival and reproductive capacity. In comparison to eucalypt and *Banksia* litter, *K. glabrescens* litter is small and fine (approximately 3–4 mm long, 1 mm wide) and *D. elastica* appears to tolerate relatively high amounts of this litter at some sites (probably due to the orchid being able to grow its leaves through the litter). The shading effect from this litter type is thereby reduced compared to that which would be caused by larger leaf litter from the nearby dominant *Banksia* species *B. attenuata* and *B. menziesii*, which have leaves that are many times larger than a single *Drakaea* leaf. Tolerance thresholds could be tested in the future via leaf litter addition within experimental plots containing adult *Drakaea* plants.

Surrounding vegetation density will also interrupt sunlight availability, particularly to species such as *D. elastica* that are low to the ground. Competition from weeds (Scade *et al.* 2006) and density of surrounding natural vegetation (McKendrick 1995) has been shown to significantly reduce survival of terrestrial orchid seedlings. Habitats supporting *D. elastica* typically have an open understorey with little cover of low native vegetation. Consequently, weed invasion in these habitats will significantly increase the cover of ground-level vegetation. In southwestern Australia, anthropogenic disturbance and inappropriate fire regimes can significantly alter natural vegetation dynamics leading to weed invasion and a permanent alteration in the biophysical and biotic

environment (Milberg and Lamont 1995; Fisher *et al.* 2009). Experiments involving caging or fencing areas of vegetation could be used to determine the rates of weed invasion into these habitats, and the role of both native and introduced herbivores in maintaining openness of the understorey.

In European orchids, canopy shading has been shown to affect flowering (Jacquemyn *et al.* 2010b), floral characteristics such as the number of flowers per inflorescence (Shefferson *et al.* 2006) and can lead to reduced reproductive output (Jacquemyn *et al.* 2008; Jacquemyn *et al.* 2010a). Further, shading can also affect dormancy and long-term survival of adult plants (Willems *et al.* 2001; Shefferson *et al.* 2005). *Drakaea elastica* tended to occur at microsites with an open canopy with predicted occurrence decreasing as canopy cover increases. While dense shading may be detrimental, low to moderate canopy cover can offer some protection to plants from direct solar radiation, particularly in microsites consisting primarily of bare sand where desiccation may pose a problem. Carstairs and Coates (1994) determined that survival rate of individual *D. elastica* plants was higher in sheltered sites compared to unsheltered sites. This might explain the observed pattern of a high probability of occurrence in close proximity to *K. glabrescens* plants at some sites. This results in plants occupying microsites with low litter cover on the one hand and partial shading on the other.

Growing in an open understorey may also ensure regular pollination as these orchids use a specialised sexual mimicry system where the male pollinator needs to be able to establish clear olfactory and visual cues. *Drakaea* attract their pollinators by sexual mimicry of female wasps (Stoutamire 1974; Peakall 1990; Phillips *et al.* 2010), whereby the orchid mimics the sex pheromones of female thynnine wasps (Hymenoptera, Thynnidae) (Schiestl *et al.* 1999; Schiestl *et al.* 2003). Male thynnine wasps, the sole pollinators of *Drakaea*, patrol through open areas of the understorey in search of females (Alcock 1981; Chapter 3). Consequently, there is the potential for dense, ground-level vegetation to disrupt the pheromone signal, or pose a physical barrier, thus leading to unsuccessful pollinator attraction to the flower. For example, (Petit and Dickson 2005) demonstrated that *Caladenia behrii* plants flowering within the foliage of shrubs suffered reduced pollination.

Implications for conservation

The results of this study inform best *in situ* conservation practice and guide efforts to identify suitable habitat for reintroductions. The findings of our microhabitat models show that an open understorey with bare sand (>40% cover; Figure 2.6) are critical for this species, with important implications for habitat management. Maintenance of an open understorey in disturbed habitats may require implementation of weed control measures such as herbicide application. This has been successfully applied in regional reserves in the vicinity of *D. elastica* where weed control has resulted in remarkable recovery in native vegetation (Botanic Gardens and Parks Authority 2011).

A number of *D. elastica* populations experience high rates of herbivory, with some populations experiencing the loss of up to 100% of flowers (range 51.8–100%, mean = $79.43 \pm 8.74\%$; Appendix Table A2.1). Fencing has been implemented at some *D. elastica* populations to exclude vertebrate herbivores such as western grey kangaroos (*Macropus fuliginosus* Desmarest) and introduced European rabbits (*Oryctolagus cuniculus* Linnaeus) (Department of Environment and Conservation 2009). However, while fencing and caging plants has been shown to directly reduce herbivory in orchids (McKendrick 1995; Faast and Facelli 2009), long-term exclusion of mammalian herbivores from populations of *D. elastica* may be detrimental, as kangaroo trails and moderate grazing may assist in maintaining an open understorey with bare sand patches. Furthermore, the effect of caging and fencing on fruit-set and population dynamics of needs to be experimentally tested across multiple years, as some caging methods have the potential to reduce pollination success (Faast and Facelli 2009), by restricting access to flowers by pollinators. It is clear that managing herbivory while at the same time maintaining an open understory will require a concerted and long-term management program to achieve the optimal balance that maximizes the survival of *D. elastica* populations.

This study has provided important insights into the microhabitat requirements of the endangered *D. elastica*. Expanded investigation of the habitat requirements limiting populations of *D. elastica* at the patch and landscape scale may further help to elucidate the key environmental factors that limit the distribution of the species. As a species dependent on specialized interactions, both above and below ground, it will be essential

to take into account these interactions in developing the most effective conservation strategies for this species.

Table 2.1. Details of sites sampled for microhabitat variables to describe the microhabitat requirements of *Drakaea elastica*. Sites are listed from north to south. Exact population locations have been withheld for conservation reasons.

Site	Region	<i>N</i> leaves	<i>N</i> occupied quadrats	<i>N</i> random quadrats
Paganoni NR (PAG) ^a	North	92	30	30
Serpentine River NR (SR) ^a	North	324	50	50
Lakes Road (LR) ^a	North	64	24	25
Carabungup NR (CAR) ^a	North	218	40	40
East Capel 1 (EC1) ^a	South	322	20	20
East Capel 2 (EC2) ^b	South	198	40	40
East Capel 3 (EC3) ^{a,b}	South	168	30	30
Capel NR 1 (CNR1) ^{a,b}	South	378	40	40
Capel NR 2 (CNR2) ^{a,b}	South	32	15	15
South of Capel NR (SC) ^b	South	53	30	30
Southeast Capel (SEC) ^{a,b}	South	210	36	36
Lindberg Road (LR)	South	119	20	20
Total		2178	375	376

NR = Nature Reserve. ^a Sites sampled for percentage canopy cover (*N northern quadrats* = 104 occupied, 145 random; *N southern quadrats* = 143 occupied, 102 random). ^b Southern sites sampled for distance to nearest *Kunzea glabrescens* (*N occupied quadrats* = 144, *N random quadrats* = 210).

Table 2.2. Details and parameter estimates for the most parsimonious set of generalised linear mixed models (GLMMs) predicting the occurrence of *Drakaea elastica* in the Capel region (southern sites). Models presented have a combined $w_i > 0.99$. Site is included as a random factor in all models. Variable² denotes a quadratic term; K = number of parameters; Δ_i = difference in Akaike's Information Criterion (corrected for small samples) between each model and the best model; w_i = Akaike weight; Dev. = Residual deviance; Dist. = Distance.

Parameters														
Model	Intercept		Bare sand		Bare sand ²		Canopy cover		Dist. to <i>Kunzea</i>		Δ_i	w_i	Dev.	K
	Coeff.	SE	Coeff.	SE	Coeff.	SE	Coeff.	SE	Coeff.	SE				
27	-1.208	0.516	0.102	0.020	-0.001	0.000	-0.009	0.007	-0.001	0.003	0.00	0.996	232.7	6
16	-0.415	0.457	0.035	0.006	NA	NA	-0.012	0.007	-0.002	0.003	11.2	0.004	246.0	5

Table 2.3. Details and parameter estimates for the most parsimonious set of generalised linear mixed models (GLMMs) predicting the occurrence of *Drakaea elastica* in the Mandurah region (northern sites). Models presented have a combined $w_i > 0.99$. Site is included as a random factor in all models. Variable² denotes a quadratic term; K = number of parameters; Δ_i = difference in Akaike's Information Criterion (corrected for small samples) between each model and the best model; w_i = Akaike weight; Dev. = Residual deviance; Dist. = Distance.

Parameters														
Model	Intercept		Bare sand		Bare sand ²		Canopy cover		Dist. to <i>Kunzea</i>		Δ_i	w_i	Dev.	K
	Coeff.	SE	Coeff.	SE	Coeff.	SE	Coeff.	SE	Coeff.	SE				
16	-0.533	1.009	0.057	0.010	NA	NA	-0.035	0.011	-0.016	0.004	0.00	0.527	174.0	5
27	-0.805	0.971	0.092	0.028	-0.000	0.000	-0.033	0.011	-0.016	0.004	0.22	0.473	172.1	6

Table 2.4. Microhabitat variables recorded within each quadrat used in the description of microhabitat requirements of *Drakaea elastica*.

Microhabitat variable	Units
Elevation	Metres a.s.l.
Bare sand	Percentage area of quadrat (nearest 5%)
<i>Kunzea glabrescens</i> litter cover	Percentage area of quadrat (nearest 5%)
Litter cover (<i>Banksia</i> , <i>Allocasuarina</i> , <i>Eucalyptus</i>)	Percentage area of quadrat (nearest 5%)
Grass and herb cover	Percentage area of quadrat (nearest 5%)
Low shrub cover 0-0.5 m	Percentage area of quadrat (nearest 5%)
Medium shrub cover to 1 m	Percentage area of quadrat (nearest 5%)
Canopy / Tall shrub cover ≥ 2 m	Percentage area of quadrat (nearest 5%)
Distance to nearest <i>K. glabrescens</i> plant	Distance (cm, maximum 2 m)

Table 2.5. Ranking of all 31 candidate generalised linear mixed models (GLMMs) predicting the occurrence of *Drakaea elastica* as a function of microhabitat variables in the Mandurah region, southwestern Australia. Site is included as a random factor in all models. * denotes an interaction; Variable² denotes a quadratic term; K = number of parameters; ΔAIC_C = difference in Akaike's Information Criterion (corrected for small samples) between each model and the best model; AIC weight = Akaike weight. Dist. = Distance.

Model	Deviance	K	ΔAIC_C	AIC weight
Bare sand + Canopy + Dist. to <i>Kunzea</i>	174.0	5	0.00	0.527
Bare sand ² + Canopy + Dist. to <i>Kunzea</i>	172.1	6	0.22	0.473
Litter + Canopy + Dist. to <i>Kunzea</i>	196.9	5	22.90	0.000
Bare sand ² + Litter + Low shrubs + Dist. to <i>Kunzea</i>	195.3	7	25.53	0.000
Bare sand + Dist. to <i>Kunzea</i>	207.4	4	31.29	0.000
Bare sand ² + Dist. to <i>Kunzea</i>	206.2	5	32.19	0.000
Bare sand + Dist. to <i>Kunzea</i> ²	207.2	5	33.19	0.000
Bare sand * Canopy	233.2	5	59.15	0.000
Litter + Dist. to <i>Kunzea</i>	235.3	4	59.19	0.000
Litter * Canopy	234.6	5	60.55	0.000
Bare sand + Litter + Canopy	244.6	5	70.55	0.000
Bare sand + Low shrubs + Canopy	245.2	5	71.15	0.000
Bare sand ² + Litter + Canopy	244.0	6	72.04	0.000
Bare sand + Canopy ²	250.3	5	76.25	0.000
Bare sand + Canopy	256.1	4	79.97	0.000
Bare sand ² + Canopy	254.4	5	80.35	0.000
Litter ² + Canopy	255.6	5	81.55	0.000
Litter + Canopy	261.7	4	85.57	0.000
Dist. to <i>Kunzea</i>	264.9	3	86.72	0.000
Dist. to <i>Kunzea</i> ²	264.8	4	88.69	0.000
Low shrubs + Canopy	265.9	4	89.77	0.000
Canopy	290.0	3	111.80	0.000
Bare sand + Litter + Low shrubs	307.5	5	133.42	0.000
Bare sand ² + Low shrubs	321.5	5	147.42	0.000
Bare sand + Litter	323.8	4	147.65	0.000
Bare sand + Low shrubs	324.6	4	148.45	0.000
Bare sand ²	338.1	4	161.95	0.000
Bare sand	341.2	3	162.99	0.000
Litter	351.6	3	173.39	0.000
Low shrubs	372.1	3	193.89	0.000
Herbs	398.6	3	220.39	0.000

Table 2.6. Ranking of all 31 candidate generalised linear mixed models (GLMMs) predicting the occurrence of *Drakaea elastica* as a function of microhabitat variables in the Capel region, southwestern Australia. Site is included as a random factor in all models. * denotes an interaction; Variable² denotes a quadratic term; K = number of parameters; ΔAIC_c = difference in Akaike's Information Criterion (corrected for small samples) between each model and the best model; AIC weight = Akaike weight. Dist. = Distance.

Model	Deviance	K	ΔAIC_c	AIC weight
Bare sand ² + Canopy + Dist. to <i>Kunzea</i>	232.7	6	0.00	0.996
Bare sand + Canopy + Dist. to <i>Kunzea</i>	246.0	5	11.19	0.004
Bare sand ² + Canopy	257.2	5	22.35	0.000
Bare sand ² + Litter + Canopy	257.1	6	24.35	0.000
Litter + Canopy + Dist. to <i>Kunzea</i>	271.7	5	36.89	0.000
Bare sand + Low shrubs + Canopy	275.5	5	40.65	0.000
Bare sand * Canopy	276.3	5	41.45	0.000
Bare sand + Canopy	280.0	4	43.07	0.000
Bare sand + Litter + Canopy	279.4	5	44.55	0.000
Bare sand + Canopy ²	279.9	5	45.05	0.000
Litter ² + Canopy	308.5	5	73.65	0.000
Litter + Canopy	312.2	4	75.27	0.000
Litter * Canopy	311.6	5	76.75	0.000
Low shrubs + Canopy	315.9	4	78.97	0.000
Canopy	327.7	3	88.70	0.000
Bare sand ² + Litter + Low shrubs + Dist. to <i>Kunzea</i>	394.8	7	164.01	0.000
Bare sand ² + Dist. to <i>Kunzea</i>	400.0	5	165.06	0.000
Bare sand + Dist. to <i>Kunzea</i> ²	406.4	5	171.46	0.000
Bare sand + Dist. to <i>Kunzea</i>	420.8	4	183.81	0.000
Litter + Dist. to <i>Kunzea</i>	442.1	4	205.11	0.000
Dist. to <i>Kunzea</i> ²	466.7	4	229.71	0.000
Dist. to <i>Kunzea</i>	485.8	3	246.77	0.000
Bare sand ² + Low shrubs	509.8	5	274.83	0.000
Bare sand ²	513.6	4	276.59	0.000
Bare sand + Litter + Low shrubs	521.3	5	286.33	0.000
Bare sand + Litter	531.7	4	294.69	0.000
Bare sand + Low shrubs	540.4	4	303.39	0.000
Bare sand	549.4	3	310.35	0.000
Litter	567.1	3	328.05	0.000
Low shrubs	612.7	3	373.65	0.000
Herbs	632.0	3	392.95	0.000



Figure 2.1. (A) A *Kunzea glabrescens* thicket, the typical habitat of *Drakaea elastica*. Note the open canopy and open understory with patches of bare sand. (B) Close-up of *Drakaea elastica* flower, and (C) the distinctive glossy green leaves.

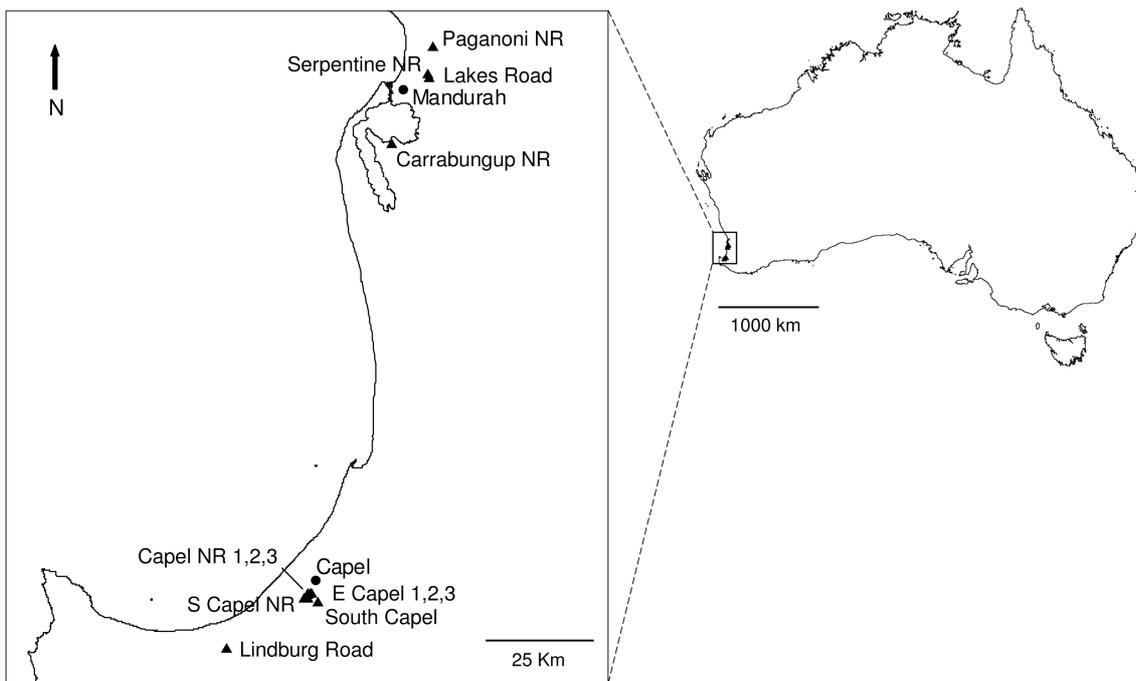


Figure 2.2. Location of study sites (solid triangles) where investigation of the microhabitat requirements of *Drakaea elastica* was conducted. NR = Nature Reserve. Solid circles indicate regions referred to in the text.

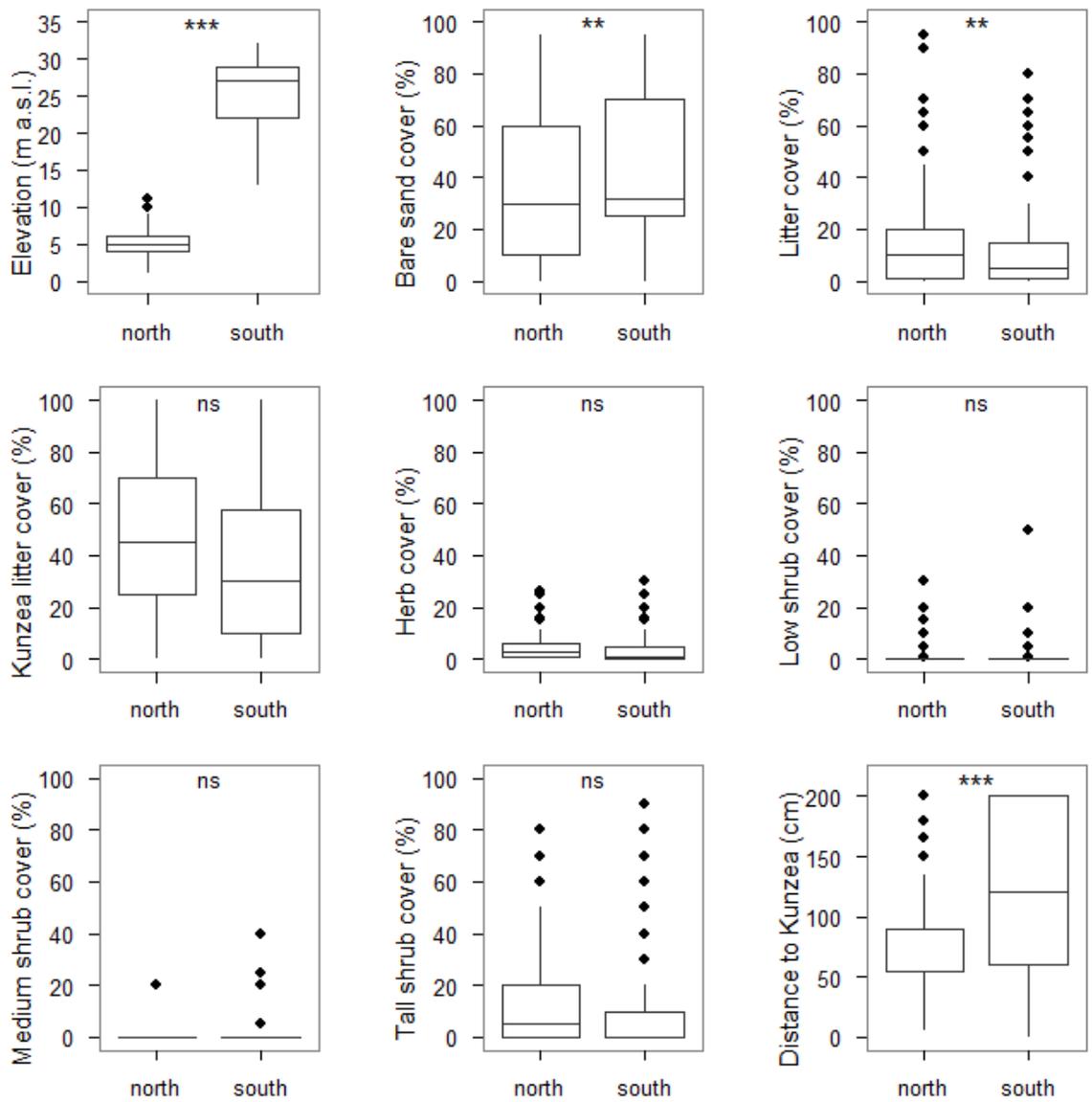


Figure 2.3. Box plots of pooled microhabitat variables at microsites occupied by *Drakaea elastica* at northern and southern sites. Results of Mann-Whitney U -tests: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant.

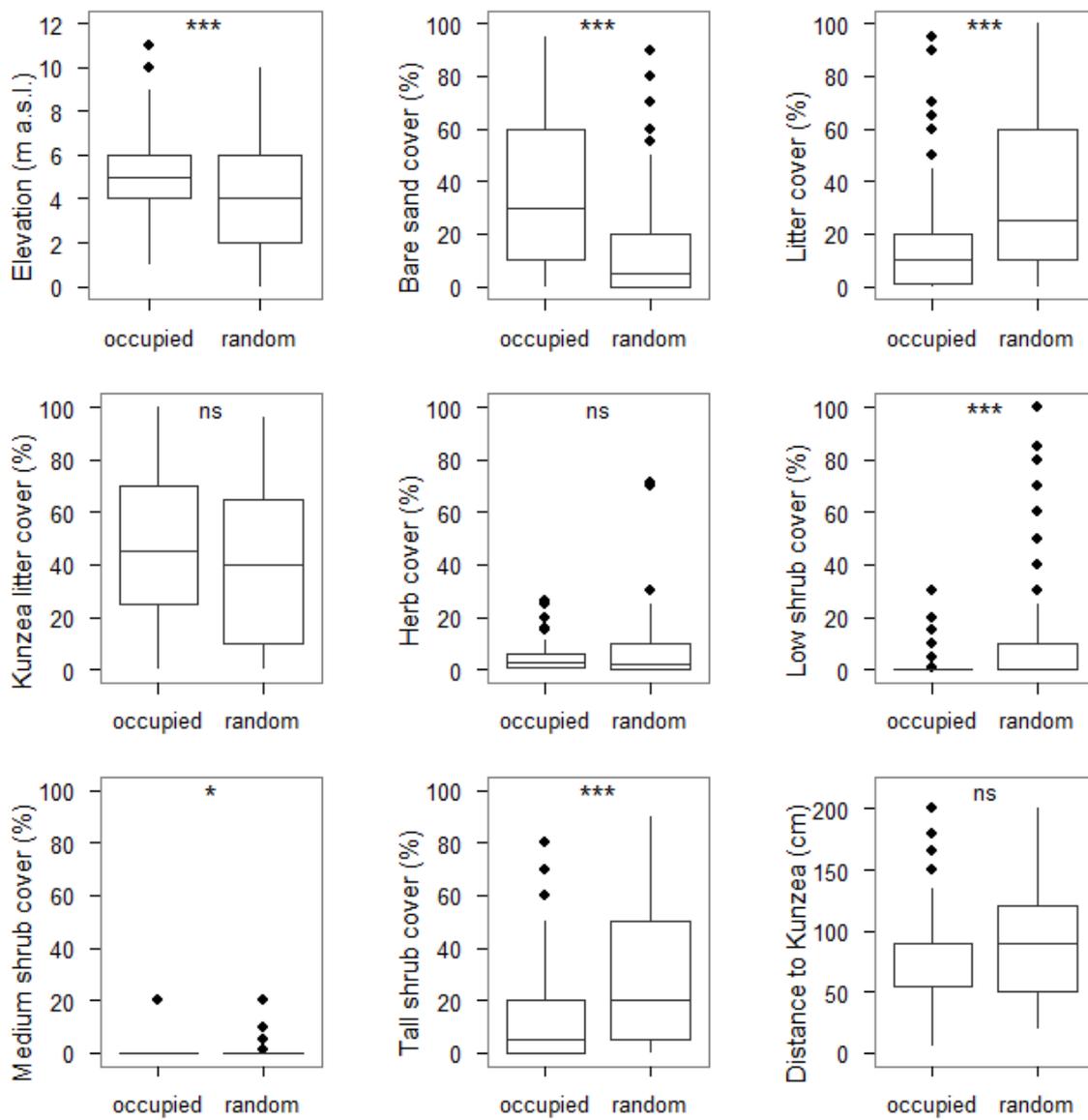


Figure 2.4. Box plots of microhabitat variables at microsites occupied by *Drakaea elastica* and random microsites at northern sites. Data presented are pooled across all sites. Results of generalized linear mixed models (GLMMs): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant.

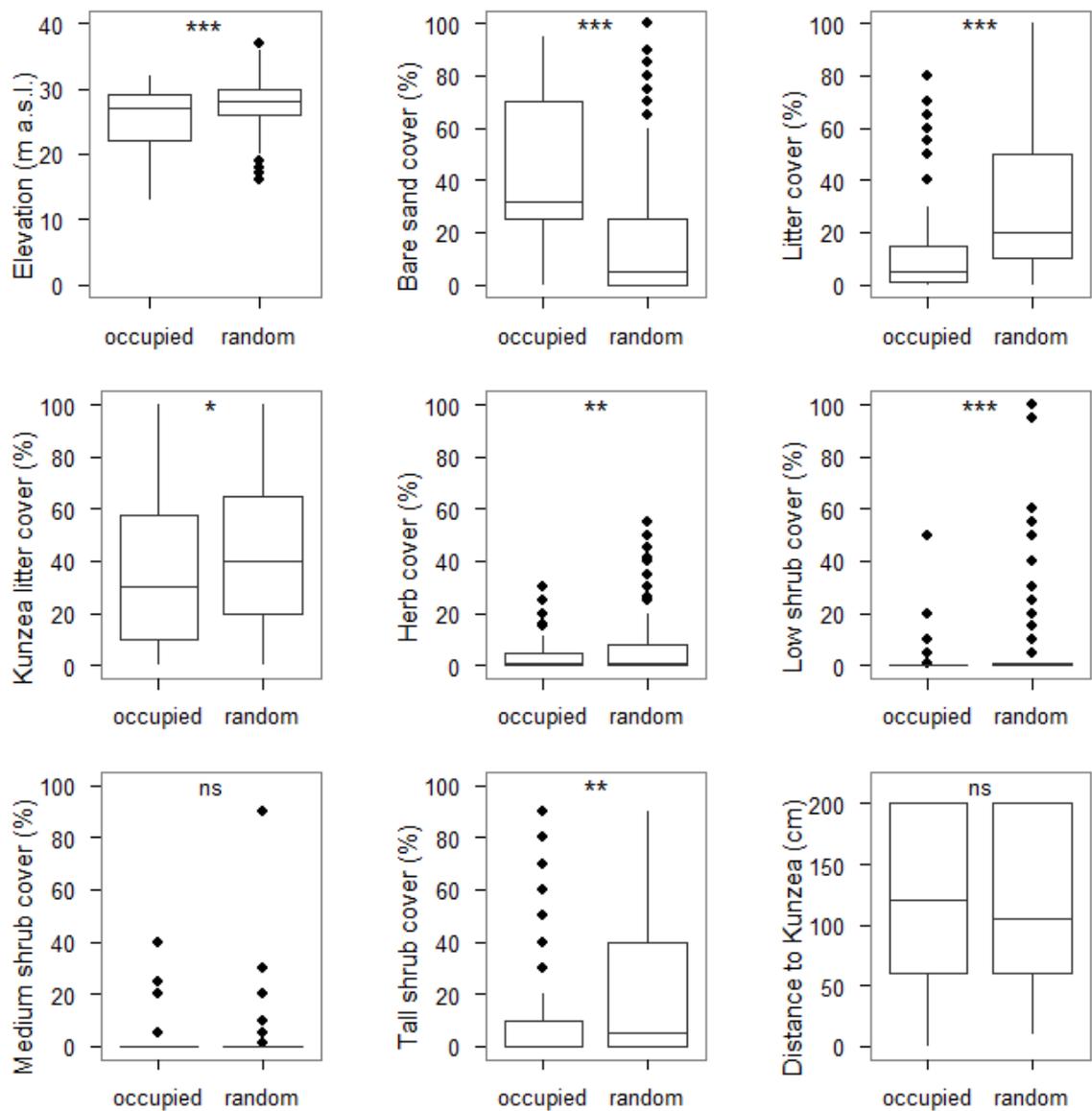


Figure 2.5. Box plots of microhabitat variables at microsites occupied by *Drakaea elastica* and random microsites at southern sites. Data presented are pooled across all sites. Results of generalized linear mixed models (GLMMs): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant.

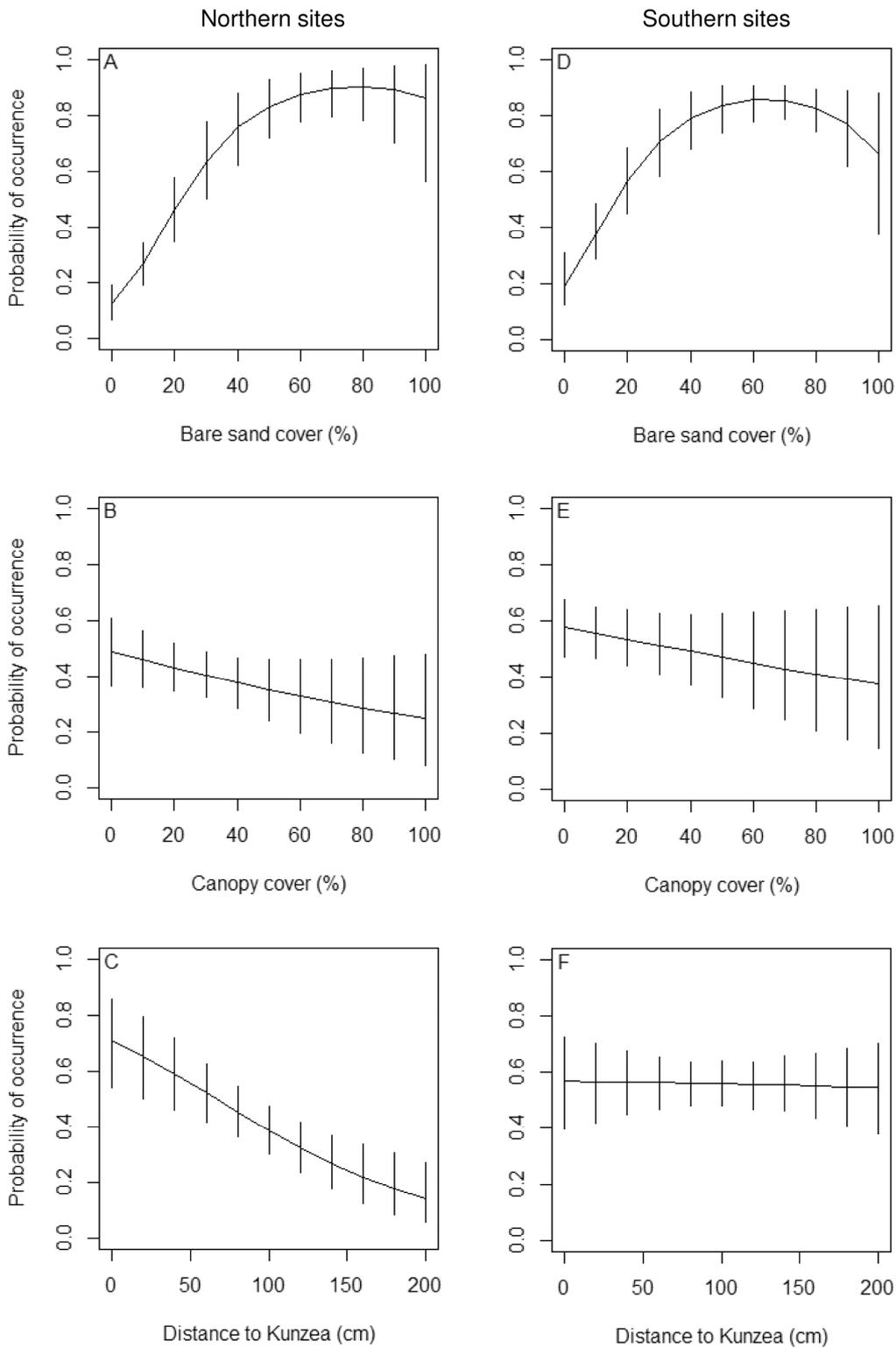


Figure 2.6. Estimates of *Drakaea elastica* occurrence probability in relation to the microhabitat variables percentage of bare sand (quadratic) at (A) northern and (D) southern sites; canopy cover at (B) northern and (E) southern sites and; distance to the nearest *Kunzea glabrescens* plant at (C) northern and (F) southern sites. Predictions are based on the most parsimonious generalized linear mixed model (GLMM) for each region. Bars represent 95% confidence intervals from 1000 bootstrap repeats.

APPENDIX

Table A2.1. Summary of *Drakaea elastica* populations surveyed for flowering, herbivory and fruit set in 2009.

Site	N total	N leaves ^B	N flowers	% flowering	N browsed	% browsed	N failed ^C	% failed	N fruiting ^D	% fruit set
East Capel 1 ^A	322	247	75	23.29	27	36.00	0	0.00	15	20.00
Lindburg Road	119	92	27	22.69	27	100.00	0	0.00	0	0.00
East Capel 2	198	139	59	29.80	35	59.32	20	33.90	2	3.39
Capel NR 2	32	25	7	21.88	5	71.43	0	0.00	1	14.29
Capel NR 1	378	271	110	27.51	57	51.82	35	31.82	3	2.73
Gavin's Road	3	1	2	66.67	2	100.00	0	0.00	0	0.00
Southeast Capel	210	142	67	31.90	63	94.03	2	2.99	0	0.00
Mean±SE	180.29±52.90	131.0±38.70	49.57±14.86	31.50±5.95	31.50±10.40	79.43±8.74	9.50±6.02	11.45±6.79	1.0±0.52	3.40±2.26

^A This population had been entirely fenced to exclude mammalian herbivores and is excluded from calculations of rates of herbivory and fruit set.

^B Includes only vegetative ramets.

^C Includes flowers that had failed, most often these were aborted as buds prior to anthesis.

^D Includes both flowers that had formed capsules and those which had been pollinated but were yet to form a capsule at the time of survey. Not included are flowers that were still open but had not been pollinated.

Chapter 3 – Mate-searching behaviour of common and rare wasps and the implications for pollen movement of the sexually deceptive orchids they pollinate

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ABSTRACT

Pollinator behaviour directly affects patterns of pollen movement and outcrossing rates in plants. In orchids pollinated by sexual deception of insects, patterns of pollen movement are primarily determined by the mate-searching behaviour of the deceived males. Here, using a capture-mark-recapture study (CMR) and dietary analysis, we compare mate-searching behaviour in relation to local abundance of two pollinator species and explore the implications for pollen movement in sexually deceptive *Drakaea* (Orchidaceae). *Drakaea* are pollinated solely by the sexual deception of male thynnine wasps. The rare *Drakaea elastica* and widespread *D. livida* occur sympatrically and are pollinated by the rare but locally common *Zaspilothynnus gilesi*, and the widespread and abundant *Z. nigripes*, respectively. Local abundance was significantly different with *Z. nigripes* twice as abundant as *Z. gilesi*. For the 653 marked wasps, there was no significant difference in median movement distance between *Z. gilesi* and *Z. nigripes*. However, the maximum movement distance was twice as high for *Z. gilesi* (556 m) compared with *Z. nigripes* (267 m). This is up to three times greater than previously reported for thynnines in CMR studies. Recapture rates were six times higher in *Z. gilesi* (57%) compared to *Z. nigripes* (9%). Pollen loads and wasp longevity were similar, suggesting that this difference in recapture rate arises due to differences in the number of males moving at a scale >500 m rather than through diet or mortality. Differences in the frequency of longer movements may arise due to variation in the spatial distribution of the wingless females. We predict that

pollen movement will largely be restricted to within populations of *Drakaea* (<500 m), with few movements between populations (>500 m).

INTRODUCTION

Pollinator behaviour directly influences patterns of pollen movement and the rate of outcrossing in plants, which are important for gene flow and seed fitness (Groom 1998; Ghazoul 2005). Pollination by food-foraging animals predominantly leads to short distance pollen movement, as they tend to move between neighbouring flowers or inflorescences (Pyke 1978; Pyke 1981). On the other hand, pollen movement patterns may be different for pollinators displaying territorial and courtship behaviour compared with food foraging behaviour. Consequently, plants pollinated by sexual deception offer an interesting opportunity to test the consequences of mate-searching behaviour on pollen movement (Peakall and Beattie 1996).

Pollination by sexual deception is one of the most specialised pollination systems. It involves the attraction of male insects by floral mimicry of the species-specific sex pheromones of female insects (Schiestl *et al.* 1999; Schiestl *et al.* 2003). Pollination is achieved when male insects are sexually attracted to the flower, often involving attempted copulation with the labellum, which brings them into contact with the pollinium and stigma (Stoutamire 1975; Peakall 1990). Sexual deception is primarily restricted to the Orchidaceae, with the exception of recently discovered cases from a South African daisy (Ellis and Johnson 2010) and a European iris (Vereecken *et al.* 2012). Sexual deception has been confirmed in orchids from Australia, Europe, Central and South America and southern Africa, utilising a wide taxonomic diversity of insect pollinators, within the Hymenoptera and Diptera (Gaskett 2011). Australia is recognised as one centre of diversity of sexually-deceptive pollination systems with at least 150 confirmed cases involving multiple orchid genera (Phillips *et al.* 2009; Gaskett 2011; Griffiths *et al.* 2011; Phillips *et al.* 2011b), the vast majority of which are pollinated by thynnine wasps (Phillips *et al.* 2009; Gaskett 2011). Thynnine wasps represent a diverse, predominantly Australian insect group, with an estimated 1500–2000 species, many of which are undescribed (Griffiths *et al.* 2011). Male thynnines patrol in search of wingless females that they carry *in copula* to a food source, usually nectar or exudations from scale insects (Hemiptera: Psyllidae) (Burrell 1935; Given 1953; Ridsdill Smith 1970b).

In species pollinated by sexual deception, patterns of flower visitation and pollen movement are predicted to reflect the optimal mate-searching behaviour of the

pollinating male insect (Ghazoul 2005). Intense male competition arises from the low number of sexually receptive females relative to males at any point in time (Alcock 2000; Alcock and Kemp 2005; Goh and Morse 2010) and is a characteristic of thynnine wasps. Consequently, male insects may search in such a way as to increase their chance of encountering unmated females (Alcock *et al.* 1978; Goh and Morse 2010). However, the mate-searching behaviour adopted may be partially determined by the spatial distribution of females. For example, if females are patchy in the landscape, then males may devote more time to searching in areas where females have previously emerged. By contrast, when females are more uniformly-distributed males may search more evenly in the landscape. To test if mate-searching behaviour of thynnines is affected by local abundance requires studying sympatric species of similar size with similar ecological requirements.

Here we compare pollinator mate-searching behaviour in relation to local pollinator abundance and explore the potential implications for pollen movement in sexually deceptive *Drakaea* (Orchidaceae). We supplement this with a dietary study of the pollinators to ensure that movement patterns and local abundance are not simply affected by differences in diet. *Drakaea elastica* Lindl. and *Drakaea livida* J.Drumm. occur sympatrically in southwestern Australia, but *D. elastica* is rare and threatened while *D. livida* is relatively common (Hopper and Brown 2007). *Drakaea elastica* is pollinated by the rare but locally common *Zaspilothynnus gilesi* Turner (Hymenoptera: Thynnidae), while *D. livida* is pollinated by the widespread and abundant *Z. nigripes* Guerin (Phillips *et al.* 2013). The wasps themselves have no dependence or feeding association on *Drakaea* orchids, and are only attracted to orchids by sexual deception. We predict that the rare *Z. gilesi* will display more restricted movement patterns, whereas the common *Z. nigripes* will show a more even distribution of movement distances.

METHODS

Study system

We used a capture-mark-recapture (CMR) study to compare the mate-searching behaviour of the rare *Z. gilesi* and the common *Z. nigripes* (Figure 3.1). Both species of *Zaspilothynnus* are within the same size range (~20–30 mm in length). They frequently

co-occur and have a similar flight phenology, allowing direct comparison of mate searching behaviour. The few thynnines for which reproduction has been studied have been found to be parasitoids on scarab beetle larvae (Coleoptera: Scarabaeidae) (Janvier 1933; Burrell 1935; Given 1953; Ridsdill Smith 1970b; Ridsdill Smith 1970a; Ridsdill Smith 1971). The apterous females spend most of their lives underground, only emerging to attract a male (Given 1953; Ridsdill Smith 1970a; Ridsdill Smith 1971). Consequently, there is always an excess of males ready to mate relative to females. Nectar-feeding thynnines are most often observed feeding on open-flowered plants such as many Myrtaceae that are accessible to a broad array of pollinators (Phillips *et al.* 2009; Menz *et al.* 2011).

Within continuous habitats, *Drakaea* typically form scattered sub-populations (<500 m), restricted to low-lying open sandy microhabitats, leading to a naturally patchy distribution (Phillips *et al.* 2011a). The rare *Drakaea elastica* is endemic to the Swan Coastal Plain, Western Australia, and is listed as critically endangered under the Australian Federal Environmental Protection and Biodiversity Conservation Act (EPBC) (Brown *et al.* 1998), whereas *D. livida* is widespread and relatively common in the south-west of Western Australia (Hopper and Brown 2007) (Figure 3.1). *Drakaea elastica* is restricted to areas of grey sand within mixed *Banksia* spp. and *Kunzea glabrescens* Toelken woodland (Brown *et al.* 1998). Natural vegetation in this region is highly fragmented due to land clearing for housing development and agriculture (Australia 2000). As a result, the majority of the remaining populations of this species are small (<50 plants) (Department of Environment and Conservation 2009) and disjunct (>1 km apart). In many of these populations, *D. elastica* occurs sympatrically with *D. livida*.

Study site

The CMR study was carried out between 7 October and 3 November 2010 in a remnant *Banksia* spp. and *Kunzea glabrescens* woodland approximately 12 km south-west of Capel (33°38'36"S, 115°29'54"E), Western Australia. Willow peppermint trees, *Agonis flexuosa* (Willd.) Sweet, contributed to a large part of the overstorey, with cover values of up to 70% in the northern half of the study area. The 80 x 450 m study site was a portion of a longer, linear habitat remnant connected to a reserve otherwise surrounded by pastures. The study site was selected because (i) the rare *Z. gilesi* and common *Z.*

nigripes were found to co-occur there in relatively large numbers, based on preliminary site-selection surveys, and (ii) there were no naturally-occurring *Drakaea* orchids at the site (thus avoiding potential interference from orchid-wasp interactions at orchids growing *in situ*). The remnant has an open understorey and prior to the CMR trial males of both wasp species were observed to patrol regularly in search of females throughout the study site.

Ethics statement

All necessary permits to conduct this research were obtained from the Western Australian Department of Environment and Conservation. No animal ethics approval was required for this research.

Capture, marking and recapture

Wasps were attracted using the baiting method developed by Stoutamire (1983) and Peakall (1990), where fresh orchid flowers are presented for short time intervals to lure male wasps. Orchids were picked and stored in a portable refrigerator. Depending on fresh flower availability, baiting for the two thynnine species *Z. gilesi* and *Z. nigripes* was undertaken simultaneously, with orchid baits placed at 108 and 88 random locations respectively, over the 27 day study period. Each location was only baited once for 10 minutes. When both orchid species were used simultaneously, they were placed approximately 30 cm apart. Baiting was restricted to warm (>18°C) and sunny days between 09:00 and 16:00 hrs, when thynnine wasps are most active (Peakall 1990). Up to 10 locations were visited per day, with a minimum distance of 30 m between consecutive locations visited. Bait flowers were kept in a sealed container between baiting locations to avoid wasps following the flowers and to overcome habituation to the floral odour by the wasp.

When arrival rates of wasps at the orchid baits were relatively low (1–3 individuals in a 10-minute period), individuals were marked on the thorax with a queen bee tag (2.5 mm diameter), attached using the glue provided with the tags (sourced from: <http://www.penders.net.au>). When arrival rates at the orchid baits were higher (4–32 individuals in a 10-minute period) it was not feasible to tag individuals on first capture using queen bee tags. Instead, each individual was marked on the thorax using one or a

combination of coloured paint pen (Uniball Posca) marks unique to each location. Upon first recapture, the paint pen mark was supplemented with a queen bee tag. There was no loss of information in this process as all single- and multi-coloured paint pen marks were clear at recapture allowing accurate identification of origin. After marking, wasps were released at the point of capture and observed to fly away from the area until lost from sight (>10 m). Marking of individuals continued for the duration of the study.

Sweep-netting for patrolling males was conducted for twelve 15-minute periods on a single day following the baiting. Sweep-netting sites were approximately 30 m apart along a sandy track running NW–SE through the study area, and extended into adjacent woodland for up to 250 m to the NW and SE beyond the boundary of the baiting area.

No baiting was conducted in adjacent pasture during the study, as the two species of thynnine wasp were not observed to mate-search in these areas. However, to confirm this for *Z. gilesi* at the study site, we conducted 48 baiting trials with *D. elastica* in the adjacent pasture up to 400 m from the study remnant in November 2011. This resulted in just a single male *Z. gilesi* being detected.

Movement distances and patrolling range

Locations of marking and recapture points were recorded using a hand-held GPS. A *G*-test was used to test for a difference in the distribution of recapture frequencies between *Z. gilesi* and *Z. nigripes* (individuals marked but not recaptured, or recaptured 1, 2, or 3–7 times). Mann-Whitney *U*-tests were used to test for a difference in median abundance of wasps per minute across baiting locations, and median number of days between marking and final recapture. All analyses were conducted in GenAIEx 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012) unless otherwise specified.

Distances between mark and recapture site were calculated in ArcView v3.3 (ESRI Inc.). To test for differences in the frequency distributions of movement distances between *Z. gilesi* and *Z. nigripes*, we implemented a kernel density estimate comparison procedure using the `kde.compare` function (Langlois *et al.* 2012) in R v2.14.0 (R Development Core Team 2011) which was adapted from the `sm.density.compare` function from the R package ‘`sm`’ (Bowman and Azzalini 2010). Kernel density estimation is a non-parametric procedure that produces a smoothed estimate of the

frequency distribution of movement distances for each species. The `kde.compare` function compares the area between these two curves to the areas between pairs of curves resulting from a user-specified number of random permutations (here 5000) of the species labels in the data using the `sm.density.compare` function (Bowman and Azzalini 2010). The `kde.compare` function expands on the `sm.density.compare` function by incorporating automatic bandwidth estimation for the kernel density estimates via the Sheather-Jones bandwidth estimation procedure (Sheather and Jones 1991), as implemented in the R function 'dpik' from the package 'KernSmooth' (Wand 2011), and increasing the number of points evaluated to produce the accompanying plot thereby resulting in a more detailed figure. Significance is calculated as the percentage of the random permutations that yield an area between the curves greater than that between the curves estimated from the data as grouped by species. Mann-Whitney *U*-tests were used to compare the median distances moved between *Z. gilesi* and *Z. nigripes* for baiting-only recapture records, and baiting combined with sweep-netting records.

Pollinator diet

Male wasps were swabbed for pollen to determine (i) which food plants the wasps were visiting and (ii) if both species were visiting the same food plant species. During the capture-mark-recapture experiment, 20 males of each species were swabbed for pollen using a gel containing fuchsin stain, as described by Wooller *et al.* (1983). Each wasp was swabbed on the top and underside of the thorax. Pollen carried by the wasps was identified using a compound microscope by comparing samples to a reference collection of pollen from plants flowering at the study site. Pollen load on each wasp was categorised by estimating the number of grains from each plant species, as 1, 1–10, 10–100, 100–1000, and ≥ 1000 grains. Only plant species represented by ≥ 10 pollen grains on an individual wasp were considered in the dietary analysis. Relative loads of each pollen species were compared between wasp species using *G*-tests.

RESULTS

Marking and recapture

We captured and marked a total of 147 individuals of the rare *Z. gilesi* from 108 baiting trials (22% zero responses; range: 0–11 wasps per trial), and 506 individuals of the common *Z. nigripes* from 88 baiting trials (17% zero responses; range: 0–32 wasps per trial), spread over 14 individual days of sampling. In the 88 paired experiments there were no cases where a wasp alighted on an orchid other than the species it typically pollinates (N responses = 653). Considering only locations where wasps were detected, median abundance of *Z. gilesi* (0.2 wasps per minute) was significantly lower than *Z. nigripes* (0.4 wasps per minute) ($Z = 3.385$, $P = 0.001$; Figure 3.2).

For *Z. gilesi*, we recaptured 83 of the 147 marked individuals (56%), with 46 (55%) of these recaptured once, 23 (28%) recaptured twice, eight (10%) recaptured three times, and six (7%) recaptured four to seven times (144 recapture events in total, from the baiting trials and sweep-netting combined). The median time between capture and final recapture for *Z. gilesi* was six days (range 0–23 days).

For *Z. nigripes*, we recaptured 48 of the 506 individuals (9%), with 36 (75%) of these recaptured once, eight (17%) recaptured twice, one (2%) recaptured three times, and three (6%) recaptured four to five times (68 recapture events in total, from the baiting trials and sweep-netting combined). The median time between capture and final recapture for *Z. nigripes* was nine days (range 0–25 days).

The distribution of recapture frequencies (G -test; individuals marked but not recaptured, or recaptured one, two, or three to seven times) differed significantly between the rare *Z. gilesi* and the common *Z. nigripes* ($G = 141.12$, d.f. = 3, $P = 0.001$; Figure 3.3), with *Z. gilesi* recaptured more frequently. Interestingly, the proportion of same day recaptures was markedly higher for *Z. gilesi* (45%) compared to *Z. nigripes* (5%). Median number of days between marking and final recapture was significantly higher for *Z. nigripes* than *Z. gilesi* ($Z = 3.46$, $P = 0.001$). This indicates that the lower recapture rate in *Z. nigripes* is unlikely to be due to higher mortality.

Movement distances

For *Z. gilesi*, recapture distances for the baiting trials ranged from 0–300 m (median = 38 m, $N = 132$), and 0–556 m (median = 81.5 m, $N = 12$) from the sweep-netting (Figures 3.4a and 3.5a). For *Z. nigripes*, recapture distances ranged from 0–150 m (median = 46.5 m, $N = 58$) for the baiting trials, and 0–267 m (median = 49.5 m, $N = 10$) from the sweep-netting (Figures 3.4b and 3.5b). The median recapture distances from both the baiting trials and the sweep-netting did not differ significantly between the rare *Z. gilesi* and the common *Z. nigripes* ($Z = 0.01$, $P = 0.99$; $Z = 0.59$, $P = 0.55$ respectively). Based on the kernel density comparison procedure (Langlois *et al.* 2012), frequency distributions of recapture distances of *Z. gilesi* and *Z. nigripes* were not significantly different (Figure 3.6; baiting only, $P = 0.098$; baiting and sweep-netting, $P = 0.127$).

Pollinator diet

Only three plant species were represented by ≥ 10 pollen grains on any one individual. These were *Agonis flexuosa*, *Eucalyptus marginata* Sm. and an unidentified herbaceous species of Asteraceae. Of the 40 wasps sampled, 38 carried mixed pollen loads with ≥ 10 pollen grains from multiple plant species. Of the 20 *Z. gilesi* sampled, 90% were carrying *E. marginata* pollen, 100% were carrying *A. flexuosa* pollen, and 5% were carrying Asteraceae pollen. Of the 20 *Z. nigripes* sampled, 100% were carrying *E. marginata* pollen, 100% were carrying *A. flexuosa* pollen, and 5% were carrying Asteraceae pollen. Pollen load frequencies for both *E. marginata* and *A. flexuosa* were not significantly different between the two wasp species ($G = 0.633$, d.f. = 1, $P = 0.89$; $G = 0.038$, d.f. = 1, $P = 0.99$, respectively).

DISCUSSION

Using a capture-mark-recapture (CMR) framework, we tested whether two sympatric orchid pollinators exhibit different patterns of local abundance, mate-searching behaviour and nectar resource use. For both species the majority of movements were concentrated in localized areas, indicating high fidelity to patrolling areas during mate searching. This confirms previous observations of male thynnines repeatedly patrolling set paths (Alcock 1981). The median movement distances of 38 m and 46.5 m (*Z.*

nigripes and *Z. gilesi*, respectively; length = 20–30 mm) are greater than that previously documented for smaller bodied (<20 mm) thynnine pollinators of sexually deceptive orchids (14.8–24 m) (Peakall 1990; Peakall and Beattie 1996; Whitehead and Peakall 2012a). Further, the maximum distance moved (556 m, *Z. gilesi*) was approximately three times greater than previously reported for thynnines in a CMR study (161 m, *Neozeleboria cryptoides*, length = 9 mm) (Whitehead and Peakall 2012a). In contrast, the bee *Colletes cunicularius* (length = 10 mm), the pollinator of four species of European *Ophrys* orchids (Paulus and Gack 1990), moved on average only 5.2 m in search of mates in the vicinity of a large nesting aggregation (Peakall and Schiestl 2004). These comparisons demonstrate that mate-searching behaviour of pollinators and subsequent pollen movement will vary between sexually deceptive systems (Peakall and Schiestl 2004).

Despite similar movement distributions, there were also significant differences in recapture rate and the number of same-day recaptures between the two species. The abundant *Z. nigripes* had a significantly lower recapture rate (9%) compared to the less abundant *Z. gilesi* (57%). Given that there was no evidence for differences in mortality, this lower recapture rate may be due to male *Z. nigripes* searching for females at a scale of >500 m (representing the extent of the study area). This may be due to variation in distributions of females and resources in the landscape between the two species. Male insects are predicted to display an optimal mate-search strategy that increases their chance of encountering unmated females (Alcock *et al.* 1978; Peakall and Beattie 1996; Goh and Morse 2010). If females are rare or patchy in the landscape, then males may devote more time to searching in areas where females have previously emerged, with occasional longer distance movements in search of females elsewhere. Conversely, in the case of common species such as *Z. nigripes*, females may be more uniformly encountered in the landscape, meaning that mate-searching/patrolling of males is less restricted to a particular local area.

The observation of markedly higher same-day recaptures for *Z. gilesi* compared to *Z. nigripes*, despite similar movement distributions, could be due to male *Z. nigripes* learning to avoid the orchid mimic. The thynnine wasp *N. cryptoides* is known to exhibit short-term (≤ 24 hours) patch avoidance following attraction to a synthetic pheromone bait (Whitehead and Peakall 2012b). In periods of low female availability, such as may characterise *Z. gilesi*, males may more readily respond to the orchid,

regardless of the accuracy of the pheromone or morphological mimicry. This could be experimentally tested by presenting captive male wasps with orchid flowers, in the presence or absence of females. Additionally, response to male competition could be tested by presenting orchid flowers to males at varying orchid to male ratios. Direct comparison of multiple thynnine species under a controlled environment may elucidate interspecific differences in male learning and response to orchid flowers.

Patterns of local abundance were significantly different between *Z. gilesi* and *Z. nigripes*. Utilisation of different food sources could potentially lead to variation in the local abundance of pollinators. However, dietary analysis in this study showed that both wasps were visiting the same plant species as nectar sources. This suggests that different nectar preferences are unlikely to be responsible for the observed differences in local abundance between *Z. gilesi* and *Z. nigripes*. The visitation of *A. flexuosa* and *E. marginata* supports the observation that larger-bodied thynnines regularly feed on nectar from open-flowered Myrtaceae (Phillips *et al.* 2009). While frequent visitation of *Agonis flexuosa* may be partially due to its high abundance at the study site, *E. marginata* was rare at the study site. Given the similarity in plant species visited, differences in larval host availability may be more important in regulating local abundance of these parasitic wasps than nectar availability.

Consequences for pollinator ecology and pollen movement in rare orchids

We found no difference in nectar plant visitation between the two species, suggesting that another part of the lifecycle is limiting the abundance of *Z. gilesi*. There have been very few studies on host choice in thynnines (Ridsdill Smith 1970b; Ridsdill Smith 1970a; Ridsdill Smith 1971), and no studies of the host associations of *Zaspilothynnus* wasps. Given the similarity in other aspects of their ecology, availability of suitable larval hosts could be contributing to the difference in overall abundance between *Z. gilesi* and *Z. nigripes*. For example, *Z. nigripes* may be less host-specific than *Z. gilesi*, or might utilise a far more common and widespread host. Thus, host specificity and abundance may play a major role in limiting populations of *Z. gilesi* and other rare thynnine species and requires further investigation.

Investigation of the behaviour and ecology of thynnine wasps yields insights that are relevant for the conservation of *Drakaea* orchids. Our study of the movement patterns

of *Z. gilesi* revealed that the majority of *D. elastica* pollen movement will be within 100 m, with the possibility for rare longer distance movements in continuous habitats. Where remnant populations of *D. elastica* are small and highly fragmented, we predict that there will be little pollen movement between these remnant populations (>1 km apart), given the inability of the agricultural matrix to support *Z. gilesi*. Direct investigation of orchid pollen movement will be required to validate these predictions and understand population connectivity via pollen flow in this species. Further, the long-distance movements of these wasps may provide the opportunity for long-distance pollen movement of the food plants. It would be interesting to combine observation of food-foraging and mate-searching to elucidate their relative roles in pollen movement for both orchids and food plants.

Knowledge of the behavioural ecology of these wasps is needed to understand how movements interact with habitat and nectar resource use at the landscape scale. Capture-mark-recapture studies of pollinators are usually focused on the local-scale and by themselves do not necessarily provide information of landscape-scale movements. Resolving patterns of landscape use could be achieved through the use of radio-telemetry, where recent advances leading to miniaturisation of transmitters has allowed long distance movements of individual pollinators to be tracked (Pasquet *et al.* 2008; Wikelski *et al.* 2010; Hagen *et al.* 2011). However, commercially available radio-tags are not yet small enough for application on many pollinator species.

The potential for habitat requirements to vary across multiple spatial scales will have implications for population dynamics of these wasps and ultimately orchid pollen movement and fruit set. For example, at the local scale, populations may be dependent on the presence of nectar plants, whereas at the landscape scale, occupancy may be affected by the proportion of remnant vegetation. Further insight into landscape use and foraging movements would require the study of copulating pairs and individuals at food sources. If large-bodied thynnines move relatively long distances to forage on nectar, this may offer the opportunity to connect fragmented populations of both plants and pollinators through the provision of habitat corridors containing nectar-producing plants (Townsend and Levey 2005; Dixon 2009; Menz *et al.* 2011).

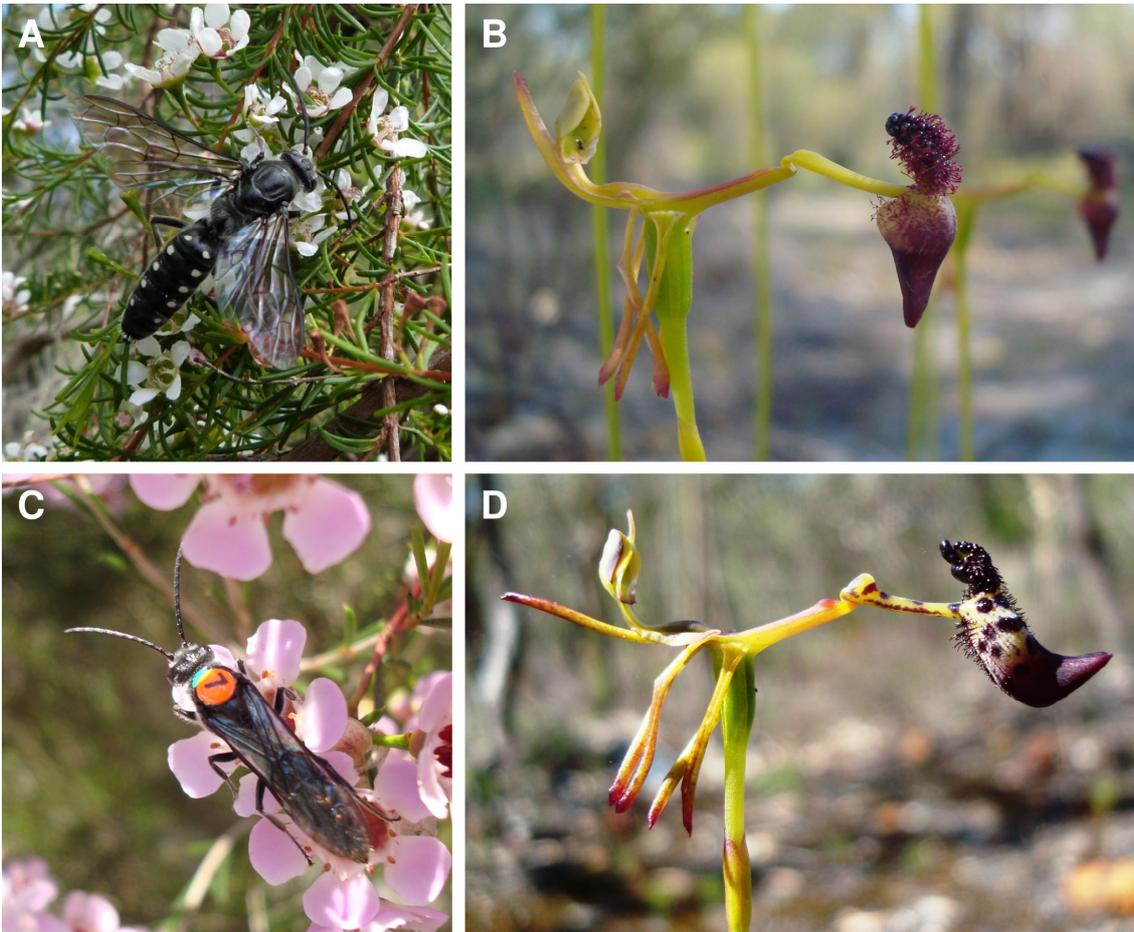


Figure 3.1. Male *Zaspilothynnus* wasps and the *Drakaea* they pollinate. (A) The thynnine wasp *Zaspilothynnus gilesi*, the pollinator of the rare *Drakaea elastica* (Orchidaceae) (B), feeding on nectar of *Astartea fascicularis* (Myrtaceae); (C) *Zaspilothynnus nigripes*, the pollinator of the common *Drakaea livida* (Orchidaceae) (D), marked with green paint pen and an orange queen bee tag, feeding on *Chamelaucium uncinatum* (Myrtaceae). Photographs: (A), (B) and (D) MHM Menz, (C) J Hardwick.

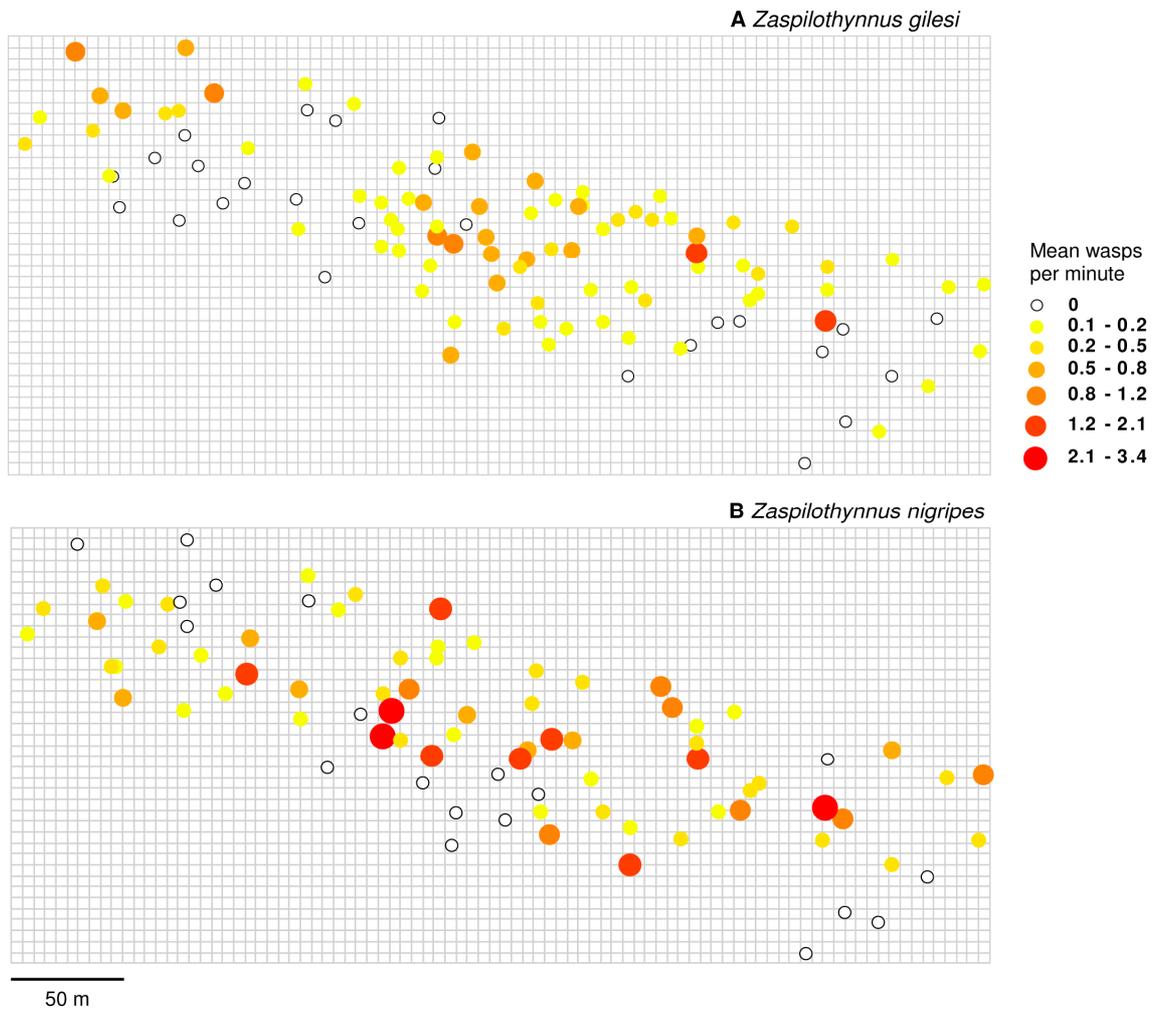


Figure 3.2. Mean number of male *Zaspilothynnus* wasps per minute attracted to orchid bait flowers. (A) *Zaspilothynnus gilesi* and (B) *Zaspilothynnus nigripes*. Open circles represent baiting locations where no wasps were caught.

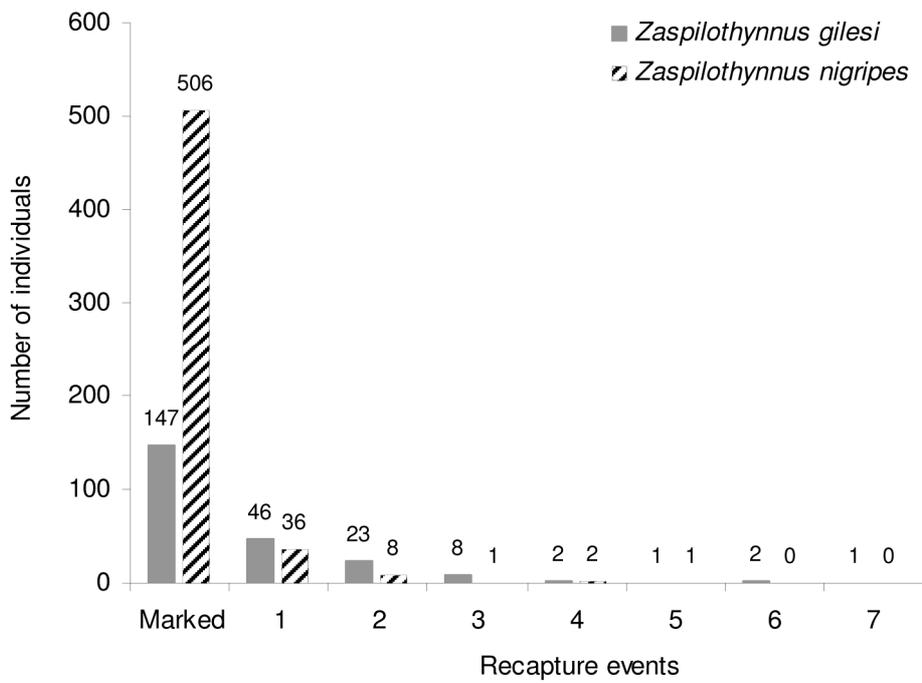


Figure 3.3. Comparison of number of male *Zaspilothynnus* wasps marked and the number of times recaptured.

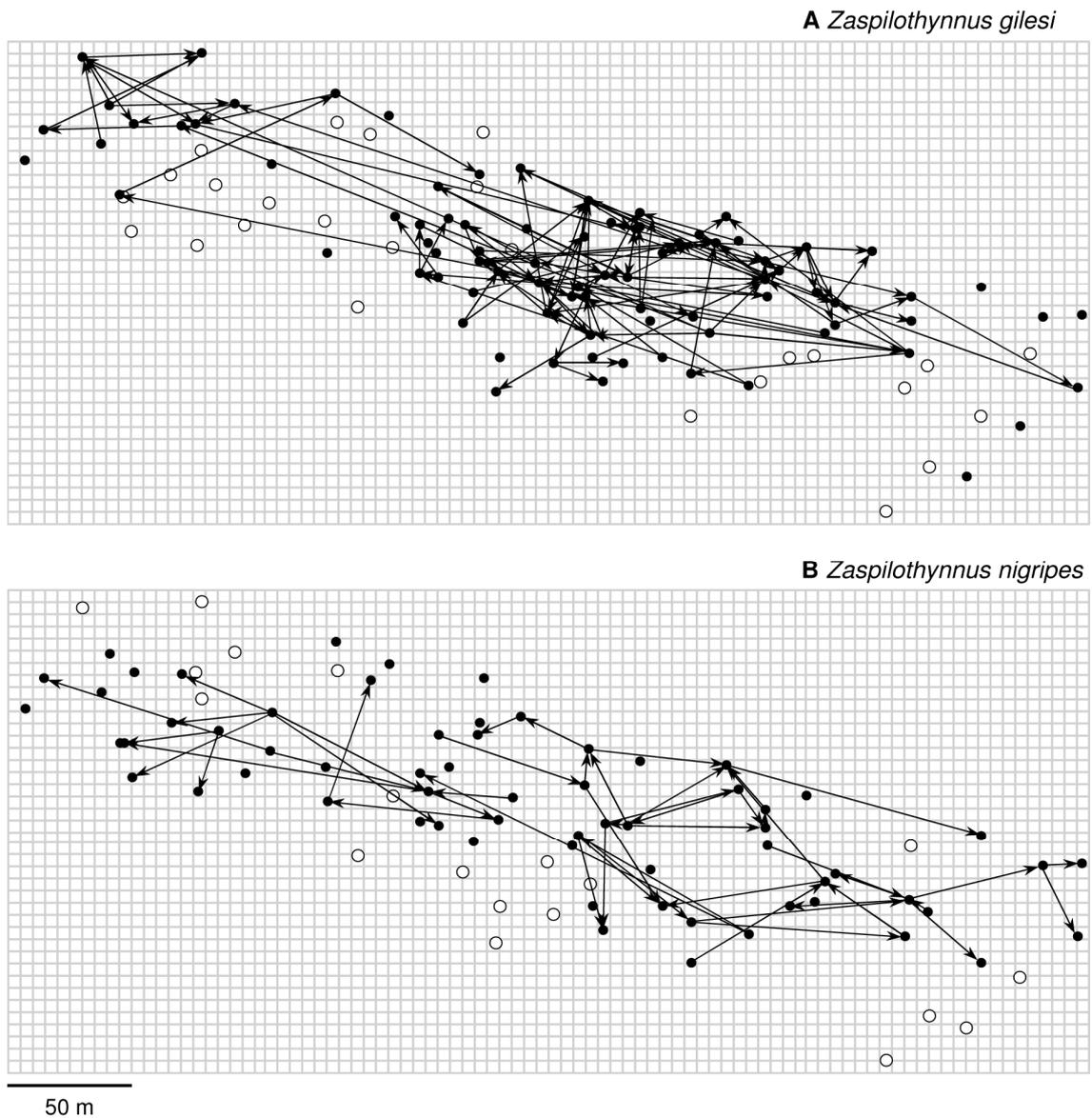


Figure 3.4. Movement vectors of male *Zaspilothynnus* wasps attracted to orchid bait flowers. (A) *Zaspilothynnus gilesi* and (B) *Zaspilothynnus nigripes*. Open circles represent baiting locations where no wasps were caught.

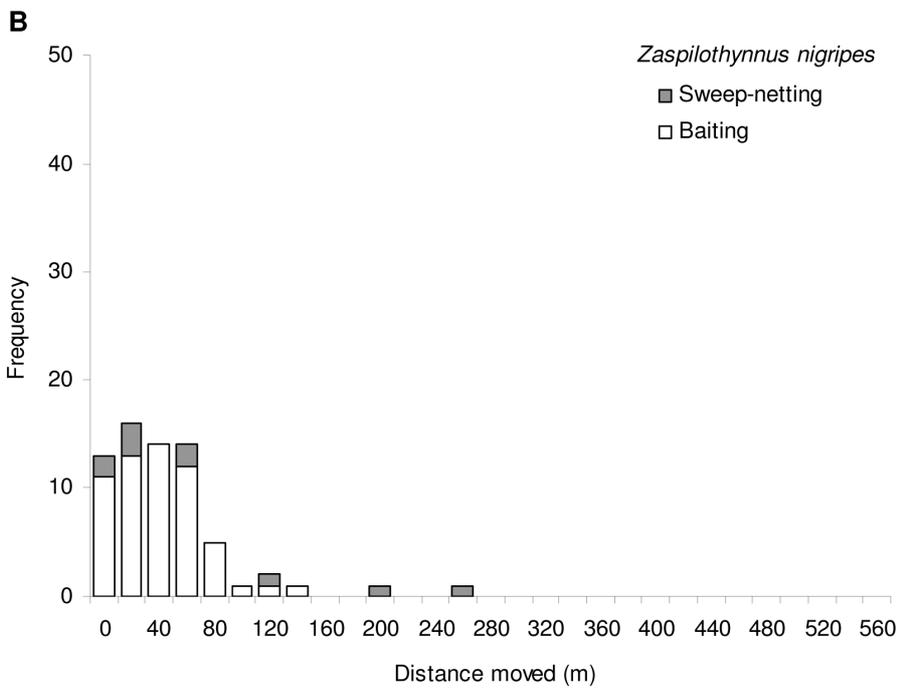
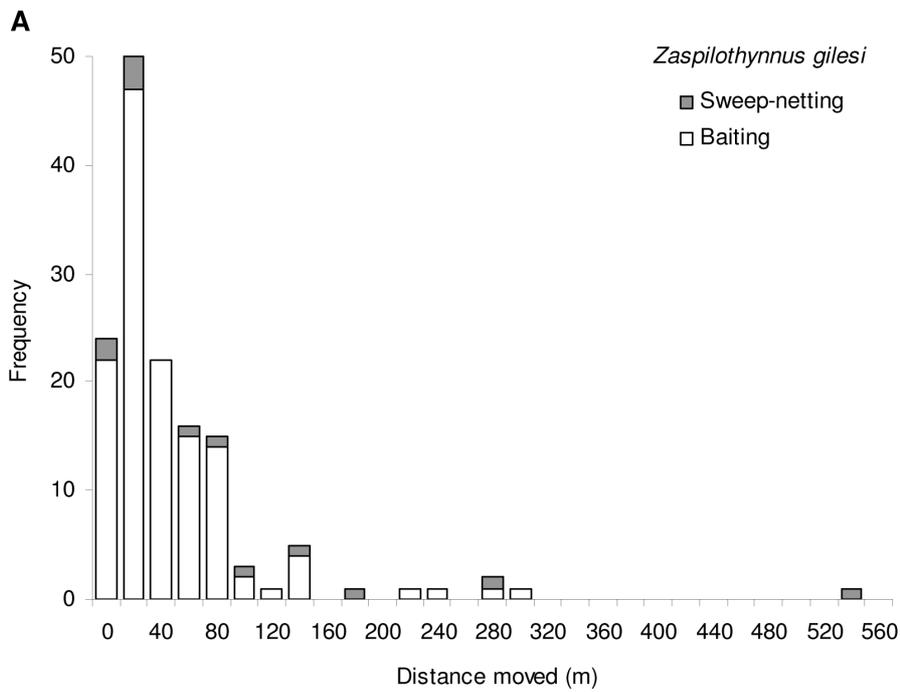


Figure 3.5. Movement distances of male *Zaspilothynnus* wasps attracted to orchid bait flowers. (A) *Zaspilothynnus gilesi* and (B) *Zaspilothynnus nigripes*. Open bars represent movements recorded from baiting trials and shaded bars from sweep-netting.

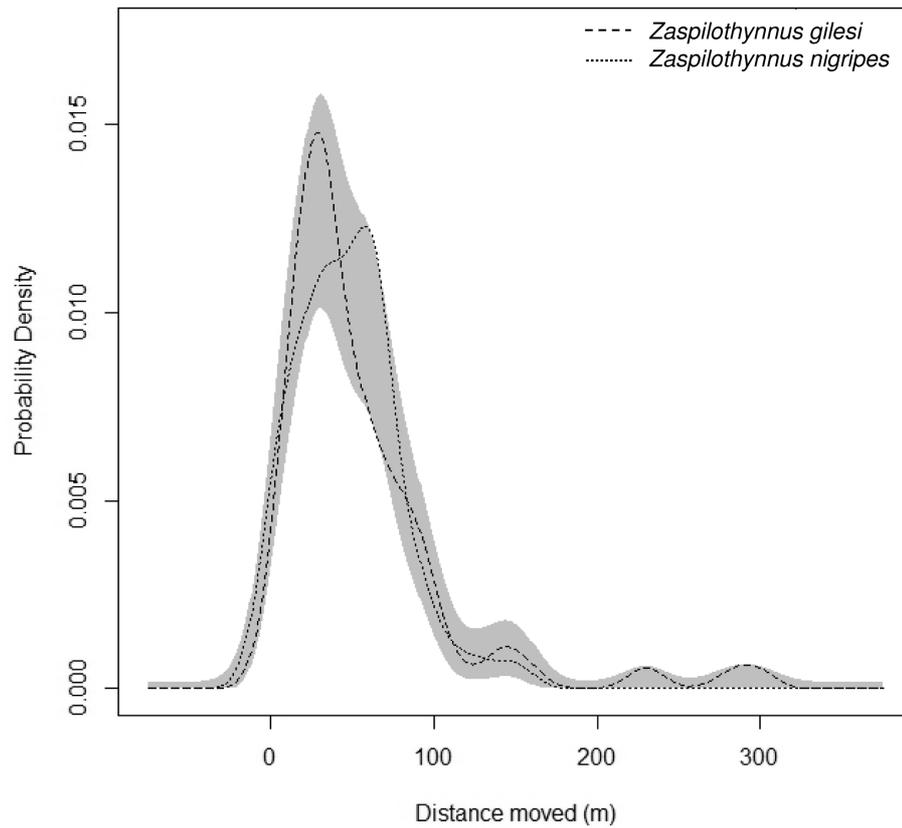


Figure 3.6. Probability density plots of movement distances of male *Zaspilothynnus* wasps lured to orchid baits. The shaded area represents a null model of no difference between the two frequency distribution curves. Significance values are based on 5000 random permutations.

Chapter 4 – Ecological, genetic and chemical analysis reveals hidden complexity in a rare species with specific pollinators

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ABSTRACT

Highly specific ecological interactions can pose significant conservation challenges. In the case of specialised plant-pollinator interactions, local differences in pollinator use may indicate the presence of cryptic taxa. In this study, we combined pollination experiments in the field, floral odour chemical analysis and population genetic analysis of the rare sexually deceptive orchid *Drakaea elastica* and its sole pollinator, the thynnine wasp *Zaspilothynnus gilesi* to investigate the potential for differences in pollinator use by orchid populations. Our findings revealed hidden complexity within both the orchid and pollinators. Pollinator baiting revealed that *D. elastica* flowers from some populations attract different forms of *Z. gilesi*. Gas chromatography / mass spectrometry – electroantennographic detection (GC/MS–EAD) results indicate that there is a single active compound that differs between the pheromone bouquets of orchids from the north and south of the species range. Consistent differences in the pollinators attracted to individual flowers indicates that they represent different ‘chemotypes’ and thus possess a degree of pollinator-mediated reproductive isolation. While population genetic differentiation was low ($F_{ST} = 0.026$), this was significant on a regional scale. Analysis of population genetic structure revealed two distinct genetic clusters, with overlap, indicating some admixture. There was no significant evidence for isolation by distance across the species range. This indicates, that at least historically, populations of *D. elastica* were connected by gene flow; most likely through long distance seed dispersal events. Spatial autocorrelation analysis showed significant patterns of fine-scale positive spatial genetic structure most likely driven by the majority

of seed falling close to the maternal plant. Clonality in *D. elastica* appears to be restricted to less than 0.2 m. Results of this study indicate that there is more than one management unit within *D. elastica* and mixed populations may occur. Consequently, all populations should be conserved until these boundaries can be further resolved using alternative population genetic markers such as might be found in chloroplast DNA, and single labellum extracts of flowers to identify individual floral chemotypes.

INTRODUCTION

Local adaptation to pollinators has been demonstrated for a number of plant species, in particular, plants that engage in specialised pollinator interactions. Known examples tend to involve species that display distinct ecotypes or morphological variants (Johnson 1997; Anderson and Johnson 2007; Anderson *et al.* 2009; Newman *et al.* 2012). However, in plants that attract their pollinators primarily via chemical cues, such as many orchids, these entities are likely to remain morphologically cryptic (Bower 2006; Bower and Brown 2009; Peakall *et al.* 2010). Cryptic species tend to be morphologically indistinguishable, and may be prevalent in groups that attract mates via non-visual cues (Bickford *et al.* 2007). In rare species, the presence of cryptic variation presents a unique set of conservation challenges, as the newly recognised entities will require a re-assessment of their conservation status and management programs (Schönrogge *et al.* 2002; Bickford *et al.* 2007). Further, in the case of specialised plant-pollinator interactions, this will require the consideration of multiple species in conservation planning.

The Orchidaceae is one of the most diverse plant families estimated to contain over 26,000 species worldwide (Joppa *et al.* 2011). Globally, there are more threatened orchid species than in any other plant family (Swarts and Dixon 2009). Threatening processes are primarily anthropogenic and result directly from habitat destruction, modification and fragmentation, and over-collection (Swarts and Dixon 2009). The tendency to exist in naturally patchy populations (Phillips *et al.* 2011b) and form specialised ecological interactions (Schiestl and Schlüter 2009; Phillips *et al.* 2011a) makes orchids particularly susceptible to disturbance and can pose challenges for conservation and restoration (Swarts and Dixon 2009; Menz *et al.* 2011). In extreme cases, loss of a pollinator can have significant detrimental consequences on a plant's population growth rate (Anderson *et al.* 2011).

Pollination by sexual deception is one of the most specialised plant pollination systems known and is exploited primarily by the Orchidaceae. Sexual deception involves the attraction of male insects (usually Hymenoptera) to flowers by mimicking the species-specific sex pheromones released by female insects (Schiestl *et al.* 1999; Schiestl *et al.* 2003). Pollination is achieved when male insects are sexually attracted to the flower and attempt to copulate with the flower (pseudocopulation) where they come into contact

with the pollinium and stigma (Stoutamire 1975; Peakall 1990). Australia is recognised as one centre of diversity of sexually deceptive orchids with at least 150 species from nine genera (Phillips *et al.* 2009; Gaskett 2011; Griffiths *et al.* 2011; Phillips *et al.* 2011b). Sexual deception has also been recorded in orchids from most other continents including Europe, both Central and South America (Gaskett 2011).

Drakaea elastica Lindl. is a rare and threatened sexually deceptive terrestrial orchid, pollinated solely by males of the thynnine wasp species *Zaspilothynnus gilesi* Turner (Hymenoptera, Thynnidae) (Phillips 2010). *Drakaea elastica* is morphologically similar across its range (Hopper and Brown 2007). However, *Z. gilesi* is also reported to contain three variants that are distinguished by slight morphological differences and colouration (Turner 1910). Whilst investigating the distribution and mate-searching behaviour of male *Z. gilesi* as part of another study (unpublished data, Chapter 3) it became apparent that there were differences in the pollinator attracted by some *D. elastica* flowers. Consistent differences in pollinator attraction by flowers can signify the presence of different ‘chemotypes’, which may indicate chemical-mediated reproductive isolation (Bower 1996; Bower 2006; Bower and Brown 2009; Peakall *et al.* 2010; Xu *et al.* 2011; Bohman *et al.* 2012b; Waterman *et al.* 2012).

This study had the following objectives: Firstly, we investigated population level and regional differences in pollinator attraction and possible chemotype variation in *D. elastica*. Secondly, using microsatellite markers, we investigated the extent of genetic variation within and between populations, and the presence of any patterns of regional genetic structuring. Thirdly, the presence of fine scale spatial genetic structure and levels of clonality are explored to infer patterns of seed and pollen dispersal between populations. Finally, we evaluate the consequences of the ecological and genetic findings for the conservation of *D. elastica*.

METHODS

Study species

Drakaea elastica is a rare and threatened orchid, endemic to the Swan Coastal Plain, southwest Western Australia (Brown *et al.* 1998) and is listed as critically endangered under the Australian Federal Environmental Protection and Biodiversity Conservation

Act (EPBC). The total population is estimated at less than 5,000 plants (Conservation 2011) that persist at approximately 42 locations (1–1,500 leaves) comprising seven major populations, situated primarily within conservation reserves (Conservation 2011) (Figure 4.1). These populations are located in two regions, approximately 110 km apart, with four near the town of Mandurah (CARA, LAKES, PAG and SERP) and three near Capel (CAPEL, LIND and SCAPEL) (Figure 4.1). Approximately 60% of the locations from where *D. elastica* is known, harbour less than 50 leaves (Conservation 2011).

Drakaea elastica produces only a single leaf and often forms small colonies. *Drakaea glyptodon* is known to form small colonies via clonal reproduction of daughter tubers (Peakall 1990). Prior to this study it was not known if these colonies of *D. elastica* constituted either clones or sexually produced recruits, or both. This has implications for the conservation status of the species as population counts treat each of these leaves as individual genets. This may potentially lead to the overestimation of true population sizes and consequently an underestimation of the conservation status of the species (Hogbin *et al.* 2000).

Within continuous habitats, *Drakaea* orchids typically form scattered populations that are restricted to low-lying open sandy microhabitats, which leads to a naturally patchy distribution (Phillips 2010). *Drakaea elastica* is relatively habitat specific, occupying areas of open, grey sandy soil and fine leaf litter within mixed stands of *Kunzea glabrescens* and *Banksia* woodland (Brown *et al.* 1998; Department of Environment and Conservation 2009; Chapter 2). Continued land clearing is further threatening the species by reducing and fragmenting suitable habitat (Brown *et al.* 1998; Department of Environment and Conservation 2009). Other threats include grazing of leaves and flowers by herbivores, competition with weeds, increasing density of ground-level vegetation and inappropriate fire regimes (Department of Environment and Conservation 2009). Across its range *D. elastica* has similar habitat and microhabitat requirements (Chapter 2) and uses the same mycorrhizal endophyte, which is essential for germination and growth (Phillips *et al.* 2011a). While *D. elastica* requires pollinators for pollination, they are self compatible (Phillips 2010). Pollinator-mediated self-pollination may occur occasionally, as observed in other species of sexually deceptive orchids that are pollinated by thynnine wasps (Peakall and Beattie 1996).

Pollinator behavioural experiments

Following the observation that some *D. elastica* flowers attract different forms of *Z. gilesi*, pollinator baiting experiments were designed to test for differences in pollinator attraction between flowers. Despite the known presence of *Z. gilesi* in the Capel region, the *D. elastica* flowers tested there from the Mandurah region failed to attract the wasp over 132 two minute baiting trials at 15 sites in 2009.

Pollinator baiting was conducted following the methods developed by (Stoutamire 1983) and (Peakall 1990) whereby male wasps can be attracted using presentations of picked orchid flowers. Initially, in 2009 baiting experiments were undertaken in the Mandurah region, however *Z. gilesi* is rare and only occurs in low densities. Subsequently, pollinator behavioural experiments in following years were only conducted in the Capel region as *Z. gilesi* is known to be locally abundant (M.H.M Menz and R.D. Phillips unpublished data). Pollinator baiting was conducted from October to November 2011 and 2012 within a habitat remnant at Ruabon, near Capel (Figure 4.1). This site was selected as *Z. gilesi* wasps occur here reliably and there are no *D. elastica* growing in the vicinity that may interfere with wasp responses to artificially presented flowers.

Due to the conservation significance of *D. elastica*, multiple samples from some populations were not possible. Nonetheless, at least six individual flowers were tested from two populations, one from each of the Mandurah and Capel regions (LAKES and CAPEL), and single flowers from three other populations from the Mandurah region. Populations were selected to determine potential regional variation in the pollinator attracted. To maintain freshness, orchid flowers were stored in a portable refrigerator held at approximately 4°C. Baiting was undertaken following a similar method to the sequential experimental design as described in (Bower 1996; Bower and Brown 2009). Initially, ‘foreign’ Mandurah orchids were presented to the wasps for a period of five minutes. Following this, the ‘local’ Capel orchids were presented as a control for a further five minutes. Flowers from a maximum of two ‘foreign’ populations were presented at any one time. The number of male wasps attempting to copulate with the flowers per minute was recorded. In 2012, baiting was undertaken in a sequential fashion as described above, using three minute trials. Here, we compared the hierarchy of attraction (Peakall 1990) to single flowers from the Mandurah and Capel regions.

Wasps were recorded as approaching the flower to within 5 cm, landing on the flower, or copulating with the flower. Flowers were kept in a sealed container between experiments. Locations were moved between experiments to avoid the well known decrease in wasp responses over time (Peakall 1990; Whitehead and Peakall 2012b) and was restricted to warm (>18°C) and sunny days between 09:00 and 16:00 hours when wasps are most active (Peakall 1990).

Floral odour extraction and GC-EAD

In addition to the pollinator baiting, we used gas chromatography/mass spectrometry and electroantennographic detection (GC/MS–EAD) to identify active semiochemicals in the pheromone bouquet of *D. elastica*. Labella from *D. elastica* flowers were collected from a greenhouse population, arising from the Mandurah region, which since 2007 has been maintained under greenhouse cultivation at the Botanic Gardens and Parks Authority (BGPA), Perth and from wild flowers in the Capel population. Floral odour extraction methods followed that described in (Bohman *et al.* 2012a; Bohman *et al.* 2012b). Briefly, labella were washed in 100 µl of distilled dichloromethane (DCM) for five minutes. The solvent extract was then transferred to a sample vial and stored at -20°C. Male *Z. gilesi* wasps were collected by sweep-netting and baiting with *D. elastica* flowers from Mandurah and Capel respectively. Live wasps were stored in a refrigerator at 4°C prior to analyses.

GC/MS-EAD data were recorded using a HP GCD 1800A equipped with a BPX5 column [(5% phenyl dimethylpolysiloxane), 30 m x 0.25 mm x 0.25 µm film thickness, SGE Australia], using helium as a carrier gas. A GC effluent splitter was used to split the flow to the MS and EAD. The split for EAD was passed through a Syntech effluent conditioner (Syntech, Kirchzarten, Germany) containing a heated transfer line, with the outlet placed in a purified and humidified airstream where the electrodes holding the antenna were presented. For each EAD run, an excised antenna with the tip cut off was mounted on the holder consisting of two electrodes using electrode gel. The electrode was connected to a PC via a Syntech Intelligent Data Acquisition Controller (IDAC2) for recording of EAD signals in the Syntech software package GC-EAD/2011 (<http://gcead.sourceforge.net/download.html>).

Population genetic sample collection and study sites

Leaf samples for genetic analysis were collected from 13 locations within the seven major extant regional populations (including the BGPA glasshouse population), from across the core of the range of *D. elastica* (Figure 4.1). Only populations of greater than 50 leaves were included in the sampling to investigate the population genetic variability present within the major extant populations across the species range (Figure 4.1).

Populations were defined as patches of *D. elastica* plants that were growing within 500 m of one another. Samples were collected across the extent of each population. In order to avoid sampling within potential clones, only a single sample consisting of a section of leaf material was taken from each colony with samples taken from colonies at least 20 cm apart. The location of each leaf sample taken was recorded with a hand-held GPS. Samples were kept in the field at 4°C in a portable refrigerator and stored at -80°C on returning to the laboratory.

Microsatellite cross-transferability validation

DNA extraction and amplification of microsatellite loci was performed as *per* (Anthony *et al.* 2010). A set of 15 microsatellite markers originally isolated from *Drakaea glyptodon* were tested for cross-transferability to *D. elastica* (Anthony *et al.* 2010). Only those markers that amplified, that were repeatable and easy to score were used. As there are potential issues with cross-transferability of markers between species (Peakall *et al.* 1998), the following steps were taken to validate the successful transfer of loci from *D. glyptodon* to *D. elastica*. Firstly, a search was undertaken for outlier loci with a high likelihood of null alleles using MICRO-CHECKER v2.2.3 (van Oosterhout *et al.* 2004). Secondly, GENEPOP 4.0.10 (Raymond and Rousset 1995; Rousset 2008) was used to perform exact tests (Guo and Thompson 1992) for deviation from Hardy-Weinberg Equilibrium (HWE) at each locus across all sampled populations and to test for linkage disequilibrium between loci (Slatkin 1994). A Bonferroni correction was applied to significance levels for multiple tests (Rice 1989). The interpretation of these analyses was made mindful of the assumption that they assume HWE, which may not apply in the case of this self compatible species.

Population genetic variability

GenAlEx 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012) was used for all analyses unless otherwise stated. The number of alleles (N_a), effective number of alleles (N_e), fixation index (F), observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated for each locus and population. Analysis of molecular variance (AMOVA) was used to; partition genetic variation within and among populations and regions; to estimate overall and pairwise population genetic differentiation (F_{ST}); differentiation among regions (F_{RT}); and among populations within regions (F_{SR}) (Wright 1965) following (Excoffier *et al.* 1992; Peakall *et al.* 1995). Tests for departure from the null hypothesis of no genetic differentiation were performed by random permutation (999 permutations). Regions were initially defined geographically to coincide with the remaining strongholds of *D. elastica* situated in the Mandurah and Capel areas (Figure 4.1).

The standardised measures of genetic differentiation F'_{ST} , F'_{RT} and F'_{SR} (Hedrick 2005) were also calculated by AMOVA following the method described in (Meirmans 2006). Standardised estimators of genetic differentiation allow for the comparison of F_{ST} between genetic markers with differing levels of genetic variation (Meirmans and Hedrick 2011; Phillips *et al.* 2012). The standardised estimator of genetic variation Jost's D_{est} (Jost 2008) was calculated following the formula presented in (Meirmans and Hedrick 2011). Jost's D_{est} is based on the effective number of alleles instead of heterozygosity, as with other measures of genetic differentiation (Jost 2008).

Isolation by distance at the population level was tested using a Mantel test (Mantel 1967). This analysis requires the input of two pairwise matrices of geographic distance (in metres) and genetic distance (F_{ST} by AMOVA) between populations. Departure from the null hypothesis of no significant relationship between genetic and geographic distances was performed by random permutation (10,000 permutations) (Smouse *et al.* 1986; Smouse and Long 1992).

The program STRUCTURE v2.3.4 was used to assign individuals to clusters based on multilocus genotype data (Pritchard *et al.* 2000). STRUCTURE implements the Bayesian Markov Chain Monte Carlo (MCMC) method to assign individuals to k clusters. We used the admixture model, with allele frequencies correlated among

populations as recommended by (Falush *et al.* 2003) for populations which may exhibit subtle structure. Burn-in was set to 10,000 iterations, followed by 100,000 MCMC iterations and replicated 10 times for each value of k , from one to eight. The program STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used to determine the optimal value of k , based on the level at which the mean logarithm of the probability of the data [$\ln P(k)$] reached a plateau, and based on the second order rate of change of the likelihood function (Δk) as *per* (Evanno *et al.* 2005).

Clonality and spatial autocorrelation analysis

To investigate the presence of clonality in *D. elastica*, a total of 15 colonies were randomly selected from wild populations. In addition, 14 colonies from the BGPA greenhouse collection were also sampled. Clusters consisted of two to 10 leaves (mean = 3.93) in the wild populations and two to eight leaves (mean = 3.64) in the greenhouse population. All leaves from each cluster were sampled and genotyped. Pairwise distances between leaves within each cluster were measured to the nearest 1 cm.

Fine-scale spatial autocorrelation analysis was conducted following the methods described in (Smouse and Peakall 1999; Peakall *et al.* 2003; Smouse *et al.* 2008). This analysis computes an autocorrelation coefficient (r , range -1 to 1) based on two input matrices of pairwise geographic and multilocus genetic distance between individuals for a number of specified geographic distance classes (Peakall *et al.* 2003). Distances ranged from 0.1 m, representing the within colony distance, to within populations (100 m). The spatial autocorrelation analysis was run in two stages, the first included ramets sampled as putative clones, and the second with these samples removed. Pairwise multilocus genetic distance was calculated following (Smouse and Peakall 1999). The 95% confidence intervals around the hypothesis of no spatial genetic structure ($r = 0$) were generated using random permutations (1,000 permutations). The 95% confidence intervals around estimates of r were calculated by 1,000 bootstrap repeats (Peakall *et al.* 2003).

RESULTS

Pollinator behavioural experiments

Results of 23 sequential trials in the Mandurah region in 2009 resulted in only four responses from male *Zaspilothynnus gilesi* wasps. Of these, three responses were to *D. elastica* flowers from Mandurah, and one was to *D. elastica* flowers from Capel. The wasp approached the Capel flowers, but did not land, conversely all responses to Mandurah orchids resulted in attempted copulation with the flower.

The pollinator baiting experiments in 2011 revealed that Capel *Z. gilesi* respond, and attempt copulation with *D. elastica* from Capel ($N = 16$) (Figure 4.2). In contrast *D. elastica* from the Lakes, Serpentine River, and Paganoni areas in the vicinity of Mandurah elicit no response from Capel *Z. gilesi* (Figure 4.2). Unexpectedly, Capel *Z. gilesi* also attempted copulation with the flower from Carabungup Nature Reserve ($N = 28$) (Figure 4.2), approximately 13 km south of Mandurah. The median number of wasps attracted per minute was not significantly different between the *D. elastica* flowers from Capel and Carabungup (Mann-Whitney *U*-test, $Z = 1.40$, ns). Wasps were recorded alighting on the labellum and attempting to copulate with the flower, and some were observed to contact the stigma, criteria previously used to define effective pollinators in *Drakaea* by (Phillips 2010). Wasps were also captured carrying *Drakaea* pollen.

The pollinator baiting experiments at Capel in 2012 (15 trials) resulted in a total of 114 responses and supported the results from 2011. Of these, 99.1% ($N = 113$) were to the Capel *D. elastica* flower and 0.9% ($N = 1$) was to the Mandurah *D. elastica* flower. Responses to the Capel flower resulted in 53.1% ($N = 60$) attempted copulations, 20.4% ($N = 23$) landings and 26.5% ($N = 30$) approaches (within 5 cm). The single response to the Mandurah flower resulted in an attempted copulation.

Turner (1910) recognised two variants of *Z. gilesi*, which he defined as *a* and *b*. Males of variant *b* are recognised from nominate *Z. gilesi* by possessing larger abdominal spots, and ferruginous femora (Turner 1910). Capel *Z. gilesi* responding to *D. elastica* from Capel and Carabungup NR were identified as the nominate form, while those responding to orchids at Lakes Road as appeared to correspond to variant *b*, based on

the morphological descriptions in (Turner 1910). These wasps at Lakes Road were also observed to pseudocopulate with *D. elastica* and contact the stigma. *Zaspilothynnus gilesi* of both the nominate form and variant *b* have been observed to visit wild orchids.

Floral odour and GC-EAD

Antennae of males *Z. gilesi* from Capel responded to compounds in the floral extracts from Mandurah and Capel *D. elastica*. There was however a different EAD response to the compounds in the odour samples. Wasp antennae responded to the following compounds present in both Mandurah and Capel *D. elastica* floral extracts: ‘Compound 1’ (retention time 8.2 min), ‘Compound 2’ (retention time 11.3 min), ‘Compound 4’ (retention time 13.5 min) and ‘Compound 5’ (retention time 14.7 min) (Figure 4.3). However, there was an additional EAD response to ‘Compound 3’ (retention time 13.3 min) in the Mandurah *D. elastica* extract, which is not present in the Capel *D. elastica* (Figure 4.3). All EAD responses were repeated on at least three different runs to confirm the results.

Microsatellite cross-transferability validation

Of the 15 microsatellite loci isolated from *D. glyptodon* (Anthony *et al.* 2010), ten amplified with polymorphic alleles, one was monomorphic, one was unable to be scored, and three did not amplify for all the individuals used in the trial. Eight of the remaining 10 loci were selected for the study. Two of these loci (A116 and A108) showed evidence of null alleles at most populations, high fixation index (*F*) values (0.294 and 0.618 respectively) and evidence of linkage disequilibrium. Consequently, these two loci were excluded from all subsequent analyses, which resulted in six loci being retained for further analysis.

In *D. glyptodon*, these loci are known to exhibit genotypes typical of diploids (Anthony *et al.* 2010). However, more than two alleles were identified in some individuals for some loci, across all of the eight loci (8.54%, *N* = 492 ramets sampled across populations). Triple banding (three alleles) appeared in at least one individual in six of the seven populations. Triple bands may represent triploid individuals, or alternatively, this may be due to genetic mosaicism (Reusch and Boström 2010). As such, samples displaying triple banding patterns were excluded from further analysis. These patterns

of triple banding were repeatable, present in all individuals of a clonal collection, and not due to accidental mixture of DNA and PCR cross-contamination.

Population genetic variability

The final population genetic analyses were based on the 331 individual *D. elastica* samples that exhibited diploid banding patterns. These were sampled from seven populations and genotyped using six polymorphic microsatellite loci. At the locus level, the number of alleles (N_a) ranged from seven (C110) to 28 (B102) with a mean of 10.4 (Table 4.1). Expected heterozygosity (H_E) ranged from 0.543 (C110) to 0.876 (B102) (mean = 0.780) (Table 4.1). Following a Bonferroni correction, significant linkage disequilibrium ($P < 0005$) was detected in only five out of 105 tests, with no consistent pairs of linked loci.

Across loci and within populations, the mean number of alleles (N_a) ranged from five to 23 (Table 2). Mean levels of H_O for populations ranged from 0.673 to 0.827, and levels of H_E ranged from 0.741 to 0.828 (Table 4.2). Lower H_O than H_E was observed at all seven populations giving positive F values (Table 4.2).

Pairwise estimates of population genetic differentiation were low, but significant in all except two cases (Table 4.3; $F_{ST} = 0.026$, $F'_{ST} = 0.126$, $D_{est} = 0.106$). The Principal Components Analysis (PCoA) explained 96.06% of the variation in the first three axes (83.35% in the first two) and revealed north–south population groupings. This reflected the geographic distribution of the populations with one exception; the population at Carrabungup Nature Reserve (CARA) clustered with the southern (Capel) populations of *D. elastica* and not with those from the north (Mandurah), to which it is geographically close (Figure 4.1). Based on the pollinator behavioural experiments and the PCoA, the population CARA was repositioned within the southern region for a subsequent AMOVA. The resulting AMOVA attributed 2% of variation among regions, 1% among populations within regions and 97% within populations (Table 4). A regional AMOVA with populations grouped geographically attributed only 1% of variation among regions, 2% among populations within regions and 97% within populations. Significant genetic differentiation was found among regions ($F_{RT} = 0.019$, $F'_{RT} = 0.1$, $P = 0.001$), and among populations between regions ($F_{SR} = 0.013$, $F'_{SR} = 0.066$, $P = 0.001$)

(Table 4.4). The Mantel test revealed no evidence of isolation by distance between populations (F_{ST} , $R_{xy} = -0.134$, $P = 0.251$).

Based on the STRUCTURE results, the optimal number of clusters was at $k = 2$, where $\ln P(k)$ reached a plateau (Appendix Figure A4.1). Additionally, Δk peaked at $k = 2$ (Appendix Figure A4.2), and minimal additional information was gained by increasing k (Appendix Figures A4.2, A4.3). In corroboration with results of the PCoA, populations showed evidence of two geographic clusters with some overlap (Figure 4.4). Mean membership values (q -mean) for Cluster 1 ranged from 0.814 to 0.861 for the Mandurah populations, and from 0.164 to 0.272 for the Capel populations. Conversely, q -mean for Cluster 2 ranged from 0.139 to 0.187 for the Mandurah populations and 0.728 to 0.836 for the Capel populations (Figure 4.4). The population CARA, while geographically closer to the Mandurah region shows a pattern of admixture, with a q -mean of 0.549 and 0.451 for Cluster 1 and 2, respectively.

Spatial autocorrelation analysis and clonality

There were no cases where discrete multilocus genotypes were shared among populations and no cases of genotype sharing among samples greater than 20 cm apart. Across the extent of the study, of the 110 leaves sampled within colonies of less than 20 cm as putative clones, 87.3% ($N = 96$) shared multilocus genotypes indicative of clonality. Further, no individuals sampled more than 12.5 cm apart were identified as clones. The median proportion of clones across colonies was significantly higher in the wild samples compared to greenhouse samples (Mann-Whitney U -test, $Z = -3.457$, $P = 0.001$).

Spatial autocorrelation analysis revealed significant fine-scale spatial genetic structure in the Capel population for both data sets, with and without putative clones (Figure 4.5). The first distance class (0.1 m) represents within-colony comparisons and showed significant positive spatial genetic structure in both cases (Figure 4.5). Results that included putative clones showed significant positive spatial structure up to a distance of 1 m (Figure 4.5A). When putative clones were removed from the analysis, significant positive spatial structure remained (Figure 4.5B). However, r was reduced in the first distance class from 0.857 to 0.393, with significant positive spatial structure up to a

distance of 10 m and with an x -intercept of 17.16 m (full results of autocorrelation analysis are presented in Appendix Table A4.1).

DISCUSSION

A recent taxonomic revision of *Drakaea* detected no morphological differences between populations of *D. elastica* across its range (Hopper and Brown 2007). This study provides evidence of two distinct yet morphologically indistinguishable entities within the rare orchid *Drakaea elastica*. Pollinator baiting revealed differences in the pollinator attracted by different *D. elastica* flowers, which was corroborated by differences in the response of these wasps to chemicals within the odour bouquet of *D. elastica*. While overall population genetic differentiation was low ($F_{ST} = 0.026$), this difference reflects both regional and population level differentiation. The STRUCTURE analysis revealed two distinct genetic clusters, with some overlap. The previously unknown *D. elastica* chemotypes may only be in the early stages of speciation with defined morphological and genetic differences yet to be revealed (Bower 1996).

Pollinator differences and chemical variation

An unexpected finding was that the population at Carabungup (CARA) was genetically clustered with the Capel population, 160 km to the south. This was despite its close geographic proximity to populations in the Mandurah region (13 km apart). This result was further supported by the pollinator baiting experiments, which revealed that the *D. elastica* flower tested from the Carabungup population attracted and elicited pseudocopulation from *Z. gilesi* in the Capel region. Additionally, the *D. elastica* flowers tested from the Mandurah region elicited only a single response from *Z. gilesi* at Capel. However, this pseudocopulatory response was potentially a mistake, as it occurred during a period of high wasp activity and the wasp didn't initially approach the flower. This does however indicate that there is little morphological distinction between the orchids and the method of pollinator discrimination is primarily chemical.

This difference in attractiveness was supported by floral odour analysis which determined that *Z. gilesi* from Capel respond differently to floral extracts from Mandurah *D. elastica*. There are four EAD-active compounds common to *D. elastica* floral extracts from both Mandurah and Capel. However, the floral odour bouquet of

Mandurah *D. elastica* flowers contains an additional EAD-active compound to those from Capel. Given the pollinator behavioural responses, it is likely that this compound is inhibitory to Capel *Z. gilesi*. The identity of most of these compounds is unknown, though the investigation of these is part of an ongoing research program. At least one of the EAD-active compounds in the floral odour of *D. elastica* represents a novel pyrazine. This is related to the compounds recently identified as important components of the sex pheromone system of thynnines and are now known to be involved in pollinator attraction to the orchid *Drakaea livida* (Bohman *et al.* 2012a; Bohman *et al.* 2012b). Consequently, the flowers tested appear to represent distinct ‘chemotypes’.

One case of multiple chemotypes within a species of *Drakaea* has recently been identified (Bohman *et al.* 2012a; Bohman *et al.* 2012b). Morphologically cryptic species have been revealed in both European *Ophrys* (Paulus and Gack 1990), Australian *Chiloglottis* (Bower 1996; Bower 2006; Bower and Brown 2009; Peakall *et al.* 2010) and *Caladenia* (Phillips *et al.* 2009) orchids through the use of pollinator choice experiments and chemical odour analysis. In orchids, previously reported population-level differences in the pollinator attracted have subsequently been recognised to represent different orchid species (Bower 2006; Bower and Brown 2009; Peakall *et al.* 2010). Speciation in sexually deceptive orchids is hypothesised to be driven primarily by the process of pollinator-switching based on floral odour variation (Nilsson 1992; Bower 1996; Peakall *et al.* 2010; Ayasse *et al.* 2011; Xu *et al.* 2011). Therefore, it is likely that these entities will remain morphologically and even genetically cryptic during early stages of speciation (Bower 1996).

Zaspilothynnus gilesi from the Mandurah and Capel regions were identified as two distinct variants based on colour of the femora and the size of the abdominal spots, recognised by (Turner 1910). While Turner (1910) did not recognise these as distinct species, these reported morphological differences are consistent between the two. The status of these two variants requires taxonomic investigation to confirm if they represent two separate species. These two forms occur sympatrically in the greater Perth-Mandurah area (M.H.M. Menz and R.D. Phillips unpublished data). Given the highly specific nature of pollination relationships in *Drakaea* (1.1 pollinators per species, (Phillips 2010) these two entities within *Z. gilesi* need to be managed separately, as part of the overall conservation of this threatened interaction.

Population genetic variability

The unexpected pollinator and chemistry findings are tentatively supported by the genetic evidence. Even with the relatively low levels of population genetic differentiation recorded in this study, it still provided an indication of significant regional genetic structure. Orchids are typically known for their patterns of low population genetic differentiation (Forrest *et al.* 2004; Tremblay *et al.* 2005). In a recent meta-analysis, (Phillips *et al.* 2012) found that mean genetic differentiation in the Orchidaceae ($F_{ST} = 0.146$) based on allozyme loci is the third lowest reported for well studied plant families. The low level of overall population genetic differentiation for microsatellite loci reported in this study ($F_{ST} = 0.026$) is low for orchids in general (Phillips *et al.* 2012). However, this is similar to that reported for *D. glyptodon* ($F_{ST} = 0.027$, R.D. Phillips unpublished data) and *Caladenia huegelii* [$F_{ST} = 0.047$ (Swarts *et al.* 2009)].

Population genetic studies on orchids across a scale greater than 250 km tend to show patterns of isolation by distance (Phillips *et al.* 2012). However, the lack of evidence of isolation by distance in this study confers with results from other population genetic studies on sexually deceptive orchids over similar scales in southwestern Australia (Swarts *et al.* 2009). The low level of population genetic differentiation and lack of isolation by distance in this study may result from these populations being historically connected by pollen and seed dispersal prior to habitat fragmentation, with genetic divergence between populations through genetic drift yet to arise. This pattern of low geographic genetic differentiation is consistent with the hypothesis that large-scale population connectivity in orchids is primarily driven by the long distance dispersal potential of their dust-like seed (Arditti and Ghani 2000; Phillips *et al.* 2012). These rare long distance seed dispersal events are revealed by the propensity for vagrancy of orchids (Arditti and Ghani 2000).

Additionally, pollinator behaviour affects patterns of pollen movement and outcrossing in plants (Ghazoul 2005; Trapnell and Hamrick 2005). It is hypothesised that pollination by sexual deception results in increased outcrossing due to deceived pollinators leaving an area (Peakall 1990; Peakall and Beattie 1996). However, it will ultimately be determined by the mate-searching behaviour of the pollinator. The genetic evidence supports the predominance of outcrossing in this system (mean \pm SE, $F = 0.048 \pm$

0.017). A capture-mark-recapture study on the mate-searching behaviour of *Z. gilesi* reported a median movement distance of 38 m, and a maximum of 556 m (Chapter 3). While this maximum is up to five times greater than that recorded for other thynnine pollinators of sexually deceptive orchids (Peakall 1990; Peakall and Beattie 1996; Whitehead and Peakall 2012a; Whitehead and Peakall 2012b); M.H.M. Menz unpublished data), the majority of movements were less than 100 m (Chapter 3). Consequently, the majority of pollen movements will be within populations of *D. elastica* with few movements between populations in continuous habitats (Chapter 3). Thus, population connectivity via pollen will be limited in isolated or fragmented populations.

Fine-scale spatial genetic structure and clonality

Significant fine-scale spatial genetic structure was detected up to a distance of 10 m. Similar patterns of fine-scale spatial genetic structuring have been reported in other studies on both terrestrial (Peakall and Beattie 1996; Chung *et al.* 2004a; Chung *et al.* 2004b; Chung *et al.* 2005) and epiphytic orchids (Tremblay and Ackerman 2001; Trapnell *et al.* 2004). Exclusion of identified clones based on multilocus genotypes still resulted in significant positive fine-scale genetic structure. The observed pattern of positive fine scale spatial genetic structure is not expected from the pollinator movement data (Chapter 3). Consequently, this pattern is likely driven by local-scale seed dispersal and availability of the mycorrhizal endophyte. Despite the potential for long-distance seed dispersal (Arditti and Ghani 2000), experimental studies have shown that the majority of orchid seeds fall close to the maternal plant, usually within 10 m (Ackerman *et al.* 1996; Murren and Ellison 1998; Jacquemyn *et al.* 2007; Jersáková and Malinová 2007). Further, orchid mycorrhizal endophytes are known to be patchily distributed, even at the scale of centimeters (Brundrett *et al.* 2003; Phillips *et al.* 2011a). This has been hypothesised to result in an increased chance of seedling germination in close proximity to the maternal plant (Diez 2007; Swarts *et al.* 2009).

Conservation implications

Of particular interest is that within this system there may in fact be up to four distinct entities involved. In this study we have presented evidence of two distinct chemotypes within *D. elastica* that elicit different pollinator responses. Further, *Z. gilesi* from the

Mandurah and Capel regions appear to correspond to two distinct morphological variants as described by (Turner 1910). Collective evidence from this study suggests that all populations of *D. elastica* need to be conserved until these cryptic entities are further understood using alternative methods. Other methods are available that could be explored. For example, alternative genetic markers such as may be found in chloroplast DNA may provide further resolution (Ebert and Peakall 2009). Additionally, single labellum extracts of flowers may allow identification of individual cryptic floral chemotypes within populations. Population level differences in pollinator attraction in *D. elastica* could lead to pollinator-mediated reproductive isolation, as seen in a number of other orchid species (Bower 1996; Bower 2006; Bower and Brown 2009; Peakall *et al.* 2010; Xu *et al.* 2011; Bohman *et al.* 2012a; Bohman *et al.* 2012b). However, the nature of cryptic species may mean these distinctions are difficult to resolve.

Conservation management actions such as seed collection should only involve wild open-pollinated seed and hand-pollinations should be avoided due to the potential for mixed populations containing individuals of both chemotypes. Planned translocations and introductions resulting from conservation programs should take place only within areas known to harbour the similar chemotype.

Importantly, if clonal structure is not accounted for it will lead to an overestimation of the effective population size (Sydes and Peakall 1998). Further, there is a need to understand the demography of populations, patterns of recruitment and incidence of clone formation in *D. elastica* as part of understanding the overall resilience of the species in a highly fragmented landscape. High levels of clonality and longevity in orchids could potentially confer some resilience to lower pollinator abundance (Bond 1994; Pauw and Bond 2011; Pauw and Hawkins 2011), but could also temporarily mask demographic processes that could ultimately lead to population extinction (Kuussaari *et al.* 2009). Consequently, pollination success, and not just population size should be taken into account when developing management plans for *D. elastica*.

Highly specific pollination interactions can pose significant conservation challenges as practitioners need intimate knowledge of all species involved (Kearns *et al.* 1998; Menz *et al.* 2011). The revelation of individual level differentiation in the pollinator attracted adds further complexity to the conservation of this interaction as both *D. elastica* and *Z. gilesi* now need to be treated as containing two separate entities. Additionally, there is

the potential for mixed populations of *D. elastica* that include individuals of both cryptic chemotypes. Confirmation of this will require more extensive chemical sampling of single labellum extracts from multiple individuals within each population. The occurrence of a relatively large number of rare species of sexually deceptive orchids, which often occur in patchy populations, indicates that cryptic entities within these may be more broadly occurring than originally considered.

Table 4.1. Average genetic variation of six polymorphic microsatellite loci isolated from *Drakaea glyptodon* and successfully transferred to *Drakaea elastica* see (Anthony *et al.* 2010). Presented are locus code and GenBank accession number, number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_O), expected heterozygosity (H_E), fixation index (F).

Locus	N_a	Mean N_e	H_O	H_E	F
D3 (GU560054)	14	4.325	0.742**	0.763	0.031
B109 (GU560051)	24	6.363	0.820*	0.837	0.019
B102 (GU560048)	28	8.347	0.776***	0.876	0.114
A114 (GU560046)	13	5.125	0.734**	0.802	0.082
C110 (GU560053)	7	2.383	0.532*	0.540	0.025
D104 (GU560057)	23	7.251	0.844***	0.860	0.019
Mean	10.357	5.632	0.741	0.780	0.048

* Significant ($P < 0.05$) departure from HWE at 1 population, ** 2 populations, *** 3 populations.

Table 4.2. Genetic variation from seven populations of *Drakaea elastica* for six polymorphic microsatellite loci. Number of individuals sampled (N), Estimated population size (number of leaves including juveniles) (N Est.), number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F). Populations are arranged north to south and arranged by geographic region. NR = Nature Reserve.

Population (CODE)	N	N Est.	N_a range	Mean N_e	H_O	H_E	F
Paganoni NR (PAG)	22	1476	5-14	5.307	0.717	0.741	0.028
Serpentine River NR (SERP)	69	420	5-17	5.979	0.732*	0.765	0.051
Lakes Road (LAKES)	43	64	6-15	5.643	0.760*	0.771	0.009
Carabungup NR (CARA)	22	819	5-12	4.979	0.736*	0.778	0.061
Capel (CAPEL)	135	1284	7-23	6.013	0.753***	0.811	0.071
Capel south (SCAPEL)	22	210	7-13	6.374	0.827***	0.828	-0.006
Lindberg Road (LIND)	17	119	10-17	5.174	0.673**	0.769	0.120

* Significant ($P < 0.05$) departure from HWE at 1 locus, ** 2 loci, *** ≥ 3 loci.

Table 4.3. Pairwise comparisons of F_{ST} (below diagonal) and D_{est} (above diagonal) between populations of *Drakaea elastica*.

	PAG	SERPNR	LAKES	CARA	CAPEL	SCAPEL	LIND
PAG		0.064	0.022*	0.051	0.125	0.169	0.236
SERPNR	0.019		0.034	0.114	0.131	0.126	0.180
LAKES	0.008*	0.011		0.070	0.113	0.114	0.225
CARA	0.016	0.029	0.021		0.050	0.092	0.137
CAPEL	0.030	0.031	0.028	0.010		0.014*	0.109
SCAPEL	0.039	0.029	0.028	0.016	0.003*		0.066
LIND	0.078	0.061	0.075	0.039	0.036	0.024	

* Not significant ($P > 0.05$).

Table 4.4. Regional analysis of molecular variance (AMOVA) for seven populations of *Drakaea elastica*. Regions are based on geographic placement of the populations, aside from CARA which is placed with the southern populations. d.f. = degrees of freedom.

Source of variation	d.f.	Variance component	% of variation	<i>P</i>
Among regions	1	0.047	2	0.001
Among populations	5	0.036	1	
Within populations	655	2.404	97	
Total	661	2.486	100	

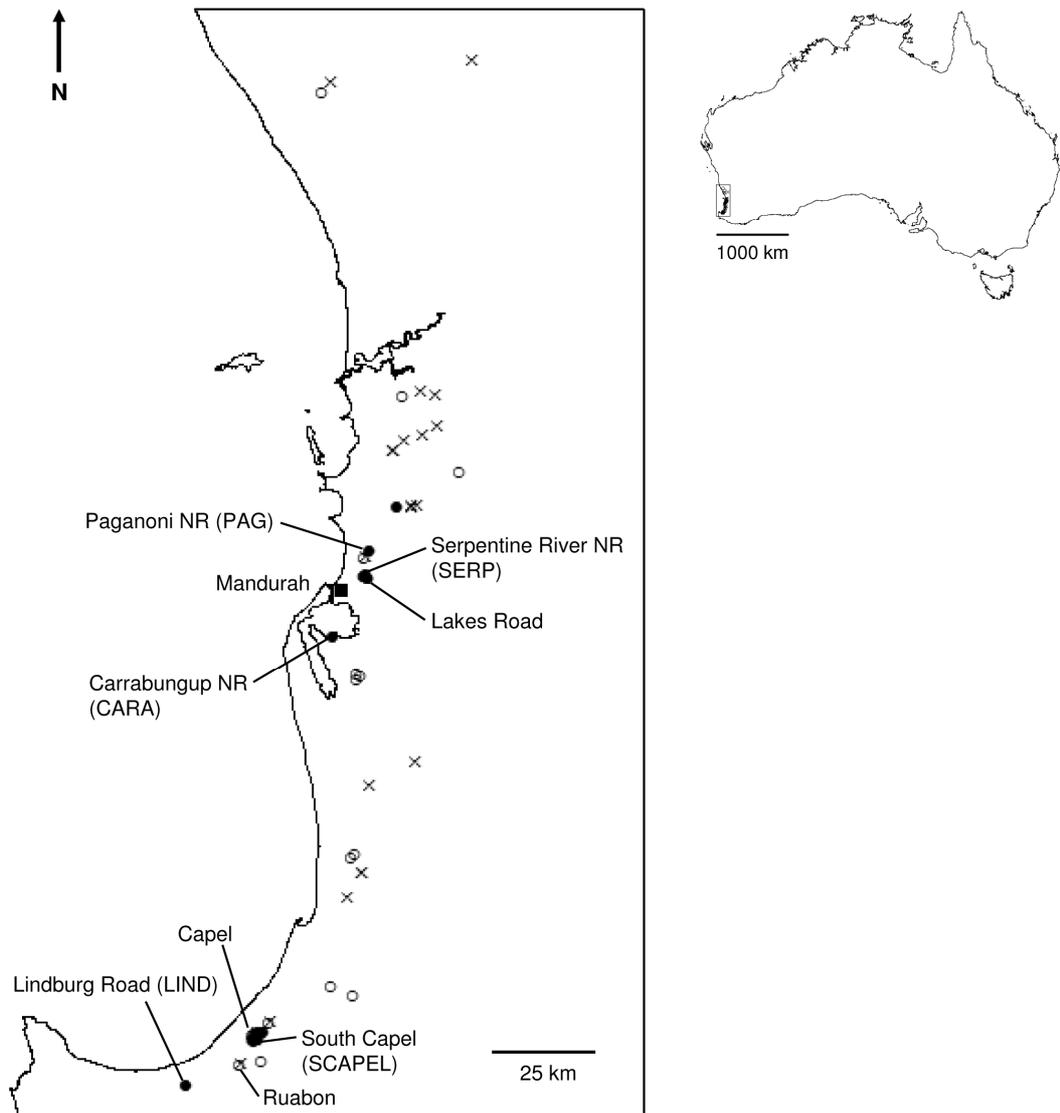


Figure 4.1. Location of all known populations of *Drakaea elastica* and place names referred to in the text. Solid circles indicate populations with greater than 50 leaves (including juveniles); open circles indicate populations with less than 50 leaves (including juveniles), crosses indicate extinct populations; and solid squares indicate to place names referred to in the text.

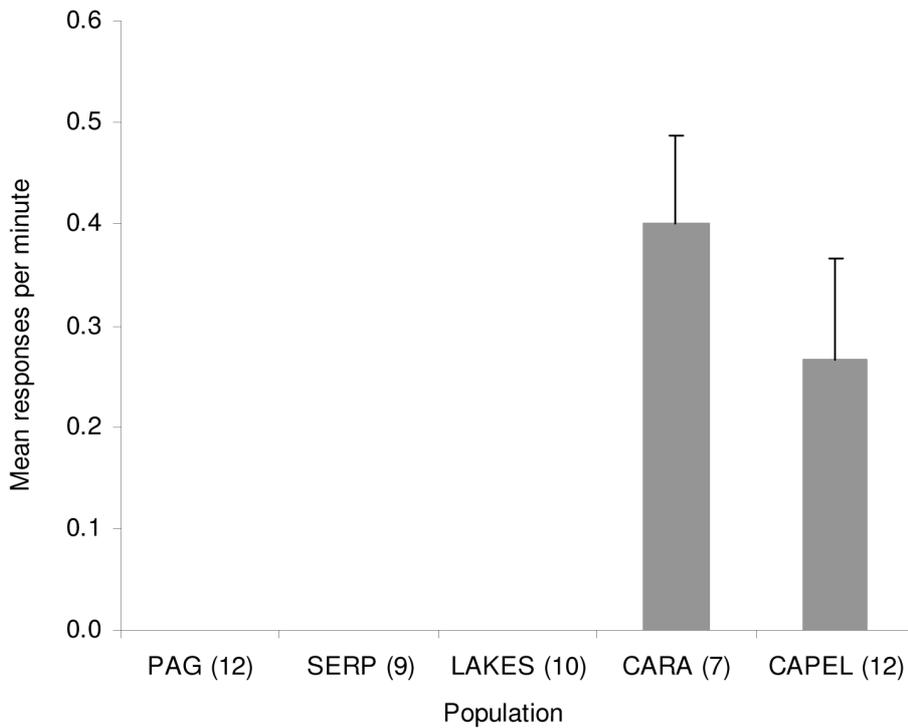


Figure 4.2. Mean responses per minute of male *Zaspilothynnus gilesi* wasps to artificially presented fresh *Drakaea elastica* orchid flowers from five populations. Mean responses per minute during sequential trials where the ‘foreign’ orchid is presented for the first five minutes, followed by addition of the ‘local’ Capel orchid. Populations are sorted geographically from north to south: Paganoni Nature Reserve (PAG), Serpentine River Nature Reserve (SERP), Lakes Road (LAKES), Carabungup Nature Reserve (CARA), and Capel (CAPEL). Number of baiting trials is presented in parentheses.

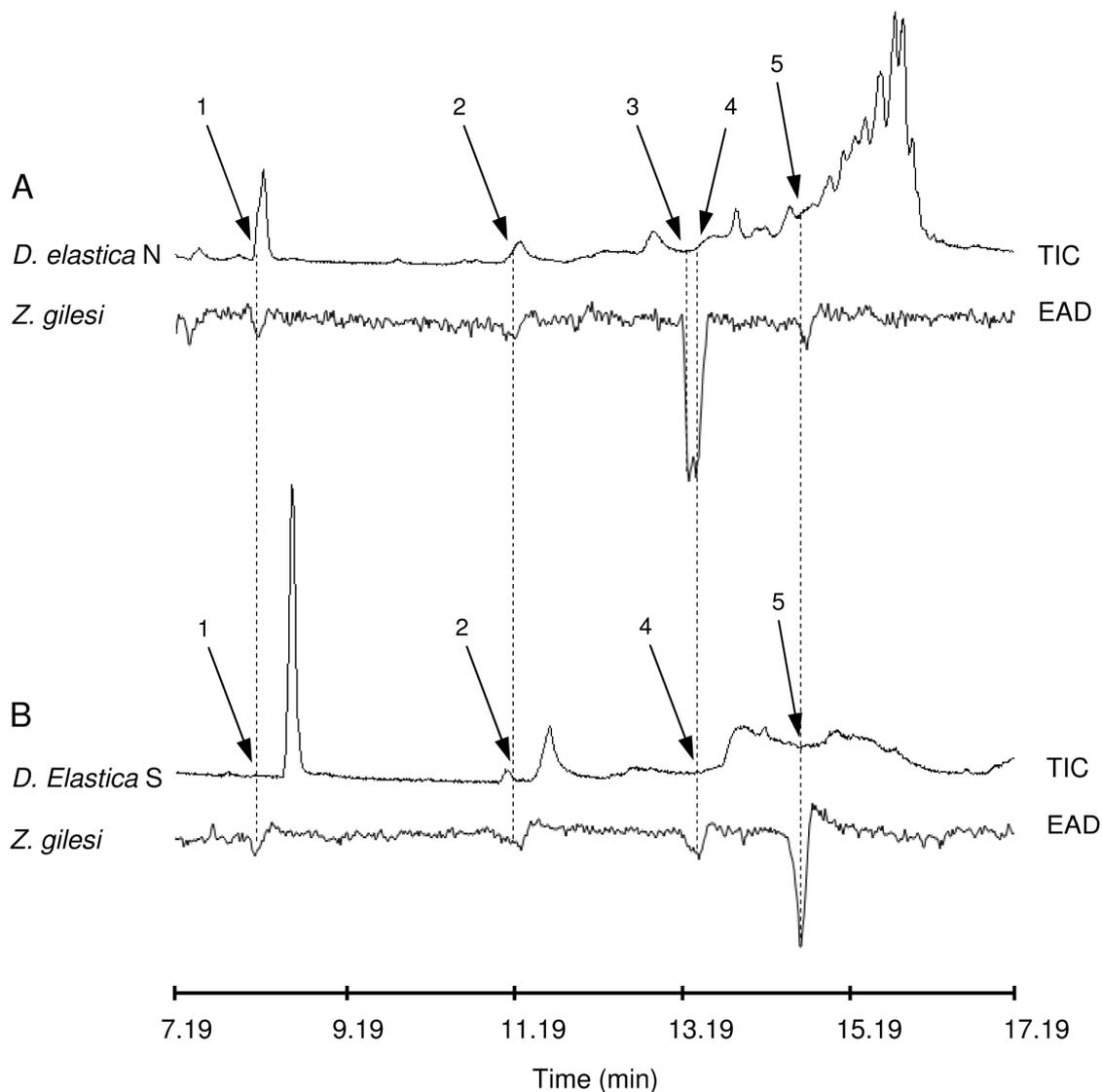


Figure 4.3. Gas chromatography/mass spectrometry with electroantennographic detection recordings of *Zaspilothynnus gilesi* from Capel (south) to floral extracts of *Drakaea elastica* flowers from (A) Mandurah (LAKES) and (B) Capel. Total ion chromatogram (TIC) peaks represent compounds present in the floral extracts. Peaks do not necessarily represent the active compounds. Electroantennographic detector (EAD) peaks represent wasp olfactory responses to specific compounds.

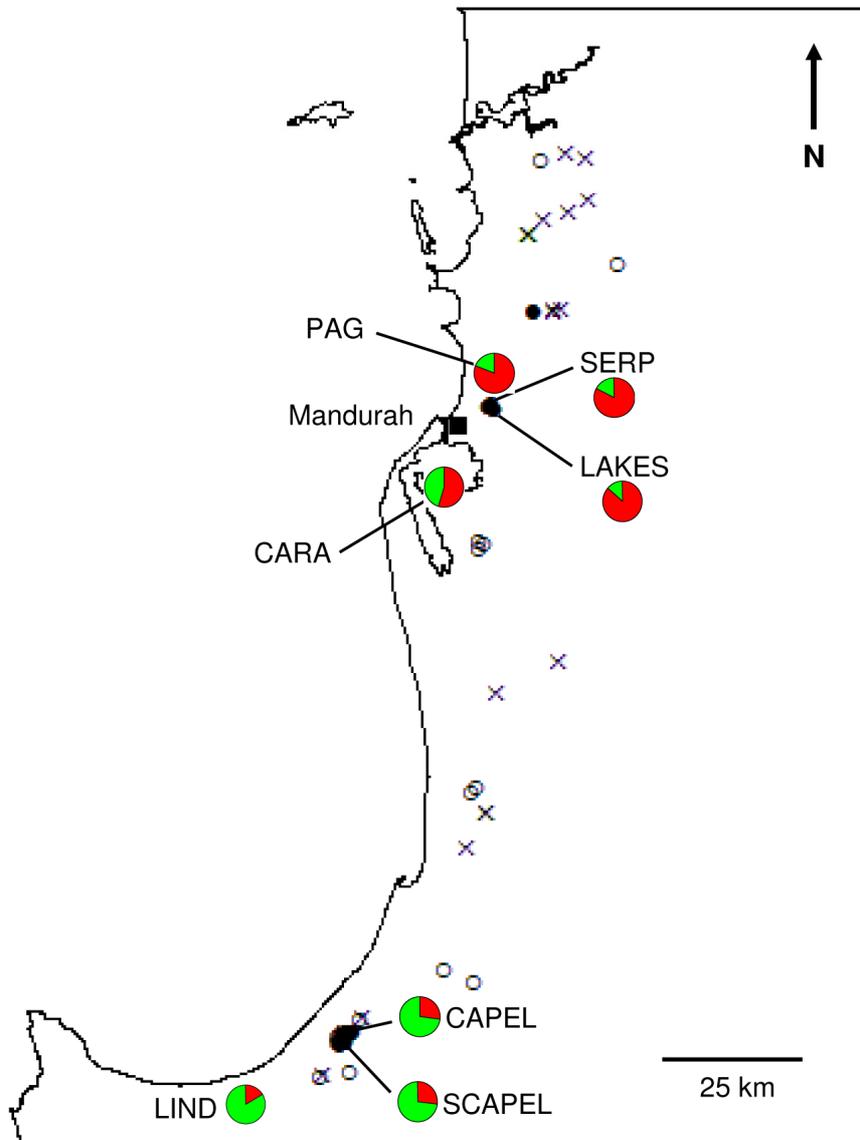
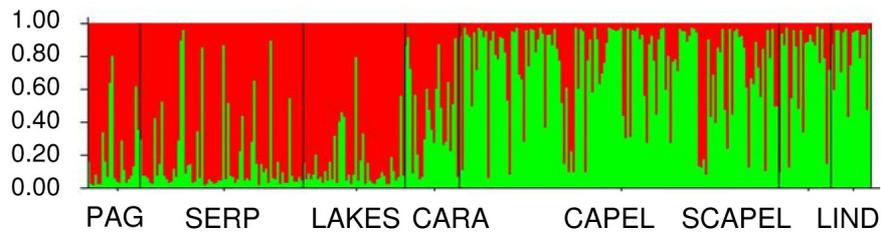


Figure 4.4. Genetic clustering of seven study populations of the rare orchid *Drakaea elastica*. Coloured bars show the proportion of each individual multilocus genotype represented in each cluster ($k = 2$). Pie charts denote membership of each population in each of the two clusters.

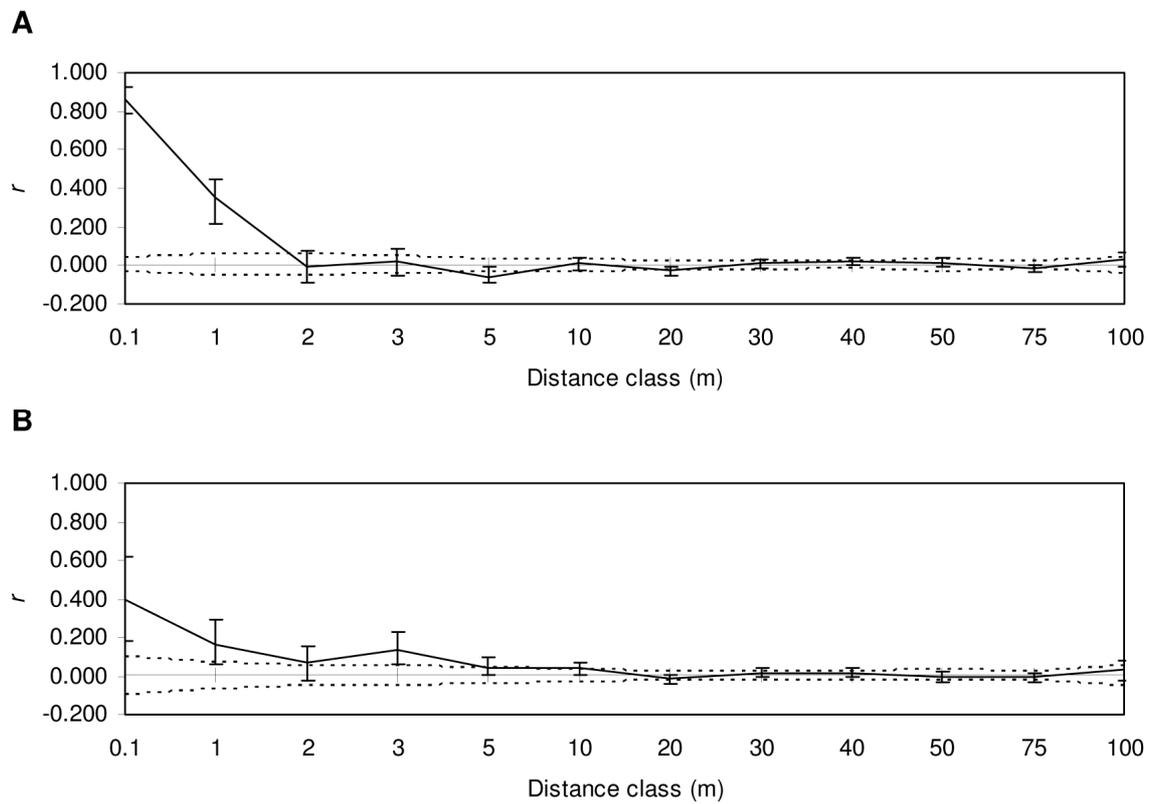


Figure 4.5. Correlogram plots showing the results of spatial autocorrelation analyses between all genotyped individual *Drakaea elastica* from the Capel population. 95% error bars are based on 1000 bootstrap repeats. (A) Including putative clones ($N = 176$); (B) Without putative clones ($N = 130$). Correlograms depict the autocorrelation coefficient r for each distance class. Dotted lines represent the 95% confidence interval about the null hypothesis of no spatial genetic structure based on 1000 permutations.

APPENDIX

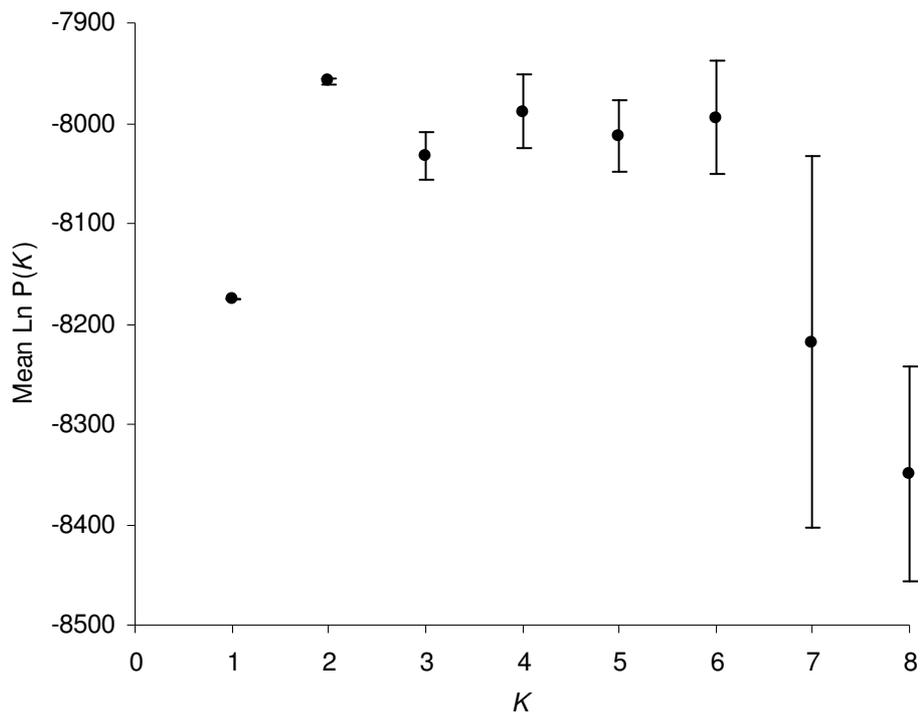


Figure A4.1. Mean log likelihood of k [Ln P(K)] for k clusters.

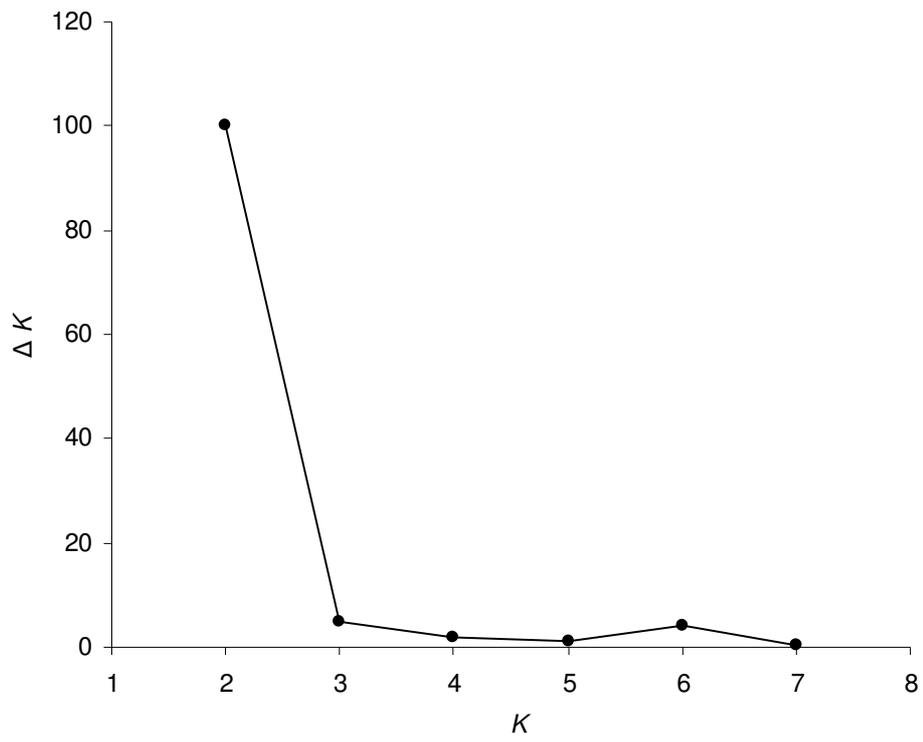


Figure A4.2. Second order rate of change of the likelihood distribution (Δk) as a function of k clusters.

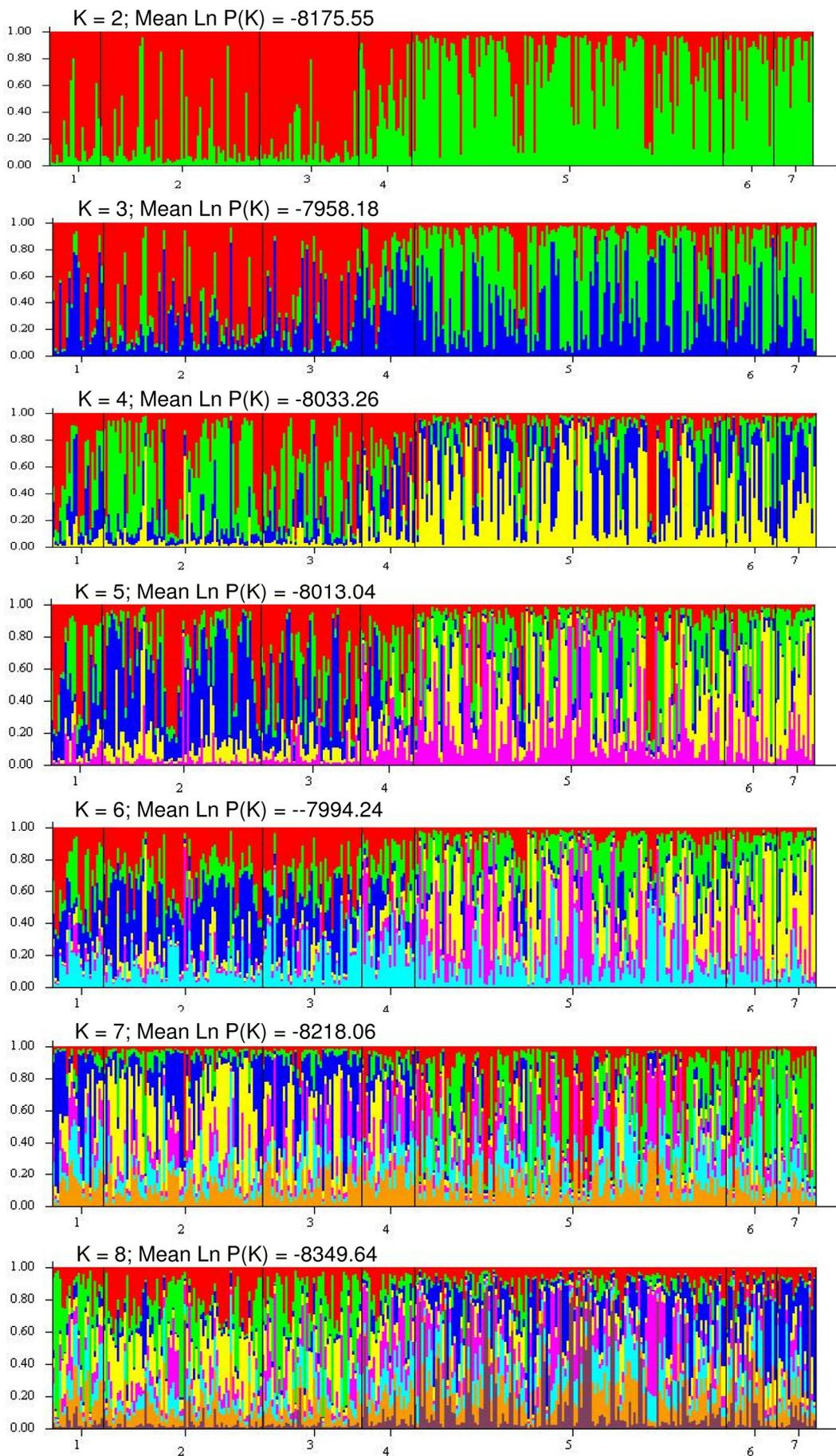


Figure A4.3. Results of Structure analysis for *Drakaea elastica* for $k = 2$ to 8 .

Table A4.1. Results of fine-scale spatial genetic autocorrelation analysis for the Capel population of *Drakaea elastica* with and without putative clones. The autocorrelation coefficient r is presented for 12 distance classes, from within colony (0.1 m) to within population (100 m). N indicates the number of pairwise comparisons, U and L are the upper and lower limits for the 95% confidence interval around the null hypothesis of no spatial structure estimated by random permutation. Ur and Lr represent 95% error bars for estimates of r calculated by bootstrapping. P indicates the probability of a one-tailed test for positive autocorrelation.

Distance Class (End Point m)	0.1	1	2	3	5	10	20	30	40	50	75	100	Intercept
With putative clones													
N	118	40	50	70	120	169	348	313	315	176	313	70	
r	0.857	0.355	-0.010	0.017	-0.057	0.010	-0.028	0.009	0.024	0.017	-0.013	0.031	
U	0.038	0.061	0.055	0.045	0.034	0.030	0.018	0.019	0.019	0.027	0.020	0.044	
L	-0.032	-0.053	-0.052	-0.043	-0.031	-0.030	-0.021	-0.021	-0.020	-0.029	-0.019	-0.043	
Ur	0.926	0.450	0.075	0.084	-0.008	0.041	-0.010	0.029	0.043	0.044	0.004	0.068	
Lr	0.788	0.213	-0.085	-0.050	-0.093	-0.022	-0.048	-0.013	0.005	-0.007	-0.030	-0.005	
$P(r\text{-rand} \geq r\text{-data})$	0.001	0.001	0.609	0.219	0.999	0.240	0.994	0.176	0.008	0.121	0.910	0.075	1.973
Without putative clones													
N	11	24	37	43	61	123	262	248	223	158	149	36	
r	0.393	0.161	0.066	0.138	0.046	0.038	-0.015	0.018	0.015	-0.004	-0.008	0.029	
U	0.098	0.068	0.054	0.049	0.042	0.029	0.019	0.022	0.021	0.028	0.024	0.053	
L	-0.093	-0.065	-0.047	-0.048	-0.040	-0.030	-0.021	-0.020	-0.021	-0.026	-0.026	-0.051	
Ur	0.622	0.295	0.150	0.226	0.097	0.071	0.008	0.040	0.038	0.023	0.016	0.081	
Lr	0.177	0.058	-0.020	0.061	0.000	0.005	-0.038	-0.006	-0.008	-0.030	-0.034	-0.020	
$P(r\text{-rand} \geq r\text{-data})$	0.001	0.001	0.011	0.001	0.017	0.009	0.940	0.060	0.070	0.618	0.726	0.128	17.156

Chapter 5 – General Discussion

Their tendency to form specialised ecological interactions (Swarts and Dixon 2009) makes orchids excellent models for investigating the ecological requirements and other considerations required for developing effective conservation programs for rare and specialised species. Further, given the large number of rare and narrow endemic orchid species worldwide, targeting strategically chosen model systems may offer insights that are broadly applicable. Surprisingly, to date, few studies have employed multidisciplinary approaches to identify the often multiple ecological factors limiting the distributions of rare orchids (Swarts 2007; Swarts and Dixon 2009; Phillips 2010). This study investigated the microhabitat requirements, pollination ecology and population genetic structure of the rare *Drakaea elastica* to inform *in situ* conservation and provide guidance for reintroduction and habitat restoration programs. As one representative of a genus where 50% of the species are declared rare flora (Phillips 2010), the findings of this study will be readily transferable. Furthermore, beyond the study genus, there are many other endangered orchids within the Southwest Australian biodiversity hotspot where the lessons from *Drakaea* may be broadly applicable.

Microhabitat requirements

A major limitation to plant recruitment is the availability of suitable microsites for germination and growth (Eriksson and Ehrlén 1992; Münzbergová and Herben 2005). Consequently, identification of the microhabitat requirements of rare plants can provide insight into the ecological processes limiting populations (Fiedler *et al.* 2007). While a growing number of studies have investigated the microhabitat requirements of rare plants (e.g. Menges and Kimmich 1996; Maliakal-Witt *et al.* 2005; Thompson *et al.* 2006; Schafer *et al.* 2010; Sommers *et al.* 2011), there appear to be very few equivalent studies on orchids. Two examples include the Australian *Thelymitra epipactioides*, which was associated with slightly disturbed microhabitats with an open understorey (Calder *et al.* 1989), and *Platanthera leucophaea* of North America, which occupies sites with varying soil characteristics, depending on substrate (Bowles *et al.* 2005). Thus, this thesis appears to represent one of the few studies to investigate the structural microhabitat characteristics of adult orchids (see also Ackerman *et al.* 1996; Kull 1998).

A regional analysis of the microhabitat requirements of adult *D. elastica* plants revealed variation in microsite characteristics at occupied microsites between regions (Chapter 2). However, there were general trends in common between the regions, indicating conservatism in microhabitat requirements within the species. Habitat modelling identified bare sand as an important predictor of *D. elastica* occurrence in both regions. Further, the requirement for bare sand is associated with the preference for microsites with partial canopy cover (Chapter 2). This microsite preference is not solely linked to the specific microhabitat requirements of the mycorrhizal endophyte, such as soil organic matter and litter cover (Phillips *et al.* 2011a), since the mycorrhizal endophyte is known to occur at microsites unoccupied by adult *Drakaea* (Phillips *et al.* 2011a).

Although an intuitive finding given the habitat preference of *D. elastica*, the formal confirmation of a bare sand habitat requirement in this study has important conservation implications. Not least because sandy bare patches are increasingly at risk of being overgrown by introduced weeds. Furthermore, this risk is exacerbated by the high frequency of control burning that is implemented in southwestern Australia, a process that serves to encourage weed invasion (see below).

Pollinator movement

Pollinator behaviour directly influences patterns of pollen movement and the rate of outcrossing in plants, which are important for gene flow and seed fitness (Groom 1998; Ghazoul 2005). In addition to providing insight into the ecology of pollinators, pollinator behavioural studies can be used to predict potential patterns of pollen movement. In plants pollinated by sexual deception, patterns of pollen movement are affected by the mate-searching movements of male insects, which will vary depending on the pollinator species exploited.

A capture-mark-recapture (CMR) study on *Z. gilesi*, the pollinator of *D. elastica* revealed the majority of wasp movements were less than 100 m, with the capability for flights in excess of 500 m (556 m, Chapter 3). Thus, while movement of pollen may well connect populations in close proximity (<1 km) within continuous habitats, the behaviour of *Z. gilesi* indicates that the majority of pollen movement would be within populations (<500 m, Chapter 3). In comparison to the common *Z. nigripes*, the median movement distance was not significantly different between the two species (Chapter 3).

In addition, there was no difference in mortality, or the food plant species utilised between thynnine species (Chapter 3). However, the recapture rate was significantly higher in *Z. gilesi* (Chapter 3). The difference in recapture rate may arise due to variation in the spatial distribution and density of the wingless females in each species, as male insects are predicted to display an optimal mate-searching strategy to increase their chance of encountering mates (Alcock *et al.* 1978; Peakall and Beattie 1996; Goh and Morse 2010).

An interesting area for further research is the patchiness of pollinator visitation and pollination success within and between populations of sexually-deceptive orchids. For orchids pollinated by large thynnines, which frequently move up to 100 m, we would predict that patchiness in pollination success may arise through site-specific patch avoidance following a deception event, instead of fine-scale overlap of plants and pollinators. (Peakall and Beattie 1996) hypothesised that sexual deception will result in long distance pollen movement and increased outcrossing, as males are known to leave the vicinity following attempted copulation with an orchid flower. However, (Whitehead and Peakall 2012b) showed that patch-avoidance in thynnines may only operate over a short time interval (<24 hours), followed by increased detection probability over longer time intervals at a site where wasps had been previously caught. This increased detection probability indicates that males show local site fidelity where they have previously encountered a female pheromone signal, potentially increasing pollination success of orchids in the vicinity of previously visited locations. However, this contrasts with the results for *Z. nigripes*, where recapture rate was very low (9%, Chapter 3). Here, males may be searching for mates more widely in the landscape instead of revisiting locations where they had previously detected a pheromone signal.

Conversely, in some sexually deceptive systems, close spatial overlap between orchid and pollinator populations will be crucial to ensuring pollinator visitation. For example, *Colletes cunicularius* bees, the pollinator of a number of species of *Ophrys* (Paulus and Gack 1990) searches for females in the vicinity of nesting aggregations (Ayasse *et al.* 2001). A CMR study on this species revealed an average movement distance of only 5.2 m (Peakall and Schiestl 2004), suggesting that males tend to search for mates across a very restricted range. A similar pattern has been observed in the Australian orchid *Leporella fimbriata*, which is pollinated by male *Myrmecia urens* ants, where the average recorded pollen movement distance for *L. fimbriata* was only 3 m (Peakall

1989b). In systems such as these, pollination success could be expected to show significant patchiness, both within and between populations, depending on proximity to pollinator populations. In rare orchids reliant on pollinators with small home ranges, close proximity to pollinator populations will be critical for maintaining fruit set and outcrossing.

In sexually-deceptive orchids the spatial patterns of pollen movement will likely reflect the mate-searching movements of the pollinator. In cases such as *Z. gilesi*, that is rare at the landscape level, but locally abundant within suitable sites, the majority of pollen movements may be restricted to within populations. Alternatively, in cases such as *Z. nigripes*, which is common across the landscape, a broader search for mates might promote inter-population pollen movement in the orchid species it pollinates (*D. livida*). Conversely, the extremely low recapture rate in *Z. nigripes* may result in increased pollen loss, due to the lower probability that an insect carrying pollen will encounter an orchid. This study demonstrates the complexity of predicting pollinator movements (and associated pollen flow) even between sympatric members of the same wasp genus and cautions against making generalisations across even closely related species of orchids and pollinators.

Population genetics and pollinator specificity

In comparison to most other flowering plant families, orchids tend to exhibit low levels of population genetic differentiation (Forrest *et al.* 2004; Tremblay *et al.* 2005; Phillips *et al.* 2012). Consistent with these general patterns, population genetic analysis revealed only weak genetic structuring across the distribution of *D. elastica*; however, this still provided a significant signal of regional genetic structure (Chapter 4). This provides some support for the unexpected result of individual-level differences in the pollinator attracted by some *D. elastica* flowers in the field, which was corroborated by differing GC/MS-EAD responses of pollinators in the laboratory (Chapter 4). Further, despite being generally similar in appearance, based on subtle morphological variation these pollinators appear to represent two variants of *Z. gilesi* as described by Turner (1910).

In species that form specialised pollination interactions, such as many orchids, population-level variation in the pollinator species used may lead to pollinator-mediated reproductive isolation (Bower 1996; Bower 2006; Bower and Brown 2009; Peakall *et*

al. 2010; Xu *et al.* 2011). Due to the highly specific nature of the pollination interaction, slight differences in floral odour compounds or different blends can elicit responses from different pollinators (Mant *et al.* 2002; Schiestl and Mant 2002; Schiestl and Peakall 2005; Schiestl and Schlüter 2009; Peakall *et al.* 2010; Vereecken *et al.* 2010). I hypothesise that these ‘chemotypes’ of *D. elastica* may represent the early stages of reproductive isolation in this orchid leading to divergence and speciation. Unfortunately, due to the conservation significance of *D. elastica* we were only able to test a limited number of individual flowers. Consequently, we cannot rule out the existence of mixed populations containing both chemotypes just as multiple species of *Drakaea* can occur sympatrically. The coexistence of multiple chemotypes within taxa of sexually deceptive orchids has been shown in eastern-Australian *Chiloglottis*, whereby pollinator-mediated reproductive isolation is maintained via variation in floral odour (Peakall *et al.* 2010).

The ability to reliably attract and elicit attempted copulation by relevant thynnines to black plastic pin-heads coated in synthetic allomones has been well established for *Chiloglottis*, and has proved to be a particularly useful technique for understanding the chemical basis for pollinator attraction and pollinator switching in these orchids (see (Schiestl *et al.* 1999; Mant *et al.* 2002; Schiestl *et al.* 2003; Schiestl and Peakall 2005; Peakall *et al.* 2010). Recent and ongoing research on floral odour chemistry in *Drakaea* has revealed the presence of cryptic chemotypes in the common *D. livida* (Bohman *et al.* 2012a; Bohman *et al.* 2012b), a phenomenon that may be more widespread within the genus. Studies such as this provide an exciting avenue for the investigation of chemical-mediated reproductive isolation in orchids, and the likely mechanisms of speciation through variation in floral odour compounds. As in *Chiloglottis*, *Drakaea* pollinators can be attracted to black plastic pin-heads coated in synthesised odour compounds (R. Peakall pers. comm.). This confirms that notwithstanding the apparent similarity of the *Drakaea* labellum to the flightless female wasp (Peakall 1990), that pollinator attraction is not dependent on close morphological mimicry of the female wasp. The ultimate confirmation that chemistry is more important than morphology has recently been demonstrated by R.D. Phillips *et al.* (unpublished data), whereby two morphologically distinct orchids, one *Drakaea* and one *Caladenia* species both attract the same pollinator by sexual deception. It follows, given that precise morphological mimicry is not required that *Drakaea* may contain multiple undiscovered cryptic species

that lack morphological divergence. Such a condition has already been uncovered in the sister genus *Chiloglottis* (Peakall *et al.* 2010).

Weak population genetic structuring with some admixture between regional clusters of *D. elastica* (Chapter 4) suggests high population genetic connectivity, either through pollen or seed dispersal. Furthermore, this finding indicates that despite the patchy distribution of the orchids in the landscape, ‘genetic populations’ have been characterized by large effective population sizes, at least historically. Seed rather than pollen movement has most likely been the major contributor to this high connectivity given that the majority of pollen movements are predicted to occur within populations of *Drakaea* (Chapter 3). In the future, seed rather than pollen flow may be particularly crucial for maintaining gene flow between the remaining populations, since *Z. gilesi* tends not to occur in the human modified matrix between most extant *D. elastica* populations (Chapter 3, unpublished data).

Despite the landscape-scale genetic evidence for extensive seed flow, at the local scale of centimetres to metres there is evidence that local restrictions of seed dispersal, not pollen dispersal, is the major contributor to the strong positive fine-scale (<10 m) spatial genetic structure in *D. elastica* (Chapter 4). Similar findings of local positive fine-scale genetic structure likely driven by local-scale seed dispersal has been reported in other orchids (Murren and Ellison 1998; Jacquemyn *et al.* 2007; Jersáková and Malinová 2007). This fine-scale genetic structure may be further reinforced by a patchy distribution of the mycorrhizal endophyte. A study by Phillips *et al.* (2011a) determined that the distribution of the fungus associated with *D. elastica* may vary at a scale of centimeters, potentially increasing germination probability in close proximity to the maternal plant (Diez 2007; Swarts and Dixon 2009), thus exaggerating the fine-scale genetic structure. In addition to affecting germination, the spatial distribution of suitable microsites where adult plants can establish may also influence the patterns of fine-scale spatial population genetic structure.

Implications for conservation and management of *D. elastica*

Drakaea elastica has a specific microhabitat requirement for sites with an open understorey with patches of bare sand. At some populations, weed invasion may be a critical factor affecting the availability of suitable microsites. There is little remaining

intact natural habitat for the establishment of additional populations of *D. elastica*. However, if *Kunzea glabrescens* thickets can be restored the fungus may colonise. For example, (Collins *et al.* 2007) showed that some orchid mycorrhizal fungi were able to recolonise restored jarrah (*Eucalyptus marginata*) woodland in southwestern Australia. Alternatively, it may be possible to reinstate the mycorrhizal endophyte through soil inoculation (Hollick *et al.* 2007). In the Capel region the pollinator *Z. gilesi* is locally common and known to occur more broadly than in *K. glabrescens* thickets. However, *Z. gilesi* tends not to occur in pasture; hence remnant vegetation will be crucial to population persistence. By contrast, in the Mandurah region, *Z. gilesi* appears to be rare, potentially making these *D. elastica* populations vulnerable to pollinator loss. This may in turn limit the value of natural or artificial establishment of new populations since such populations may not be self-sustaining.

An unexpected finding in this study was the presence of multiple entities within both *D. elastica*, and the pollinator *Z. gilesi* (Chapter 4). In effect, conservation programs designed to protect this plant-pollinator interaction could be dealing with up to four cryptic entities.

Based on multiple lines of evidence, I can infer the presence of two distinct chemotypes within *D. elastica*. Given the inherent level of complexity within the species, all remaining populations of *D. elastica* need to be conserved. Further, the likelihood that some populations may contain both floral chemotypes (Figure 5.1) means that until this can be further resolved, it is not possible to delineate populations as distinct entities. While microsatellite markers provided some support for the presence of regional clustering, there was still admixture between clusters (Chapter 4). The low level of population genetic differentiation may be due to these chemotypes being in the early stages of speciation and as yet have not yet significantly diverged, or the presence of populations containing mixed chemotypes (Figure 5.1). Further, microsatellite markers may not provide the appropriate resolution for identifying these chemotypes via molecular techniques. Other genetic markers such as those found within chloroplast DNA may provide further resolution.

As these putative chemotypes are morphologically indistinguishable, further resolution of the distribution of these will require fine-scale chemical identification involving single labellum extracts, once there is sufficient understanding of the chemistry

involved. However, presently for single labellum extracts we lack the high-resolution required to detect the biologically active compounds which are only found at very low levels.

In addition to the prospect of two *Drakaea* taxa, it is possible that the two variants of *Z. gilesi* (Turner 1910) in fact represent two distinct species. Consequently, management of the pollinator populations will differ depending on the abundance and ecology of each form.

Of particular interest is the population located at Carabungup Nature Reserve. While geographically close to other *D. elastica* populations (13 km), the flower sampled from this population attracted the same pollinator as flowers from sites up to 100 km away, rather than local populations (Chapter 4). Further, this population showed the greatest level of admixture between the two genetic clusters (Chapter 4). This population also falls within the distribution of both variants of *Z. gilesi*. Consequently, this population potentially contains both chemotypes, warranting further, careful investigation (Figure 5.1). If indeed this population contains both chemotypes then this may be a particularly important population to protect, since it may be the most resilient to future unpredictable shifts in pollinator distributions due to global change.

Much of the habitat fragmentation on the Swan Coastal Plain is relatively recent (<50 years), consequently, the pattern of low genetic differentiation between populations of *D. elastica* almost certainly reflects historic, not current patterns of gene flow between populations. Ensuring exchange of propagules between extant populations presents a long-term challenge for the conservation of *D. elastica*, as the majority of remaining populations are small and highly fragmented, while being situated within an ecologically hostile landscape matrix. While *Z. gilesi* is known to rarely occur in pasture landscapes (unpublished data), further investigation into the patterns of landscape use by this species will elucidate the potential for pollen movement between fragmented populations of *D. elastica*.

Opportunities for further research on *Drakaea elastica*

Two key aspects of the ecology of *D. elastica* that are in need of further investigation are the biology of the pollinator and the physiology of the orchid-fungus interaction. As

highlighted in Chapter 3, the ecological requirements of pollinators are an often-neglected aspect in plant conservation and restoration programs (Kearns *et al.* 1998; Dixon 2009; Winfree 2010; Menz *et al.* 2011). Numerous Australian sexually-deceptive orchids, including many rare and endangered species, utilise thynnine wasps as their exclusive pollinators (Phillips *et al.* 2009; Gaskett 2011), however, we know very little about the life-cycle of these insects, which is largely spent underground. The available evidence, based on just a few studies indicates all the species investigated are parasites on scarab beetle larvae and feed on nectar and/or honeydew as adults (Burrell 1935; Given 1953; Ridsdill Smith 1970b; Ridsdill Smith 1970a; Ridsdill Smith 1971; Alcock 1981; Phillips *et al.* 2009). Further research on the biology of thynnines, in particular host choice, will be critical for conserving pollinator populations.

Another gap in knowledge is the physiology of the *Tulasnella* mycorrhizal endophyte utilised by *D. elastica*. Nurfadilah (2010) investigated the response of this mycorrhiza to different Carbon, Nitrogen and Phosphorous sources in the laboratory. It was determined that this *Tulasnella* utilises similar C, N and P sources as other orchid mycorrhiza (Nurfadilah 2010). However, this *Tulasnella* is extremely slow growing (Phillips 2010) and thus could potentially be outcompeted for nutrient sources by other faster-growing fungi. If true, this hypothesis might explain the strong preference of *Drakaea* for bare open sandy habitats (Phillips *et al.* 2011a; Chapter 2).

Conservation of plant-pollinator interactions in rare plants

The present study has shed new light on the extent and patterns of thynnine wasp movements. Expansion of similar CMR studies to the landscape scale will be crucial to evaluate the response of pollinators to habitat fragmentation, in addition to providing clues as to how landscape connectivity can be improved. The ability to track individual pollinators will be crucial to this endeavor. The application of emerging technologies for studying the movements of individual insects opens exciting new opportunities to conduct such studies. The use of harmonic radar has allowed for the investigation of landscape-scale foraging movements of insect pollinators (Riley *et al.* 1996; Osborne *et al.* 1999; Osborne *et al.* 2008). However, this technology is not readily accessible due to cost and equipment constraints. Far more promising for general use is the miniaturisation of radio-tracking equipment that has already allowed for the tracking of larger individual pollinators (Pasquet *et al.* 2008; Wikelski *et al.* 2010; Hagen *et al.*

2011). For thynnines, there remain size constraints, as radio-tags are still too large (Chapter 3), but we can expect considerable technological progress that will allow smaller and smaller electronic tags to be used in the future.

Until such a time as these new tools become available, we can continue to employ techniques such as colour-marking and bee tags (Peakall 1990; Peakall and Beattie 1996; Peakall and Schiestl 2004; Chapter 3) to provide insight into the ability of orchid pollinators to move between habitat fragments. The recent application of MicroDots for tagging pollinators has created the opportunity to individually mark even very small insects (<1 cm) (Whitehead and Peakall 2012a; Whitehead and Peakall 2012b). Further, the colour-labeling of orchid pollen (Peakall 1989a; Ortiz-Barney and Ackerman 1999; Salguero-Faria and Ackerman 1999; Johnson *et al.* 2005; Kropf and Renner 2008) and use of microtags (Nilsson *et al.* 1992) provides a direct measure of pollen movement between orchid populations.

Notwithstanding the potential importance of pollen flow, the dust-like seeds of orchids likely means that seeds, not pollen are crucial for maintaining population connectivity over broad scales (Arditti and Ghani 2000). This hypothesis is supported by the inherently low level of population genetic differentiation typically observed in orchids (Phillips *et al.* 2012). However, much remains to be learnt about how landscape-scale processes such as landscape fragmentation may interfere with population connectivity through disruption of seed dispersal events in orchids. Despite the potential for long-distance seed dispersal, in highly fragmented landscapes, we might expect gene-flow to be reduced between fragmented populations due to the removal of intervening ‘stepping-stone’ populations (Phillips *et al.* 2012).

In some systems the distribution of mutualist partners has been identified as a good predictor of a species occurrence in habitat suitability models (Gutiérrez *et al.* 2005; Araújo and Luoto 2007). For example, (Gutiérrez *et al.* 2005) showed that inclusion of the distribution of the mutualist ant partner in habitat suitability models was a good predictor of the occurrence and abundance of the Lycaenid butterfly *Plebejus argus*. While parallel declines in plants and pollinators have been shown in some systems (Biesmeijer *et al.* 2006) this may not be the case for non-mutualistic pollination systems. In some cases, response to environmental change may lead to spatial or temporal mismatches between species (Hegland *et al.* 2009) which may be a significant

threat to specialist interactions (Schweiger *et al.* 2010). In plant-pollinator interactions such that of *Drakaea*, the plant and insect partners may have different habitat requirements. This could potentially lead to differences in species occurrence and variation in response to changing environments. Consequently, the degree of overlap in ecological requirements in plant-pollinator associations will be a rich area for future investigation, and can provide insight into the evolution of specialised mutualisms. Further, this could also shed light on the potential resilience of these relationships to changing environmental conditions.

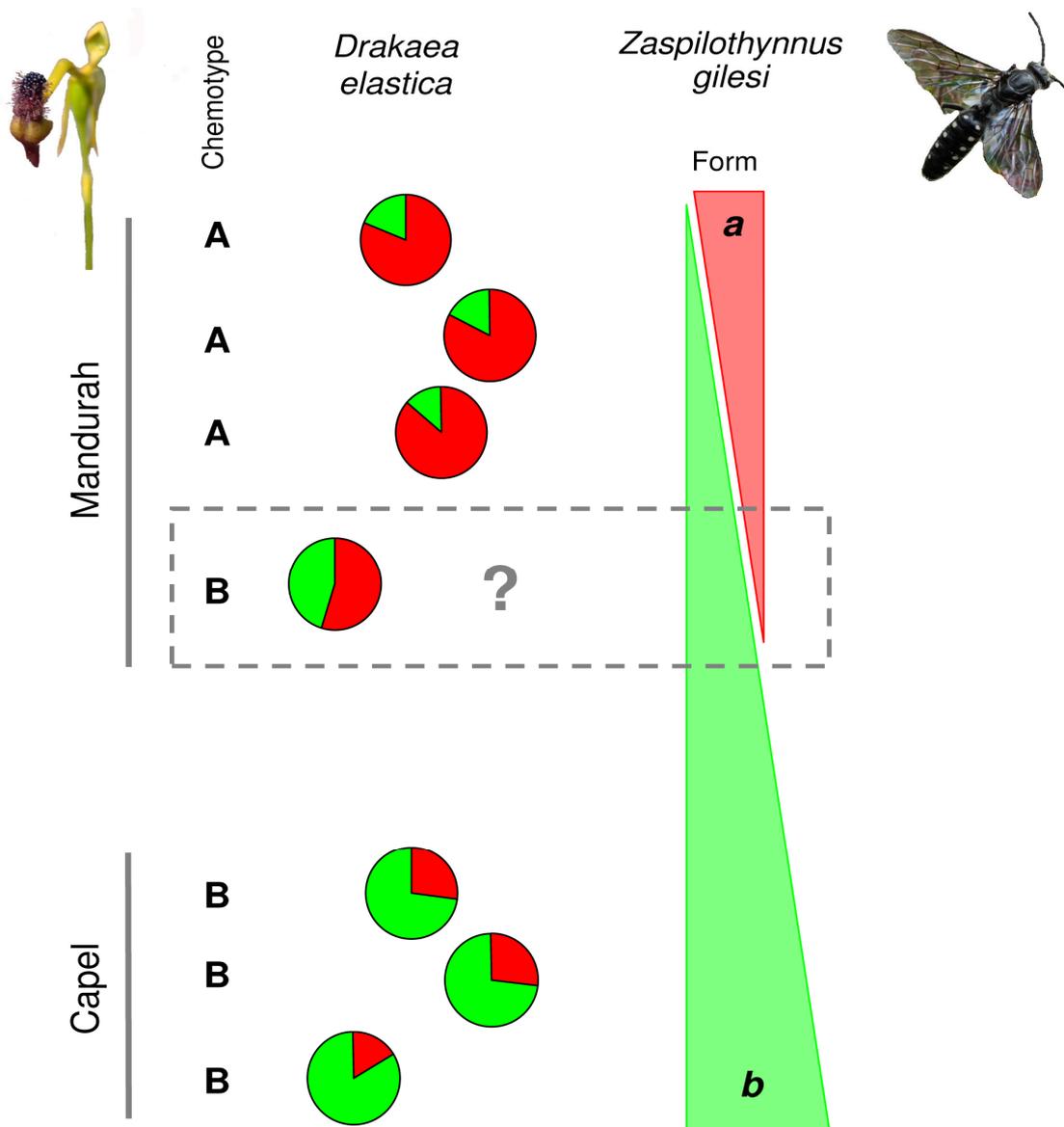


Figure 5.1. Schematic representation of geographic overlap in the two forms of *Drakaea elastica* and *Zaspilothynnus gilesi*. Chemotype refers to the known presence of either chemotype A or B in each population studied (Chapter 4). Chemotype was determined for at least one individual in each population, by pollinator baiting (Chapter 4). Pie charts represent proportional membership of *D. elastica* populations to each of two genetic clusters, based on microsatellite markers (Chapter 4). Two forms of *Z. gilesi* are recognised based on morphology described by Turner (1910). Both forms of *Z. gilesi* are known to occur in part of the range of *D. elastica* (M.H.M. Menz and R.D. Phillips unpublished data and specimens from the Western Australian Museum). The dotted square represents the population at Carrabungup Nature Reserve, which is within the range of both forms of *Z. gilesi* and where chemotype B of *D. elastica* is known to occur. This population is the most likely candidate for containing both chemotypes of *D. elastica*.

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Review

The precipitous decline of the ortolan bunting *Emberiza hortulana*: time to build on scientific evidence to inform conservation management

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Abstract In recent decades there has been a marked decline in most ortolan bunting *Emberiza hortulana* populations in temperate Europe, with many regional populations now extinct or on the brink of extinction. In contrast, Mediterranean and, as far as we know, eastern European populations seem to have remained relatively stable. The causes of decline remain unclear but include: habitat loss and degradation, and related reduction in prey availability; climate change on the breeding grounds; altered population dynamics; illegal captures during migration; and environmental change in wintering areas. We review the current knowledge of the biology of the ortolan bunting and discuss the proposed causes of decline in relation to the different population trends in temperate and Mediterranean Europe. We suggest new avenues of research to identify the factors limiting ortolan bunting populations. The main evidence-based conservation measure that is likely to enhance habitat quality is the creation of patches of bare ground to produce sparsely vegetated foraging grounds in invertebrate-rich grassy habitats close to breeding areas.

Keywords Agricultural intensification, *Emberiza hortulana*, farmland birds, habitat degradation, migration, ortolan bunting, species decline, threatened species

Introduction

Migratory birds, in particular long-distance migrants, are vulnerable to environmental change in multiple regions (Sanderson et al., 2006; Both et al., 2010). The

ortolan bunting *Emberiza hortulana* is the only long-distance trans-Saharan migrant among old world buntings (Cramp & Perrins, 1994; Glutz von Blotzheim & Bauer, 1997). The species has undergone the second most pronounced decline of any bird species in temperate Western Europe in recent decades, with an estimated 82% population reduction between 1980 and 2008 (Klvanova et al., 2010), although the decline began earlier in some places (Conrads, 1977; Lang et al., 1990; Meier-Peithmann, 1992; Dale, 1997). Ortolan bunting populations have recently crashed across northern Europe and Scandinavia (van Noorden, 1991, 1999; Vepsäläinen et al., 2005; Ottvall et al., 2008) and the species has effectively become extinct as a breeding species within the last decade in Belgium, The Netherlands (van Noorden, 1991, 1999; Vieuxtemps & Jacob, 2002; van Dijk et al., 2005) and Switzerland (Revaz et al., 2005; Menz et al., 2009b), with mostly unpaired singing males remaining in these populations. The species has apparently remained stable in Eastern Europe (BirdLife International, 2004), and the only notable increase has been in Catalonia, in the Mediterranean (Pons, 2004; Brotons et al., 2008).

Although the life history of the ortolan bunting is generally well resolved we lack consolidated information about the species' key ecological requirements and most conservation action for the species is based on expert opinion rather than scientific evidence. Thus, there is uncertainty about the optimal conservation measures to implement. Given the challenges of integrating research programmes across regions and countries clear direction is required for appropriate conservation research for the ortolan bunting. In this review we: (1) synthesize existing knowledge of the biology of the ortolan bunting, (2) discuss the proposed causes of the species' decline, (3) propose priorities for future research to inform conservation action, and (4) provide preliminary evidence-based management recommendations from the information currently available (Pullin & Knight, 2001).

Literature searches were primarily on the ISI Web of Science, the Ornithological Worldwide Literature database (OWL, 2010), and reference lists from published articles. The review of threatening processes considered articles published after 1950 as this is believed to be the year in which many population declines began (Lang et al., 1990; Meier-Peithmann, 1992; Dale, 1997).

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Ecology of the ortolan bunting

Habitat requirements

In Mediterranean and sub-Mediterranean Europe the species breeds primarily in open shrubland and steppe-like habitat, particularly on south-facing slopes (Cramp & Perrins, 1994; Glutz von Blotzheim & Bauer, 1997; Fonderflick et al., 2005; Brotons et al., 2008). Here, the species favours areas with shrub and tree cover of c. 20–30% (Kölsch, 1959; Keusch, 1991; Menz et al., 2009a) and rarely occurs where tree cover exceeds 30–50% (Fonderflick et al., 2005; Fonderflick, 2006). In temperate Europe the species breeds primarily in agricultural habitats, particularly areas of small-scale cultivation, set-asides, short-rotation coppice and shrublands in historically burnt habitats, with these habitats often co-occurring (e.g. Berg & Pärt, 1994; Dale & Hagen, 1997; Berg, 2002; Dale & Olsen, 2002; Goławski & Dombrowski, 2002; Revaz et al., 2005). In farmland the species favours field margins with structural elements such as isolated trees, hedges and nearby forest margins (Meier-Peithmann, 1992; Grützmänn et al., 2002), a characteristic shared by several farmland bunting species (Brambilla et al., 2008, 2009).

Within both natural and agricultural landscapes the ortolan bunting breeds primarily in relatively warm, dry areas, with well-drained soils and an annual rainfall below 600–700 mm (Cramp & Perrins, 1994; Grützmänn et al., 2002), avoiding wet habitats (e.g. Nævra, 2002; Dale & Manceau, 2003; Hänel, 2004; Deutsch, 2007). Exceptions include populations occurring in areas with extremely well-drained soils and steep, sloping topography (Conrads, 1977). Ortolan buntings nest on the ground, typically producing only one brood per season, with exceptional replacement clutches and second broods (Garling, 1943; Conrads, 1969; Hänel, 2004).

Ortolan bunting populations typically consist of loose aggregations of breeding pairs (Vepsäläinen et al., 2007). Local colonization, extinction, and population fluctuations are often observed (Glitz, 1967; Dale & Steifetten, 2011), with areas seemingly isolated from other populations also colonized (van Noorden, 1991, 1999; Revaz et al., 2005). This could indicate the existence of a broadscale metapopulation structure, with areas being settled or abandoned as habitat suitability fluctuates following major disturbance events (Brotons et al., 2005). Short-term population increases have been observed in response to fire (Brotons et al., 2005, 2008), clearing of vegetation by a storm, forestry interventions, or cultivation (Conrads & Kipp, 1980; Nævra, 2002). Sparse vegetation and a large proportion of bare ground are the most noticeable common features of these habitats (Nævra, 2002). The species often becomes locally common after disturbance (Sposimo, 1988; Pons, 2004; Revaz et al., 2005) with, for example, populations peaking

3–4 years after fire (Pons & Clavero, 2010). This relationship is particularly strong in Mediterranean and, to a lesser extent, sub-Mediterranean biomes, where occurrence of fires is still commonplace, and it is also noticeable in temperate Europe where the species also nests on historic burns (e.g. Dale & Olsen, 2002; Revaz et al., 2005). This indicates that the ortolan bunting behaves like a pioneer species, typically colonizing the early stages of vegetation succession.

Diet

The ortolan bunting has a varied diet, including both plant (seeds) and animal matter (Cramp & Perrins, 1994; Glutz von Blotzheim & Bauer, 1997), although the diet of the chicks is restricted to a few dominant prey orders: Lepidoptera, particularly Tortricidae larvae, and Coleoptera in the north of its range (Conrads, 1968, 1969; Hänel, 2004), and Orthoptera, particularly Tettigoniidae, in the south (Kunz, 1950; Keusch & Mosimann, 1984). In Switzerland, Tettigoniidae made up nearly 70% of the total items provisioned to nestlings, a much higher percentage than in the sympatric rock bunting *Emberiza cia*, which has a more diverse diet (Keusch & Mosimann, 1984). In the north of the range caterpillars are fed to nestlings in the early stages of development, with diet switching towards larger prey in later developmental stages until post-fledging (Kunze, 1954; Knoblauch, 1968; Conrads, 1969; Hänel, 2004).

Foraging ecology

Ortolan buntings forage primarily in patches of bare ground within sparsely vegetated habitats (Stolt, 1974; Gnielka, 1987; Boitier, 2001; Menz et al., 2009b). However, prey such as caterpillars are also collected from fields (Conrads, 1969), or gleaned from tree crown foliage, particularly oaks *Quercus* spp. (Knoblauch, 1968; Conrads, 1969; Stolt, 1974; Gnielka, 1987), which harbour a relatively high density of caterpillars compared to other tree species (Naef-Daenzer, 2000). Adult males sometimes forage in the same oaks used as song posts (Hänel, 2004). Consequently, song post selection may function as a signal of territory quality, as oaks appear to be over-represented as song posts, compared to local availability of other tree species (M.H.M. Menz, pers. obs.).

In Switzerland most Tettigoniidae fed to nestlings are captured on the ground (Keusch & Mosimann, 1984) and in Germany Coleoptera are collected on paths or in cereal fields (Knoblauch, 1968). Tettigoniidae are most abundant in relatively dense steppe grass or bushes (Arlettaz et al., 1997). However, ortolan buntings do not necessarily forage in habitats with highest prey abundance but rather in those with a high proportion of bare ground (Menz et al., 2009b), as observed in other ground foraging birds (Wilson et al.,

2005; Schaub et al., 2010). Prey accessibility, therefore, rather than abundance, drives foraging habitat selection (Menz et al., 2009b; Schaub et al., 2010).

In temperate Europe foraging often takes place in cultivated fields, sometimes a distance away from breeding areas (Dale, 2000; Dale & Olsen, 2002). Cereal fields, particularly oats, are important for replenishing body fat prior to and upon return from migration, when birds feed on seeds and sprouting plants (Keusch, 1991; Grützmänn et al., 2002). Oat is probably favoured because of the high energy content of the grain (Glutz von Blotzheim, 1989; Diaz, 1990).

Threats and reasons for decline

Habitat loss and degradation, and reduction in prey availability

Agricultural intensification has resulted in land-use changes such as homogenization of agricultural landscapes, loss of structural heterogeneity and an increased use of pesticides (Newton, 2004). A reduction of crop diversity and the transition in cultivation from summer to winter cereals may have contributed to the decline of the ortolan bunting, as such changes will limit the amount of bare ground in cultivated fields. Conversion of rye, and especially oat, to maize cultures has been reported to affect the species negatively (Maes et al., 1985; Ikemeyer & von Bülow, 1995; van Noorden, 1999; Grützmänn et al., 2002; Deutsch, 2007; Berg, 2008), although this has not been quantified. Creation of monoculture agricultural habitats by destruction of structural habitat elements such as tree lines and hedges may be detrimental, as these provide song posts and foraging opportunities (Knoblauch, 1968; Vepsäläinen et al., 2005). Urbanization has often encroached into small-scale agricultural areas that had typically been preserved close to villages (van Noorden, 1999), increasing disturbance near to breeding areas (Steiner & Hüni-Luft, 1971).

One of the principal reasons for the observed decline of the ortolan bunting in temperate Europe is probably a reduction in prey availability/accessibility driven by habitat deterioration on the breeding grounds, primarily via changes in agricultural practices (Claessens, 1992; Kutzenberger, 1994; van Noorden, 1999; Grützmänn et al., 2002; Revaz et al., 2005; Vepsäläinen et al., 2005; Deutsch, 2007). A reduction in patches of bare ground within foraging areas will result in decreased prey availability (Wilson et al., 2005; Schaub et al., 2010) in two ways. Firstly, agricultural intensification includes increased application of fertilizers, which closes the vegetation and suppresses patches of bare ground, and the use of pesticides eliminates invertebrate prey. Secondly, areas of bare ground also vanish following vegetation encroachment through natural succession after abandonment of traditional agricultural practices such as extensive grazing and burning of dry grass (Stolt, 1974; Dale,

1997; Nævra, 2002; Revaz et al., 2005; Wilson et al., 2005; Sirami et al., 2007; Menz et al., 2009b; de Groot et al., 2010). There is increasing evidence that reduction in structural heterogeneity and bare ground is threatening a number of ground-foraging farmland bird species (Wilson et al., 2005; Schaub et al., 2010).

Climate change on the breeding grounds

Climate change has been proposed as a possible cause of the decline of the ortolan bunting (Knoblauch, 1954; Helb, 1974; Maes et al., 1985; Grützmänn et al., 2002; Vieuxtemps & Jacob, 2002). A low tolerance to cold temperatures (Wallgren, 1952, 1954) may increase the risk of physiological stress on breeding birds during inclement weather. Microclimate at the nest site is also likely to have consequences for the growth and survival of the nestlings (Conrads, 1977; Lang et al., 1990; Dale, 2000; Dale & Olsen, 2002; Grützmänn et al., 2002; Vepsäläinen et al., 2005), as observed in other bird species (Ullrich, 1971). Poor weather during the breeding season, such as cold, rainy springs, also lowers reproductive success through nestling mortality from food limitation (Ruge et al., 1970; Fonderflick & Thévenot, 2002), something also observed in other species (Arlettaz et al., 2010).

The earlier growing season predicted under some climate change scenarios may lead to reduced foraging opportunities for ortolan buntings returning to their breeding grounds because of vegetation closure (Lang, 2007). The relatively short nestling phase (9–14 days; Cramp & Perrins, 1994; Glutz von Blotzheim & Bauer, 1997) means chicks require a large amount of invertebrate prey in a short period (Meier-Peithmann, 1992). Ortolan buntings may have evolved a reproductive phenology to coincide with peak prey availability, as seen in some other bird and mammal species (Blondel et al., 1991; Arlettaz & Fournier, 1993; van Noordwijk et al., 1995; Arlettaz et al., 1997, 2001). Chicks usually hatch in c. mid June, when Tettigoniidae are abundant and at a profitable size (Kunz, 1950; Arlettaz et al., 2001). Conrads (1968, 1977) noted breeding was synchronous with sprouting of oak leaves and the appearance of large numbers of defoliating caterpillars, although this was not quantified. Given the short breeding season and nestling phase, and their relatively late return from Africa (Claverie, 1955), altered climate regimes may see a mismatch between breeding period and prey availability (Both et al., 2010).

Altered population structure and dynamics

Studies from multiple regions have reported 29–60% of singing ortolan bunting males remain unpaired during the breeding season (Conrads, 1968; Dale, 2001; Fonderflick & Thévenot, 2002; Steifetten & Dale, 2006; Berg, 2008). However, even in declining and fluctuating populations

breeding success appears to remain stable (Maes, 1989; Steifetten & Dale, 2006). In small and isolated populations unpaired males may be all that remain prior to population extinction (Dale, 2001; Vieuxtemps & Jacob, 2002; Donald, 2007; Menz et al., 2009b). In Norway populations are limited by a drastic reduction in the number of breeding pairs because of females dispersing away from the population (Dale, 2001; Steifetten & Dale, 2006), resulting in the male-biased sex-ratio seen in declining populations (Dale et al., 2006). In relatively isolated populations there is little opportunity for recruitment of females from elsewhere (Steifetten & Dale, 2006). Declines and local population fluctuations are also driven by males undertaking relatively long-distance breeding dispersal in search of females (Dale et al., 2005; Dale & Christiansen, 2010; Dale & Steifetten, 2011). Loss of females from a population can only be mitigated by increasing the availability and suitability of habitat patches within breeding areas, which requires detailed knowledge of the species' habitat and foraging requirements (Steifetten & Dale, 2006).

Illegal captures during migration

The ability to constitute fat reserves rapidly before autumn migration seems to be an idiosyncrasy of the ortolan bunting, a characteristic known for centuries in gastronomic circles around Europe (Bastien, 1798; Kumerloeve, 1954; Claverie, 1955). The fact that the ortolan bunting is the only species of bunting in Western Europe that undertakes long-distance migration may indicate specific adaptations for storing fat reserves. Historically, large numbers of ortolan buntings were trapped during the autumn and, to a lesser extent, spring migration, mostly in southern Europe (Claessens, 1992). Small traps known as *matoles* are used that are baited exclusively with nearly ripe oat stalks (Claverie, 1955). It is likely that the species has become a delicacy because of its propensity to lay down fat relatively quickly when fed grain (oat and millet) *ad libitum* in captivity (Claverie, 1955; Claessens, 1992; Dale, 1997; Steifetten & Dale, 2006). After fire a wild form of oat occurs *en masse* in some Mediterranean habitats (R. Arlettaz, unpubl. data) and may have constituted an important food source prior to the expansion of agriculture.

In some areas of south-west France, despite the species now being protected, trapping and fattening continues unabated, with an estimated 50,000 birds illegally captured per year until at least the early 1990s (Claessens, 1992). No studies have been conducted on the consequences of this regionally intensive poaching on the demography of temperate European populations. Although it is unlikely that birds from all declining European populations of the ortolan bunting cross these areas during migration, in a wide-scale metapopulation system these losses may affect overall population dynamics and thus also have regional consequences for distant populations.

Environmental changes in wintering areas

Although the migration phenology is well documented for Europe (Stolt, 1977; Cramp & Perrins, 1994; Stolt & Fransson, 1995; Yosef & Tryjanowski, 2002; Bairlein et al., 2009), the wintering areas of the ortolan bunting are poorly known. Habitat changes in wintering areas because of climate variation and/or anthropogenic impacts on land (e.g. pesticides, Vepsäläinen et al., 2005; Zwarts et al., 2009) may also contribute to the observed population declines (Conrads, 1977; Kutzenberger, 1994; Busche, 2005; Vepsäläinen et al., 2005; Lang, 2007). However, the fact that some populations in the Mediterranean have been recently expanding (Brotons et al., 2008) seems to indicate that the problem may lie primarily with the quality of the breeding grounds in Europe, possibly compounded by environmental changes in African wintering areas. Identification of the wintering areas of this species is imperative for understanding factors that may be affecting the species outside the breeding season and whether birds from temperate and Mediterranean populations winter in different areas.

Discussion

Knowledge gaps and recommendations for future research

Further information on the diet of the ortolan bunting across its range, particularly quantification of nestling diet in relation to prey availability in the main foraging habitats, is required for a full understanding of the species' ecological requirements. In particular, quantification of the abundance and availability (the latter being abundance modified by accessibility) of major invertebrate groups in relation to the stages of vegetation succession following events such as fire may provide information on why the species colonizes these disturbed habitats during specific time windows (Pons & Claverie, 2010). Understanding the relationships between timing of breeding and prey phenology/availability would also elucidate the potential effects of weather and climate variation on reproductive output. Shifts in insect phenology could potentially lead to a mismatch between breeding season and prey availability, a phenomenon that may particularly affect long-distance migrants (Both et al., 2010).

More data are required on survival and movement patterns in areas where populations are stable (Pons, 2004). As most detailed studies on population structure and dynamics have been conducted in northern Europe (particularly Norway: Dale, 2001; Steifetten & Dale, 2006; Dale & Steifetten, 2011), a comparison between eastern European, Mediterranean and temperate populations would facilitate an understanding of the demographic

factors limiting populations, such as the propensity for females to disperse away from certain areas. Investigation into the extent of continued poaching would elucidate the potential effects this may have on the demography of temperate European populations as a whole.

Climate variation has already affected the ecology and distribution of some bird species (Arlettaz et al., 2010; Both et al., 2010). It is uncertain what effect predicted climate change scenarios would have on the ortolan bunting, especially given the paucity of data on future precipitation regimes (Easterling et al., 2000) and the impact of weather on the species' reproductive success. Studies at the edges of the species' range could provide insights into possible colonization of higher latitudes and altitudes. In Mediterranean Europe temperature increases may lead to abandonment of the warmest areas. Such studies are needed to disentangle the future effects of climate modification and ecosystem changes. Identification of the species' African wintering grounds by use of new light-weight tracking techniques such as geolocators (Bächler et al., 2010) would facilitate the assessment of any potential environmental issues that may be contributing to the decline of the species outside its breeding areas and elucidate the connectivity of European breeding populations.

Conservation recommendations

Until we know more about the specific factors limiting ortolan bunting populations, we recommend application of evidence-based conservation measures (Pullin & Knight, 2001) to counteract vegetation encroachment and increase the proportion of patches of bare ground within vegetated patches close to ortolan bunting breeding areas. This could be achieved through extensive grazing, controlled fire (Wilson et al., 2005; Schaub et al., 2010) and forestry interventions such as short-rotation coppicing (Berg, 2002). However, attention should be paid to protecting sufficient dense grass sward to support prey populations. Patches of bare ground in cultivated fields close to breeding areas may also be produced by spring sowing, decreasing seed sowing density or increasing the distance between rows in sown fields. Although prescribed fire may be a cost-effective management option that is already used in several countries to counteract vegetation encroachment (Montané et al., 2009), further research is required to determine the potential detrimental effects of prescribed burning on other aspects of biodiversity. In southern Switzerland extensive grazing, forestry measures (coppicing), sowing of oat fields and controlled fire have recently been applied simultaneously to halt the decline of a rare butterfly species and the ortolan bunting (E. Revaz & R. Arlettaz, unpubl. data). Thus in certain habitats conservation measures targeting the ortolan bunting may have broader benefits for biodiversity.

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Biographical sketches

MYLES MENZ has a broad interest in landscape ecology and conservation biology of birds, insects and plants, with particular interest in pollination ecology and the restoration of plant-pollinator mutualisms. RAPHAËL ARLETTAZ has wide interests in biodiversity conservation. His research is aimed at providing the necessary rigorous evidence-based guidance to maintain and restore ecosystems and their emblematic species, especially vertebrates and invertebrates in agro-ecosystems and Alpine ecosystems. He is also committed to bridging the great divide that exists between research and action in conservation biology, developing integrated research-implementation programmes, mostly within Switzerland.

Hurdles and Opportunities for Landscape-Scale Restoration

Myles H. M. Menz,^{1,2*} Kingsley W. Dixon,^{1,2} Richard J. Hobbs²

A priority outcome from the 2012 United Nations Rio+20 Conference on Sustainable Development (1) was the target to restore, by 2020, 150 million ha of disturbed and degraded land globally (2). An initiative of this scale is estimated to cost U.S. \$18 billion per year and to provide U.S. \$84 billion per year to the global economy (2). Although such initiatives have transformative potential because of their scope and backing, they require technology and knowledge capacity to deliver proven, scalable restoration (3). Restoration processes must achieve the greatest value for money, as far as socioeconomic and biodiversity conservation outcomes, while avoiding costly and simplistic plantings (4).

Although we recognize that preventing loss and damage in the first place is a far better investment than restoration after damage has occurred, we propose a four-point plan to ensure that restoration sustains and enhances ecological values: (i) identify focal regions with high restoration demands, (ii) identify knowledge gaps and prioritize research needs to focus resources on building capacity, (iii) create restoration knowledge hubs to aggregate and disseminate knowledge at the science-practice interface, and (iv) ensure political viability by establishing economic and social values of functioning restored ecosystems. These points are interrelated and may occur in parallel.

Ecological restoration, not just a matter of planting trees (5), involves assisting the recovery of a damaged or destroyed ecosystem (6). Landscape-scale restoration includes large, contiguous, or fragmented areas (equal to or greater than several km²). Restoration often takes place in an unpredictable socio-ecological context, involving multiple stakeholders and interests, where local actions aggregate into a broader context that considers landscape flows and connectivity (see the figure). A realistic assessment of prior knowledge, technological capacity, financial viability, and

social license is needed for understanding scientific and practical constraints to achieving global restoration targets. Recent examples, such as China's Great Green Wall and Grain for Green programs, although politically viable, could threaten ecosystem services through ill-placed restoration (7). In such situations, the scientific support behind less-popular options may have been ignored or simply may not be available. A key limitation is the lack of information on successes and failures in landscape-scale restoration projects (8) to guide more effective practice. Early engagement with science will be critical, such as Future Earth: Research for Sustainability, launched at Rio+20, which proposes coordination and facilitation of global science (9).

Identification of Focal Regions

Success of landscape-scale restoration projects will be more likely in some ecosystems and regions than others (10). We should set

Gaps in knowledge must be identified, capacities developed, and research translated into policy and practice.

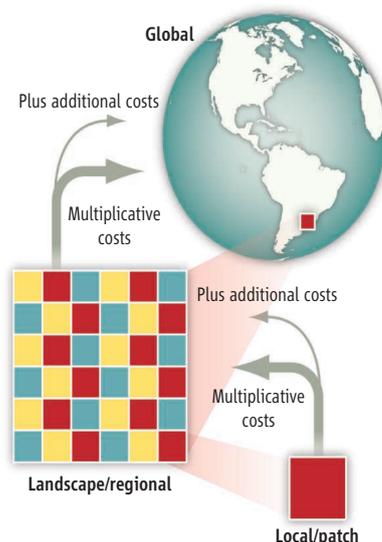
realistic goals (11–13) and identify ecosystems where resources are best positioned to achieve the most cost-effective results to maximize ecosystem services and biodiversity gains. Professional societies, governments, the private sector, and nongovernmental organizations must collaborate through umbrella organizations such as the International Union for Conservation of Nature (IUCN) to set standards and prioritize ecosystems and regions for resource allocation. Such an approach has identified 2 billion ha globally that provide forest restoration opportunities (14) where ecosystem services could be delivered through cost-effective natural and assisted regeneration.

Drylands also provide landscape-scale restoration opportunities. Drylands, extensive in many parts of the world, have been identified as ecosystems that will suffer greatly from climate change, with desertification likely to affect 30% of the world's population (15). Many drylands are major resource hubs that provide financial capacity to fund and implement research and restoration (15).

Prioritization of Research Needs

Once areas have been prioritized and funding has been secured, key knowledge gaps for achieving landscape-scale restoration should be identified. There are few ecosystems for which we have sufficient knowledge to achieve restoration success beyond the local scale. A range of scalable, proven, and cost-effective capacities will be needed, e.g., the scaling-up of resources such as seed banks, to facilitate landscape-scale restoration (3). Approaches are being developed to prioritize actions depending on landscape conditions and likely effectiveness (16, 17).

Although long-term cost-effectiveness of most interventions remains unclear and may be potentially costly, some regional-scale projects may be relatively inexpensive. Linking restoration initiatives with evolving knowledge will allow for targeted, cost-effective interventions (12), while avoiding actions that may make things worse in the longer term [e.g., (18)]. For example, wetland systems where altered water flows have caused system decline can



Scaling up restoration. Costs multiply as local patches are added, each requiring site treatment, seed or plant input, management, and so on. Additional costs and knowledge are necessitated by landscape and regional structures and processes (e.g., hydrological management or transaction costs among different land uses). Economies of scale may be possible. Scaling up to the global level requires multiplicative and additional costs relating to social and political requirements.

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be restarted by reinstating prior conditions, such as in the Mesopotamian Marshes (19). Dryland restoration can be kick-started by providing simple physical barriers to water movement (20).

There is growing awareness of the interdisciplinary science packages required for restoration (16) [e.g., microbiology (21), seed science (3), and pollination ecology (22, 23)], integrated with socioeconomic expertise (24). There needs to be a balance between generally applicable approaches and solutions tailored to region- or ecosystem-specific requirements. Interdisciplinary actions—facilitated by umbrella organizations such as the Society for Ecological Restoration, IUCN, and Conservation International—would provide project carriage beyond the typical 3- to 5-year funding cycles. Although funding agencies and universities need to play a key role in supporting these programs, lobbying by umbrella organizations would be an important step in establishing the process. Successful collaborative initiatives [e.g., (25)] provide solid footing for future programs.

Restoration Knowledge Hubs

Effective information transfer is paramount to the success of landscape-scale restoration projects and to avoid repeating costly mistakes while closing the science-practice gap (13). Attempts to bridge this gap are often local in focus. Restoration ecologists need to take responsibility for translating their science into on-the-ground actions (13, 26). Successful science-practice communication must be two-way to achieve the greatest benefit, as practitioners are valuable for identifying knowledge gaps and guiding research. Evidence-based literature and information repositories should be developed for the restoration sciences (27).

Few restoration initiatives provide for dialogue with the restoration sciences. The Australian government is spending A\$1 billion (U.S. \$1 billion) to restore 18 million ha of degraded land, yet, is silent on links to science or provision of investment in research (28). This is despite the acknowledgment of the southwest Australian biodiversity hot spot as a region where restoration need far outstrips scientific knowledge (3).

Professional scientific associations can compile scientific knowledge and restoration practice and act as information clearing-houses (13). The Global Restoration Network (29) provides a Web-based hub for information on restoration projects. Initiatives such as Future Earth provide hope for linking technology, innovation, and science (30). Gov-

ernments and funders of ecological restoration must develop practice-based templates for global capacity-building and measures for streamlining knowledge dissemination.

Political Viability of Restoration

Landscape-scale restoration projects are likely to work best when initiators are motivated by both environmental and social issues or either one [e.g., (31)]. Good science is required to ensure that the programs are a success, although achieving long-term, dual ecological and social goals remains challenging [e.g., (32, 33)]. Restoration will provide economic benefits worldwide (34), particularly if ecosystem services are matched with biodiversity conservation, including nonmarket services (35, 36). Creating rigorous economic valuation and efficient markets for the wide range of ecosystem services is a critical step, with much still to be done.

Net benefits of sustainable, ecologically resilient restoration (13, 37) must be communicated in a compelling way to policymakers and practitioners if longer-term funding opportunities are to be realized, particularly support for science programs to fill knowledge gaps. Scientists need to shift from a focus on journal writing and professional conferences to reach a broader community and political audience who will make the call on restoration funding. Such a dialogue must remain science-based. For example, evaluation of cost-effectiveness based on ecosystem service return showed that many dry forest restoration approaches may be economic failures (38). Robust analyses like this are important for identifying false, politically damaging assumptions of restoration programs, e.g., China's Great Green Wall (7).

However, in some cases, given the social and ecological values of restoration, costs need not be considered a hurdle, but a challenge to improve our technology in developing more cost-effective techniques.

Not a Magic Bullet

Restoration is often viewed simplistically, as if science and practice were well established. Restoration ecology is not a magic bullet that provides instant ecosystems of the desired type, but an emerging science less than four decades old. In many cases, restoration projects fall short of reinstating functional ecosystems akin to their natural reference sites (10). For example, restoration projects developed in exchange for habitat destruction elsewhere are becoming more prevalent, resulting in losses of high-

quality ecosystems that we are not yet able to restore (10). Deciding on useful targets in a period of rapid environmental change is another area of discussion in restoration ecology (11, 13).

Restoration knowledge hubs are most often associated with developed, boreal economies. For landscape-scale restoration to be effective, science funding and technology development need to realize targets that go beyond such local scales. Restoration is but one tool; with effective management and prevention of further damage to natural areas, restoration would become less urgent.

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Reconnecting plants and pollinators: challenges in the restoration of pollination mutualisms

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Ecological restoration of plant–pollinator interactions has received surprisingly little attention, despite animal-mediated pollination underpinning reproduction of the majority of higher plants. Here, we offer a conceptual and practical framework for the ecological restoration of pollination mutualisms. Through the use of targeted restoration plantings to attract and sustain pollinators and increased knowledge of the ecological requirements of pollinators, we propose that pollination could be successfully restored in degraded ecosystems. The challenge for pollination biologists is to integrate their findings with those of plant restoration ecologists to ensure sustainable pollination in restored ecosystems.

Pollinator loss and the need for pollination restoration

Approximately 90% of flowering plant species globally are reliant on biotic pollination for reproduction and maintaining genetic viability [1]. Because of the economic implications of reduced crop yield due to pollination failure, the purported decline of pollination services in agri-environments has received considerable scientific attention (e.g. [2–8]). Consequently, there is a growing literature on restoring pollination services within agricultural settings [9–12]. Given that crop plants represent <0.1% of angiosperm species globally [13,14], this represents a considerable bias towards restoration of pollination services in agricultural landscapes compared with restoration of pollination in non-agricultural habitats.

In human-modified landscapes, habitat degradation, loss and fragmentation can cause declines in plant and/or pollinator populations, potentially leading to pollination limitation [4,15]. Furthermore, the majority of plant populations surveyed to date experienced pollination limitation of plant sexual reproduction [16], although the magnitude of pollination limitation could be overestimated by studies

that fail to take into account that not all pollination events result in fruit-set [17]. Given the importance of pollination and its sensitivity to human disturbance, it is surprising how little focus has been placed on restoration of animal-mediated pollination in natural habitats [18,19]. Based on a search of the terms ‘pollinat*’ and ‘restoration’ in ISI Web of Science, only five papers have used plant–pollinator communities as a metric for determining functional success in the restoration of natural landscapes [20–24], out of 22,137 papers devoted to pollination biology (pollinat* in ISI Web of Science). The few previous studies comparing plant–pollinator networks between restored and natural sites have highlighted lower network complexity and robustness in restored sites [21,24]. Failure to understand, manage and promote pollinators could lead to decline or collapse in ecological restoration. With ecological restoration estimated to be a trillion dollar global activity [25], establishing animal-mediated pollination will be of widespread importance for ensuring resilience in restored plant communities.

Glossary

Allee effect: reduced per capita reproductive output or demographic viability in small populations.

Asymmetric interactions: occur when a specialist interacts with a generalist, such as a specialist plant interacting with a generalist pollinator.

Bridging plants: a type of keystone plant that provides nectar and pollen resources during bottlenecks of resource availability.

Framework plants: a type of keystone plant that provides considerable nectar and/or pollen resources to a large number of pollinator species.

Generalist plant: plant species pollinated by a large number and often a broad taxonomic range of pollinator species.

Generalist pollinator: pollinator species that visit a large number and often a broad taxonomic range of plant species.

Pollination network: all the interactions between plants and pollinators within a given area or time period. Analogous to (or a type of) food web.

Specialist plant: plant species that are pollinated by one or a small number of pollinator species from a restricted taxonomic range.

Specialist pollinator: pollinator species that visit one or a small number of plant species from a restricted taxonomic range.

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Owing to the paucity of information on pollinator restoration, here we review findings from recent community and population level pollination ecology studies to assess the challenges involved in reconnecting plants and pollinators, with particular emphasis on natural area restoration. We discuss several of the key issues surrounding the development of a pollination-based restoration program: (i) structural characteristics of plant–pollinator networks; (ii) selecting plants to restore pollinators; (iii) ecological requirements of pollinators; (iv) landscape structure and facilitation of pollinator movement; and (v) variation among biogeographic regions.

Not all restoration projects have the same objective. For example, if the aim is to restore native plant reproduction, it might not be crucial that the species composition of the pollinator community differs from the target natural ecosystem. As long as all functional pollinator groups are retained and plants are consistently producing viable seed across years, then pollination could still be considered to have been functionally restored [26]. However, a far more intimate knowledge of the ecological requirements of the whole system is required if the goal is reestablishment of the original pollinator community or the restoration of both plants and pollinators.

Structural characteristics of plant–pollinator networks

Inherent characteristics of pollination networks can affect how they respond to ecological perturbations and ultimately impact their ease of restoration. Pollinator communities typically comprise a small number of rare, highly specialized species, many moderately specialized species and a few common, generalist species, which provide the majority of animal-mediated pollination [27,28]. There are consistent structural characteristics of pollination networks, including high levels of asymmetry (i.e. specialist plants interacting with generalist pollinators) [27,29–32] and nestedness (specialists interacting with a subset of species that generalists interact with) [31,33]. These features of pollination networks confer resilience to disturbance, because as long as the core of generalists is retained, most plants will have pollinators. The loss of a rare specialist pollinator is unlikely to result in the loss of a plant [30,34].

General topological patterns of network structure, such as asymmetry and nestedness appear to vary little temporally [35–37]. However, species composition and pair-wise relationships among species can show high temporal variation [35–38]. Similarly, the food plants used by a given pollinator species often change between seasons and years due to shifts in the composition of the flowering community. This means that species that appear to be dietary specialists in a short-term study might prove to be generalists if the study is extended over multiple years or a greater number of observations are made [35,37]. These characteristics suggest that restoration for maximal pollinator diversity is important, so that species can continue providing functional replacements for each other over time [35–38].

Although generalist pollinators visit many plant species [39], they might not be as effective at transferring pollen as pollinators specifically adapted to visiting that plant [40,41]. For example, several typically bird pollinated plants suffered reduced seed set when birds were excluded,

allowing visitation only by bees (e.g. [41,42]). Furthermore, a recent study has shown that when pollen transfer is considered relative to pollinator visitation, levels of specialization in plant–pollinator networks increase [43].

Selecting plants to restore pollinators

Several studies have proposed that when restoring natural areas, plant species should be planted that attract and sustain pollinators for the duration that they require nectar and/or pollen [18,44–47]. These have been referred to as framework and bridging plants depending on how they function quantitatively and temporally to support the pollinator community [18].

‘Framework’ plants support pollinator communities by providing considerable nectar and/or pollen resources to numerous pollinator species and individuals [18]. Consequently, the use of framework plants might sustain a pollinator community that could also service smaller or less attractive members of the plant community [48–53]. For example, restoration research in agricultural landscapes has shown that a disproportionate number of visits by bumblebees are to a small number of plant species [9–12,54]. Identification and planting of these framework species can be used to promote effective pollinator restoration (Box 1) and potentially facilitate the reproduction of less attractive plants within the restoration palette.

A risk in the use of exceptionally nectar- or pollen-rich plants is that they might compete via pollination, rather than facilitate the pollination of less attractive plants [50,52,55]. A key challenge is that it is currently difficult to predict if plant species that share pollinators will interact competitively or facilitatively. However, there is some evidence that the directionality of these interactions can depend on relative plant abundance [50], possibly because individual pollinators can temporarily specialize on the more abundant species at the expense of rarer species [50,56]. Therefore, careful consideration needs to be given to relative abundances of the different plants when seeking to enhance pollinator visitation to targeted plant species.

‘Bridging’ plants provide nectar and pollen resources during otherwise resource-limited times [18,57]. The use of bridging plants is most important in communities with pollinators that require pollen or nectar all or most of the year, such as some vertebrate pollinators [58,59], and social and/or multivoltine bee species such as bumblebees (*Bombus* spp.; Figure 1). Bridging plants can be particularly important for pollinators with relatively specific food requirements. For example, the honey possum (*Tarsipes rostratus*, Tarsipedidae) only consumes the pollen and nectar of vertebrate-pollinated plants making it reliant on one or few species of food plants during some seasons [58]. The necessity for bridging plants varies between ecosystems. For example, in some environments, such as tropical forests, species of pollen-feeding or nectarivorous insects can be active throughout the year necessitating a year-round flower supply [60]. Conversely, in some environments with short growing seasons, bridging species might not be needed because flowering is intense during the growing season and insects persist during the non-growing season as eggs or larvae [61].

Box 1. Case study: restoration of pollination services in the Central Valley of California

In the Central Valley of California, land is managed intensively for row crop, vineyard and orchard production in large monoculture fields. Wild bee communities and the pollination services they provide to a variety of crops are greatly diminished in such landscapes, compared with diversified, organic plantings grown in more heterogeneous landscapes that include regions of natural habitat [6,112]. However, growers are increasingly planting native plant hedgerows along field edges to restore a range of ecological functions, including pollination services (Figure 1). We used a large dataset on plant–bee interactions from farms and natural area sites in this region [113] to select plants that would support the 20 most important crop pollinators (based on [5,6,114,115]) across their flight seasons. First, we identified the plant species visited by the largest number of individuals and the species of these crop–pollinators (framework plants). Second, we compared the blooming periods of these plants against the flight seasons of the crop–pollinating bee species to identify any gaps in the provisioning of floral resources. We added plant species that both bloom during those gaps and were visited by our crop–pollinating species (bridging species). Finally, we removed plant species that were impossible to cultivate or were “weedy” in habit (and therefore unlikely to be accepted by grower partners), identifying substitute species whenever possible.

Our conservation partners (Xerces Society, Audubon Society) identified interested growers and convinced them to use this planting palette in their hedgerow restoration planting. From 2006, we began monitoring five pre-restoration sites, along with 11 control sites, matched in pre-restoration vegetation, adjacent land use and landscape context (a “beyond Before-After-Control-Impact design” [116]). Although we monitor the pollinator communities at these sites annually, we do not expect to see strong differences among hedgerow and control sites (which are unmanaged, often weedy, field edges) until hedgerows mature in several more years. Meanwhile, we are also studying mature hedgerows (established more than a decade ago) that contain many of the same plant species, but that were developed to promote natural enemies and pest control services rather than pollinator communities and pollination services [117]. A promising initial finding is greater bee diversity (but not abundance, which is dominated across all sites by several superabundant halictid species) on the mature hedgerows compared with controls. In addition, at hedgerow sites, both honey bees and native bee species preferred foraging on native hedgerow shrubs relative to exotic weeds co-flowering at these sites, and native plants were visited by more species and individuals than exotic plants (L. Morandin and C. Kremen unpublished data).



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Figure 1. Hedgerow restoration in the Central Valley, California. The image on the left depicts the initial stages of hedgerow restoration (M. Vaughan, Xerces Society), whereas the image on the right shows a mature hedgerow, approximately 10 years following restoration (L. Morandin, University of California Berkeley).

Plant populations in restored areas need to be large enough to avoid Allee effects, (see [Glossary](#)), the commonly observed reduction in fecundity of plants in small populations [62–65]. Experiments using supplemental hand-pollinations across a range of population sizes have shown that these Allee effects are often due to pollen-limitation of seed production [66,67]. Pollen deficits can arise when pollinators are less likely to visit plants in small populations or when plants in these populations receive insufficient compatible pollen on stigmas [68]. Some plants appear to be resilient to Allee effects even though they depend on pollinators (compare with [69]); knowledge about the reproductive biology of these species might provide clues as to which plant–pollinator interactions are easier to restore.

Studies on the restoration of animal-mediated pollination should focus on both the ecological requirements of the target plant community and the associated pollinators. Once candidate framework and bridging plants have been identified, researchers should identify which of these plants supports the greatest abundance and diversity of pollinators for the given area.

Ecological requirements of pollinators

Pollinator colonization and persistence in restored natural areas requires that the ecological needs of pollinators are met either entirely within the restoration site or within foraging distance of the restoration site. Pollinators depend on several factors for the completion of their life cycle, such as the availability of food sources, nesting material and nest sites [4,70–72]. For example, solitary bees can be limited by the availability of nest sites [73,74], butterflies require both larval host plants and nectar resources as adults [75], thynnid wasps (Hymenoptera: Thynnidae; [Figure 1](#)), which are important pollinators of Australian orchids [76], require a carbohydrate source as adults (i.e. nectar, secretions from larval psyllids: Hemiptera: Psyllidae, or honeydew) [76] and scarab beetle larvae (Coleoptera: Scarabaeidae), which they parasitize [77,78]. Although measures such as providing artificial nest sites can facilitate colonization and increase bee populations [73,74], there is little known about the importance of life cycle requirements other than nectar and pollen for the successful restoration of most pollinator species.



Figure 1. Examples of plants and pollinators demonstrating different levels of specialization. (a) Flowers of *Lapeirousia oreogena* (Iridaceae) are pollinated exclusively by an undescribed long-proboscid fly species (*Prosoeca* sp., Nemestrinidae) (South Africa). (b) A New Holland honeyeater (*Phylidonyris novaehollandiae*, Meliphagidae), a generalist bird pollinator, feeding on the specialist plant *Banksia coccinea* (Proteaceae), which is pollinated by vertebrates (southwestern Australia). (c) The generalist pollinator *Bombus* sp. (Apidae) on *Perovskia atriplicifolia* (Lamiaceae) (North America). (d) The sexually deceptive *Drakaea gracilis* (Orchidaceae) attracts only a single species of pollinating thynnid wasp (an undescribed species of *Thynnoides*, Thynnidae), a generalist nectarivore (southwestern Australia). (e) A gerbil (*Gerbillurus paeba*) feeding on flowers of *Massonia depressa* (Hyacinthaceae), a lily specialized on pollination by small, ground-dwelling mammals (South Africa). Photographs: (a) and (e) S.D. Johnson, (b) D. McGinn, (c) L. Mandle, (d) B. and B. Wells.

Pollinator life histories vary in their susceptibility to disturbance. For example, above-ground nesting and social bee species have been shown to be more negatively affected by isolation from remnant native habitat than below-ground nesting and solitary bee species [72]. Furthermore, invertebrate pollinators with narrow habitat requirements, slower development, fewer generations per year and lower mobility have been shown to experience greater declines in some countries [79]. These trends might indi-

cate that pollinator species with more complex life cycles, or life cycles that do not facilitate rapid colonization or recovery from local extinction might be more difficult to restore.

Landscape structure and facilitation of pollinator movement

Anthropogenic habitat removal and subsequent landscape fragmentation can alter pollination and pollinator commu-

nities [57,80–83]. Habitat fragmentation can result in decreased pollinator abundance and diversity in small fragments [81]. However, pollinators exhibit a broad range of responses to habitat fragmentation and ecosystem disturbance [81,84,85]. For example, some bee species are negatively affected by human disturbance [72,81], whereas others might benefit [86]. A common consequence of habitat fragmentation is reduction in plant population size [64], which can lead to a decline in levels of pollination, seed set and recruitment regardless of any effect from the pollinator community (e.g. [15,66,67,87]).

Ideally, knowledge of dispersal, subsequent colonization capability, minimum habitat area requirements and potential barriers to dispersal [82,83,88] of the focal pollinator groups would be desirable before undertaking restoration. To facilitate colonization of restored sites, consideration needs to be given to the layout of restoration plantings in relation to the ability of pollinators to use and cross the landscape matrix. In cases where pollinators forage widely and are able to traverse a variety of habitats, initial restoration of animal-mediated pollination might occur through foraging by wide-ranging generalist pollinators, such as Australian honeyeaters (Meliphagidae; [45]; Figure 1), bumblebees (Figure 1) and the honey bee *Apis mellifera* [89–92].

In cases where pollinators are able to move through a hostile matrix, remnant plants, such as individual trees can serve as stepping stones, increasing landscape and genetic connectivity (e.g. [93,94]). The creation of a stepping stone habitat could form the initial step of restoration programs to facilitate dispersal of pollinators between otherwise isolated fragments to promote diverse pollinator communities at the landscape scale.

Alternatively, pollinators with limited dispersal capability can require contiguous links of favorable habitat (corridors) to facilitate movement into restored sites. Depending on the landscape and the target organisms, corridors can refer to either strips of vegetation in an agricultural landscape, or strips of open vegetation within a forested matrix. Connection of plant populations by corridors has been shown to facilitate pollinator movement [95]. Furthermore, in agricultural and silviculture dominated landscapes, for some plants, pollen transfer has been shown to be significantly higher between populations connected by corridors than those that were not [96,97]. In some cases, pollen transfer declined with increasing distance to the source, indicating limitations to the use of corridors [98]. Furthermore, the efficacy of corridors for facilitating dispersal can vary between taxonomic groups [98]. The abundance of wild bees along linear habitat corridors in an agricultural landscape has been shown to decline with distance to remnant semi-natural habitat, whereas hoverflies showed the reverse trend [98]. However, corridors might be the only viable option when the majority of the landscape is dedicated to agriculture and large-scale revegetation is impossible.

Corridors can potentially create undesirable side effects such as facilitating the spread of invasive or edge species [99]. An alternative is to enlarge the existing habitat area to reduce the negative effects of small fragment size. This could have a more pronounced effect on increasing population

sizes within a patch, compared with corridors, particularly when patches increase beyond the minimum area required for supporting viable pollinator populations [100].

The natural colonization of a restored site and the complexity of the pollinator community present can be strongly influenced by its proximity to remnant habitat [20,21,26]. For relatively immobile pollinator species, pollination restoration might require habitat remnants to be directly connected by favorable habitat patches, such as vegetated corridors. For example, foraging ranges of bees can vary from less than a hundred meters for small-bodied species to several kilometers for large-bodied species [91,92]. Where pollination restoration can be achieved by highly vagile generalist species, stepping stone plantings might be sufficient to facilitate colonization. In the presence of a hostile landscape matrix, colonization could be facilitated by direct connection via corridors. Consequently, the landscape context of a restoration site and the ecology of the fauna will both have considerable bearing on whether or not corridors or stepping stones are effective or needed in facilitating pollinator colonization.

Captive breeding and reintroduction programs might be effective in cases where pollinators have been extirpated and natural colonization processes are unlikely through lack of source populations or low dispersal [18], particularly for restoring habitat patches that are isolated from sources of recruits. However, there could be many challenges associated with restoring certain pollinators that have highly specific ecological requirements. To our knowledge, there are no published studies of reintroduction or captive breeding of native pollinators primarily for reinstating pollination in natural or restored areas.

Variation among biogeographic regions

The optimal techniques for facilitating pollination in restored environments could differ markedly between habitats and biogeographic regions. For example, there are significant geographical differences in levels of specialization and diversity of plant–pollinator interactions [101,102]. Among the comparatively well-studied floras, the Cape Region stands out as a region characterized by remarkably high levels of both plant and pollinator specialization [103]. This suggests that pollinators and pollination, if lost from this region, would be comparatively difficult to restore. Furthermore, pollinator functional groups (*sensu* [102]) might show varying levels of morphological and ecological specialization between regions depending on the evolutionary and ecological histories of the plants and pollinators involved [104,105]. Areas where there is high functional diversity are likely to require an increased diversity of targeted plantings to assist in the restoration of these more complex pollinator communities.

Plants and pollinators from landscapes of different ecological and evolutionary history are likely to have varying reproductive and dispersal behaviors and resilience to habitat fragmentation [106,107]. For example, the old, geologically diverse but stable landscapes of the Cape Floristic Region and the Southwest Australian Floristic Region have facilitated the evolution of diverse heathland floras characterized by a high incidence of naturally frag-

Box 2. Incorporating the difficulty of restoring plant species into plant selection

Owing to differences in the ease of restoring plant species, the most attractive plant species will not necessarily be the most efficient for achieving rapid restoration of pollinators. Difficulty of restoring each plant species might be caused by factors such as limitation of propagule sources, difficulty of establishment (susceptibility to disease, lack of vigor) and poor long-term establishment. We use examples from another system that has been well-researched in terms of restoration practices, the biodiverse *Banksia* woodland of the Southwest Australian Floristic Region, to illustrate how the characteristics of the species in each of these boxes can be used to guide the restoration process (Figure I). Although Panel 1 contains few plant species, these are the highest priority for restoration due to ease of restoration and the large number of pollinator species that they support. For example, the canopy forming *Eucalyptus marginata* (Myrtaceae) can attract over 80 species of nectar- and pollen-feeding insects at a single site [119]. Panel 2 contains plant species with a higher level of pollination specialization. Therefore, in restoration, plant species in this category should be chosen for minimal overlap

with the generalists from Panel 1 to maximize pollinator diversity. For example, in the Southwest Australian Floristic Region (SWAFR), the kangaroo paw species, *Anigozanthos humilis* (Haemodoraceae) is readily restored and is visited by several honeyeater species [120], birds that are specialized on a subset of the plant community [47]. Panel 3 contains plant species that are difficult to restore but, if restoration is successful, will attract a wide range of pollinators. This option could be pursued if the species attracted a suite of pollinators not already accounted for in Panel 1 species. In the SWAFR example, *Philotheca spicata* (Rutaceae) is difficult to restore (Alcoa World Alumina, personal communication) but attracts a range of understory Hymenoptera. Panel 4 represents species that should only be targeted for restoration if they are plants of special conservation concern, such as rare or threatened species. For example, many orchids have specialized mycorrhizal and pollinator relationships making restoration challenging [76,121]. However, because of their popularity and high degree of threat, orchid conservation is a relatively high priority in the conservation community.

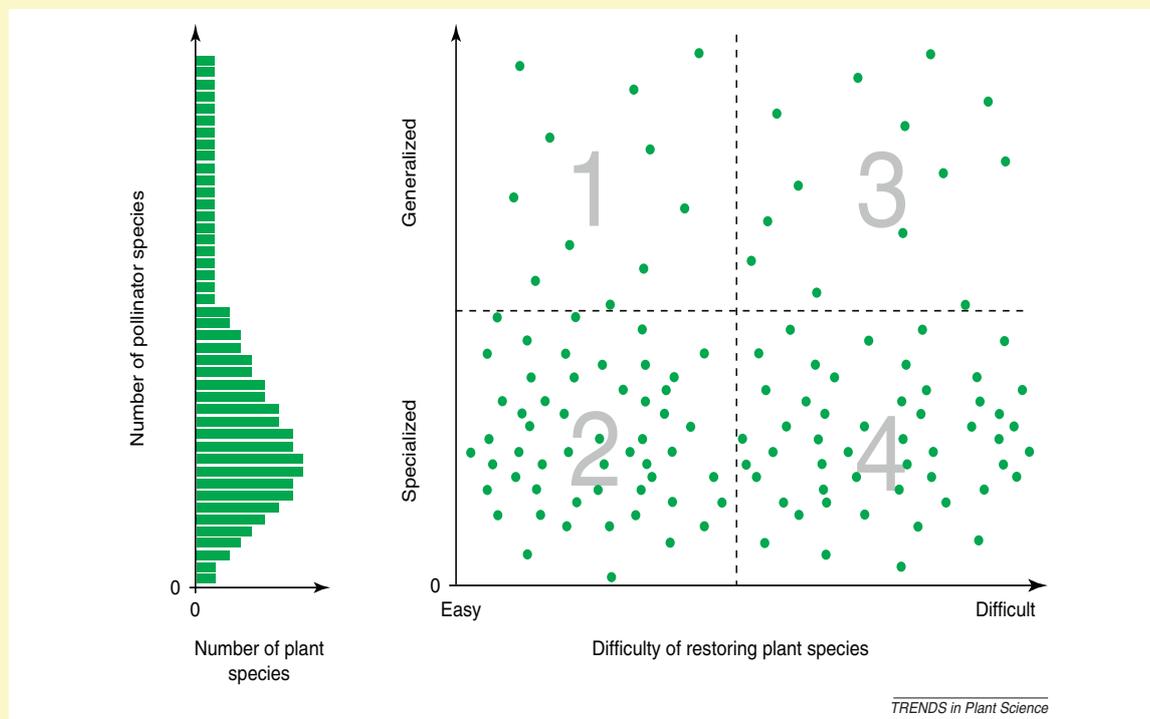


Figure I. A framework for the choice of plant species in restoration, based on specialization of pollination system versus ease of restoration. The scatterplot depicts a hypothetical plant community, with each point representing a single plant species. The difficulty of restoring each plant species has been plotted against the number of species of visiting pollinators. Panels 1 and 3 depict generalist plants that receive visits from many pollinator species, and Panels 2 and 4 depict plants with more specialized pollination (fewer visitor species). The relative proportions of plant species that have specialized versus generalized pollination systems (based on the number of visitors) has been based on an actual plant–pollinator network [118], as shown in the histogram on the left, which shows the number of plant species in the community exhibiting each level of specialization (expressed as the number of species of visiting pollinator).

mented plant populations [107,108]. We predict that in these landscapes, small-bodied generalist pollinator species might be able to access a range of species within a small area and have naturally small foraging ranges. Similarly, more specialized small-bodied species might have restricted dispersal so that they remain in the relatively small, disjunct patches of suitable habitat. At the other extreme, species from environments with episodic and patchy flowering, such as deserts, might contain species with large foraging ranges. Although these remain predictions, it highlights the caution required when

extrapolating restoration practices between environments or biogeographic regions.

Concluding remarks: future research directions

Restoration of pollination systems provides an example where management at the local scale could potentially have profound effects on the diversity of pollination interactions at the landscape scale [109]. Future research should resolve patterns of dispersal by pollinators and how this process can be facilitated through planting flora that attract and sustain a variety of pollinator species, with

the ultimate aim of colonization and persistence in restoration sites. The stage of restoration in which specific framework and bridging species are planted will need to be determined based on plant phenology and pollinator requirements. Recent evidence suggests that when pollen transfer is taken into account in plant–pollinator visitation webs, levels of specialization increase [43], highlighting the need for research on the ability of different pollinators to transfer pollen effectively to target plant species.

Further research is required to determine the extent of the impacts of invasive pollinators on the success of pollinator restoration projects, through competition and transmission of diseases [110]. Given that invasive pollinators are known to be a problem on multiple continents [111], such research would have broad implications. By designing restoration plantings to favor native pollinators, restoration sites could represent an opportunity to create pollinator communities free from invasive pollinators.

To maximize the efficiency of the restoration process, practitioners should, when choosing plant species for restoration of pollination networks, consider not only the pollinator species attracted but the ease of restoring the plant species (Box 2). Likewise, choosing the pollinators to target for restoration should involve both consideration of the efficacy of the pollinator and how readily they can be attracted to the site. As such, the final challenge for pollination biologists is to integrate their findings with the work of plant restoration ecologists to achieve the highest degree of ecosystem function.

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